Caryolan-1-ol, an antifungal volatile produced by *Streptomyces* spp., inhibits the endomembrane system of fungi

Gyeongjun Cho¹, Junheon Kim², Chung Gyoo Park¹, Corey Nislow³, David M. Weller⁴ and Youn-Sig Kwak¹

¹Division of Applied Life Science (BK21Plus), Institute of Agriculture and Life Science, Gyeongsang National University, Jinju 52828, Republic of Korea
²Forest Insect Pests and Diseases Division, National Institute of Forest Science, Seoul 02455, Republic of Korea
³Pharmaceutical Sciences, University of British Columbia, Vancouver, British Columbia, Canada
⁴US Department of Agriculture, Agricultural Research Service, Wheat Health, Genetics, and Quality Research, Pullman, WA, USA

Received: 29 March 2017
Accepted: 22 June 2017

**1. Introduction**

The genus *Streptomyces* belongs to the phylum Actinobacteria and constitutes a group of soil-dwelling bacteria. *Streptomyces* exhibits morphological differentiation and is able to grow aerial hyphae and spore chains. Certain *Streptomyces* species and isolates are able to inhibit plant and animal pathogens from a wide range of terrestrial and aquatic environments [1]. This environmental flexibility has been attributed to the capacity of *Streptomyces* spp. to produce a wide diversity of secondary metabolites, which have been sources for new drugs with antiviral, antitumoral, antihypertensive, antibiotic and immunosuppressant activity [2–4]. These metabolites have been found in approximately 500 different groups of compounds from thousands of different strains of *Streptomyces*. About 10 400 of the known 33 500 bioactive microbial metabolites produced by microorganisms have been isolated from *Streptomyces* [5] and more than 60% of all antibiotics discovered have come from *Streptomyces* [6]. The genus *Streptomyces* is the largest source of bioactive metabolites, but the proportion of newly discovered metabolites from *Streptomyces* is decreasing [5]. For this reason, volatiles produced by *Streptomyces* have recently attracted much attention for their biomedical potential.

Volatile compounds are easily converted from liquids or solids into gas as a consequence of their low molecular weight (less than 300 Da) and high vapour pressure (0.01 kPa at 20 °C). Because these properties allow them to diffuse easily, many bacterial volatiles function in competition, signalling and cooperation, but...
production is subject to environmental conditions. Bacterial volatiles belong to distinct compound classes, e.g. alkenes, alcohols, ketones, terpenes, benzenoids, pyrazines, acids and esters [7]. The biosynthesis of volatiles is generally linked to catabolic pathways including glycolysis, proteolysis and lipolysis [8,9]. Some volatiles also influence differentiation, growth, movement and stress response of plants, animals and fungi. For this reason, bacterial volatiles are of considerable research interest for a variety of applications. The volatiles dimethyl disulfide and dimethyl trisulfide have been reported to have powerful antifungal activity [10,11], however their exact mode(s) of action have not been determined at the molecular level [12–15].

In 1996, the Saccharomyces cerevisiae genome project was completed and a deletion mutant collection was developed. The development of molecular genetics has made the functional effect of chemicals in genetics easier to study using S. cerevisiae. Homozygous profiling (HOP) uses a pool of approximately 4700 diploid strains that deletes both copies of non-essential genes and predicts target pathway information of chemicals [16]. The pathways of drugs such as wortmannin, benomyl, tunicamycin, rapamycin, sulfometuron methyl, flucnazole, cycloheximide, FK506, caffeine, hydroxyurea and benomyl, tunicamycin, rapamycin, sulfometuron methyl, flucnazole, cycloheximide, FK506, caffeine, hydroxyurea were predicted in S4–7 and S8 (GenBank Accession: GCA_000932225.1 for S4–7, CP015362 for S8). Geosmin (RT 14.2 min), caryolan-1-ol (RT 16.4 min) and torreyol (RT 17.2 min) were predicted in S4–7 and S8 (RT 14.2 min) [17], methyl methanesulfonate [18] and their potential targets have been discovered using HOP.

