농학박사 학위논문

해양방선균 유래 Hydroxyquinolines과 Diketopiperazines의 *Candida albicans* 저해활성 및 작용기전 연구

Studies on the Antimicrobial Activity and Mechanism of Hydroxyquinolines and Diketopiperazines from Marine Actinomycetes against *Candida albicans*

2019년 8월

서울대학교 대학원
농생명공학부 응용생명화학전공
김 희 규

A Dissertation for the Degree of Doctor of Philosophy

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August 2019

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지도교수 오 기 봉

이 논문을 농학박사학위논문으로 제출함

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농생명공학부 응용생명화학전공

김 희 규

김희규의 박사학위논문을 인준함

2019년 7월

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Studies on the Antimicrobial Activity and Mechanisms of Hydroxyquinolines and Diketopiperazines from Marine Actinomycetes against *Candida albicans*

Advisor: Ki-Bong Oh

A Dissertation Submitted in Partial Fulfillment
of the Requirement for the Degree of

DOCTOR OF PHILOSOPHY

to the Faculty of
Applied Life Chemistry Major
Department of Agricultural Biotechnology

at

SEOUL NATIONAL UNIVERSITY

by

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Date Approved

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Abstract

The first part deals with the isolation and purification of 2-alkyl-4-hydroxyquinoline which possess inhibitory activity against yeast-to-hyphae transition derived from *Streptomyces* sp. MBTG13. *Candida albicans*, the opportunistic pathogenic fungus in humans, has an ability of reversible morphogenetic transitions between budding yeast and filamentous hyphal forms in response to environmental signals. Filamentous growth of cells is essential for pathogenicity of *C. albicans*. Four of 2-alkyl-4-hydroxyquinoline derivatives (**1**–**4**) were isolated from a semisolid rice culture of *Streptomyces* sp. MBTG13. Compound **1** is identified as an inhibitor of filamentous growth in *Candida albicans*, with an IC_{50} value of 11.4 μg/mL. Growth experiments show that this compound do not inhibit yeast cell growth, but inhibits hyphal growth inductions. Filamentous growth of *C. albicans* is mainly depended on activation of the mitogen-activated protein kinase or cyclic AMP-protein kinase A (cAMP-PKA) pathway. Based on the results of gene expression analyses, *HWP1* and *ALS3* (hypha-specific genes positively regulated by Efg1, an important regulator of cell wall dynamics), included cAMP-PKA pathway, are inhibited by compound **1** These results indicate that compound **1** acts on the Efg1-mediated cAMP-PKA pathway and regulates hyphal growth in *Candida albicans*.

The second part deals with the isolation and purification of diketopiperazines which possess inhibitory activity against isocitrate lyase (ICL) enzyme from *C. albicans*. ICL, the key enzyme of glyoxylate cycle, plays an important role in the pathogenesis of microorganisms during infection. An *icl*-deletion mutant of *Candida albicans* exhibited reduced virulence in mice compared with the wild-type. Five of diketopiperazines (**5**-**9**) are isolated from *Streptomyces puniceus* Act1085. Compound **5** is identified as an *C. albicans* ICL inhibitor, with IC_{50} value of 27 μg/mL. Based on the growth phenotype of the *icl*-deletion mutants

and *ICL* expression analyses, compound **5** inhibits the *ICL* gene transcription in *C. albicans* under C_2-carbon-utilizing conditions.

Keywords: *Streptomyces*, *Candida albicans*, hydroxyquinolines, diketopiperazines, filamentous growth, isocitrate lyase, cAMP-PKA pathway

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List of Abbreviations

AD	Alzheimer's disease
BCRP	Breast cancer resistance protein
BLAST	Basic local alignment search tool
cAMP	Cyclic adenosine monophosphate
C. albicans	*Candida albicans*
cDNA	Complementary DNA
COSY	Correlation spectroscopy
CLSI	Clinical and Laboratory Standards Institute
DMSO	Dimethyl sulfoxide
E. coli	*Escherchia coli*
EMEA	Europe, the middle east and africa
EtOAc	Ethyl acetate
EU	European Union
FDA	Food and drug administration
GTP	Guanosine triphosphate
HIV-1	Human immunodeficiency virus type
HMBC	Heteronuclear multiple-bond correlation spectroscopy
HSQC	Heteronuclear single-quantum correlation spectroscopy
HPLC	High performance liquid chromatography
ICL	Isocitrate lyase
NMR	Nuclear magnetic resonance spectrometer
MAPK	Mitogen-activated protein kinase
MeOH	Methanol
MIC	Minimum inhibitory concentration
MS	Mass spectrometer
mRNA	Messeenger RNA

NCIRF	National center for interuniversity research facilities
PDA	Potato dextrose agar
RPMI	Roswell Park Memorial Institute
RT-PCR	Reverse transcription-polymerase chain reaction
TCA	Tricarboxylic acid
US	United states
WHO	World health organization

Part I

Inhibitory Effects of 2-Alkyl-4-Hydroxyquinolines on Yeast-to-Hyphae Transition of *Candida albicans*

Introduction

Candida albicans and pathogenicity

C. albicans is the serious human fungal pathogenic microorganisms and a ubiquitous commensal of human gastrointestinal tract, mouth and skin (Ghannoum et al., 2010; Findley et al., 2013; Hoffmann et al., 2013). This strain becomes pathogenic when human host is immunocompromised or infected with candidiasis in bloodstream which has mortality rate of 40% approximately (Lu et al., 2014). Although modern medical therapies such as antibiotics, cancer chemotherapy, organ transplantation have been developed and applied, the number of patients suffering from infectious disease of *C. albicans* has increased (Noble et al., 2017). Also, several researches have shown that *Candida* species were ranked as the third or fourth most common cause of invasive bloodstream infections in hospitals located in United States (Wisplinghoff et al., 2004; Pfaller and Diekema, 2007).

The important ability of *C. albicans* is its switch between yeast and hyphal growth form as dimorphism. *C. albicans* undergoes a reversible morphogenetic transformation in response to environmental signals such as serum, high temperature (37°C), high ratio of CO_2 to O_2, neutral pH, and nutrient poor media (Sudbery 2011). These features of *C. albicans* are a virulence determinant, as hyphal type of cells is strongly involved in the infection process. The hyphae forms release several hydrolytic enzymes to invade and damage epithelial and endothelial cells. Moreover, hyphae express various virulence factors related to cell type including adhesion, tissue-degrading enzymes, antioxidant defense proteins, and cytolytic peptide toxin (Lane et al., 2001; Nantel et al., 2002; Kadosh and Johnson, 2005).

In *C. albicans*, several signaling pathways related to yeast-to-hyphae morphological transition have been identified (Fig. 1.1). Among these signaling pathways, mitogen-activated protein kinase (MAPK) pathway and cyclic AMP

(cAMP)-dependent protein kinase A (PKA) pathway were reported as major complex network of signaling pathway for hyphae development. Yeast-to-hyphae morphological transition is induced by activating hypha-specific genes such as *ALS3*, *ECE1*, *HGC1*, *HWP1*, *HYR1*, *RBT1* and *RBT4* (Kadosh and Johnson, 2005).

MAPK pathway is composed of the Cst20p (p21-activated kinase; PAK), Hst7p (MAP kinase; MEK) and Cek1p (MAPK) (Liu, 2001). Mutants of these genes all show a defect in filamentous growth of *C. albicans* (Kohler and Fink, 1996). MAPK signaling pathway is controlled by Cph1, a transcription factor, at its downstream.

In cAMP-PKA pathway, catalytic subunits are encoded by *TPK1* and *TPK2* genes and regulatory subunit is encoded by *BCY1* gene (Giacometti et al., 2009). Cyclic AMP functions as an intracellular regulator and performs many cellular processes in both prokaryotic and eukaryotic organisms. cAMP-PKA pathway regulates hyphal development in various fungi including *C. albicans* (Lengeler et al., 2000). The function of cAMP is determined by its endogenous levels that are controlled by phosphodiesterase enzymes and adenylate cyclase (Dhillon et al., 2003). *CYR1*, a single adenylate cyclase gene in *C. albicans*, is necessary to only filamentous growth but not yeast growth. *CAP1*, the adenylate cyclase-associated protein gene, participates in process of hyphal development and germ tube formation for the pathogenesis of candidiasis. In mice, *cap1*-deletion mutants are not exhibit pathogenesis (Bahn et al., 2001; Bahn and Sundstrom, 2003; Zou et al., 2010). Efg1, a transcription factor, acts on downstream of the cAMP-PKA pathway and induces the hyphal-specific genes in *C. albicans*.

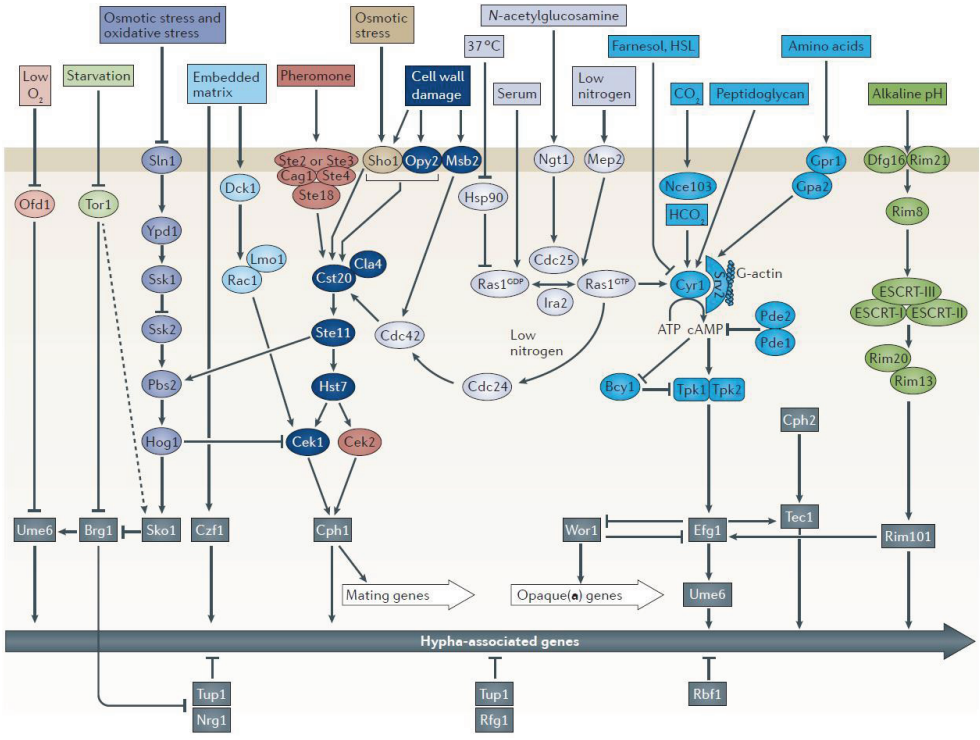
Ras1 affects upstream of two main signaling pathways, MAPK pathway and cAMP-PKA pathway, as a major regulator of hyphal formation (Sudbery, 2011). Ras proteins belong to the small GTPase superfamily which regulate a wide variety of processes in the cell, including growth, cell differentiation, and cell movement. Ras proteins are controlled by cycling between the active GTP-bound

and inactive GTP-bound forms (Bourne et al., 1990). In *C. albicans*, Ras1 is not essential for survival (Feng et al., 1999). *ras*-deletion mutants showed the defect of filamentous growth in response to hyphal inducing conditions.

To attenuate *C. albicans* virulence and treat candidiasis, researches on inhibition of morphogenesis has been pursued for the development of prophylaxis and/or therapy (Vila et al., 2017). Based on the efforts of searching the inhibitor of hyphae morphogenesis, azole, geldamycin, histone deacetylase inhibitors and propranolol were discovered but these compounds had a poor potential of further developments for antifungal drugs (Shareck and Belhumeur, 2011). Also, a small number of simple molecules possesses inhibitory activity of *C. albicans* morphological transition with moderate toxicity at concentration 1–100 mM (Toenjes et al., 2005). Up to date, filastatin and diazaspiro-decane were identified as the representative inhibitor of hyphal morphogenesis (Fazly et al., 2013; Pierce et al., 2015). Considering these results, it is necessary to find inhibitor of hyphae growth other than filastatin and diazaspiro-decane.

Figure 1.1 Signaling pathway leading to hyphae-associated genes in *C. albicans*

Numerous host signals and fungal signaling pathways have been determined in the regulation of cell type, such as yeast and hyphae in *C. albicans*. (Noble et al., 2017)


Osmotic stress and oxidative stress
Osmotic stress
N-acetylglucosamine
37 °C
Farnesol, HSL
Amino acids
Low O2
Starvation
Embedded matrix
Pheromone
Cell wall damage
Serum
Low nitrogen
CO2
Peptidoglycan
Alkaline pH
Sln1
Ste2 or Ste3
Cag1 Ste4
Ste18
Sho1
Opy2
Msb2
Hsp90
Ngt1
Mep2
Nce103
HCO3
Cpr1
Cpa2
Dfg16
Rim21
Rim8
Ofd1
Tor1
Ypd1
Dck1
Lmo1
Rac1
Cst20
Cla4
Cdc25
Ras1GDP
Ras1GTP
Ira2
Cyr1
CAP
G-actin
ATP cAMP
Pde2
Pde1
ESCRT-III
ESCRT-I
ESCRT-II
Rim20
Rim13
Ssk1
Ssk2
Ste11
Cdc42
Low nitrogen
Pbs2
Hst7
Cdc24
Bcy1
Tpk1
Tpk2
Hog1
Cek1
Cek2
Cph2
Ume6
Brg1
Sko1
Czf1
Cph1
Wor1
Efg1
Tec1
Rim101
Mating genes
Opaque(a) genes
Ume6
Hypha-associated genes
Tup1
Nrg1
Tup1
Rfg1
Rbf1

Natural products derived from marine organisms

Since prehistoric time, natural products have been used as medicine for alleviating pains caused by several diseases. Natural products have been founded in plants, animals, microorganisms, and recently marine microorganisms and contributing to development of drugs and supplying basic scaffolds of drugs until now. Unfortunately, the number of novel natural products that show interesting biological activity was steadily decreased after late 1960s. Also, researches and industry of drug development encountered a major problem such as reduction of new class compounds discovery, a wide range of resistance and inefficient drug discovery and development process in terms of costs. To solve problems, fresh strategies of drugs discovery emerged from combinatorial chemistry, example high-throughput screening, and computational design of drugs (Fenical et al., 2006). These approaches have limitation because it is based on the natural products derived from terrestrial organisms.

Several researchers are interested in marine environments which cover approximately 75 percent of the earth planet. It is expected that marine environments have a different ecosystem because ocean is unexplored area until late 1990s. Following rapid development of technologies about collecting samples from deep ocean, a wide variety of marine organisms have been collected and investigated their biological activities. The deep ocean environments are recognized as quiet unique habitat, which is characterized by no light, extreme low temperatures (< 4°C), high pressure (up to 110 MPa) and low nutrient input (Nagano et al., 2010). These environments enforce that marine organisms have flexible bodies and sedentary life style for survival. Thus, these organisms have been evolved the ability to make toxic compounds or to acquire them from other organism (Haefner, 2003). The huge diversity of marine organisms has not been explored extensively. It would be expected that a wide range of organisms produced high diversity and unique structure of secondary metabolites. Thus,

marine organisms are attractive with regard to finding a new class compounds. Actually, various marine natural products have been reported by several research groups. In previous review (Hu et al., 2015; Pereira and Aires-de-Sousa, 2018), more than 28,000 marine natural products have been reported from marine organisms and among them, 4,196 compounds have been reported to have diverse biological activities by 2015.

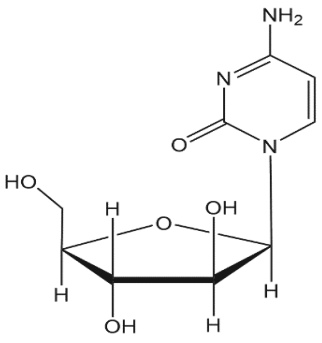
Until now, four drugs derived marine organisms with US FDA approval or EU registrations were reported (Fig. 1.2). Cytarabine (Ara-C) which is arabinosyl cytosine of cytosine arabinoside was the first FDA-approved marine-derived drugs at 1965 (Newman et al., 2009). It was reported that this compound originally isolated from the Caribbean sponge *Tethya crypta* and used in treatment of acute leukemia but it has cytotoxicity. Vidarabine, which is purine nucleoside, was isolated from the Carribbean sponge *Tethya crypta* and *Streptomyces antibioticus*. This compound was received the FDA approval in 1976 as an antiviral agent (Montaser and Luesch, 2011). Ziconotide is composed of 25-amino acid peptide, originally isolated from venom of marine snail *Conus magus* (Olivera, 2000). This compound received FDA approval in 2004 and affected to several chronic pain in patients with cancer or AIDS. Trabectedin (ET-743) which is a tetrahydroisoquinoline alkaloids derived from *Ecteinascidia turbinate* is first marine natural compound registered in the European Union (EU) (Wright et al., 1990). Patients with soft tissue sarcoma or relapsed platinum-sensitive ovarian cancer had been treated with this compound (Yap et al., 2009; Verweij, 2009).

Actinomycetes which is gram-positive bacteria with high content of GC nucleosides, were rich source of bioactive metabolites with unique structure. Since terrestrial actinomycetes were screened for the purpose of searching novel metabolites in the early 1950s, a wide-range of metabolites have been identified and developed into drugs such as antimicrobial (vancomycin, amphotericin B and erythromycin), anticancer (bleomycin, mitoxantrone and daunorubicin), and

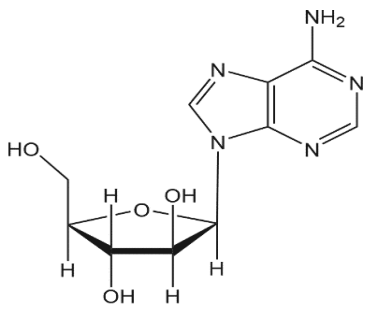
immunosuppressive (rapamycin) (Zotchev, 2012). Among marine organisms, actinomycetes have an advantage such as available fermentation, gene manipulation for high production and efficient storage as cell stocks. Also, development of sampling and fermentation techniques allows isolation of marine actinomycete producing unique compounds (Jensen et al., 2005). Overall, these features support that marine actinomycete is a promising resource for searching bioactive metabolites.

