Anethole potentiates dodecanol's fungicidal activity by reducing PDR5 expression in budding yeast

Anethole potentiates dodecanol's fungicidal activity by reducing *PDR5* expression in budding yeast

Ken-ichi Fujita, Takayuki Ishikura, Yui Jono, Yoshihiro Yamaguchi, Akira Ogita, Isao Kubo, Toshio Tanaka

Anethole potentiates dodecanol's fungicidal activity by reducing *PDR5* expression in budding yeast

Ken-ichi Fujita[a,*], Takayuki Ishikura[a], Yui Jouno[a], Yoshihiro Yamaguchi [a,b], Akira Ogita[a,c], Isao Kubo[d], Toshio Tanaka[a]

[a] *Graduate School of Science, Osaka City University, Sumiyoshi-ku, Osaka, Japan*

[b] *Advanced Research Institute for Natural Science and Technology, Osaka City University, Sumiyoshi-ku, Osaka, Japan*

[c] *Research Center for Urban Health and Sports, Osaka City University, Sumiyoshi-ku,Osaka, Japan*

[d] *Department of Environmental Science, Policy and Management, University of California, Berkeley, CA, USA*

* Corresponding author at: Graduate School of Science, Osaka City University, Osaka, Sugimoto 3-3-138, Sumiyoshi-ku, Osaka 558-8585, Japan.

E-mail address: kfujita@sci.osaka-cu.ac.jp (K. Fujita).

Abstract

Background: *trans*-Anethole (anethole), a major component of anise oil, has a broad antimicrobial spectrum and a weaker antimicrobial potency than other available antibiotics. When combined with polygodial, nagilactone E, and *n*-dodecanol, anethole has been shown to exhibit synergistic antifungal activity against a budding yeast, *Saccharomyces cerevisiae*, and a human opportunistic pathogenic yeast, *Candida albicans*. However, the mechanism underlying this synergistic effect of anethole has not been characterized.

Methods: We studied this mechanism using dodecanol-treated *S. cerevisiae* cells and focusing on genes related to multidrug efflux.

Results: Although dodecanol transiently reduced the number of colony forming units, this recovered to levels similar to those of untreated cells with continued incubation beyond 24 h. Reverse transcription polymerase chain reaction analysis revealed overexpression of an ATP-binding cassette (ABC) transporter gene, *PDR5*, in addition to a slight increase in *PDR11*, *PDR12*, and *PDR15* transcriptions in dodecanol-treated cells. In the presence of anethole, these effects were attenuated and the fungicidal activity of dodecanol was extended. Dodecanol showed longer lasting fungicidal activity against a Δ*pdr5*. In addition, Δ*pdr3* and Δ*lge1*, lack transcription factors of *PDR5 and PDR3*, were partly and completely susceptible to dodecanol, respectively. Furthermore, combination of anethole with fluconazole was also found to exhibit

synergy on *C. albicans*.

Conclusions: These results indicated that although anethole reduced the transcription of several transporters, *PDR5* expression was particularly relevant to dodecanol efflux.

General significance: Anethole is expected to be a promising candidate drug for the inhibition of efflux by reducing the transcription of several ABC transporters.

Keywords: S. cerevisiae, Multidrug resistance, *PDR5*, Anethole, Antifungal, Dodecanol

Abbreviations: pleiotropic drug resistance (PDR), ATP-binding cassette (ABC), reverse transcription polymerase chain reaction (RT-PCR), colony-forming units (CFU), minimum growth inhibitory concentration (MIC), minimum fungicidal concentration (MFC), fractional inhibitory concentration (FIC), fractional fungicidal concentration (FFC), major facilitator superfamily (MFS).

1. Introduction

The development of antifungal antibiotics with novel modes of action and fewer adverse effects in humans is urgently required because of an increase in opportunistic fungal infections in immunocompromised patients (e.g., due to immunosuppressant therapy after organ transplant, acquired immune deficiency syndrome, or leukemia) and the elderly [1]. Current antifungal targets are limited to the functions and structures unique to fungi, namely ergosterol, the cell wall, and cytosine deaminase. Polyene macrolide antifungals, typified by amphotericin B [2], directly bind ergosterol in the plasma membrane and then form pores. These drugs have excellent antifungal potencies and spectra, but can cause nephropathy as an adverse effect. Azole antifungals such as miconazole, fluconazole, and itraconazole inhibit ergosterol biosynthesis [3]. Their adverse effects include liver damage and menstrual abnormalities [4]. In addition, clinical isolates have been reported to show resistance to these antifungals, especially azoles [5]. In susceptible fungal cells, a synthetic fluorinated analogue of cytosine, fluorocytosine, is converted to 5-fluorouracil by cytosolic cytosine deaminase, which human cells do not express [6]. However, fluorocytosine-resistant fungal strains have frequently been reported in clinical isolates [7]. The most recently developed echinocandins, consisting of micafungin, anidulafungin and caspofungin, inhibit β-1,3-glucan synthase thereby weakening fungal cell wall [8]. The occurrence of *Candida* clinical isolates with lower susceptibility to echinocandins has been also reported, which is possibly due to its broad

clinical use [9]. Therefore, strategies for overcoming drug-resistance should be developed to improve antifungal chemotherapy.

trans-Anethole (anethole), a chief component of anise and fennel oils, has been reported to exhibit antimicrobial activity against bacteria, yeasts, and filamentous fungi [10-12]. We recently revealed that anethole-induced growth inhibition and morphological changes in a filamentous fungus, *Mucor mucedo*, depended on cell wall fragility that was caused by chitin synthase inhibition [12]. In addition, we also found that anethole showed fungicidal activities against a human opportunistic pathogenic fungus, *Aspergillus fumigatus*, and a budding yeast, *Saccharomyces cerevisiae*, that were accompanied by generation of reactive oxygen species and DNA fragmentation, indicating apoptotic-like cell death [13]. Although the antimicrobial potency of anethole is weaker than those of other available antifungals, anethole synergistically enhanced the antifungal activities of polygodial, nagilactone E, 2*E*-undecenal, and dodecanol against a budding yeast, *S. cerevisiae*, and a human pathogenic fungus, *Candida albicans* [14-17].

Anise oil is frequently used as a food additive in seasoning and herbal tea [18], indicating that anethole exhibits low toxicity in humans. If the mechanisms underlying the synergistic effects of anethole can be elucidated, this may inform the development of combination antifungal chemotherapies using lower drug doses, thereby reducing the risk of adverse effects.

Anethole exhibits synergistic effects in combination with several antifungals that are

chemically unrelated and act by different mechanisms [14-16]. We hypothesized that this phenomenon was associated with a reduction in multidrug resistance. The present study therefore focused on multidrug efflux pumps, which are closely associated with the mechanism underlying drug resistance, in order to analyze the synergistic fungicidal effects of anethole and a model drug, *n*-dodecanol (dodecanol), in budding yeast, *S. cerevisiae*. This yeast is protected against xenobiotics by multidrug efflux pumps, including the pleiotropic drug resistance (PDR) transporters [19]; these belong to the family of ATP-binding cassette (ABC) transporters. Fungal pathogen drug resistance is often caused by the overexpression of ABC transporters [20].

Dodecanol was selected for this study because it shows unusual antifungal effects on *S. cerevisiae* cells [14]. This compound produces a transient fungicidal effect during short-term exposure but over the longer term, yeast cell proliferation is gradually restored. This growth eventually recovers completely and attains the same level observed under control conditions. The present study investigated the ability of anethole to restrict this recovery and extend the fungicidal activity of dodecanol.

In this study, we analyzed yeast gene expression using reverse transcription polymerase chain reaction (RT-PCR) and found that dodecanol induced overexpression of *PDR5* and that anethole attenuated dodecanol-induced *PDR5* overexpression.

2. Materials and methods

2.1 Chemicals

All chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO), unless otherwise stated. Drugs (including anethole) were diluted with *N*,*N*-dimethylformamide prior to the following experiments.

