Interaction of fluoxetine and lipid membranes: effect of cholesterol

NGUYEN QUAN TRINH, DAO NGOC PHUONG UYEN, NGUYEN THAO TRANG
School of Biotechnology, International University - Vietnam National University-HCMC, Vietnam
Email: ntrang@hcmiu.edu.vn

Abstract: In this study, the influence of cholesterol on the interaction between fluoxetine, a SSRI antidepressant, and 1, 2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) large unilamellar vesicles (LUVs) was investigated using FTIR to provide insights into the drug’s interaction with lipid membrane. Infrared spectroscopic bands assigned to the CH$_2$ stretching and PO$_2^-$ stretching vibrations revealed that fluoxetine at a concentration of 1.14 mM influences both nonpolar and polar parts of DOPC bilayers. The conformational changes, in both phosphate and alkyl chain regions, of the pure DOPC and DOPC containing 10 mol% cholesterol LUVs upon the introduction of fluoxetine were observed. However, with 20 mol% and 28 mol% cholesterol incorporated into the DOPC LUVs, the lipid conformations remain unchanged in the presence of fluoxetine. This suggests that cholesterol at higher concentration, suppresses the partitioning of fluoxetine into DOPC bilayers. These results showed that the interactions of fluoxetine and lipid membrane play a significant role in the drug partitioning and this, in turn, could affect the drug action.

Keywords: Antidepressant, Fluoxetine, FTIR, Lipid Conformation, Cholesterol

Introduction:
The selective serotonin reuptake inhibitors (SSRIs) are the newest antidepressants in trade that have fewer side effects than older antidepressants such as tricyclic antidepressants (TCA) and monoamine oxidase inhibitors (MAOIs)[1]. Although mechanisms of SSRIs which they exert the therapeutic effects are poorly understood, generally they are the first line of therapy [2]. SSRIs decrease symptoms of depression by blocking the reabsorption of serotonin by certain nerve cells in the brain [3]. This leads to increases of serotonin concentration in synapses, thereby facilitating serotonergic neurotransmission. Fluoxetine (Prozac), an antidepressant of SSRIs class, is one of the latest of the new generation [4]. In medical aspect, the drug treats not only depression, obsessive – convulsive disorder, bulimia nervosa but also many other disorders. Nowadays, fluoxetine is commonly used in the world because of their acceptable side effects to the depressed patients compared to the other antidepressants. Fluoxetine can be considered an amphipathic molecule with pK$_a$ of 10.1 [5], at physiological pH, exist a polar head and a nonpolar body. It is well documented that fluoxetine suppresses the activity of the TREK-1 potassium channel which is embedded in cell membranes [6]. TREK-1 activity has been implicated in mood regulation and is proposed to be a therapeutic target of fluoxetine, which has been shown to inhibit the channel activity [7-9]. The C-terminal of TREK-1 channels plays a role in the mechanosensitivity of the channels. When the C-terminal tail is fully bound to the plasma membrane, the TREK-1 potassium channel opens more; when the tail is unbound from the plasma membrane, the ion channel closes[10]. Fluoxetine inhibits TREK-1 by cutting off the C-terminal domain of the channel, resulting in inhibition of TREK-1 channel activity [11]. A highly membrane permeability is associated with amphipathic drugs, which makes the interactions of the drug with biomembranes[12, 13]. Fluoxetine partitions into the cell membrane. Given its chemical and functional features essential for its interaction with cell membrane components (i.e. phospholipids), such as a protonable nitrogen atom, a hydrogen bond group and a relatively high hydrophobicity, fluoxetine could be able to incorporate into and alter the membrane structure, which in turn affects the protein function and efficacy of the drug as well[14]. Thus, it is of great importance to study the interaction of fluoxetine and phospholipids bilayer in order to fully understand the drug’s behavior and mode of action.