Figure 1.2. Marine-derived natural products approved by the FDA or the EMEA

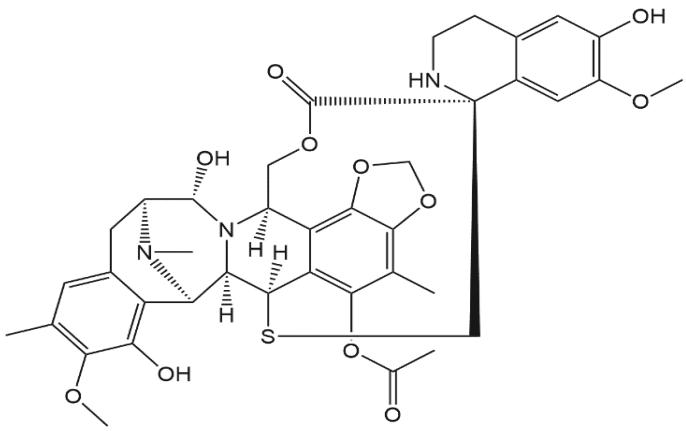
Cytarabine, vidarabine and ziconotide are approved by the FDA (Montaser and Luesch, 2011; Olivera, 2000). Trabectedin is approved by the EMEA (Wright et al., 1990).



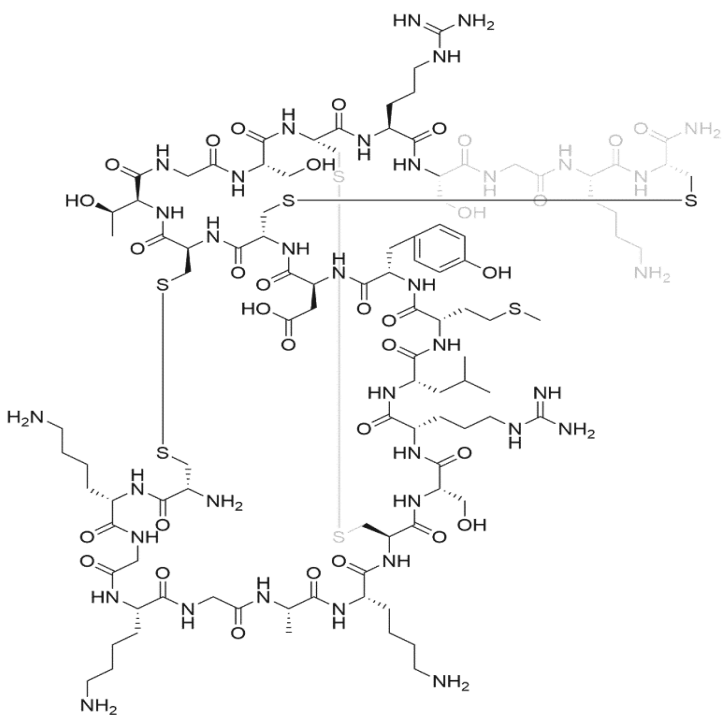
Cytarabine



Vidarabine



Trabectedin



Ziconotide

Quinoline derivatives as potential drugs

Quinoline, a heterocyclic aromatic compound with the chemical formula C_9H_7N, is a double-ring structure that contains a benzene ring fused to pyridine at two adjacent carbon atoms. Quinoline and its derivatives derived from plants, animals, and microorganisms show diverse biological activities and simple structures, so that many researchers have been focused on developing potential applications related to pharmaceuticals. Also, quinolone and its derivatives are critical structures in medical chemistry and used as "parental" compounds to synthesize molecules. In microbial source, actinomycetes of the genus *Streptomyces* produce most of quinoline derivatives (Raynes et al., 1995).

Various quinoline and its derivatives possess diverse biological activities such as antimalarial (Narender et al., 2005; Burnett et al., 2007; Cross et al., 2010), antimicrobial (Kumar et al., 2009; Maity et al., 2012; Desai et al., 2016), anti-tuberculosis (Nayyar et al., 2006), and anticancer activities (Sharma et al., 2017). Recently, mechanism of quinolone and its derivatives action in tyrosine kinase, proteasome, tubulin polymerization topoisomerase and DNA repair are identified (Jain et al., 2016). Various functional groups such as nitro, halogen, and methoxy are a crucial role in applications of medicinal chemistry. The addition of nitro, halogen, oxygen and other substitution to quinolone ring lead to the significant alteration in optical properties, distribution of charge, biological activities and molecular structure (Lakshmi et al., 2011; Romano et al., 2012). In case of gemifloxacin and trovafloxacin, the change of C-7 position in quinolone core determines the potency and target preference (Domagala et al., 1998). Also, this substituent position controls the pharmacokinetic features of the drugs with a basic nitrogen.

The quinoline derivatives have been identified as several biological agents. Representatively, quinololines are historically among the most important antimalarial drugs, such as quinine, chloroquine, bisqinoline, mefloquine,

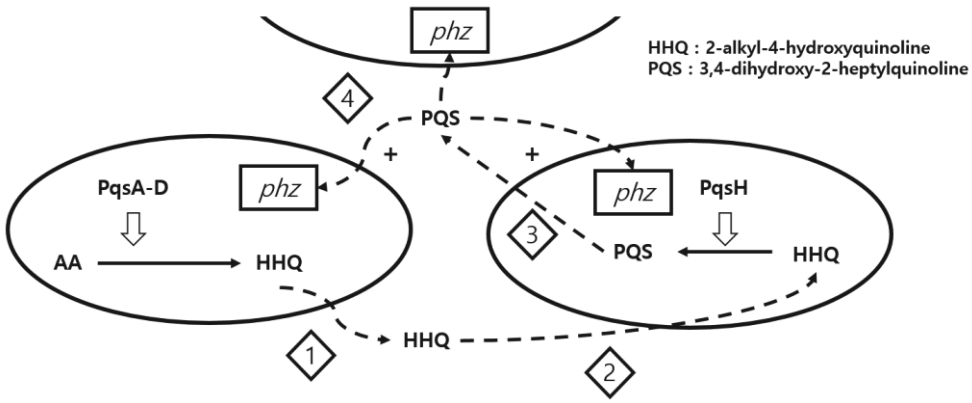
amodiaquinine and primaquine. When chloroquine was first chemically synthesized and applied to patients suffered from malaria, the World Health Organization (WHO) declared a malaria war. Following resistance of chloroquine was steadily increased, many research groups had been tried to overcome resistance and developed strategies which is the addition of various length linker and chemical on two 4-aminoquinoline moieties. As a result, a series of bisquinoline screened against chloroquinine sensitive strains and resistance strains and determined as alternative agents of malaria (Raynes et al., 1995). Also, quinolines and its derivatives plays a crucial role in anticancer drugs development such as campothecin. Campothecin is a cytotoxic quinolone alkaloid and first isolated from the bark and stem of *Camptotheca acuminata* (Wall et al., 1996). This compound possesses inhibitory activities of topoisomerase by interacting with Asp533 at the catalytic site of the enzyme. Several modification trials to develop drugs based on camptothecin lead to discovering topotecan and irinotecan which apply to cancer chemotherapy (Srivastava et al., 2005). 8-Hydroxyquinoline derivatives are identified as agent of cancer and Alzheimer's disease (AD) (Hung and Barnham, 2012). Because 8-hydroxyquinoline is identified as a metal chelator, this compound can be efficiently applied on various pharmacological applications such as being an iron and copper chelator for a ligand of metalloproteins, an anti-human immunodeficiency virus (HIV) agent, an antifungal agent and neuroprotection. Clioquinol (5-chloro-7-iodo-8-hydroxyquinoline), a metal-protein-attenuating compound, has reached on phase II clinical trial for the treatment of AD and leukemia, myelodysplasia (Crouch and Barnham, 2012).

Among quinoline alkaloids, 2-alkyl-4-hydroxyquinoline is identified as quorum-sensing molecules, involved in cell-to-cell communication (Déziel et al., 2004). When cell encounter specific environmental agents such as cell density, flux, mass transfer and spatial distribution, 2-alkyl-4-hydroxyquinolines are synthesized based on pqsABCD operon in *P. aeruginosa*. Déziel group suggests that 2-alkyl-4-

hydroxyquinoline and 3,4-dihydroxy-2-heptylquinoline-mediated cell-to-cell communication model, including steps as follows: 1. 2-alkyl-4-hydroxyquinolone is synthesized and released by bacterial cells. 2. extracellular 2-alkyl-4-hydroxyquinoline is absorbed by neighboring bacteria and converted to 3,4-dihydroxy-2-heptylquinoline, possibly in the periplasm. 3. 3,4-dihydroxy-2-heptylquinoline is released to act as a signaling molecule for other cells. 4. 3,4-dihydroxy-2-heptylquinoline activates target gene expression, such as the *phz1* operon which play roles in the synthesis of pyocanin (Fig. 1.3). The features of this compound have been considered as one of noteworthy biological activities of quinoline derivatives.

Figure 1.3. Cell-to-Cell communication model mediated by HHQ and PQS in *P. aeruginosa*

The pathway of cell-to-cell communication are indicated by dotted lines. AA means anthranillic acid.


phz
HHQ : 2-alkyl-4-hydroxyquinoline
PQS : 3,4-dihydroxy-2-heptylquinoline
4
PQS
+
+
PqsA-D
phz
phz
PqsH
3
AA
HHQ
PQS
HHQ
HHQ
1
2

Materials and Methods

General experimental procedures

^{1}H, ^{13}C, and 2D NMR (COSY, HMBC, and HSQC) spectra were measured using a Bruker Avance 600 MHz spectrometer at the National Center for Interuniversity Research Facilities (NCIRF) located in Seoul National University. Mass spectrometric data were obtained at the Korea Basic Science Institute (Daegu, Republic of Korea) and were acquired using a JMS 700 mass spectrometer (JEOL, Tokyo, Japan) using meta-nitrobenzyl alcohol as a matrix for fast atom bombardment mass spectrometry. HPLC was performed using a Spectrasystem p2000 (Thermo Scientific, Waltham, MA, USA) equipped with a Spectrasystem RI-150 refractive index detector. All organic solvents used in the experiment purchased from Fisher Scientific (FairLawn, NJ, USA)

Taxonomic analysis of the producing bacteria

The bacterial strain, *Streptomyces* sp. (strain number: MBTG13), was isolated from a marine sediment sample from the shoreline of Jeju Island, Republic of Korea. The strain was identified using standard molecular biological protocols by DNA amplification and sequencing of the internal transcribed spacer region. The genomic DNA extraction was performed using an i-Genomic BYF DNA Extraction Mini Kit (Intron Biotechnology, Seoul, Republic of Korea) according to the manufacturer's protocol. The nucleotide sequence of MBTG13 has been assigned as accession number MK408429 in the GenBank database.

Culture conditions of the producing bacteria

Strain MBTG13 was cultivated on YPG agar plate (5 g of yeast extract, 5 g of peptone, 10 g of glucose, and 16.0 g of agar in 1 L of artificial seawater) for 5 days at 28°C. It formed brown-yellow colonies produced white spore on plates. Agar

plugs (1 cm × 1 cm, five pieces each) were inoculated into 100 mL of YPG media for 5 days to acquire seed culture. For the purpose of large-scale fermentation, seed cultures separately were transferred to 24 flasks, each containing 2 g of peptone, 2 g of yeast extract, and 200 g of rice with 200 mL of artificial seawater after autoclave sterilization (121°C for 15 min). The fermentation in rice media was conducted under static conditions for 6 weeks at 28°C.

Antimicrobial activity assay

The antibacterial activity assay was carried out according to the Clinical and Laboratory Standards Institute (CLSI) method (CLSI, 2018). Gram-positive bacteria (*Staphylococcus aureus* ATCC25923, *Enterococcus faecalis* ATCC19433, *Enterococcus faecium* ATCC19434) and Gram-negative bacteria (*Klebsiella pneumoniae* ATCC10031, *Salmonella enterica* ATCC14028, *Escherchia coli* ATCC25922) were cultured overnight in Mueller Hinton broth (MHB) at 37°C. The cells collected by centrifugation, and washed two times with sterile distilled water. Each test compound was dissolved in dimethyl sulfoxide (DMSO) and diluted with MBH to prepare serial twofold dilutions in the range of 128–0.06 μg/mL. The final DMSO concentration was maintained at 1% by adding DMSO to the medium as guided by the CLSI (CLSI, 2018). In each well of a 96-well plate, 190 μL of MBH containing the test compound was mixed with 10 μL of the broth containing approximately 10^6 colony-forming units (cfu)/mL of test bacterium (final concentration: 5×10^4 cfu/mL) adjusted to match the turbidity of a 0.5 MacFarland standard. The plates were incubated for 24 h at 37°C. The MIC was defined as the lowest concentration of test compound that prevented cell growth. Ampicillin and tetracycline were used as reference compounds.

The antifungal activity assay was performed in accordance with the guidelines in CLSI document M38 (CLSI, 2018). *C. albicans* SC5314 was cultured on Potato dextrose agar (PDA). After incubation for 48 h at 28°C, yeast cells were

harvested by centrifugation and washed twice with sterile distilled water. *Aspergillus fumigatus* HIC6094, *Trichophyton rubrum* NBRC9185, and *Trichophyton mentagrophytes* IFM40996 were plated on PDA and incubated for 2 weeks at 28°C. Spores were harvested and washed two times with sterile distilled water. Stock solutions of the compound were prepared in DMSO. Each stock solution was diluted in RPMI 1640 broth (Difco, Livonia, MI, USA) with the concentration range of 0.06–128 μg/mL. The final DMSO concentration was maintained at 1% by adding DMSO to the broth. Harvested cells were adjusted to 0.5 MacFarland standard in RPMI 1640 broth and the diluted cells were added to each well of a 96-well plate with test compound solution. The final inoculum concentration was 10^4 cells/mL in each well. The plates were incubated for 24 h (for *C. albicans*), 48 h (for *A. fumigatus*), and 96 h (for *T. rubrum* and *T. mentagrophytes*) at 37°C. The MIC value was determined as the lowest concentration of test compound that fully inhibited cell growth. A culture with DMSO (1%) was used as a solvent control, and a culture supplemented with amphotericin B was used as a positive control.

Candida albicans strains and culture media

C. albicans strain SC5314 was used for most experiments. To prepare cells, *C. albicans* cultures were grown at 28°C with shaking in YPD (1% yeast extract, 2% peptone, and 2% dextrose) medium to the exponential phase, harvested by centrifugation, washed, resuspended in distilled water, and stored for 48 h at 4°C before use. The hypha-inducing media used in this study was composed of GS medium (5 g of glucose, 0.26 g of $Na_2HPO_4 \cdot 12H_2O$, 0.66 g of KH_2PO_4, 0.88 g of $MgSO_4 \cdot 7H_2O$, 0.33 g of NH_4Cl, and 16 μg of biotin per L) (Oh et al., 2001).

Candida albicans growth assay

C. albicans SC5314 cells were pregrown in YPD medium at 28°C up to the

exponential phase, washed, and starved in water for 48 h at 4°C. Stock solutions of compounds **1**–**4** (4 mg/mL) were prepared in DMSO and added to test media at the prescribed concentrations (final DMSO concentration: 1%). For the yeast cell growth assay, 1×10^6 cells/mL were inoculated in GS medium containing test compounds at different concentrations, and the cultures were incubated at 28°C for 24 h. The number of cells at each specific time interval was determined by measuring the OD_{660} of vigorously vortexed cultures.

Morphological transition inhibition assay

For the hyphal formation assay, approximately 5×10^6 cells/mL were added to GS medium containing test compounds at different concentrations and incubated at 37°C for 4 h. GS medium with 1% DMSO was used as a negative control. At each time point, the morphology of the cells was determined by light microscopy. A minimum of 200 cells were counted for each sample. Assays were carried out three times, each with three replicates.

Oligonucleotides

For PCR reactions, oligonucleotides were purchased from Cosmogenetech Korea Co. (Seoul, Korea). The sequence of oligonucleotides is shown in Table 1.1.

Table 1.1. List of used oligonucelotides

Primer Name	Sequence
CPH1-For	5′-GAAATGTGGCGCCGATGCAA-3′
CPH1-Rev	5′-ACCCGGCATTAGCAGTAGAT-3′
EFG1-For	5′-ACAGGCAATGCTAGCCAACA-3′
EFG1-Rev	5′-GCAGCAGTAGTAGTAGCAGC-3′
GAP1-For	5′-TTAAGTACTGGTGGACCAGC-3′
GAP1- Rev	5′-CAAACCCACTTTGAGCAAC-3′
HWP1-For	5′-GTGACAATCCTCTCAACCT-3′
HWP1-Rev	5′-GAGAGGTTTCACCGGCAGGA-3′
ALS3-For	5′-CCACTTCACAATCCCAT-3′
ALS3-Rev	5′-CAGCAGTAGTAGTAACAGTAGTAGTTTCATC-3′
GPD1-For	5′-AGTATGTGGAGCTTTACTGGGA-3′
GPD1-Rev	5′-CAGAAACACCAGCAACATCTTC-3′

Semi-quantitative RT-PCR

C. albicans strain SC5314 was cultured in YPD medium at 28°C on rotary shakers for 24 h, harvested by centrifugation, washed, resuspended in distilled water, and incubated at 4°C for 48 h before use. GS medium containing compound **1** (25, 50, and 100 μg/mL) was added to *C. albicans* cells, and each sample was incubated at 37°C for 2 h. Total RNA was isolated from the incubated cells and purified using an RNeasy Mini Kit (Qiagen, San Diego, CA, USA). cDNA synthesis was performed using a Superscript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) for RT-PCR, and oligo $[dT]_{20}$ (50 mM) was used to prime the cDNA synthesis reaction. Semi-quantitative RT-PCR was conducted with gene-specific primers for major components of the signaling pathways (Table 1.1). Signaling pathway components are coded as follows: *CPH1* (transcription factor), MAPK pathway; *EFG1* (transcription factor), cAMP-protein kinase A complex (PKA) pathway; *GAP1*, which encodes a general amino acid permease and is positively regulated by Cph1; *HWP1* and *ALS3*, which encode hyphal wall protein 1 and agglutinin-like protein 3, respectively, and are activated by Efg1. The housekeeping gene *GPD1* (encoding glyceraldehyde-3-phosphate dehydrogenase) was used as a loading control (Davis-Hanna et al. 2008). PCR was performed according to the manufacturer's instructions: initial denaturation at 98°C for 5 min, followed by 30 cycles of denaturation at 98°C for 10 sec, annealing at 55°C for 30 sec, and extension at 72°C for 1 min, with a final extension at 72°C for 5 min. ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used for densitometric analyses of the mRNA expression levels.

