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Physicochemical Solubility of and Biological Sensitivity to Long-Chain Alcohols Determine the Cutoff Chain Length in Biological Activity[S]

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ABSTRACT

The cutoff phenomenon associated with the effectiveness of long-chain alcohols in the induction of anesthesia is also observed for various antimicrobial activities, although the mechanism has remained unknown for over eight decades. The minimum inhibitory concentrations at 25°C for budding yeast growth exponentially decreased with increasing chain length of *n*-alcohols (C_2–C_{12}), whereas alcohols $\geq C_{13}$ lost the inhibitory effect. Thus, growth inhibition by *n*-alcohols obeys the Meyer-Overton correlation up to C_{12} and exhibits a cutoff phenomenon. The densities of *n*-alcohols are low, and the melting point and hydrophobicity increase with chain length. C_{13} and C_{14} inhibited yeast growth at 39.8°C, above their melting points. Alcohols $\leq C_{14}$ inhibited thermophilic bacterial growth at 50°C, whereas C_{16} inhibited it at 67.5°C, above their melting points. Thus, the high melting points of long-chain alcohols contribute to the cutoff phenomenon. C_{14} did not effectively inhibit yeast growth in a static culture at 39.8°C, in contrast to a shaking culture, in which the low density–dependent concentration gradient was eliminated. The duration of the transient growth inhibition of yeast by C_{12} was prolonged by sonication, which prevented hydrophobic aggregation. Therefore, a nonuniform distribution owing to low density and high hydrophobicity contributes to the cutoff. C_{14} inhibited the growth at 25°C of the *pdr1,3,5* mutant, defective in multidrug efflux pumps, whereas C_{12} did not inhibit the growth of yeast overexpressing *PDR5*, indicating that the sensitivity to long-chain alcohols contributed to the cutoff. A balance between the physicochemical solubility of and the biological sensitivity to long-chain alcohols determines the cutoff chain length.

Introduction

The mechanism of anesthesia remains unclear because of the difficulty in explaining how various anesthetics cause similar anesthetic effects (Urban et al., 2006). The Meyer-Overton correlation—the potency of various anesthetics correlates with the lipophilicity/hydrophobicity—paved the way for the elucidation of this mechanism (Meyer, 1899; Overton, 1901). Initially, anesthetics were presumed to target membrane lipids as lipophilic sites according to the correlation. These are the so-called lipid theories (Antkowiak, 2001). The anesthetic "cutoff" is an unresolved phenomenon in which the anesthetic effects of *n*-alcohols increase with their chain lengths (lipophilicity/hydrophobicity), but long-chain alcohols ($\geq C_{13}$) abruptly lose their effects (Meyer and Hemmi, 1935; Pringle et al., 1981). This reproducible lack of correlation between the anesthetic potency and lipophilicity/hydrophobicity of long-chain alcohols contradicted the Meyer-Overton correlation, suggesting that the lipid theories are insufficient to explain the anesthetic mechanism.

Anesthetics and *n*-alcohols inhibited firefly luciferase activity in the absence of membrane lipids, suggesting a direct interaction between anesthetics and proteins (Ueda, 1965; Franks and Lieb, 1985). The inhibitory effects of *n*-alcohols increased with chain lengths, except for long-chain alcohols, indicating that the Meyer-Overton correlation and cutoff are also observed in luciferase inhibition. The protein theory was proposed to explain the Meyer-Overton correlation, the luciferase inhibition by anesthetics and *n*-alcohols, the cutoff effect, and other observations (Franks and Lieb, 1985, 1990). A simple hypothesis explaining the cutoff assumes that target proteins have hydrophobic anesthetic-binding pockets/clefts that fit various anesthetics but not long-chain compounds larger than the cutoff length. Accordingly, several membrane proteins with pockets are considered as target candidates (Urban et al., 2006). However, several reports raised questions about such pocket/clefts (Chiou et al., 1990; Eckenhoff et al., 1999; Cantor, 2001; Peoples and Ren, 2002; Mohr et al., 2005; Williams et al., 2007). *n*-Alcohols $\leq C_{10}$ decrease the phase-transition temperature of the membrane model in response to chain length, whereas alcohols $\geq C_{12}$ increase it. Thus, the Meyer-Overton correlation and cutoff may be explained by a

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ABBREVIATIONS: ABC, ATP-binding cassette; DMF, *N*,*N*-dimethylformamide; GABA, γ-aminobutyric acid; HydC, hydrated concentration; logP, logarithm of octanol-water partition coefficient; MIC, minimum inhibitory concentration; MixC, mixed concentration; NMDA, *N*-methyl-D-asparate receptor; PDR, pleiotropic drug resistance; SD-U, synthetic complete dextrose medium minus uracil; TMA, trimethyl ammonium compounds; YPD, yeast extract peptone dextrose; YT, yeast extract tryptone.

membrane alteration in the absence of protein, although the biological significance of the phase-transition temperature on anesthesia remains unknown (Kamaya et al., 1984). Recently, the cutoff phenomenon was observed in various biological systems, such as the sodium channel, gramicidin A channel formation, NMDA and GABA receptors, and cytokine production (Ingólfsson and Andersen, 2011; Carignan et al., 2013). These analyses explained the correlation between the biological phenomena and chain length at which the cutoff is observed (the cutoff chain length), but not the causation. At present, there remains no unified view to explain the cutoff.

n-Alcohols inhibit the growth of various microorganisms, and the inhibition potency increases with chain length, but long-chain alcohols lose this inhibitory effect (Gill and Ratledge, 1972; Teh, 1974; Kato and Shibasaki, 1980; Kubo et al., 1995, 2003; Mukherjee et al., 2013). Therefore, the Meyer-Overton correlation and cutoff phenomenon are also observed in antimicrobial activity, indicating that these effects are not specific to anesthesia in animals and various biological systems. Moreover, the chain lengths for the antimicrobial cutoff ($\geq C_{13}$) are similar to those for anesthesia. Thus, the cutoff phenomenon may be caused either by the target common to organisms or the physicochemical properties of *n*-alcohols.

