JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Interaction of Four Monoterpenes Contained in Essential Oils with Model Membranes: Implications for Their Antibacterial Activity

MARIATERESA CRISTANI,[†] MANUELA D'ARRIGO,[†] GIUSEPPINA MANDALARI,[†] FRANCESCO CASTELLI,[‡] MARIA GRAZIA SARPIETRO,[‡] DOROTEA MICIELI,[‡] VINCENZA VENUTI,[†] GIUSEPPE BISIGNANO,[†] ANTONELLA SAIJA,[†] AND DOMENICO TROMBETTA*,[†]

Department Farmaco-Biologico, School of Pharmacy, University of Messina, Contrada Annunziata, 98168 Messina, Italy, and Department of Chemical Sciences, University of Catania, Viale A. Doria 6, 95125 Catania, Italy

The present article reports the antimicrobial efficacy of four monoterpenes (thymol, carvacrol, *p*-cymene, and γ-terpinene) against the Gram-positive bacterium *Staphylococcus aureus* and the Gram-negative bacterium *Escherichia coli*. For a better understanding of their mechanism of action, the damage caused by these four monoterpenes on biomembranes was evaluated by monitoring the release, following exposure to the compounds under study, of the water-soluble fluorescent marker carboxyfluorescein (CF) from large unilamellar vesicles (LUVs) with different lipidic composition (phosphatidylcholine, PC, phosphatidylcholine/phosphatidylserine, PC/PS, 9:1; phosphatidylcholine/stearylamine, PC/SA, 9:1). Furthermore, the interaction of these terpenes with dimyristoylphosphatidylcholine multilamellar vesicles as model membranes was monitored by means of differential scanning calorimetry (DSC) technique. Finally, the results were related also with the relative lipophilicity and water solubility of the compounds examined. We observed that thymol is considerably more toxic against *S. aureus* than the other three terpenes, while carvacrol and *p*-cymene are the most inhibitory against *E. coli*. Thymol and carvacrol, but not γ-terpinene and *p*-cymene, caused a concentration-dependent CF leakage from all kinds of LUVs employed; in particular, thymol was more effective on PC and PC/SA LUVS than on PC/PS vesicles, while carvacrol challenge evoked a CF leakage from PC/PS LUVs similar to that induced from PC/SA LUVs, and lower than that measured with PC vesicles. Concerning DSC experiments, these four terpenes caused a decrease in T_m and (especially carvacrol and *p*-cymene) ΔH values, very likely acting as substitutional impurities. Taken together, our findings lead us to speculate that the antimicrobial effect of thymol, carvacrol, *p*-cymene, and γ-terpinene may result, partially at least, from a gross perturbation of the lipidic fraction of the plasmic membrane of the microorganism. In addition to being related to the physicochemical characteristics of the compounds (such as lipophilicity and water solubility), this effect seems to be dependent on the lipidic composition and net surface charge of the microbic membranes. Furthermore, the compounds might cross the cell membranes, thus penetrating into the interior of the cell and interacting with intracellular sites critical for antibacterial activity.

KEYWORDS: Thymol; carvacrol; *p*-cymene; γ-terpinene; *Staphylococcus aureus*; *Escherichia coli*; phospholipid liposomes; differential scanning calorimetry; carboxyfluorescein

INTRODUCTION

Essential oils are mixtures of compounds obtained from spices, aromatic herbs, fruits, and flowers and characterized by their aroma (*1*, *2*). Analysis of essential oils shows that, of the different compounds forming them, terpenoids are the most abundant, present both as sesquiterpenes and especially as monoterpenes.

An extensive body of research has demonstrated that essential oils and their main components possess a wide spectrum of biological activity, which may be of great importance in several fields, from food chemistry to pharmacology and pharmaceutics. In particular, several essential oils have been studied for their

* Corresponding author. Telephone: 0039 90 6766458. Fax: 0039 90 3533142. E-mail: dtrombetta@pharma.unime.it.
[†] University of Messina.
[‡] University of Catania.

10.1021/jf070094x CCC: $37.00
Published on Web 06/30/2007

antimicrobial activity against a wide variety of microorganisms, including Gram-positive and Gram-negative bacteria and fungi (*3*−*5*). For example, the essential oils obtained from oregano and thyme, traditional Mediterranean spices, are well-known for their antimicrobial activity (*6*−*8*) and are characterized by a very high content of monoterpenes, both hydrocarbons (such as γ-terpinene, ranging 2−52%, and *p*-cymene, ranging from trace to 52%) and oxygenated compounds (mainly thymol, ranging from trace to 64%, and carvacrol, ranging from trace to− 80%) (*9*−*11*).

The antimicrobial activity of the essential oils can be explained by the lipophilic character of the monoterpenes contained in them. The monoterpenes act by disrupting microbial cytoplasmic membrane, which thus loses its high impermeability for protons and bigger ions; if disturbance of membrane integrity occurs, then its functions are compromised not only as a barrier but also as a matrix for enzymes and as an energy transducer (*12*−*15*). It is interesting to mention that bacteria predominantly respond to environmental conditions and particular antimicrobial agents such as thymol and carvacrol by modulating membrane fluidity (*16*). However, specific mechanisms involved in the antimicrobial action of monoterpenes remain poorly characterized.

In the present article, we report the antimicrobial efficacy of four major monoterpenes contained in essential oils (thymol, carvacrol, *p*-cymene, and γ-terpinene) against the Gram-positive bacterium *Staphylococcus aureus* and the Gram-negative bacterium *Escherichia coli*. To better understand the mechanism of action of these four monoterpenes, their damaging effect on the biomembranes was evaluated by monitoring the release, following exposure to the compounds under study, of the water-soluble fluorescent marker carboxyfluorescein (CF) from unilamellar vesicles (LUVs) with different lipidic composition. The lipids, the main constituents of cell membranes, provide the membrane with its barrier function and influence a variety of processes in the bacterial cell. However, since the lipidic composition of membranes is widely different in the world of bacteria (*17*, *18*) and may influence the susceptibility to the compounds tested, the LUVs employed in our study were constituted of phosphatidylcholine (PC), phosphatidylcholine/phosphatidylserine (PC/PS, 9:1), or phosphatidylcholine/stearylamine (PC/SA, 9:1) to thus verify if the net surface charge of the target membrane (neutral for PC or DMPC LUVs, positive for PC/SA LUVs, and negative for PC/PS LUVs) can influence CF leakage following challenge with the monoterpenes studied.

Moreover, to increase knowledge on compound interaction with lipid membranes we have investigated the action of terpenoids on the membrane microenvironment. With this in view, differential scanning calorimetry (DSC) technique on pure dimyristoylphosphatidylcholine (DMPC) liposomes, as model lipidic membranes, was employed to evaluate: (1) the melting temperature depression caused by the dissolution of the terpenes tested in the DMPC membrane and (2) the migration of the terpenes under study through the aqueous medium, their interaction with empty liposomes, and transmembrane transfer. Finally, the results were related also with the relative lipophilicity and water solubility of the compounds examined.