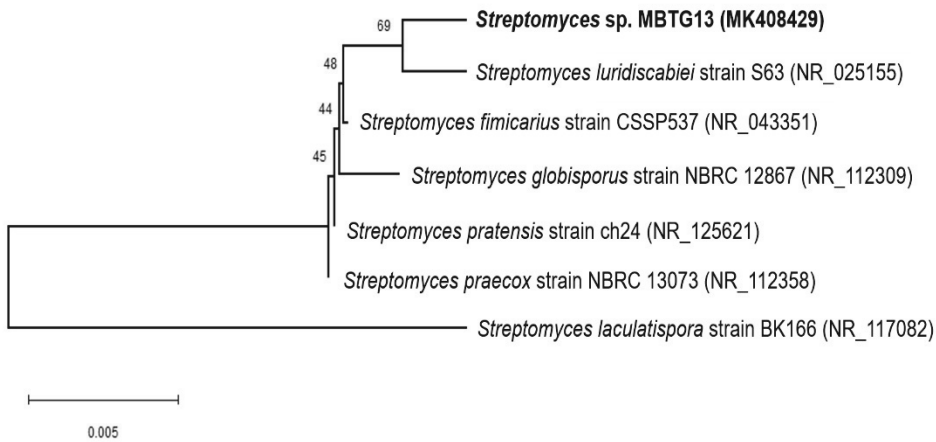
Results

Identification of MBTG13 by phylogenetic analysis

The complete sequence of the 16S rDNA of MBTG13 were determined by polymerase chain reaction (PCR). This sequence was aligned and compared in basic logic alignment search tool (BLAST). As a result of sequence comparison, this sequence was matched to *Streptomyces* species with high similarity. Among them, *Streptomyces luridiscabiei* (GenBank accession number: NR_025155) showed the highest similarity (sequence identity 99%). Thus, this strain was named as *Streptomyce*s sp. MBTG13 (GenBank accession number: MK408429). The phylogenetic tree constructed by neighbor-joining and maximum likelihood methods based on the 16S rDNA sequence data showed the evolutionary relationships of strain MBTG13 with a group of known *Streptoymyces* species (Fig 1.4).

Figure 1.4. Neighbor-joining phylogenetic tree generated by 16S rDNA sequence analysis

The phylogenetic tree was constructed using the neighbor-joining program of MEGA X software. Bootstrap was carried out with 1,000 replicates. The Kimura two-parameter model was used for measuring distance. Bar indicates 0.5% sequence divergence.


69
48
44
45
Streptomyces sp. MBTG13 (MK408429)
Streptomyces luridiscabiei strain S63 (NR_025155)
Streptomyces fimicarius strain CSSP537 (NR_043351)
Streptomyces globisporus strain NBRC 12867 (NR_112309)
Streptomyces pratensis strain ch24 (NR_125621)
Streptomyces praecox strain NBRC 13073 (NR_112358)
Streptomyces laculatispora strain BK166 (NR_117082)
0.005

Isolation and structure determination of compounds 1-4

A total of 510 g of rice fermentation culture was repeatedly extracted by MeOH (1 L × 3) and dichloromethane (1 L × 3). The organic solvents were evaporated to dryness under reduced pressure to gain 8.5 g of total extracts. A total extract was successively partitioned between n-butanol (1.6 g) and H_2O (6.2 g). The n-butanol fraction was repartitioned using H_2O-MeOH (15:85) (1.1 g) and n-hexane. The H_2O-MeOH fraction was subjected to C_{18} reverse-phase vacuum flash chromatography using a gradient of MeOH and H_2O to give seven fractions (five fractions in the gradient, H_2O-MeOH, from 0:100 to 100:0, acetone, and finally EtOAc). On the basis of the results of biological analyses, the fraction eluted with H_2O-MeOH (20:80) (1.1 g) was separated and purified by semi-preparative reverse-phase HPLC (Agilient C_{18} column, 10.0 × 250 mm; H_2O-MeOH, 30:70 with 0.1% trifluoroacetic acid; 2.0 mL/min) to obtain compounds **1** (t_R: 16 min, 24.2 mg), **2** (t_R: 24 min, 3.5 mg), **3** (t_R: 26 min, 2.5 mg), and **4** (t_R: 31 min, 2.7 mg) (Fig. 1.5). By combined spectroscopic analyses, including ^1H, ^{13}C NMR, two-dimensional (2D) NMR spectral analyses (COSY, HSQC, and HMBC), and UV data, compounds **1**–**4** were identified as 2-*n*-heptyl-4-hydroxyquinoline (**1**) (Hays et al., 1945; Kitamura et al., 1986), 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide (**2**) (Hays et al., 1945), 2-*n*-octyl-4-hydroxyquinoline (**3**) (Supong et al., 2016), and 3-*n*-heptyl-3-hydroxy-1,2,3,4-tetrahydroquinoline-2,4-dione (**4**) (Kitamura et al., 1986) (Fig. 1.6). The spectroscopic data for these compounds were in good agreement with previous reports.

Figure 1.5. Flow chart of compounds 1-4 isolation

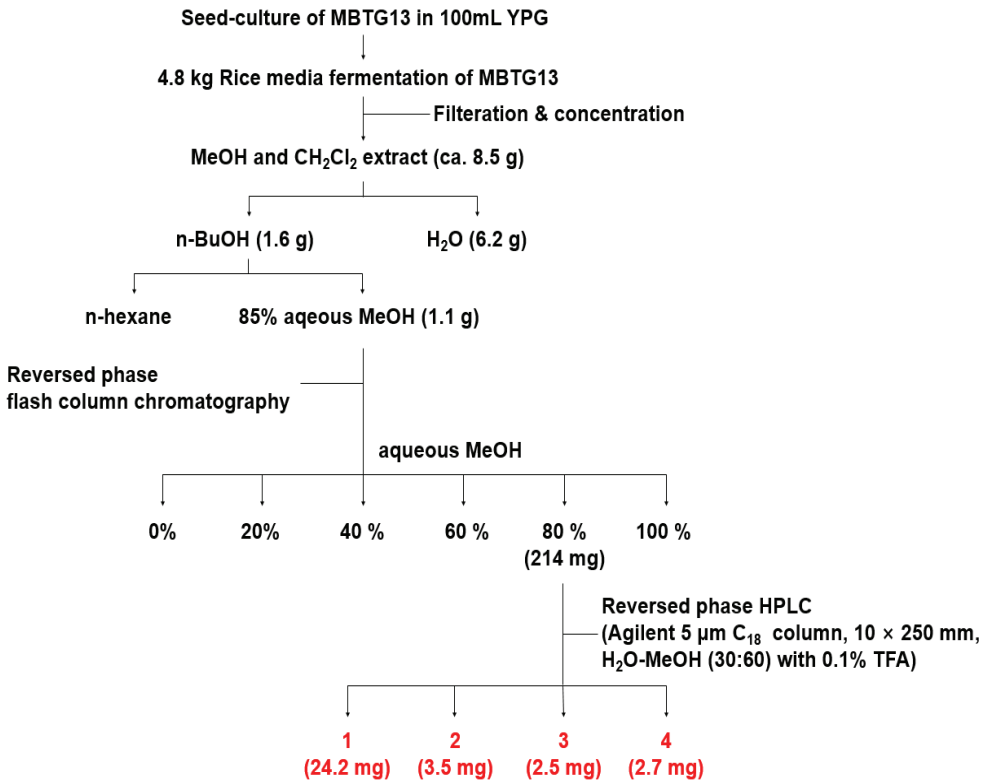
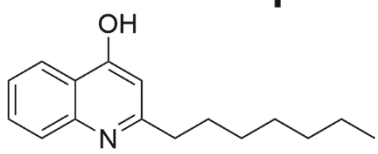
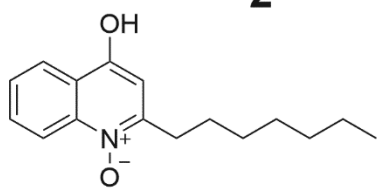

Seed-culture of MBTG13 in 100mL YPG
4.8 kg Rice media fermentation of MBTG13
Filteration & concentration
MeOH and CH_2Cl_2 extract (ca. 8.5 g)
n-BuOH (1.6 g)
H_2O (6.2 g)
n-hexane
85% aqeous MeOH (1.1 g)
Reversed phase
flash column chromatography
aqueous MeOH
0%
20%
40 %
60 %
80 %
(214 mg)
100 %
Reversed phase HPLC
(Agilent 5 µm C_{18} column, 10 × 250 mm,
H_2O-MeOH (30:60) with 0.1% TFA)
1
(24.2 mg)
2
(3.5 mg)
3
(2.5 mg)
4
(2.7 mg)

Figure 1.6. Structures of compounds 1-4 isolated from *Streptomyces* sp. MBTG13

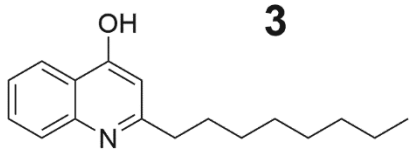
1



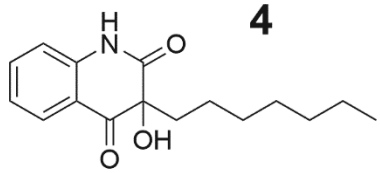
2



3



4



Effects of compounds 1-4 on pathogenic microorganism growth

Because compound **1** was initially reported as an antibacterial compound (Hays et al., 1945), the antimicrobial activities of the isolated compounds were first evaluated against phylogenetically diverse pathogenic bacterial strains, including *S. aureus* ATCC25923, *E. faecalis* ATCC19433, *E. faecium* ATCC19434, *S. enterica* ATCC14028, *K. pneumoniae* ATCC10031, and *E. coli* ATCC25922, using ampicillin and tetracycline as positive control compounds. Compound **1** displayed weak antibacterial activity against *S. aureus* ATCC 25923, *E. faecalis* ATCC19433, and *E. coli* ATCC25922, with minimum inhibitory concentration (MIC) values of 128, 128, and 64 μg/mL, respectively. Compound **2** broadly inhibited most of the tested bacterial pathogens, except *K. pneumoniae* and *E. coli*, with MIC values in the range of 16–32 μg/mL (Table 1.2). The antifungal activities of compounds **1**–**4** were also evaluated against pathogenic fungal strains, including *C. albicans* SC5314, *A. fumigatus* HIC6094, *T. rubrum* NBRC9185, and *T. mentagrophytes* IFM40996, using amphotericin B as a positive control compound. However, compounds **1**–**4** did not exhibit inhibitory activity against the tested fungi (MIC > 128 μg/mL) (Table 1.3).

Table 1.2. Antibacterial activity assay

Compound	Minimum Inhibitory Concentration (MIC) (μg/mL)					
	Gram(+) Bacteria			Gram(-) Bacteria		
	A	B	C	D	E	F
1	>128	128	>128	>128	64	>128
2	16	32	32	>128	>128	>128
3	>128	>128	>128	>128	>128	>128
4	128	>128	>128	>128	>128	>128
Ampicillin	0.07	0.13	0.13	0.13	>128	16
Tetracycline					0.5	

A: *Staphylococcus aureus* ATCC25923, B: *Enterococcus faecalis* ATCC19433, C: *Enterococcus faecium* ATCC19434, D: *Salmonella enterica* ATCC14028, E: *Klebsiella pneumoniae* ATCC10031, F: *Escherichia coli* ATCC25922. Ampicillin and tetracycline were used as control compounds.

Table 1.3. Antifungal activity assay

Compound	Minimum Inhibitory Concentration (MIC) (μg/mL)			
	A	**B**	**C**	**D**
1	>128	>128	>128	>128
2	>128	>128	>128	>128
3	>128	>128	>128	>128
4	>128	>128	>128	>128
Amphotericin B	0.5	1	1	1

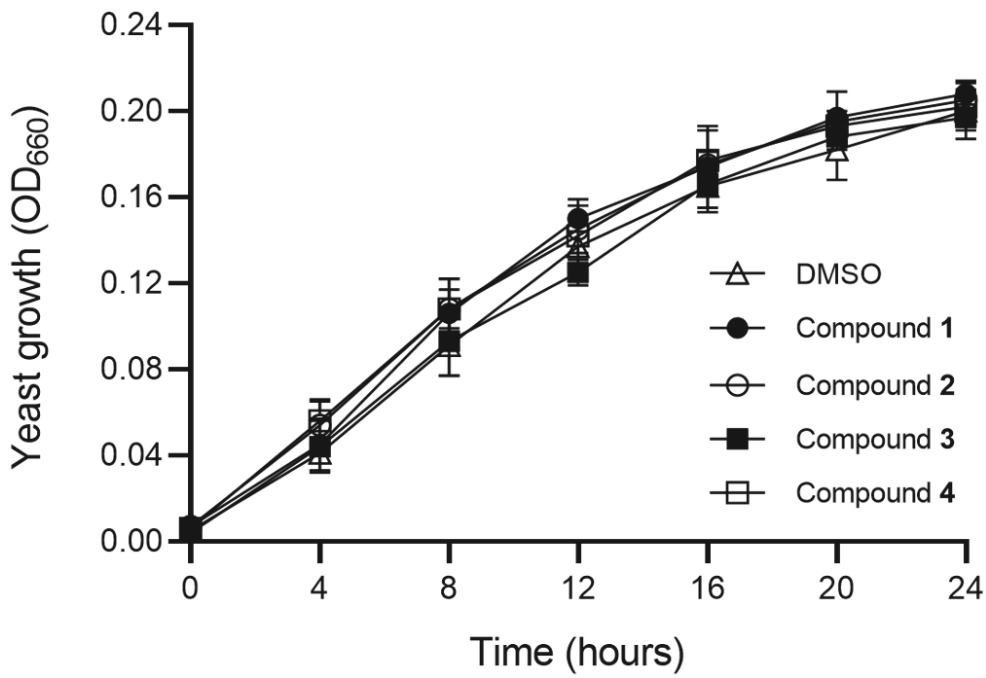
A: *Candida albicans* SC5314, B: *Aspergillus fumigatus* HIC6094, C: *Trichophyton rubrum* NBRC9185, D: *Trichophyton mentagrophytes* IFM40996. Amphotericin B was used as positive control.

Effects of compounds 1-4 on *C. albicans* proliferation

To evaluate the effects of compounds **1**-**4** on *C. albicans* yeast growth, the cells were grown in glucose salt (GS) medium supplemented with 100 μg/mL of test compound at 28°C, and the optical density at 660 nm (OD_{660}) of each sample was measured at each specific time interval. Tested cell were normally grown independent upon compounds **1**–**4**. These results shown that compounds **1**–**4** at 100 μg/mL did not inhibit yeast cell growth in *C. albicans* (Fig. 1.6).

Figure 1.7. Effects of compounds 1-4 on *C. albicans* SC5314 growth

Effects of compounds **1**–**4** (each 100 μg/mL) on yeast cell growth in *C. albicans*. Glucose salt (GS) medium with 1% dimethyl sulfoxide (DMSO) was used as control. The number of cells at each specific time point at 28°C was assessed by measuring the optical density absorption at 660 nm (OD_{660}).

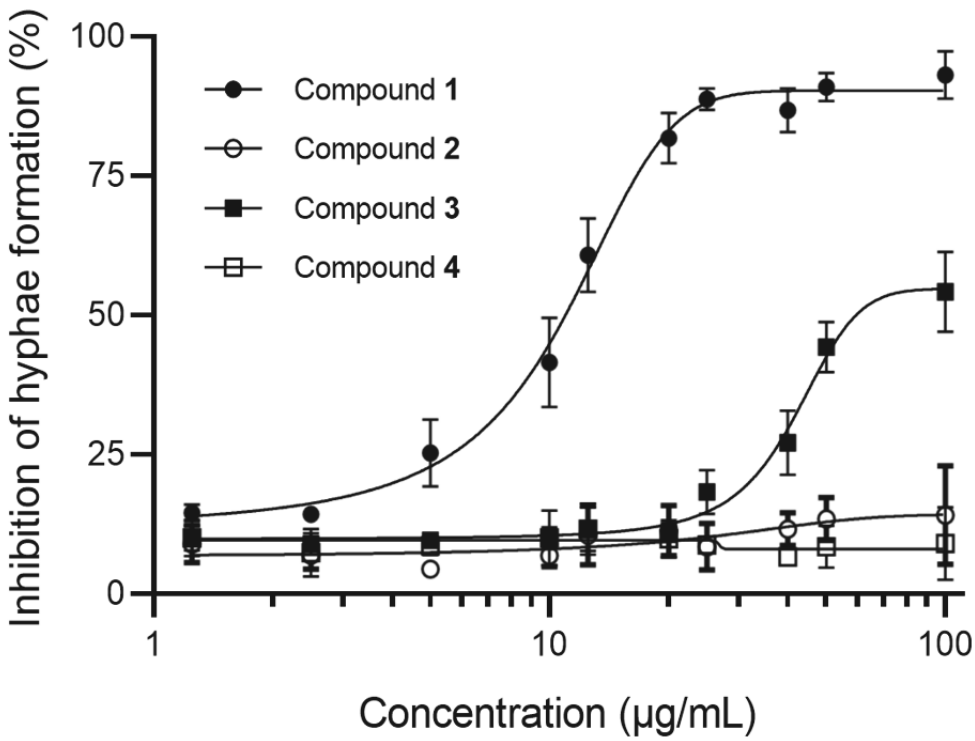

0.24
0.20
0.16
0.12
0.08
0.04
0.00
Yeast growth (OD660)
0
4
8
12
16
20
24
Time (hours)
DMSO
Compound 1
Compound 2
Compound 3
Compound 4

Inhibitory activity of yeast-to-hyphae transition in *C. albicans*

To evaluate the effects of compounds **1**–**4** on the hyphal growth of *C. albicans*, approximately 5×10^6 cells/mL were added to GS medium supplemented with the test compounds at different concentrations and incubated at 37°C. At each time point, the morphology of approximately 200 cells was determined by light microscopy (Fig. 1.8). Under these conditions, >90% of *C. albicans* cells are converted to the hyphal form after 4 h of incubation. Cultures treated with compounds **1**–**4** exhibited concentration-dependent inhibition of the hyphal form of *C. albicans*. Among these compounds, compound **1** exhibited inhibitory activity, with an IC_{50} of 11.4 μg/mL. Oxidation of 4-hydroxyquinoline moiety (**2**) led to a loss of inhibitory activity compared to compound **1** (Fig. 1.6). With regard to the effect of alkyl chain length, for a given compound **1**, extension of the chain by one methyl unit (**3**) resulted in a dramatic decrease in inhibitory effect upon hyphal formation at the highest concentration tested (50% at 100 μg/mL).

Figure 1.8. Effects of compounds 1-4 on yeast-to-hyphae transition of SC5314

Effects of compounds **1**–**4** on hyphal formation in *C. albicans*. Cells (5×10^6 cells/mL) were grown in GS medium containing different concentrations of test compound at 37°C. At least 200 cells were counted for each sample after 4 h of cultivation. Data are presented as the mean fold changes ± SD of three independent experiments.


