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Enhancement in Skin Permeation of 5-Aminolevulinic Acid Using *l*-Menthol and its Derivatives

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Enhancing effect of *l*-menthol and its derivatives, *l*-menthyl formate, *l*-menthyl acetate, and *l*-menthyl propionate, on skin permeation of 5-aminolevulinic acid (ALA) through Yucatan micropig full-thickness skin was investigated using a Franz-type diffusion cell. ALA solutions were prepared using ethanol-water mixed solvents with *l*-menthol or the derivative. Skin permeation coefficients (Kp) of ALA with more than 3.0 wt% of *l*-menthol was significantly larger than that without *l*-menthol. In addition, Kp of ALA with the derivative increased as follows: *l*-menthol ≈ *l*-menthyl propionate < *l*-menthyl formate < *l*-menthyl acetate. These results suggest that *l*-menthol and the derivative are effective to enhance ALA skin permeation.

Keywords skin permeation; 5-aminolevurinic acid; *l*-menthol; *l*-menthyl alkylate; and skin permeation coefficient

INTRODUCTION

5-Aminolevulinic acid-based photodynamic therapy (ALA-PDT) is one of the most effective treatments of skin diseases such as cutaneous carcinoma and intractable acne (Babilas, Landthaler, & Szeimies, 2006; Blume & Oseroff, 2007; Itoh, Ninomiya, Tajima, & Ishibashi, 2001; Kimura, Itoh, Tokuoka, & Kawashimam, 2004). In ALA-PDT, ALA is administered and is localized in skin diseases, followed by accumulation of an endogenous photosensitizer, protoporphyrin IX (PpIX). When PpIX is photoexcited by light with a specific wavelength ranging from 600 to 700 nm, the energy is transferred to molecular oxygen, which forms cytotoxic singlet oxygen, leading to photochemical and photobiological processes that cause irreversible photodamage to the skin diseases (Bonnett, 2000; Castano, Demidova, & Hamblin, 2004; Kurwa & Barlow, 1990).

Skin has an outermost thin layer, stratum corneum, and underlying viable epidermis and dermis. Since the stratum corneum is highly hydrophobic, dry, and a percutaneous barrier, hydrophilic compounds are inferior to hydrophobic compounds in permeating through the stratum corneum (Hikima & Maibach). When ALA is topically applied to skin diseases in ALA-PDT, it is hard for ALA to permeate through the stratum corneum because of its hydrophilicity. In order to enhance ALA-PDT efficacy for skin diseases, therefore, skin permeation of ALA needs to be improved.

l-Menthol is well known to remarkably enhance skin permeation of hydrophilic drugs (Kamal, Iimura, Nabekura, & Kitagawa, 2006; Kobayashi et al., 1993; Kunta et al., 1997; Liu et al., 2005). Fluidity of intercellular lipid lamellar structures of stratum corneum, especially of a hydrophilic domain of the

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lipid lamellar structure, increases with addition of *l*-menthol (Hosokai et al., 1998; Walker & Smith, 1996). The perturbation of the rigid packing of the hydrophilic domain elevates diffusion coefficients of hydrophilic drugs and enhances their skin permeation through the lipid lamella. Moreover, alkylmenthol and acylmenthol derivatives were investigated for their ability to enhance skin permeation. In particular, *o*-ethylmenthol showed high efficiency at its low concentration and caused relatively little skin irritation (Nakamura et al., 1996; Obata, Maruyama, & Takayama, 2006). Nevertheless, few studies document the enhancement in skin permeation of ALA by *l*-menthol and its derivatives.

In the present study, we focused on ALA skin permeation with *l*-menthol and its derivatives, *l*-menthyl formate, *l*-menthyl acetate, and *l*-menthyl propionate, as a permeation enhancer. We determined in vitro skin permeation coefficients of ALA with *l*-menthol or the derivative through Yucatan micropig (YMP) full-thickness skin using a Franz-type diffusion cell, and investigated their ability to enhance ALA skin permeation.

