JOURNAL OF NATURAL PRODUCTS

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Influence of the Active Compounds of *Perilla frutescens* Leaves on Lipid Membranes

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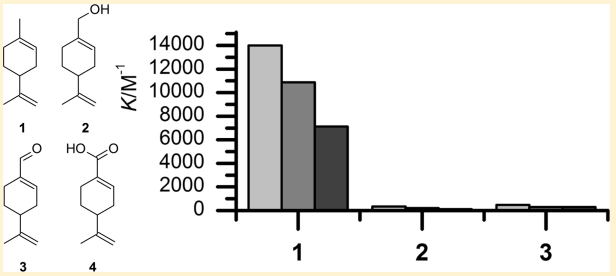
Supporting Information

ABSTRACT: The leaves of the annual plant *Perilla frutescens* are used widely as a spice and a preservative in Asian food as well as in traditional medicine. The active compounds in the leaves are the cyclic monoterpene limonene (**1**) and its bio-oxidation products, perillaldehyde (**2**), perillyl alcohol (**3**), and perillic acid (**4**). These compounds are known to be biologically active and exhibit antimicrobial, anticancer, and anti-inflammatory effects that could all be membrane mediated. In order to assess the possible biophysical effects of these compounds on membranes quantitatively, the influence of limonene and its bio-oxidation products has been investigated on a membrane model composed of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) using differential scanning calorimetry (DSC), isothermal titration calorimetry (ITC), and electron paramagnetic resonance spectroscopy (EPR). It was found that limonene (**1**), perillyl alcohol (**2**), and perillaldehyde (**3**) partitioned into the DMPC membrane, whereas perillic acid (**4**) did not. The DSC results demonstrated that all the partitioning compounds strongly perturbed the phase transition of DMPC, whereas no perturbation of the local membrane order was detected by EPR spectroscopy. The results of the study showed that limonene (**1**) and its bio-oxidation products affect membranes in rather subtle ways.



The leaves of *Perilla frutescens* L. ex B.D. Jacks. (Lamiaceae) are used as a popular garnish in Asian food because of their aromatic, basil-mint-like taste and pleasant green or purple colors. In Japanese food, these leaves are known as *shiso* and are used traditionally in dishes with fish or shellfish, such as sushi.[1] Purple shiso leaves are also utilized as a coloring agent and a preservative in pickled foodstuffs, such as vegetables and plums. Furthermore, the leaves are used in traditional medicine, specifically for the treatment of a variety of ailments, such as colds,[2] food poisoning,[2] food allergy,[3] and depression.[4] A steam-distillate of the leaves has been shown to have antimicrobial activity, especially against *Salmonella choleraesuis*, one of the major bacteria causing food poisoning from raw fish.[2]

Analysis of the steam-distillate has shown that the main constituents of *P. frutescens* leaves are perillaldehyde (**3**) (about 75%) and limonene (**1**) (12%),[2,5] whereas other studies have suggested the compounds perillaketone (1-(furanyl)-4-methyl-1-petanone),[5,6] elsholtziaketone (2-(4-methylpent-2-enal)3-methylfuran), and elemicin (5-allyl-1,2,3-trimethoxybenzene) to be present in the leaves.[7] In work by Koezuka et al.,[7] four different chemotypes were described corresponding to the above-mentioned compounds and with there being a genetic control of the production of these compounds. The chemotype having a high content of perillaldehyde (**3**) is preferred both as a spice and for use in traditional medicine.[7]

In order to obtain more information about the possible mechanism behind the biological effects of *shiso*, a range of biophysical techniques were used to study the interaction between the main compounds of the steam-distillate of the *P. frutescens* leaves and a simple biomembrane model. Besides the primary compounds, limonene (**1**) and perillaldehyde (**3**), two other oxidation products in the bioconversion of limonene (review by Duetz et al.[8]) were investigated.

