Structure-activity relationships of antifungal phenylpropanoid derivatives and their synergy with n-dodecanol and fluconazole

Y. Tsukuda, N. Mizuhara, Y. Usuki, Y. Yamaguchi, A. Ogita, T. Tanaka, K. Fujita

Y. Tsukuda, N. Mizuhara, Y. Usuki, Y. Yamaguchi, A. Ogita, T. Tanaka, K. Fujita. (2022). Structure-activity relationships of antifungal phenylpropanoid derivatives and their synergy with n-dodecanol and fluconazole. *Letters in Applied Microbiology*. 74, 377-384. https://doi.org/10.1111/lam.13613.

PROF. AKIRA OGITA (Orcid ID : 0000-0002-1694-2356)

DR. KEN-ICHI FUJITA (Orcid ID : 0000-0001-8496-7435)

Article type : Original Article

Original Article

Structure-activity relationships of antifungal phenylpropanoid derivatives and their synergy with *n*-dodecanol and fluconazole

Y. Tsukuda, N. Mizuhara, Y. Usuki, Y. Yamaguchi, A. Ogita, T. Tanaka, K. Fujita*

Graduate School of Science, Osaka City University, Osaka, Japan

Running headline: **SAR of antifungal phenylpropanoids**

*Correspondence

Ken-ichi Fujita, Graduate School of Science, Osaka City University, 3-3-138 Sugimoto, Sumiyoshi-ku, Osaka 558-8585, Japan

E-mail: kfujita@osaka-cu.ac.jp

Significance and Impact of the Study:

Combination therapies that utilize adjuvant antibiotic and nonantibiotic drugs to potentiate antibiotic activity are emerging as an attractive approach to counteract fungal resistance to drugs. In this study, we investigated the structure-activity relationships of phenylpropanoids and related derivatives that restrict drug resistance by inhibiting their efflux. A desirable synergy was observed when *n*-dodecanol or fluconazole was combined with a phenylpropanoid anethole and *p*-alkylanisoles having alkyl chains with a length of C2–C6. Our results provide a foundation for the practical application of combination therapies against antifungal resistance.

Abstract

trans-Anethole (anethole) is a phenylpropanoid; with other drugs, it exhibits synergistic activity against several fungi and is expected to be used in new therapies that cause fewer patient side effects. However, the detailed substructure(s) of the molecule responsible for this synergy has not been fully elucidated. We investigated the structure-activity relationships of phenylpropanoids and related derivatives, with particular attention on the methoxy group and the double bond of the propenyl group in anethole, as well as the length of the *p*-alkyl chain in *p*-alkylanisoles. Antifungal potency was largely related to *p*-alkyl chain length and the methoxy group of anethole, but not to the double bond of its propenyl group. Production of reactive oxygen species also played a role in these fungicidal activities. Inhibition of drug efflux was associated with the length of the *p*-alkyl chain and the double bond of the propenyl group in anethole, but not with the methoxy group. Although a desirable synergy was observed between *n*-dodecanol and anethole or *p*-alkylanisoles with a length of C2-C6 in alkyl chains, it cannot be explained away as being solely due to the inhibition of drug efflux. Similar results were obtained when phenylpropanoid derivatives were combined with fluconazole against *Candida albicans*.

Keywords

antifungal activity, *Candida albicans*, drug resistance, multidrug efflux pump, phenylpropanoid, *Saccharomyces cerevisiae*, *trans*-anethole

Introduction

Recent advances in medicine, particularly the identification of new drugs and the development of therapeutic protocols, have increased the lifespans of immunosuppressed patients, including those with cancer, transplants, and HIV. However, an ever-increasing immunocompromised population has led to a dramatic expansion in the incidence of human invasive mycosis, producing a global public health issue (Nicola *et al*. 2019). Current antifungal agents for the treatment of systemic infections are limited to four types: polyenes, which complex with ergosterol inducing an efflux of cytoplasmic content from cells; azoles, which inhibit the biosynthesis of ergosterol; 5-fluorocytosines, which inhibit nucleic acid synthesis; and echinocandins, which inhibit the biosynthesis of the fungal cell wall component β-1,3-glucan (Balkis *et al*. 2002). The emergence of fungi resistant to existing antifungals, especially azoles and echinocandins, poses a serious challenge. Although the development of antibiotics aimed at novel targets may resolve these issues, developing antifungals is a complicated task, as fungi are closely related to humans, so that many potential drug targets are shared by fungi and their human hosts. Therefore, antifungal agents can pose a high risk of toxicity to humans (Selitrennikoff 2001).

Phenylpropanoid *trans*-anethole (anethole; Fig. 1), a major constituent of anise and fennel oils, is widely used as a flavouring agent in the food, cosmetic, perfume, and pharmaceutical industries. Various studies conducted during the last few years have revealed that anethole exerts multiple beneficial effects, including anti-inflammatory, anticarcinogenic, chemopreventive, antidiabetic, immunomodulatory, neuroprotective, and antithrombotic effects (Aprotosoaie *et al*. 2016). In addition, its constituents have been shown to have antifungal activities against several fungi (Fujita *et al*. 2014; Yutani *et al*. 2011).