MATERIALS AND METHODS

Chemicals Used. Carvacrol (98%), *p*-cymene (99%), γ-terpinene (97%), and thymol (99.5%) were obtained from Sigma-Aldrich (Milan, Italy). Synthetic L-α-dimyristoylphosphatidylcholine was obtained from Genzyme (Liestal, Switzerland). L-α-Phosphatidylcholine, phosphatidylserine, and stearylamine were purchased from Fluka (Milan, Italy). Crude carboxyfluorescein (Fluka) was purified by the method described by Mercadal et al. (*19*). All other chemicals were of reagent grade.

Microbiological Studies. Two American Type Culture Collection (ATCC) standard strains were used: *Staphylococcus aureus* ATCC 6538P and *Escherichia coli* ATCC 15221.

The antimicrobial activity was evaluated by determining the minimum inhibitory concentration (MIC) according to the M7-A5 guidelines, established by the National Committee for Clinical Laboratory Standards (*20*). The MIC values (minimum concentration that inhibits the inoculum growth) against the bacterial strains were determined on 96 well culture plates by a microdilution method using Mueller-Hinton broth (Becton-Dickinson, Milan, Italy). Eight twofold dilutions of the samples were carried out starting from the concentration of 0.5% w/v (5% of Tween 20, used to dissolve the terpene and thus to ensure its contact with microorganisms). All preparations were sterilized with a 0.22-μm filter. The wells were inoculated with a microorganism suspension at a density of 10^5 cells/mL. The plates were incubated at 37 °C for 24 h. After incubation, the plates were observed to determine the MICs. Proper blanks were prepared simultaneously. Samples were tested in triplicate.

Carboxyfluorescein Studies. In the present study, we monitored the release of CF trapped in LUVs with different lipidic composition (PC; PC/PS, 9:1; PC/SA, 9:1) following exposure to the terpenes under investigation (*21*, *22*). Briefly, multilamellar liposomes (MLVs) were obtained by freshly prepared chloroform/methanol concentrated lipid solutions. The solvent was removed under nitrogen in a rotoevaporator, and the resulting film was kept overnight under vacuum to remove the residual solvent. The dried film was then suspended in buffer (150 mM NaCl/5 mM Hepes, pH 7.4) containing 20 mM CF, and the dispersion was vortexed intermittently for 20 min at room temperature. The LUVs were prepared by submitting the previously prepared MLV dispersion (approximately 1 mg mL^{-1}) to extrusion (19 times) through 100-nm polycarbonate membranes (Avestin, Inc., Ottawa, Canada) in an extruder system (LiposoFast Basic, Avestin) (*23*, *24*). Free CF was removed by passing the dispersion through a 1 × 30 cm column of Sephadex G-50 where the vesicles eluted with the void volume. Aliquots of eluted liposomes were used to determine the amount of phospholipid by the phosphorus assay (*25*).

Aliquots of the liposomal stock preparations (diluted to about 20 μg/mL) were incubated with various concentrations of the terpene to be tested at room temperature, and fluorescence was recorded continuously for 15 min (wavelength of excitation, $\lambda_{ex} = 490$ nm; wavelength of emission, $\lambda_{em} = 520$ nm). At the end of each experiment, total CF was determined after lysing the liposomes with 10% Triton X-100 (150 μL), after which the mixture was heated at 100 °C for 5 min and mixed vigorously for 2 min at room temperature. All experiments were carried out in triplicate.

The rates of CF leakage are expressed as percent of total trapped CF released

$$\%CF_{released} = \left(\frac{F - F_0}{F_t - F_0}\right) \times 100$$

where F is the fluorescence intensity measured at a specified time, F_0 is the fluorescence intensity measured at zero time, and F_t is the total fluorescence measured after Triton disruption. F_t is corrected for the dilution introduced by the addition of Triton. Incubation of liposomes with higher concentrations of Triton did not affect the value of F_t, indicating that the procedure resulted in a complete release of dye from the liposomes.

MLV Preparation. MLVs were prepared in the presence and absence of carvacrol, *p*-cymene, γ-terpinene, or thymol. Aliquots of a chloroform stock solution of lipid were delivered in glass tubes to have the same amount of lipid (0.010325 mmol). The solvent was removed under nitrogen flow, and the resulting film was freeze-dried to remove the residual solvent. Then an exactly weighed amount of each terpene, corresponding to a well-defined molar fraction, was added to the lipid film to avoid any loss of terpenes during the under vacuum process. Liposomes were prepared by adding to the films 168 μL of 50 mM Tris buffer solution (pH = 7.4), heating at 37 °C, temperature above the gel−liquid crystalline phase transition, and vortexing three times

for 1 min. The samples were shaken for 1 h in a water bath at 37 °C to homogenize the liposomes, permitting the compounds to partition between lipid and aqueous phases. Then, 120 μL of lipid suspensions (0.007375 mmol of lipid) was transferred in a 160-μL DSC aluminum pan, hermetically sealed, and submitted to DSC analysis.

Differential Scanning Calorimetry. A Mettler Toledo STARe system equipped with a DSC-822e calorimetric cell and Mettler TA-STARe software was used. The scan heating rate employed was 2 °C/min at a temperature range of 5−37 °C. The sensitivity was automatically chosen as the maximum possible by the calorimetric system, and the reference pan was filled with Tris buffer solution. To measure transition temperature and enthalpy changes, the calorimetric system was calibrated by using indium, stearic acid, and cyclohexane following the procedure of the Mettler TA STARe Software.

To check the reproducibility of results, the calorimetric scan was repeated four times; aliquots of all samples were then extracted from the calorimetric aluminum pans and used to determine, by the phosphorus assay (*25*), the exact amount of phospholipids present in each sample.

Permeation Experiments. The ability of the examined terpenes to dissolve in the aqueous phase, migrate through it, and be absorbed on the external lipidic layer of MLVs and be transferred successively to the inner bilayers was investigated by carrying out the following "kinetic" experiment. A fixed amount of terpene (corresponding to a 0.12 molar fraction with respect to the phospholipid) was weighed at the bottom of a 160-μL DSC aluminum crucible, and then 120 μL (0.007375 mmol) of DMPC aqueous dispersion (MLVs) was added. The samples, hermetically sealed in the pans, were gently shaken for 10 s and then submitted to subsequent calorimetric scan cycles by using the following procedure:

(1) A scan between 5 and 37 °C, to detect the interaction between the compounds and the model membrane during the sample heating;

(2) An isothermal period (1 h) at 37 °C to allow the terpene to dissolve in the aqueous medium and possibly interact and permeate the lipid bilayers, which at 37 °C exist at the disordered state;

(3) A cooling scan between 37 and 5 °C, at the rate of 4 °C/min, to bring the lipid layers back to an ordered state before restarting the heating program (step 1).

This procedure was run at least six times and repeated after a longer incubation time (24 h) to follow eventual variations of the transitional temperature of the DMPC calorimetric peak due to the uptake of the terpene by the model membrane. If an interaction followed by a full lipid bilayer penetration did occur, an effect similar to that obtained by MLVs containing 0.12 molar fraction of terpene and prepared as reported in MLV preparation should be observed.