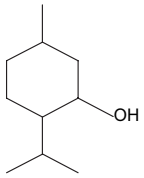
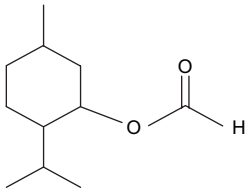
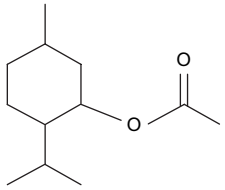
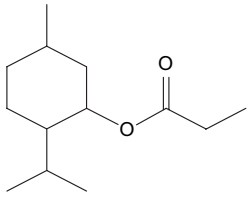
EXPERIMENTAL

Materials

5-Aminolevulinic acid hydrochloride (ALA, 98%) was produced by a fermentation method (Nishikawa et al., 1999). *l*-Menthol (99%) purchased from Aldrich Co., and *l*-menthyl formate, *l*-menthyl acetate, and *l*-menthyl propionate (98%) provided from T. Hasegawa Co., Ltd. were used without further purification. Their molecular structures and molecular weights are summarized in Table 1. To prepare liposome dispersion, moreover, *l*-α-dipalmitoylphosphatidylcholine (DPPC, 99.7%) and cholesterol (99%) were purchased from NOF. Co. and SIGMA, respectively. They were used without further purification. Pyrene (98%) used as a fluorescence probe was purchased from WAKO Pure Chemical Industries, Ltd., and was used after triple extraction with ethanol.

The inorganic/organic values (I/O) of *l*-menthol and its derivatives also are shown in Table 1. I/O is a useful index for evaluating hydrophobicity of a substance (Fujimoto, 1985).

TABLE 1
l-Menthol and its Derivatives Used in this Study

	Molecular Structure	Molecular Weight	I/O*
l-Menthol		156.27	0.58
l-Menthyl formate		184.28	0.33
l-Menthyl acetate		198.30	0.30
l-Menthyl propionate		212.33	0.28

*Inorganic and organic value.

Total hydrophilicity (inorganic value) and total hydrophobicity (organic value) of a substance are calculated from the summation of hydrophilicity and hydrophobicity assigned to each element and functional group of the substance, respectively, and I/O is estimated as a ratio of total hydrophilicity to total hydrophobicity. The hydrophobicity of the substance then increases with a decrease in its I/O. Table 1 indicates that the hydrophobicity of *l*-menthol and its derivatives used in this study increases in the following order: *l*-menthol < *l*-menthyl formate < *l*-menthyl acetate < *l*-menthyl propionate.

Measurement of In Vitro Skin Permeation of ALA

In vitro skin permeation of ALA with *l*-menthol and the derivative through Yucatan micropig (YMP) full-thickness skin (Charles River Laboratories Japan, Inc.) was determined with a Franz-type diffusion cell having donor and receptor compartments. The available diffusion area of the skin was 1.33 cm^2, and the volume of the receptor compartment was 17 ml. The skin was mounted in the Franz-type diffusion cell. A 10 wt% (104 mg/ml) of ALA solution was prepared using ethanol-distilled water mixed solvents with *l*-menthol or the derivative. The composition of each solvent used in this study is listed in Table 2 (A: with various *l*-menthol concentrations, B: with the same mole of *l*-menthol and its derivatives). The ALA solution was added in the donor compartment, and 17 ml of phosphate buffered saline (pH: 7.4) was poured into the receptor compartment. After the cell stood for a certain time at 37°C, with the buffered saline stirred with a magnetic stirrer, 200 μl of buffered saline in the receptor compartment was taken out. The concentration of ALA permeated through the YMP skin into the buffered saline was measured, and the cumulative amount of permeated ALA per unit skin area was calculated at each permeation time. Moreover, permeability coefficients (Kp) of ALA were estimated according to Equation 1 with flux (J) corresponding to a slope of initial linear relationship between the cumulative amount of permeated ALA per unit skin area and the permeation time (Azzi et al., 2006).

$$Kp = \frac{J}{C_D} \tag{1}$$

where C_D was the initial concentration of ALA in the donor compartment.

Measurement of ALA Concentration

The concentration of ALA permeated through the YMP skin into the buffered saline was measured by the fluorometric method described in previous paper (Okayama, Fujii, & Miura, 1990). A 50 μl of buffered saline with ALA permeated was treated with a mixture of 3.5 ml of acethylacetone, ethanol, and water (15:10:75 in volume ratio) containing 4 g of sodium chloride per liter, and then treated with 450 μl of 85 ml/l aqueous formaline solution. The treated solution was heated in boiling water for 30 min and was cooled in a water bath for 5 min. These reactions eventually gave us the fluorescent ALA derivative,

TABLE 2
Composition of Each Solvent Used in this Study

A: with Various *l*-Menthol Concentrations

	Composition (wt%)		
	l-Menthol	Ethanol	Distilled Water
	0.0	40	60
	1.0	40	59
	3.0	40	57
	5.0	40	55

B: with the Same Mole of *l*-Menthol and its Derivatives

	Composition (wt%)		
	l-Menthol and *l*-Menthol Derivative*	Ethanol	Distilled Water
l-Menthol	3.0	40	57.0
l-Menthyl formate	3.5	40	56.5
l-Menthyl acetate	3.8	40	56.2
l-Menthyl propionate	4.1	40	55.9

*Molality: 0.20 mol/kg.

