Catalytic Analysis of the Validamycin Glycosyltransferase (ValG) and Enzymatic Production of 4''-*epi*-Validamycin A

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ValG is a glycosyltransferase (GT) that is responsible for the glucosylation of validoxylamine A to validamycin A. To explore the potential utilization of ValG as a tool for the production of validamycin analogues, a number of nucleotidyldiphosphate-sugars were evaluated as alternative substrates for ValG. The results indicated that in addition to its natural substrate, UDP-glucose, ValG also efficiently utilized UDP-galactose as sugar donor and resulted in the production of an unnatural compound, 4''-*epi*-validamycin A. The new compound demonstrated a moderate growth inhibitory activity against the plant fungal pathogen *Rhizoctonia solani* (=*Pellicularia sasakii*). A comparative analysis of ValG with its homologous proteins revealed that ValG contains an unusual DTG motif, in place of the DXD motif proposed for metal ion binding and/or NDP-sugar binding and commonly found in other glycosyltransferases. Site-directed mutagenesis of the DTG motif of ValG to DCD altered its preferences for metal ion binding, but did not seem to affect its substrate specificity.

Combinatorial biocatalysis is an emerging technology in the field of drug discovery and development. Some of the approaches include the use of enzymatic, chemoenzymatic, and microbial transformations to generate libraries of new chemical entities from lead compounds.[1–3] Since many bioactive natural products contain sugar moieties, which play a critical role in their pharmacological activities,[4,5] glycosylation processes of natural products have become a prime subject of investigation.[6]

Glycosyltransferases (GTs) catalyze the transfer of a sugar moiety from a nucleotidyldiphosphate (NDP)-sugar to an acceptor, which could be a growing oligosaccharide, a lipid, a protein, or a small molecule. On the basis of tertiary structure analysis, GTs have been divided into two superfamilies, known as GT-A and GT-B.[7] Members of the GT-A superfamily contain two dissimilar domains, one involved in the recognition of the NDP-sugar and the other in the recognition of the acceptor molecule. Most of the GTs in the Leloir pathway that reside in the Golgi apparatus and the endoplasmic reticulum belong to this family. The GT-B superfamily is remarkably diverse and contains members that are extremely promiscuous to their NDP-sugar donors.[8,9] The GT-B family consists mostly of prokaryotic enzymes that glycosylate secondary metabolites to produce active natural products, as well as some glycosyltranferases from the primary pathways.

Validamycin A (**2**), a commercially used agricultural antifungal antibiotic, is a microbial-derived pseudotrisaccharide, which contains a core aminocyclitol moiety, validoxylamine A (**1**), and glucose. The core aminocyclitol moiety is derived from two units of 2-*epi*-5-*epi*-valiolone, each of which is a cyclization product of the C$_7$-sugar phosphate sedoheptulose 7-phosphate. The glucose attachment is critical for the intake of the drug by the fungal cells through the common oligosaccharide transport system. Inside the cells, the compound is hydrolyzed to **1** and serves as a competitive inhibitor of trehalase.[10] In fungi, trehalose is commonly used as a storage carbohydrate, which can be hydrolyzed by trehalase to two molecules of glucose for energy supply and other physiological purposes. Therefore, inhibition of the trehalase activity is detrimental to fungal growth.
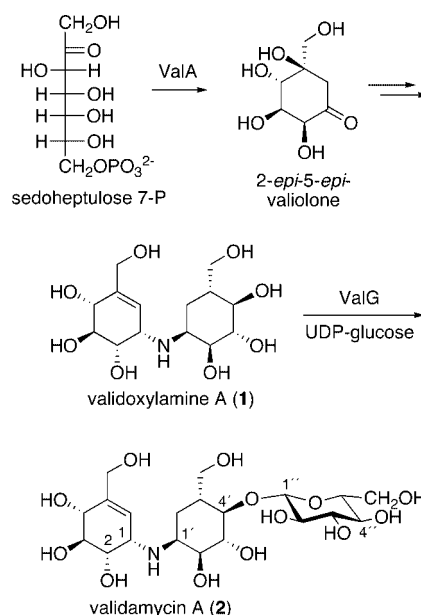

Figure 1. Biosynthesis of validamycin A.