2.2 Yeast strains and culture

The wild-type strain of *S. cerevisiae*, ATCC 7754, was obtained from the American Type Culture Collection (Manassas, VA). The parental strain of *S. cerevisiae* BY4741 (*MATa, ura3-Δ0, leu2-Δ0, met15-Δ0,* and *his3-Δ1*) and its deletion strains (Δ*pdr1*, Δ*pdr3*, Δ*pdr5*, Δ*pdr11*, Δ*pdr12*, Δ*pdr15*, Δ*yor1*, Δ*snq2*, Δ*lge1*, Δ*psd1*, and Δ*ssa1*) were purchased from OPEN Biosystems (Lafayette, CO). *C. albicans* IFO 1061 was obtained from Institute for Fermentation, Osaka (Osaka, Japan). Exponentially growing yeast cells (10^6 cells/ml) were incubated in 3 ml of 2.5% malt extract (ME; Oriental Yeast Co., Tokyo, Japan) broth without shaking at 30°C, unless otherwise stated. For the determination of minimum inhibitory concentrations (MICs), the assay tubes were incubated without shaking at 30°C for 48 h. The MIC is the lowest concentration of test compound that demonstrated no visible growth. The minimum fungicidal concentrations (MFCs) were examined as follows. After determining the MIC, a 30 μL of aliquot was taken from each clear tube and added into 3 mL of YPD (1% yeast extract, 2% polypeptone and 2%

123 glucose) medium. After 48-h incubation, the MFC was determined as the lowest concentration of
124 the test compounds in which no recovery of microorganisms was observed. Cell viability was
125 determined by counting the colony-forming units (CFU) on YPD agar plates [21]. All assays for
126 MIC, MFC, time-kill studies and RT-PCR were performed at least three times on separate
127 occasions. Representative time-kill curves obtained in the CFU assays were shown in Figures.
128 *2.3 RT-PCR*
129 The relative expression level of each gene related to drug efflux pumps was compared using
130 RT-PCR. Exponentially growing cells (10^6 cells/ml) were incubated with or without drugs in ME
131 broth without shaking at 30°C for 4 h; the cells were then harvested by centrifugation. Total
132 mRNA was isolated from the cells using the RNeasy Kit (QIAGEN, Tokyo, Japan). The total
133 mRNA concentration was estimated at 260 nm. Reverse transcription was carried out using
134 ReverTra Ace®, 5 × RT Buffer, RNase inhibitor, 2 mM dNTPs Mixture (TOYOBO, Osaka,
135 Japan), and CDS-primer. The mixture was incubated under the following conditions: 30°C for 10
136 min, 42°C for 60 min, and 99°C for 5 min. PCR was then performed using the resultant cDNA,
137 10 × PCR Buffer, rTaq DNA polymerase (Bio-Rad), dNTPs, and the forward and reverse primers
138 described below. All fragments were amplified by incubation 94°C for 2 min followed by 40
139 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s, followed by a final extension at 72°C
140 for 5 min using. The primers employed were designed using Primer 3 software

141 (http://frodo.wi.mit.edu/primer3/) and were: *ACT1*-forward, 5′-ATGGTCGGTATGGGTCAAAA-
142 3′; *ACT1*-reverse, 5′-AACCAGCGTAAATTGGAACG-3′; *PDR1*-forward, 5′-
143 GGAGCGAAGCTTTTGACAAC-3′; *PDR1*-reverse, 5′-CTGCAGAAATGGTGCTCGTA-3′;
144 *PDR3*-forward, 5′-GTTTGGGCATGTTTGGACTT-3′; *PDR3*-reverse, 5′-
145 CCCGGTTCAACTTCTTTCAA-3′; *PDR5*-forward, 5′-GTTGCCTAAACCCAGGTGAA-3′;
146 *PDR5*-reverse, 5′-ATTGCTACTTCCGCCAAATG-3′; *PDR10*-forward, 5′-
147 CCAGTCTTTCCCCAGATCAA-3′; *PDR10*-reverse, 5′-CGCGACTAGCCAATTTCTTC-3′;
148 *PDR11*-forward, 5′-CCAGTCTTTCCCCAGATCAA-3′; *PDR11*-reverse, 5′-
149 CGCGACTAGCCAATTTCTTC-3′; *PDR12*-forward, 5′-AACCTGTTGAGGGAGGAGGT-3′;
150 *PDR12*-reverse, 5′-GTTGAAAGAAGCAGGCAAGG-3′; and *PDR15*-forward, 5′-
151 TACGGACATGGAAGGTGTGA-3′; *PDR15*-reverse, 5′-GGTCTCCCAAGAACAACCAA-3′.
152 Each amplified DNA sample was electrophoresed on 1% agarose gel, stained with GelRed
153 (Biotium, Inc., Hayward, CA), and visualized under UV light. The relative expression levels of
154 each gene were quantified using Fujifilm Multi Gauge Version 2.1. Data are means ± standard
155 deviations of triplicate experiments.
156 *2.4 Acquisition of dodecanol-resistant strains*
157 *S. cerevisiae* ATCC7754 cells were incubated in 2.5% ME broth with 250 μM dodecanol
158 for 48 h. After incubation, cells were harvested and spread on an ME agar plate. Colonies formed

159 on the plate represented spontaneous revertants.

160 *2.4 FIC and FFC indices*

161 Combination studies were performed by a broth checkerboard method [22]. A series of 2-fold
162 dilutions of one compound were tested in combination with 2-fold dilutions of the other compounds.
163 The assays were performed in triplicate on separate occasions. Fractional inhibitory concentration
164 (FIC) indices were calculated from checkerboard data. The FICs for these combinations were
165 calculated as (MICa combination/MICa alone) + (MICb combination/MICb alone), where a and b
166 were two compounds tested. The FIC presented are significant values obtained from the
167 checkerboard matrix. FIC indices were used to define the interaction of combined compounds:
168 synergistic (X <0.5), additive (1 <X>0.5), indifferent (4 <X>1), or antagonistic (X>4). Fractional
169 fungicidal concentration (FFC) indices were also calculated from the checkerboard data of MFC.
170

3. Results and discussion

Anethole is a major component of the essential oils derived from aniseed and fennel seeds by steam distillation [23]. This phenylpropanoid has been reported to exhibit a wide variety of biological effects such as antioxidant, anti-inflammatory [24], antinociceptive [25], and anesthetic [26] activities. MIC and MFC of anethole against *S. cerevisiae* were 625 and 1250 μM, respectively (Fig. 1). It also induced complete cell death in *S. cerevisiae* at 1250 μM, as shown in Fig. 2. This study determined cell viability by counting CFUs. After a 72-h incubation, no revertant cells were observed in the presence of 1250 μM anethole. In the presence of 625 μM anethole, the viable cell number was reduced at 24 h and had recovered by 72 h. This indicated the possibility that some cells had become acclimatized to anethole-induced stress or had reacquired their reproductive capability. Anethole did not significantly affect the growth of yeast cells at 312 μM (Fig. 2).

Primary aliphatic alcohols (*n*-alkanols) also show broad-spectrum antimicrobial activity against bacteria and fungi [27-29]. These alcohols exhibited fungicidal activity against a food-borne yeast, *Zygosaccharomyces bailii*, and the human opportunistic fungus, *C. albicans*, in addition to *S. cerevisiae* [30-32]. This activity was enhanced as the carbon chain lengthened, with the maximum fungicidal activity against *S. cerevisiae* observed for the 11-carbon undecanol [30]. However, *n*-alkanols with a carbon chain length of more than 12 do not show fungicidal activity against *S. cerevisiae* after long-term incubations [30]. This ‘cut off’ phenomenon is observed at different

189 carbon chain lengths, depending on the microorganism studied [33]. MIC and MFC of dodecanol
190 against *S. cerevisiae* were 125 and >1250 μM, respectively (Fig. 1). We could not distinguish
191 between turbidity arising from yeast cells and that arising from dodecanol, because of the limited
192 solubility of dodecanol at concentrations above 1250 μM in the water-based medium. In fact, 12-
193 carbon dodecanol did not show any fungicidal activity after 48 h, even at the highest concentrations
194 tested in this study, as shown in Fig. 2. However, a transient reduction in cell viability was observed
195 after 24 h.

196 Essential oils are composed of a variety of phenylpropanoids and their derivatives. Crude oils
197 were reported to show synergistic antimicrobial activities against bacteria and fungi, including
198 pathogens, in combination with other antibiotics and preservatives [34]. However, there have been
199 few reports of the synergistic antimicrobial activities of purified constituents of essential oils. A
200 derivative of phenylpropanoid, cinnamaldehyde, was reported to show synergistic antifungal
201 effects on *A. fumigatus* when used in combination with fluconazole [35]. The synergistic antifungal
202 and fungicidal effects of anethole combined with dodecanol were evaluated using FIC and FFC
203 indices, respectively. The FIC index was 0.56 indicating weak synergy or additive effect (Fig. 1).
204 On the other hand, the FFC index was assumed to be <0.31 indicating synergy as the MFC of
205 dodecanol was >1250 μM (Fig. 1). These effects were also confirmed based on time-kill study. As
206 shown in Fig. 2, 312 μM anethole did not affect yeast viability. However, the combination of 312

μM anethole and 250 μM dodecanol induced rapid loss of cell viability within 2 h, as shown in Fig. 2, and there was no recovery of viability for up to 72 h. This was a significant delay in the recovery of cell viability observed in the presence of 250 μM dodecanol alone. These results suggested that anethole extended the fungicidal effect of dodecanol on *S. cerevisiae* at concentrations which did not affect growth of this yeast.