2-methyldeneamin-3,5-diacetyl-4,6-dimethylphenylpropionic acid. The fluorescence intensity of the ALA derivative was measured with a fluorescence spectrophotometer (V-530, Jasco Co.). The excitation and emission wavelengths were 363 and 473 nm, respectively.

Measurement of Micropolarity of Liposomal Bilayer Membranes

Liposomes, of which the bilayer membrane included pyrene as a fluorescence probe, were prepared using Bangham's method (Bangham, Standish, & Weissmann, 1965). A 2.45×10^{-5} mol of DPPC, 1.05×10^{-5} mol of cholesterol, and 6.00×10^{-7} mol of pyrene were dissolved in 5 ml of chloroform in a test tube. Chloroform was evaporated under N_2 gas, and a thin lipid film was formed on the wall of the test tube. To remove the residual solvent completely, the test tube was allowed to stand in a desiccator under reduced pressure. Since ethanol disrupts liposome structures, 10 ml of distilled water was added into the test tube. The test tube was kept for 5 min at 60°C, agitated on a vortex mixer for a few minutes to give liposomes. The liposome dispersion was extruded through Millipore filter (Whatman Co.) of 10 μm pore diameter with an extruder (Lipex Biomembranes Inc.) to obtain homogeneously sized liposome dispersion. Liposome formation was confirmed with phase-contrast and polarized microscopy.

Taking account of the fact that the solubility of *l*-menthol in water is less than 0.1 wt%, we added 1.0 wt% of *l*-menthol or its derivative into the homogeneously sized liposome dispersion, stirring the dispersion for 24 hr at 37°C by a shaker to solubilize *l*-menthol or the derivative into the liposomal bilayer membrane. Before and after the solubilization, the fluorescence spectrum of pyrene in the liposomal bilayer membrane was measured with the same fluorescence spectrophotometer as shown above. The excitation wavelength was 335 nm, and the emission wavelength was in the range from 350 to 650 nm. The fluorescence peak intensity ratio of pyrene at 375 and 395 nm (I_1/I_3) reflects micropolarity around pyrene; the micropolarity increases with an increase in I_1/I_3 (Benrraou, Bales, & Zana, 2003; Thomas, 1980).

RESULTS AND DISCUSSION

Figure 1 shows changes in cumulative amounts of permeated ALA per unit skin area with permeation time at various *l*-menthol concentrations. The cumulative amount of ALA permeated with 1.0 wt% of *l*-menthol slightly increased with increasing permeation time. This tendency was similar to that without *l*-menthol. The amounts of ALA permeated with 3.0 and 5.0 wt% of *l*-menthol drastically increased with an increase in permeation time after the 5-hr permeation. In the 30-hr permeation, moreover, the cumulative amounts in these systems were eventually about 5 times larger than that in the system without *l*-menthol.

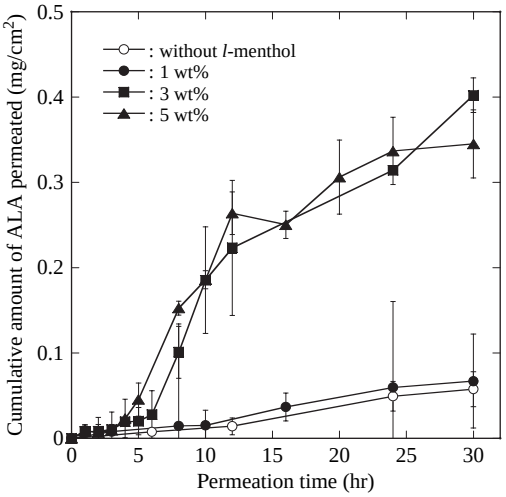


FIGURE 1. Changes in cumulative amounts of ALA permeated through YMP skins with permeation time at various concentration of *l*-menthol. Values are mean ± *SD* (n = 3–6).