In a previous study, we found that anethole has antifungal activity against *Mucor mucedo,* causing morphological abnormalities by its inhibition of the biosynthesis of cell wall component chitin. Anethole also acts against human opportunistic pathogenic fungus, as well as *Aspergillus fumigatus* and the budding yeast *Saccharomyces cerevisiae*, via the generation of reactive oxygen species and DNA fragmentation,

producing apoptosis-like cell death (Fujita *et al*. 2014; Yutani *et al*. 2011). In addition, although its effect was not as potent as that seen with other available antifungal drugs, anethole markedly enhanced the antifungal activities of polygodial, nagilactone E, *n*-dodecanol (dodecanol) against *S. cerevisiae* and also the antifungal activity of fluconazole against human pathogenic *Candida albicans* (Fujita *et al*. 2007; Kubo and Himejima 1992; Kubo *et al*. 1993; Kubo and Kubo 1995; Ueda *et al*. 2021). Interestingly, anethole enhanced antifungal activity by suppressing drug efflux by inhibiting the ATP-binding cassette (ABC) transporter Pdr5p, which plays a role in fungal drug resistance (Fujita *et al*. 2017; Oyama *et al*. 2020). The properties of anethole indicate its potential for use as a novel antifungal agent which poses a low risk for adverse effects in humans. However, the detailed substructures of the molecule associated with these activities remain unclear.

The objective of this study was to assess the possibility of utilizing new, naturally derived antifungal agents. Therefore, we investigated the structure-activity relationships among phenylpropanoids and related derivatives, with particular reference to the double bond between the propenyl and methoxy groups of anethole as well as to the length of its *p*-alkyl chain, using synthetic analogues of the constituent. Structure-activity relationships were elucidated mainly in relation to antifungal activity, synergy when combined with the model drug dodecanol, and ability to restrict rhodamine 6G (R6G) efflux.

Results and discussion

Although anethole, a major component of anise oil, displays a broad antimicrobial spectrum, its antimicrobial potency is weaker than those of other available antibiotics (Fujita *et al*. 2017). We first assessed the antifungal effects of anethole and its derivatives against the model fungus *S. cerevisiae*. The minimum inhibitory and minimum fungicidal concentrations (MIC and MFC, respectively) of anethole, following a 48-h incubation period, were 0.625 and 1.25 mmol l^{-1}, respectively (Table 1a). Other phenylpropanoids, including estragole, *p*-propylphenol, and propylbenzene, exhibited growth inhibitory and fungicidal activities at slightly higher concentrations than those of anethole. The MIC and MFC of propylbenzene were 4-fold higher than those of anethole, indicating that the methoxy group is preferable for antifungal and fungicidal activities. Anisole, which lacks the *p*-alkyl chain, had no antifungal or fungicidal activity at concentrations of up to 5.0 mmol l^{-1}, while the MIC and MFC of *p*-alkylanisoles with alkyl groups, varying in length from C1 to C6, decreased as the alkyl chain lengthened. On the other hand, concentrations of up to 5.0 mmol l^{-1} of *p*-alkylanisols with alkyl chains more than C7 did not affect the growth of *S. cerevisiae*, indicating that their activities were similar to that of anisole. These results indicated that the length of *p*-alkyl groups (Figs. 1e and f) contributes to the antifungal potency of anethole rather than the double bond of the propenyl group (Figs. 1a, b and R=C3 in f).

Anethole induces cell death in fungi, including *S. cerevisiae* and the human pathogenic *Aspergillus fumigatus*, via oxidative stress caused by a typical mitochondrial apoptosis-like cascade (Fujita *et al*. 2014). Therefore, we measured the levels of cellular reactive oxygen species (ROS) production in *S. cerevisiae* treated with MICs and MFCs of anethole, its derivatives, and *p*-alkylanisoles. The increased ROS production induced in *S. cerevisiae* by H_2O_2 at its MIC of 10 mmol l^{-1} was used as the positive control (Fig. 2). MFCs of the phenylpropanoids anethole, estragole, *p*-propylphenol, and propylbenzene also promoted ROS levels similar to those induced by the MIC of H_2O_2. This indicated that the level of ROS production was not affected by the presence or absence of a methoxy group or a double bond in the

propenyl group of phenylpropanoids. In addition, the MFCs of *p*-alkylanisoles with C2-C6 hydrocarbons induced cellular ROS production at levels that were also similar to those induced by the MIC of H_2O_2, except for *p*-hexylanisole (C6, $p<0.05$), the MFC of which induced the greatest oxidative stress among all derivatives tested. These results demonstrated that fungicidal action of phenylpropanoids and *p*-alkylanisoles, with C2-C6 hydrocarbon side chains, was possibly dependent on oxidative stress. On the other hand, ROS levels induced by the MICs of all tested derivatives, including anethole, were lower than that induced by the MIC of H_2O_2 ($p<0.05$). In addition to anisole, its derivatives with C1, C7, and C8 alkyl chains did not induce cellular ROS production. ROS production was not correlated with the antifungal activity of the derivatives (Table 1a). Thus, antifungal activities, as well as production levels of cellular ROS, appeared to be only partially associated with the alkyl chain lengths of *p*-alkylanisoles.

Pdr5p, a multi-drug efflux pump, belongs to a family of ABC transporters in *S. cerevisiae* (Balzi *et al*. 1994). This pump is vital for the efflux of hydrophobic molecules, including most antibiotics. Anethole restricts the expression of the *PDR5* gene, which encodes Pdr5p, as well as the expression of other genes that encode drug efflux pumps (Fujita *et al*. 2017). Therefore, to confirm the effect of phenylpropanoids, anisole, and its derivatives on drug efflux, we quantified the glucose-induced efflux of the fluorescent probe rhodamine 6G (R6G). Anethole, even at half MIC, inhibited R6G efflux from cells to a level that was 28 ± 1.8% of R6G efflux in control cells ($p<0.05$) (Fig. 3). Although the antifungal activity of estragole was weaker than that of anethole, both estragole and anethole similarly inhibited efflux. Both *p*-propylphenol and propylbenzene showed similar inhibition (61 ± 0.53 and 57 ± 2.0% of control, respectively), but were weaker than of anethole. Treatment with anisole and *p*-alkyl derivatives with C1-C6 hydrocarbon chains indicated that R6G efflux was suppressed as an increasing in the number of hydrocarbons. By contrast, the inhibitory activity of *p*-heptylanisole (C7) was weaker than that of *p*-alkylanisoles with C2-C6 hydrocarbon chains ($p<0.05$). Moreover, *p*-Octylanisole (C8) did not inhibit R6G efflux. When combined with each of several antifungal agents, anethole displayed a synergistic