As part of our ongoing study on the biosynthesis of validamycin, we have identified the complete biosynthetic gene cluster in *Streptomyces hygroscopicus* subsp. *jinggangensis* 5008.[11] Among the proteins believed to be directly involved in the biosynthesis, ValG has been characterized both *in vivo* and *in vitro* as a glycosyltransferase that catalyzes the conversion of **1** to **2** using UDP-glucose as the sugar donor (Figure 1).[11] Interestingly, ValG belongs to the GT-A family of glycosyltransferases, even though it functions in secondary metabolism. ValG is also unique in that it has an unusual DTG motif instead of the DXD motif common in closely related proteins. While generally there is no significant identity between different GT families, the acidic DXD motif is highly conserved in almost all GTs[12] and is predicted to participate in the coordination of a divalent metal ion (most commonly Mn^{2+}) and/or in the binding of the NDP-sugar. Mutagenesis of a mannosyltransferase has shown that altering either of these aspartates completely eliminates the enzymatic activity without causing the protein to misfold or denature.[13] In this study, we investigated

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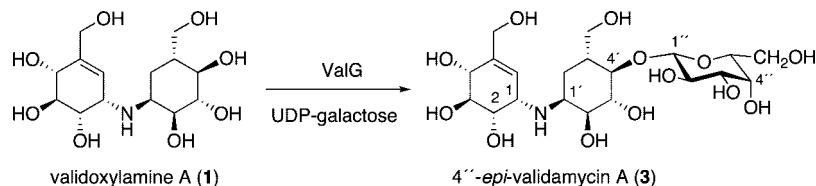
Figure 2. Bioconversion of **1** to **3** using the glycosyltransferase ValG.

the utilization of ValG as a tool for generating analogues of validamycin by testing a number of commercially available NDP-sugars as substrates. The involvement of the DTG sequence of ValG in its catalytic activity or substrate specificity was explored by replacing the DTG sequence with a DCD motif.

Results and Discussion

Enzymatic Assays of ValG with Different NDP-Sugars. To explore the possibility of employing ValG as a tool for generating analogues of validamycin, a number of commercially available NDP-sugars were tested as sugar donors. The recombinant histidine-tagged protein was prepared heterologously in *E. coli* BL21Gold(DE3)pLysS and purified on a BD TALON affinity column. The enzyme was incubated with validoxylamine A as a sugar acceptor and either UDP-glucose, UDP-galactose, UDP-*N*-acetylglucosamine, UDP-glucuronic acid, or GDP-mannose as a sugar donor. The reactions were carried out in the presence of Mg^{2+} for 1−6 h and monitored by TLC. UDP-glucose is the natural sugar donor of ValG, which converts validoxylamine A (**1**) to validamycin A (**2**), and was used in this study as a positive control to validate the activity of the enzyme. The results revealed that, in addition to UDP-glucose, ValG also utilizes UDP-galactose as substrate to produce a new validamycin analogue, 4″-*epi*-validamycin A (**3**) (Figure 2), whereas incubations with UDP-*N*-acetylglucosamine, UDP-glucuronic acid, or GDP-mannose did not give any products at a detectable level.

Interestingly, in contrast to the conversion of **1** to **2**, a complete conversion of **1** to **3** can be achieved only when a purified ValG was used. When a cell-free extract of *E. coli* harboring *valG* was used for the enzyme reaction, only part of **1** was converted to **3** after 3 h, and most of the product was hydrolyzed back to **1** after 6 h (as monitored by TLC and MS). We hypothesized that this hydrolysis was due to an endogenous β-galactosidase activity of *E. coli* origin. Therefore, both **2** and **3** were incubated individually with either cell-free extract of *E. coli* harboring the *valG* gene or cell-free extract of *E. coli* harboring the pRSET-B vector. The reactions were carried out with or without an addition of uridyl-diphosphate (UDP). Validoxylamine A (**1**) was not produced during any of the incubations with **2**, whereas a significant amount of **1** was produced in incubations with **3**. This activity was not dependent on the presence of additional UDP (data not shown).