To investigate the mechanism underlying this effect, the relative expression levels of multidrug efflux pump-related PDR genes were estimated using RT-PCR. *S. cerevisiae* cells have been reported to possess at least 16 ABC multidrug transport proteins [36]. In this study, *PDR5*, *PDR10*, *PDR11*, *PDR12*, *PDR15*, and *PDR18* were selected for analysis of gene expression levels, in addition to their transcription factors, *PDR1* and *PDR3*. Yeast cells were treated with or without drugs for 4 h prior to RNA extraction and analysis. As shown in Fig. 3, cells treated with dodecanol showed high expression levels of *PDR5* mRNA. The expression of *PDR11*, *PDR12*, and *PDR15* was also slightly increased. The mRNA level of another PDR efflux pump, *PDR10*, was not affected by treatment with dodecanol. Conversely, the levels of *PDR8* and *PDR18* mRNAs were slightly reduced by exposure to dodecanol. These results indicated that dodecanol strongly promoted the gene expression of a multidrug efflux pump, *PDR5*. The protein encoded by *PDR5*, Pdr5p, is one of the most extensively characterized pumps involved in the efflux of harmful drugs for cellular detoxification [17, 19]. Previous investigations have revealed factors involved in

positive and negative regulation of *PDR5* expression [37-41]. The transcription factors, Pdr1p and Pdr3p, promote the expression of *PDR5* [38, 42]. Pdr1p and Pdr3p possess a Zn(II)2Cys6 binuclear cluster DNA binding domain, which binds to the promoter region of *PDR5* and promotes its expression [38, 42]. The expression levels of *PDR1* and *PDR3* were slightly increased in cells treated with dodecanol (Fig. 3). In contrast to findings obtained with dodecanol, anethole reduced *PDR5* expression, as compared with the level observed in control cells (Fig. 3). Surprisingly, yeast cells exposed to anethole combined with dodecanol showed lower levels of *PDR5* mRNA than control cells. This treatment also reduced the expression of *PDR1* and *PDR3*, as compared with the level observed in cells treated with dodecanol alone. Furthermore, the levels of *PDR11*, *PDR12*, and *PDR15* mRNAs were slightly reduced. *PDR11* expression is regulated by Pdr1p [43], while *PDR10* and *PDR15* are regulated by both Pdr1p and Pdr3p [42, 44]. Pdr12p is required for the efflux of weak acids and its gene expression is regulated by a Zn(II)2Cys6 zinc finger transcription factor, War1p [45]. The results shown in Fig. 4 could not fully elucidate the roles of *PDR1* and/or *PDR3* on the regulation of *PDR* genes in yeast cells treated with anethole but they did indicate that the dodecanol-induced increase in *PDR5* expression was attenuated in the presence of anethole. In addition, our results indicated the possibility that anethole regulated the expression of genes encoding several multidrug efflux pumps, other than *PDR5*.

We evaluated the antifungal activities of dodecanol against several yeast strains lacking PDR-

243 related genes. Dodecanol did not show antifungal activity against the wild-type or parental strain
244 after 48 h because of the recovery of cell viability (Fig. 2). Therefore, MIC were determined by
245 measuring the culture turbidity after 48-h incubations. The MIC of dodecanol was 1000 μM in the
246 Δ*pdr5* strain, cultured at an initial cell density of 10^7 cells/ml. We did not determine the MIC values
247 for the other deletion strains tested in this study, or for the parental strain, as described above.
248 Therefore, the MIC values of these strains were assumed to be >1250 μM. These results suggested
249 that the dodecanol-induced overexpression of *PDR5* was involved in its transient antifungal effect.
250 We also investigated the effects of 250 μM dodecanol on the growth of several *PDR*-related
251 deletion mutant strains cultured at an initial cell density of 10^6 cells/ml. As shown in Fig. 4, a
252 temporary decrease in CFU was observed in all strains tested, with the exception of Δ*pdr5*. The
253 number of Δ*pdr5* CFU did not recover until 72-h. The Δ*pdr3* CFU showed a partial recovery.
254 Complete recovery of CFU was observed in the other deletion strains examined and in the parental
255 strain, at 48 h except Δ*pdr3*. These results suggested that resistance against dodecanol required the
256 expression of *PDR3* and *PDR5*. *PDR5* transcription was previously reported to be strongly
257 enhanced in cells containing hyperactive *PDR1* dominant alleles through the binding of
258 transcriptional regulatory proteins to three sites in the *PDR5* promoter region [46]. However, in
259 relation to dodecanol resistance, Pdr1p is probably not involved in the transcriptional control of
260 *PDR5*. Expression of other major multidrug efflux pump genes *YOR1* and *SNQ2*, which are

involved in the exhaust of organic anions and metal cations, respectively, is partially promoted by the presence of Pdr1p [47]. The growth of Δ*yor1* and Δ*snq2* cells were also recovered after 72-h treatment with dodecanol (Fig. 4 lower panel). On the other hand, loss of mitochondrial DNA (ρ^0) leads to a strong induction of *PDR5* gene expression that is strictly Pdr3p-dependent [48]. *LGE1* deletion fails to fully induce *PDR5* transcription in ρ^0 cells [49]. The Hsp70 protein Ssa1p has been reported to be as a negative regulator of *PDR3* transcription factor [50]. Thus, we also examined the effect of dodecanol on the growth of Δ*lge1*, Δ*psd1* and Δ*ssa1* (Fig. 4 lower panel). The growth recovery of Δ*lge1* cells were not observed in treatment with dodecanol among the three strains tested.

Next, we evaluated the expression levels of *ACT1*, *PDR1*, *PDR3* and *PDR5* in the cells of Δ*pdr1*, Δ*pdr3,* Δ*lge1*, and their parental strain treated with or without dodedcanol (Fig. 5). The expressions of *ACT1* were not affected by dodecanol in the strains tested. The expression levels of *PDR3* and *PDR5* were similar in dodecanol-treated cells of Δ*pdr1* and the parental strain. While the expressions of *PDR5* were significantly reduced by treatment with dodecanol in Δ*pdr3* and Δ*lge1*. The reduction levels in Δ*lge1* were more potent than those in Δ*pdr3*. These results were supported by no growth recovery in Δ*lge1* cells treated with dodecanol (Fig. 4) and then indicated that dodecanol stress might go through mitochondria. Anethole induces apoptotic-like cell death of *S. cerevisiae* due to generation of reactive oxygen species [13]. ROS generation is mainly derived

279 from mitochondria [13]. Thus, anethole might cut off the signal transduction around mitochondria
280 thereby finally restricting *PDR5* expression. Furthermore, the expressions of *PDR1* were reduced
281 by treatment with dodecanol in Δ*pdr3* and Δ*lge1*. Conversely, *PDR1* deletion did not affect *PDR3*
282 expression. *PDR3* and *LGE1* deletion might directly or indirectly regulate *PDR1* expression. Our
283 results obtained above could not explain the reason why the *PDR5* expression was weakly restricted
284 in Δ*pdr3* than Δ*lge1* and *PDR1* expression was also done in Δ*pdr3* and Δ*lge1*.

285 When Δ*pdr5* cells were treated with 125 μM dodecanol, complete loss of cell viability was
286 retained at 48 h, as shown in Fig. 6. A slight elevation of CFU was observed at 72 h. This indicated
287 the possibility of drug efflux involving transporters other than Pdr5p. In addition, the fungicidal
288 profile of anethole was similar in the presence and absence of the *PDR5* deletion (data not shown).
289 This indicates that anethole may not be effluxed by Pdr5p.