antifungal effect against *S. cerevisiae* by restricting drug efflux associated with the detoxification of antifungal drugs (Fujita *et al*. 2017). Data for R6G showed that the methoxy group of anethole plays an important role in inhibiting drug efflux. In addition, we found that restriction of drug efflux by anethole required the presence of a double bond, regardless of its location in the *p*-alkyl chain. Interestingly, although the inhibition of R6G efflux was potentiated by *p*-alkylanisoles containing alkyl groups with increasing hydrocarbon chain lengths of up to C6, such inhibition was extremely decreased for derivatives containing alkyl groups with more than C7 ($p<0.05$), suggesting a cut-off point between the C6 and C7 alkyl chains. These data indicated that the length of the alkyl chain in the alkyl groups of derivatives, which imparts appropriate hydrophobicity to molecules, may be associated with the potency of drug efflux inhibition.

Anethole has been reported to increase the fungistatic activity of a model drug dodecanol, found in essential oils widely used in surfactants and pharmaceuticals, to a fungicidal effect by blocking the growth recovery process, as well as the drug efflux which prevents drug-induced damage (Fujita *et al*. 2007). Therefore, we investigated whether combining dodecanol with anethole-related derivatives would lead to synergistic antifungal activities similar to those displayed by anethole and dodecanol against *S. cerevisiae*. We found that *p*-propylphenol and propylbenzene exhibited synergy with dodecanol (Table 2a). Although the combination of estragole and dodecanol was evaluated for additive effects, the fractional inhibitory concentration (FIC) index measured using the checkerboard test was 0.56, indicating that the effect was only nearly synergistic. Combined dodecanol and anisole was not as effective as the MIC of anisole in combination with 2.5 mmol l^{-1}. *p*-Alkylanisoles with C2, C3, and C6 alkyl groups, which showed a synergistic effect. The FIC index of *p*-butylanisole (C4) was 0.625, which only approximated a synergistic effect. The MICs of combinations with *p*-alkylanisoles gradually decreased as an increase in their alkyl chain length. Among the *p*-alkylanisoles, the greatest effect was observed with *p*-hexylanisoles (C6). Significant synergy between *p*-heptylanisole (C7) and *p*-octylanisole (C8) was scored as “not

applicable" because these agents had no antifungal activity up to 5 mmol l^{-1} or growth inhibitory effects, despite being combined with dodecanol.

When combined with fluconazole, a common azole antifungal in clinical practice, anethole showed synergistic antifungal activity against the human pathogen *C. albicans* (Fujita *et al*. 2017). Overexpression of drug efflux pumps has been frequently reported in fluconazole-resistant *C. albicans* (Arendrup and Patterson 2017). We confirmed the effect of phenylpropanoids and *p*-alkylanisoles, both with and without fluconazole, on fungal growth. In this experiment, the assay for fungicidal activity was not performed because fluconazole has only fungistatic activity against *C. albicans* (Charlier *et al*. 2006) (Table 1b). The MIC values of *p*-alkylanisoles decreased with increasing alkyl chain length. However, *p*-alkylanisoles with alkyl chains with more than C7 had no antifungal activity at less than 5 mmol l^{-1}. As shown in Table 2b, the minimum FIC index was observed in combination with anethole. The dose of fluconazole required to inhibit *C. albicans* was reduced when combined with *p*-alkylanisoles, although the same effect was not observed in combination with *p*-alkylanisoles containing C2-C4 alkyl groups. The minimum effective dose of fluconazole (1/64 MIC, 0.078 μg ml^{-1}) was observed when it was combined with estragole, propylbenzene, *p*-propylanisole, or *p*-butylanisole (Table 2b). On the other hand, the FIC index indicated that the MIC of fluconazole in combination with anethole was only 1/4 MIC (1.25 μg ml^{-1}) of fluconazole alone (5.0 μg ml^{-1}). These results indicated that combinations with estragole, propylbenzene, *p*-propylanisole, or *p*-butylanisole reduce the required dosage of fluconazole; this is clinically significant, because the use of high dosages is considered to be one of the causes of drug resistance.

To our knowledge, our study is the first to investigate the role of structure-activity relationships in antifungals as well as the synergy of combining antifungals and phenylpropanoids, their derivatives, and *p*-alkylanisoles. The potency of fungicidal activity and ROS production in the derivatives largely involved *p*-alkylanisoles containing *p*-alkyl groups with adequate carbon chain lengths, rather than the double bonds of the propenyl and methoxy groups in phenylpropanoids. The inhibition exerted by phenylpropanoids on

drug efflux was also associated with the presence of double bonds and methoxy groups. Surprisingly, these effects gradually increased as an increasing in *p*-alkyl chain length of the *p*-alkylanisoles. Although *p*-alkylanisoles with C7 or more hydrocarbons did not exhibit antifungal activity, the highest activity was shown by a derivative with C6. Additional alkyl chain length has been deemed essential for regulating hydrophobicity, as octyl-functionalized nanoparticles are highly hydrophobic, whereas methyl-functionalized nanoparticles are hydrophilic (Chen *et al.* 2021). As drug efflux pumps are embedded in plasma membranes, adequate hydrophobicity (XLogP3=4.6 for *p*-hexylanisole) is necessary for their interaction with derivatives. Furthermore, the length of the hydrophobic alkyl (tail) chain of the hydrophilic hydroxyl group (head) (Kubo *et al*., 2003) determines the antifungal activity of aliphatic primary alcohols. Although the mechanism by which various activities are inhibited when the alkyl chain exceeds a certain length remains unknown, it is evident that for phenylpropanoids and their derivatives, they depend on the length of the alkyl chain.