Chemical Structure Determination of 4″-*epi*-Validamycin A. For a complete characterization of 4″-*epi*-validamycin A (**3**), a large-scale enzyme reaction of purified ValG with validoxylamine A (**1**) and UDP-galactose was carried out and the product was purified by ion-exchange chromatography. The high-resolution mass spectrum of **3** revealed a pseudomolecular ion *m/z* 498 [M + H]⁺, which is identical to that of validamycin A (**2**). However, the ¹H and ¹³C NMR spectra of **3** are slightly different from those of **2**.[14] In particular, the C-4″ proton of **3** appeared as a small doublet (*J* = 3 Hz), as opposed to the large doublet (*J* = 12 Hz) of **2** (Figure 3), which confirmed the identity of a galactosyl moiety in **3**. On the other hand, the coupling constant of the anomeric proton (H-1″) of **3** is 7.5 Hz, which is consistent with that of **2**, indicating a β-glycosidyl linkage of galactose. Furthermore, a correlation between H-1″ (δ_H 4.42 ppm) and C-4′ (δ_C 83.8 ppm) in the HMBC analysis provides evidence that the sugar moiety attaches at the C-4′ position of **3**, which is consistent with the regioselectivity of
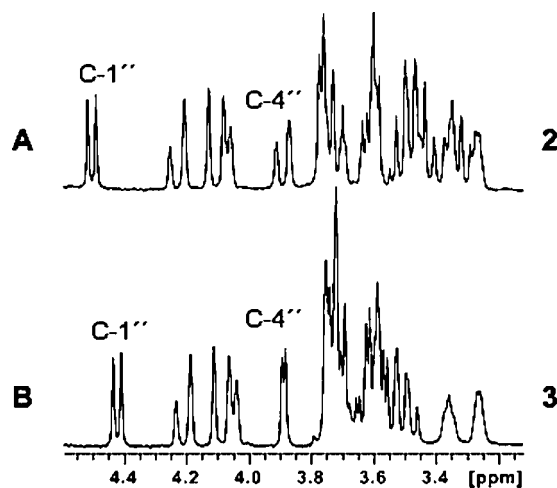

Figure 3. Partial ¹H NMR spectra of validamycin A (**2**) and 4″-*epi*-validamycin A (**3**).

ValG.[11] Complete structure elucidation and assignments of the protons and carbons were based on direct comparisons of the ¹H and ¹³C NMR spectroscopic data with those for **2**, as well as 2D NMR, including ¹H−¹H COSY, HSQC, and HMBC.

Fungal Growth Inhibitory Assay of 4″-*epi*-Validamycin A. It has been reported that **1** has a stronger *in vitro* inhibitory activity against trehalase than **2**. However, **2** is more active *in vivo*, as it is efficiently taken up by the fungal cells and then hydrolyzed by an intracellular β-glucosidase to give the active pharmacophore **1**.[15] Given that **3** is readily hydrolyzed by the *E. coli* enzymes to give **1**, it was anticipated that **3** might be comparably active to or more active than **2** if a similar β-galactosidase activity is present in the fungal cells. To compare their fungal growth inhibitory activity, **2** and **3** were tested against *Pellicularia sasakii* using an agar plug assay.[16] Agar plugs with diameter of 5 mm and containing *P. sasakii* mycelia were placed in the center of agar plates that contain 1 mg of each compound, and the growth of the pathogen was monitored after 2 days. Active compounds were determined on the basis of their ability to inhibit the expansion of the fungal mycelia on the Petri dishes. Consistent with the reported results, validamycin A (**2**) demonstrated a potent fungal growth inhibitory activity (Figure 4A), which validated the sensitivity of the assay. While **3** demonstrated a moderate growth inhibitory activity against *P. sasakii*, it is unexpectedly less active than **2** (Figure 4A and B). On the other hand, the negative control (Figure 4C) showed extensive fungal mycelial expansion. The lower activity of **3** may be due to a number of reasons, including less efficient uptake by the fungal cells and/or less effective intracellular hydrolysis by the fungal enzyme.