290 The occurrence of antimicrobial drug resistance in pathogens is thought to reflect a process of
291 adaptive evolution, generally as a result of genetic mutations [51]. Thus, cell suspensions were
292 exposed to 125 μM dodecanol for 48 h prior to identifying colonies of spontaneous revertants. The
293 antifungal profile of anethole against 96% of these revertants was similar to that of the dodecanol-
294 susceptible parental strain (data not shown). Namely, when the revertants were re-exposed to
295 dodecanol, a complete loss of growth and the recovery of growth were observed after 24- and 48-
296 h incubations, respectively, in 96% of the colonies obtained (data not shown), indicating no

297 acquisition of dodecanol-resistance. Anethole (312 μM) in combination with 250 μM dodecanol
298 completely induced cell death in the dodecanol-susceptible parental strain, but only weakly
299 inhibited the growth of 4% of the revertant colonies (Fig. 7). The size of most of these revertant
300 colonies were significantly smaller than that of the dodecanol-susceptible parental strain when
301 grown on ME agar plates indicating the occurrence of the petite colonies (data not shown). The
302 petite mutants, which lack mitochondrial DNA and are respiration deficient, are resistant to due to
303 overexpression of multidrug exhaust pumps [52]. Thus, dodecanol resistance might be due to at
304 least *PDR5* overexpression in the revertants. In addition, these results indicated that 4% of the
305 spontaneous revertants showed resistance against dodecanol when rechallenged. Regrettably,
306 anethole could not produce synergistic antifungal effects against all of the revertants obtained.
307 Conversely, co-treatment with anethole could contribute to restricting the occurrence of dodecanol-
308 resistant cells.

309 Although anethole synergistically enhanced the antifungal activities of several natural products
310 from plants against a human pathogenic *C. albicans* [14-16], the synergy of antifungals clinically
311 used has not been reported in combination with anethole. *C. albicans* possesses *CDR1*and *CDR2*
312 as homologs of *PDR5* and azole antifungals were reported to be exhausted by the products from
313 *CDR1* and *CDR2* [53]. Although *C. albicans* is unable to survive lacking mitochondrial DNA [54],
314 it is possible to obtain respiratory deficient strains due to mutations in mitochondrial DNA [55].

315 Furthermore, it has been reported that there is a relationship between upregulation of a major
316 facilitator superfamily (MFS) transporter gene *MDR1* with its resulting higher azole resistance
317 and uncoupled mitochondrial oxidative phosphorylation in *C. albicans* [56]. The restriction of
318 MFS transporters gene expression are rather expected to show synergy in combination with
319 anethole in the case of *C. albicans*.

320 We examined the effect of anethole on the MIC of fluconazole against *C. albicans*. The MIC
321 of fluconazole was lowered from 2500 to 312 ng/ml when 100 μM of anethole was combined (Fig.
322 8). As a result, the activity of fluconazole was increased 8-fold and the FIC index was namely
323 0.375 indicating synergy.

324 **4. Conclusions**

325 The results obtained in this study suggest that anethole exhibits a synergistic fungicidal effect
326 in combination with dodecanol via restriction of *PDR5* expression. *PDR5* encodes Pdr5p, which
327 plays a major role in the efflux of harmful drugs for cellular detoxification. However, anethole
328 reduced the expression of several other efflux pumps, in addition to *PDR5*. Therefore, it is highly
329 possible that anethole potentiates the antifungal effects of a range of drugs by reducing the
330 expression of efflux pump genes and thus inhibiting drug efflux.

331

332 **Funding information**

333 This work was partly funded by Japan Society for the Promotion of Science, Grants-in-Aid for
334 Scientific Research (C) 25460128 and 16K08299.

335

336 **Acknowledgements**

337 We are grateful to Daisuke Yamaguchi for performing assays with anethole at an earlier stage
338 of the work and Chika Yamawaki for technical assistance.

339

References

1. M.H. Miceli, J.A. Díaz, S.A. Lee. Emerging opportunistic yeast infections. Lancet Infect. Dis. 11 (2011) 142-151.
2. A. Mark, M.D. Miller. Reversible hepatotoxicity related to amphotericin B. Can. Med. Assoc. J. 131 (1984) 1245–1247.
3. M. Borgers, H. Degreef, G. Cauwenbergh. Fungal infections of the skin: Infection process and antimycotic therapy. Curr. Drug. Targets 6 (2005) 849–862.
4. R.J. Rodriguez, D. Acosta Jr. Comparison of ketoconazole- and fluconazole-induced hepatotoxicity in a primary culture system of rat hepatocytes. Toxicology 96 (1995) 83–92.
5. M. Masiá Canuto, F. Gutiérrez Roder. Antifungal drug resistance to azoles and polyenes. Lancet Infect. Dis. 2 (2002) 550–563.
6. A. Loyse, F. Dromer, J. Day, O. Lortholary, T.S. Harrison. Flucytosine and cryptococcosis: time to urgently address the worldwide accessibility of a 50-year-old antifungal. J. Antimicrob. Chemother. 68 (2013) 2435–2444.
7. A. Polak, P.G. Hartman. Antifungal chemotherapy--are we winning? Prog. Drug Res. 37 (1991) 181–269.
8. P. Mazur P, N. Morin, W. Baginsky, M. el-Sherbeini, J.A. Clemas, J.B. Nielsen, F. Foor. Differential expression and function of two homologous subunits of yeast 1,3-β-D-glucan

358 synthase. Mol. Cell. Biol. 15 (1995) 5671-5681.
359 9. S.S. Gonçalves, A.C. Souza A. Chowdhary, J.F. Meis, A.L. Colombo. Epidemiology and
360 molecular mechanisms of antifungal resistance in *Candida* and *Aspergillus*. Mycoses. 59
361 (2016) 198–219.
362 10. H. Hitokoto, S. Morozumi, T. Wauke, S. Sakai, H. Kurata. Inhibitory effects of spices on
363 growth and toxin production of toxigenic fungi. Appl. Environ. Microbiol. 39 (1980) 818–
364 822.
365 11. I. Kosalec, S. Pepeljnjak, D. Kustrak. Antifungal activity of fluid extract and essential oil
366 from anise fruits (*Pimpinella anisum* L., Apiaceae). Acta Pharm. 55 (2005) 377–385.
367 12. M. Yutani, Y. Hashimoto, A. Ogita, I. Kubo, T. Tanaka, K. Fujita. Morphological changes of
368 the filamentous fungus *Mucor mucedo* and inhibition of chitin synthase activity induced by
369 anethole. Phytother. Res. 25 (2011) 1707–1713.
370 13. K. Fujita, M. Tatsumi, A. Ogita, I. Kubo, T. Tanaka. Anethole induces apoptotic cell death
371 accompanied with reactive oxygen species production and DNA fragmentation in Aspergillus
372 fumigatus and *Saccharomyces cerevisiae*. FEBS J. 281 (2014) 1304–1313.
373 14. K. Fujita, T. Fujita, I. Kubo. Anethole, a potential antimicrobial synergist, converts a fungistatic
374 dodecanol to a fungicidal agent. Phytother. Res. 21 (2007) 47–51.
375 15. I. Kubo, M. Himejima. Potentiation of antifungal activity of sesquiterpene dialdehydes against

376 *Candida albicans* and two other fungi. Experientia 48 (1992) 1162–1164.

377 16. I. Kubo, H. Muroi, M. Himejima. Combination effects of antifungal nagilactones against

378 *Candida albicans* and two other fungi with phenylpropanoids. J. Nat. Prod. 56 (1993) 220–226.

379 17. A. Kubo, I. Kubo. Antimicrobial agents from *Tanacetum balsamita*. J. Nat. Prod. 58 (1995)

380 1565–1569.

381 18. R.M. Soares, R.F. Lima, A. de Freitas Pires, E.P. Souza, A.M. Assreuy, D.N. Criddle. Effects

382 of anethole and structural analogues on the contractility of rat isolated aorta: Involvement of

383 voltage-dependent Ca^{2+}-channels. Life Sci. 81 (2007) 1085–1093.

384 19. R.P. Gupta, P. Kueppers, L. Schmitt, R. Ernst. The multidrug transporter Pdr5: a molecular

385 diode? Biol. Chem. 392 (2011) 53–60.

386 20. R. Prasad, A. Goffeau. Yeast ATP-binding cassette transporters conferring multidrug resistance.

387 Annu. Rev. Microbiol. 66 (2012) 39–63.

388 21. C.K. Kang, K. Yamada, Y. Usuki, A. Ogita, K. Fujita, T. Tanaka. Visualization analysis of the

389 vacuole-targeting fungicidal activity of amphotericin B against the parent strain and an

390 ergosterol-less mutant of *Saccharomyces cerevisiae*. Microbiology 159 (2013) 939–947.

391 22. C.W. Norden, H. Wenzel, E. Keleti. Comparison of techniques for measurement of in vitro

392 antibiotic synergism. J. Infec. Dis. 140 (1979) 441-443.

393 23. S.K. O'Shea, D.D. Von Riesen, L.L. Rossi. Isolation and analysis of essential oils from spices.