Synergistic therapies that utilize adjuvant antibiotic and nonantibiotic drugs to potentiate antibiotic activity are emerging as an attractive approach to overcome fungal drug resistance (Kalan and Wright 2011). Our assays were mainly performed using the model yeast *S. cerevisiae*. However, our findings related to antifungal synergy between phenylpropanoids as well as their derivatives and fluconazole indicate that the antifungal effects of anethole and its derivatives, especially in enhancing the inhibition of drug efflux, are likely to apply to pathogenic yeasts, such as *C. albican*s. Our results contribute to the foundation of the practical application of combination therapies aimed at overcoming antifungal resistance.

Materials and Methods

Strains and culture conditions

Saccharomyces cerevisiae BY4741 was purchased from the Yeast Knock Out Strain Collection (Thermo Scientific Open Biosystems, Waltham, MA, USA). *Candida albicans* NBRC 1061 was obtained from the National Institute of Technology and Evaluation (Tokyo, Japan). *S. cerevisiae* and *C. albicans* were grown in YPD broth, consisting of 1% Bacto yeast extract, 2% Bactopeptone, 2% D-glucose, and 2.5% malt extract broth (Oriental Yeast, Tokyo, Japan), respectively, at 30°C for 16 h without shaking.

Chemicals

Anethole, *p*-propylanisole, 2',7'-dichloro-fluorescein diacetate (DCFH-DA), and R6G were purchased from Sigma-Aldrich (St. Louis, MO, USA). *N*,*N*-dimethylformamide (DMF), propylbenzene, anisole, and *p*-ethylanisole were obtained from Wako Pure Chemicals (Osaka, Japan). *n*-Dodecanol, estragole, and *p*-propylphenol were obtained from Tokyo Chemical Industry (Tokyo, Japan). All other reagents were of analytical grade. No commercially available *p*-alkylanisole derivatives with more than four carbons in the alkyl side chain were converted from *p*-alkylphenols via methylation of the hydroxy groups with methyl iodide, as described in the Supporting Information. Drugs were dissolved in DMF prior to use and R6G was diluted with ethanol prior to use.

Antifungal assay

Assays were performed as described (Yamawaki *et al*., 2018; Yamawaki *et al*., 2019). Serial, two-fold dilutions of the compounds were prepared in DMF. A 30-µl aliquot of 100 × stock solution was added to 3 ml of YPD broth in a test tube (diameter 10 mm). Yeast cells were inoculated at 10^6 colony-forming units (CFU) ml^{-1}. Cultures were incubated without shaking for 48 h. The MIC was defined as the lowest concentration of the test compound that showed no visible growth. After the MIC was determined, an aliquot was withdrawn from each culture and diluted 100-fold in drug-free YPD broth.

Following 48 h of incubation, MFC was determined as the lowest concentration of the test compound at which no recovery of yeast cells was observed.

Measurement of reactive oxygen species (ROS)

Cellular ROS generation was measured using the DCFH-DA method, which is dependent on intracellular deacetylation and oxidation of 2',7'-dichlorofluorescein (DCF) to the fluorescent compound, 2,7′-dichloro-fluorescein (DCF) (Fujita *et al*., 2014). This probe is highly reactive with hydrogen peroxide and has been used to evaluate ROS generation in yeast. Following preincubation (1×10^7 CFU ml^{-1}) in YPD medium with 40 μmol l^{-1} of DCFH-DA at 30°C for 60 min, 3 ml of cell suspension was withdrawn and further treated with phenylpropanoids including their derivatives at MIC and MFC against *S. cerevisiae* other than 10 mM H_2O_2 for 60 min, then washed and resuspended in 330 μl of PBS buffer. Fluorescence intensity of the cell suspension was measured using GENios (TECAN, Grödig, AT) (excitation at 485 nm and emission at 535 nm). Arbitrary units were based on fluorescence intensity per 10^7 cells. H_2O_2 was used as the positive control.

Rhodamine 6G efflux

Assays were performed as previously described (Yamawaki *et al*., 2019). Yeast cells cultured overnight in YPD broth were centrifuged at 5,000 × *g* for 5 min at 27°C. Cells were washed twice with phosphate-buffered saline (PBS) and resuspended in PBS. Cell suspensions were incubated for 18 h with shaking at 30°C, pelleted at 5,000 × *g* for 5 min at 27°C, and resuspended to a density of 5×10^8 cells ml^{-1} in PBS. R6G was added at 10 μmol l^{-1} and yeast were incubated for 60 min at 30°C. Cells that sufficiently incorporated R6G were washed and resuspended at 1.5×10^8 cells ml^{-1} in PBS. Phenylpropanoids including their derivatives at 0.5 MIC against *S. cerevisiae* and 10 mmol l^{-1} glucose were added to this suspension. Three-ml aliquots of the suspensions were withdrawn at the indicated times; cells were pelleted at 5,000

× *g* for 1 min at 27°C. Fluorescence intensity in the supernatant was measured using GENios with 485 nm excitation and 535 nm emission filters.

Evaluation of synergy

The action of derivatives combined with antimicrobials fluconazole or dodecanol was assessed using checkerboard arrays, as described above. Binary mixtures were prepared using the MIC of each antimicrobial as the maximum concentration in the mixture. The MICs of individual antimicrobials and combined antimicrobials were defined as the lowest concentrations that inhibited visible fungal growth. Each assay was conducted in triplicate. MIC data were transformed into fractional inhibitory concentration (FIC) indices. FIC indices were estimated following previously reported methods with minor modifications and interpreted as: synergy when FIC index ≤ 0.5; additive or indifferent effects when FIC index > 0.5 and < 1; and antagonism when FIC index > 1 (Konaté *et al*. 2012).

