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ORIGINAL ARTICLE

Physiological role of germicidins in spore germination and hyphal elongation in *Streptomyces coelicolor* A3(2)

Yuu Aoki[1,4], Daisuke Matsumoto[2], Hiroshi Kawaide[3] and Masahiro Natsume[3]

Four germicidin homologs were isolated from a liquid culture of *Streptomyces coelicolor* A3(2). These were identified as germicidins A, B and C, and surugapyrone A (germicidin D). Absolute stereochemistry of the chiral center in germicidins A and C is determined to be *S*. All germicidins inhibited germination of *S. coelicolor* A3(2) spores above $1 \mu g \, ml^{-1}$. *S. coelicolor* A3(2) spores collected from a single petri dish (9 cm i.d.) contained $5.4 \mu g$ of germicidin A ($\sim 2.7 \times 10^{-14}$ g per spore), which accounts for 2.3% of the spore extract, and contents of germicidins B, C and D were 0.2–$0.8 \mu g$. The activity of the spore extract corresponded well with the sum of the activity of each germicidin, which was estimated from the content and dose–response curve, which indicates that germicidins functions as self-germination inhibitors in *S. coelicolor* A3(2). Inhibitory action of germicidin A on spore germination was reversible and germicidin A inhibited not only spore germination but also hyphal elongation.

INTRODUCTION

Regulation of spore germination is an important step for survival of microorganisms. Hirsch and Ensign[1] examined the germination process of *S. viridochromogenes* spores morphologically and physiologically, and found that germination was accompanied by loss of spore refractility and a decrease in the optical density (OD) at 600 nm of a spore suspension during the first hour of incubation. They also found that a potent germination inhibitor was released from the germinated spores,[2] and investigated its properties and partially purified it.[3] The germination inhibitor was isolated from liquid cultures by Petersen *et al.*[4] and was named germicidin (**1**; absolute stereochemistry was unknown). Germicidin at $40 \, pg \, ml^{-1}$ (200 pM) inhibited the first event in spore germination, namely, a decrease in the OD of a spore suspension, and was detected in the culture supernatant of spores during germination. However, there is no information on whether germicidin is a self-inhibitor of spore germination, that is, whether its action is reversible and specific for spore.

Yoshida and Kobayashi[5] observed morphogenesis of the pathogenic *Streptomyces* sp. causing root tumor of melon by optical and scanning electron microscopy. They found that spores swell for the first hour, germination begins at 3 h and most spores germinate after 24 h

of incubation. However, about 10–20% of the spores remained ungerminated.[6] These spores can be activated by heat shock treatment at 40 °C for 20 min and this treatment was more effective in 0.025% SDS or 2% yeast extract solution, which caused almost all spores to germinate.[6]

On the basis of the results of Yoshida *et al.*, we predicted the presence of a germination self-inhibitor in the spores of this tumor-causing *Streptomyces* sp. We first searched for the inhibitor in liquid cultures because *Streptomyces* spores are very small and mass collection is laborious; we identified anthranilic acid as an inhibitor.[7] Anthranilic acid inhibited spore germination in a reversible manner and did not affect hyphal growth. Anthranilic acid was detected in the culture supernatant during germination, but its activity and content in spores were low. We thus judged that the contribution of anthranilic acid to the inhibition of spore germination is small. We then searched for an inhibitor in agar cultures, isolated 0.25 mg of an inhibitor from 3500 cultured petri dishes, and named this inhibitor hypnosin (**5**).[8,9] Hypnosin selectively inhibits spore germination and its inhibitory activity is removable by washing hypnosin-treated spores in water. Hypnosin showed activity at a concentration equivalent to that in the culture supernatant during germination; thus, we identified it as the self-inhibitor of the phytopathogenic *Streptomyces* sp.