Transmembrane Transfer Kinetics. Sometimes lipophilic media instead of aqueous ones can support the absorption of bioactive compounds by biological membranes. Therefore, it is interesting to investigate how the examined terpenes dispersed in MLVs (containing a 0.12 molar fraction with respect to the aqueous lipid dispersion; loaded MLVs) can be transferred to pure DMPC MLVs (empty MLVs). If the compound transfer occurs, an effect similar to that observed in MLVs prepared in the presence of 0.06 molar fraction of terpene will be obtained. This effect is due to the emptying of loaded MLVs and the contemporary terpene transfer and absorption inside empty MLVs. These kinetic experiments were carried out by transferring 60 μL of empty MLVs in a 160-μL calorimetric pan where 60 μL of loaded MLVs had previously been placed. The sample was hermetically sealed in the pans, gently shaken for 10 s, and then submitted to subsequent calorimetric cycles by using the same step procedure reported in the previous section.

Relative Lipophilicity Determination. The relative lipophilicity (R_m) of the terpenes under investigation was measured by reversed-phase high-performance thin-layer chromatography (*26*). Briefly, Whatman KC 18F plates were used as the nonpolar stationary phase. The plates were dried at 105 °C for 1 h before use. The mobile phase was a 2:1 (v/v) mixture of acetone and water. Each compound was dissolved in absolute ethanol (100 mg/mL), and 2 μL of solution was applied to the plate. The experiments were repeated five times with different disposition of the compounds on the plate. The retardation factor (R_f) values were expressed as the mean of the five determinations. The R_m values were calculated from the experimental R_f values according to the formula $R_m = \log[(1/R_f) - 1]$. Higher R_m values indicate higher lipophilicity.

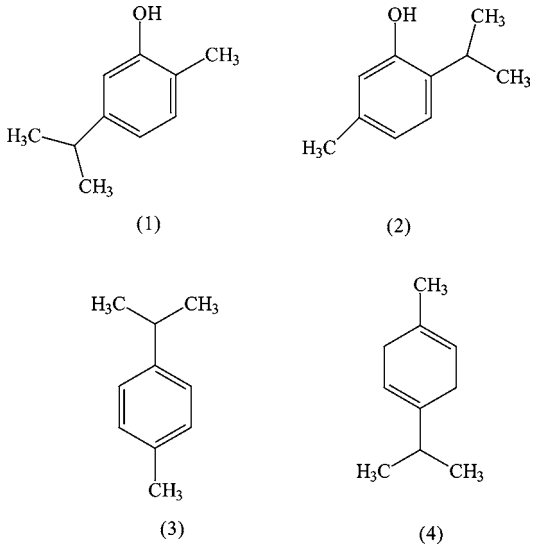

Figure 1. Chemical structure of (**1**) carvacrol, (**2**) thymol, (**3**) *p*-cymene, and (**4**) γ-terpinene.

Table 1. Antimicrobial Activity of Thymol, Carvacrol, *p*-Cymene, and γ-Terpinene on Two ATCC Microbial Strains[a]

	MIC (mg/mL)	
compounds	*S. aureus* ATCC 6538P	*E. coli* ATCC 15221
thymol	0.31	5.0
carvacrol	1.25	2.5
p-cymene	1.25	2.5
γ-terpinene	2.50	5.0

[a] Results are expressed as MIC.

RESULTS AND DISCUSSION

In the present study, we have evaluated the antibacterial activity of four major terpenes (thymol, carvacrol, *p*-cymene, and γ-terpinene) contained in oregano essential oils (**Figure 1**). The results, presented in **Table 1**, show that each of the terpenes tested inhibits the growth of the two microbial strains employed, the Gram-positive *S. aureus* ATCC 6538P and the Gram-negative *E. coli* ATCC 15221. However, the Gram-positive *S. aureus* appears more sensitive than the Gram-negative *E. coli* toward all compounds characterized. The MIC values demonstrate that thymol is considerably more toxic against *S. aureus* than the other three terpenes, while carvacrol and *p*-cymene are the most inhibitory against *E. coli*.

To characterize the mechanisms subserving their observed cytotoxic effect, these four monoterpenes have been tested for their capability to interact with model membranes through the aid of some biophysical tools.

Compound−membrane interaction is a dynamic phenomenon, bilaterally controlled not only by the compound chemical structure but also by the membrane organization; it can be affected by compound internalization and absorption at the membrane−water interface and in turn can be modified as a consequence of compound incorporation within membrane structure.

A first series of experiments was carried out to determine if challenge with the terpenes under study can cause a leakage of CF entrapped in LUVs with different lipids. Curves reported

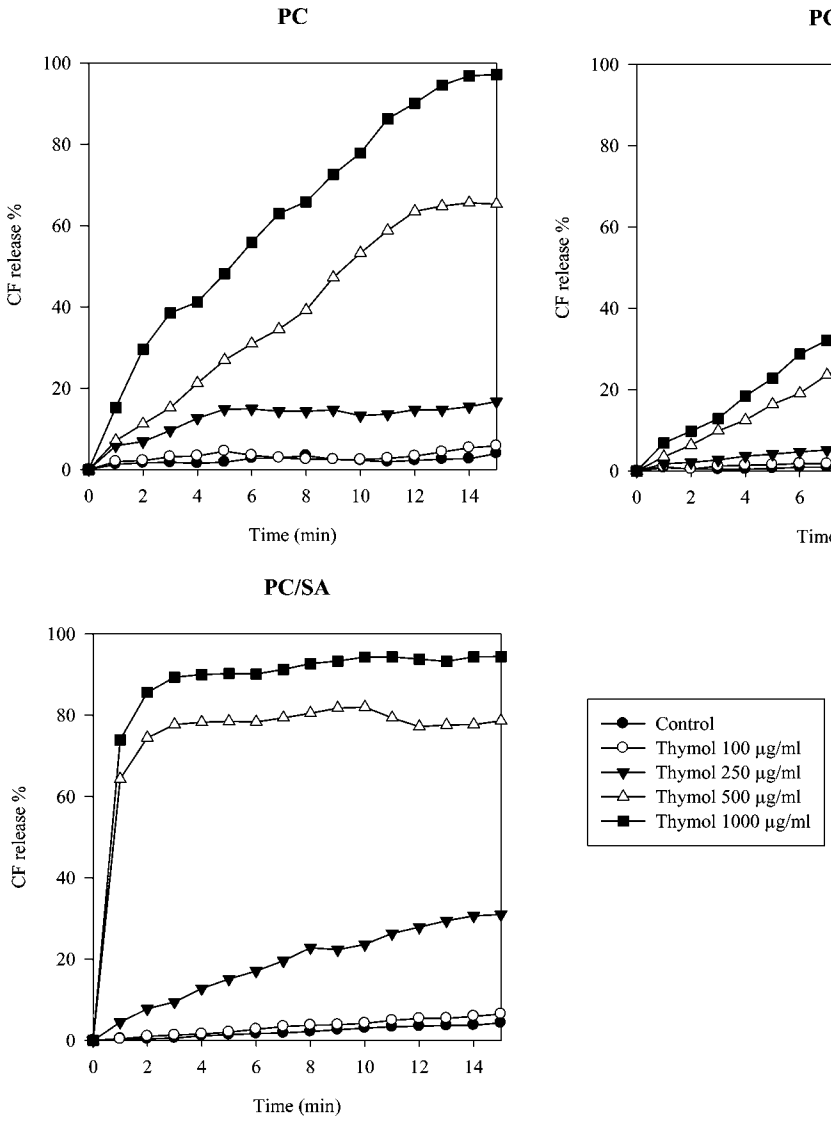

Figure 2. Effect of thymol challenge on CF release from CF-encapsulated large unilamellar vesicles with different lipid composition. PC: phospatidylcholine; PS: phospatidylserine; SA: stearylamine.

in **Figures 2** and **3** illustrate the time course of the terpene-induced CF release; the results expressed as % of total trapped CF released in 15 min are shown in **Table 2**.