394 J. Chem. Educ. 89 (2012) 665–668.
395 24. R.S. Freire, S.M. Morais, F.E. Catunda-Junior, D.C.S.N. Pinheiro DCSN. Synthesis and
396 antioxidant, anti-inflammatory and gastroprotector activities of anethole and related
397 compounds. Bioorg. Med. Chem. 12 (2005) 4353–4358.
398 25. C.F. Estevão-Silva, R. Kummer, F.C. Fachini-Queiroz, R. Grespan, G.A. Nogueira de Melo, S.
399 Baroni, R.K. Cuman, C.A. Bersani-Amado. Anethole and eugenol reduce in vitro and in vivo
400 leukocyte migration induced by fMLP, LTB4, and carrageenan. J. Nat. Med. 68 (2014) 567–
401 575.
402 26. C. Ghelardini, N. Galeotti, G. Mazzanti. Local anaesthetic activity of monoterpenes and
403 phenylpropanes of essential oils. Planta Med. 67 (2001) 564–566.
404 27. E.M. Lansford Jr., I.D. Hill, W. Shive. Reversal by ribonucleosides of bacterial growth
405 inhibition caused by alcohol. J. Biol. Chem. 235 (1960) 3551–3555.
406 28. I. Kubo, H. Muroi, H. Himejima, A. Kubo. Antibacterial activity of long-chain alcohols: the
407 role of hydrophobic alkyl groups. Bioorg. Med. Chem. Lett. 3 (1993) 1305–1308
408 29. I. Kubo, H. Muroi, M. Himejima. Antimicrobial activity of green tea flavor components and
409 their combination effects. J. Agric. Food Chem. 40 (1992) 245–248.
410 30. I. Kubo, T. Fujita, A. Kubo, K.I. Fujita. Modes of antifungal action of alkanols against
411 *Saccharomyces cerevisiae*. Bioorg. Med. Chem. 11 (2003) 1117–1122.

412 31. K. Fujita, T. Fujita, I. Kubo. 2008. Antifungal activity of alkanols against *Zygosaccharomyces*
413 *bailii* and their effects on fungal plasma membrane. Phytother. Res. 22 (2008) 1349–1355.
414 32. H. Gershon, L. Shanks. Antifungal properties of *n*-alkanols, alpha, omega-*n*-alkanediols, and
415 omega-chloro-alpha-alkanols. J. Pharm. Sci. 69 (1980) 381–384.
416 33. P. Balgavý, F. Devínsky. Cut-off effects in biological activities of surfactants. Adv. Colloid
417 Interface Sci. 66 (1996) 23–63.
418 34. I.H. Bassolé, H.R. Juliani. Essential oils in combination and their antimicrobial properties.
419 Molecules 17 (2012) 3989–4006.
420 35. M.S. Khan, I. Ahmad. Antifungal activity of essential oils and their synergy with fluconazole
421 against drug-resistant strains of *Aspergillus fumigatus* and *Trichophyton rubrum*. Appl.
422 Microbiol. Biotechnol. 90 (2011) 1083–1094.
423 36. T. Chinen, Y. Ota, Y. Nagumo, H. Masumoto, T. Usui. Construction of multidrug-sensitive yeast
424 with high sporulation efficiency. Biosci. Biotechnol. Biochem. 75 (2011) 1588–1593.
425 37. S. Meyers, W. Schauer, E. Balzi, M. Wagner, A. Goffeau, J. Golin. Interaction of the yeast
426 pleiotropic drug resistance genes PDR1 and PDR5. Curr. Genet. 21 (1992) 431–436.
427 38. D.J. Katzmann, P.E. Burnett, J. Golin, Y. Mahé, W.S. Moye-Rowley. Transcriptional control of
428 the yeast PDR5 gene by the PDR3 gene product. Mol. Cell. Biol. 14 (1994) 4653–4661.
429 39. K. Hellauer, B. Akache, S. MacPherson, E. Sirard, B. Turcotte. Zinc cluster protein Rdr1 is a

transcriptional repressor of the PDR5 gene encoding a multidrug transporter. J. Biol. Chem. 278 (2002) 17671–17676.

40. C. Gao, L. Wang, E. Milgrom, W.C. Shen. On the mechanism of constitutive Pdr1 activator-mediated PDR5 transcription in *Saccharomyces cerevisiae*: evidence for enhanced recruitment of coactivators and altered nucleosome structure. J. Biol. Chem. 279 (2004) 42677–42686.

41. A.K. Souid, C. Gao, L. Wang, E. Milgrom, W.C. Shen. ELM1 is required for multidrug resistance in *Saccharomyces cerevisiae*. Genetics 173 (2006) 1919–1937.

42. S. Paul, W.S. Moye-Rowley. Multidrug resistance in fungi: regulation of transporter-encoding gene expression. Front. Physiol. 5 (2014) 143.

43. B. Rogers, A. Decottignies, M. Kolaczkowski, E. Carvajal, E. Balzi, A. Goffeau. The pleitropic drug ABC transporters from *Saccharomyces cerevisiae*. J. Mol. Microbiol. Biotechnol. 3 (2001) 207–214.

44. H. Wolfger, Y. Mahé, A. Parle-McDermott, A. Delahodde, K. Kuchler. The yeast ATP binding cassette (ABC) protein genes PDR10 and PDR15 are novel targets for the Pdr1 and Pdr3 transcriptional regulators. FEBS Lett. 418 (1997) 269–274.

45. Y. Nygård, D. Mojzita, M. Toivari, M. Penttilä, M.G. Wiebe, L. Ruohonen. The diverse role of Pdr12 in resistance to weak organic acids. Yeast 31 (2014) 219–232.

46. D.J. Katzmann, T.C. Hallstrom, Y. Mahé, W.S. Moye-Rowley. Multiple Pdr1p/Pdr3p binding

448 sites are essential for normal expression of the ATP binding cassette transporter protein-
449 encoding gene PDR5. J. Biol. Chem. 271 (1996) 23049–23054.
450 47. A. Kolaczkowska, M. Kolaczkowski, A. Goffeau, W.S. Moye-Rowley. Compensatory
451 activation of the multidrug transporters Pdr5p, Snq2p, and Yor1p by Pdr1p in *Saccharomyces*
452 *cerevisiae*. FEBS Lett. 582 (2008) 977-983.
453 48. S. Paul, W.S. Moye-Rowley. Multidrug resistance in fungi: regulation of transporter-encoding
454 gene expression. Front Physiol. 5 (2014) 143.
455 49. X. Zhang, A. Kolaczkowska, F. Devaux, S.L. Panwar, T.C. Hallstrom, C. Jacq, W.S. Moye-
456 Rowley. Transcriptional regulation by Lge1p requires a function independent of its role in
457 histone H2B ubiquitination. J. Biol. Chem. 280 (2005) 2759-2570.
458 50. P. Shahi, K. Gulshan, W.S. Moye-Rowley. Negative transcriptional regulation of multidrug
459 resistance gene expression by an Hsp70 protein. J. Biol. Chem. 282 (2007) 26822–26831.
460 51. M. Huang, M. McClellan, J. Berman, K.C. Kao. Evolutionary dynamics of *Candida albicans*
461 during in vitro evolution. Eukaryot. Cell 10 (2011) 1413–1421.
462 52. W.S. Moye-Rowley. Retrograde regulation of multidrug resistance in *Saccharomyces*
463 *cerevisiae*. Gene 354 (2005) 15-21.
464 53. A. Mane, P. Vidhate, C. Kusro, V. Waman, V. Saxena, U. Kulkarni-Kale, A. Risbud. Molecular
465 mechanisms associated with fluconazole resistance in clinical *Candida albicans* isolates from

India. Mycoses 59 (2016) 93-100.

54. X.J. Chen, G.D. Clark-Walker. The petite mutation in yeasts: 50 years on. Int. Rev. Cytol. 194 (2000) 197-238.

55. C. Gyurko, U. Lendenmann, R.F. Troxler, F.G. Oppenheim. *Candida albicans* mutants deficient in respiration are resistant to the small cationic salivary antimicrobial peptide histatin 5. Antimicrob. Agents Chemother. 44(2) (2000) 348–354.

56. S. Cheng, C.J. Clancy, K.T. Nguyen, W. Clapp, M.H.A. Nguyen. A *Candida albicans* petite mutant strain with uncoupled oxidative phosphorylation overexpresses *MDR1* and has diminished susceptibility to fluconazole and voriconazole. Antimicrob. Agents Chemother. 51 (2007) 1855–1858.