Trimethyl ammonium compounds (TMAs), one type of quaternary ammonium compound, are representative surfactants that have a cationic hydrophilic region and a hydrophobic alkyl chain and induce alcohol-like reactions in yeast, such as the inhibition of translation initiation and actin polarization. TMA-induced reactions also exhibit the Meyer-Overton correlation, but not the cutoff, even though the alkyl chain is C_{18} (Uesono et al., 2011). Dendritic tricarboxylato compounds with hydrophilic tricarboxyl groups and a long hydrophobic alkyl chain ($\leq C_{22}$) also do not exhibit the cutoff for antimicrobial activities (Williams et al., 2007). Because the water solubility of TMAs (TOXNET, https://toxnet.nlm.nih.gov) and tricarboxylato compounds is far higher than that of long-chain alcohols, the physicochemical property of solubility may account for the cutoff of long-chain alcohols. Here, we focused on the causation between the physicochemical properties of *n*-alcohols and the cutoff in microorganisms.

Materials and Methods

Strains, Media, and Plasmids. Standard genetic manipulation of yeast and DNA manipulation were performed as described previously (Guthrie and Fink, 1991). The media used for the budding yeast *Saccharomyces cerevisiae* were YPD (yeast extract peptone dextrose: 1% yeast extract, 2% polypeptone, and 2% glucose) and SD-U (synthetic complete dextrose medium minus uracil: 2% glucose, 0.67% yeast nitrogen base, 0.5% casamino acids, 20 mg/l tryptophan, and 20 mg/l adenine sulfate). Sodium phosphate buffer (pH 6.0) was added into these media at the final concentration of 10 mM. The thermophilic bacterium *Geobacillus stearothermophilus* (NBRC12550) was purchased from Biologic Resource Center, NITE (Chiba, Japan). The medium used for the bacteria was 2×YT (1.6% Bacto tryptone, 1% yeast extract, 0.5% NaCl) containing 3 mM $MgSO_4$ (Msadek et al., 1998). For overexpression of *PDR5* (pleiotropic drug resistance 5), the *PDR5* fragment was cloned into the yeast multicopy vector YEp24 (YEp24-PDR5), a gift from Y. Tsujimoto (Tsujimoto et al., 2015). The *pdr5* knockout strain and the *pdr1, pdr3,* and *pdr5* triple-knockout strain were a gift from M. Mizunuma (Tutulan-Cunita et al., 2005). The yeast strains used in this study are listed in Table 1.

Chemicals. The *n*-alcohols, ethanol (C_2), 1-propanol (C_3), 1-butanol (C_4), 1-hexanol (C_6), 1-octanol (C_8), 1-decanol (C_{10}), 1-tridecanol (C_{13}), 1-tetradecanol (C_{14}), 1-hexadecanol (C_{16}), oleyl alcohol ($C_{18:1}$), and Sudan III were purchased from Wako Pure Chemicals (Tokyo, Japan). 1-Dodecanol (C_{12}) and 1-octadecanol (C_{18}) were purchased from Tokyo Chemical Industry (Tokyo, Japan).

Determination of the Minimum Inhibitory Concentration. The minimum inhibitory concentration (MICs) of microorganisms were determined as described previously (Kubo et al., 1995). Microbial growths were performed in test tubes, microtubes, and 96-well microtiter plates (Corning, Corning, NY), and the growths (OD_{600}) were monitored by a UV spectrophotometer (Amersham Ultrospec 1100 Pro). Logarithmic cultures of the yeast strains grown in YPD (pH 6.0) and the yeast strains harboring the plasmids grown in SD-U (pH 6.0) at 25°C for 24 hours were inoculated into YPD (pH 6.0) (OD_{600} = 0.05; 7.5×10^5 cells/ml) and incubated at the indicated temperatures. Logarithmic cultures of thermophilic bacterium grown in 2×YT containing $MgSO_4$ at 50°C were inoculated into the same medium (OD_{600} = 0.05; 2×10^7 cells/ml) and incubated at the indicated temperatures. The long-chain alcohols ($\geq C_6$) were first dissolved in *N,N*-dimethylformamide (DMF), and serial 2-fold dilutions were performed in DMF. The diluted solutions (5 μl) were added to the sterile media (final 500 μl) containing the cells in test tubes and microtubes at the indicated concentrations of alcohols. The final concentration (1%) of DMF did not affect the microbial growth. The short-chain alcohols ($\leq C_4$) were added directly to the media containing the cells, and serial 2-fold dilutions were performed in the same media. These samples were incubated with or without shaking for the indicated times and at the indicated temperatures. For the 96-well microtiter plates, 100 μl of the above media containing the dilution series of long-chain alcohols was used without dilution on the plate, and then incubated without shaking. The MIC of the test compound was the lowest concentration that completely inhibited microbial growth, as judged by visual observation or spectrophotometric determination.

Sonication. The medium containing the alcohol aggregates (1 mM C_{12}, 1% DMF) was sonicated in a microtube (pulse, 5 seconds; output level, 5), using an ultrasonic disruptor (UD201; TOMY, Tokyo, Japan) and then transferred to a test tube for monitoring of the growths (OD_{600}). To visualize the alcohol behavior in water by sonication, we used Sudan III dye that is a poorly soluble in water but soluble in C_{12}. The C_{12} solution containing 1% Sudan III was mixed with water (10 mM of C_{12}) and sonicated in a microtube, and then transferred to a test tube.

Data Analysis. Statistical analyses of yeast growth were performed by one-way analysis of variance with the Tukey-Kramer multiple comparison test, using the R statistical software package (ver. 3.5.0; R Development Core Team 2018).

TABLE 1
Yeast strains used in this study

Strain	Genotype	Sources
BY4741	MATa *his3Δ1 leu2Δ0 ura3Δ0 met15Δ0*	EUROSCARF collection
W303-1A	MATa *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1can1-100 ssd1-d2*	Our strain collection
YYU701	W303-1B MATα *pdr5Δ::LEU2*	Tutulan-Cunita et al. (2005)
YYU702	W303-1A MATa *pdr1Δ::hisG pdr3Δ::hisG pdr5Δ:: LEU*	Tutulan-Cunita et al. (2005)