The basal rate of CF leakage from liposomes ranged from 2 to 5% for PC and PC/SA LUVs and from 0 to 3% for PC/PS LUVs. Thymol and carvacrol, tested at the same concentrations, caused a concentration-dependent CF leakage from all kinds of LUVs employed, the extent of dye release slowly increasing during the 15-min observation period. Conversely, only a slight CF release was observed from LUVs challenged with *p*-cymene and γ-terpinene. The present results lead to the hypothesis that the antimicrobial activity of monoterpenes may be the result of the perturbation of the lipidic fraction of the microorganism plasmic membrane. Consistent with these findings, it has been demonstrated that also the antibacterial activity of other phytochemicals, such as *Melaleuca alternifolia* essential oil (*27*), α,β-unsaturated aldehydes (*22*, *28*), and other bioactive terpenes (*29*), results from modifications of cell membrane characteristics.

When PC LUVs were employed, the effectiveness in damaging lipidic membranes was in the order thymol = carvacrol $\gg$ *p*-cymene = γ-terpinene. However, thymol was more effective on PC and PC/SA LUVS than on PC/PS vesicles, while carvacrol challenge evoked a CF leakage from PC/PS LUVs similar to that induced from PC/SA LUVs and lower than that measured with PC vesicles.

The study of the thermotropic phase transition of DMPC MLVs by DSC is a precise method to investigate the influence of bioactive compounds on lipid membrane organization. The presence of compounds interacting with phospholipid bilayers may cause perturbations that can disturb membrane fluidity and depend on the localization of the perturbing molecule in the lipid bilayer. In fact, certain molecules that act as "substitutional impurities" of a membrane, taking the place of lipid molecules, cause transition temperature (T_m) and enthalpy difference (ΔH) variations. Instead, other molecules act as "interstitial impurities", by intercalating among the flexible acyl chain of lipids, and cause T_m variations without ΔH change, according to the temperature depression of melting point for an ideal solution (*30*−*34*).

The effect of the compounds on the phase transition of DMPV MLVs was investigated on samples containing increasing amounts (0.0, 0.015, 0.03, 0.045, 0.06, 0.09, and 0.12 molar fractions) of the studied terpenes.

As evidenced in the calorimetric curves (data not shown), the pure DMPC MLV calorimetric curve is characterized by a main transition peak at 24.6 °C, between an ordered gel state

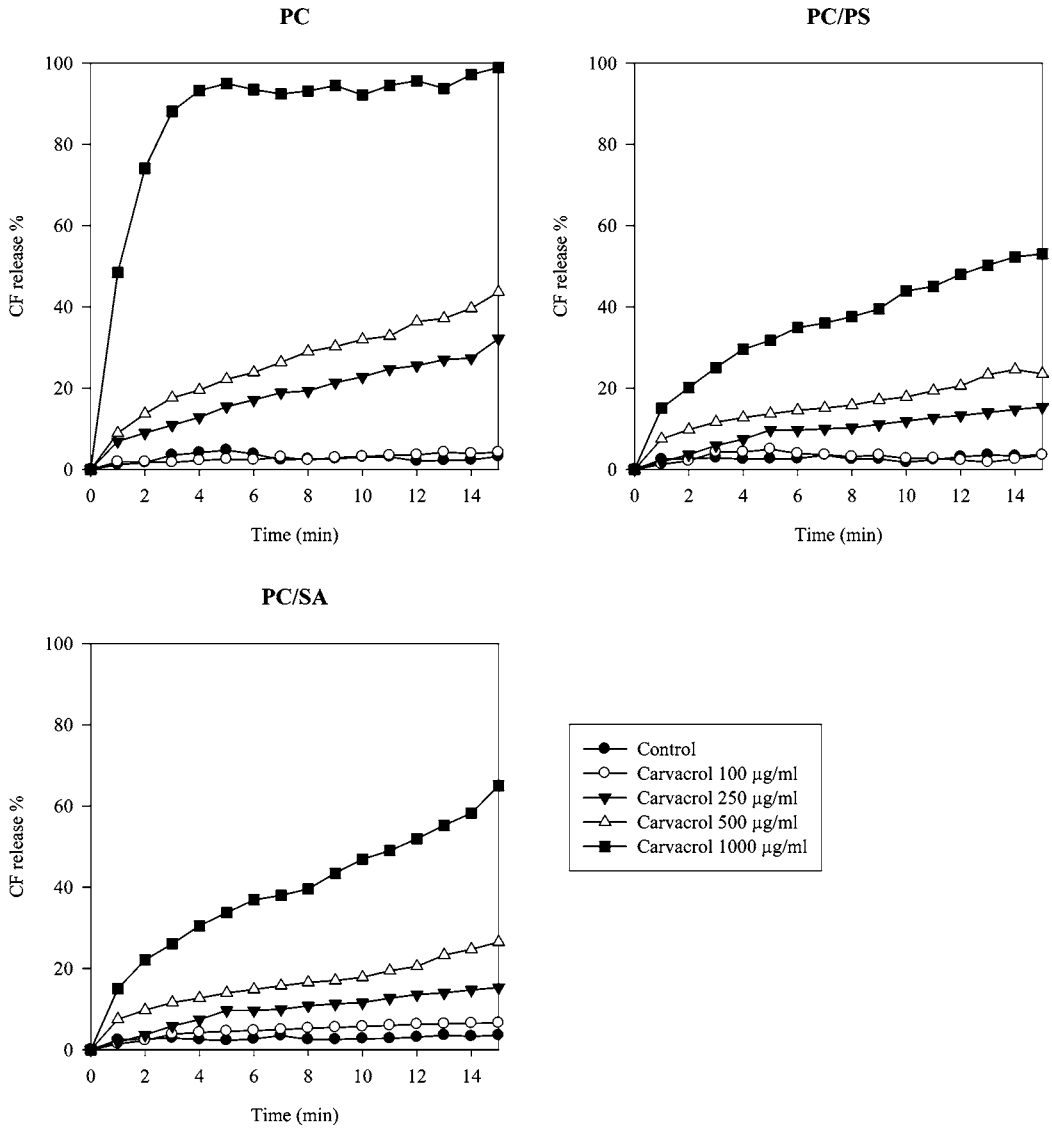

Figure 3. Effect of carvacrol challenge on CF release from CF-encapsulated large unilamellar vesicles with different lipid composition. PC: phospatidylcholine; PS: phospatidylserine; SA: stearylamine.

and a disordered liquid crystalline state, and a small pretransition peak at about 16.5 °C related to the alkyl chain tilt (*35*−*37*). All the terpenes caused a shift toward lower temperature and a broadening of the main peak of the calorimetric curve, in a more evident way as the molar fraction of terpene increased. Thymol and carvacrol, at molar fractions higher than 0.09, produced also a phase separation, which is evidenced by the split of the main peak into two peaks and indicates the presence of terpene-rich and terpene-poor regions. Furthermore, all the terpenes determine the disappearance of the pretransition peak, very likely because of their direct interaction with the DMPC bilayer surface and a perturbation of the acyl chain tilt.