Site-Directed Mutagenesis of ValG. BLAST analysis of ValG showed that it is a member of the glycosyltransferase family 2 (GT-2), classified by the CAZy system. This family is the largest and evolutionarily the most ancient of the inverting enzyme families with over 8000 members to date, and it utilizes a vast array of potential sugar donors and acceptors. The SpsA from *Bacillus subtilis* is one of the few well-studied glycosyltransferases with crystal structures in this most widespread family.[17] The N-terminal domains of this family often display a much higher similarity since
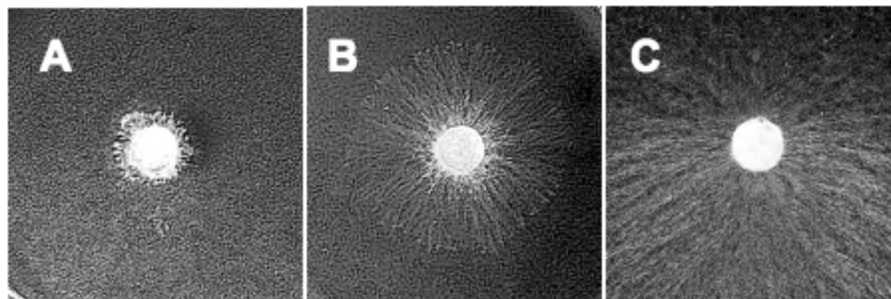
Figure 4. Fungal growth inhibitory assays of validamycin A (A), 4″-*epi*-validamycin A (B), and the negative control, water (C).
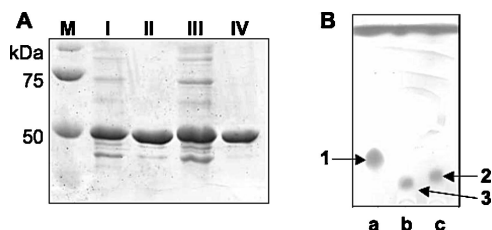

Figure 5. (A) SDS-PAGE of ValG and ValG mutant: M, protein marker; I, cell-free extract of *E. coli* harboring *valG*; II, purified ValG; III, cell-free extract of *E. coli* harboring mutated *valG*; IV, purified ValG mutant. (B) TLC analysis (^{n}PrOH/AcOH/H_2O, 4:1: 1) of ValG reactions: a, validoxylamine A; b, glycosylation product of validoxylamine A with UDP-galactose; c, glycosylation product of validoxylamine A with UDP-glucose.

they bind a common nucleotidyl moiety, and the C-terminal domains show enormous diversity probably because of the variety of sugar acceptors. Alignment of ValG showed that the N-terminal domain (amino acid residues 12−110) has high homology with the sugar donor-binding domain of SpsA, and the C-terminal domain shows very low similarity to other proteins.

Structurally, ValG belongs to the GT-A superfamily. A general feature of all enzymes of the GT-A fold family is the presence of a common DXD motif, which interacts with a divalent metal ion and/or an NDP-sugar. In the DXD motif, the third aspartate residue binds the divalent metal ion, typically Mn^{2+}, and the middle amino acid residue binds the hydroxyl groups on the ribose moiety of the nucleotide moiety. Alignments of the amino acid sequence of ValG with its homologous proteins revealed that instead of having the common DXD motif at the position 99−101, ValG contains a DTG sequence. Using Hydrophobic Cluster Analysis (HCA),[18] which is commonly used for predicting structural similarities in protein sequences with low levels of identity, we were able to predict that the DTG sequence in ValG is indeed located at the DXD motif position in other glycosyltransferases. To investigate the role of this DTG sequence, as opposed to the common DXD motif, the $D_{99}/T_{100}/G_{101}$ motif was replaced with DCD residues by site-directed mutagenesis. An overlapping PCR method was used for this experiment, and the mutated gene was confirmed by DNA sequencing. The original DNA sequence ACTGGT encoding threonine (T) and glycine (G) was replaced with TGCGAC for cysteine (C) and aspartic acid (D).

Effects of DTG → DCD Mutation on ValG Catalytic Activity. Recombinant wild-type and mutant forms of ValG were produced in *E. coli* BL21 Gold(DE3)pLysS, purified by nickel columns, and tested for their catalytic activity using the suite of different sugar donors described above. While the enzyme maintained its catalytic activity with UDP-glucose and UDP-galactose (Figure 5), no other NDP-sugars were recognized as alternative substrates. On the other hand, compared to the native ValG, the mutated enzyme appeared to have different preferences toward divalent metal ions.
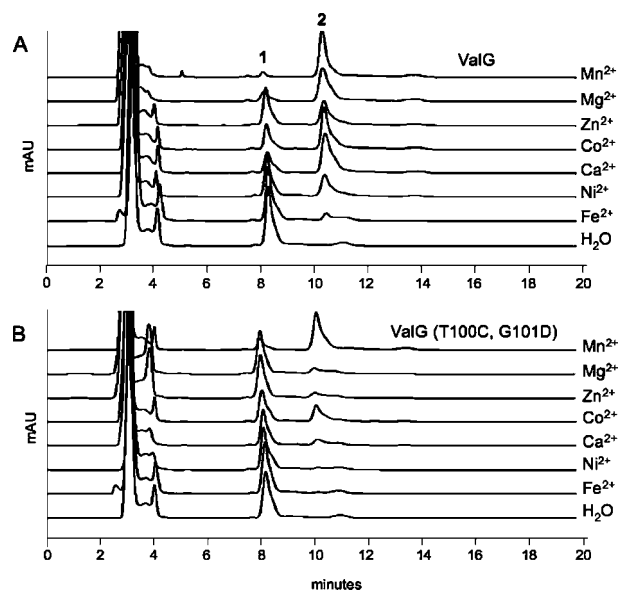
Figure 6. HPLC analyses of the reaction products of ValG (A) and mutated ValG (B) with different divalent metal ions.