Kp of ALA calculated by Equation (1) is summarized in Figure 2. With and without 1.0 wt% *l*-menthol, Kp was almost constant. Moreover, Kp in the case with 3.0 and 5.0 wt% of *l*-menthol was about 10 times as large as that without *l*-menthol. These findings consider, therefore, that more than 3.0 wt% of *l*-menthol is significantly effective to enhance the ALA permeation through the YMP skin.

Intercellular lipid lamellar structures in stratum corneum act as a barrier for permeation of most drugs, especially as a hydrophobic barrier for hydrophilic and ionized drug permeation (Hikima & Maibach, 2006). In general, *l*-menthol affects a hydrophilic domain of the intercellular lipid lamella structure and makes the hydrophilic domain fluid, leading to promotion in skin permeation of hydrophilic drugs (Hosokai et al., 1998;

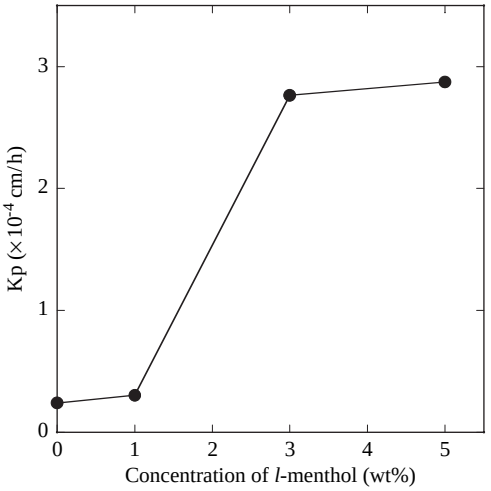


FIGURE 2. Relationship Kp of ALA and *l*-menthol concentration.

Walker & Smith, 1996). According as the partition coefficient (log P) of ALA between 1-octanol and phosphate buffered saline phases is –2.04 (Kosobe, Tokuoka, Ochiai, & Kawashima, 2006), ALA seems to be hydrophilic enough to be able to pass through the hydrophilic domain in the intercellular lipid lamellar structure. In this study, therefore, the increase in fluidity of the hydrophilic domain by *l*-menthol enhances the skin permeation of ALA.

Figure 3 refers to relationship between cumulative amounts of ALA permeated with *l*-menthol or its derivative and permeation time. The cumulative amount of ALA permeated with the derivative also increased with increasing permeation time and was significantly larger than that without them. In comparison, at the same permeation time over 16 hr, moreover, the cumulative amount of ALA is obviously the largest in the case with *l*-menthyl acetate. Nevertheless, there is not much difference in cumulative amount of ALA permeated with the derivate in the initial permeation time, especially *l*-menthyl formate and *l*-menthyl acetate. This would be caused by the small difference in I/O, or hydrophobicity, of the derivative, as shown in Table 1.

Kp of ALA permeated with *l*-menthol and the derivative also was calculated. Change in Kp with I/O of *l*-menthol and the derivative is depicted in Figure 4. Kp in any system was larger than that without *l*-menthol and the derivative (dashed line). In addition, Kp increased with decreasing I/O, or with increasing hydrophobicity, of *l*-menthol and the derivative, reaching the maximum in the system with *l*-menthyl acetate. This finding suggests that there is an optimal hydrophobicity of *l*-menthol derivative for enhancing ALA skin permeation. This tendency was observed in the effect of cyclohexanol derivatives on ketoprofen skin permeation (Obata et al., 2000).

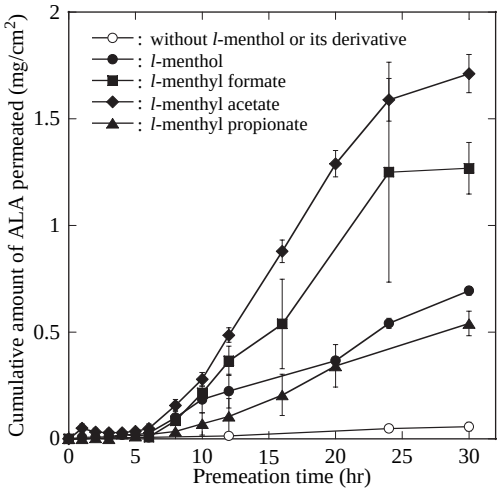


FIGURE 3. Changes in cumulative amounts of ALA permeated through YMP skin with permeation time in cases with *l*-menthol and its derivatives. Values are mean ± *SD* (*n* = 3–6).