The thermodynamic data obtained from the calorimetric curves are reported in **Figure 4**. In **Figure 4A**, the T_m has been plotted as $\Delta T/T^0_m$ ($\Delta T = T_m - T^0_m$, where T_m is the value of transition temperatures of each terpene/DMPC MLV dispersion, and T^0_m is the transition temperature of pure DMPC MLVs), as a function of the terpene molar fraction in the lipid aqueous dispersion. The terpenes under investigation markedly decrease the DMPC T_m. Up to 0.06 molar fraction the four terpenes elicited similar effects, but at higher molar fractions the effect exerted by carvacrol and thymol was stronger than that of *p*-cymene and γ-terpinene. As shown in **Figure 4B**, reporting the enthalpy variation as $\Delta\Delta H/\Delta H^0$ (where $\Delta\Delta H = \Delta H - \Delta H^0$, ΔH is the value of enthalpy change of each terpene-loaded DMPC MLVs, and ΔH^0 is the enthalpy change of pure DMPC MLVs) as a function of terpene molar fraction, all terpenes, and especially carvacrol and *p*-cymene, induced a significant decrease in enthalpy variation.

Thus, it is evident that the monoterpenes investigated are able to interact with phospholipidic membranes. Our findings may be largely explained in terms of a fluidifying effect due to the introduction of these lipophilic molecules into the ordered structure of the lipid bilayer. Since these four terpenes caused a decrease in T_m and (especially carvacrol and *p*-cymene) of ΔH values, we can suppose that these compounds act as substitutional impurities.

Following the procedure reported in previous research (*29*, *38*, *39*), a series of permeation experiments were carried out to determine whether the terpenes are able to dissolve in the aqueous medium, reach the vesicle surface, and penetrate it. Briefly, the terpenes (0.12 molar fraction) were placed in contact with pure DMPC MLVs at a temperature higher than the DMPC T_m, and at 1 h intervals, the variations in the DSC curves with respect to that of pure DMPC vesicles were noted. The terpenes cause a broadening and shift toward lower temperature of the

Table 2. CF Release from Liposomes with Different Lipid Composition Following Exposure to Thymol, Carvacrol, *p*-Cymene, and γ-Terpinene[a]

		CF release in 15 min % increase vs control	lipid composition	
compounds	concn (μg/mL)	PC	PC/SA (9:1)	PC/PS (9:1)
thymol	100	5.80	6.50	3.03
	250	16.72	30.98	10.11
	500	65.32	78.64	34.95
	1000	97.20	94.44	64.85
carvacrol	100	4.20	6.60	3.50
	250	32.15	15.31	15.31
	500	43.65	26.50	23.05
	1000	98.90	65.02	53.03
p-cymene	100	6.45	5.96	6.59
	250	6.75	6.06	7.52
	500	7.27	9.85	8.56
	1000	9.67	10.25	9.85
γ-terpinene	100	5.05	5.45	4.95
	250	5.58	5.58	5.31
	500	7.56	5.84	6.85
	1000	8.12	7.21	7.12

[a] Data are expressed as percentage increase vs control of the total CF released in a 15-min period. PC: phosphatidylcholine; SA: stearylamine; PS: phosphatidylserine.

main peak and a gradual disappearance of the pretransition peak (curves not shown). The different behavior of the four terpenes studied is evident in **Figure 5**, which reports the T_m, as $\Delta T/T^0_m$, as a function of the incubation time; in this figure, t_{inf} represents the maximum interaction between MLVs and the terpenes, and it is obtained from the calorimetric curve of DMPC MLV prepared in the presence of a 0.12 molar fraction of terpene. All terpenes studied cause a decrease in T_m, thus demonstrating their capability to dissolve in the aqueous medium and reach and interact with lipidic bilayers. All four terpenes tend to reach the maximum interaction. However, carvacrol shows the fastest kinetic of interaction, which can be related with its lipophilicity; in fact, carvacrol is the less lipophilic among the four terpenes, and thus it is more able to migrate through the aqueous medium and, hence, to reach the MLV surface and interact with the bilayers.

Transmembrane transfer kinetic experiments were carried out by mixing equimolar amounts of MLVs containing 0.12 molar fraction of the terpene (loaded MLVs) with pure DMPC MLVs (empty MLVs) for increasing incubation times. In **Figure 6**, the T_m, as $\Delta T/T^0_m$, is reported as a function of the incubation time and compared with that of MLVs containing a 0.06 molar fraction of the terpene (t_{inf}), used as reference concentration value. In fact, when the compound transfers from loaded MLVs (prepared in the presence of 0.12 molar fraction of the compound) to pure MLVs, the loaded vesicles lose half of the enclosed compound, while the pure vesicles accumulate the free compound, thus obtaining, at the steady state of the transfer process, a uniform population of MLVs containing a 0.06 molar fraction of the compound. This figure indicates that all the terpenes quickly transfer from loaded MLVs to empty MLVs. Thymol shows a more gradual transfer kinetic compared to the other compounds. Thymol and γ-terpinene transfer completely to MLVs and reach a homogeneous concentration inside all the MLVs similar to that observed in the preparation in organic phase (t_{inf}); on the contrary, carvacrol and *p*-cymene do not reach the maximum effect because of an incomplete equilibrium between loaded and empty vesicles.

The amphipathicity of phenolic compounds such as thymol and carvacrol can explain their interactions with biomembranes