[1]Science of Plant and Animal Production Course, United Graduate School of Agricultural Science, Tokyo University of Agriculture and Technology, Tokyo, Japan; [2]Computational Science and Engineering Solution Division, Technical Computing Solutions Unit, Fujitsu, Chiba, Japan and [3]Division of Bioregulation and Biointeraction, Graduate School of Agriculture, Tokyo University of Agriculture and Technology, Tokyo, Japan
[4]Current address: Life Science Research Institute, Kumiai Chemical Industry Co., Ltd., Kikugawa, Shizuoka 439-0031, Japan.
Correspondence: Professor M Natsume, Division of Bioregulation and Biointeraction, Graduate School of Agriculture, Tokyo University of Agriculture and Technology, 3-5-8, Saiwai-cho, Fuchu, Tokyo 183-8509, Japan.
E-mail: natsume@cc.tuat.ac.jp
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Song *et al.*[10] reported that the translational product of *sco7221* ORF in *S. coelicolor* A3(2) yielded four germicidin homologs. Their interests were focused on the selectivity of substrates and products of the enzyme, a type III polyketide synthase, and their report included no description of the physiological role of these homologs in the producing organism. We have therefore examined the germicidin content of the spores and their effect on germination of *S. coelicolor* spores.

MATERIALS AND METHODS

Microorganisms and media

S. coelicolor A3(2) was supplied by Dr Keith F Chater of the John Innes Centre, UK. A yeast-starch medium[5,6] consisting of yeast extract 0.2% and soluble starch 1.0% (pH 7.3) was used for the production of germicidins by *S. coelicolor* A3(2). For the collection of spores from *S. coelicolor* A3(2), a yeast extract-malt extract agar medium consisting of yeast extract 0.4%, malt extract 1.0%, glucose 0.4% and agar 2.0% (pH 7.3) was used, based on our previous results for aerial mycelium formation.[11]

Production, isolation and structural elucidation of germicidins

S. coelicolor A3(2) was inoculated into 500 ml of yeast-starch medium in a 2-l Erlenmeyer flask and incubated on a rotary shaker (180 r.p.m.) at 28 °C for 7 days. The culture broth was treated as shown in Figure 1. Final purification was performed with repeated preparative HPLC (column: Develosil ODS-UG 5, 5-μm particle size, 20×250 mm (Nomura Chemical, Aichi, Japan); solvent: 40–70% aq. MeOH containing 0.2% acetic acid (isocratic elution); flow rate: 10 ml min^{-1}; detection: UV at 290 nm).

The structures of germicidin homologs were confirmed by ^{1}H and ^{13}C NMR spectra measured by a JNM-A600 spectrometer (JEOL, Tokyo, Japan) using CDCl$_3$ or CD$_3$OD solutions and by mass spectra that were recorded by an LC-ESI-MS, JMS-T100LC AccuTOF spectrometer (JEOL, Tokyo, Japan) equipped with an Agilent 1100 LC system (Agilent, Tokyo, Japan). Optical rotation was measured by DIP-360 polarimeter (JASCO, Tokyo, Japan). Conformation search was performed with CONFLEX 3 program at MM2 force field. Geometry optimization and calculation of heat of formation were carried out with AM1 or PM5 method in SCIGRESS Version 2 (Fujitu, Chiba, Japan).
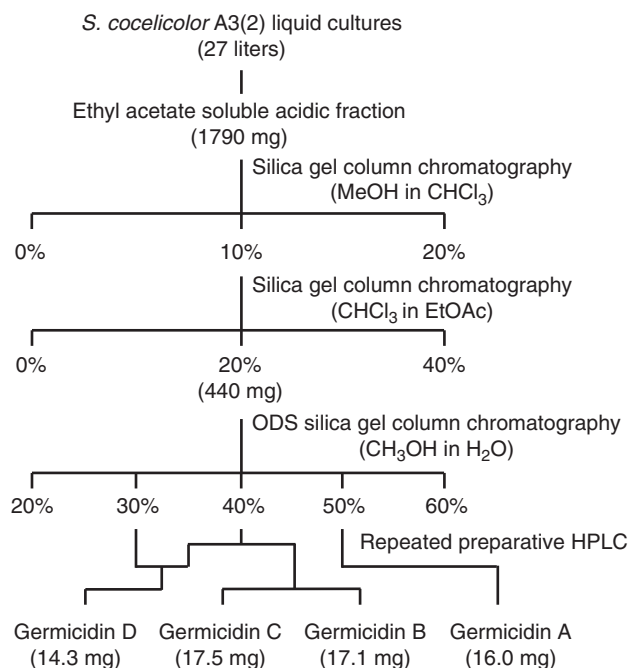
Figure 1 Isolation scheme of germicidins from *Streptomyces coelicolor* A3(2) liquid cultures.