Statistical analysis

Statistical evaluation was performed using Student's *t*-test. Results with *p*-values below 0.05 were considered statistically significant.

Supporting Information

Supporting data. Syntheses of several *p*-alkylanisoles and their structural elucidation.

Acknowledgments

We are grateful to K. Uemura for the preliminary research concerning antifungal activities. This research was partly funded by the Japan Society for the Promotion of Science, Grants-in-Aid for Scientific Research (C) K19K05799 and 21K07029.

Conflict of Interest

The authors declare no competing interests.

Author contributions

Y.Y., A.O., T. T., and K.F. conceived and supervised the study; Y.T., Y.U., A.O., and K.F. designed the experiments; Y.T. and N.M. performed experiments for antifungal characterization and chemical syntheses; and Y.T., Y.U., A.O., and K.F. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

References

Aprotosoaie, A.C., Costache, I.I. and Miron, A. (2016) Anethole and Its Role in Chronic Diseases. *Adv Exp Med Biol* **929**, 247-267.

Arendrup, M. C. and Patterson, T. F. (2017) Multidrug-resistant *Candida*: Epidemiology, molecular mechanisms, and treatment. *J Infect Dis* **216**, S445-S451.

Balkis, M.M., Leidich, S.D., Mukherjee, P.K. and Ghannoum, M.A. (2002) Mechanisms of Fungal Resistance. *Drugs* **62**, 1025-1040.

Balzi, E., Wang, M., Leterme, S., Dyck, L.V. and Goffeau, A. (1994) PDR5, a novel yeast multidrug resistance conferring transporter controlled by the transcription regulator PDR1. *J Biol Chem* **269**, 2206-2214.

Charlier, C., Hart, E., Lefort, A., Ribaud, P., Dromer, F., Denning, D.W. and Lortholary, O. (2006) Fluconazole for the management of invasive candidiasis: where do we stand after 15 years? *J Antimicrob Chemother* **57**, 384-410.

Chen, W., Karde, V., Cheng, T.N.H., Ramli, S.S. and Heng, J.Y.Y. (2021) Surface hydrophobicity: effect of alkyl chain length and network homogeneity. *Front Chem Sci Eng* **15**, 90-98.

Fujita, K., Fujita, T. and Kubo, I. (2007) Anethole, a potential antimicrobial synergist, converts a fungistatic dodecanol to a fungicidal agent. *Phytother Res* **21**, 47-51.

Fujita, K.I., Ishikura, T., Jono, Y., Yamaguchi, Y., Ogita, A., Kubo, I. and Tanaka, T. (2017) Anethole potentiates dodecanol's fungicidal activity by reducing *RDR5* expression in budding yeast. *Biochim Biophys Acta* **1861**, 477-484.

Fujita, K.I., Tatsumi, M., Ogita, A., Kubo, I. and Tanaka, T. (2014) Anethole induces apoptotic cell death accompanied with reactive oxygen species production and DNA fragmentation in *Aspergillus fumigatus* and *Saccharomyces cerevisiae*. *FEBS J* **281**, 1304-1313.

Kalan, L. and Wright, G.D. (2011) Antibiotic adjuvants: multicomponent anti-infective strategies. *Expert Rev Mol Med* **13**, E5.

Konaté, K., Mavoungou, J.F., Lepengué, A.N., Aworet-Samseny, R.R.R., Hilou, A., Souza, A., Dicko, M.H. and M'Batchi, B. (2012) Antibacterial activity against β-lactamase producing Methicillin and Ampicillin-resistants Staphylococcus aureus: fractional Inhibitory Concentration Index (FICI) determination. *Ann Clin Microbiol Antimicrob* **11**, 18.

Kubo, I., Fujita, T., Kubo, A. and Fujita, K.I. (2003) Modes of antifungal action of alkanols against *Saccharomyces cerevisiae*. *Bioorg Med Chem* **11**, 1117-1122.

Kubo, I. and Himejima, M. (1992) Potentiation of antifungal activity of sesquiterpene dialdehydes against *Candida albicans* and two other fungi. *Experientia* **48**, 1162-1164.

Kubo, A. and Kubo, I. (1995) Antimicrobial agents from *Tanacetum balsamita*. *J Nat Prod* **58**, 1565-1569.

Kubo, I., Muroi, H. and Himejima, M. (1993) Combination effects of antifungal nagilactones against *Candida albicans* and two other fungi with phenylpropanoids. *J Nat Prod* **56**, 220-226.

Nicola, A.M., Albuquerque, P., Paes, H.C., Fernandes, L., Costa, F.F., Kioshima, E.S., Abadio, A.K.R., Bocca, A.L. and Felipe, M.S. (2019) Antifungal drugs: New insights in research & development. *Pharmacol Ther* **195**, 21-38.

Oyama, M., Tamaki, H., Yamaguchi, Y., Ogita, A., Tanaka, T. and Fujita, K.I. (2020) Deletion of the golgi Ca^{2+}-ATPase *PMR1* gene potentiates antifungal effects of dodecanol that depend on intracellular Ca^{2+} accumulation in budding yeast. *FEMS Yeast Res* **20**, foaa003.

Selitrennikoff, C.P. (2001) Antifungal Proteins. *Appl. Environ. Microbiol* **67**, 2883-2894.

Ueda, Y., Tahara, Y.O., Miyata, M., Ogita, A., Yamaguchi, Y., Tanaka, T. and Fujita, K.I. (2021) Involvement of a multidrug efflux pump and alterations in cell surface structure in the synergistic antifungal activity of nagilactone E and anethole against budding yeast *Saccharomyces cerevisiae*. *Antibiotics* **10**, 537.