Major constituents of membranes surrounding mammalian cells are phospholipids and cholesterol. The major component of phospholipid is comprised of a polar head group and two non-polar acyl chains as a tail. Based on the hydrophilic head groups, phospholipids are further divided into different groups including phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine[15]. Phosphatidylcholines (PC), which is electrically neutral incorporated choline as a head group, is more common than others [16]. In addition, the two acyl chains may be saturated, unsaturated or one chain saturated and the other unsaturated. In this study, di-unsaturated 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) (Fig.1) is used. This phospholipid is applied as the mimicking mammalian cell membrane environment in many researches investigating drug-lipid interaction [17-19]. Beside of phospholipid components in biomembranes, cholesterol (Fig.1) is the significant molecule in regulating the membrane...
parameters [20]. The typical concentrations of cholesterol are around 20-30 mol% and up to 50 mol% in red blood cells [21] and about 70 mol% in the ocular lens membranes [22]. This compound has a hydrophilic hydroxyl group that interacts with the hydrophilic head group of phospholipid; also, the bulky steroid group interacts with the hydrophobic acyl chains of lipids. These interactions influence the fluidity and packing of the lipid membrane [15]. Therefore, cholesterol modulates membrane permeability and fluidity by changing their available area and formation of domains of composition [20]. Cholesterol effects to the lipid membranes by fluidizing which makes lipid bilayers from the liquid-crystalline state becomes more ordered [23]; also, the concentration of cholesterol in lipid bilayers dictates the function it performs in the membranes [24]. Raffy et al.[25] and Shi-ichiro et al.[26] reported cholesterol could occupy empty space causing the structural changes that occur within the lipid bilayers, preventing drug molecules from partitioning into the lipid membrane. At molecular level, when increasing cholesterol content, the effect of increased head group spacing was pronounced [24]. At low concentration of cholesterol (≤ 10 mol%) and at the temperature above the chain melting temperature of phospholipids, in mixtures of saturated phosphatidylycerines and cholesterol such as DMPC/cholesterol and DPPC/cholesterol, the liquid-crystalline phase is characterized by a large increase in the average orientational order of the lipid chains and an increase in head group spacing with increasing cholesterol concentration in this range [27]. In animal cell membranes with typical concentrations of around 20-30 mol%, cholesterol was found to reduce the rate of trans/gauche isomerizations in bilayers [28] and decrease the orientational order [29]. In the study of saturated phosphatidylycine-cholesterol interaction, cholesterol strongly orders the acyl chains, the addition of cholesterol leads to considerable ordering of the PC acyl chains [30]. After this point, the interaction between phospholipids and cholesterol clearly affects the lipid conformation, leading to an influence on the lipid-drug interaction.

It is known that Fourier-transform infrared (FTIR) spectroscopy is very useful in probing the structure and dynamics of biological molecules with a rapidly expanding area [31]. This technique is especially powerful for detecting conformational changes by recording spectral differences in lipid bilayer [32-34]. In the investigation of Lichtenberger et al. [35], FTIR spectra clearly indicated significant vibrational changes of PC in the presence of nonsteroidal anti-inflammatory drugs. Also, the IR result demonstrated the trans/gauche rotamer content of lipid hydrocarbon chains and the change in the polar headgroups in a study of thermotropic phase behavior and organization of anionic phospholipid tetramyristoyl diphasphatidylglycerol of Lewis and partners [36]. There is no systematic research on fluoxetine-lipid interactions using Fourier transform infrared (FTIR) spectroscopy. The first and up-to-date techniques for study on fluoxetine-lipid interaction are different scanning calorimetry (DSC) and spin labeling EPR, which has been used by Momo and coworkers in 2005 [14]. It is proved that fluoxetine is a perturbing agent, able to interact strongly with the negatively charged phosphate group of the multilamellar liposomes composed of phosphatidylycholines, indicating a possible accumulation near the interfacial region, leading to destabilizing the membrane integrity. In the present study, the influence of cholesterol at different concentrations, 10 mol%, 20 mol% and 28 mol%, on the conformational changes of the phospholipid bilayer, 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), induced by the partitioning of fluoxetine was investigated using Fourier transform infrared spectroscopy.

**Figure 1: Molecular structure of fluoxetine, 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and cholesterol**
Interaction of fluoxetine and lipid membranes: effect of cholesterol

Materials and Methods

1. Materials
Fluoxetine hydrochloride was purchased from Sigma Aldrich (USA). 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) dissolved in chloroform was obtained from Avanti Polar Lipids (Alabaster, AL, USA) at 20 mg/ml and was used without further purification. Cholesterol was from Sigma Aldrich (USA). HEPES buffer (10 mM (2-hydroxyethyl)-1-piperazine-ethanesulfonic acid) (Sigma Aldrich, USA), 50 mM NaCl was adjusted to a pH of 7.4.

2. Methods
2.1. Preparation of Liposomes
Cholesterol was dissolved in chloroform at stock solution of 5 mg/ml. Chloroform solution of DOPC/Cholesterol compositions in molar ratios were prepared (100/0 mol/mol (0 mol% cho), 90/10 mol/mol (10 mol% cho), 80/20 mol/mol (20 mol% cho), 72/28 mol/mol (28 mol% cho) in different 1.5mL vials. The residual organic solvent was gently evaporated under a stream of nitrogen and kept in a vacuum chamber for at least 8h at room temperature. The dried lipid film was hydrated in HEPES buffer. The sonication was applied above the phase transition temperature (Tm = -17°C) to form multimamellar vesicles (MLVs). Lipid suspensions were extruded 30 cycles through polycarbonate membrane 0.1µm pore size in order to form large unilamellar vesicles (LUVs).