The aims of this study were to identify volatiles in Streptomyces with antifungal activity and to describe their mechanism(s) of action.

2. Results

2.1. Antifungal volatiles emitted by Streptomyces spp.

To measure the antifungal activity of Streptomyces volatiles, Botrytis cinerea, Colletotrichum gloeosporioides, Fusarium oxysporum, Gibberella moniliformis, Phytophthora nicotinae, Rhizoctonia cerealis and R. solani were exposed to volatiles from Streptomyces strains S2, S4–7 and S8 (electronic supplementary material, figure S1). Mycelial growth of B. cinerea, C. gloeosporioides, P. nicotinae and R. solani was clearly inhibited by the S4–7 and S8 volatiles. Grey mould disease, caused by B. cinerea, of strawberry was significantly reduced when berries were stored with S4–7 cultured plates at room temperature (figure 1). With the treatment of strain S4–7, B. cinerea disease sign ratio was 0.00 ± 0.00, while that of the control was 0.76 ± 0.40 (p = 0.026, t-test).

2.2. Analysis of Streptomyces spp. volatiles’ main components

The volatiles produced by strains S2, S4–7 and S8 were identified by solid phase micro-extraction (SPME) and gas chromatography–mass spectrometry (GC-MS) analysis. The volatiles of S4–7 and S8 contain three main compounds with the same retention times (RTs)—14.2, 16.4 and 17.0 min, respectively. However, S2 produced only one volatile in common with strains S4–7 and S8 (RT 14.2 min) (electronic supplementary material, figure S2). Mass spectra of the three compounds matched geosmin (RT 14.2 min), caryolan-1-ol (RT 16.4 min) and torreyol (RT 17.2 min) (electronic supplementary material, table S1) in the mass spectral library. Geosmin is produced by many Streptomyces spp. and has a distinct earthy odour.

The main volatile components were predicted to be sesquiterpenes due to the three-isoprene terpene units in their chemical structures. Analysis of the KEGG database showed that S4–7 had a sesquiterpene synthetic pathway through the MEP/DOXY pathway (electronic supplementary material, figure S3). By antiSMASH, terpene synthesis gene clusters were predicted in S4–7 and S8 (GenBank Accession: GCA_000932225.1 for S4–7, CP015362 for S8). Geosmin synthase, caryolan-1-ol synthase and putative sesquiterpene cyclase were selected (table 1). Streptomyces griseus has been reported to produce caryolan-1-ol [19]. The GC-MS retention times (figure 2a) and mass spectra patterns (figure 2b) of naturally produced caryolan-1-ol and synthesized caryolan-1-ol were identical.

2.3. Antifungal activity of caryolan-1-ol

Geosmin is known to have no antibiotic activity [20], but no information is available about the bioactivity of caryolan-1-ol. The collected mixture A, with caryolan-1-ol and the unknown sesquiterpene (electronic supplementary material, table S2) as main constituents (electronic supplementary material, table S1) in the mass spectral library.
material, figure S4), inhibited *B. cinerea* mycelial growth at 20 and 40 μl of mixture A. Synthetic caryolan-1-ol (0.005–0.075 μmol ml⁻¹) inhibited mycelial growth in a dose-dependent manner (figure 3). Half maximal inhibitory concentration (IC₅₀) of the synthetic caryolan-1-ol was 0.026 μmol ml⁻¹ for *B. cinerea* after 4 days of exposure.

### 2.4. Mode of action of caryolan-1-ol antifungal activity

For HOP assay, growth of the diploid strain *S. cerevisiae* BY4743 was measured with different concentrations of caryolan-1-ol (electronic supplementary material, figure S5). *Saccharomyces cerevisiae* growth was reduced by 4.28%, 11.80% and 30.80% by 0.063, 0.125 and 0.250 μmol ml⁻¹ caryolan-1-ol, respectively. A threshold for the HOP screen with caryolan-1-ol (0.188 μmol ml⁻¹) was defined with a fitness defect of 3.3 or greater (figure 4). Based on these criteria, caryolan-1-ol affected 33 deletion strains (electronic supplementary material, table S3). Based on Gene Ontology (GO) enrichment, caryolan-1-ol affected the following biological functions: lipid synthesis (*sur4*, *scs7* and *bro1*); DNA replication stress response (*ste11*, *mgs1*, *cot1* and *csg2*); and transported organelles related to endosomes, vesicles, vacuoles; Golgi and endoplasmic reticulum (*did4*, *vid22*, *vps4*, *sip3*, *cot1*, *vma9*, *vps52*, *eug1*, *vms1*, *rer1* and *csg2*).