477 **Figure captions**

478

479 **Fig. 1.** Resulting isobologram of the MICs and MFCs obtained with combinations of anethole and
480 dodecanol against *S. cerevisiae*. Exponentially growing cells of *S. cerevisiae* (1 x 10^6 cells/ml) were
481 incubated in 2.5% ME broth at 30°C. Data are indicated as MICs or MFCs. The dotted line indicates
482 an additive effect and belonging to area left under of the dotted line shows a synergistic effect on
483 the MIC.

484

485 **Fig. 2.** Effects of anethole and/or dodecanol on *S. cerevisiae* viability. Exponentially growing cells
486 of *S. cerevisiae* were incubated in 2.5% ME broth at 30°C with (upper panel) no drug (filled circles)
487 or anethole at 313 μM (filled diamonds), 625 μM (filled triangles), or 1250 μM (filled squares);
488 (middle panel) no drug (filled circles) or dodecanol at 125 μM (open triangle) or 250 μM (open
489 squares); and (lower panel) no drug (filled circles), 313 μM anethole + 125 μM dodecanol (open
490 circles), and 313 μM anethole + 250 μM dodecanol (open diamonds). Data are means ± standard
491 deviations of triplicate experiments.

492

493 **Fig. 3.** Relative expression of genes related to drug efflux pumps. Exponentially growing *S.*
494 *cerevisiae* cells were incubated with 312.5 μM anethole (Anethole), 32 μM dodecanol (Dodecanol),

495 or these treatments in combination of 312.5 μM anethole and 32 μM dodecanol (Combination) in
496 2.5% ME medium at 30°C for 4 h prior to extraction of total RNA. Control indicates cells incubated
497 without drugs. After incubation, the mRNA level of each gene was determined using reverse
498 transcription polymerase chain reaction. Actin (*ACT1*) cDNA amplification was used to normalize
499 the signal. The results quantitated are shown in a lower panel. Data are means ± standard deviations
500 of triplicate experiments.
501
502 **Fig. 4.** Effect of PDR-related gene deletion on the antifungal activity of dodecanol. Upper panel:
503 Exponentially growing cells of parent (filled circles), Δ*pdr1* (filled squares), Δ*pdr3* (filled
504 triangles), Δ*pdr5* (open squares), Δ*pdr11* (open triangles), Δ*pdr12*, (open circles), and Δ*pdr15*
505 (filled diamonds) strains were incubated in 2.5% ME broth supplemented with 250 μM dodecanol
506 at 30°C. Lower panel: Cells were incubated in 2.5% ME broth supplemented with 250 μM
507 dodecanol at 30°C. Viability after 72-h treatment with dodecanol was also shown. Data are means
508 ± standard deviations of triplicate experiments.
509
510 **Fig. 5.** Relative expression of *ACT1*, *PDR1*, *PDR3* and *PDR5* in Δ*pdr1*, Δ*pdr3* and Δ*lge1* and their
511 parental strain. Exponentially growing cells were incubated in 2.5% ME broth supplemented with
512 32 μM dodecanol at 30°C for 4 h prior to extraction of total RNA. After incubation, the mRNA

level of each gene was determined using reverse transcription polymerase chain reaction. Data are means ± standard deviations of triplicate experiments.

Fig. 6. Fungicidal effects of anethole, dodecanol, and their combination on the parental and Δ*pdr5* *S. cerevisiae* strains. Exponentially growing cells were incubated in 2.5% ME broth at 30°C in the presence of no drug (filled circles), 313 μM anethole (filled diamonds), 125 μM dodecanol (filled triangles), or 313 μM anethole + 125 μM dodecanol (open circles). Data are means ± standard deviations of triplicate experiments.

Fig. 7. Fungicidal effects of anethole, dodecanol, and their combination on one dodecanol-resistant strain obtained from dodecanol-treated cells. Exponentially growing *S. cerevisiae* revertant cells were incubated in 2.5% ME broth at 30°C in the presence of no drug (filled circles); anethole at 313 μM (filled diamonds), 625 μM (filled triangles), or 1250 μM (filled squares); dodecanol at 125 μM (open triangles) or 250 μM (open squares); 313 μM anethole + 125 μM dodecanol (open circles) or 313 μM anethole + 250 μM dodecanol (open diamonds). Data are means ± standard deviations of triplicate experiments.

Fig. 8. Resulting isobologram of the MICs obtained with combinations of anethole and fluconazole

against *C. albicans*. Exponentially growing cells of *C. albicans* were incubated in 2.5% ME broth at 30°C. Data are indicated as MICs. The dotted line indicates an additive effect and belonging to area left under of the dotted line shows a synergistic effect on the MIC.

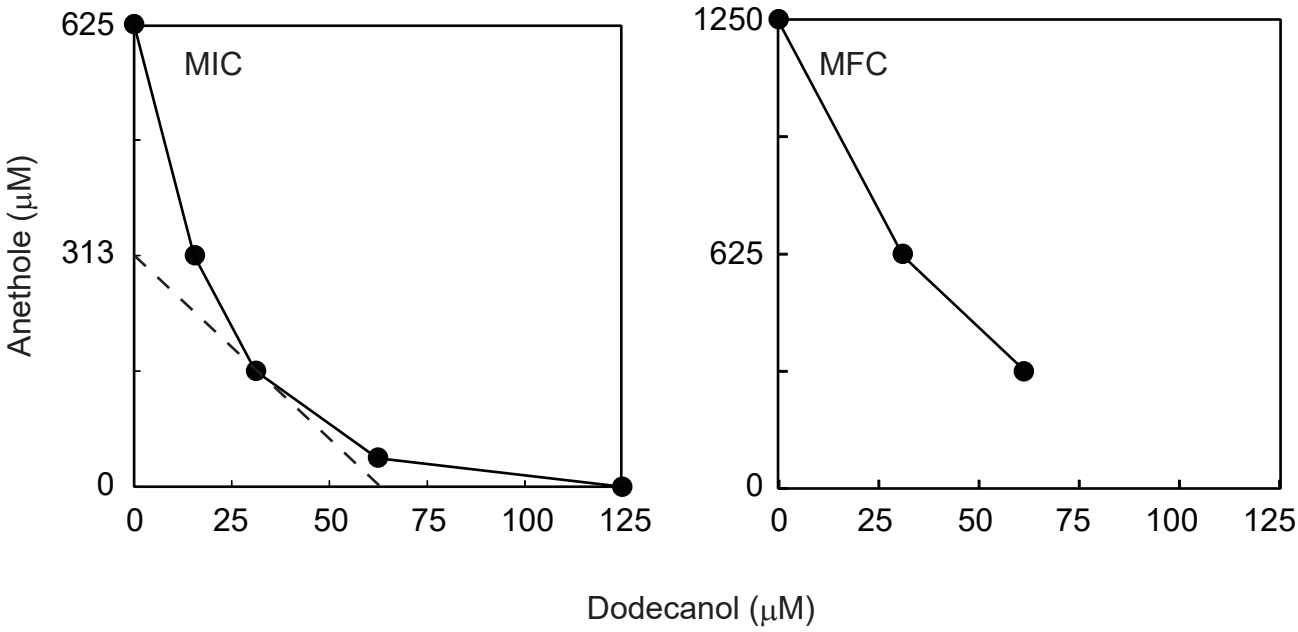

Fig. 1. Fujita et al.