In addition to *P. frutescens*, limonene (**1**) is also found in many other plants and fruits. Especially high contents are found in the peel of some citrus fruits.[8,9] Compound **1** is used industrially for many different purposes, e.g., as a solvent, in paint and paint remover, and as a starting material for organic synthesis.[10] It is present also in large quantities in some food additives, such as orange oil, due to its pleasant orange-like smell and taste.[11] Limonene (**1**) has been shown to have antibacterial,[12] antifungal,[11,13] and anticarcinogenic effects[14] as well as being toxic to insects.[15] Furthermore, it has been used as a skin penetration enhancer in transdermal drug delivery.[16]

Perillyl alcohol (**2**) is the first bio-oxidation product of **1** in many bacteria, fungi, and plants[8] and in humans.[17] Substantial amounts of **2** are found in cherries and spearmint. Compound **2** is shown to have an antimicrobial effect[18] and to induce apoptosis in cancer cells.[19] These effects may, at least in part, be due to the Na/K-ATPase inhibitory effect described recently.[20] Due to the possible anticancer effects of **2**, it has been subjected to several phase I clinical trials[21] as well as a phase IIa study investigating this compound as a skin cancer chemopreventive agent.[22]

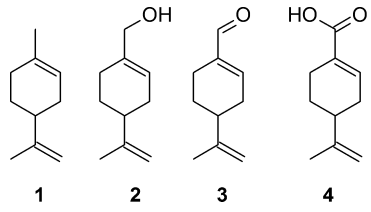
Received: September 5, 2011
Published: January 24, 2012

Perillaldehyde (**3**) is the bio-oxidation product of **2** in many organisms,[8] and it seems to have some of the same properties as **2**, namely, antibacterial effects[2] and the capability to induce apoptosis in cancer cells.[19]

The final bio-oxidation product used was perillic acid (**4**), which is being marketed as a preservative for cosmetics due to its antimicrobial effects.[23] This substance is also cytotoxic to cancer cells[19] and has been shown to inhibit protein prenylation.[17] It has been suggested that the effects of the other compounds (**1**–**3**) are due to the inhibition of the protein prenylation by **4** caused by their oxidation. However, it has been shown that the anticancer effect of **2** occurs at markedly lower concentrations than the inhibition of the protein prenylation,[24] and the Na/K-ATPase inhibitory effect of **4** is significantly less potent than that of **2**.[20] The wide range of unspecific biological effects of **1**–**4** suggests that their main effect may be through a physicochemical action on biological membranes.

The fluid lipid-bilayer component of cell membranes is composed of mainly phospholipids and proteins, and it plays a vital role for controlling the biological processes taking place at or in the biological membrane.[25,26] This includes maintaining the concentrations or gradients of solutes and water in the cell or organelles and dividing the cell into compartments for biochemical differentiation. The cell membrane also influences the function of proteins embedded in the membrane. This includes channels and pumps, like Na/K-ATPase.[27] Many small molecules, such as alcohols, anesthetics, and a variety of amphiphilic drugs, are known to interact with this membrane and exert their action by influencing its properties.[28−30]

Although the anticancer and antimicrobial effects of the cyclic terpenoids studied in the present paper have been claimed to be mediated by biological membranes,[31−33] or at least be controlled by membranes in their capacity as guardians of the cell interior, very little quantitative information is available about the biophysical effects of the interactions between these substances and the cell membrane. The membrane partition coefficient for **1** into *E. coli* lipids has been measured,[34] and a single calorimetry study of the effect of **1** on a model membrane has been published.[35] Recently, a molecular dynamics study of the interaction between the investigated compounds and a DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine) lipid bilayer showed that **1**–**3** are all associated with the DMPC bilayer, and all induce a slight ordering of the membrane.[36]



In the present work, various biophysical techniques have been used to investigate the interactions between a membrane model in the form of lipid bilayers composed of DMPC and the terpenoids **1**–**4**. In particular, isothermal titration calorimetry (ITC), differential scanning calorimetry (DSC), and electron paramagnetic resonance spectroscopy (EPR) have been employed to assess quantitatively the biophysical effects of these interactions.

RESULTS AND DISCUSSION

Isothermal titration calorimetry may be used to determine the membrane partition coefficient, K, and the thermodynamic functions, ΔH, ΔG^0, and ΔS^0, as well as the change in the heat capacity, ΔC_P, of the terpenoids investigated upon interaction with the membrane. The partition coefficient, K, provides precise information on the distribution of the terpenoids between the membrane and the water phase, whereas the thermodynamics functions provide details about the energetics of the partitioning and hence indirect insight into the permeation process (see Heerklotz and Seelig[37] for more details regarding the experimental technique).