Yamawaki, C., Oyama, M., Yamaguchi, Y., Ogita, A., Tanaka, T. and Fujita, K.I. (2019) Curcumin potentiates the fungicidal effect of dodecanol by inhibiting drug efflux in wild-type budding yeast. *Lett Appl Microbiol* **68**, 17-23.

Yamawaki, C., Yamaguchi, Y., Ogita, A., Tanaka, T. and Fujita K.I. (2018) Dehydrozingerone exhibits synergistic antifungal activities in combination with dodecanol against budding yeast via the restriction of multidrug resistance. *Planta Med Int Open* **5**, e61-e67.

Yutani, M., Hashimoto, Y., Ogita, A., Kubo, I., Tanaka, T. and Fujita, K.I. (2011) Morphological changes of the filamentous fungus *Mucor mucedo* and inhibition of chitin synthase activity induced by anethole. *Phytother Res* **25**, 1707-1713.

Figure legends

Figure 1. Chemical structures of phenylpropanoids, anisole, and their related derivatives. *trans*-Anethole (a), estragole (b), *p*-propylphenol (c), propylbenzene (d), anisole (e), and its analogs with different alkyl chain lengths (f): *p*-methylanisole (R=C1); *p*-ethylanisole (R=C2); *p*-propylanisole (R=C3); *p*-butylanisole (R=C4); *p*-hexylanisole (R=C6); *p*-heptylanisole (R=C7); and *p*-octylanisole (R=C8).

Figure 2. Effects of phenylpropanoids, anisole, and their related derivatives on ROS production in *S. cerevisiae*. Cells (10^7 per ml) were incubated in YPD broth with MICs (open column) and MFCs (closed column) of the drugs listed in Table 1 at 30˚C for 1 h after preincubation with DCFH-DA for 1 h. Control denotes treatment without drugs. H_2O_2 (10 mmol l^{-1}) was used as a positive control. Data are expressed as means ± standard deviations (n = 3). Asterisks indicate compounds that showed no fungicidal activity at evaluated concentrations of up to 5 mmol l^{-1}.

Figure 3. Effects of phenylpropanoids, anisole, and their derivatives on relative efflux of rhodamine 6G (R6G) in *S. cerevisiae*. Cells were incubated without shaking at 30°C in PBS containing 10 mmol l^{-1} glucose with each drug at 0.5 MIC listed in Table 1. Relative R6G efflux was measured after 60-min drug incubations. Data are expressed as means ± standard deviations of triplicate experiments.

Table 1. MICs and MFCs of phenylpropanoids, anisole, and their related derivatives.

Compounds		MIC* (mmol l^{-1})	MFC† (mmol l^{-1})
(a) MIC and MFC of each compound on *S. cerevisiae*.			
trans-anethole		0.625	1.25
estragole		1.25	2.5
p-propylphenol		1.25	2.5
propylbenzene		2.5	5.0
anisole		> 5.0	> 5.0
p-methylanisole	(C1)	> 5.0	> 5.0
p-ethylanisole	(C2)	2.5	5.0
p-propylanisole	(C3)	0.625	1.25
p-butylanisole	(C4)	0.313	0.625
p-hexylanisole	(C6)	0.156	0.313
p-heptylanisole	(C7)	> 5.0	> 5.0
p-octylanisole	(C8)	> 5.0	> 5.0
(b) MIC of each compound on *C. albicans*.‡			
trans-anethole		1.25	–
estragole		1.25	–
p-propylphenol		0.625	–
propylbenzene		5.0	–
anisole		> 5.0	–
p-methylanisole	(C1)	> 5.0	–
p-ethylanisole	(C2)	1.25	–
p-propylanisole	(C3)	1.25	–
p-butylanisole	(C4)	0.625	–
p-hexylanisole	(C6)	0.625	–
p-heptylanisole	(C7)	> 5.0	–
p-octylanisole	(C8)	> 5.0	–

At least three independent assays were performed with the same results.

*Minimum inhibitory concentration.

†Minimum fungicidal concentration.

‡The fungicidal effects against *C. albicans* were not tested.

Table 2. Synergistic antifungal activity of anethole-related derivatives and other compounds.

Compounds	Effective MICs dodecanol (mmol l^{-1}) or fluconazole (μg ml^{-1}) /anethole-related derivatives (mmol l^{-1})	FIC index	Outcome
(a) Synergy of dodecanol and anethole-related derivatives on *S. cerevisiae*.			
n-dodecanol	0.625 / –	–	–
trans-anethole	0.156 / 0.156	0.5	synergy
estragole	0.313 / 0.078	0.56	additive
p-propylphenol	0.156 / 0.156	0.375	synergy
propylbenzene	0.156 / 0.625	0.5	synergy
anisole	0.156 / 2.5	N.A. (< 0.75)	N.A. (additive)
p-methylanisole (C1)	0.156 / 0.313	N.A. (< 0.313)	N.A. (synergy)
p-ethylanisole (C2)	0.156 / 0.156	0.313	synergy
p-propylanisole (C3)	0.156 / 0.156	0.5	synergy
p-butylanisole (C4)	0.313 / 0.039	0.625	additive
p-hexylanisole (C6)	0.156 / 0.019	0.375	synergy
p-heptylanisole (C7)	N.A.	N.A.	N.A.
p-octylanisole (C8)	N.A.	N.A.	N.A.
(b) Synergy of fluconazole and anethole-related derivatives on *C. albicans.*			
fluconazole	5.0 / –	–	–
trans-anethole	1.25 / 0.078	0.188	synergy
estragole	0.078 / 0.625	0.515	additive
p-propylphenol	1.25 / 0.078	0.375	synergy
propylbenzene	0.078 / 2.5	0.515	additive
anisole	N.A.	N.A.	N.A.
p-methylanisole (C1)	N.A.	N.A.	N.A.
p-ethylanisole (C2)	0.156 / 0.313	0.281	synergy
p-propylanisole (C3)	0.078 / 0.313	0.266	synergy
p-butylanisole (C4)	0.078 / 0.313	0.515	additive
p-hexylanisole (C6)	1.25 / 0.313	0.75	additive
p-heptylanisole (C7)	N.A.	N.A.	N.A.
p-octylanisole (C8)	N.A.	N.A.	N.A.