Using network analysis and GO provided by the Saccharomyces Genome Database (SGD), the 15 most sensitive deletion strains corresponded to genes associated with the sphingolipid metabolic process (*sur4*, *scs7* and *bro1*); cellular response to osmotic stress (*smp1* and *ste11*); and late endosome vacuole transport (*bro1*, *did4*) (figures 5 and 6). Comprehensive GO process results were categorized in the electronic supplementary material, figure S6. The GO component ‘Endomembrane system’ was shared by 14 of the 33 significantly sensitive strains (table 2). Collectively, these observations suggested that caryolan-1-ol affects fungal growth and probably acts by affecting membrane lipid processes and the intercellular transport system in fungi (figure 6).

### 3. Discussion

This study was designed to characterize antifungal volatiles in *Streptomyces*, to identify new antifungal volatile components
and to analyse the genetic effects of these volatile components on fungi. The antifungal test demonstrated that *Streptomyces* produces antifungal volatiles against a variety of phytopathogenic fungi in different classes. Hora & Baker [14] reported that volatiles of certain *Streptomyces* strains inhibited growth of *Trichoderma viride*, *Zygorhynchus vuilleminii*, *Gonatobotrys simplex* and *Cladosporium* sp. The grey mould pathogen *B. cinerea* is visibly ashy when spores are generated. However, the pathogen on strawberry that was exposed to S4–7 volatiles did not form spores and its growth was inhibited. GC-MS analysis using strains S2, S4–7 and S8 indicated that the three major components are terpenes: geosmin, an unknown sesquiterpene and caryolan-1-ol. In the secondary metabolism analysis of the genome, three terpene synthetic clusters corresponding to the GC-MS analyses were also confirmed. These results indicate that caryolan-1-ol is produced by strains S4–7 and S8; the GC-MS results comparing S4–7 volatiles and synthetic caryolan-1-ol were an identical match. Our results support previous studies that showed caryolan-1-ol was released by specific *Streptomyces* strains, including *S. griseus* [19,21]. In addition, caryolan-1-ol has been found in several plants and fungi [22–28]. These types of caryolan-1-ol can consist of 32 enantiomers, but only three have been documented: (+)-caryolan-1-ol (*S. griseus*),

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**Figure 2.** GC-MS (GC-2010 coupled with GCMS-QP2010 plus) analysis of S4–7 volatiles and synthetic caryolan-1-ol. (i) Geosmin; (ii and iv) caryolan-1-ol; (iii) unknown sesquiterpene. (a) GC of total ion current of synthetic caryolan-1-ol. S4–7 volatile and synthetic caryolan-1-ol have identical retention times (24 min). (b) Mass spectra comparison of S4–7 volatiles and synthetic caryolan-1-ol. Synthetic caryolan-1-ol (iv) mass spectrum coincides with S4–7 caryolan-1-ol (ii).
(-)-caryolan-1-ol (Zingiber officinale and Hardwickia pinnata) and 1R-caryolan-1-ol (Aspergillus niger). Since the S4–7 and S8 genomes have (+)-caryolan-1-ol synthase, they may produce only (+)-caryolan-1-ol.

Although the synthesized caryolan-1-ol has not been characterized as a type of optical isomer, B. cinerea growth was inhibited by the isolated and the synthesized caryolan-1-ol. These similar antifungal activities between the Streptomyces

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**Figure 3.** Growth inhibition of B. cinerea by synthetic caryolan-1-ol. (a) Growth ratio using box-and-whisker plots. (b) Growth of B. cinerea at 4 days. Acetone (10 μl) was used as a control treatment. Synthetic caryolan-1-ol inhibited the growth and spore formation.