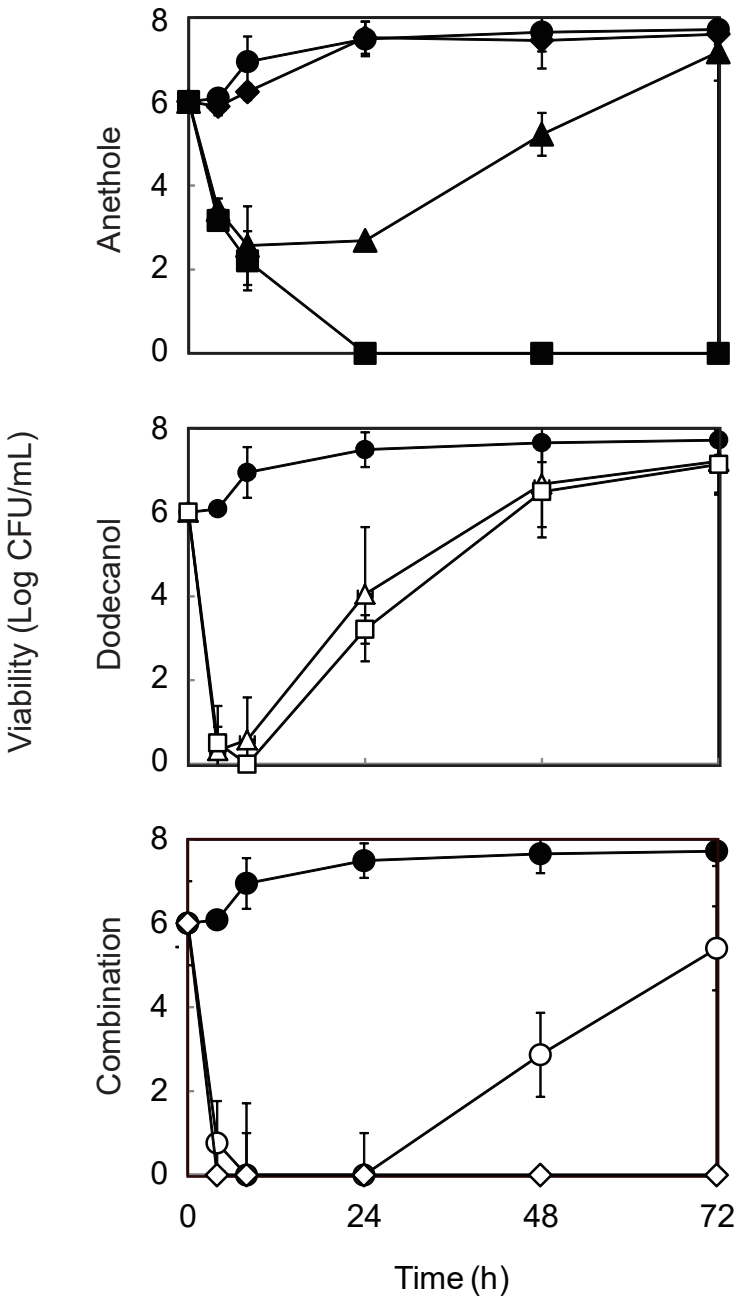

Fig. 2. Fujita et al.

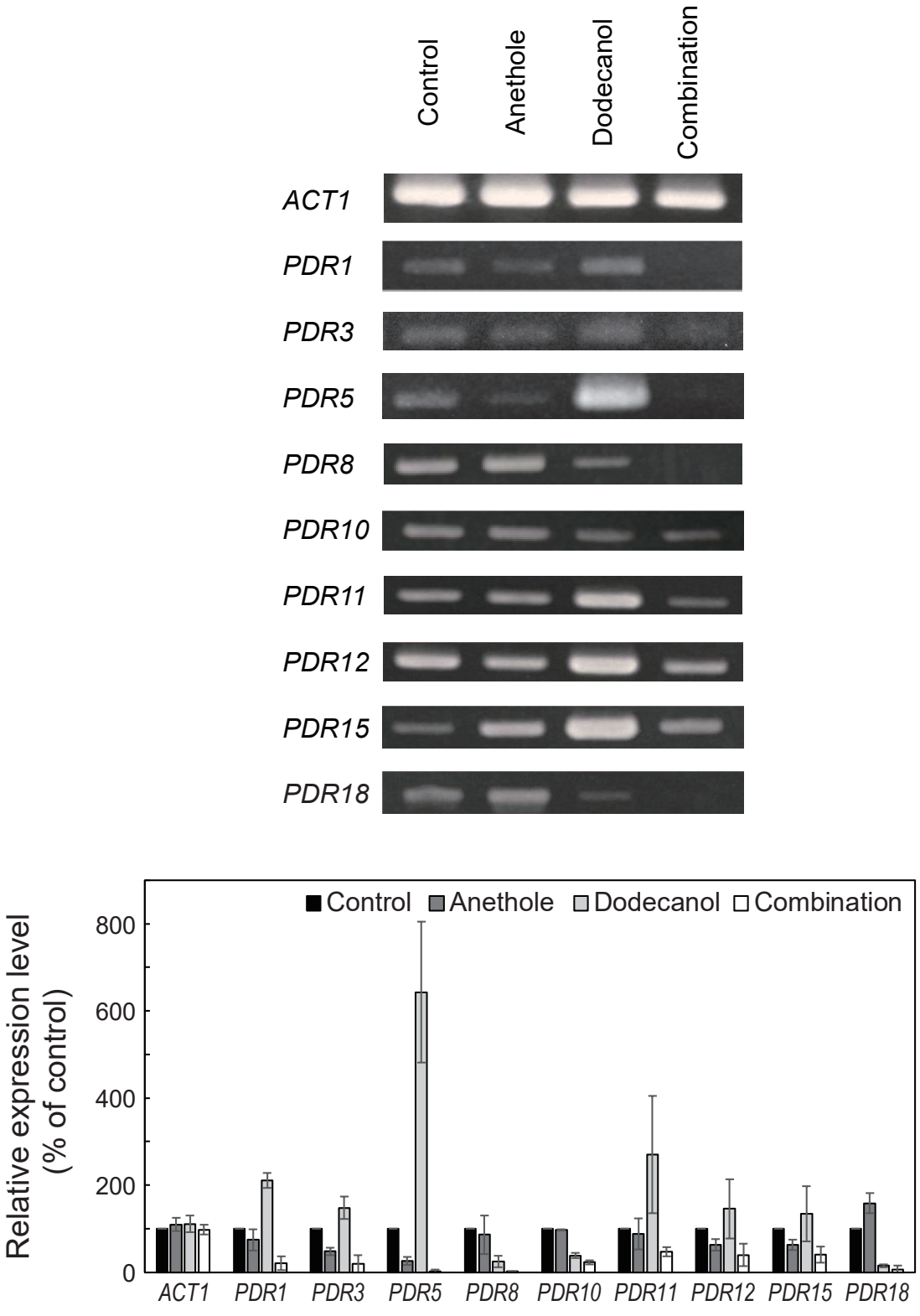

Fig. 3. Fujita et al.

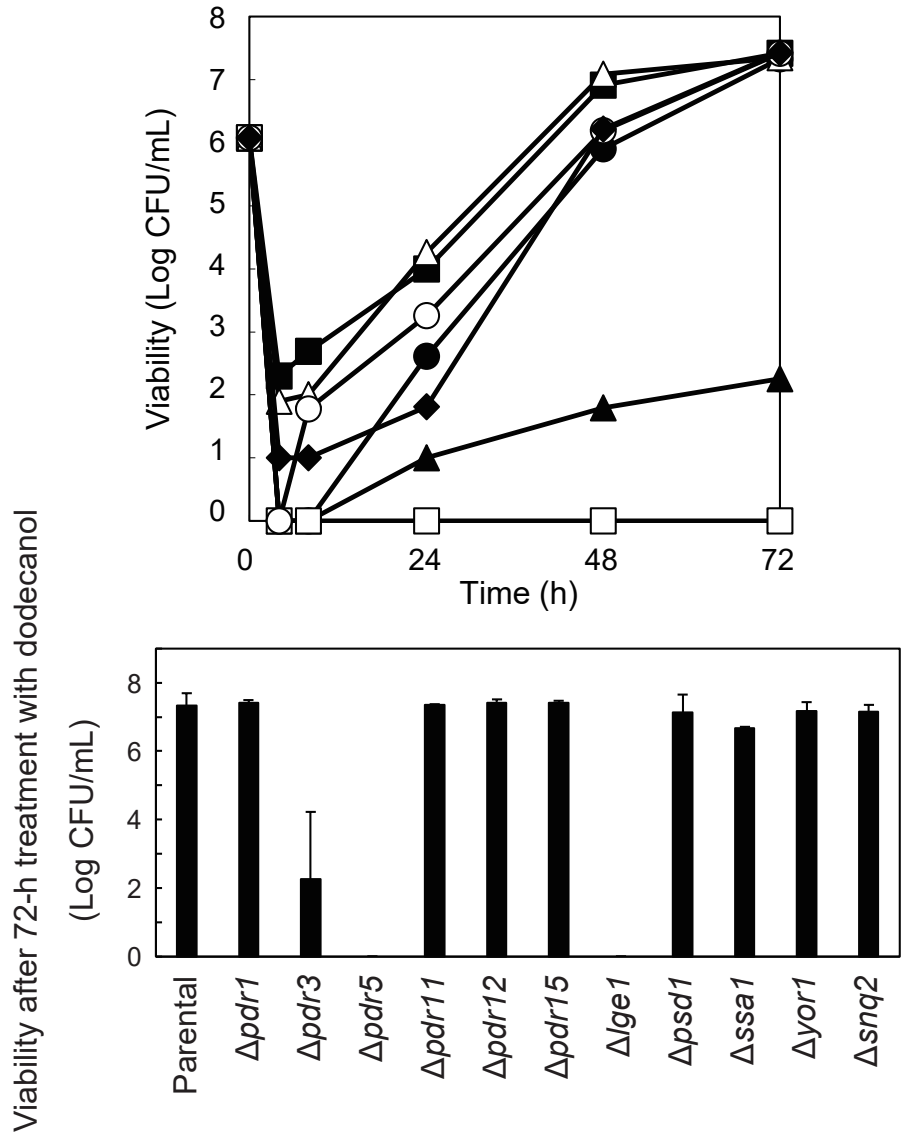

Fig. 4. Fujita et al.