Results

A Comparison between the Aqueous Solubility of Alcohols on the Basis of Chain Length and Minimum Inhibitory Concentrations against Yeast. At 25°C for 24 hours, the MICs of budding yeast (*S. cerevisiae*) growth exponentially decreased with increasing chain lengths of C_2–C_{12}, whereas longer alcohols ($\geq C_{13}$) lost their inhibitory effect, even though these were added in excess ($\geq$10 mM) (Fig. 1, red circles). Thus, the inhibition of yeast growth by *n*-alcohols obeys the Meyer-Overton correlation up to C_{12} and exhibits the cutoff phenomenon, as reported previously (Gill and Ratledge, 1972; Teh, 1974; Kato and Shibasaki, 1980; Kubo et al., 1995, 2003). Comparing the MICs with the aqueous solubility (S_W) of *n*-alcohols (Bell, 1973), the MICs of the alcohols $\leq C_{10}$ were lower than the solubility, but the MIC of C_{12} was higher than the solubility (Fig. 1). Similar trends were observed for the inhibition of luciferase, the NMDA receptor, and immunomodulatory activity (Franks and Lieb, 1985; Peoples and Ren, 2002; Carignan et al., 2013), suggesting that the correlation with S_W is not specific for yeast growth inhibition. This result suggests that the aqueous solubility of shorter-chain alcohols ($\leq C_{10}$) reaches the MIC, but those of the longer-chain alcohols ($\geq C_{12}$) do not. According to this hypothesis, the C_{12} alcohol is considered to be the cutoff compounds; however, C_{12} inhibited growth. To explain this finding, we determine whether the aqueous solubility of *n*-alcohols affects the biological activity.

Physicochemical Properties to Determine the Solubility of *n*-Alcohols. The solubility of long-chain *n*-alcohols in water is very low because of their physicochemical properties. First, all densities of the *n*-alcohols are lower than that of water (Supplemental Table 1). Water molecules interact with each other through hydrogen bonds, forming a hydrogen bond network. The aqueous solubility of an *n*-alcohol is determined by the hydrogen bonds between the hydroxyl group of the *n*-alcohol and water molecules. In addition to hydrogen bonds, short-chain alcohols ($\leq C_3$) are trapped in the hydrogen bond network and are efficiently miscible in water (Fig. 2A, C_2), whereas long-chain alcohols ($\geq C_4$) are not trapped because they would destroy the network. Consequently, long-chain alcohols ($\geq C_4$) separate from water and float owing to their specific densities (Fig. 2A, lower panel C_4–C_{12}). Second, in the upper layer, van der Waals forces among the alkyl chains increase with chain length, resulting in an increase in the melting point (Supplemental Table 1). For example, C_{14} is solid owing to the formation of a crystal structure at 25°C, which is lower than its mp (37.7°C) (Fig. 2A). Third, the hydrophobicity/lipophilicity of *n*-alcohols, expressed as the octanol-water partition coefficient (logP), increases with an increase in the hydrophobic alkyl chain length and a decrease in the relative abundance of hydrogen bonds (Supplemental Table 1). Therefore, long-chain alcohols aggregated and separated rapidly from the medium after mixing owing to the hydrophobic interactions defined by the chain lengths (Fig. 2A, 0.5 minutes). Because the liquid alcohols (C_4–C_{12}) have similar densities (Supplemental Table 1), the aggregation/separation rates depend on the hydrophobicity, rather than the density and melting point.

In the lower layer (Fig. 2B), long-chain alcohol molecules hydrogen-bond with water molecules and exist in a hydrated state (hydrated concentration: HydC). HydC exponentially decreases with chain length because of the decrease in the relative contribution of hydrogen bonds, regardless of the solid/liquid state (Fig. 2B, black circles). In addition,

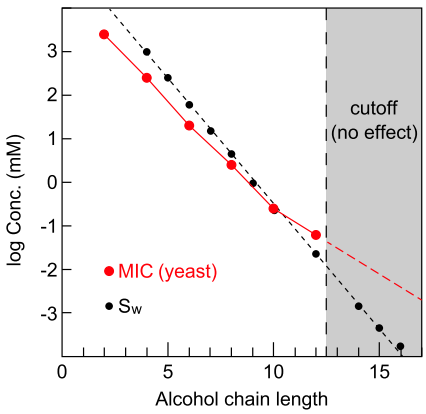

Fig. 1. A comparison between the aqueous solubilities of *n*-alcohols and the minimum inhibitory concentrations of yeast. The MICs of *n*-alcohols C_2–C_{18} were measured in yeast (BY4741) after 24 hours in YPD with shaking at 25°C. The alcohols (C_{13}–C_{18}) did not inhibit yeast growth, even at 10 mM. The area containing the cutoff alcohols (C_{13}–C_{18}) is indicated with the gray zone. The log MICs (C_2–C_{12}) (red circles) and the log aqueous solubility (S_W) of *n*-alcohols C_2–C_{16} (Bell, 1973) (black circles) are plotted against the number of carbons in the alkyl chain. The dotted red line indicates the extrapolated the MICs of yeast. The MICs of three independent experiments were identical.

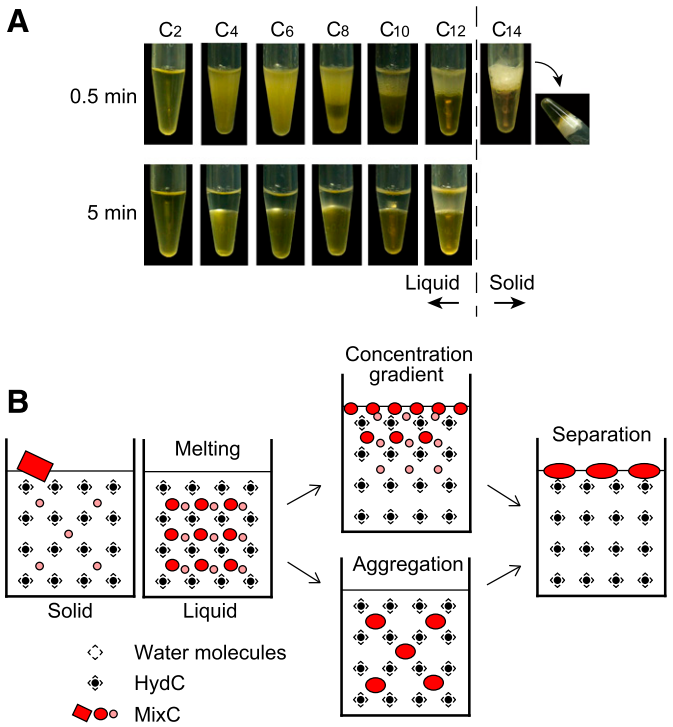

Fig. 2. States of *n*-alcohols in aqueous solutions. (A) *n*-Alcohols C_2–C_{14} (500 μl) were mixed with equal amounts of YPD using a microtube mixer at top speed for 5 seconds and were then observed after 0.5 and 5 minutes at 25°C. C_2 to C_{12} alcohols were liquid, but C_{14} was solid at 25°C. (B) States of the long-chain alcohols (solid/liquid) in the aqueous solution after mixing. The long-chain alcohol molecules hydrogen-bond with water molecules and exist in the hydrated state. The long-chain alcohol droplets/particles ($\geq C_4$) simply mix with water without stable hydrogen bonds and exist in the mixed state. The MixC is unstable because of its low density (concentration gradient) and high hydrophobicity (aggregation).