Spore germination assay

Germination inhibitory activity of spores was calculated from OD at 595 nm of spore suspensions after 48 h incubation in a 96-well microplate in the presence or absence of germicidins, hypnosin and spore extract as previously reported.[7,8]

Quantitative estimation of germicidins and hypnosin in *S. coelicolor* spores

Spores of *S. coelicolor* A3(2) were collected by the glass-bead-based method originally described by DeJong and McCoy[12] and modified by Hirsch and Ensign;[1] spores were collected with glass beads (4-mm diameter) by rolling them over *S. coelicolor* A3(2) that had been cultured on yeast extract-malt extract agar medium. Spores and beads were then soaked in a mixture of H$_2$O–MeOH–CHCl$_3$ (4:10:5) for 48 h and the mixture was filtered to obtain an extract. The extract was concentrated *in vacuo* to remove organic solvent and the residual aqueous solution was partitioned with ethyl acetate at pH 3. The extract was applied to a Sep-Pak Plus C18 cartridge (360 mg of adsorbent, Waters, Milford, MA, USA) and the cartridge was washed with H$_2$O, then eluted with 40% aq. MeOH. The eluate was analyzed by a JEOL JMS-T100LC AccuTOF spectrometer equipped with an Agilent 1100 LC system (HPLC conditions, column: Develosil ODS-UG 5, 5-μm particle size, 2×150 mm; solvent: 40% aq. MeOH (0–5 min), a linear increase from 40% aq. MeOH to MeOH (5–17 min), MeOH (17–25 min) in the presence of 0.1% trifluoroacetic acid; flow rate: 0.2 ml min^{-1}).

Effect of germicidin A and hypnosin on hyphal growth and reversibility of germination inhibitory activity of germicidin A

These experiments were performed similarly to a previous report.[8]

RESULTS AND DISCUSSION

Preparation of germicidins

Four germicidin homologs (**1–4**) were isolated from 27 l of *S. coelicolor* A3(2) liquid cultures by monitoring them in each purification steps with their protonated molecules at *m/z* 197, 183 and 169 by LC-ESI-MS analysis (Figure 1). Yields of germicidin homologs (14–18 mg) were ~10 times higher than those in *S. viridochromogenes* NRRL B-1511 (1–2 mg from 25 l).[4] These homologs were identified as germicidins A (**1**, called 'germicidin' by Petersen *et al.*[4]), B (**2**) and C (**3**),[10] and surugapyrone A (**4**)[13] (we describe this compound as germicidin D for the sake of shorthand) by spectroscopic analysis (Figure 2).

We could not find the isogermicidins A and B reported by Song *et al.*[10] in our liquid cultures or spore extracts by LC-ESI-MS analysis of each fraction of ODS silica gel column chromatography (data not shown). This may be attributable to the difference in the culture medium used because the biosynthetic starter unit of isogermicidins is 3-methylbutyryl- or *n*-butyryl-CoA, which are different from those of germicidins A-D; Song *et al.* used supplemented minimal medium, which contains casamino acid as the amino acid source, whereas we cultured in yeast-starch medium, which contains yeast extract. A relaxed substrate specificity of the type III polyketide synthase[14] would account for the different results.