The ITC data are summarized in Figure 1. The value of K for **1** was found to be very large (10 900 M^{-1} at 35 °C), suggesting

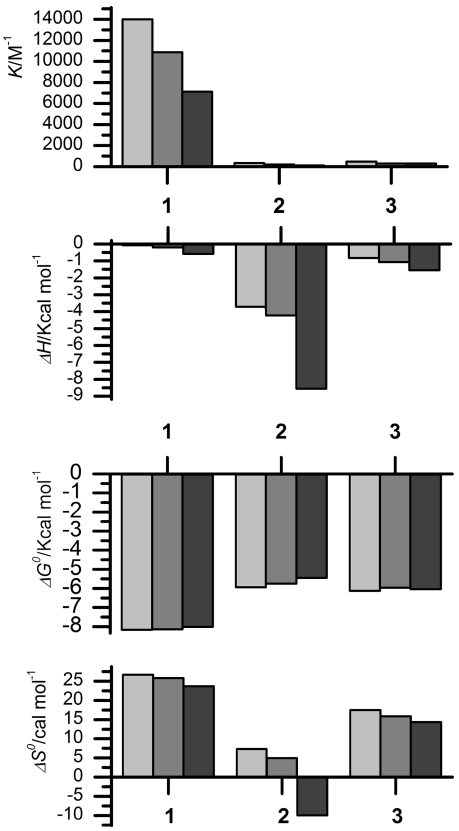


Figure 1. Membrane partition coefficient, K, and thermodynamic functions (ΔH, ΔG^0, and ΔS^0) characterizing the partitioning of compounds **1**–**3** from the water phase into DMPC vesicles at 30 °C (light gray), 35 °C (gray), and 40 °C (dark gray). Values at 30 °C are from Witzke et al.[36]

that almost all of this compound was in the membrane. Although the values of K for **2** and **3** are much smaller (respectively 305 and 215 M^{-1} at 35 °C), they indicate that most of these compounds are also associated with the DMPC membrane. The small value of ΔH (−200 cal mol^{-1} at 35 °C) obtained and the negative value of ΔC_P (−51.2 cal K^{-1} mol^{-1}) for **1** are characteristic for transfer of a hydrophobic compound into an organic phase,[37] consistent with the hydrophobic nature of **1**. The transfer of **2** and **3** from the aqueous phase to the membrane is also associated with a negative ΔC_P (−485 and −70 cal K^{-1} mol^{-1}, respectively), but with a much larger (negative) enthalpy of transfer (respectively −4200 and −1100 cal mol^{-1} at 35 °C) compared to **1**. This difference can be

dx.doi.org/10.1021/np200713q | J. Nat. Prod. 2012, 75, 160−166

explained by the fact that **2** and **3** both have the possibility of forming hydrogen bonds between their oxygen atoms and the water or to DMPC. As the larger part of the enthalpy stems from rearrangements in the water structure (i.e., making and breaking of hydrogen bonds), a large negative ΔH of transfer is consistent with the larger hydration sphere expected for **2** and **3**. The large negative ΔH for **2** is also consistent with the hydrogen bonding found between DMPC and **2** by molecular dynamics simulations.[36]

The membrane partition coefficient and the thermodynamic functions for **4** could not be determined by ITC, consistent with preliminary experiments suggesting K to be very small in this case. In order to investigate this weak partitioning equilibrium further, the protocol devised by Zhang and Rowe[38] for determining very small K values (the so-called "solvent null" method) was used. This method has been used successfully to study weakly partitioning compounds such as short-chain alcohols.[30] However, this protocol also showed no measurable partitioning of **4** into the DMPC membrane.[36] Therefore the effects of **4** on membranes was not investigated further.

In order to investigate the physical chemical properties of DMPC membranes subjected to the terpenoids, their effects on the phase transition of the DMPC membrane were studied using DSC. This is a widely used technique to obtain information on small molecule−membrane interactions[39−41] and provides information on the phase-transition temperature and the enthalpy (ΔH) of the membrane phase transition. By observing how small terpenoid molecules perturb the phase-transition properties, indirect information my be gained on the interaction of the small molecules with the model membrane.

A fully hydrated DMPC membrane displays two phase transitions, which can be observed readily by DSC.[41] At low temperature, the DMPC bilayers exist in a solid-like state, the gel or L_β phase. At around 13 °C there is a transition (the so-called "pretransition") into the so-called "ripple" or P_β phase. The ripple phase is also a solid phase, which at 24 °C melts into a liquid phase, the fluid or L_α phase. This transition is known as the "main phase transition". Both phase transitions are sensitive to the addition of small molecules and to the way they affect the structure of the membrane.[40,41]

The thermograms of multilamellar DMPC vesicles with increasing mole fractions of **1** are shown in Figure 2. The thermograms obtained for the systems with **2** and **3** were similar to those for **1**, although the effects on the phase transition were slightly smaller, making it possible to observe the phase transition at x = 0.2, which was not the case for **1**. (Thermograms of **2** and **3** are shown in Figure S1, Supporting Information.)