long-chain alcohol droplets/particles $\geq C_4$ simply mix with water without stable hydrogen bonds and exist in a mixed state (mixed concentration: MixC). MixC is unstable because the droplets/particles separate from water and aggregate owing to high hydrophobicity and float on water owing to the low density (Fig. 2B, red/pink figures). For example, in the lower layer, the solubility of the supersaturated C_{16} solution gradually decreased over 20 days, and reached an equilibrium concentration (Krause and Lange, 1965). The gradual decrease resulted from a decrease in the unstable MixC, and the equilibrium concentration corresponded to HydC (Fig. 2B).

The equilibrated solubility of organic nonelectrolytes in water is estimated by the equations for liquid (eq. 1) and the solid solutes (eq. 2) (Jain and Yalkowsky, 2001).

$$\log S^{liquid} = 0.5 - \log P_{OW} \quad (1)$$

$$\log S^{solid} = 0.5 - 0.01(mp - 25) - \log P_{OW} \quad (2)$$

where S^{liquid} is the solubility of the liquid in water, S^{solid} is the solubility of the solid in water, $\log P_{OW}$ is the octanol-water partition coefficient (Supplemental Table 1), mp is melting point, and 25 is room temperature. These are applicable to the equilibrated solubility (HydC) of long-chain alcohols in water. According to the equations, the HydC of the solid alcohol is estimated by eq. 2 at room temperature. When the solid alcohol is melted at high temperature, its HydC is estimated by eq. 1. Thus, the HydC of the melted alcohol is higher than that of the solid alcohol. Indeed, the solubility of solid alcohols increases at high temperatures compared with the solubility of liquid alcohols (Krause and Lange, 1965). Consequently, the hydrophobicity (partition coefficient) and melting point are important factors for estimating the HydC, although an effective method to estimate the unstable MixC remains unknown.

Important for organisms living in an aqueous solution is the total solubility in the lower, which consists of both the stable HydC and unstable MixC. These properties of *n*-alcohols are observed even at the low concentrations used for assessing anesthetic and antimicrobial activities. Importantly, the total solubility of long-chain alcohols is largely affected by physicochemical conditions. We examined the causation between these physicochemical properties of *n*-alcohols and the cutoff phenomenon.

Low Solubility owing to the High Melting Point of Long-Chain Alcohols Contributes to the Cutoff. Alcohols $\leq C_{12}$ (mp: ≤24°C) with anesthetic and antimicrobial activities are liquid at 25°C, whereas alcohols $\geq C_{13}$ (mp: ≥32.5°C) without those activities are solid (Supplemental Table 1), suggesting that the solid phase may influence the cutoff. Because the solubility of solid alcohols is increased by melting (Krause and Lange, 1965) (Fig. 2B, Melting), we measured the MICs of *n*-alcohols on yeast growth at a high temperature. Alcohols C_{13} (mp: 32.5°C) and C_{14} (mp: 37.7°C) inhibited yeast growth at 39.8°C when they were melted, but other alcohols ($\geq C_{16}$, mp: ≥49.3°C) did not (Fig. 3A), indicating that the melting of long-chain alcohols avoids the cutoff. However, the MIC of C_{14} was higher than that predicted by the Meyer-Overton correlation. Because C_{14} may be partially melted and the alcohols $\geq C_{16}$ are not melted at 39.8°C, limiting temperature for yeast growth, a higher temperature is required to precisely evaluate these MICs. Therefore, we used *Geobacillus stearothermophilus*, a Gram positive bacterium that can grow at ~70°C (Fig. 3B). At 50°C, the MICs exponentially decreased with chain length (C_2–C_{14}), whereas alcohols $\geq C_{16}$ lost their inhibitory effect. Thus, growth inhibition also obeys the Meyer-Overton correlation and exhibits the cutoff phenomenon at 50°C. At 67.5°C, C_{16} also inhibited growth according to the correlation. These results indicate that the cutoff chain length increases with an increase in temperature regardless of the species. Destabilizing the solid-state structures of the long-chain alcohols with high temperatures increases the number of free molecules, thereby increasing the total solubility and allowing the MIC to be reached. Consequently, the low solubility resulting from the high melting points of long-chain alcohols contributes to the cutoff.

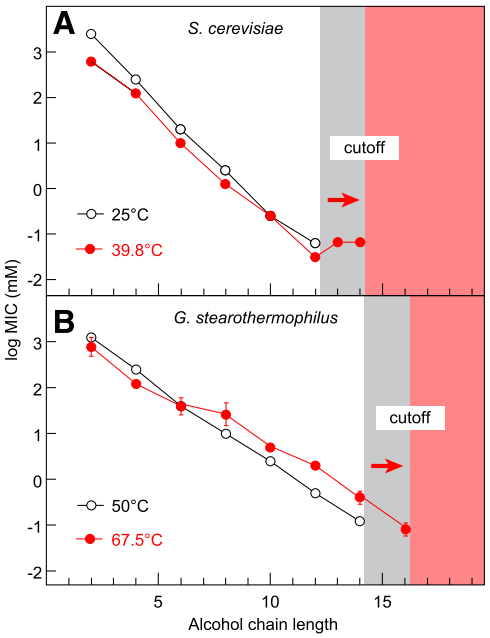

Fig. 3. Effects of temperature on the cutoff chain length of *n*-alcohols. The log MICs of *n*-alcohols (C_2–C_{18}) for each microorganism are plotted against the number of carbons in the alkyl chain, except for the cutoff alcohols. (A) The MICs of the yeast strain (BY4741) after 24 hours in YPD with shaking at the indicated temperatures. (B) The MICs of the thermophilic bacterium *G. stearothermophilus* after 24 hours in 2×YT containing $MgSO_4$ with shaking at the indicated temperatures. The areas containing the cutoff alcohols with no growth inhibitory effect are indicated by the gray zones at low temperatures (25°C and 50°C) and the red zone at high temperatures (39.8°C and 67.5°C). Data are the means ± S.D. of triplicate experiments.