**Figure 4.** Genes affected by caryolan-1-ol. Thirty-three deletion strains with a fitness defect score of 3.3 or greater were selected.
mixture of volatiles and the synthesized caryolan-1-ol indicated that the antifungal activity was caused by the caryolan-1-ol. Inhibition by caryolan-1-ol coincided well with an earlier report on unknown volatiles from *S. griseus* affecting fungal growth and sporulation of *Gloeosporium aridum* [12].

To investigate the biological functions of caryolan-1-ol, the screening of affected genes was conducted by HOP assay. It was shown that 33 homozygous deletion strains were susceptible to caryolan-1-ol with a fitness defect score of 3.3 or higher. Fourteen of the 33 strains were deleted for genes associated with the endomembrane system, whose role it is to separate the structural compartments or organelles, including the nuclear envelope, endoplasmic reticulum, Golgi apparatus, vacuoles, vesicles, Spitzenkörper and plasma membrane. The fitness defects in the top 15 strains were related to sphingolipid metabolic processes, response of osmotic stress and late endosome to vacuole transport. The cellular process results suggested that these genes were related to sphingolipid synthesis, vesicle transport, vesicle trafficking and maintenance.

Sphingolipids, which are classified as ceramides, sphingomyelins and glycosphingolipids, are especially important because they constitute 40% of plasma membranes in yeast [29,30]. This supports our observation from the HOP results that caryolan-1-ol inhibited the sensitivity strains that were deleted for osmotic stress response genes (*smp1* and *ste11*). It has been documented that sphingolipids are involved in numerous cellular processes including cell senescence, cell differentiation, apoptosis, cell-cycle arrest, cell proliferation, etc. [31] *sur4* (ola3) synthesizes a ceramide molecule which is the backbone of sphingolipids [32,33]. Trajkovic *et al.* [34] reported that ceramide is required for budding exosome into multi-vesicular endosome formation in fungi. The selected *scs7* and *csg2* are known to synthesize α-hydroxy-phytoceramide and mannosyl-inositol-phosphoryl-ceramide (MIPC), which are made from ceramide and phytoceramide [33,35]. The MIPC is required for proper localization of plasma membrane proteins using vesicle trafficking as well as maintenance of cell morphology and vacuoles [36]. Based on these

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**Figure 5.** Network analysis of high defect score for the top 15 genes. Visualization was performed by GeneMANIA (http://genemania.org). Genes are functionally grouped into sphingolipid, cellular response to osmotic stress and late endosome to vacuole transport.

**Figure 6.** Caryolan-1-ol affects very long chain fatty acid and ceramide-based lipid biosynthesis pathways. The illustration shows the sphingolipid synthesis pathway in yeast. Blue lines show synthetic pathway and red letters indicate genes with high fitness defect score (greater than 3.3) in figure 4.
previous studies, caryolan-1-ol may also interrupt other GO processes such as vesicle transport, vesicle trafficking and maintenance of location that are linked to sphingolipid metabolism. The vesicle supply at the fungal hypha tip and Spitzenkörper are key components of the endomembrane system. Botrytis cinerea growth inhibition probably occurred due to fungal Spitzenkörper damage.

In conclusion, this is the first report on the biological role of caryolan-1-ol, a volatile produced by Streptomyces. It has antifungal properties that are caused by perturbation of the endomembrane system in fungi. Further work will help dissect the mechanism by which this volatile affects sphingolipid metabolism, vesicle formation, vesicle trafficking and membrane localization.

4. Material and methods

4.1. Antifungal test of Streptomyces volatiles

Streptomyces sp. strain S2, Streptomyces sp. strain S8, and S. griseus strain S4–7 were isolated from the rhizosphere of a 15-year old strawberry field [37]. Streptomyces sp. strains S2 and S8 were isolated from the turfgrass rhizosphere [38]. Streptomyces spp. were cultured for 10 days at 27 °C in potato dextrose peptone (PDK) agar media (per 1 l: potato dextrose: 10 g; peptone: 10 g; agar: 20 g) in 90 °C15 mm Petri dishes. Botrytis cinerea, C. gloeosporioides, F. oxysporum, G. moniliformis, P. nicotinae, R. cerealis and R. solani were inoculated on PDK agar media in 90 °C15 mm Petri dishes.