Inhibition of hyphae formation (%)
100
75
50
25
0
1
10
100
Concentration (µg/mL)
Compound 1
Compound 2
Compound 3
Compound 4

Effects of compound 1 on gene expression related to hypha-inducing signaling pathways

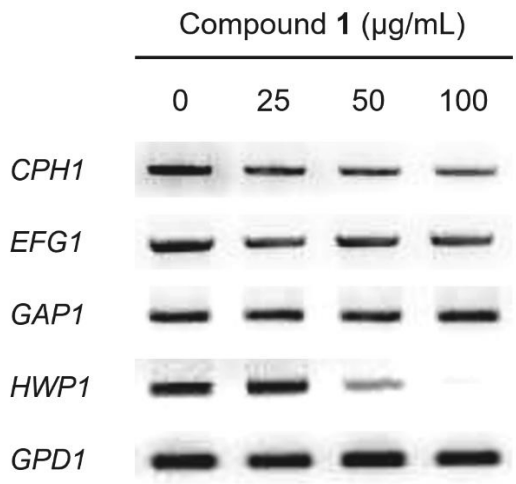
In *C. albicans*, hyphal development is mainly regulated by the mitogen-activated protein kinase (MAPK) and cyclic AMP-protein kinase A (cAMP-PKA) pathways, and active form of Ras1 is required for the regulation of both pathways (Sudbery, 2011). The transcription factors Efg1 and Cph1 are activated by distinct upstream signaling pathways. In the case of Efg1, the pathway is based on cAMP, while in the case of Cph1 the pathway depends on a MAPK signaling pathway, with Ras1 stimulating both pathways. To investigate the effect of compound **1** on hypha-inducing signaling pathways in *C. albicans*, the mRNA expression levels of *CPH1*, *EFG1*, *GAP1*, and *HWP1* were examined. Semi-quantitative reverse transcription (RT)-PCR showed that the mRNA expression of *CPH1* and *EFG1* was constant independent on treatment of compound **1** in cells (Fig. 1.9a). The transcript level of *GAP1*, which encodes a general amino acid permease and is positively regulated by the transcription factor Cph1 (Biswas et al., 2007) was also unchanged. Importantly, a complete loss of *HWP1* mRNA expression occurred with 100 μg/mL of compound 1. *HWP1*, which encodes a glycosylphosphatidylinositol-anchored cell wall protein (Staab et al., 1999), is a downstream component of the cAMP-dependent PKA pathway and is positively regulated by the transcription factor Efg1, which is an important regulator of cell wall dynamics (Sharkey et al., 1999).

Fig. 1.8b shows the relationship between *HWP1* transcript levels and hypha formation in *C. albicans* were grown in GS medium treated with increasing concentrations of compound **1**. The level of *HWP1* transcript was undetectable in yeast cells grown in GS medium at 28°C for 2 h. In contrast, under hyphal growth-inducing conditions, the *HWP1* transcript level was increased with a decrease in concentration of compound **1**, mainly due to the predominant growth of the hyphal population. (Fig. 1.9b) These results suggest that compound 1 affects to the cAMP-Efg1 pathway, but not the MAPK-Cph1 pathway.

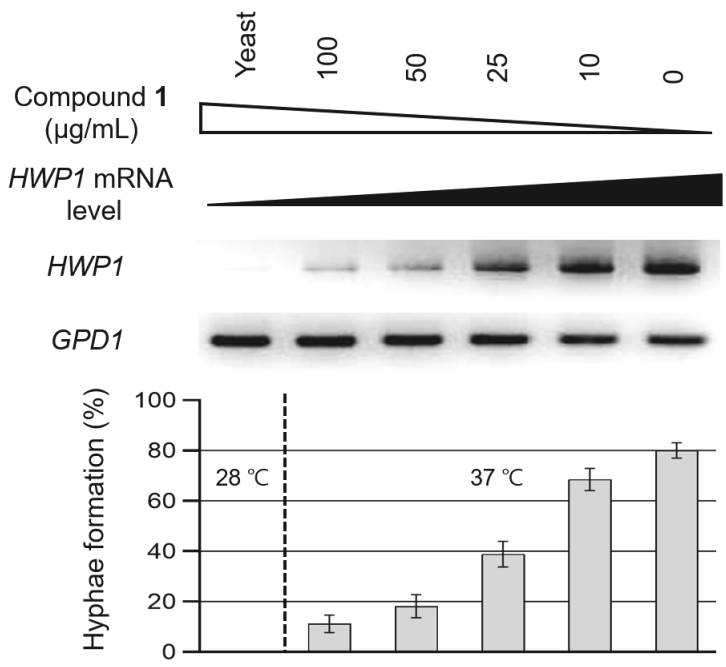
Figure 1.9. Semi-quantitative RT-PCR analysis of mRNAs related to the hyphal-inducing signaling pathway in *C. albicans* SC5314 cells

(a) Relative expression of mRNAs related to the hyphal-inducing signaling pathway in *C. albicans*. Cells were incubated in the absence or presence of compound **1** at 37 ℃ for 2 h, followed by RNA isolation and cDNA synthesis. (b) Relationship between *HWP1* transcript level and hyphal formation in *C. albicans* cultures grown in GS medium treated with increasing concentrations of compound **1** at 37 ℃ for 2 h. *C. albicans* yeast cells were grown in GS medium without compound **1** at 28 ℃ for 2 h.

(a)


Compound 1 (μg/mL)
0
25
50
100
CPH1
EFG1
GAP1
HWP1
GPD1

(b)

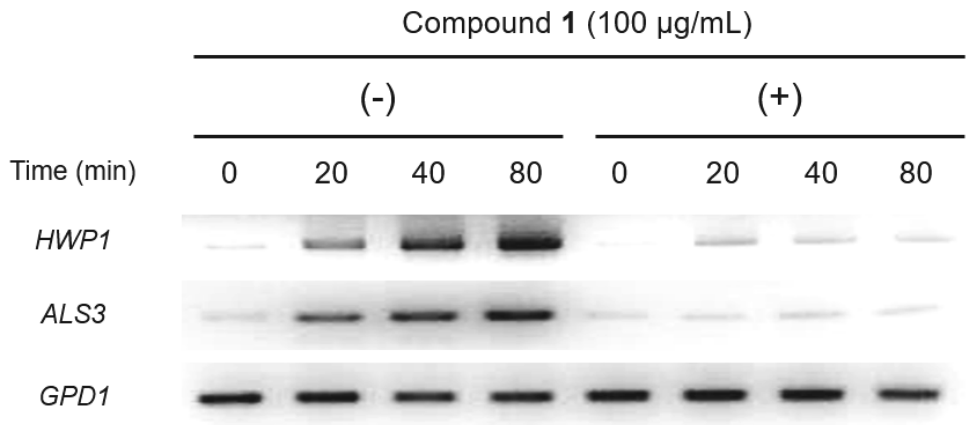

Yeast
100
50
25
10
0
Compound 1 (μg/mL)
HWP1 mRNA level
HWP1
GPD1
Hyphae formation (%)
100
80
60
40
20
0
28 ℃
37 ℃

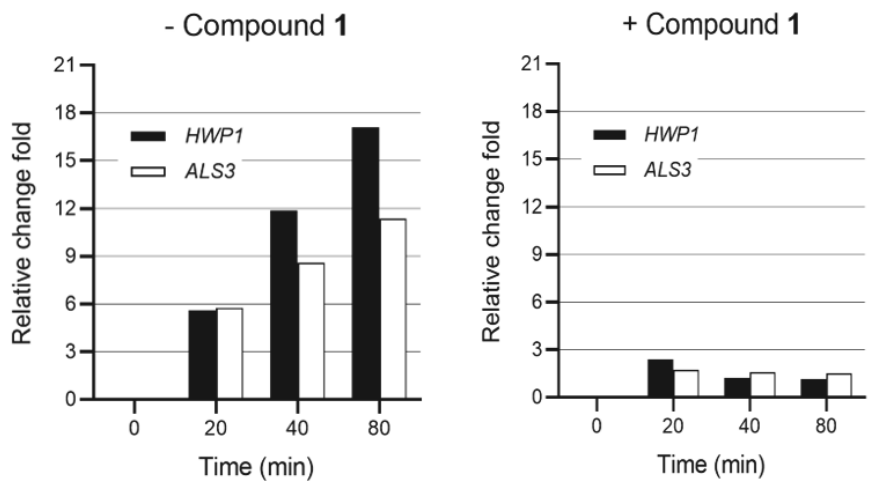
Expression of hyphae-specific *HWP1* and *ALS3* in cAMP-PKA pathway

Gene-specific studies and genome-wide analyses have revealed only a small number of hypha-specific genes in *C. albicans* (Kadosh and Johnson, 2005). These include *ALS3*, *ECE1*, *HGC1*, *HWP1*, *HYR1*, *RBT1*, and *RBT4*. The most highly expressed gene encodes *HWP1*, which is one of a small set of hypha-specific genes in *C. albicans* that include *ALS3* (encoding agglutinin-like protein 3) and *ECE1* (encoding cell elongation protein 1). Among them, *HWP1* and *ALS3* encode adhesins and are activated by the transcription regulator Efg1 during hypha formation (Argimón et al., 2007). Thus, we investigated whether compound **1** also affected *HWP1* and *ALS3* mRNA levels (Fig. 1.10). In the absence of compound **1**, *HWP1* and *ALS3* transcripts were undetectable at time zero, but they were strongly expressed from 20 to 80 min. Importantly, compound **1** (100 μg/mL) reduced the magnitude of *HWP1* and *ALS3* mRNA expression in compound **1**-treated cells, when compared with untreated cells.

Figure 1.10. Kinetic analysis of hypha-specific *HWP1* and *ALS3* mRNA levels

C. albicans cells were incubated in the absence or presence of 100 μg/mL compound **1** at 37 °C in GS medium. Cells were then harvested at 0-, 20-, 40-, and 80-min post-incubation. ImageJ software was used for densitometric analysis of mRNA expression level.


Compound 1 (100 μg/mL)
(-)
(+)
Time (min)
0
20
40
80
0
20
40
80
HWP1
ALS3
GPD1


- Compound 1
+ Compound 1
Relative change fold
HWP1
ALS3
0
3
6
9
12
15
18
21
0
20
40
80
Time (min)

Discussion

A chemical investigation of a semisolid rice culture extract of the marine-derived actinomycete *Streptomyces* sp. MBTG13 led to the isolation of 2-alkyl-4-hydroxyquinoline derivatives (**1**–**4**). Their structures were determined based on measured spectroscopic data and were consistent with previous reports. Compounds **1** and **2** exhibited weak to moderate antibacterial activity against pathogenic bacteria with MIC value of 128 and 16–32 μg/mL. Compound **1** was earlier isolated from *Pseudomonas aeruginosa* and had antibacterial activity (Hays et al., 1945). In addition, compounds **1**, **2**, and **4** were isolated from *Pseudomonas methanica* KY4634 as 5-lipoxygenase inhibitors (Kitamura et al., 1986). Recently, it was reported that compounds **1**–**3** exhibited antimalarial activity against *Plasmodium falciparum* (Supong et al., 2016). In first part of this study, it was identified that compounds **1** and **3** reduced the pathogenicity of *C. albicans* by inhibiting hyphal growth, without inhibiting yeast cell growth. Interestingly, structures of these compounds were similar to quorum sensing signal molecules, such as farnesoic acid and farnesol (Hornby et al., 2001; Oh et al., 2001). Therefore, these compounds were analyzed to confirm that they are inhibitors of hyphal growth induction in *C. albicans*. These molecules are secreted into the medium as the cells proliferate and are involved in morphogenesis. Therefore, it was confirmed that these compounds function on inhibition of the morphological transition in *C. albicans* under hypha-inducing conditions. Based on the results of growth curves and hypha inhibition analyses, 2-alkyl-4-hydroxyquinoline derivatives regulate the hyphal formation process of *C. albicans* without interfering with its yeast form proliferation. The inhibitory potency and selectivity of these compounds are sensitively dependent upon the chain length and to substitutions on the 4-hydroxyquinoline template. Compound **3** and **4** have unstable structures compared to compound **1** and **2** because these compounds have charged residues at oxygen in 4-hydroxyquinoline template. This may have affected the inhibitory

activity of hyphal growth.

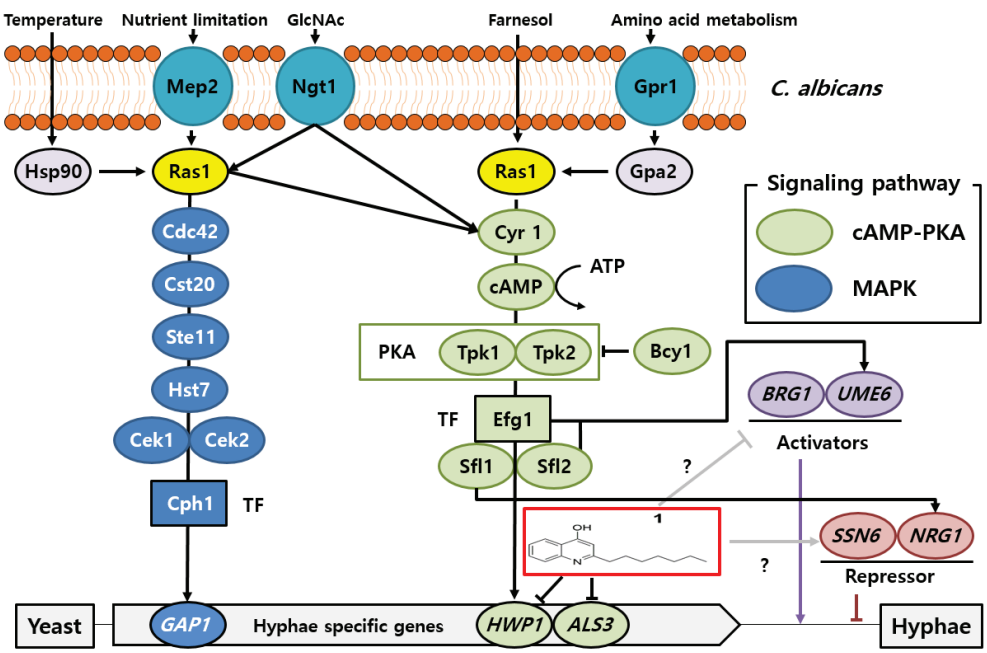
In *C. albicans*, the positive regulation of hypha-specific gene expression is mainly mediated through the MAPK and cAMP-PKA signaling pathways (Sudbery, 2011; Noble et al., 2017). Transcription factors Efg1 and Cph1 are activated by distinct upstream signaling pathways. In the case of Efg1, the pathway is based on cAMP, while in the case of Cph1 the pathway depends on MAPK signaling. To investigate the networks by which the morphogenesis of *C. albicans* is controlled by compound **1**, the expression levels of these major hypha-inducing signaling pathway components in compound **1**-treated cells with untreated control cells were compared. When cells were exposed to compound **1**, the mRNA expression of *HWP1* and *ALS3*, hypha-specific genes that are positively regulated by an important regulator of cell wall dynamics (Efg1), were significantly inhibited by the addition of compound **1**. However, the transcription of *CPH1*, *EFG1*, and *GAP1* mRNA was unchanged.

Efg1 and Cph1 are transcription factors whose activity is regulated on a post-transcriptional level. Threonine-206, a phosphorylation site for protein kinase A (PKA) within an Efg1p domain, is essential to promote hyphal induction by environmental factors (Bockmühl et al., 2001). Previous studies have examined the role of Efg1 and Cph1 in the repression of hyphal growth by farnesol, a related compound. It has been found that even though these proteins are clearly involved in mediating the farnesol effect, there is no significant change in *EFG1* or *CPH1* mRNA (Kebaara et al., 2008; Langford et al., 2013). In my results, *EFG1* mRNA levels are regulated during hyphal development, but they were not affected by compound **1**, since the magnitude of the changes were similar in the presence and absence of compound **1** (Fig. 1.9). These results are consistent with the previous study (Kebaara et al., 2008). Together with these results, compound **1** does not regulate *EFG1* mRNA levels, but at this time, the possibility that post-translational regulation of Efg1 is affected by compound **1** was not concluded (Fig. 1.11). First

part of this study is the first report demonstrating inhibitory activity of *C. albicans* filamentation by compounds **1**–**4**, and suggests that the 2-alkyl-4-hydroxyquinoline class of compounds may be valuable as antifungal agents to suppress virulence in *C. albicans*.

Figure 1.11. Proposed model underlying the mechanism by which compound 1 inhibits hyphal growth in *C. albicans*

HWP1 and *ALS3* mRNA which are regulated by transcription factor Efg1-mediated cAMP-PKA pathway are inhibited by compound **1**. Not only MAPK pathway but also upstream of Efg1-mediated cAMP-PKA pathway are not affected by compound **1**. TF means transcription factor.


Temperature
Nutrient limitation
GlcNAc
Farnesol
Amino acid metabolism
Mep2
Ngt1
Gpr1
C. albicans
Hsp90
Ras1
Ras1
Gpa2
Signaling pathway
cAMP-PKA
MAPK
Cdc42
Cyr 1
ATP
Cst20
cAMP
Ste11
PKA
Tpk1
Tpk2
Bcy1
Hst7
BRG1
UME6
Activators
TF
Efg1
Cek1
Cek2
Sfl1
Sfl2
?
Cph1
TF
SSN6
NRG1
?
Repressor
Yeast
GAP1
Hyphae specific genes
HWP1
ALS3
Hyphae

Part II

Diketopiperazines from Marine-Derived *Streptomyces puniceus* Inhibit Isocitrate Lyase of *Candida albicans*

Introduction

Importance of isocitrate lyase as antifungal target

The glyoxylate cycle, present in archaea, bacteria, fungi, plants and nematodes, functions on cell metabolic process as anaplerotic pathway for the tricarboxylic acid (TCA) cycle (Vanni et al., 1990). This cycle consists of citrate synthase, aconitase which are initial steps of TCA cycle, followed isocitrate lyase (ICL), malate synthase (MLS) and malate dehydrogenease (Fig. 2.1). In glyoxylate cycle, ICL cleaves isocitrate to glyoxylate and succinate, and then MLS convert acetyl-CoA and cleaved glyoxylate to malate. These enzymes are the key components of the glyoxylate cycle which is able to bypass the CO_2-generating steps of the TCA cycle and supply the TCA cycle intermediates for gluconeogenesis or other biosynthetic process by assimilating carbon from C_2 compounds which is non-fermentable carbon source, such as acetate, ethanol and fatty acids (Dunn et al., 2009).