From the thermograms the transition temperature, T_m, and the enthalpy, ΔH, of the main phase transition were determined. The resulting data are shown in Figure 3 as functions of terpenoid mole fraction. At low concentrations of **1** (below x = 0.05), an almost constant T_m was observed, but above x = 0.05 a more significant decrease was observed in the range from 24.4 to 16.3 °C for x = 0.15. The pretransition could be observed only for x = 0.01 and x = 0.025, and it was found to be shifted slightly upward from 12.7 °C at x = 0 to 14.5 °C at x = 0.025. The ΔH of the main phase transition showed a small increase for x = 0.01 (from 5.3 to 5.6 kcal mol^{-1}) and thereafter a strong decrease to 1.7 kcal mol^{-1} for x = 0.15. At the same time, a broadening of the peak set in, and at higher concentrations of **1** the phase transition could not be discerned.

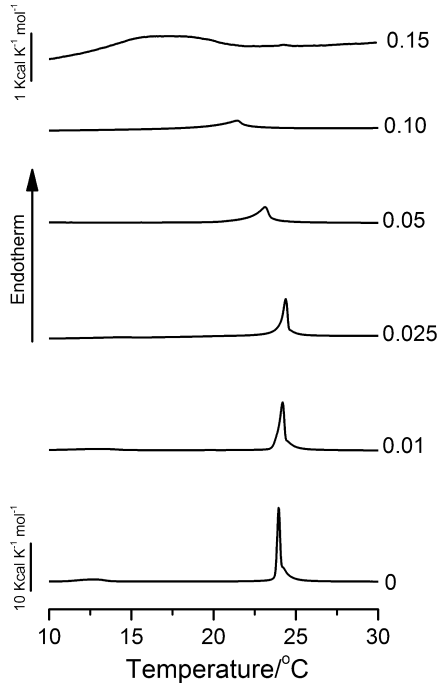


Figure 2. Thermograms of 15 mM multilamellar DMPC vesicles for varying concentrations of limonene (**1**). Figures to the right denote the mole fraction, x, of **1**. Note the scaling of the data for x = 0.15.

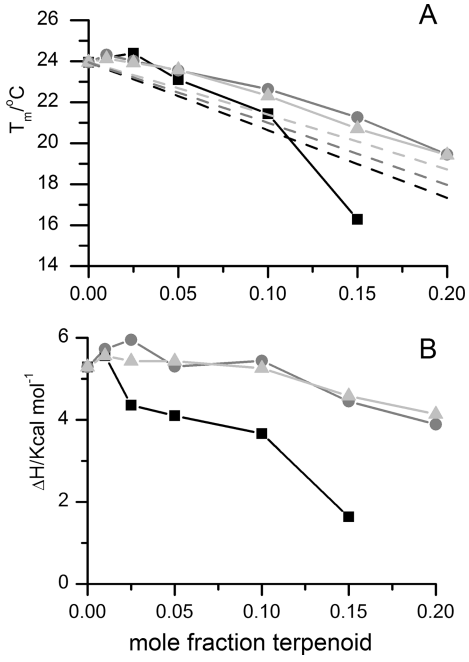


Figure 3. Phase transition temperature, T_m (A), and the enthalpy, ΔH (B), of the main phase transition for 15 mM DMPC vesicles in the presence of the terpenoids investigated as a function of the mole fraction of these compounds. Black line with squares: limonene (**1**); gray line with circles: perillyl alcohol (**2**); and light gray line with triangles: perillaldehyde (**3**). The dashed lines in A are the T_m values calculated from the freezing point depression by eq 1 for various values of K. Light gray: K = 200 M^{-1}, gray: K = 500 M^{-1}, and black: K = 14 000 M^{-1}; see text for further details.

The influence of **2** and **3** on the DMPC membrane phase behavior is very similar to that exerted by **1** (see Figure 3). For both compounds, almost no change in T_m was observed for

dx.doi.org/10.1021/np200713q | J. Nat. Prod. 2012, 75, 160−166

x = 0.01. At higher mole fractions of **2** or **3**, a decrease of T_m to 19.4 °C at x = 0.2 was observed in each case. The enthalpy of the phase transition showed for **2** a slight increase for x = 0.01 and x = 0.025 (ΔH being 5.7 and 5.9 kcal mol^{-1}, respectively), and thereafter it exhibited a decrease to 3.9 kcal mol^{-1} at x = 0.2. Also, for these compounds a significant broadening of the phase transition was observed, making the detection of the phase transition at x > 0.2 impossible.