Concentration Gradient owing to the Low Density of Long-Chain Alcohols Contributes to the Cutoff. Because long-chain alcohols ($\geq C_4$) were concentrated in the upper layer of the medium owing to their low specific densities, the concentrations would be lower at the bottom in the static culture (Fig. 2B, concentration gradient). In the shaking culture, the concentration gradient formed by the low density would be eliminated. To test the effect of the gradient on growth, we used budding yeast that grows both at the bottom of the static culture and in the entire area of the shaking culture. As shown in Fig. 4A, yeast cells grew at the bottom of the static culture containing C_{14} (4×MIC) at 39.8°C when it was melted, but not in the shaking culture. This observation suggests that the concentration of C_{14} was lower in the bottom of the static culture than the shaking culture. No growth was observed in either of the cultures containing C_2 (1×MIC) at 25°C (Fig. 4A). Thus, C_{14} would form a gradient of the unstable MixC lower at the bottom, whereas the water-miscible C_2 would not form a MixC gradient. Although C_{10}

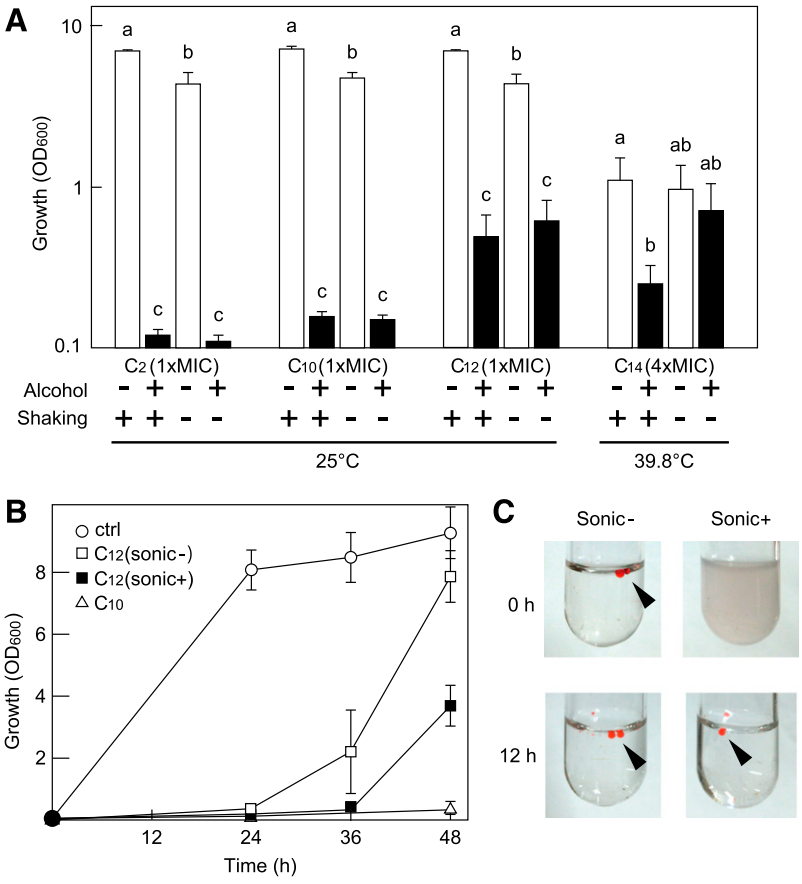

Fig. 4. Effects of culture methods on the growth inhibitory activity of long-chain alcohols. (A) The BY4741 cells were inoculated (OD_{600} = 0.05) into 500 μl of YPD containing C_2 (2.5 M: 1×MIC), C_{10} (250 μM in 1% DMF: 1xMIC), C_{12} (63 μM in 1% DMF: 1×MIC), C_{14} (250 μM in 1% DMF: 4×MIC) (black bars), YPD without alcohols (for C2), and YPD containing 1% DMF (for C_{10}, C_{12}, and C_{14}) (white bars). Growth (OD_{600}) was monitored after 24 hours at the indicated temperatures in the shaking/static cultures of the microtubes. Data are the means ± S.D. ($n \geq 3$). Different letters denote significant differences using analysis of variance and Tukey-Kramer test at $P < 0.05$. (B) The OD_{600}s of BY4741 was monitored at the indicated times in the shaking YPD cultures (500 μl in test tubes) containing 1% DMF (circles), C_{10} (250 μM in 1% DMF: 1×MIC) (triangles), C_{12} (1000 μM in 1% DMF: 16×MIC) without sonication (squares), and C_{12} (16×MIC) with sonication (black squares). Data are the means ± S.D. ($n \geq 3$). (C) The C_{12} solutions containing 1% Sudan III were mixed with water (final 10 mM) and incubated for 12 hours with shaking. The nonsonicated (left) and sonicated solution (right) were indicated. The arrows indicate the aggregates of C_{12}.

should also form a gradient, no growth difference was observed in either culture containing C_{10} (1×MIC), suggesting that the HydC alone of C_{10} reaches the MIC at the bottom of the static culture, but that of C_{14} does not. Thus, the increase in the MixC of C_{14} by shaking increases the total solubility with the HydC, thereby reaching the MIC. Consequently, the concentration gradient owing to the low density of long-chain alcohols is involved in the cutoff.

Compared with C_2 and C_{10}, C_{12} (1×MIC) did not completely inhibit growth in a microtube, suggesting that the HydC of C_{12} also does not reach the MIC (Fig. 4A). However, no apparent difference in growth inhibition was observed in either culture, which differs from the case of C_{14}, possibly because growth at 25°C is faster than at 39.8°C, a severe growth condition. Therefore, the gradient elimination alone of C_{12} does not efficiently inhibit fast growth at 25°C. Presumably, the efficient inhibition of fast growth by C_{12} requires a mechanism other than gradient elimination, as described below.