The role of glyoxylate cycle is demonstrated by analyzing *icl*-deletion mutants. In *Magnaporthe grisea* which is the rice blast pathogen, strongly expressed *ICL* gene was detected during infection. When the *icl*-deletion mutant of *M. grisea* exposed to rice, rice blast symptoms such as appressorium formation, conidiogenesis and cuticle penetration were reduced (Wang et al., 2003). *Mycobacterium tuberculosis* is one of the most pathogenic actinobacterium in humans and kills more than three million people annually via tuberculosis. During intracellular infection by *M. tuberculosis*, its express ICL protein (Sturgill-Koszycki et al., 1997). When *M. tuberculosis* infected the lungs of mice, *icl*-mutant of *M. tuberculosis* had no effect on their growth for 2 weeks. After 16 weeks, *icl*-mutant of *M. tuberculosis* reveal a reduced ability to sustain the infection (McKinney et al., 2000). Also, *icl* or *aceA* deletion mutant had no effect on bacterial growth in C_2-carbon-utilizing conditions, while an *icl aceA* double-

deletion mutant did not grow on same condition. When *icl aceA* double-deletion mutant inoculated into mice, this mutant did not infect the lungs and spleen (Muñoz-Elías and McKinney, 2005). Therefore, ICL activity is essential for *M. tuberculosis* survival of the host such as mice.

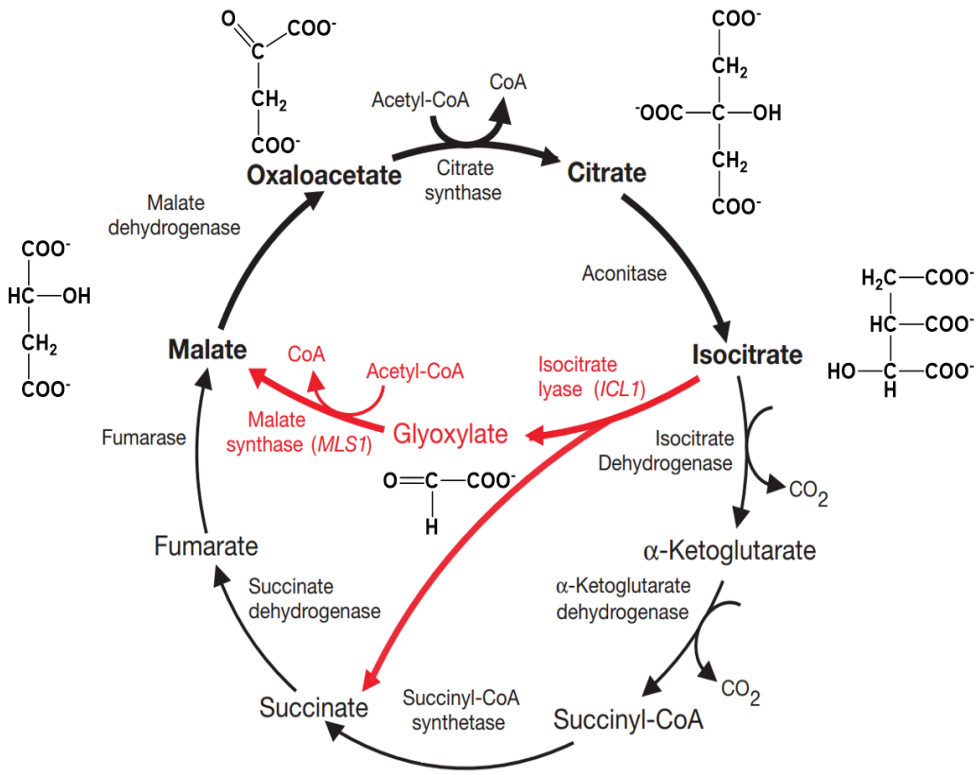
In immunocompromised patients, *C. albicans* is responsible for life-threatening systemic infection and human muscosal infection by surviving in macrophage. All steps of the glyoxylate cycle in *C. albicans* which grow in macrophage were induced (Lorenz et al., 2004). ICL and MLS were strongly induced in *C. albicans* during phagocytosis (Lorenz and Fink, 2001). In *C. albicans* obtained from patients suffering from candidiasis, ICL and MLS enzymes had high activity (Lattif et al., 2006). Furthermore, several reports provide evidences that *ICL* and *MLS*-deficient mutant of pathogenic microorganisms were significantly less virulent (McKinney et al., 2000; Muñoz-Elías and McKinney, 2005; Dunn et al., 2009). This cycle is involved in microbial pathogenesis by allowing the growth of pathogenic microorganisms on C_2 substrates. Also, *C. albicans* utilizes the glyoxylate cycle for pathogenesis in response to glucose-deficient environment. Regardingly, several reports demonstrated an important role of ICL in fungal virulence (Lorenz and Fink, 2001; Ramirez and Lorenz, 2007). In mice, an *icl*-deletion of *C. albicans* was unable to to utilize C_2 carbon substrates and had diminished virulence compared with wild-type strain. As this cycle does not exist in mammalian cells, ICL appears to be a prospective target for the development of antifungal drugs (Lorenz and Fink, 2001).

Up to date, several potent ICL inhibitor such as itaconate, bromopyruvate, nitropropionate, and malate have been identified (McFadden and Purohit (1977); Greene et al., 1998; Ko et al., 2004). Because these inhibitors detected toxicity and no specific effect, it is unsuitable for pharmacological utilization. For example, itaconate causes hypertonicity of blood pressure in cats and inhibits the growth or rats (Finkelstein et al., 1947; Booth et al., 1952). Nitropropionate shows

neurotoxicity and non-specific effects which inhibited ICL but also succinate dehydrogenase, a crucial enzyme of TCA cycle (Alston et al., 1977). Bromopyruvate inhibits glycolysis which is major metabolic process (Shoshan, 2012). For identification of more effective ICL inhibitors, numerous studies have been conducted (Ansari et al., 2014; Cheah et al., 2018; Shukla et al., 2018).

Figure 2.1. Schematic model of the glyoxylate cycle and TCA cycle.

Glyoxylate-specific process and enzymes are indicated in red (Lorenz and Fink, 2001).


Oxaloacetate
Acetyl-CoA
CoA
Citrate synthase
Citrate
Aconitase
Isocitrate
Isocitrate Dehydrogenase
CO2
α-Ketoglutarate
α-Ketoglutarate dehydrogenase
CO2
Succinyl-CoA
Succinyl-CoA synthetase
Succinate
Succinate dehydrogenase
Fumarate
Fumarase
Malate
Malate dehydrogenase
Malate synthase (MLS1)
CoA
Acetyl-CoA
Glyoxylate
Isocitrate lyase (ICL1)

Biological activities of diketopiperazines

Diketopiperazines are small cyclic dipeptides, consist of two amino acids with or without additional modification. As a result of 2 *cis*-amid bonds between two rings, 2 H-bond acceptor and 2 H-bond donor sites is generated, which is important for binding to enzymes and receptors. The *cis*-amide functional group of the diketopiperazine ring forms an intermolecular hydrogen bonds (N−H...O) between adjacent molecules, so that diketopiperazines can be formed and regulated higher-ordered super molecular structures that are important in crystal engineering and as liquid gelators (Palacin et al., 1997; McBride, 1998; Xie et al., 2009). Diketopiperazines are semi-rigid molecules with conformationally constrained heterocycle. Nevertheless, this compounds are flexible because the six-membered ring can exist in an essentially flat conformation or a slightly puckered boat form (Borthwick, 2012). Six positions to add substituents and four positions to control their stereochemistry increase the structural diversity of diketopiperazines. This compounds are produced by a variety of organisms, such as bacteria (*Bacillus* sp. and *Streptomyces* sp.), fungi (*Alternaria alternate* and *Penicillium* sp.) and marine sponges (e.g., *Dysidea fragilis*) (Huang et al., 2010). This class compounds have been focused on the development of new drugs because it shows a common scaffold which easily modify to adapt effective pharmacological characteristics, and relatively easy acquirement via conventional procedures (Martins and Carvalho, 2007).

Diketopiperazines possess a number of diverse biological properties, such as antimicrobial activity, antitumor activity antiviral activity, inhibition of quorum-sensing signaling, plant-growth promotion, and inhibition of aflatoxin production. For instance, cyclo(indole-*N*-isoprenyl-Trp-Val), isolated from marine-derived Ascomycota, inhibited the growth of *Pyricularia oryzae*, a pathogen of monocot plants (Byun et al., 2003). Cyclo(L-His-L-Phe) showed antitumor activity by causing significantly reduction of the viability of HeLa and MCF-7 (McCleland et

al., 2004). Cyclo(L-Pro-L-Tyr) and cyclo (L-Phe-L-Val) regulate the LuxR-based quorum-sensing system by interacting to LuxR-binding site (Holden et al., 1999). The C-C chemokine receptor type 5 (CCR5) is a target protein as antiviral agents because human immunodeficiency virus type 1 (HIV-1) used this protein as receptor to enter target immunological cells (Murphy, 2001). Aplaviroc is a CCR5 inhibitor that belongs to a class of diketopiperazines as a drug of HIV infection (Nakata et al., 2005). Tryptostatins A is a diketopiperazine, produced by the marine fungal strain of *Aspergillus fumigatus*. Tryptostatins A have attracted attention as potential anticancer drugs because this compound is an inhibitor of BCRP/ABCG2 which is the breast cancer resistance protein (Woehlecke et al., 2003)

Material and methods

General experimental procedures

^{1}H, ^{13}C, and 2D NMR (COSY, HMBC, and HSQC) spectra were measured using a Bruker Avance 600 MHz spectrometer at the National Center for Interuniversity Research Facilities (NCIRF) located in Seoul National University. Mass spectrometric data were obtained at the Korea Basic Science Institute (Daegu, Republic of Korea) and were acquired using a JMS 700 mass spectrometer (JEOL, Tokyo, Japan) using meta-nitrobenzyl alcohol as a matrix for fast atom bombardment mass spectrometry. HPLC analysis was carried out by Shimadzu SCL-10A control system connected with a RID-10A refractive index detector (Shimadzu, Japan) and UV-Vis SPD-10A detector (Shimadzu, Japan). All organic solvents were analytical reagent grade and purchased from Fisher Scientific (FairLawn, NJ, USA).

Bacterial and fungal strains

S. puniceus Act1085, isolated from seafloor sediment from the shoreline of Jeju Island, Republic of Korea, was used as a diketopiperazine producing strain (Park et al., 2017). *C. albicans* SC5314 (ATCC MYA-2876) (wild-type), MRC10 (Δ*icl*), MRC11 (Δ*icl* + ICL), ATCC10261, ATCC18804, and ATCC11006 were used for growth assay. *C. albicans* ATCC10231 was the source or *ICL* gene. The *icl* mutant strains were provided by Prof. Michael C. Lorenz (The University of Texas Health Science Center at Houston, USA) (Ramirez MA and Lorenz MC, 2007). These fungal strains were cultured in yeast nitrogen base (YNB) medium (Difco Laboratories, Detroit, MI, USA) containing 2% glucose at 28°C shaking incubator. Bacterial and fungal strains were maintained as frozen cell glycerol stocks prior to use.

Fermentation of the producing bacteria

Frozen stock of *S. puniceus* Act1085 was pre-cultured on a GTYB agar plate (2 g of tryptone, 1 g of yeast extract, 1 g of beef extract, 10 g of glucose, and 16 g of agar in 1 L of artificial seawater) for 5 days at 28°C. The bacteria produced brown color colonies with white spores. Spores of Act1085 were harvested according to the methods described reference and put into 2 L flasks containing 300 mL MTYB medium (2 g of tryptone, 1 g of yeast extract, 1 g of beef extract, 10 g of mannitol in 1 L distilled water) after autoclave sterilization (121°C for 15 min). These cultures were incubated at 28°C under static conditions.

Preparation of recombinant isocitrate lyase of *C. albicans*

Expression and purification of recombinant ICL protein from the genomic DNA of *C. albicans* ATCC10231 was carried out using a method described previously (Shin, 2005). Briefly, the gene for ICL was amplified by PCR using two synthetic primers: 5′-AGAATTCCTACCATGCCTTACACTCC-3′ (forward) and 5′-CTTCGTCGA CTCAAAATTAAGCCTTG-3′ (reverse). The PCR product was cloned into the pBAD/Thio-TOPO vector (Invitrogen, Carlsbad, CA, USA) and transformed into *Escherichia coli* TOP10 (Invitrogen). After induction of protein expression with 0.02% arabinose at 25°C for 8 h, cells were lysed by lysozyme treatment and sonication, and the recombinant protein was purified using a Ni-NTA affinity column (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Protein concentration was determined by the method of Bradford using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA) and bovine serum albumin as a standard (Bradford, 1976).

ICL inhibitory activity assay

The ICL inhibitory activity of test compounds was evaluated according to a procedure documented previously (Dixon and Kornberg, 1959; Lee et al., 2014).

The principle of ICL inhibition assay is the formation of glyoxylate phenylhydrazone in the reaction solution with treatment of isocitrate and phenylhydrazine. Each test compound was dissolved in DMSO. The reaction solution containing 20 mM sodium phosphate buffer (pH 7.0), 1.27 mM threo-DL (+) isocitrate, 3.75 mM $MgCl_2$, 4.1 mM phenylhydrazine and 2.5 μg/mL purified ICL was incubated at 37°C for 30 min with/without a prescribed concentration of the compound dissolved in DMSO (final concentration, 0.5%). The increase of intensity by formation of glyoxylate phenylhydrazone was recorded by a spectrophotometer (Shimadzu, Japan) using wavelength of 324 nm. The ICL inhibitory activity was calculated as a percentage relative to the solvent-treated control with three times. The concentration ranges of 256 to 1 μg/mL of compound in the reaction mixture. IC_{50} values were calculated by non-linear regression analysis (Graph Pad ver. 8.0, Prism) (Miller et al., 2014). 3-Nitropropionate, a known ICL inhibitor, was used as a positive control (Sharma et al., 2000).

Kinetic analysis

Dixon plot was used to determine the type of enzyme inhibition. The kinetic analysis was performed at designated concentration of phenylhydrazine (0.13, 0.26, 0.53, 1.05 and 2.10 mM) and ICL as substrate and inhibitor, respectively. Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) of inhibitor were determined by non-linear regression analysis (Graph Pad ver. 8.0, Prism). The inhibition constants (K_i) was determined via interpretation of the Dixon plots.

MIC assay

C. albicans strains were cultured in YNB broth containing 2% glucose at 28°C for 24 h, centrifuged at 12,000 rpm for 1 min, and washed twice with sterile distilled water. Each test compound was dissolved in DMSO and diluted into each YNB broth containing 2% glucose or 2% potassium acetate to make a dilution series of

256 μg/mL to 1 μg/mL. Additional DMSO added to the medium so that its final concentration was 0.5%. The 20 μL of fungus culture was poured into a 96-well assay plate to give a final concentration of 1×10^4 cells/mL and reach a final volume of 100 μL. Culture plates were placed on the 28°C incubator for 3 days. Positive control was amphotericin B which is a known antifungal compound.

Growth phenotype

C. albicans SC5314 (wild-type), MRC10 (Δ*icl*) and MRC11 (Δ*icl* + ICL) was cultured in YNB broth (2% glucose) at 28°C on rotary shakers for 24 h, harvested by centrifugation, and washed twice with sterile distilled water. Cells were added to YNB media containing 2% glucose, 2% potassium acetate, or 2% potassium acetate plus cyclo(L-Phe-L-Val) (8, 16, and 32 μg/mL) and incubated for 4 h at 28°C.

Semi-quantitative RT-PCR

The total RNA was extracted by the RNase mini kit (Qiagen, Germany) with manufacturer's manual. Complementary DNA (cDNA) synthesis was carried out by the Superscript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) with oligo$[dT]_{20}$ (50 μM) primers. Semi-quantitative RT-PCR was conducted with ICL primers. The housekeeping gene *GPDH* (glycerol-3-phosphate dehydrogenase) was served as loading control. The reversely transcribed cDNA was subject to PCR under the following conditions: an initial denaturation at 98°C for 5 min, followed by 35 cycles of denaturation at 98°C for 10 s, annealing temperature at 55°C for 30 s and elongation at 72°C for 1 min, with a final extension at 72°C for 5 min.

Oligonucleotides

For PCR reactions, oligonucleotides were purchased from Cosmogenetech Korea Co. (Seoul, Korea). The sequence of used oligonucleotides is shown in Table 2.1.

Table 2.1. Lists of used oligonucleotides.

Primer Name	Sequence
ICL-For	5′- ATGCCTTACACTCCTATTGACATTCAAAA-3′
ICL-Rev	5′- TAGATTCAGCTTCAGCCATCAAAGC-3′
GPDH-For	5′-ATCACCATCTTCCAGGAG-3′
GPDH-Rev	5′- ATGGACTGTGGTCATGAG-3′

Results

Isolation and structure elucidation of compounds 5-9

The culture broth (40 L) was filtered with filter paper to remove mycelia and the filtered culture broth was sequentially fractionated with equal volumes of *n*-hexane, EtOAc, and *n*-butanol. On the basis of results from ICL activity assay, the EtOAc extract (1.7 g) was chosen for further separation. The EtOAc extract subjected to reverse-phase flash column chromatography packed with YMC Gel ODS-A (S-75 μm) and eluted with MeOH-H_2O (gradient from 0:100 to 100:0), acetone and EtOAc, affording seven fractions. Based on the bioactivity analyses, the H_2O-MeOH (60:40) (325 mg) fraction was isolated by semi-preparative reverse-phase HPLC (Agilent C_{18} column, 10.0 × 250 mm; 2 mL/min flow rate; UV 254, 365 nm detection; H_2O-MeOH = 40:60 in 50 min) to yield compound **5** (t_R = 37.2 min, 6.4 mg), **6** (t_R = 10.7 min, 5.2 mg), **7** (t_R= 21.3 min, 10.3 mg), **8** (t_R = 28.5 min, 7.4 mg), and **9** (t_R = 8.3 min, 7.2 mg) (Fig. 2.2).

Using combined spectroscopic analyses, including ^{1}H, ^{13}C nuclear magnetic resonance (NMR), 2D NMR spectral analyses (COSY, HMQC, and HMBC), and UV data, the isolated compounds **5**-**9** were identified as diketopiperazines: cyclo(L-Phe-L-Val) (**5**) (Schmidtz et al., 1983), cyclo(L-Pro-L-Val) (**6**) (Huang et al., 1995), cyclo(L-Pro-L-Leu) (**7**) (Huang et al., 1995), cyclo(L-Pro-L-Tyr) (**8**) (Barrow and Sun, 1994), and cyclo(L-Phe-L-Pro) (**9**) (Barrow and Sun, 1994) (Fig. 2.3). The spectroscopic data obtained from the isolated compounds were consistent with the previously reported values.