To test the antifungal activity of the volatiles of Streptomyces spp., fungal plates and Streptomyces spp. plates without the lids were put into a 2.7 l airtight container and incubated at 27 °C. Fungal mycelial growth was measured every day in the same container with four replicates for each treatment.

4.2. Test for controlling postharvest disease by Streptomyces volatiles

Nine strawberries and six 5-day old S4–7 PDK plates were in the airtight containers. The PDK plates were located in the bottom of the container and the strawberries were put on top of the PDK plates. The containers were incubated at room temperature for 5 days. The pixels with no signs were counted using a quick selection tool and histogram bar window to display the number of pixels in PHOTOSHOP CS5. The sign ratio could be described using the following equation:

\[
\text{Sign ratio} = \frac{\text{normal pixels of 1 day}}{\text{normal pixels of 5 days}}
\]

The statistical analyses of sign ratios were performed using the Shapiro–Wilk’s normality test, Bartlett test of homogeneity of variances and two sample t-tests using R program v. 3.2.3.

4.3. Gas chromatography–mass spectrometry

Streptomyces spp. were cultured at 27 °C for 10 days in PDK agar media in 60 × 15 mm Petri dishes. The Petri dishes containing the Streptomyces cultures were put into a glass jar, and the volatiles from the Streptomyces spp. were collected using SPME at 80 °C for 30 min. The fibres of SPME were composed of divinylbenzene/carboxen/polydimethylsiloxane. The volatiles
collected from the SPME fibres were analysed by electron impact gas chromatography–mass spectrometry (EI-GC-MS; GC2010 plus-GCMS-TQ8030; Shimadzu, Tokyo, Japan) equipped with a Rtx-5MS column (30 m × 0.25 mm i.d. × 0.25 μm film thickness, Restek Co., PA, USA). The carrier gas was programmed as 1.1 ml min⁻¹ for an initial 2 min, and then gradually decreased to 0.5 ml min⁻¹ for 20 min and held there. The oven temperature was maintained at 50°C for 2 min, and then increased to 250°C at 10°C min⁻¹ with that temperature being held for 8 min. The temperatures of the interface and ion source were set at 230°C and 280°C, respectively.

Volatiles collected from *Streptomyces* spp. and the synthesized caryolan-1-ol was analysed by GC-MS (GC-2010 coupled with GCMS-QP2010 plus; Shimadzu, Tokyo, Japan) using HP-Innowax (30 m × 0.25 mm × 0.25 μm film thickness; J&W Scientific). The oven temperature was maintained at 40°C for 1 min, then raised to 250°C at 6°C min⁻¹ and held for 4 min. Helium was the carrier gas at a flow rate of 1.0 ml min⁻¹.

4.4. Secondary metabolism pathway analysis of the S4-7 and S8 genomes

The S4-7 and the S8 genomic sequences were examined by CLGENOMICS (v. 1.53; ChunLab, Seoul, South Korea), which supports the secondary metabolism pathway analysis using the KEGG database. Gene cluster analysis used the secondary metabolism finder tool antiSMASH (https://antismash.secondarymetabolites.org/).

4.5. Mass collection of volatiles of *Streptomyces griseus* S4-7

*Streptomyces griseus* S4-7 was cultured for 10 days at 27°C in PDK agar media in 90 × 15 mm Petri dishes. To collect a large amount of the *S. griseus* S4-7 volatile compounds, 20 Petri dishes were inoculated with *S. griseus* S4-7 and then put into a container (6 l). The volatiles were collected with an absorbent, Super Q 100 (approx. 100 mg, 2 cm in length, 6 mm OD in a glass tube; Alltech, Deerfield, Illinois, USA), connected to an air pump. Charcoal-filtered air was introduced into the glass tube; Alltech, Deerfield, Illinois, USA), connected to an air pump. Charcoal-filtered air was introduced into the glass tube. Alltech, Deerfield, Illinois, USA), connected to an air pump. Charcoal-filtered air was introduced into the glass tube. The oven temperature was maintained at 40°C for 1 min, then raised to 250°C at 6°C min⁻¹ and held for 4 min. Helium was the carrier gas at a flow rate of 1.0 ml min⁻¹.