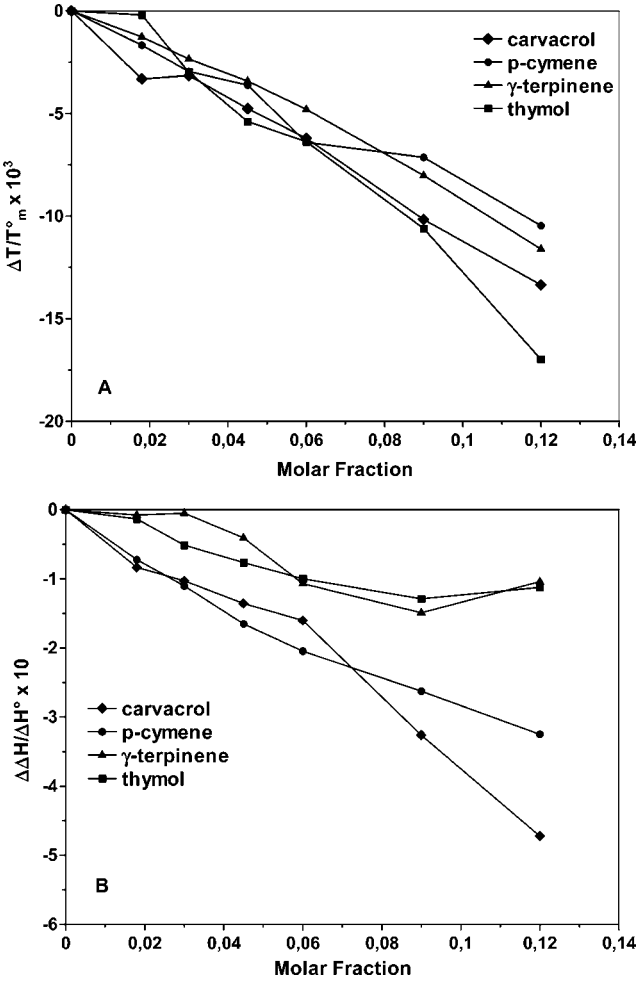

Figure 4. (**A**) Transition temperature variation (as $\Delta T/T^0_m$; $\Delta T = T_m - T^0_m$, where T_m is the transition temperature of DMPC MLVs containing terpene and T^0_m is the transition temperature of pure DMPC MLVs). (**B**) Transition enthalpy variation (as $\Delta\Delta H/\Delta H^0$; $\Delta\Delta H = \Delta H - \Delta H^0$, where ΔH is the enthalpy change of DMPC MLVs containing terpene and ΔH^0 is the enthalpy change of pure DMPC MLVs), in heating mode, as a function of terpene molar fractions.

and thus the antimicrobial activity (*40*). In fact, the hydrophilic part of the molecule interacts with the polar part of the membrane, while the hydrophobic benzene ring and the aliphatic side chains are buried in the hydrophobic inner part of the bacterial membrane. Furthermore, the involvement of the hydroxyl group in the formation of hydrogen bonds and the acidity of these phenolic compounds may have other possible explanations. However, in our experiments thymol and carvacrol show a calorimetric behavior similar, respectively, to that of the non-hydroxyclic monoterpenes γ-terpinene and *p*-cymene, thus ruling out that these are the main mechanism involved in the membrane changes elicited by terpene exposure.

These findings are consistent with the relative lipophilicity and water solubility of the four terpenes examined; in fact, as demonstrated by results reported in **Table 3**, the lipophilicity order was *p*-cymene > γ-terpinene $\gg$ thymol > carvacrol, and, unlike thymol, *p*-cymene, γ-terpinene, and carvacrol are absolutely insoluble in water (*41*).

Taken together, the present results allow us to speculate that the antibacterial activity of the terpenes under study may be caused, partially at least, by their penetration in the lipid assemblies and by a consequent perturbation of the lipid fraction of the plasmic membranes; however, it is significantly influenced

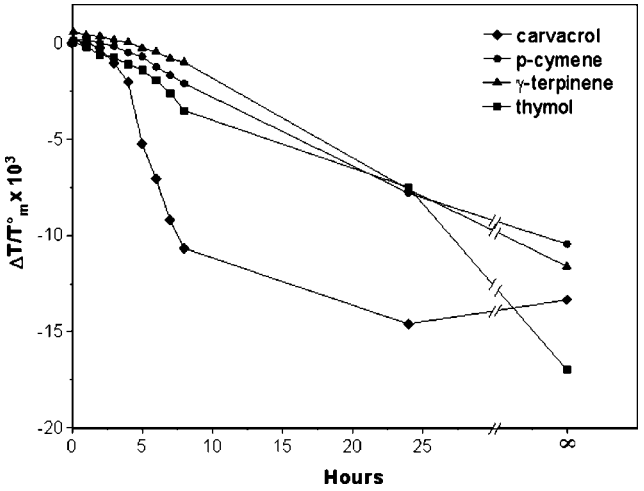

Figure 5. Transitional temperature variations ($\Delta T/T^0_m$), for increasing incubation times, of empty DMPC MLVs left in the presence of a 0.12 molar fraction of terpenes. The t_{inf} values represent the effect exerted by 0.12 molar fraction of terpene on MLVs to be considered as the maximum interaction between compound and vesicles.

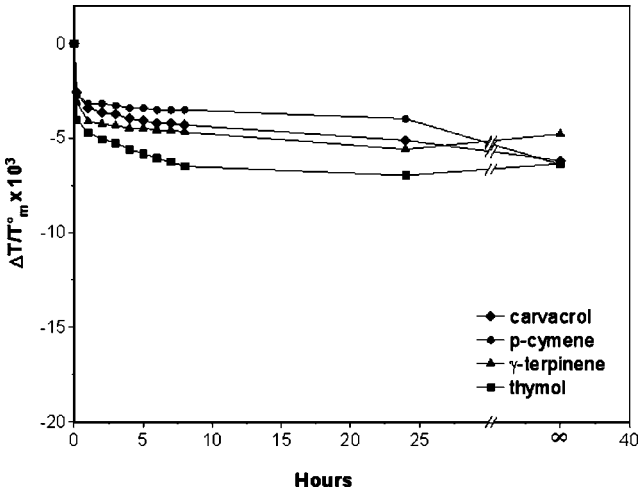

Figure 6. Transitional temperature variations ($\Delta T/T^0_m$), for increasing incubation times, of empty DMPC MLVs left in the presence of equimolar DMPC MLVs loaded with a 0.12 molar fraction of terpenes. The t_{inf} values represent the effect exerted by 0.06 molar fraction of terpene on MLVs to be considered as the maximum interaction between compound and vesicles.

Table 3. Relative Lipophilicity (R_m) Values and Water Solubility of Thymol, γ-Terpinene, Carvacrol, and *p*-Cymene

compounds		water solubility[a]
thymol	0.204	lightly soluble[b]
γ-terpinene	–0.204	practically insoluble[c]
carvacrol	0.311	practically insoluble[c]
p-cymene	–0.234	practically insoluble[c]

[a] From ref 41. [b] 100–1000 mL of solvent/g of solute. [c] More than 10 000 mL of solvent/g of solute.

by their physicochemical characteristics and by the composition of bacterial membranes. More particularly, several points need to be discussed.

First, among the terpenes tested, thymol showed the strongest toxic activity against *S. aureus*; it possesses the highest capacity to increase the permeability of PC LUVs, together with the ability to migrate across an aqueous medium and to interact with phospholipidic membranes. Furthermore, thymol possesses discrete lipophilic characteristics and a detectable water solubility. Thus, its antimicrobial activity may be potentiated by the fact that it can migrate across the aqueous extracellular medium, interact with, and damage lipidic membranes.

Second, *p*-cymene and carvacrol are more active against the Gram-negative *E. coli*; their calorimetric behavior shows that they markedly affect membrane lipid composition, taking the place of lipid molecules, and are strongly absorbed by lipidic membranes, so that they are not released and transferred to other bilayers. Thus, the cytotoxicity of monoterpenes against Gram-negative bacteria seems to be related to their capability to deeply interact with and affect the molecular structure of lipidic bilayers.

Third, it is evident that the antimicrobial activity of terpenes is related to their ability to affect not only permeability but also other functions of cell membranes. In fact, γ-terpinene and *p*-cymene are cytotoxic for Gram-positive and Gram-negative bacteria but do not affect CF release from all kinds of LUVs employed. On the other hand, as also suggested by other authors (*42*, *43*), we cannot exclude that the interaction with other targets of the microbial cell might play a key role in the observed antimicrobial effect of the monoterpenes under study. The results of permeation experiments and DSC measurements suggest a real transfer of monoterpenes through lipidic bilayers; thus, these compounds might be able to permeate through cellular membranes and interact with intracellular components.