At least three independent assays were performed with the same results.

N.A., not applicable, as these compounds alone did not provide definitive MIC measurements.

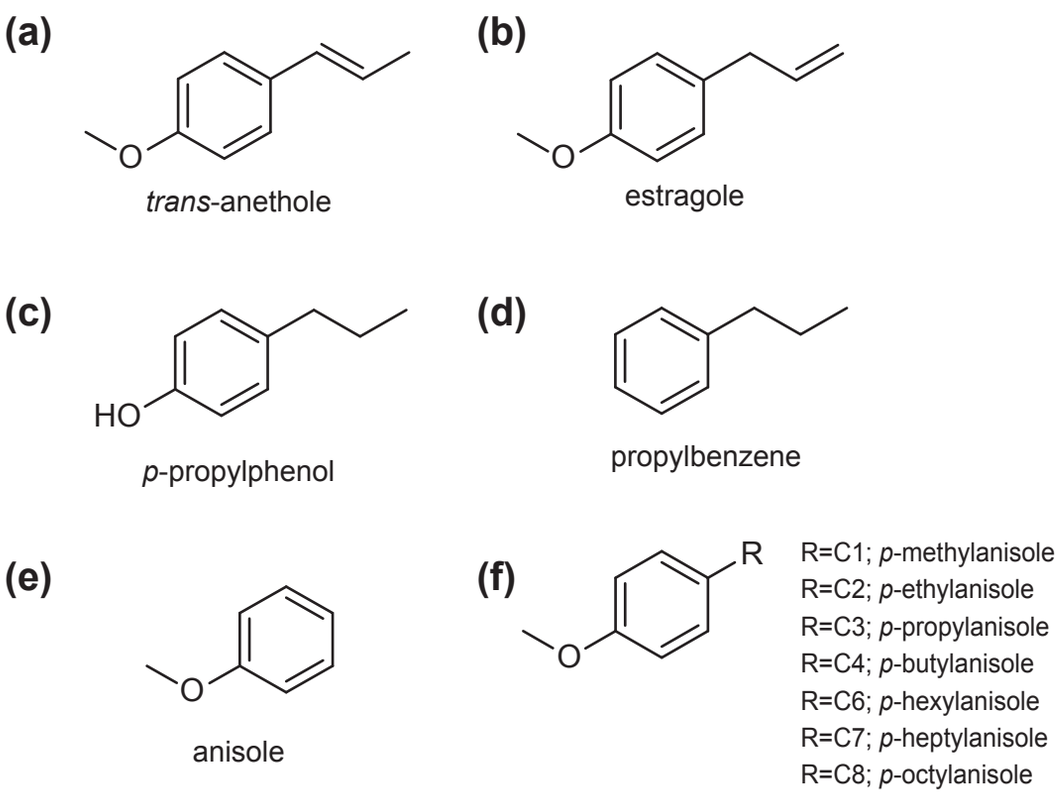

Fig. 1. Tsukuda et al.