Chirality of the asymmetric carbon in germicidins A and C is determined to be *S* (Figure 2) by comparing the sign of [α]$_D$ of them (germicidin A: [α]$_D$ +22° (MeOH; *c* 0.10); C: [α]$_D$ +26° (MeOH; *c* 0.30) with those of myxopyronins A (**6a**) and B (**6b**) (myxopyronin A: [α]$_D$ −73° (MeOH; *c* 0.3); B: [α]$_D$ −75° (MeOH; *c* 0.2))[15] with the assistance of computational chemistry; conformation search and calculation of heat of formation of model compounds had the consequence that the side chain of myxopyronin does not exert influence on the sign of optical rotation. That is, stable conformers were sought by rotating the dihedral angle C4-C3-C1′-O in **7a** with CONFLEX program and four conformers were obtained. Geometry of each conformer was optimized with AM1 method in SCIGRESS, and dihedral angle and heat of formation were calculated. As a result,
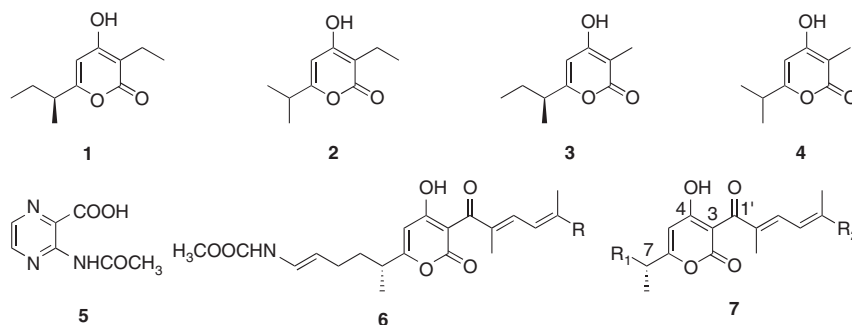
Figure 2 Structures of compounds. **1**: germicidin A, **2**: germicidin B, **3**: germicidin C, **4**: surugapyrone A (germicidin D), **5**: hypnosin, **6**: myxopyronin A (**6a**, R=n-C_3H_7) and B (**6b**, R=n-C_4H_9), **7**: model compound A (**7a**, R_1=CH_3, R_2=n-C_3H_7) and B (**7b**, R_1=n-C_3H_7, R_2=CH_3) for computer chemistry.

two conformers with the same heat of formation and absolute value of the dihedral angle were predominant (>99.8%); conformer A: dihedral angle; +14.43°, heat of formation; −149.2 kcal mol^{-1}, conformer B: dihedral angle; −14.73°, heat of formation; −149.2 kcal mol^{-1}. The geometry optimization with PM5 method also came to the same conclusion; abundance ratio of two predominant conformers; >99.6%, dihedral angle; +15.50°/−15.54°, heat of formation; −169.3 kcal mol^{-1}. The geometry optimization of **7b** with AM1 or PM5 method on the initial conformation setting the dihedral angle with +10° or −10° produced the same results; AM1 method: dihedral angle; +22.04/−22.05°, heat of formation; −143.1 kcal mol^{-1}, PM5 method: dihedral angle; +16.40°/−16.12°, heat of formation; −164.3 kcal mol^{-1}. These results show that cross-conjugated chromophore and α-pyrone ring of myxopyronin is not planar but two stable and predominant conformers balance the effect of twisted conformation on optical rotation. The sign of optical rotation thus is not affected by the side chain of myxopyronin and it depend only on the chirality at C7.

The absolute stereochemistry of germicidins A (**1**) and C (**3**) thus determined was opposite to that of phomapyrone C isolated from the phytopathogenic fungus *Leptosphaeria maculans*,[16] which has the same planar structure as germicidin C (**3**) by comparing their specific optical rotations (germicidin A: [α]$_D$ +19° (CHCl$_3$; *c* 0.13); germicidin C: [α]$_D$ +17° (CHCl$_3$; *c* 0.11); phomapyrone C: [α]$_D$ −16.1° (CHCl$_3$; *c* 0.28)[16]). Some cases have been reported of a prokaryote producing the enantiomer of a eukaryote's metabolite; a diketopiperazine produced by a fungus, *Emericella heterothallica* is the enantiomer produced by *Streptomyces noursei*.[17] An algal morphogenesis inducer, thallusin, produced by a marine bacterium, has a terpenoid skeletal structure that is the enantiomer of that produced by fungi or plants.[18–20] As the absolute stereochemistry must be regulated by the three dimensional conformation of the biosynthetic enzyme, comparative research of the enzymes in *Streptomyces* and fungi is of much interest.

Germination inhibitory activity of germicidins and hypnosin
Germination inhibitory activity of the four germicidins and hypnosin was examined with *S. coelicolor* A3(2) spores. All germicidin homologs inhibited spore germination above 1 μg ml^{-1} and no obvious difference was observed among homologs; IC$_{50}$ values of germicidins calculated by probit method are in the range from 20 (germicidin D) to 90 μg ml^{-1} (germicidin A; Figure 3).