Finally, Gram-positive bacteria possess a permeable cell wall that usually does not restrict the penetration of antimicrobials; the major component of a Gram-positive cell wall is peptidoglycan covalently linked to teichoic and teichuronic acids, which give the wall a net negative charge (*44*). However, antimicrobial monoterpenes are thought to target the bacterial cytoplasmic membrane. Unlike *E. coli* cytoplasmic membranes, those of the Gram-positive bacterium *S. aureus* contain the positively charged lipid lysylphosphatidylglycerol at levels between 15 and 40% (*45*). Several authors have demonstrated that the membrane charge of *S. aureus* can influence the efficacy of cationic and anionic antimicrobial agents (*46*, *47*). In our experiments, thymol was more effective than carvacrol against the Gram-positive bacterium *S. aureus* and in eliciting CF release from PC/SA vesicles. Thus, we can suppose that the antimicrobial effect of monoterpenes is influenced by the net surface charge of microbic cell membrane.

Furthermore, the outer layer of the Gram-negative outer membrane is composed primarily of lipopolysaccharide molecules and forms a hydrophilic permeability barrier providing protection against the effects of highly hydrophobic compounds (*48*−*50*). This may explain the low sensibility of *E. coli* to the cytotoxic effect of the four lipophilic monoterpenes tested.

In conclusion, the present data support the hypothesis that the antibacterial effect of thymol, carvacrol, γ-terpinene, and *p*-cymene may be due, partially at least, to a perturbation of the lipidic fraction of microorganism plasmic membrane, resulting in alterations of membrane properties. In addition to being related to physicochemical characteristics of the compounds (such as lipophilicity and water solubility), this effect appears to be dependent also on net surface charge of microbic membranes. Furthermore, the compounds might cross the cell membranes, thus penetrating into the interior of the cell and interacting with intracellular sites critical for antibacterial activity.

LITERATURE CITED

(1) Kohlert, C.; van Rensen, I.; Marz, R.; Schindler, G.; Graefe, E. U.; Veit, M. Bioavailability and pharmacokinetics of natural volatile terpenes in animals and humans. *Planta Med.* **2000**, *66*, 495−505.

(2) Loza-Tavera, H. Monoterpenes in essential oils. Biosynthesis and properties. *Adv. Exp. Med. Biol.* **1999**, *464*, 49−62.

(3) Dorman, H. J.; Deans, S. G. Antimicrobial agents from plants: Antibacterial activity of plant volatile oils. *J. Appl. Microbiol.* **2000**, *88*, 308−316.

(4) Cowan, M. M. Plant products as antimicrobial agents. *Clin. Microbiol. Rev.* **1999**, *12*, 564−582.

(5) Burt, S. Essential oils: Their antibacterial properties and potential applications in foods. *Int. J. Food Microbiol.* **2004**, *94*, 223−253.

(6) Lambert, R. J. W.; Skandamis, P. N.; Coote, P. J.; Nychas, G. J. A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. *J. Appl. Microbiol.* **2001**, *91*, 453−462.

(7) Marino, M.; Bersani, C.; Comi, G. Antimicrobial activity of the essential oils of *Thymus vulgaris* L. measured using a bioimpedometric method. *J. Food Prot.* **1999**, *62*, 1017−1023.

(8) Kulevanova, S.; Kaftandzieva, A.; Dimitrovska, A.; Stefkov, G.; Grdanoska, T.; Panovski, N. Investigation of antimicrobial activity of essential oils of several Macedonian Thymus L. species (Lamiaceae). *Boll. Chim. Farm.* **2000**, *139*, 276−280.

(9) Azaz, A. D.; Irtem, H. A.; Kurkcuoglu, M.; Baser, K. H. Composition and the in vitro antimicrobial activities of the essential oils of some Thymus species. *Z. Naturforsch., C: Biosci.* **2004**, *59*, 75−80.

(10) Baratta, T. M.; Dorman, D. J. H.; Deans, G. S.; Biondi, D. M.; Ruberto, G. Chemical composition, antimicrobial and antioxidative activity of laurel, sage, rosemary, oregano and coriander essential oils. *J. Essent. Oil Res.* **1998**, *10*, 618−627.

(11) Russo, M.; Galletti, G. C.; Bocchini, P.; Carnacini, A. Essential oil chemical composition of wild populations of Italian oregano spice (*Origanum vulgare* ssp. *hirtum* (Link) Ietswaart): A preliminary evaluation of their use in chemotaxonomy by cluster analysis. 1. Inflorescences. *J Agric. Food Chem.* **1998**, *46*, 3741−3746.

(12) Sikkema, J.; Debont, J. A. M.; Poolman, B. Interactions of cyclic hydrocarbons with biological membranes. *J. Biol. Chem.* **1994**, *269*, 8022−8028.

(13) Sikkema, J.; de Bont, J. A.; Poolman, B. Mechanisms of membrane toxicity of hydrocarbons. *Microbiol. Rev.* **1995**, *59*, 201−222.

(14) Turina Adel, V.; Nolan, M. V.; Zygadlo, J. A.; Perillo, M. A. Natural terpenes: Self-assembly and membrane partitioning. *Biophys. Chem.* **2006**, *122*, 101−113.

(15) Gill, A. O.; Holley, R. A. Disruption of *Escherichia coli*, *Listeria monocytogenes* and *Lactobacillus sakei* cellular membranes by plant oil aromatics. *Int. J. Food Microbiol.* **2006**, *108*, 1−9.

(16) Di Pasqua, R.; Hoskins, N.; Betts, G.; Mauriello, G. Changes in membrane fatty acids composition of microbial cells induced by addiction of thymol, carvacrol, limonene, cinnamaldehyde, and eugenol in the growing media. *J. Agric. Food Chem.* **2006**, *54*, 2745−2749.

(17) Huijbregts, R. P.; de Kroon, A. I.; de Kruijff, B. Topology and transport of membrane lipids in bacteria. *Biochim. Biophys. Acta* **2000**, *1469*, 43−61.

(18) Lohner, K.; Latal, A.; Degovics, G.; Garidel, P. Packing characteristics of a model system mimicking cytoplasmic bacterial membranes. *Chem. Phys. Lipids* **2001**, *111*, 177−192.

(19) Mercadal, M.; Domingo, J. C.; Bermudez, M.; Mora, M.; De Madariaga, M. A. N-Palmitoylphosphatidylethanolamine stabilizes liposomes in the presence of human serum: Effect of lipidic composition and system characterization. *Biochim. Biophys. Acta* **1995**, *1235*, 281−288.

(20) *Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically;* Approved standard M7-A5; National Committee for Clinical Laboratory Standards: Villanova, PA, 2000.

(21) Hirakura, Y.; Alvarez-Bravo, J.; Kurata, S.; Natori, S.; Kirino, Y. Selective interaction of synthetic antimicrobial peptides derived from sapecin B with lipid bilayers. *J. Biochem.* **1996**, *120*, 1130−1140.

(22) Trombetta, D.; Saija, A.; Bisignano, G.; Arena, S.; Caruso, S.; Mazzanti, G.; Uccella, N.; Castelli, F. Study on the mechanisms of the antibacterial action of some plant α,β-unsaturated aldehydes. *Lett. Appl. Microbiol.* **2002**, *35*, 285−290.