The DSC experiments hence demonstrated that terpenoids **1**−**3** do have a strong influence on the phase transition of DMPC membranes. In all cases, the main phase transition temperature, T_m, was lowered and the value of the transition enthalpy, ΔH, significantly diminished. These observations indicated that these terpenoids perturb the fatty acid part of the phospholipids.[41]

One possible interpretation of the DSC results could be made in the context of a freezing point depression of the membrane, as observed for several alcohols.[42] If it is assumed that the partitioning of the terpenoids into the gel phase membrane is negligible and an ideal solution of each terpenoid occurs in the lipid in the fluid phase, the change in transition temperature, ΔT_m, can be calculated as follows:

$$\Delta T_m = -\left(\frac{RT_0^2}{\Delta H_0}\right) x_{\text{terpenoid}}^{\text{mem}} \quad (1)$$

where R is the universal gas constant, T_0 is the phase transition temperature of the pure DMPC membrane, ΔH_0 is the melting enthalpy of the pure DMPC membrane, and $x_{\text{terpenoid}}^{\text{mem}}$ is the *membrane* mole fraction of the terpeneoids. The $x_{\text{terpenoid}}^{\text{mem}}$ could, in principle, be calculated from the known total mole fractions and the membrane partition coefficient, K. However, it is known from the ITC data that K exhibits a strong variation with temperature. For **1**, this is not a major problem, as the large K values imply that almost all of this compound is in the membrane at all temperatures and the difference between the total mole fraction and the membrane mole fraction is very small. For **2** and **3**, their lower K values imply that there is a notable difference between the mole fractions and that this will vary with temperature.

In order to compare the melting points found with the freezing point depression model, the melting points for K = 14 000 (for comparison with **1**) and for K = 200 and K = 500 (for comparison with **2** and **3**) were calculated. These latter values of K represent reasonable lower and upper limits of the K values for **2** and **3**. The calculated melting points are plotted in Figure 3A. When the experimentally found T_m are compared with T_m calculated from eq 1, some clear discrepancies were noted, especially for **1**. However given all the assumptions in the model, the effects of **2** and **3** seem, at least in part, to be described by the freezing point model, whereas **1** is not. This is consistent with the stronger influences on T_m and especially on ΔH found for **1**.

The observed initial nonlinearity in both T_m and ΔH is unusual, but a similar phenomenon has been observed for farnesol−DMPC interactions.[43] The origin of this behavior is likely to be found in the cooperative coupling of the individual lamellae in the multilamellar vesicles, as recently described by Parry et al.[44]

The strong effect of **1** on lipid membranes found in the present work is in contrast to the observations by El Maghraby et al.[35] At x = 0.7 they were still able to observe both the pretransition and the main phase transition of a 5.8 mM suspension of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), whereas no phase transition for x > 0.15 was observed in the present study. Although several differences exist between the two studies (e.g., different chain lengths of the phospholipids and different lipid concentrations), we believe the difference to be caused by the fact that El Maghraby et al. chose to cosolubilize **1** and the lipid and then remove the solvent under vacuum. In our hands, limonene (**1**) evaporated when it was placed under a vacuum, and therefore the compounds were added to preformed vesicles as a concentrated MeOH solution. This may explain the observed differences beyond the possible influence of the acyl chain length.

In order to obtain information on more local effects of the terpenoids on the DMPC membrane, electron paramagnetic resonance spectroscopy was used. With this technique, which is sensitive to the intramolecular motion of an added spin label, details can be obtained on the local properties of the membrane. From the EPR spectra the local order parameter, S, can be determined. This describes the organization of the membrane by the time-averaged fluctuation of the acyl chain segment relative to the bilayer normal, hence providing information on the degree of acyl chain order.[45] Furthermore, it is possible to determine the rotational correlation time, τ. This can be viewed as a measure of the fluidity of the membrane,[46] since τ is inversely proportional (through the Stokes−Einstein equation) to the microviscosity of the membrane. In the present work fluidity has been defined as the inverse of the local viscosity of the membrane. For τ < 3 ns, the correlation time and the order parameter can be determined independently from the EPR spectrum.[46]

The order parameters were calculated from the spectra of the methyl-5-doxyl stearic (Me-5-DSA) spin label in the membrane at 35 °C, and they are shown as a function of terpenoid concentration in Figure 4 (spectra for x = 0 and x = 0.15 are shown in Figure S2, Supporting Information). Surprisingly, in the light of the strong influence of the terpenoids on the phase transition revealed by the calorimetry studies, almost no variation was found in S.