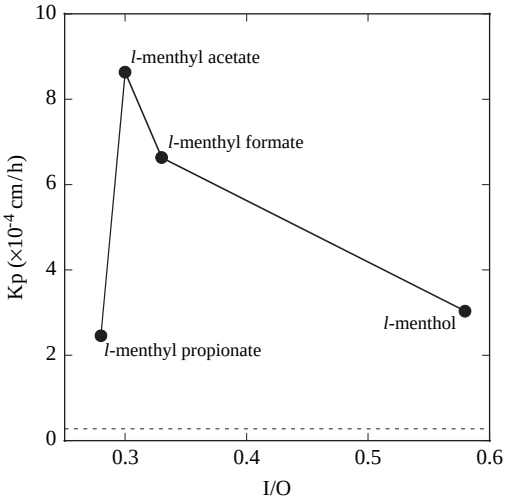


FIGURE 4. Change in Kp of ALA with I/O of *l*-menthol and its derivatives. Dashed line is Kp of ALA without additives.

Hydrophobicity of a substance is generally essential for its partition into skin phases. Kitagawa and Li (1999) examined skin permeation of benzoic acid and its 4-alkyl substituted derivatives. They demonstrated that the benzoic acid derivative possessing more hydrophobic substituents elevated its partition to the skin phase. According to their demonstration, therefore, the increment in Kp of ALA with an increase in hydrophobicity from *l*-menthol to *l*-menthyl acetate, except for *l*-menthyl propionate, may be caused by promotion in their partitions into the YMP skin phase and in perturbation of the hydrophilic domain in the intercellular lipid lamellar structure of the skin.

Table 1 indicates that *l*-menthyl propionate is the most hydrophobic among the derivatives used in this study. Hence, *l*-menthyl propionate is expected to be partitioned more readily into the skin phase and to be effective for enhancing ALA skin permeation. Contrary to our expectation, however, Kp of ALA in the case with *l*-menthyl propionate was as small as that with *l*-menthol, as shown in Figure 4.

Hydrophobicity of a substance also has an influence on its solubilization site in amphiphilic molecular aggregates such as surfactant micelles, vesicles, liquid crystals, and so on. For example, solubilization sites of more hydrophobic substances are transferred from a hydrophilic domain to a hydrophobic one in surfactant micellar aggregates (Attwood & Florence, 1983). Intercellular lipid lamellar structures of stratum corneum may solubilize substances in the same manner.

We tried to deduce solubilization sites of *l*-menthol and its derivatives in intercellular lipid lamellar structures from measuring micropolarity of inside liposomal bilayer membranes including *l*-menthol or the derivative (Abe, Tokuoka, Uchiyama, & Ogino, 1990). Figure 5 refers to relationship between I_1/I_3 of pyrene in the liposomal bilayer membranes including *l*-menthol or the derivative and their I/O. Here, since pyrene is solubilized around a boundary between a hydrophilic and a

hydrophobic domain (palisade layer) in surfactant micellar aggregates (Ghosh, Maki, & Petrin, 1986), I_1/I_3 reflects micropolarity around a boundary between hydrophilic and hydrophobic domains in the liposomal bilayer membrane. As can be seen in Figure 5, the micropolarity around the boundary was independent of the addition of *l*-menthol, but increased with an increase in hydrophobicity of the derivative. Since *l*-menthol possessing less hydrophobicity is solubilized in the hydrophilic domain, the micropolarity around the boundary is not affected by *l*-menthol. In contrast with *l*-menthol, the *l*-menthol derivative, having more hydrophobicity, penetrates more deeply into the hydrophobic domain and spreads intermolecular distance of lipids forming the liposomal bilayer membrane; thereby, bulk water molecules penetrate into the hydrophobic domain, leading to the increase in micropolarity around the boundary. To summarize these findings, the solubilization site of *l*-menthol and the derivative into the intercellular lipid lamellar structures may be deduced as illustrated in Figure 6.