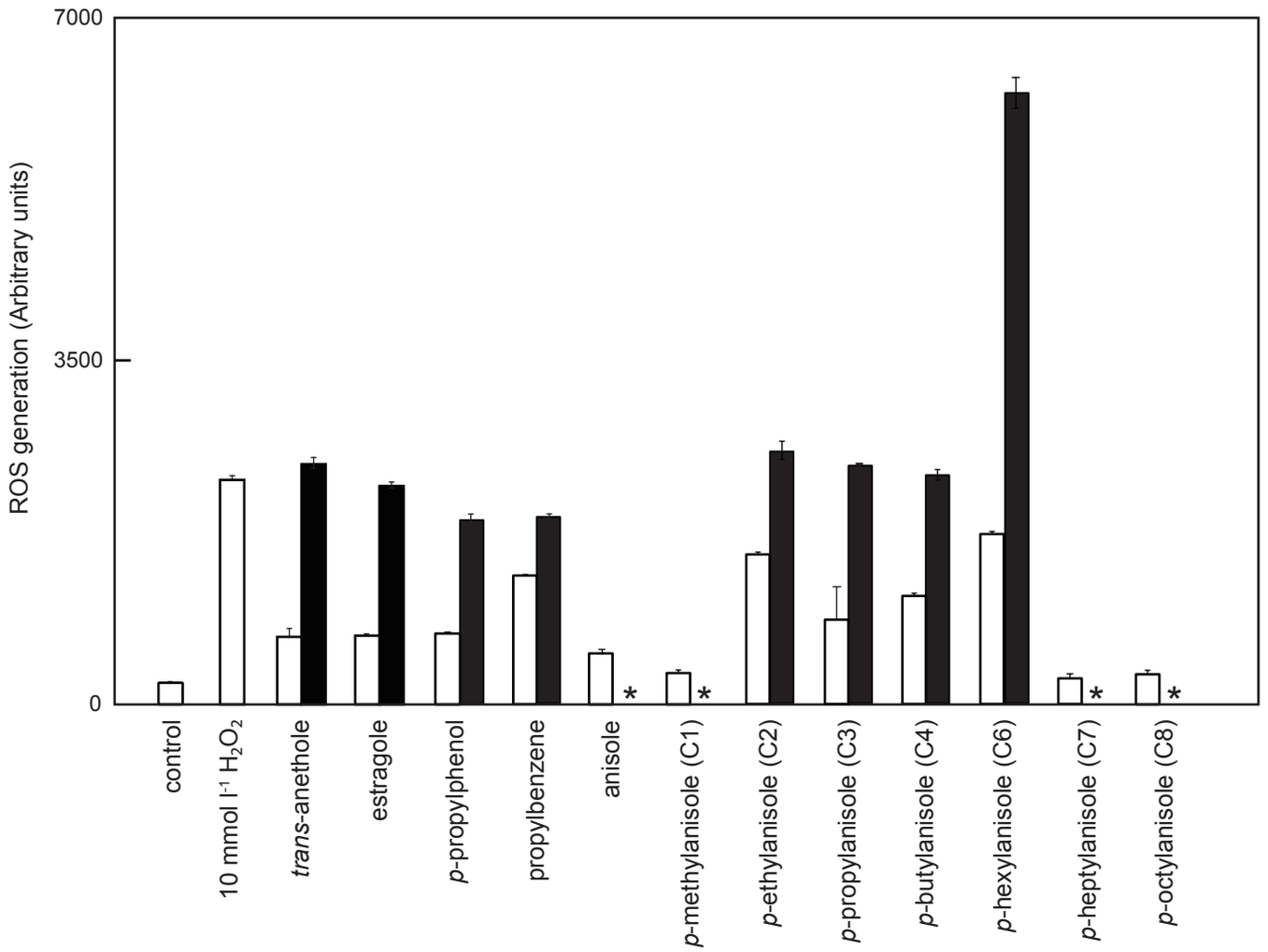

Fig. 2. Tsukuda et al.

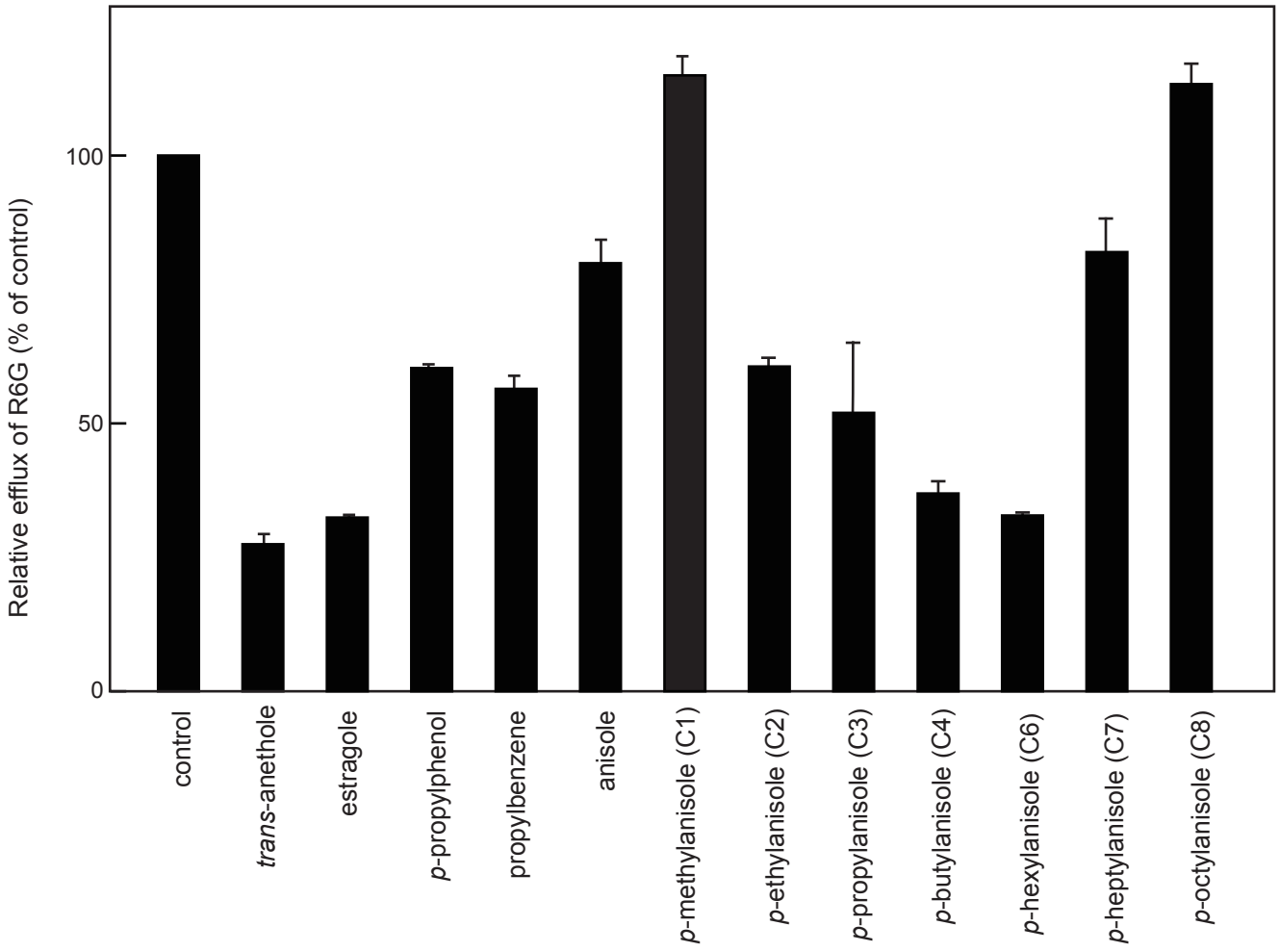

Fig. 3. Tsukuda et al.