Nonuniform Distribution owing to the High Hydrophobicity of Long-Chain Alcohols Contributes to the Cutoff. Long-chain alcohols aggregate through hydrophobic interactions even in a shaking culture (Fig. 2B, Aggregation), thereby gradually decreasing the local MixC, which allows microbial growth. Indeed, C_{12} inhibited yeast growth for 24 hours in the shaking culture at 25°C but did not inhibit growth after 48 hours (16×MIC), whereas C_{10} (1×MIC) inhibited growth after 72 hours, indicating that growth inhibition by C_{12} is transient (Fig. 4B). Therefore, C_{12} is also the cutoff compound if it is judged by long-term treatments, as expected in Fig. 1. This observation is consistent with previous reports (Kubo et al., 2003; Fujita et al., 2017). Presumably, the HydC alone of C_{10} would reach the MIC, but that of C_{12} would not, although the total solubility (HydC + MixC) reaches the MIC. The decrease in the MixC by aggregation gradually reduces the total solubility even in the shaking culture, thereby causing transient growth inhibition. Therefore, if the hydrophobic aggregation of C_{12} is prevented and the MixC does not decrease, the growth remains inhibited. To confirm this, we sonicated the medium containing C_{12} to prevent aggregation. C_{12} in the aqueous solution was visualized with Sudan III, except for very small droplets. The sonicated C_{12} dispersed but gradually aggregated after 12 hours in a shaking aqueous solution, whereas it did not disperse without sonication (Fig. 4C). Yeast cells were inoculated into the sonicated medium and cultured in it with shaking. The duration of growth inhibition was prolonged in the sonicated medium compared with the nonsonicated medium (Fig. 4B). This increase in the duration of inhibition occurs because the prevention of aggregation delays the decrease in MixC. Therefore, the duration of the total solubility (HydC + MixC) reaching the MIC is extended, resulting in a prolonged duration of growth inhibition, regardless of the cellular states. Therefore, nonuniform distribution owing to the high hydrophobicity of long-chain alcohols contributes to the cutoff.

Biological Sensitivity to *n*-Alcohols Contributes to the Cutoff. A variety of chain lengths for *n*-alcohols inhibit the growth of various microorganisms under similar physicochemical conditions, although the reason remains unknown (Gill and Ratledge, 1972; Teh, 1974; Kato and Shibasaki, 1980; Kubo et al., 1995, 2003). This observation

suggests that a factor other than the physicochemical properties of *n*-alcohols is also involved in the cutoff. Long-chain alcohols will inhibit the growth of microorganisms if the solubility reaches the MIC; otherwise, they would be cutoff compounds. If the cutoff chain length were determined by a balance between the solubility and the MIC, the cutoff chain length would be extended by a reduction in the MIC without a change in the physicochemical conditions. C_{12} inhibited the growth of BY4741, a wild-type strain, in 96-well plates at 24 hours, but not at 48 hours (Table 2). This transient inhibition is consistent with the observation in Fig. 4B. However, at 48 hours, C_{12} continued to inhibit the growth of W303, another wild-type strain, indicating that W303 is more sensitive to C_{12} than BY4741 (Table 2). Because W303 is defective in the *SSD1* gene for cell wall formation (Ibeas et al., 2001), the cell wall is involved in the sensitivity to long-chain alcohols.

The yeast Pdr5 is a multidrug efflux pump [ATP-binding cassette (ABC) transporter] that extrudes various lipophilic drugs; therefore, the *pdr5* mutant is sensitive to drugs, decreasing the MICs (Balzi et al., 1994; Bissinger and Kuchler, 1994; Hirata et al., 1994). If Pdr5 extrudes the lipophilic long-chain alcohols, the mutant may be sensitive to alcohols. The MIC of C_{12} was variable at 48 hours but tended to be lower in the *pdr5* mutant than in the wild-type parent (W303), although the MICs of both strains were the same at 24 hours (Table 2). This result is consistent with a previous report that the fungicidal activity of C_{12} is higher in the *pdr5* mutant than in the wild-type strain (Fujita et al., 2017). Various MICs of C_{12} at 48 hours in budding yeast were also reported previously (Kubo et al., 2003). Presumably, various MICs of long-chain alcohols observed in these mutants are attributable to the nonuniform distributions caused by their high hydrophobicity, as described in Fig. 4B. These observations indicate that the *pdr5* mutant is sensitive to long-chain alcohols. C_{13} transiently inhibited the growth of the *pdr5* mutant at 25°C but not that of W303, indicating that the *pdr5* mutant escapes the cutoff of C_{13} during a short-term incubation. The *PDR1* and *PDR3* genes are transcription factors for several ABC transporters in yeast. The *pdr1,3,5* triple mutant is more sensitive to various drugs than the *pdr5* mutant is (Delaveau et al., 1994), as demonstrated by the sensitivity to C_{13}. C_{14} transiently inhibited the growth of the *pdr1,3,5* triple mutant but not that of the *pdr5* mutant at 25°C, although the MIC was variable at 24 hours (Table 2). Therefore, the *pdr1,3,5* mutant escapes the cutoff of C_{14} during a short-term incubation. Consequently, reducing the MIC of long-chain alcohols lengthens the cutoff chain length. By contrast, the overexpression of *PDR5* confers resistance to various drugs and increases the MICs (Balzi et al., 1994; Bissinger and Kuchler, 1994; Hirata et al., 1994). The wild-type strain harboring *PDR5* on a multicopy vector was resistant to 1000 μM of C_{12} at 48 hours, indicating that the overexpression of *PDR5* shortens the cutoff chain length (Table 2). Collectively, the biological sensitivity to long-chain alcohols contributes to the cutoff.

Discussion

Increasing the physicochemical solubility of alcohols lengthens the cutoff chain length (Fig. 5A), and decreasing the MIC of alcohols lengthens the cutoff chain length (Fig. 5B). Therefore, a balance between the physicochemical properties and biological sensitivity determines the cutoff chain length. In general, when the aqueous solubility of a compound does not reach the critical concentration for the biological effect, the compound is a cutoff compound. This is similar to the hypothesis proposed by Ferguson (1939), which was supported by previous reports (Raines and Miller, 1994; Eckenhoff et al., 1999; Peoples and Ren, 2002). However, because the cutoff had not been elucidated, several hypotheses were proposed to explain the cutoff and focused on proteins and membranes. The reason for these confusions may have been the ambiguous definition of aqueous solubility. Therefore, we propose categorizing the aqueous solubility of *n*-alcohols into stable hydrated concentrations (HydC) and unstable mixed concentrations (MixC). Identifying these would be useful in explaining the various cutoff phenomena.