2.2. Preparation of Drug-Liposome Environments:
Stock solution of fluoxetine was prepared at 10 mg/mL in HEPES buffer. 1.14 mM fluoxetine was added into LUVs composed of pure DOPC and DOPC containing different cholesterol concentrations: 10 mol%, 20 mol%, 28 mol%. The final concentration of pure DOPC and DOPC/Chol in the suspensions was 2.5 mM. All suspensions were vortexed for 5 minutes followed by incubated in 30 minutes at room temperature.

2.3. FTIR Measurements:
The IR spectra were collected on a Bruker Tensor 27 FTIR spectrometer. The scan range of 4000-1000 cm⁻¹ was used with a 64 scan-average and a spectral resolution of 2 cm⁻¹. The wavenumber accuracy is better than 0.01 cm⁻¹ at 2000 cm⁻¹ (Bruker Optics TENSOR 27 FT-IR).The lipid solutions were measured on surface of a Zn-Se crystal (Pike Tech., USA). All experiments were carried out at room temperature (25°C).
IR spectra of the pure DOPC and DOPC containing cholesterol suspensions were collected in the absence and presence of fluoxetine. All spectra were obtained after subtraction of the background spectra measured with the HEPES buffer. The IR signal of CH₂ stretching frequency regions (2800-3000 cm⁻¹) of the acyl chain and the PO₂ stretching bands (1000-1300 cm⁻¹) of the lipid headgroups were collected. PeakFit version 4.12 software was used to find the component peaks from the spectrum. Data fitting was carried out in which the IR spectrum was then deconvoluted into component peaks using Gaussian mathematical function [37, 38].

Results and Discussion
It is well known that cholesterol plays a key role in biomembrane which in turn is thought to modify the membrane fusion properties [2]. Cholesterol is incorporated in the lipid bilayer at temperatures above the lipid chain-melting transition causing a large increase in the average orientational order of the tail region and increased head group spacing [39]. Cholesterol molecules insert themselves in the membrane with the same orientation as the phospholipid molecules[40]. The polar head (hydrophilic group) of cholesterol is aligned with the polar head of the phospholipids, and, the hydrophobic region of cholesterol binds to hydrophobic acyl chains of lipids. These cause the lipid conformation changes in the presence of high cholesterol concentration [41, 42]. In this study, 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) was used as a model liposome in investigating the influence of cholesterol on the conformational changes of the lipid induced by the partitioning of fluoxetine. DOPC exists in the liquid-crystalline state [43] at room temperature (Tm = -17°C). When cholesterol was mixed with DOPC liposomes, it was embedded in the bilayer phospholipid easily [44]. The liquid phase at high cholesterol concentrations has been denoted a liquid ordered phase, demonstrating a high degree of the acyl chain order in this lamellar liquid crystalline phase [45]. In addition, cholesterol could occupy empty spaces, causing an increase the packing of phospholipids. Thus, when increasing concentration of cholesterol in lipid bilayers, it could reduce permeability of cell membrane and prevent drug molecules from partitioning into the lipid membrane.

1. The changes of lipid conformation in the presence of cholesterol
1.1. CH₂ stretching vibration in DOPC liposome and DOPC/Cholesterol (DOPC/Chol) liposome
The frequency of the methylene stretching vibration ν(CH₂) provides a sensitive qualitative measure of the conformational order of the lipid acyl chains [46-49]. The frequencies of CH₂ symmetric (νs,CH₂) and asymmetric stretch (νas,CH₂) are around 2850 cm⁻¹ and 2920 cm⁻¹, respectively, upon a transition of the lipid from the ordered gel to the disordered liquid crystalline phase [31, 32]. These wavenumbers typically respond to changes of the trans/gauche isomerization [50]. In the investigation of lipid conformation changes in the presence of cholesterol, the IR signal was measured in order to illustrate the changes of gauche conformer of acyl chains and