4.6. Synthesis of caryolan-1-ol

Caryolan-1-ol was synthesized by modifying the method of Iwamuro et al. [39]. β-Carophyllene (3.0 g; 15 mmol, TCI, Tokyo, Japan) was dissolved in acetic acid (100 ml) with BHT (butylated hydroxytoluene; Sigma-Aldrich). The solution was passed twice through a column packed with Amberlyst 15 (Alfa Aesar, Lancaster, UK) at a rate of 2–3 ml min⁻¹. The solution was diluted with ether and then washed five times with 2N NaOH, followed by washing with water and brine, and dried. After removing the solvent, the residue was subjected to silica gel column chromatography (Wakogel-200) to isolate the caryolan-1-ol (45% yield, 53% purity). Further purification using a fraction collector was done and 94.0% pure caryolan-1-ol (100 mg) was obtained. The caryolan-1-ol was confirmed via nuclear magnetic resonance (NMR) and GC-MS. NMR spectra agreed with those published in previous reports [40,41]. 1H and 13C NMR (500 and 125 MHz, respectively) analysis was conducted with a Bruker DRX-500 spectrometer using TMS in CDCl₃ as an internal standard at GNU’s Center for Research Facilities.

4.7. Antifungal test of collected volatiles and synthetic caryolan-1-ol

To test antifungal activity, the volatile mixture concentration of S4-7 was prepared at 5 μg μl⁻¹ and 40, 20 and 10 μl samples used. The synthetic caryolan-1-ol was diluted to 0.075, 0.038, 0.019, 0.009 and 0.005 μmol ml⁻¹. A paper disc (8 mm) treated with a fraction of volatiles mixture or synthesized caryolan-1-ol was put in the lid of the Petri dish cover and *B. cinerea* was grown on the agar at 27°C. After 4 days of incubation, mycelial growth was measured.

4.8. Homozygous profiling assay

To discover drug effects, the IC₂₀ is generally used for the HOP assay [42]. The caryolan-1-ol IC₂₀ was determined by the following approach. *S. cerevisiae* BY4743 was inoculated in yeast extract peptone dextrose (YPD; 10 g yeast extract, 20 g peptone, 20 g dextrose per l) broth in a 24-well plate. Synthesized caryolan-1-ol was added at 250, 125 and 62.5 nmol. Growth of BY4741 was measured by absorbance at 600 nm every 15 min for 24 h at 30°C. The IC₂₀ was calculated to be 188 nmol ml⁻¹. The homozygous diploid deletion strains pool (approx. 4800 strains) was inoculated in the caryolan-1-ol (188 nmol ml⁻¹) amended and non-amended YPD broth at 30°C for 24 h. Total genomic DNA of the pools was extracted using the YeaStar™ Genomic DNA kit (Zymo Research Co, USA) and its uptags and downtags were amplified by PCR. Amplification quantification was performed with Affymetrix TAG4 arrays [42]. Caryolan-1-ol sensitive strains were selected using fitness defect score expressed as the log 2 ratios. Deletion genes of these strains were analysed by GenMania (http://genemania.org), Saccharomyces Genome Database (SGD) Gene Ontology (GO) term finder and BLAST2GO (v. 4.0.7).

Data accessibility. Additional data are available in the electronic supplementary material.

Authors’ contributions. G.C., J.K. and G.P performed MS/MS analysis and synthesized molecules. G.C. and C.N. conducted chemical-genetic experiments. W.D and Y.K. designed the research and wrote the manuscript.

Competing interests. The authors declare no competing interests.

Funding. This research was supported by the Next-Generation BioGreen 21 Program (PJ011800).
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