Figure 2.2. Flow chart of compounds 5-9 isolation

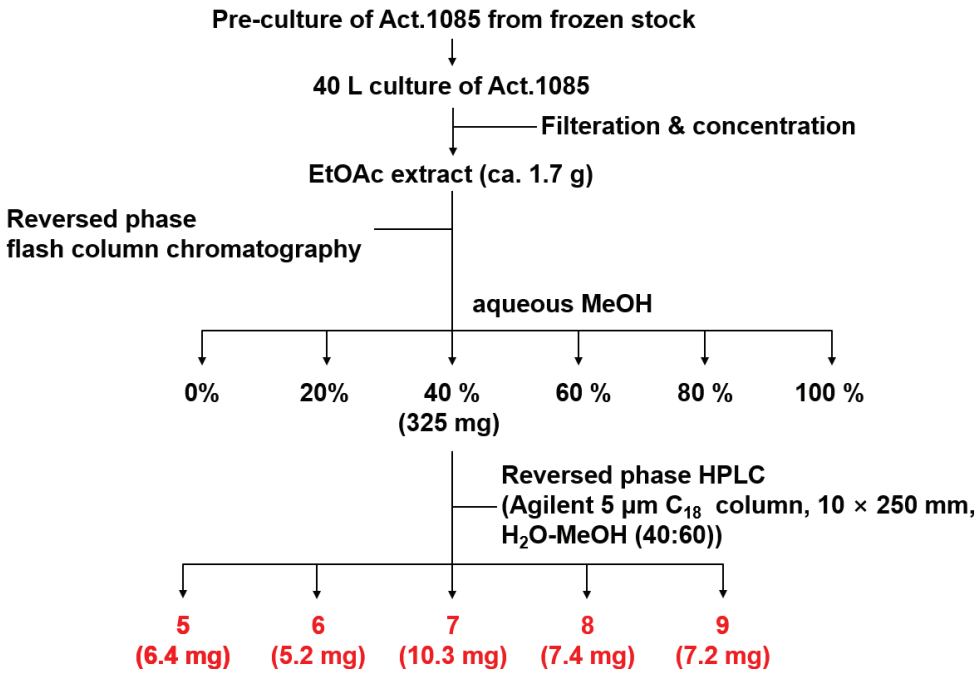
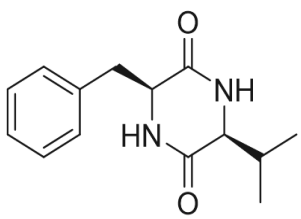
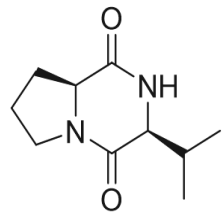

Pre-culture of Act.1085 from frozen stock
40 L culture of Act.1085
Filteration & concentration
EtOAc extract (ca. 1.7 g)
Reversed phase
flash column chromatography
aqueous MeOH
0%
20%
40 %
(325 mg)
60 %
80 %
100 %
Reversed phase HPLC
(Agilent 5 µm C18 column, 10 × 250 mm,
H2O-MeOH (40:60))
5
(6.4 mg)
6
(5.2 mg)
7
(10.3 mg)
8
(7.4 mg)
9
(7.2 mg)

Figure 2.3. Structures of compounds 5-9



5



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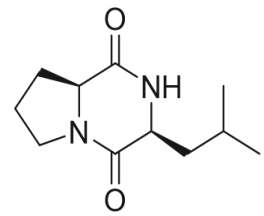

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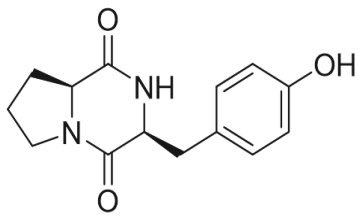

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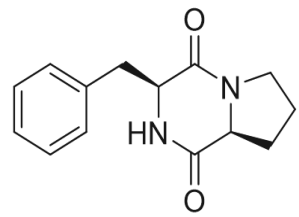


9

ICL inhibitory activity of compounds 5-9

To investigate whether isolated diketopiperazines possess ICL inhibitory activity, compounds **5-9** were evaluated for ICL inhibitory activity according to methods reported previously (Shin et al., 2005). The inhibitory concentrations (IC_{50}) values of the isolated compounds **5-9** are represented in Table 2.2. Among these, compound **6** and **7** exhibited weak inhibitory activity toward ICL enzyme, with IC_{50} values of 516.28 and 533.79 μM, respectively (Table 2.2). Compound **5** exhibited the strongest inhibitory activity of the test compounds but weaker than nitropropionate, with IC_{50} values of 109.50 and 15.95 μM, respectively (Fig. 2.4). The others were inactive under given condition.

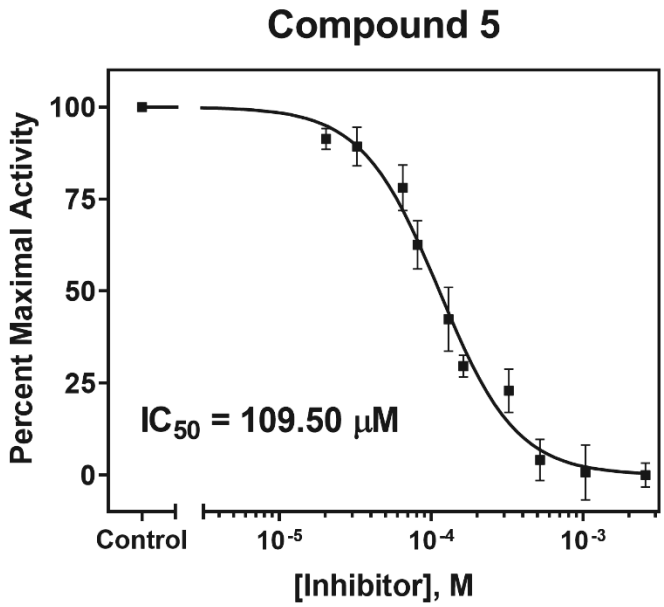
Table 2.2. Inhibitory activity of isolated diketopiperazines toward the ICL enzyme and growth of *C. albicans* SC5314.

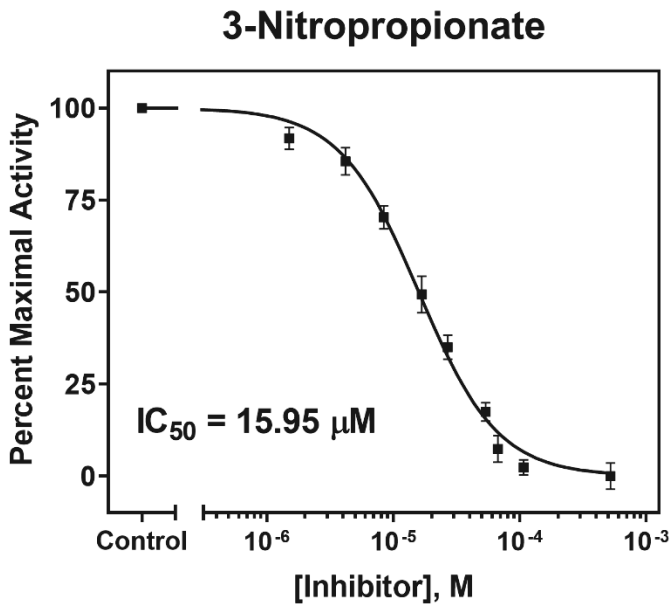
Compound	ICL IC_{50}, µM (µg/mL)	MIC (µg/mL)
		Glucose
5	109.50 ± 4.17 (27.74 ± 2.24)	>256
6	516.28 ± 9.18 (101.32± 4.22)	>256
7	533.79 ± 3.12 (112.24 ± 1.94)	>256
8	>1048.75 (>256)	>256
9	>984.16 (>256)	>256
3-Nitropropionate	15.94 ± 2.13(1.90 ± 1.57)	>256
Amphotericin B	ND [1]	1

3-Nitropropionate was used as a reference inhibitor of ICL. Amphotericin B was used as a standard antifungal drug.

Figure 2.4. Dose-dependent curves of the compound 5 and 3-nitropropionate

ICL source was *C. albicans* ATCC10231. 3-nitropropionate was used as reference compound. The data were analyzed using non-linear regression curve fitting in GraphPad software ver 8.0 (Prism). The vertical bars indicate the standard errors (n=3)


Compound 5
Percent Maximal Activity
100
75
50
25
0
IC50 = 109.50 μM
Control
10-5
10-4
10-3
[Inhibitor], M

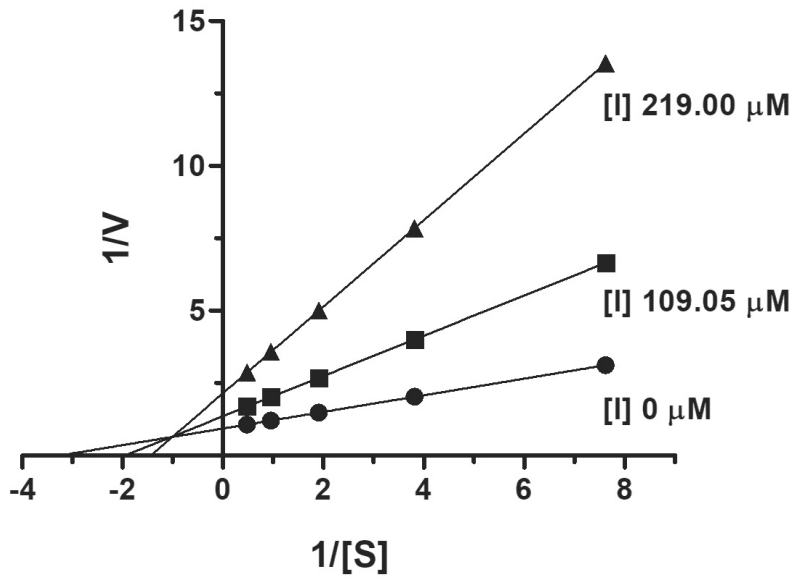

3-Nitropropionate
Percent Maximal Activity
100
75
50
25
0
IC50 = 15.95 μM
Control
10-6
10-5
10-4
10-3
[Inhibitor], M

Determination of inhibition type

To determine the kinetic parameters (K_{max} and V_{max}), reaction mixtures including ICL was incubated with designated concentrations and times of compound **5** at 37°C. Kinetic constants (K_{max} and V_{max}) were calculated from Michaelis-Menten plot and calculated kinetic constants were used for determining inhibitor constant (K_i) value in Lineweaver-Burk plot. The inhibitor constant (K_i) calculated as 64.86 μM. Based on the result of the Lineweaver-Burk plot, compound **5** behaved as mixed inhibitor (Fig. 2.5).

Figure 2.5. Dixon plot for ICL inhibition of compound 5

S and V represent the substrate concentration (mM) and reaction velocity (ΔA_{324nm}/min), respectively. Each data point represents the mean of three experiments.


15
10
5
1/V
-4
-2
0
2
4
6
8
1/[S]
[I] 219.00 μM
[I] 109.05 μM
[I] 0 μM

Growth assay of *C. albicans* strains under C_2 substrate use

To determine whether compound **5** affects C_2 substrate use, *C. albicans* strains SC5314, ATCC10231, ATCC10259, ATCC11006, and ATCC18804 were grown in YNB liquid broth containing either 2% glucose or 2% acetate as the sole carbon source. Compound **5** exhibited the inhibitory effect on *C. albicans* in acetate (minimum inhibitory concentration of 32–64 μg/mL) but no inhibitory effect on *C. albicans* in glucose (Table 2.3). These results showed that compound **5** affects ICL-mediated proliferation of the fungus under C_2-carbon-utilizing conditions.

Table 2.3. Effect of compound 5 on *C. albicans* strains grown in glucose or acetate as sole carbon source.

Strain	MIC (μg/mL)			
	Glucose		Acetate	
	Compound 5	Amph B	Compound 5	Amph B
SC5314	>256	1	32	0.5
ATCC10231	>256	1	32	0.5
ATCC10259	>256	1	64	0.5
ATCC11006	>256	0.5	32	0.5
ATCC18804	>256	1	64	0.5

C. albicans cells (1×10^4 cells/mL) were incubated with varying concentrations of compound **5** for 72 h at 28°C in YNB medium containing 2% glucose or 2% potassium acetate. Amphotericin B (Amph B) was used as a standard antifungal drug.

Effects of compound 5 on growth phenotype and *ICL* gene expression

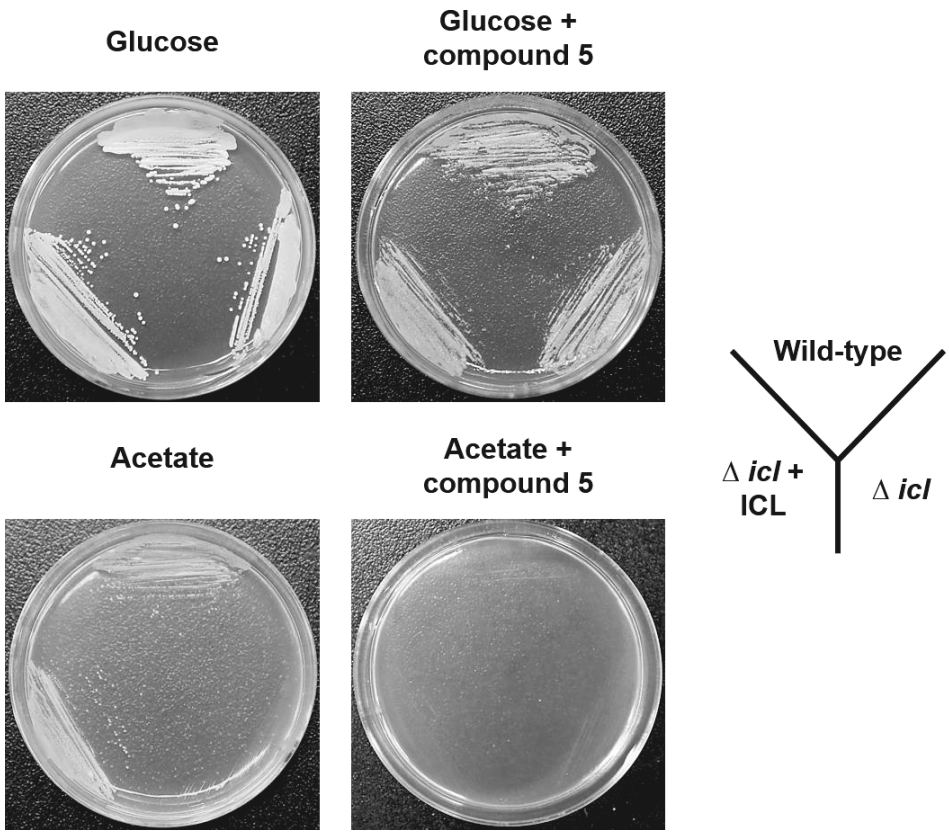
To determine whether the cell phenotype of the *icl*-deletion mutant is affected by the presence of compound **5**, a growth assay was conducted using *C. albicans* SC5314 (wild-type) and two *icl*-deletion mutants (MRC10 and MRC11). After pre-culture, these strains were streaked onto YNB agar containing 2% glucose or 2% potassium acetate with or without 32 μg/mL of compound **5**. All strains grew normally on both the plates with glucose and those with glucose plus the compound. However, MRC10 did not grow when acetate was the sole carbon source. Furthermore, none of the tested strains exhibited growth on the YNB agar plate with compound **5** (Fig. 2.6a).

The effect of compound **5** on *ICL* expression were examined by semi-quantitative reverse-transcription (RT)-PCR. No ICL-specific PCR product was detected in the cultures when SC5314 and MRC11 were grown in YNB liquid broth containing glucose. However, *ICL* was strongly induced when these cells were cultured in YNB broth containing acetate. The intensity of the PCR band corresponding to the *ICL* product decreased with increasing compound **5** concentrations in the cells grown under *ICL* expression conditions (Fig. 2.6b). *GPDH* expression was detected in all treatments regardless of compound **5** exposure. These results indicate that compound **5** inhibits *ICL* expression in *C. albicans* under C_2-carbon-utilizing conditions.

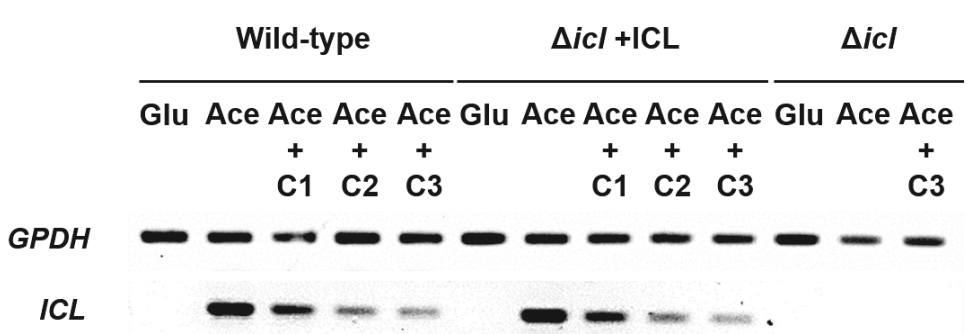
Figure 2.6. Analysis of growth phenotypes and *ICL* mRNA expression.

(a) *C. albicans* SC5314 (wild-type), MRC10 (Δ*icl*), and MRC11 (Δ*icl* + ICL) were cultured on YNB agar plates containing the indicated carbon source (2% glucose or 2% potassium acetate) with or without 32 μg/mL of compound **5** for 2 days at 28 °C. (b) Strains were grown until the mid-log phase in minimal YNB liquid medium containing 2% glucose. Cells were collected by centrifugation and transferred to the same medium containing 2% glucose (Glu), 2% potassium acetate (Ace), or 2% potassium acetate (Ace) plus compound **5** (C1: 8 μg/mL; C2: 16 μg/mL; and C3: 32 μg/mL) and cultured for 4 h at 28 °C. Total RNA was prepared from these cells, and *ICL* mRNA expression was analyzed by semi-quantitative RT-PCR. The *GPDH* housekeeping gene was evaluated as a loading control.

(a)


Glucose
Glucose + compound 5
Acetate
Acetate + compound 5
Wild-type
Δ icl + ICL
Δ icl

(b)


Wild-type
Δicl +ICL
Δicl
Glu Ace Ace + C1 Ace + C2 Ace + C3 Glu Ace Ace + C1 Ace + C2 Ace + C3 Glu Ace Ace + C3
GPDH
ICL

Discussion

The glyoxylate cycle, an anaplerotic pathway of the TCA cycle, is well documented in prokaryotes and eukaryotes. Diverse pathogenic fungus utilized glyoxylate cycle during host infection. The function of this cycle has been confirmed by analyzing mutants of pathogenic microorganisms that lack ICL and MLS, key components of glyoxylate cycle. Research on candidiasis in mice has shown that *Candida albicans*, the most serious human pathogenic fungus, requires ICL to be fully virulent. As this cycle does not operate in mammalian cells, ICL is an attractive target for the development of antimicrobial drugs. In this study, five diketopiperazine compounds were isolated from the culture of marine-derived S. puniceus Act1085 and evaluated their inhibitory activities toward ICL enzyme from *C. albicans*. These studies led to the identification of cyclo(L-Phe-L-Val) as a potent ICL inhibitor. The growth phenotype of *ICL* deletion mutants and semi-quantitative RT-PCR analyses indicated that this compound inhibits the *ICL* gene expression in *C. albicans* under C_2-carbon-utilizing conditions.