Petersen *et al*.[4] reported that IC$_{50}$ of germicidin (**1**) on *S. viridochromogenes* NRRL B-1511 spore germination was ∼5 ng ml^{-1} and that its homolog (**2**) was biologically inactive, and **2** was not present in the germination fluid. One of the reason why sensitivity of germicidin
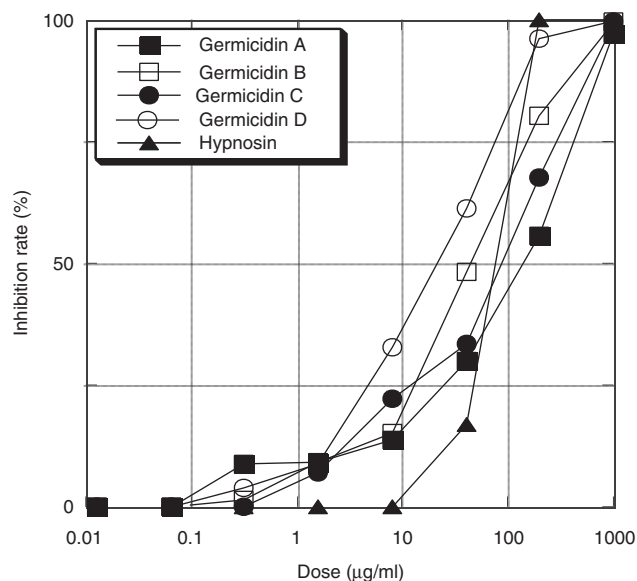
Figure 3 Germination inhibitory activities of germicidins and hypnosin on *S. coelicolor* A3(2) spores. Inhibition rate was calculated from eight samples. Error bars were omitted to facilitate visualization. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

(**1**) and structure–activity relationship of germicidin homologs is different between *S. coelicolor* and *S. viridochromogenes* spores is thought to be the difference in bioassay; Petersen *et al*. evaluate activity of germicidin by a decrease in the absorbance of the spore suspension due to swelling by water absorption; it is hour event of germination. In contrast, we examined the increase in OD due to emergence of the germ tube and its extension for 48 h. However, the dose–response curves, in which dose is expressed as petri dish equivalent, are similar between *S. coelicolor* (Figure 5) and *Streptomyces* sp. CB-1-1.[8] This can be interpreted as relatively large contents of germicidins with weak activity control germination of *S. coelicolor* spores and a small amount of hypnosin with strong activity operates in *Streptomyces* sp. CB-1-1. Our preliminary experiment showed that germicidin A did not inhibit germination of *S. viridochromogenes* JCM 4265 (=NRRL B-1511) spores at 40 μg ml^{-1} in our bioassay. Detailed comparative study of the action of germicidins on *S. coelicolor* and *S. viridochromogenes* is now in progress.

Hypnosin inhibited spore germination of *S. coelicolor* A3(2) with IC$_{50}$=100 μg ml^{-1}, which was somewhat weak compared with the activities of germicidins. *S. coelicolor* A3(2) was significantly less
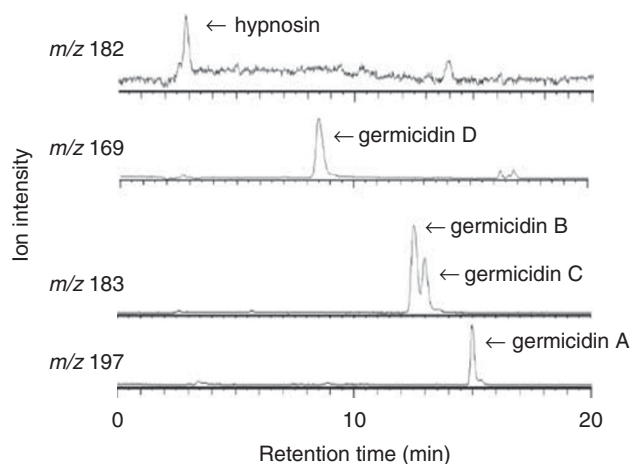
Figure 4 LC-MS analyses of germicidins and hypnosin in the extract of *S. coelicolor* A3(2) spores.

sensitive to hypnosin than *Streptomyces* sp. CB-1-1, from which hypnosin was originally isolated ($IC_{50}=0.25\,\mu g\,ml^{-1}$).[8,9]