From the relative spectral line intensity the so-called B and C parameters can be calculated (see Experimental Section, eqs 9 and 10). For isotropic movements, $|C/B| \simeq 1$, but here $|C/B|$ was found to be around 1.4, indicating an anisotropic motion around the nitroxide z-axis (which is parallel to the average orientation of the fatty acid chains of DMPC).[46] Therefore the spectra need to be analyzed in terms of two different correlation times: $\tau_\parallel$ for movement around the z-axis and $\tau_\perp$ for movement perpendicular to the z-axis. These two correlation times were calculated, and the results are shown in Figure 4 as a function of terpenoid concentration at 35 °C. These data showed almost no dependence on the presence of the terpenoids used.

In order to investigate whether the lack of effect of the terpenoids on the EPR spectra of DMPC membranes could be due to a possible insensitivity of the spin label used to detect the changes induced by these compounds, EPR spectra were measured using several other spin labels in a pure DMPC membrane and in DMPC membranes containing 15 mol % of the different terpenoids. Spectra for carbon-16-labeled Me-DSA, carbon-5-labeled doxyls stearic acid (5-DSA), and the cholestane spin label, CSL, were obtained. In all cases, no difference between the spectra with or without the added terpenoids could be discerned. (The spectra are shown in Figure S3, Supporting Information.)

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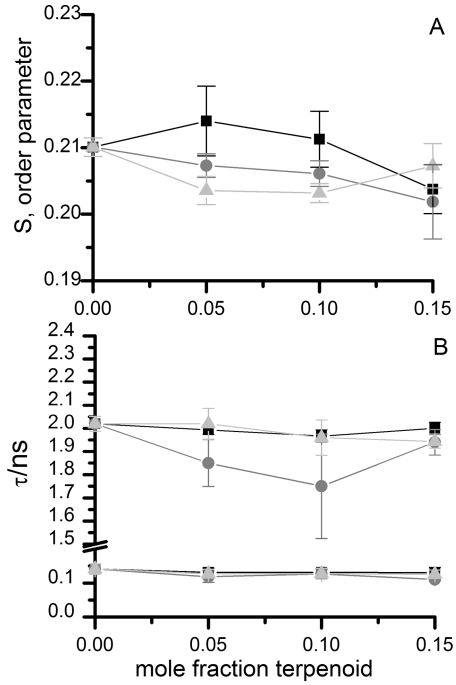

Figure 4. Order parameter, S (A), parallel rotational correlation time, $\tau_{\parallel}$ (B, lower curves), and perpendicular rotational correlation time, $\tau_{\perp}$ (B top curves), as obtained from EPR experiments. All parameters are shown as a function of terpenoid mole fraction at 35 °C. Black line with squares: limonene (**1**); gray line with circles: perillyl alcohol (**2**); and light gray line with triangles: perillaldehyde (**3**).

It was rather surprising that the presence of up to 15 mol % of a foreign molecule in the membrane did not cause any local effects, either on the structure (order parameter) or on the fluidity. A clear, concentration-dependent effect on both the order and the fluidity of a model membrane was found for several local anesthetics[47] and on the fluidity of isolated brush border membranes for a series of *n*-alcohols.[48] For these alcohols, the change in the T_m was analyzed satisfactorily by a freezing point depression analysis.[49] This is in contrast to the behavior in the present work on the terpenoids investigated, where the EPR measurements showed no effects on either the structure or fluidity, although it is known from the calorimetric measurements that the compounds were present in the membrane and strongly perturbed the lipid melting.

Although the lack of effect found on local lipid acyl chain order was somewhat surprising when comparing with the DSC data, this was not totally unexpected. In recent molecular dynamics simulations,[36] it was found that the fatty-acid chains in DMPC bilayers were only slightly ordered by the several terpenoids used, with a change in S of around 0.04. This small effect could be masked by the slightly disordering effect of the probe in the EPR experiments used[50] or could have been slightly overestimated by the molecular dynamics simulations. The seemingly small discrepancy between the EPR data and the data from the molecular dynamics simulations could also be due to the fact that the simulations determine the order parameter of the fatty-acid chains, whereas EPR experiments determine the order parameter of the spin-label.