It is well known that diketopiperazines possess a broad range of biological activities (Degrassi et al., 2002; Yan et al., 2004; Mccleland et al., 2004; Mas et al., 2006; Park et al., 2006; Borthwick, 2012). Many research group have been focused on the pharmacological potential of diketopiperazine because it has advantage for medical chemistry such as resistance of proteolysis, mimicking of peptidic pharmacophoric groups, conformational rigidity, substituent group stereochemistry, and existence of donor and acceptor groups for hydrogen bonding (favoring interactions with targets) (Martins and Carvalho, 2007). Above all things, diketopiperazines can be easily synthesized and isolated from natural products. In this work, as a result of activity-guided separation processing, five diketopiperazines, cyclo(L-Phe-L-Pro), cyclo(L-Pro-L-Leu), cyclo(L-Pro-L-Tyr), cyclo(L-Pro-L-Val), and cyclo(L-Phe-L-Val), were obtained from a marine actinomycete *S. puniceus*. Among them, cyclo(L-Pro-L-Leu), cyclo(L-Pro-L-Val)

and cyclo(L-Phe-L-Val) were exhibited ICL inhibitory activities, with IC_{50} in the range 27 – 106 μg/mL (Table 2.2). It is observed that the structures of isolated diketopiperazines with ICL inhibition activity possess an isopropyl moiety, suggesting that isopropyl moiety plays a role in inhibiting ICL activity.

Bacterium (*M. tuberculosis*) and fungus (*C. albicans*) can be virulent, causing expression of *ICL* gene which is a component of the glyoxylate cycle during the persistence infection in macrophages (McKinney et al., 2000; Ramirez and Lorenz, 2007). It was expected that inhibitors of the glyoxylate cycle block the nutrient availability and lead to the death of these pathogens in the macrophage. Based on these findings, the effect of cyclo(L-Phe-L-Val) on *C. albicans* ICL was investigated through the observation of growth phenotype and *ICL* transcript levels using *C. albicans* SC5314 (wild-type), MRC10 (Δ*icl*) and MRC11 (Δ*icl* +ICL) (Ramirez and Lorenz, 2007). Based on the growth assay, cyclo(L-Phe-L-Val) specifically inhibits ICL enzyme by identifying no growth of SC5314 and MRC11 on the YNB agar plates containing acetate plus 32 μg/mL of compound. Moreover, it is observed that icl transcript levels were reduced depend on the concentration of cyclo(L-Phe-L-Val). Taken together, second part of this study indicated that diketopiperazine class compound shows antifungal agent in terms of suppressing *C. albicans* virulence.

Reference

Alston TA, Mela L, and Bright HJ (1977) 3-Nitropropionate, the toxic substance of Indigofera, is a suicide inactivator of succinate dehydrogenase. *Proc Natl Acad Sci USA* 74, 3767–3771.

Ansari MA, Fatima Z, Ahmad K, and Hameed S (2018) Monoterpenoid perillyl alcohol impairs metabolic flexibility of *Candida albicans* by inhibiting glyoxylate cycle. *Biochem Biophys Res Commun*, 495 560–566.

Argimón S, Wishart JA, Leng R, Macaskill S, Mavor A, Alexandris T et al. (2007) Developmental regulation of an adhesion gene during cellular morphogenesis in the fungal pathogen *Candida albicans*. *Eukaryot Cell* 6, 682–692.

Bahn YS and Sundstrom P (2001) *CAP1*, an adenylate cyclase-associated protein gene, regulates bud-hypha transitions, filamentous growth, and cyclic AMP levels and is required for virulence of *Candida albicans*. *J Bacteriol* 183, 3211–3223.

Bahn YS, Staab J, and Sundstrom P (2003) Increased high-affinity phosphodiesterase *PDE2* gene expression in germ tubes counteracts *CAP1*-dependent synthesis of cyclic AMP, limits hypha production and promotes virulence of *Candida albicans*. *Mol Microbiol* 50, 391–409.

Barrow CJ and Sun HH (1994) Spiroquinazoline, a novel substance P inhibitor with a new carbon skeleton, isolated from *Aspergillus flavipes*, *J Nat Prod* 57, 471–476.

Biswas S, Van Dijck P, and Datta A (2007) Environmental sensing and signal transduction pathways regulating morphopathogenic determinants of *Candida albicans*. *Microbiol Mol Biol Rev* 71, 348–376.

Bockmühl DP, and Ernst JF (2001) A potential phosphorylation site for an A-type kinase in the Efg1 regulator protein contributes to hyphal morphogenesis of *Candida albicans*. *Genetics* 157, 1523–1530.

Booth AN, Taylor J, Wilson RH, and DeEds F (1952) The inhibitory effects of itaconic acid in vitro and in vivo. *J Biol Chem* 195, 697–702.

Borthwick AD (2012) 2,5-Diketopiperazines: Synthesis, reactions, medicinal chemistry, and bioactive natural products. *Chem Rev* 112, 3641–3716.

Bourne HR, Sanders DA, and McCormick F (1991) The GTPase superfamily: conserved structure and molecular mechanism. *Nature* 349, 117–127.

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72, 248–254.

Burnett JC, Opsenica D, Sriraghavan K, Panchal RK, Ruthel G, and Ngyuen TL (2007) Novel 4-aminoquinolines active against chloroquine-resistant and sensitive *P. falciparum* strains that also inhibit botulinum serotype A. *J Med Chem* 50, 2127–2136.

Byun HG, Zhang H, Mochizuki M, Adachi K, Shizuri Y, Lee WJ et al. (2003) Novel antifungal diketopiperazine from marine fungus. *J antibiot* 56, 102-106.

Carroll AR, Copp BR, Davis RA, Keyzers RA, and Prinsep MR (2019) Marine natural products. *Nat Prod Rep* 36, 122−173.

Cheah HL, Lim V, and Sandai D (2014) Inhibitors of the glyoxylate cycle enzyme ICL1 in *Candida albicans* for potential use as antifungal agents. *PloS One* 9, e95951.

CLSI (2017). *Reference Methods for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi*, (3rd ed.). CLSI standard M38; Clinical and Laboratory Standards Institute, USA.

CLSI (2018) *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically*, (11th ed.). CLSI standard M07; Clinical and Laboratory

Standards Institute, USA.
Cross RM, Monastyrskyi A, Mutka TS, Burrows JN, Kyle DE, and Manetsch R (2010) Optimization: structure-activity and structure-property relationship studies of 3-substituted 2-methyl-4-(1*H*)-quinolones with antimalarial activity. *J Med Chem* 53, 7076–7094.
Crouch PJ and Barnham KJ (2012) Therapeutic redistribution of metal ions to treat Alzheimer's disease. *Acc Chem Res* 45, 1604–1611.
Davis-Hanna A, Piispanen AE, Stateva LI, and Hogan DA (2008) Farnesol and dodecanol effects on the *Candida albicans* Ras1-cAMP signaling pathway and the regulation of morphogenesis. *Mol Microbiol* 67, 47–62.
Degrassi G, Aguilar C, Bosco M, Zahariev S, Pongor S, and Venturi V. (2002) Plant growth-promoting *Pseudomonas putida* WCS358 produces and secretes four cyclic dipeptides: Cross-talk with quorum sensing bacterial sensors. *Curr Microbiol* 45, 250–254.
Desai NC, Patel BY, and Dave BP (2016) Synthesis and antimicrobial activity of novel quinoline derivatives bearing pyrazoline and pyridine analogues. *Med Chem Res* 26, 109–119.
Déziel E, Lépine F, Milot S, He J, Mindrinos MN, Tompkins RG, and Rahme LG. (2004) Analysis of *Pseudomonas aeruginosa* 4-hydroxy-2-alkylquinolines (HAQs) reveals a role for 4-hydroxy-2-heptylquinoline in cell-to-cell communication. *Proc Natl Acad Sci USA* 101, 1339–1344.
Dhillon NK, Sharma S, and Khuller GK (2003) Signaling through protein kinases and transcriptional regulators in *Candida albicans*. *Crit Rev Microbiol* 29, 259–275.
Dixon GH and Kornberg HL (1959) Assay methods for key enzymes of the glyoxylate assay. *Biochem J* 72, 3P.
Domagala JM, Heifetz CL, Hutt MP, Mich TF, Nichols JB, Solomon M et al. (1988) 1-Substituted 7-[3-[(ethylamino) methyl]-1-pyrrolidinyl]-6, 8-difluoro-1, 4-dihydro-4-oxo-3-quinolinecarboxylic acids. New quantitative structure activity relationships at N1 for the quinolone antibacterials. *J Med Chem* 31, 991-1001.
Dunn MF, Ramírez-Trujillo JA, and Hernández-Lucas I (2009) Major roles of isocitrate lyase and malate synthase in bacterial and fungal pathogenesis. *Microbiology* 155, 3166–3175.
Fazly A, Jain C, Dehner AC, Issi L, Lilly EA, Ali A et al. (2013) Chemical screening identifies filastatin, a small molecule inhibitor of *Candida albicans* adhesion, morphogenesis, and pathogenesis. *Proc Nat Acad Sci USA* 110, 13594–13599.
Feng Q, Summers E, Guo B, and Fink G (1999) Ras signaling is required for serum-induced hyphal differentiation in *Candida albicans*. *J Bacteriol* 181, 6339–6346.
Fenical W and Jensen PR (2006) Developing a new resource for drug discovery: marine actinomycete bacteria. *Nat Chem Biol* 2, 666−673.
Findley K, Oh J, Yang J, Conlan S, Deming C, Meyer JA et al. (2013) Topographic diversity of fungal and bacterial communities in human skin. *Nature* 498, 367−370.
Finkelstein M, Gold H, and Paterno CA (1947) Pharmacology of itaconic acid and its sodium, magnesium, and calcium salts. *J Am Pharm Assocc* 36, 173–179.
Ghannoum MA, Jurevic RJ, Mukherjee PK, Cui F, Sikaroodi M, Naqvi A et al. (2010) Characterization of the oral fungal microbiome (mycobiome) in healthy individuals. *PLoS Pathog* 6, e1000713.
Giacometti R, Kronberg F, Biondi RM, and Passeron S (2009) Catalytic isoforms Tpk1 and Tpk2 of *Candida albicans* PKA have non-redundant roles in stress response and glycogen storage. *Yeast* 26, 273–285.
Greene JG, Sheu SS, Gross RA, and Greenamyre JT (1998) 3-Nitropropionic acid exacerbates *N*-methyl-D-aspartate toxicity in striatal culture by multiple mechanisms. *Neuroscience* 84, 503–510.

Haefner B (2003) Drugs from the deep: marine natural products as drug candidates. *Drug Discov today* 8, 536−544.

Hays EE, Wells IC, Katzman PA, Cain CK, Jacobs FA, Thayer SA et al. (1945) Antibiotic substances produced by *Pseudomonas aeruginosa. J Biol Chem* 159, 725–750.

Hoffmann C, Dollive S, Grunberg S, Chen J, Li H, Wu GD et al. (2013) Archaea and fungi of the human gut microbiome: correlations with diet and bacterial residents. *PloS One* 8, e66019.

Holden MT, Ram Chhabra S, De Nys R, Stead P, Bainton NJ, Hill PJ et al. (1999) Quorum-sensing cross talk: isolation and chemical characterization of cyclic dipeptides from *Pseudomonas aeruginosa* and other gram-negative bacteria. *Mol Microbiol* 33, 1254-1266.

Hornby JM, Jensen EC, Lisec AD, Tasto JJ, Jahnke B, Shoemaker R et al. (2001) Quorum sensing in the dimorphic fungus *Candida albicans* is mediated by farnesol. *Appl Environ Microbiol* 67, 2982–2992.

Hu Y, Chen J, Hu G, Yu J, Zhu X, Lin Y et al. (2015) Statistical research on the bioactivity of new marine natural products discovered during the 28 years from 1985 to 2012. *Mar Drugs* 13, 202−221.

Huang R, Zhou X, Xu T, Yang X, and Liu Y (2010) Diketopiperazines from marine organisms. *Chem Biodivers* 7, 2809–2829.

Huang,Q, Tezuka Y, Hatanaka Y, Kikuchi T, Nishi A, and Tubaki K (1995) Studies on metabolites of mycoparasitic fungi. III. New sesquiterpene alcohol from *Trichoderma koningii*. *Chem Pharm Bull* 43, 1035–1038.

Hung LW and Barnham KJ (2012) Modulating metals as a therapeutic strategy for Alzheimer's disease. *Future Med Chem* 4, 955–969.

Jain S, Chandra V, Jain PK, Pathak K, Pathak D, and Vaidya A. (2016) Comprehensive review on current developments of quinoline-based anticancer agents. *Arab J Chem*, in press

Jensen PR, Mincer TJ, Williams PG, and Fenical W (2005). Marine actinomycete diversity and natural product discovery. *Antonie Van Leeuwenhoek* 87, 43−48.

Kadosh D and Johnson AD (2005) Induction of the Candida albicans filamentous growth program by relief of transcriptional repression: a genome-wide analysis. *Mol Biol Cell* 16, 2903–2912.

Kebaara BW, Langford ML, Navarathna DH, Dumitru R, Nickerson KW, Atkin AL et al. (2008) *Candida albicans* Tup1 is involved in farnesol-mediated inhibition of filamentous growth induction. *Eukaryot Cell*, 7, 980–987.

Kitamura S, Hashizume K, Iida T, Miyashita E, Shirahata K, and Kase H (1986) Studies on lipoxygenase inhibitors. II. KF8940 (2-n-heptyl-4-hydroxyquinoline-N-oxide), a potent and selective inhibitor of 5-lipoxygenase, produced by *Pseudomonas methanica*. *J Antibiot* 39, 1160–1166

Ko YH, Smith BL, Wang Y, Pomper MG, Rini DA, Torbenson MS et al. (2004) Advanced cancers: Eradication in all cases using 3-bromopyruvate therapy to deplete ATP. *Biochem Biophys Res Commun* 324, 269–275.

Köhler JR and Fink GR (1996) *Candida albicans* strains heterozygous and homozygous for mutations in mitogen-activated protein kinase signaling components have defects in hyphal development. *Proc Nat Acad Sci* 93, 13223–13228.

Kumar S, Bawa S, and Gupta H (2009) Biological activities of quinoline derivatives. *Mini-Rev Med Chem* 9, 1648–1654

Lakshmi A, Balachandran V, and Janaki A (2011) Comparative vibrational spectroscopic studies, HOMO-LUMO and NBO analysis of 5,7-dibromo-8-hydroxyquinoline and 5,7-dichloro-8-hydroxyquinoline based on density functional theory. *J Mol Struct* 1004, 51–66.

Lane S, Birse C, Zhou S, Matson R, and Liu H (2001) DNA array studies demonstrate

convergent regulation of virulence factors by Cph1, Cph2, and Efg1 in *Candida albicans*. *J Biol Chem* 276, 48988–48996.

Langford ML, Hargarten JC, Patefield KD, Marta E, Blankenship JR, Fanning S et al. (2013) *Candida albicans* Czf1 and Efg1 coordinate the response to farnesol during quorum sensing, white-opaque thermal dimorphism, and cell death. *Eukaryot Cell* 12, 1281–1292.

Lattif AA, Prasad R, Banerjee U, Gupta N, Mohammad S, and Baquer NZ (2006) The glyoxylate cycle enzyme activities in the pathogenic isolates of *Candida albicans* obtained from HIV/AIDS, diabetic and burn patients. *Mycoses* 49, 85–90.

Lee SH, Won TH, Kim H, Ahn CH, Shin J, and Oh KB (2014) Suvanine sesterterpenes from a tropical sponge *Coscinoderma* sp. inhibit isocitrate lyase in the glyoxylate cycle. *Mar Drugs* 12, 5148-5159.

Lengeler KB, Davidson RC, D'souza C, Harashima T, Shen WC, Wang P et al. (2000) Signal transduction cascades regulating fungal development and virulence. *Microbiol Mol Biol Rev* 64, 746–785.

Liu H (2001) Transcriptional control of dimorphism in *Candida albicans*. *Curr Opin Microbiol* 4, 728–735.

Lorenz MC and Fink GR (2001) The glyoxylate cycle is required for fungal virulence. *Nature* 412, 83–86.

Lorenz MC, Bender JA, and Fink GR (2004) Transcriptional response of *Candida albicans* upon internalization by macrophages. *Eukaryot cell* 3, 1076–1087.

Lu Y, Su C and Liu H (2014) *Candida albicans* hyphal initiation and elongation. *Trends Microbiol* 22, 707−714.

Maity S, Khan SA, and Ahmad S (2012) Synthesis, characterization, antimicrobial and antioxidant activity of some novel Schiff base derived from quinoline. *Int J Pharm Biol Sci* 2, 90–98

Martins MB and Carvalho I (2007) Diketopiperazines: biological activity and synthesis. *Tetrahedron* 63, 9923–9932.

Mas V, Falco A, Brocal I, Perez L, Coll JM, and Estepa A (2006) Identification of selective inhibitors of VHSV from biased combinatorial libraries of *N*,*N*′-disubstituted 2,5-piperazinediones. *Antiviral Res* 72, 107–115.

McBride M (1998) Engineering layers in molecular solids with the cyclic dipeptide of (*S*)-aspartic acid. *Chem Commun* 145–146.

McCleland K, Milne PJ, Lucieto FR, Frost C, Brauns SC, Van De Venter M et al. (2004) An investigation into the biological activity of the selected histidine-containing diketopiperazines cyclo (His-Phe) and cyclo (His-Tyr). *J Pharm Pharmacol* 56, 1143-1153.

McFadden BA and Purohit S (1977) Itaconate, an isocitrate lyase-directed inhibitor in *Pseudomonas indigofera*. *J Bacteriol* 131, 136-144.

McKinney JD, Zu Bentrup KH, Muñoz-Elías EJ, Miczak A, Chen B, Chan WT et al. (2000) Persistence of *Mycobacterium tuberculosis* in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. *Nature* 406, 735–738.

Miller B, Friedman AJ, Choi H, Hogan J, McCammon JA, and Hook V (2014) The marine cyanobacterial metabolite gallinamide A is a potent and selective inhibitor of human cathepsin L. *J Nat Prod* 77, 92–99.

Montaser R and Luesch H (2011) Marine natural products: a new wave of drugs? *Future Med Chem* 3, 1475−1489.

Muñoz-Elías EJ and McKinney JD (2005) *Mycobacterium tuberculosis* isocitrate lyases 1 and 2 are jointly required for in vivo growth and virulence. *Nat Med* 11, 638–644.

Murphy PM (2001) Viral exploitation and subversion of the immune system through chemokine mimicry. *Nat Immunol* 2, 116–122.