### Quantitative estimation of germicidins and hypnosin in *S. coelicolor* spores

From 50 cultured petri dishes (9 cm i.d.) of *S. coelicolor* A3(2), $\sim 1.0\times10^{10}$ spores were collected by the glass-bead-based method and 11.9 mg of ethyl acetate-soluble extract was obtained. The content of germicidins and hypnosin in *S. coelicolor* A3(2) spores was determined by LC-ESI-MS analysis of the ethyl acetate-soluble extract. Germicidin A was the major component (5.4 μg in spores collected from a single petri dish (9-cm i.d.), $\sim 2.7\times10^{-14}$ g per spore) and it accounted for 2.3% of this spore extract (Figure 4). The content of germicidins B, C and D was 0.4×10^{-14} g, 0.1×10^{-14} g and 0.1×10^{-14} g per spore, respectively. Hypnosin also was detected in the extracts of *S. coelicolor* A3(2) spores at 0.2×10^{-14} g per spore, which is ~ 10 times higher than the amount in the spores of *Streptomyces* sp. CB-1-1.

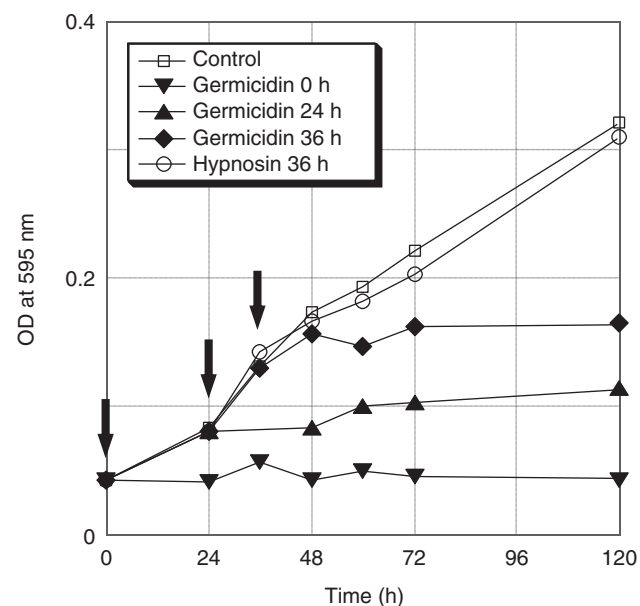### Germination inhibitory activity of the extract of *S. coelicolor* spores

Germination inhibitory activity of the spore extract of *S. coelicolor* A3(2) was examined with fivefold serial dilutions of the extract obtained from spores cultured on 10 petri dishes; the inhibition rate was determined based on the dose relative to the petri dish equivalent (Figure 5, line plot). The extract obtained from 10 petri dishes completely inhibited spore germination and inhibitory activity was detected at a dose equivalent to the extract obtained from 1/12.5 of a cultured petri dish.

Estimation of germination inhibitory activity from the amount of germicidin and hypnosin in spores

Contents of germicidins and hypnosin in the spore extract at each dose as shown in Figure 5 were calculated from the result of quantitative estimation as shown in the previous section. Inhibition rate of the given amount of each germicidin and hypnosin in the bioassay solution (200 μl) was estimated form the approximated curve for the results in Figure 3 and was overwrote as stacked bar graph in the dose–response curve of spore extract (Figure 5). Contribution of germicidins for inhibitory activity was germicidin A, germicidin C, germicidin D and germicidin B, in that order. Hypnosin was detected in *S. coelicolor* A3(2) spores, but its contribution was insubstantial.
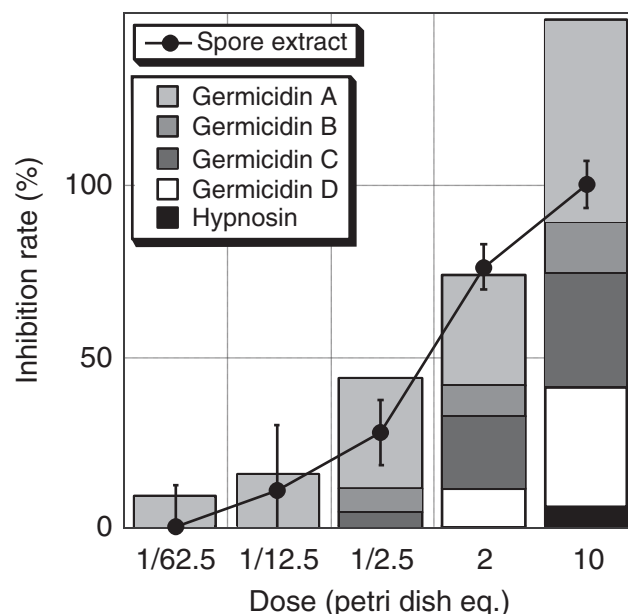
Figure 5 Germination inhibitory activity of *S. coelicolor* A3(2) spore extracts and estimated activity of endogenous germicidins and hypnosin. Line plot shows the germination inhibitory activity of the spore extract. Dose is expressed as cultured petri dish equivalent. Stacked bar graphs show the sum of estimated activity of each germicidin homologs and hypnosin. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