A comparative study between the native and mutated ValG revealed that the native DTG-bearing ValG efficiently used a number of divalent metal ions, e.g., Mn^{2+}, Mg^{2+}, Co^{2+}, and Ca^{2+}, as cofactor, whereas the DCD-bearing ValG could effectively use only Mn^{2+}, and to some extent Co^{2+}, as cofactor (Figure 6). The result is consistent with the nature of other DXD-containing glycosyltransferases, which mostly require Mn^{2+} for their catalytic activities. The DTG sequence of ValG may provide greater cofactor flexibility for the enzyme; however, it is not clear as to what extent this would benefit the host bacteria in relation to validamycin A biosynthesis.

Experimental Section

General Experimental Procedures. $[\alpha]_D$ was recorded on a P-1010 polarimeter (JASCO Corporation). ^1H and ^{13}C NMR were recorded on a Bruker DPX300 NMR spectrometer. Low-resolution electrospray ionization (ESI) mass spectra were recorded on a ThermoFinnigan liquid chromatograph-ion trap mass spectrometer. High-resolution electrospray mass spectra were recorded on Waters/Micromass LCT spectrometer. Reactions were monitored by TLC (silica gel 60 F_{254}, Merck) with detection by use of $Ce(SO_4)_2$ solution. Column chromatography was performed on Dowex 50Wx8-200 (Fluka). All chemicals were purchased from Aldrich or Sigma and were used without further purification unless otherwise noted. Reversed-phase preparative HPLC was carried out on a C_{18} column (Atlantis C_{18} 5 mm column, Waters) with CH_3CN/KH_2PO_4 buffer (pH 6.92, 1 mM) (3:97) as mobile phase at 0.6 mL/min. Cloning and overexpression of the *valG* gene and purification of the recombinant His$_6$-tagged ValG were carried out as described previously.[11]

Enzyme Assays. The enzyme assays were carried out at 30 °C for 3−12 h in a 100 μL volume of 25 mM Tris-HCl (pH 7.5), 10 mM