Cutoff lengths $\geq C_{15}$ for various bacteria growing at approximately 37°C tend to be longer than those ($\geq C_{12}$) for various fungi at 25–30°C (Teh, 1974; Kato and Shibasaki, 1980; Kubo et al., 1995, 2003). Because destabilizing the solid-state structures of C_{13} (mp: 32.5°C) and C_{14} (mp: 37.7°C) at approximately 37°C increases their dissolution and releases free molecules, the total solubility (HydC + MixC) increases and reaches the MICs, thereby lengthening the cutoff chain

TABLE 2

MICs of long-chain alcohols determined in the *pdr* mutants

A MICs (micromolar) were determined at 25°C in 96-well plates containing 100 μl of YPD and the indicated alcohols.

Time (h)	BY4741	W303-1A			*pdr5Δ*			*pdr1Δ,3Δ,5Δ*		
	C_{12}	C_{12}	C_{13}	C_{14}	C_{12}	C_{13}	C_{14}	C_{12}	C_{13}	C_{14}
24	31	31	n.d.	n.d.	31	125	n.d.	31	63	63–125*
48	n.d.	63	n.d.	n.d.	31–63*	n.d.	n.d.	31–63*	125	n.d.

B W303-1A harboring YEp24 (vector) and YEp24-PDR5 (*PDR5^{ox}*) precultured in SD-U were grown at 25°C for 48 hours in 96-well plates containing 100 μl of YPD and the indicated alcohols. The identical MICs of the independent experiments are shown ($n \geq 3$).

Time (h)	W303-1A/vector			W303-1A/*PDR5^{ox}*		
	C_{10}	C_{12}	C_{13}	C_{10}	C_{12}	C_{13}
48	250	31–63*	n.d.	250	n.d.	n.d.

n.d., MIC was not determined because the growth was observed at 1000 μM for the indicated alcohols (cutoff).
*A range indicates that various MICs were observed.

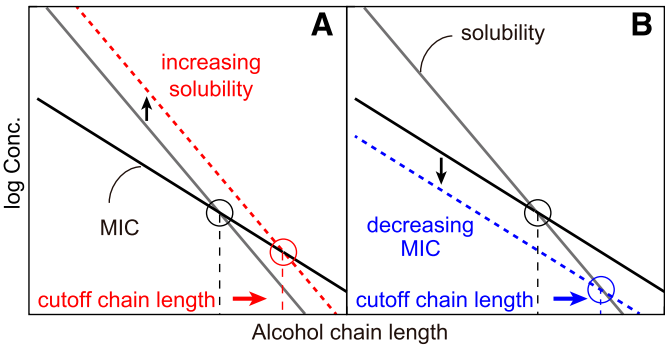

Fig. 5. Physicochemical solubility of and biological sensitivity to long-chain alcohols determine the cutoff chain length. Long-chain alcohols inhibit the growth of microorganisms when their solubilities reaches the MIC; however, when they do not, the alcohols are cutoff compounds. Increasing the solubility of alcohols lengthens the cutoff chain (A). Decreasing the MIC to alcohols lengthens the cutoff chain (B).

length in bacteria, as shown in Fig. 3. Therefore, the melting point of long-chain alcohols is responsible for the difference in the cutoff chain length between bacteria and fungi.

n-Alkanes also have antimicrobial activities and inhibitory activity against luciferase, although their cutoff chain length ($\geq C_7$) are shorter than those associated with *n*-alcohols (Gill and Ratledge, 1972; Teh and Lee, 1973; Franks and Lieb, 1985). Because the melting points of *n*-alkanes (e.g., $\leq C_{16}$: $\leq$18°C) are lower than those of *n*-alcohols (e.g., $\leq C_{16}$: $\leq$49°C), they are not important for the cutoff of liquid alkanes. The hydrophobicity (logP) of *n*-alkanes is higher than that of *n*-alcohols with the same alkyl chain (TOXNET), because *n*-alkanes lack a hydroxyl group for hydration. Therefore, the HydC (S^{liquid}) of liquid alkanes is lower than that for *n*-alcohols (eq. 1). Additionally, the densities are lower than those of *n*-alcohols (TOXNET). Because of their high hydrophobicity and low density, *n*-alkanes aggregate and separate from water more easily than do *n*-alcohols, resulting in a lower MixC. Hence, the total aqueous solubility (HydC + MixC) of *n*-alkanes is lower than that of *n*-alcohols (TOXNET). According to Fig. 5A, the low aqueous solubility of *n*-alkanes results in shorter-chain cutoffs compared with those of *n*-alcohols. By contrast, TMAs exhibit antimicrobial activity, even for C_{18} (Uesono et al., 2011). The hydrophobicity (logPs) is far lower than that of *n*-alcohols, resulting in high aqueous solubility (TOXNET), possibly because the ionic hydration of the quaternary ammonium group with water is stronger than the hydration of the alcoholic hydroxyl group. Thus, TMAs easily reach the MIC. Because the melting points of TMAs are higher than 150°C (TOXNET), hydrophobicity is more important than the melting point for the cutoff, as described for *n*-alkanes.

No growth inhibition was observed for the shaking cultures of the thermophilic bacterium treated with C_{18} (mp: 59.5°C) at 67.5°C (Fig. 3B) or of the yeast treated with $C_{18:1}$ (mp: 7°C) at 25°C (data not shown), above their melting points. Because these alcohols have densities similar to those of short-chain alcohols ($\geq C_4$) (Supplemental Table 1), their high hydrophobicity owing to the long chains should be important for the cutoff, rather than the melting points and the densities. Long-chain solid alcohols delay the growth of *Staphylococcus aureus* in a shaking culture at 37°C, below their melting points, and the time required for the delay decreases with their chain length (C_{14}: 29.7 hours, C_{16}: 17.3 hours, C_{18}: 10 hours) (Togashi et al., 2007). Therefore, solid alcohols have antimicrobial activity within short-term incubations, although they are judged as cutoff compounds in long-term incubation (Fig. 4B; Table 2). Because these alcohols (C_{14}–C_{18}) have similar densities (Supplemental Table 1), the difference in the delays is attributable to the hydrophobicity, rather than the melting points and densities. The long-chain solid alcohols also probably exist in the aqueous phase as hydrated and mixed concentrations (Fig. 2). Therefore, the duration of the delay decreases with decreases in the MixCs (the increase in the aggregation rate) determined by the chain length. Collectively, the high hydrophobicity, rather than the high melting point and low density of these alcohols, is important for the cutoff.