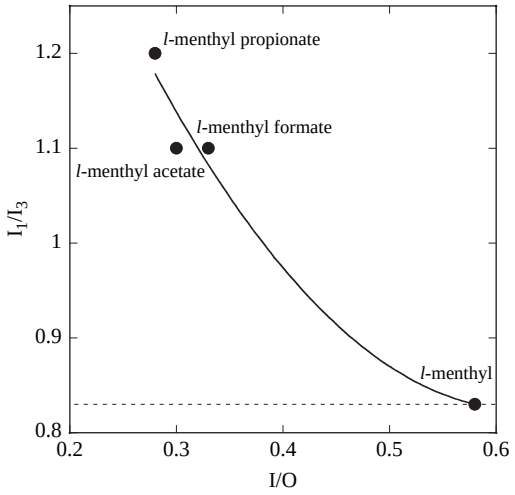


FIGURE 5. Change in I_1/I_3 of pyrene incorporated with *l*-menthol and its derivatives into liposomal bilayer membranes with their I/O. Dashed line is I_1/I_3 of pyrene incorporated without additives.

Taking this change in solubilization site of *l*-menthol and the derivative into consideration, since I_1/I_3 is the largest in the case with *l*-menthyl propionate having the most hydrophobicity, *l*-menthyl propionate may penetrate most deeply into the hydrophobic domain in the intercellular lipid lamellar structure. As a result, *l*-menthyl propionate is ineffective in making the hydrophilic domain fluid in the intercellular lipid lamellar structure, leading to a little enhancement in the ALA permeation through the YMP skin, compared with *l*-menthyl acetate.

CONCLUSION

The enhancing effect of *l*-menthol and its derivatives, *l*-menthyl formate, *l*-menthyl acetate, and *l*-menthyl propionate, on ALA skin permeation through the YMP full-thickness skin was investigated with the Franz-type diffusion cell. As a result, *l*-menthol and the derivative are effective to enhance ALA skin permeation, and this enhancing effect increased as follows: *l*-menthol ≈ *l*-menthyl propionate < *l*-menthyl formate < *l*-menthyl acetate.

However, this study is incomplete and partial on the ALA skin permeation with *l*-menthol and its derivatives. The ALA

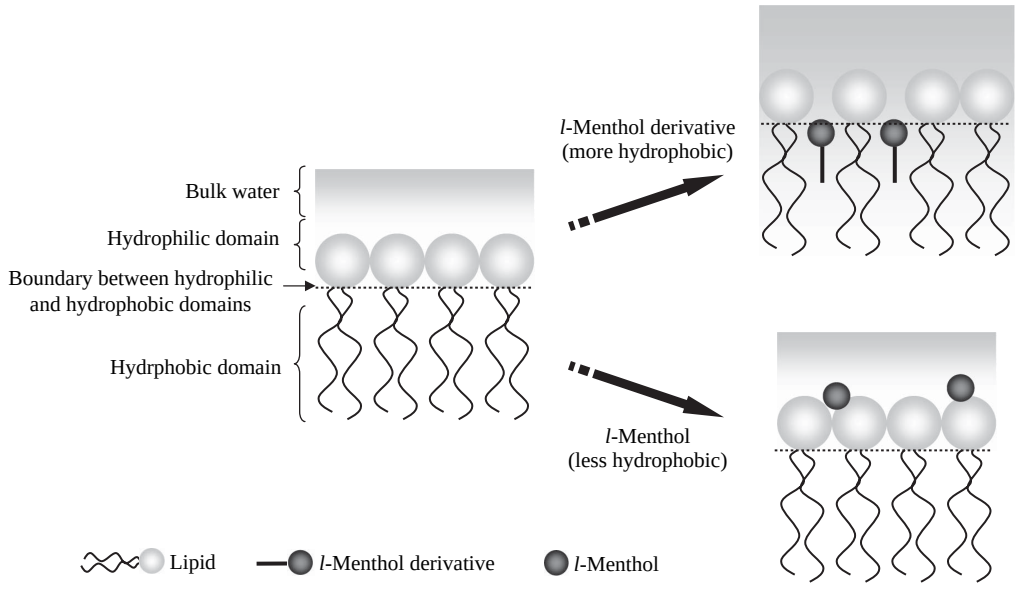


FIGURE 6. Scheme of solubilization mechanism of *l*-menthol and its derivatives into intercellular lipid lamellar structures. *l*-Menthol that is less hydrophobic is solubilized into hydrophilic domain, but *l*-menthol derivative that is more hydrophobic penetrates into hydrophobic domain in intercellular lipid lamellar structures.

amount in stratum corneum, epidermis (or combined), and in dermis or total skin and the complete mechanism on the permeation enhancing effect of *l*-menthol and the derivative on ALA permeation have not been understood yet. Hence, further studies should be required to understand the ALA permeation with *l*-menthol and the derivative in more detail.

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