In conclusion, it has been shown that the widespread secondary metabolite limonene (**1**) and its bio-oxidation products, perillyl alcohol (**2**) and perillaldehyde (**3**), all reside preferentially in the hydrophobic membrane environment, as shown by their large membrane partition coefficients. These terpenoids perturb the phase transitions of model membranes strongly, indicating that their presence in the membrane modifies the global properties of the model membrane. Interestingly the local properties, as monitored by EPR spectroscopy, were not modified significantly by the presence of these compounds. This finding suggests that **1**−**3** exert at least part of their possible biological effects, such as the inhibition of Na/K-ATPase,[20] through a membrane effect.

The final bio-oxidation product, perillic acid (**4**), did not show any partitioning into the membrane, under the experimental conditions used, indicating that the partition coefficient is at least 100 times smaller than for **2** and **3**. This could indicate that the possible biological effects of this molecule are not mediated directly by the lipid-bilayer component of the membrane, but most probably by the inhibition of protein prenylation.[17,24]

EXPERIMENTAL SECTION

General Experimental Procedures. 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine was obtained from Avanti Polar Lipids (Alabaster, AL). Limonene (**1**), perillyl alcohol (**2**), and perillic acid (**4**) were from Aldrich (Copenhagen, Denmark). Perillaldehyde (**3**) was obtained from Fluka (Copenhagen, Denmark). Sodium phosphate was from Sigma (Copenhagen, Denmark), and sodium chloride was from Merck (VWR, Copenhagen, Denmark). The spin labels and all solvents were from Aldrich and of HPLC quality or better.

Isothermal Titration Calorimetry. This technique was performed in a VP-ITC calorimeter from Microcal (Northampton, MA, USA). In general, the membrane partition coefficient, K, was determined by injection of small aliquots of the lipid suspension (concentration between 1 and 20 mM) into a ca. 300 μM solution of each terpenoid. The analysis was performed in Origin 7.0 (Origin Lab, Northampton, MA, USA) by a script written by our group. Details concerning the experimental setup and data analysis can be found in the review by Herklotz and Seelig.[37]

The model adopted for calculating the membrane partition coefficient assumes that the following holds for all concentrations:

$$\frac{C_{t,b}}{C_L} = KC_{t,f} \tag{2}$$

where $C_{t,b}$ is the concentration of bound terpenoid, C_L is the lipid concentration, and $C_{t,f}$ is the concentration of free terpenoid. K is the membrane partition coefficient. It is assumed that the terpenoid is in the form of either bound or free terpenoid, i.e., $C_{t,b} + C_{t,f} = C_{t,tot}$, where $C_{t,tot}$ is the total concentration of terpenoid. For determining K, the experimental observed integrated heat per injection, δh_i, was fitted to the following equation:

$$\delta h_i = C_t^0 \Delta H V_{cell} \frac{K}{(1 + iK\delta C_L^0)^2} \delta C_L^0 + Q_{dil} \tag{3}$$

where C_t^0 is the terpenoid concentration in the cell, ΔH is the enthalpy for transferring the terpenoid from the water into the membrane, V_{cell} is the cell volume (1.409 mL), i is the injection number, δC_L^0 is the change in lipid concentration in the cell per injection, and Q_{dil} is the heat of dilution for the injection. The latter includes both the heat of dilution for the lipid suspension and the terpenoid solution. Both the terpenoid and lipid concentrations were corrected for dilution effects. The expression in eq 3 differs from the one given in Herklotz and Seelig[37] by the term Q_{dil}. The other thermodynamic parameters were obtained from $\Delta G^0 = -RT\ln(55.5K) = \Delta H - T\Delta S^0$, where the factor of 55.5 corrects for the cratic contribution to the binding.[51] The change in heat capacity for the terpenes upon insertion into the membrane, ΔC_p, was calculated from the temperature dependence of ΔH by linear regression in Origin 7.0.

dx.doi.org/10.1021/np200713q | J. Nat. Prod. 2012, 75, 160−166

Lipid samples for ITC were prepared by hydrating dry DMPC powder in a 50 mM sodium phosphate buffer with the ion strength set to 154 mM with NaCl, and the pH was adjusted to 7.5 followed by extrusion at 35 °C through two stacked Whatman nucleopore filters with a hole diameter of 100 nm using a Lipex extruder (Northern Lipids, Vancouver, Canada).[52]

Differential Scanning Calorimetry. This technique was performed in a Calorimetric Science Corp. (Lindon, UT, USA) 4100 MC-DSC.[53] The lipid concentration was 15 mM, and the scan rate was 10 K h^{-1}. The analysis of the thermograms was performed in Origin 7.0 with the DSC module from Microcal.