(23) MacDonald, R. C.; MacDonald, R. I.; Menco, B. P. M.; Takeshita, K.; Subbarao, N. K.; Hu, L. Small-volume extrusion apparatus for preparation of large, unilamellar vesicles. *Biochim. Biophys. Acta* **1991**, *1061*, 297−303.

(24) Hope, M. J.; Bally, M. B.; Webb, G.; Cullis, P. R. Production of large unilamellar vesicles by a rapid extrusion procedure. Characterization of size distribution, trapped volume and ability to maintain a membrane potential. *Biochim. Biophys. Acta* **1985**, *812*, 55−65.

(25) Rouser, G.; Fleicher, S.; Yamamoto, A. Two dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids* **1970**, *5*, 494−496.

(26) Boyce, C. B. C.; Milborrow, B. V. A simple assessment of partition data for correlating structure and biological activity using thin-layer chromatography. *Nature* **1965**, *208*, 537−539.

(27) Cox, S. D.; Mann, C. M.; Markham, J. L.; Bell, H. C.; Gustafson, J. E.; Warmington, J. R.; Wyllie, S. G. The mode of antimicrobial action of the essential oil of *Melaleuca alternifolia* (tea tree oil). *J. Appl. Microbiol.* **2000**, *88*, 170−175.

(28) Bisignano, G.; Laganà, M. G.; Trombetta, D.; Arena, S.; Nostro A.; Uccella, N.; Mazzanti, G.; Saija, A. In vitro antibacterial activity of some aliphatic aldehydes from *Olea europaea* L. *FEMS Microbiol. Lett.* **2001**, *198*, 9−13.

(29) Trombetta, D.; Castelli, F.; Sarpietro, M. G.; Venuti, V.; Cristani, M.; Daniele, C.; Bisignano, G.; Saija, A.; Mazzanti, G. Study on the mechanisms of the antibacterial action of three monoterpenes. *Antimicrob. Agents Chemother.* **2005**, *49*, 2474−2478.

(30) Tenchov, B. On the reversibility of the phase transitions in lipid-water systems. *Chem. Phys. Lipids* **1991**, *57*, 165−177.

(31) Lee, A. G. Lipid phase transitions and phase diagrams. II. Mixtures involving lipids. *Biochim. Biophys. Acta* **1977**, *472*, 285−344.

(32) Mouritsen, O. G.; Bloom, M. Mattress model of lipid-protein interactions in membranes. *Biophys. J.* **1984**, *46*, 141−153.

(33) Cevc, G.; Marsh, D. *Phospholipid Bilayers: Physical Principles and Models*; Wiley & Sons: New York, 1987; Vol. 5, p 441.

(34) Jorgensen, K.; Ipsen, J. H.; Mouritsen, O. G.; Bennet, D.; Zuckermann, M. J. A general model for the interaction of foreign molecules with lipid membranes: Drugs and anaesthetics. *Biochim. Biophys. Acta* **1991**, *1062*, 227−238.

(35) Lo, Y. L.; Rahman, Y. E. Protein location in liposomes, a drug carrier: A prediction by differential scanning calorimetry. *J. Pharm. Sci.* **1995**, *84*, 805−814.

(36) Pedersen, T. B.; Sabra, M. C.; Frokjaer, S.; Mouritsen, O. G.; Jorgensen, K. Association of acylated cationic decapeptides with dipalmitoylphosphatidylserine-dipalmitoylphosphatidylcholine lipid membranes. *Chem. Phys. Lipids* **2001**, *113*, 83−95.

(37) Walde, P. Preparation of Vesicles (Liposomes). In *Encyclopedia of Nanoscience and Nanotechnology*; Nalwa, H. S., Ed.; American Scientific Publishers: Stevenson Ranch, CA, 2004; Vol. 9, pp 43−79.

(38) Castelli, F.; Librando, V.; Sarpietro, M. G. Calorimetric approach of the interaction and absorption of polycyclic aromatic hydrocarbons with model membranes. *Environ. Sci. Technol.* **2002**, *36*, 2717−2723.

(39) Castelli, F.; Caruso, S.; Uccella, N. Biomimesis of linolenic acid transport through model lipidic membranes by differential scanning calorimetry. *J. Agric. Food Chem.* **2003**, *51*, 851−855.

(40) Veldhuizen, E. J.; Tjeerdsma-van Bokhoven, J. L.; Zweijtzer, C.; Burt, S. A.; Haagsman, H. P. Structural requirements for the antimicrobial activity of carvacrol. *J. Agric. Food Chem.* **2006**, *54*, 1874−1879.

(41) *The Index Merck: An Encyclopedia of Chemicals, Drugs, and Biologicals*, 13th ed., Merck Research Laboratories: Whitehouse Station, NJ, 2001.

(42) Glover, R. E.; Smith, R. R.; Jones, M. V.; Jackson, S. K.; Rowlands, C. C. An EPR investigation of surfactant action on bacterial membranes. *FEMS Microbiol. Lett.* **1999**, *177*, 57−62.

(43) Denyer, S. P.; Stewart, G. S. A. B. Mechanisms of action of disinfectants. *Int. Biodeterior. Biodegrad.* **1998**, *41*, 261−268.

(44) Schaffer, C.; Messner, P. The structure of secondary cell wall polymers: How Gram-positive bacteria stick their cell walls together. *Microbiology* **2005**, *151*, 643−651.

(45) Fedtke, I.; Gotz, F.; Peschel, A. Bacterial evasion of innate host defenses − the *Staphylococcus aureus* lesson. *Int. J. Med. Microbiol.* **2004**, *294*, 189−194.

(46) Dennison, S. R.; Howe, J.; Morton, L. H. G.; Brandenburg, K.; Harris, F.; Phoenix, D. A. Interactions of an anionic antimicrobial peptide with *Staphylococcus aureus* membranes. *Biochem. Biophys. Res. Commun.* **2006**, *347*, 1006−1010.

(47) Nishi, H.; Komatsuzawa, H.; Fujiwara, T.; McCallum, N.; Sugai, M. Reduced content of lysyl-phosphatidylglycerol in the cytoplasmic membrane affects susceptibility to moenomycin, as well as vancomycin, gentamicin, and antimicrobial peptides, in *Staphylococcus aureus*. *Antimicrob. Agents Chemother*. **2004**, *48*, 4800−4807.

(48) Ikigai, H.; Nakae, T.; Hara, Y.; Shimamura, T. Bactericidal catechins damage the lipid bilayer. *Biochim. Biophys. Acta* **1993**, *1147*, 132−136.

(49) Helander, I. M.; Alakomi, H.-L.; Latva-Kala, K.; Mattila-Sandholm, T.; Pol, I.; Smid, E. J.; Gorris, L. G. M.; von Wright, A. Characterization of the action of selected essential oil components on Gram-negative bacteria. *J. Agric. Food Chem.* **1998**, *46*, 3590−3595.

(50) Hancock, R. E. W. The bacterial outer membrane as a drug barrier. *Trends Microbiol.* **1997**, *5*, 37−42.

Received for review January 12, 2007. Revised manuscript received May 8, 2007. Accepted May 13, 2007.

JF070094X