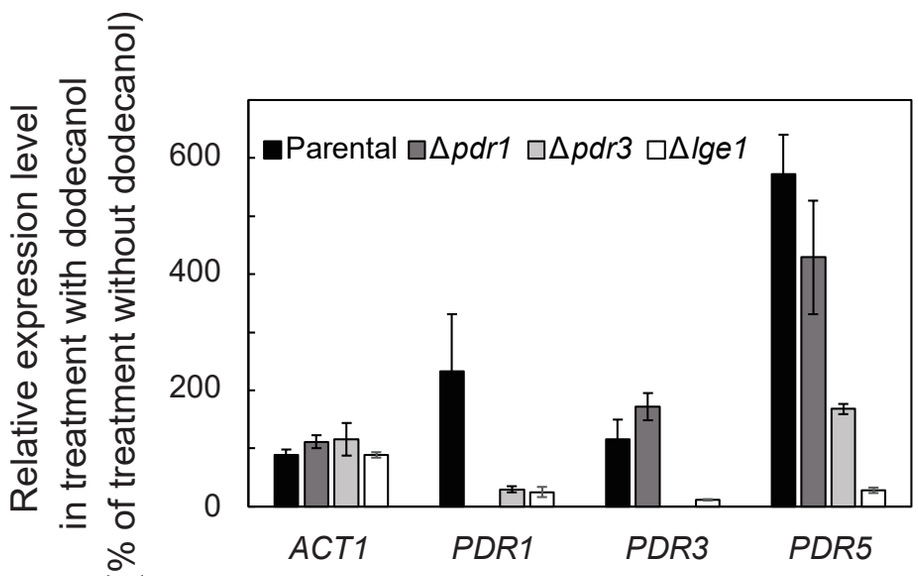

Fig. 5. Fujita et al.

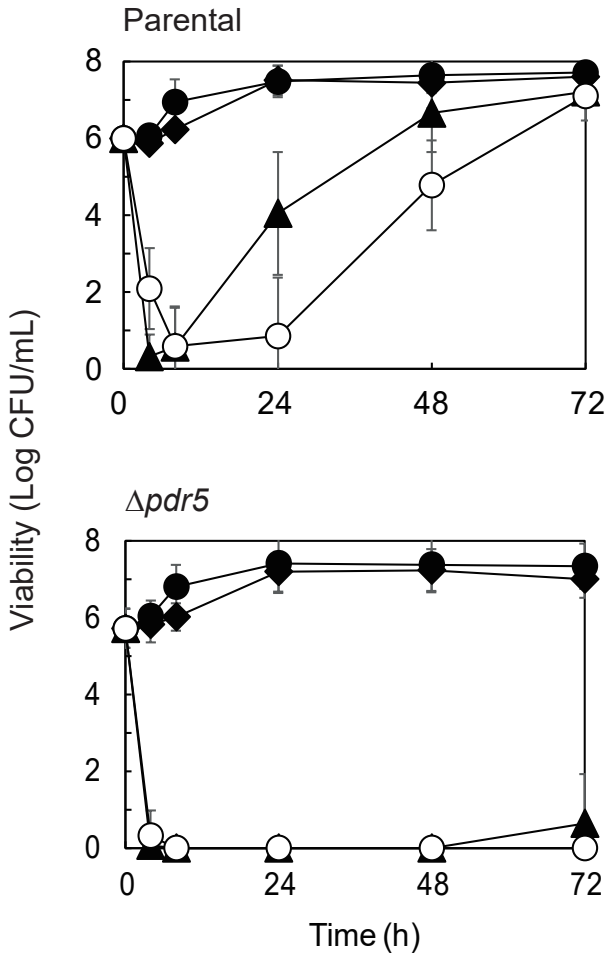

Fig. 6. Fujita et al.

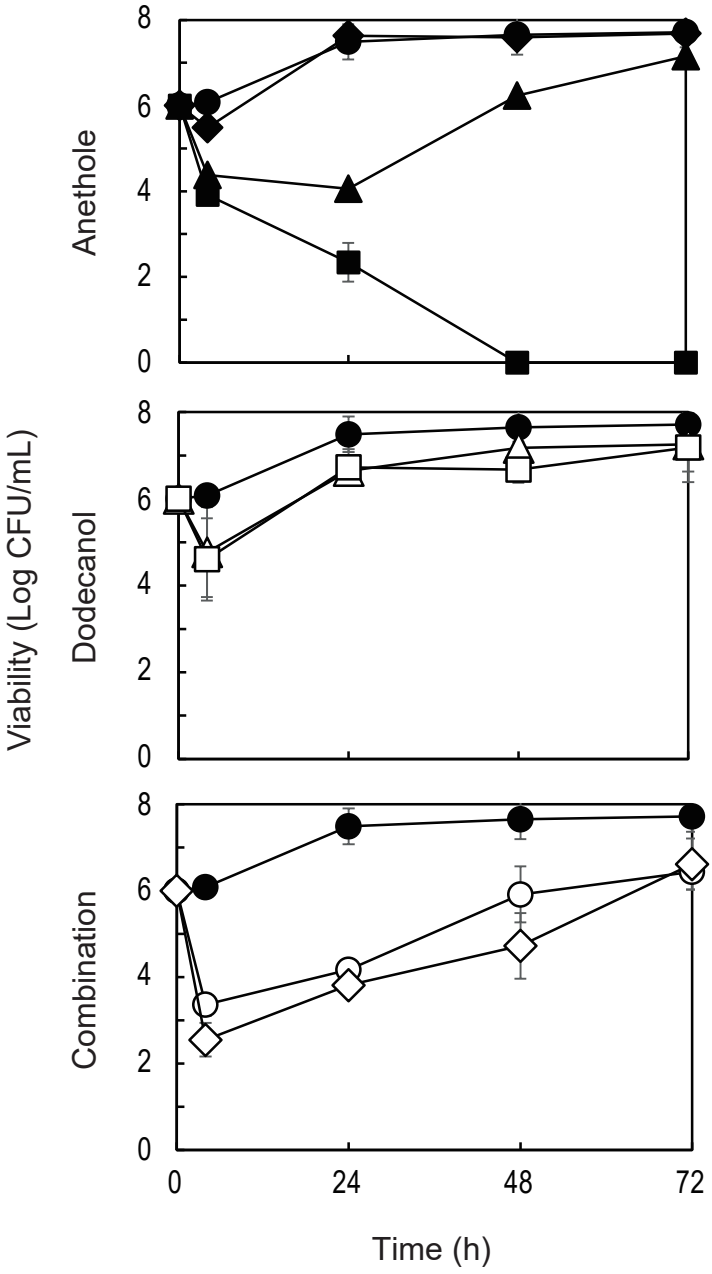

Fig. 7. Fujita et al.

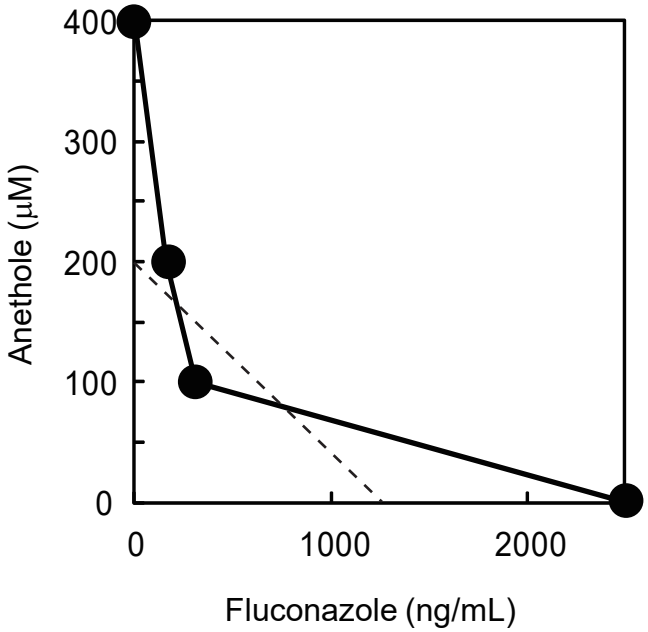

Fig. 8. Fujita et al.