The samples containing **1**−**3** were prepared by addition of a small aliquot of a concentrated methanolic solution of the terpenoids to the DMPC suspension. The buffer used was 50 mM sodium phosphate with the ion strength set to 154 mM with NaCl, and the pH was adjusted to 7.5. The amount of MeOH added was always below 10 μL, and to the x = 0 sample there was always added 10 μL of pure MeOH, which did not change the peak position and shape of the thermogram significantly (data not shown). The method of adding small aliquots of methanolic solutions was chosen over the method of mixing all components in $CHCl_3$ and then evaporating the solvent, as it was found that the terpenoids evaporated during the removal of the $CHCl_3$.

Electron Paramagnetic Resonance Spectroscopy. The EPR spectra were recorded on a Buker EMX spectrometer (Bruker, Rheinstetten, Germany) equipped with a Eurotherm VT-200 temperature controller. Samples for EPR were prepared by mixing DMPC and the appropriate spin label in $CHCl_3$. The solvent was then removed with a gentle stream of N_2, whereafter the samples were placed under reduced pressure overnight. The resulting thin lipid film was then hydrated as for the DSC measurements. For measurements, the samples where placed in sealed hematocrit tubes and placed at 4 °C overnight, leading to a small pellet of lipid in the bottom of the tube. The hematocrit tubes were then placed in a standard 4 mm o.d. EPR quartz tube, which was mounted in the spectrometer. The samples were allowed to equilibrate for 10 min at 35 °C before recording the spectra. The spectra were recorded in the X-band range (frequency ∼9.4 GHz) with a power of ∼2mW. The modulation frequency was 100 kHz with an amplitude of 1 G. The typical sweep time was 41 s. Usually five scans were summed in order to improve the signal-to-noise ratio.

The molecular order parameter, S, was calculated as[45]

$$S = 0.5407\frac{A_{\parallel} - A_{\perp}}{1/3(A_{\parallel} - 2A_{\perp})} \quad (4)$$

where $A_{\parallel}$ and $A_{\perp}$ are the hyperfine splittings measured from the EPR spectrum.

As the rotation of the Me-5-DSA spin label is anisotropic, two correlation times were needed, one parallel with the z-axis of the nitroxide (and for the Me-5-DSA parallel with the fatty-acid chains), $\tau_{\parallel}$, and one perpendicular to the z-axis, $\tau_{\perp}$. The correlation times can be found from the line intensity of the EPR spectrum:[46]

$$\tau_{\parallel} = \frac{2\tau_0\tau_{22}}{3\tau_0 - \tau_{22}} \quad (5)$$

$$\tau_{\perp} = \tau_0 \quad (6)$$

where

$$\tau_0 = 1.16 \times 10^{-9}(C - 0.0316B) \quad (7)$$

$$\tau_{22} = -0.437 \times 10^{-9}(B + 0.988C) \quad (8)$$

and B and C are found as

$$B = 0.5\Delta B_0(\sqrt{h_0/h_{+1}} - \sqrt{h_0/h_{-1}}) \quad (9)$$

$$C = 0.5\Delta B_0(\sqrt{h_0/h_{+1}} + \sqrt{h_0/h_{-1}} - 2) \quad (10)$$

where ΔB_0 is the peak width of the central line, h_0 is the height of the central line, h_1 is the height of the low-field line, and h_{-1} is the height of the high-field line.

■ ASSOCIATED CONTENT

Supporting Information

Thermograms of a DMPC membrane containing **2** and **3** are given in Figure S1. EPR spectra of Me-5-DSA in a pure DMPC membrane and a membrane with x = 0.15 of the different compounds are given in Figure S2. EPR spectra of the Me-16-DSA, 5-DSA, and CSL spin label in pure DMPC and in DMPC membranes with x = 0.15 of **1**−**3** are given in Figure S3. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ACKNOWLEDGMENTS

Dr. K. I. Pakkanen (Technical University of Denmark) is thanked for insightful comments on the manuscript. MEMPHYS−Center for Biomembrane Physics is supported by the Danish National Research Foundation.

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