Figure 6 Effects of germicidin A and hypnosin on the elevation of optical density (OD) at 595 nm by germination and hyphal elongation. Arrow indicates the time of addition of germicidin A and hypnosin ($1000\,\mu g\,ml^{-1}$). A full color version of this figure is available at *The Journal of Antibiotics* journal online.

The sum of the estimated germination inhibitory activity correlated well with the experimental inhibitory activity of the spore extract, which means that germicidins act as germination inhibitors in *S. coelicolor* A3(2) spores.

Effect of germicidin A and hypnosin on hyphal growth of *S. coelicolor* A3(2)

The process of germination and subsequent early hyphal elongation was monitored by the OD at 595 nm. The OD rose continuously for 120 min and no obvious inflection point was observed from the germination stage to the hyphal elongation phase in our assay system (Figure 6). Microscopic observation revealed that germination starts at 3 h after initiation of incubation, a considerable number of spores germinated by 12 h and is complete by 36 h. Addition of germicidin A (1000 μg ml^{-1}) at time 0 caused no change in OD. Addition of germicidin A at 24 and 36 h after incubation started preventing subsequent elevation of OD. In contrast, similar treatment with hypnosin did not inhibit this elevation. These results indicate that germicidin A inhibited not only spore germination but also hyphal elongation, but hypnosin did not act on hyphae in *S. coelicolor* A3(2), as was observed in the *Streptomyces* sp. CB-1-1.[8]

Growth inhibitory activity of germination inhibitor in *S. virido-chromogenes* was reported by Grund and Ensign;[3] in the course of purification of a germination inhibitor, they reported that four activities (inhibition of respiration, growth, germination and ATPase) were co-purified during chromatography and that the germination inhibitor is a low-molecular weight substance with antibiotic activity against some bacteria. We confirmed their observation as the action of active principle. Germicidins, however, should function in regulation of germination by preventing coordinated germination and avoiding extinction of the species because its action is reversible as shown in the next section and its content in spores is sufficient to account for the germination inhibitory activity of spore extracts.

Reversibility of the germination inhibitory activity of germicidin A

Continuous incubation of *S. coelicolor* A3(2) spores in the presence of germicidin A at 1000 μg ml^{-1} for 48 h completely inhibited spore germination (germination rate: $2.8 \pm 3.5\%$ of control). However, 3 h treatment with germicidin A and subsequent removal of germicidin A by two rounds of centrifugation and resuspension in water and additional 45 h incubation without the inhibitor allowed germination to recover ($69.5 \pm 10.9\%$ of control), which means that the action of germicidin A is reversible.

Pyrone-containing natural products exhibit a wide range of biological activities such as antibiotic, antifungal, cytotoxic, neurotoxic, phytotoxic and radical-scavenging activity.[13,14,21,22] Among those with a similar structure to germicidin, 6-pentylpyran-2-one produced by *Trichoderma* spp. inhibits hyphal growth, spore formation or spore germination of fungi.[23,24] Fistupyrone, 4-hydroxy-6-(3-methyl)-butylpyroan-2-one, produced by a *Streptomyces* sp., inhibits spore germination, appressorial formation and infection hyphae formation of a fungus, *Alternaria brassicicola*,[25] but does not have these effects on spores of *A. alternata*.[26] Its effect is fungicidal because washing of fistupyrone-treated spores did not allow recovery of viability.

Clarification of the target site and mode of action of germicidins and these rather simple pyrone compounds is a matter of great concern because they show similar biological activity in prokaryotic and eukaryotic microorganisms.

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