$MgCl_2$, 20 mM NH_4Cl, 15 mM UDP-sugar, 10 mM validoxylamine A, and 50 μL of purified ValG solution (1 mg/mL) or 50 μL of the cell-free extract of *E. coli* BL21(DE3)pLysS containing ValG. The reaction progress was monitored by TLC analysis. For a scale-up experiment, 5.71 mg of validoxylamine A was used. To terminate the reaction, EtOH was added to precipitate the protein. The supernatant was subjected to ion-exchange (Dowex 50Wx8-200 [H^+ form]) column chromatography. The column was washed with H_2O and then eluted with 0.5 M aqueous NH_4OH. The elution of the product was monitored by TLC. Fractions containing the desired product [R_f value 0.15 (^{n}PrOH/AcOH/H_2O, 4:1:1)] were pooled and lyophilized. The product was subsequently subjected to preparative HPLC to give 3.6 mg of pure 4″-*epi*-validamycin A (**3**). **3**: white powder, [α]$^{23}_D$ +49.2 (*c* 0.07, H_2O); ^{1}H NMR (D_2O, 300 MHz) δ 6.01 (1H, d, *J* = 5 Hz, H-6), 4.42 (1H, d, *J* = 7.5 Hz, H-1″), 4.21 (1H, d, *J* = 14 Hz, H-7b), 4.08 (1H, d, *J* = 14 Hz, H-7a), 4.05 (1H, brd, *J* = 7.0 Hz, H-4), 3.89 (1H, d, *J* = 3 Hz, H-4″), 3.76−3.68 (6H, overlap, H-2′, H-7′a,b, H-5″, H-6″a,b), 3.60 (1H, brd, *J* = 3 Hz, H-3″), 3.58−3.56 (3H, overlap, H-2, H-3, H-2″), 3.52 (1H, d, *J* = 2.5 Hz, H-3′), 3.48 (1H, brd, *J* = 2.5 Hz, H-4′), 3.34 (1H, m, H-1), 3.25 (1H, m, H-1′), 2.07 (1H, m, H-5′), 1.91 (1H, brd, *J* = 15 Hz, H-6′b), 1.29 (1H, brd, *J* = 15 Hz, H-6′a); ^{13}C NMR (D_2O, 75 MHz) δ 139.1 (C-5), 123.1 (C-6), 103.3 (C-1″), 83.8 (C-4′), 75.3 (C-5″), 73.6 (C-3), 73.1 (C-2′), 72.7 (C-3″), 72.6 (C-2), 71.4 (C-4), 71.2 (C-3′), 69.4 (C-2″), 68.5 (C-4″), 61.7 (C-7′), 61.5 (C-7), 60.9 (C-6″), 53.7 (C-1′), 52.3 (C-1), 37.4 (C-5′), 26.8 (C-6′); HRESIMS *m/z* 498.2169 [M + H]$^+$ (calcd for $C_{20}H_{36}NO_{13}$, 498.2187).

Site-Directed Mutagenesis of *valG*. The mutagenesis in *valG* was introduced by an overlapping PCR method. For the first step, two fragments of *valG* were amplified separately by PCR with Platinum Pfx DNA polymerase (Invitrogen) using the cosmid clone 17F2 as template. A 323 bp DNA encoding the N-terminal fragment of *valG* (fragment 1) was amplified using primers ValGPM-1F2 (5′-AAA*G-GATCC*A*CATATG*CCCGGTGCGCATCCC-3′; the *Bam*HI and *Nde*I sites are in italics) and ValGPM-1R2 (5′-TACTGCGGCCCCGCCAG-GAC*GTCGCA*GTCGAGGAAGGCCAGGAGTG-3′; the introduced mutation sites are underlined). Similarly, a 992 bp DNA encoding the C-terminal fragment of *valG* (fragment 2) was amplified using primers ValGPM-2F2 (5′-CACTCCTGGCCTTCCTCGAC*TGCGACGTC*-CTGGCGGGGCCGCAGTA-3′; the mutation sites are underlined) and ValGPM-2R2 (5′-AAA*GAATTC*AGTCACCGCGAAGAGAC-3′; the *Eco*RI site is in italics). For the second step, the whole modified *valG* was amplified using fragments 1 and 2 as templates with primers ValGPM-1F2 and ValGPM-2R2. Gel-purified PCR products were digested with *Bam*HI and *Eco*RI and subsequently ligated into *Bam*HI/*Eco*RI-digested pRSET-B. The constructs were transferred into *E. coli* DH10B and plated on LB agar plates containing 100 μg/mL ampicillin. The expected mutation was confirmed by sequencing.

Enzyme Assays with Different Metal Ions. Enzyme assays of mutated ValG with different metal ions were carried out at 30 °C for 3 h in a 100 μL volume of 25 mM Tris-HCl (pH 7.5), 1 mM divalent metal ion, 20 mM NH_4Cl, 15 mM UDP-sugars, 10 mM validoxylamine A, and 50 μL of protein solution (0.1 mg/mL). Subsequently, 100 μL of MeOH was added into the reaction, and the mixture was centrifuged for 5 min at 12 000 rpm. The supernatant was transferred into a clean Eppendorf tube and dried. The product was then dissolved in 100 μL of H_2O for HPLC analysis.

Bioassay. A 1% agar solution (14 mL) was mixed with the compounds (dissolved in 1 mL of H_2O) and plated into Petri dishes. PDA agar plugs with mycelia of *P. sasakii* were placed in the center of the plates as the indicator strain for bioassay of the compounds. The plates were incubated at 30 °C for 2 days.

Supporting Information Available: Table 1 and Figures S1−S8. This material is available free of charge via the Internet at http://pubs.acs.org.

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