Nagano Y, Nagahama T, Hatada Y, Nunoura T, Takami H, Miyazaki J et al. (2010) Fungal

diversity in deep-sea sediments–the presence of novel fungal groups. *Fungal Ecol* 3, 316−325.

Nakata H, Maeda K, Miyakawa T, Shibayama S, Matsuo M, Takaoka Y et al. (2005) Potent anti-R5 human immunodeficiency virus type 1 effects of a CCR5 antagonist, AK602/ONO4128/GW873140, in a novel human peripheral blood mononuclear cell nonobese diabetic-SCID, interleukin-2 receptor γ-chain-knocked-out AIDS mouse model. *J Virol* 79, 2087−2096.

Nantel A, Dignard D, Bachewich C, Harcus D, Marcil A, and Bouin AP (2002). Transcription profiling of *Candida albicans* cells undergoing the yeast-to-hyphal transition. *Mol Biol Cell* 13, 3452–3465.

Narender T, Shweta, Tanvir K, Rao MS, Srivastava K, and Puri SK (2005) Prenylated chalcones isolated from *Crotalaria genus* inhibits in vitro growth of the human malaria parasite *Plasmodium falciparum*. *Bioorg Med Chem Lett* 15, 2453–2455.

Nayyar A, Malde A, Coutinho E, and Jain R (2006) Synthesis, antituberculosis activity, and 3D-QSAR study of ring-substituted-2/4-quinolinecarbaldehyde derivatives. *Bioorg Med Chem* 14, 7302–7310.

Newman DJ, Cragg GM, and Battershill CN (2009) Therapeutic agents from the sea: biodiversity, chemo-evolutionary insight and advances to the end of Darwin's 200th year. *Diving Hyperb Med* 39, 216−225

Noble SM, Gianetti BA, and Witchley JN (2017) *Candida albicans* cell-type switching and functional plasticity in the mammalian host. *Nat Rev Microbiol* 15, 96−108.

Oh KB, Miyazawa H, Naito T, and Matsuoka H (2001) Purification and characterization of an autoregulatory substance capable of regulating the morphological transition in *Candida albicans*. *Proc. Natl. Acad. Sci USA* 98, 4664–4668.

Olivera BM (2000) w-Conotoxin MVIIA: from marine snail venom to analgesic drug. In Drugs from the Sea (Fusetani ed.), pp. 75–85. Karger, Switzerland.

Palacin S, Chin DN, Simanek EE, MacDonald JC, Whitesides GM, McBride MT et al. (1997) Hydrogen-bonded tapes based on symmetrically substituted diketopiperazines: a robust structural motif for the engineering of molecular solids. *J Am Chem Soc* 119, 11807–11816.

Park D, Lee K, Baek C, Kim IH, Kwon J, Lee WK et al. (2006) Cyclo(Phe-Pro) modulates the expression of *ompU* in *Vibrio* spp. *J Bacteriol* 188, 2214–2221.

Park W, Woo JK, Shin J, and Oh KB (2017). nonG, a constituent of the nonactin biosynthetic gene cluster, regulates nocardamine synthesis in *Streptomyces albus* J1074. *Biochem Biophys Res Commun* 490, 664−669.

Pereira F and Aires-de-Sousa J (2018) Computational methodologies in the exploration of marine natural product leads. *Mar Drugs* 16, 236.

Pfaller MA and Diekema DJ (2007) Epidemiology of invasive candidiasis: a persistent public health problem. *Clin Microbiol Rev* 20, 133−163.

Pierce CG, Chaturvedi AK, Lazzell AL, Powell AT, Saville SP, McHardy SF et al. (2015) A novel small molecule inhibitor of *Candida albicans* biofilm formation, filamentation and virulence with low potential for the development of resistance. *NPJ Biofilms Microbiomes* 1, 15012.

Ramirez MA and Lorenz MC (2007) Mutations in alternative carbon utilization pathways in *Candida albicans* attenuate virulence and confer pleiotropic phenotypes. *Eukaryot Cell* 6, 280–290.

Raynes K, Galatis D, Cowman AF, Tilley L, and Deady LW (1995) Synthesis and activity of some antimalarial bisquinolines. *J Med Chem* 38: 204–206.

Romano E, Castillo, MV, Pergomet JL, Zinczuk J, and Brandán SA (2012) Synthesis and structural and vibrational analysis of (5,7-dichloro-quinolin-8-yloxy) acetic acid. *J Mol Struct* 1018, 149–155.

Schmidtz FJ, Vanderah DJ, Hollenbeak KH, Enwall CE, Gopichand Y, and SenGupta PK

(1983) Metabolites from the marine sponge *Tedania ignis*. A new atisanediol and several known diketopiperazines. *J Org Chem* 48, 3941–3945.
Shareck J and Belhumeur P (2011) Modulation of morphogenesis in *Candida albicans* by various small molecules. *Eukaryot Cell* 10, 1004–1012.
Sharkey LL, Mcnemar MD, Saporito-Irwin SM, Sypherd PS, and Fonzi WA (1999) *HWP1* functions in the morphological development of *Candida albicans* downstream of EFG1, TUP1, and RBF1. *J Bacteriol* 181, 5273–5279.
Sharma V, Kumar Mehta D, and Das R. (2017) Synthetic methods of quinoline derivatives as potent anticancer agents. *Mini-Rev Med Chem* 17, 1557–1572.
Sharma V, Sharma S, Hoener Zu Bentrup K, McKinney JD, Russell DG, Jacobs WR et al. (2000) Structure of isocitrate lyase, a persistence factor of *Mycobacterium tuberculosis*. *Nat Struct Biol* 7, 663–668.
Shin DS, Kim S, Yang HC, and Oh KB (2005) Cloning and expression of isocitrate lyase, a key enzyme of the glyoxylate cycle, of *Candida albicans* for development of antifungal drugs. *J Microbiol Biotechnol* 15, 652–655.
Shoshan MC (2012) 3-Bromopyruvate: targets and outcomes. *J Bioenergy Biomembr* 44, 7–15.
Shukla R, Shukla H, Sonkar A, Pandey T, and Tripathi T (2018) Structure-based screening and molecular dynamics simulations offer novel natural compounds as potential inhibitors of *Mycobacterium tuberculosis* isocitrate lyase. *J Biomol Struct Dyn* 36, 2045–2057.
Srivastava V, Negi AS, Kumar JK, Gupta MM, and Khanuja SP (2005) Plant-based anticancer molecules: a chemical and biological profile of some important leads. *Bioorg Med Chem* 13, 5892–5908.
Staab JF, Bradway SD, Fidel PL, and Sundstrom P (1999) Adhesive and mammalian transglutaminase substrate properties of *Candida albicans* Hwp1. *Science* 283, 1535–1538.
Sturgill-Koszycki S, Haddix PL, and Russell DG (1997) The interaction between *Mycobacterium* and the macrophage analyzed by two-dimensional polyacrylamide gel electrophoresis. *Electrophoresis* 18, 2558-2565.
Sudbery PE (2011) Growth of *Candida albicans* hyphae. *Nat Rev Microbiol* 9, 737−748
Supong K, Thawai C, Supothina S, Auncharoen P, and Pittayakhajonwut P (2016) Antimicrobial and anti-oxidant activities of quinoline alkaloids from *Pseudomonas aeruginosa* BCC76810. *Phytochem Lett* 17, 100–106.
Toenjes KA, Munsee SM, Ibrahim AS, Jeffrey R, Edwards JE, and Johnson DI (2005) Small-molecule inhibitors of the budded-to-hyphal-form transition in the pathogenic yeast *Candida albicans*. *Antimicrob Agents Chemother* 49, 963–972.
Vanni P, Giachetti E, Pinzauti G, and McFadden BA (1990) Comparative structure, function and regulation of isocitrate lyase, an important assimilatory enzyme. *Comp Biochem Physiol B: Biochem* 95, 431–458.
Verweij J (2009) Soft tissue sarcoma trials: one size no longer fits all. *J Clin Oncol* 27, 3085−3087.
Vila T, Romo JA, Pierce CG, McHardy SF, Saville SP, and Lopez-Ribot JL (2017) Targeting *Candida albicans* filamentation for antifungal drug development. *Virulence*, 8, 150–158.
Wall ME, Wani MC, Cook CE, Palmer KH, McPhail AA, and Sim GA (1966) Plant antitumor agents. I. The isolation and structure of camptothecin, a novel alkaloidal leukemia and tumor inhibitor from *camptotheca acuminata*[1,2]. *J Am Chem Soc* 88, 3888–3890.
Wang ZY, Thornton CR, Kershaw MJ, Debao L, and Talbot NJ (2003) The glyoxylate cycle is required for temporal regulation of virulence by the plant pathogenic fungus *Magnaporthe grisea*. *Mol Microbiol* 47, 1601–1612.

Wisplinghoff H, Bischoff T, Tallent SM, Seifert H, Wenzel RP, and Edmond MB (2004) Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. *Clin Infect Dis* 39, 309−317.

Woehlecke H, Osada H, Herrmann A, and Lage H (2003) Reversal of breast cancer resistance protein–mediated drug resistance by tryprostatin A. *Int J Cancer* 107, 721–728.

Wright AE, Forleo DA, Gunawardana GP, Gunasekera SP, Koehn FE, and McConnell OJ (1990) Antitumor tetrahydroisoquinoline alkaloids from the colonial ascidian *Ecteinascidia turbinata*. *J Org Chem* 55, 4508−4512.

Xie Z, Zhang A, Ye L, and Feng ZG (2009) Organo-and hydrogels derived from cyclo(L-Tyr-L-Lys) and its ε-amino derivatives. *Soft Matter* 5, 1474–1482.

Yan P, Song Y, Sakuno E, Nakajima H, Nakagawa H, and Yabe K (2004) Cyclo(L-Leucyl-L-Prolyl) produced by *Achromobacter xylosoxidans* inhibits aflatoxin production by *Aspergillus parasiticus*. *Appl Environ Microbiol* 70, 7466–7473.

Yap TA, Carden CP, and Kaye SB (2009) Beyond chemotherapy: targeted therapies in ovarian cancer. *Nat Rev Cancer* 9, 167−181.

Zotchev SB (2012) Marine actinomycetes as an emerging resource for the drug development pipelines. *J Biotechnol* 158, 168−175.

Zou H, Fang HM, Zhu Y, and Wang Y (2010) *Candida albicans* Cyr1, Cap1 and G-actin form a sensor/effector apparatus for activating cAMP synthesis in hyphal growth. *Mol Microbiol* 75, 579–591.

Abstract in Korean

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해양의 독특한 환경(고압, 낮은 산소농도, 암흑상태)은 현재까지 밝혀지지 않은 다양한 종들의 생물들이 서식하고 있으며 이 다양한 생물종으로부터 구조적으로 특이성이 높으며 생리활성이 뛰어난 천연물들이 발견되고 있다. 특히 해양방선균의 경우 다른 생물종들과 달리 장기보관의 용이성, 유전자 조작으로 인한 천연물 생산성 향상이 가능한 점 및 대량배양이 가능한 점의 특징이 있기에 천연물 연구의 중요 자원으로 현재까지 활용되고 있다. 본 연구에서는 해양방선균 *Streptomyces* 균주로부터 hydroxyquinoline 계열의 물질과 diketopiperazine 계열의 대사산물을 분리 정제하였고 이들의 생리활성 측정 및 그 작용기전을 규명하였다.

첫번째 파트에서는 해양방선균인 *Streptomyces* sp. MBTG13의 쌀배지 배양액으로부터 hydroxyquinoline계열의 대사산물을 분리하였으며 이 물질의 *Candida albicans*의 균사 성장 저해 활성 및 작용기전을 규명하였다. *C. albicans*는 사람에게서 병원성을 띄는 대표적인 기회감염성 진균으로 다양한 환경 변화(혈장, 37 °C, 질소원 고갈, > pH 7.0)에 의하여 효모 및 균사형의 형태변환을 일으키는 특징을 지니고 있다. 특히 균사형의 형태변환 조절 기전은 인체 내 병원성 유발기전에 밀접한 연관이 있는 것으로 알려져있다. 본 연구에서 분리 및 정제한 hydroxyquinoline은 항박테리아 성장 저해 활성을 지니고 있음을 확인하였다. 분리한 2-alkyl-4-hydroxyquinoline이 *C. albicans*의 quorum sensing 화합물인 farnesoic acid 및 farnesol 등과 구조적 유사성을 나타내기에 위 물질이 *C. albicans*의 형태변환 저해 활성을 나타낼 것이라 가정하였고 그 저해 활성을 측정한 결과 *C. albicans*의 균사성장을 억제함을 확인하였다 (IC_{50} = 11.4 μg/mL). 균사성장 환경에서 *C. albicans* 형태 변환시 주요 신호경로인 MAPK 경로 및 cAMP-PKA 경로 내 균사성장에 필수적인 유전자

들을 대상으로 유전자 발현 분석을 통하여 2-alkyl-4-hydroxyquinoline이 cAMP-PKA 경로에 작용하는 것을 확인하였다.

두번째 파트에서는 해양방선균 *Streptomyces puniceus*로부터 diketopiperazine계열의 대사산물을 분리 및 정제하였으며 이 물질이 C. albicans내 항진균 표적분자인 isocitrate lyase (ICL) 를 저해함을 확인하였다. Glyoxylate cycle은 당류가 부족한 환경에서 미생물이 C_2 탄소원을 이용하여 에너지 획득 및 TCA 회로의 중간물질을 공급하는데 있어서 매우 중요한 대사경로이다. ICL은 이 회로의 주요 구성 효소이며 인체 병원균의 병원성유발에 중요한 역할을 하는 것이 보고되었다. 본 연구에서는 *C. albicans* 유래의 ICL 저해제를 찾고자 하였으며 그 결과 분리한 cyclo(L-Phe-L-Val)이 ICL 저해활성이 나타냄을 확인하였다 (IC_{50} = 27 μg/mL). Cyclo(L-Phe-L-Val) 처리시 ICL 유전자의 야생형과 결손 돌연변이의 성장 표현형 및 ICL 유전자 발현수준을 측정하였다. 그 결과 Cyclo(L-Phe-L-Val)은 ICL 유전자의 발현을 억제하는 작용기전을 통해 ICL 효소저해활성을 나타냄을 확인하였다.

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Publications

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Heegyu Kim, Ji-Yeon Hwang, Jongheon Shin, and Ki-Bong Oh (2019) Inhibitory effects of diketopiperazines from marine-derived *Streptomyces puniceus* on the isocitrate lyase of *Candida albicans*. *Molecules*, 24, 2111.

Chan-Hong Ahn, Tae Hyung Won, **Heegyu Kim**, Jongheon Shin, and Ki-Bong Oh (2013) Inhibition of *Candida albicans* isocitrate lyase activity by cadiolides and synoilides from the ascidian *Synoicum* sp. *Bioorg Med Chem Lett* 23, 4099−4101.

Munhyung Bae, **Heegyu Kim**, Yoonho Shin, Byung Yong Kim, Sang Kook Lee, Ki-Bong Oh, Jongheon Shin, and Dong-Chan Oh (2013) Separacenes A–D, novel polyene polyols from the marine Actinomycete, *Streptomyces* sp. *Mar Drugs* 11, 2882−2893.

Ju-Eun Jeon, Lijuan Liao, **Heegyu Kim**, Chung J Sim, Dong-Chan Oh, Ki-Bong Oh, and Jongheon Shin (2013) Cytotoxic diterpenoid pseudodimers from the Korean sponge *Phorbas gukhulensis*. *J Nat Prod* 76, 1679−1685.

Soohyun Um, Tae Joon Choi, **Heegyu Kim**, Byung Yong Kim, Seong-Hwan Kim, Sang Kook Lee, Ki-Bong Oh, Jongheon Shin, and Dong-Chan Oh (2013) Ohmyungsamycins A and B: cytotoxic and antimicrobial cyclic peptides produced by *Streptomyces* sp. from a volcanic island. *J Org Chem* 78, 12321−12329.

Jung-Kyun Woo, Chang-Kwon Kim, Seong-Hwan Kim, **Heegyu Kim**, Dong-Chan Oh, Ki-Bong Oh, and Jongheon Shin (2014) Gombaspiroketals A–C, Sesterterpenes from the Sponge *Clathria gombawuiensis*. *Org Lett* 16, 2826−2829.

So-Hyoung Lee, Kyuho Moon, **Heegyu Kim**, Jongheon Shin, Dong-Chan Oh, and Ki-Bong Oh (2014) Bahamaolide A from the marine-derived *Streptomyces* sp. CNQ343 inhibits isocitrate lyase in *Candida albicans*. *Bioorg Med Chem Lett* 24, 4291−4293.

So-Hyoung Lee, Tae Hyung Won, **Heegyu Kim**, Chan-Hong Ahn, Jongheon Shin, and Ki-Bong Oh (2014) Suvanine sesterterpenes from a tropical sponge *Coscinoderma* sp. inhibit isocitrate lyase in the glyoxylate cycle. *Mar Drugs* 12, 5148−5159.

Munhyung Bae, **Heegyu Kim**, Kyuho Moon, Sang-Jip Nam, Jongheon Shin, Ki-Bong Oh, and Dong-Chan Oh (2015) Mohangamides A and B, new dilactone-tethered pseudo-dimeric peptides inhibiting *Candida albicans* isocitrate lyase. *Org Lett* 17, 712−715.

Woo-Young Yang, Chang-Kwon Kim, Chan-Hong Ahn, **Heegyu Kim**, Jongheon Shin, and Ki-Bong Oh (2016) Flavonoid glycosides inhibit sortase A and sortase A-mediated aggregation of *Streptococcus mutans*, an oral bacterium responsible for human dental caries. *J Microbiol Biotechnol* 26, 1566−1569.

Chan-Hong Ahn, Sohyoung Lee, Eunji Cho, **Heegyu Kim**, Beomkoo Chung, Wanki Park, Jongheon Shin, and Ki-Bong Oh (2017) A farnesoic acid-responsive transcription factor, Hot1, regulates yeast-hypha morphogenesis in *Candida albicans*. *FEBS Lett* 591, 1225−1235.

Chang-Kwon Kim, Riswanto Riswanto, Tae Hyung Won, **Heegyu Kim**, Berna Elya, Chung J Sim, Dong-Chan Oh, Ki-Bong Oh, and Jongheon Shin (2017) Manzamine alkaloids from an *Acanthostrongylophora* sp. Sponge. *J Nat Prod* 80, 1575−1583.

Oh-Seok Kwon, Donghwa Kim, **Heegyu Kim**, Yeon-Ju Lee, Hyi-Seung Lee, Chung J. Sim, Dong-Chan Oh, Sang Kook Lee, Ki-Bong Oh, and Jongheon Shin (2018) Bromopyrrole Alkaloids from the Sponge *Agelas kosrae*. *Mar Drugs* 16, 513.

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