The cutoff chain length of alcohols for firefly luciferase inhibition ($\geq C_{17}$) is abnormally longer than the cutoff chain length ($\geq C_{13}$) observed in various biological systems (Ingólfsson and Andersen, 2011). Firefly luciferase is similar to rat long-chain acyl-CoA synthetase (Suzuki et al., 1990) and catalyzes the formation of fatty acyl-CoA from long-chain fatty acid as a substrate (Oba et al., 2003). Therefore, luciferase is considered to have as high an affinity for long-chain alcohols as do fatty acids. Consequently, alcohols may inhibit luciferase at lower concentrations, resulting in the high sensitivity of luciferase to long-chain alcohols. According to Fig. 5B, the high biological sensitivity lengthens the cutoff chain length for luciferase inhibition. In addition, *n*-alcohols (C_{18}) partially inhibit luciferase activity, more weakly than the inhibition by C_{16} (Ueda and Suzuki, 1998). This apparently differs from the typical cutoff phenomenon: complete loss of biological activity. The difference between C_{16} and C_{18} may reflect the effect on solubility resulting from the nonuniform distribution of *n*-alcohols in the solution, compared to the uniform distribution of luciferase.

The cutoff length of straight-chain diols ($\geq C_{14}$) for inhibition of the NMDA receptor is longer than that of *n*-alcohols ($\geq C_{10}$) (Peoples and Ren, 2002). This could be explained by Fig. 5A, because the solubilities of diols having two hydroxyl groups are higher than those of alcohols. However, the cutoff lengths for the inhibition of P2X receptor are abnormally short ($\geq C_4$) (Weight et al., 1999). This may be owing to another mechanism because it is not resistant to alcohols.

The minimum concentration required for anesthetics and alcohols to affect microorganisms with cell walls is higher than that required to affect animals without cell walls (Fig. 1; Table 2) (Pringle et al., 1981; Kubo et al., 1995, 2003; Keil et al., 1996). This observation suggests that the cell wall is involved in sensitivity to anesthetics and alcohols. The cutoff chain length ($\geq C_8$) for Gram negative bacteria are shorter than those ($\geq C_{13}$) for Gram positive bacteria (Kubo et al., 1995). The cell walls of these groups are quite different. Gram negative bacteria have double membrane structures and a thin wall, whereas Gram positive bacteria have a single membrane structure and a thick wall. Therefore, long-chain alcohols with low aqueous solubility do not reach the MIC of Gram negative bacteria protected by complex membrane structures, thereby shortening the cutoff length. This indicates that the membrane structure is a determinant of the cutoff phenomenon, rather than wall thickness. ABC transporters affected the cutoff chain length of *n*-alcohols in yeast (Table 2), indicating that the membrane state, such as the

alcohol sensitivity, possibly defined by the efflux of lipophilic long-chain alcohols, is another determinant for the cutoff. Collectively, both the structure and state of the biological membrane defining the alcohol sensitivity contribute to the cutoff.

The maximum chain length (C_{13}) for the anesthesia of a tadpole is longer than C_{12} for yeast growth inhibition (Pringle et al., 1981). This is because the alcohol sensitivity of a tadpole is higher than that of yeast with a cell wall (Table 2). However, C_{13} also had no anesthetic effect in a later study (Alifimoff et al., 1989). This discrepancy may be caused by the equilibration treatment of C_{13} performed in the later study, because the long-term treatment reduced the MixC at the bottom of the container owing to low density and high hydrophobicity (Fig. 2B; Fig. 4, B and C; Table 2). The decreased total solubility with HydC did not reach the anesthetic concentration for a tadpole, resulting in the cutoff of C_{13}. Accordingly, our theory derived from microbial experiments may also be applicable to the anesthetic cutoff of animals.

The cutoff phenomenon of long-chain alcohols can be escaped by increasing alcohol solubility. Thus, the cutoff can be explained by a mechanism other than the special pocket in proteins and the membrane alteration. Additionally, earlier explanations of the cutoff proposed complicated equations, such as the parabolic model and the bilinear model, to quantitate the structure-activity relationship (Hansch, 1969; Kubinyi, 1977). However, they may be simplified as reported previously (Meyer and Hemmi, 1935; Hansch and Dunn, 1972): There is no necessity to consider the cutoff compounds, because they do not reach biologically active concentrations. Increasing the aqueous solubility of hydrophobic compounds is the way to detect unknown biological effects. If the low solubility of high hydrophobic compounds such as long-chain alcohols is improved and the cutoff is escaped, the correlation between biological effects and the chain length (hydrophobicity) is correctly evaluated, as seen for water-soluble compounds (Williams et al., 2007; Uesono et al., 2011).

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Authorship Contributions

Participated in research design: Matsumoto, Uesono.

Conducted experiments: Matsumoto.

Performed data analysis: Matsumoto, Uesono.

Wrote or contributed to the writing of the manuscript: Matsumoto, Uesono.

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Supplementary data

Physicochemical solubility of and biological sensitivity to long-chain alcohols determine the cutoff chain length in biological activities

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Molecular Pharmacology

Supplemental Table 1

Physicochemical properties of *n*-alcohols

Chain Length	Solubility (mM)	Melting Point (°C)	Specific Density	$logP_{o/w}$	Molecular Weight
1	miscible	-97.8	0.7866	-0.77	32.04
2	miscible	-114.1	0.7893	-0.31	46.07
3	miscible	-127.0	0.8053	0.25	60.1
4	920	-88.6	0.8098	0.88	74.121
5	250	-79.0	0.8146	1.51	88.15
6	58	-44.6	0.8153	2.03	102.18
7	14	-34.6	0.8219	2.62	116.2
8	4.1	-14.7	0.8262	3.00	130.23
9	0.97	-5.0	0.8279	3.77	144.25
10	0.23	6.4	0.8297	4.57	158.28
11	0.11	19.0	0.8298	4.72	172.31
12	0.021	24.0	0.8309	5.13	186.33
13	0.0070	32.5	0.8223	5.82	200.36
14	0.00089	37.7	0.823	6.03	214.39
15	0.00045	45.0	0.829	6.24	228.417
16	0.00010	49.3	0.8187	6.83	242.45
17	0.000031	53.8	no data	7.23	256.47
18	0.0000041	59.5	0.8124	7.72	270.49

All data were cited from TOXNET (https://toxnet.nlm.nih.gov). $logP_{o/w}$: logarithm of octanol/water partition coefficient.