vibration of polar headgroup regions of both DOPC liposomes and DOPC/Cholesterol liposomes. For pure DOPC liposomes, the data fitting result revealed the frequency of CH₂ symmetric (v_s(CH₂)) and asymmetric stretch (v_a(CH₂)) were centered at 2855 cm⁻¹ and 2924 cm⁻¹, respectively (Fig. 2). In the presence of 10 mol% of cholesterol, the wavenumber shifted to the lower position, 2854 cm⁻¹ for v_s(CH₂) and had no change for v_a(CH₂), which remained at 2924 cm⁻¹ (Table 1). No further change in the presence of cholesterol at 20 mol% and 28 mol% (Table 1) was observed. In a study of Cong et al., 2009, it was reported that the IR frequency precision for their instrument is better than 0.1 cm⁻¹ at 2000 cm⁻¹. They claimed that the shifts of 0.3 cm⁻¹ in the CH₂ stretching vibrational band was significant. Thus any shift of 1 cm⁻¹ or above in this study was significant with the wavenumber accuracy of 0.01 cm⁻¹ at 2000 cm⁻¹. The decrease in the frequency of CH₂ stretching observed from the pure DOPC liposomes to DOPC/Chol denotes a decrease in gauche conformers of the lipid’s acyl chains when cholesterol was presented. This could be explained by the ordering effect of cholesterol on the lipid acyl chains. The ordering effect occurs when the cholesterol induced increased ordering (as compared to pure liposomes) of the hydrocarbon acyl chains, which is one of the generic effects cholesterol has on a lipid bilayer in the physiologically relevant liquid-crystalline phase [39]. Hence, it causes a decrease in gauche conformations of the CH₂ chains. This behavior was observed at 10 mol% cholesterol in DOPC liposomes without further change at higher concentration of cholesterol, suggesting that 10 mol% cholesterol in DOPC liposomes could be enough to induce the order of the lipid acyl chains. All these experiments were performed without any interaction with foreign molecules, demonstrating that the changes in lipid conformations were solely due the presence of cholesterol in DOPC liposomes. Therefore, the presence of cholesterol in DOPC liposomes prevents the crystallization of the lipid’s hydrocarbons, making the lipid’s acyl chains more ordered. This could suppress the partitioning of fluoxetine into the liposomes.

1.2. The phosphate headgroup region in DOPC liposome and DOPC/Chol liposomes

The change of lipid conformations was also observed at the phosphate head-group vibrational mode. The phosphate moiety of the head-group gives rise to several strong vibrations in the infrared frequency [31]. The spectral characteristics of the anti-symmetric and symmetric PO₄ stretching vibrations are very sensitive to hydrogen bonding and van der Waals forces [31]. In DOPC, asymmetric and symmetric stretching modes for the PO₄ group are found near 1228 cm⁻¹ and 1085 cm⁻¹, respectively [50]. These vibrations depend on the “free” phosphate in polar head-groups. The more “free” the phosphate ions, the more vibrational the molecules.

In this study, the frequency of symmetric PO₄ (v_s(PO₄)) stretching was centered at 1086 cm⁻¹ for pure DOPC liposomes and DOPC containing cholesterol liposomes. However, the IR signal of the asymmetric PO₄ (v_a(PO₄)) stretching were completely different for pure DOPC and DOPC/Chol liposomes. Particularly, the wavenumber of v_a(PO₄) stretching in pure DOPC liposomes was located at 1228 cm⁻¹ (Fig. 2). In the presence of 10 and 20 mol% cholesterol in DOPC liposomes, the v_a(PO₄) stretching shifted to a lower position, at 1227 cm⁻¹ (Table 1). This v_a(PO₄) stretching was decreased to 1226 cm⁻¹ for DOPC with 28 mol% cholesterol (Fig. 2). These results illustrated the vibration of PO₄ of the polar headgroup decreases with the increase in the cholesterol level incorporated in the liposomes. In the headgroup of DOPC, there exists an attraction force between the negatively charged phosphate and the positively charged amine N(CH₃)₃⁺ of the choline group, decreasing the “free” phosphate ions. Moreover, the insertion of cholesterol into the lipid bilayers also lower “free” phosphate ions by hydrogen bond formation between hydroxyl group of cholesterol and the PO₄ polar headgroup of DOPC. Therefore, higher concentration of cholesterol further decreases the “free” phosphate ions, causing the lower frequency of v_a(PO₄).

2. Effect of cholesterol on DOPC conformation in the presence of 1.14 mM fluoxetine

2.1. CH₂ stretching vibration in fluoxetine/DOPC liposome and fluoxetine/DOPC/Chol liposomes

In the presence of fluoxetine, the v(CH₂) of pure DOPC liposomes shifted to higher frequency, specifically from 2855 cm⁻¹ to 2856 cm⁻¹ (Fig. 2, 3) and from 2924 cm⁻¹ to 2925 cm⁻¹ in the v(CH₂) (Fig. 2, 3). These higher frequency shifts indicated an increase of gauche conformers, i.e. a disordering of the lipid acyl chains induced by fluoxetine. This behavior was also observed in an EPR study that the partitioning of fluoxetine into the lipid caused the disordered motion of lipid’s acyl chains [14].

The v(CH₂) stretching mode was not sensitive to the incorporation of cholesterol into the liposomes (Table 1) whereas the v(CH₂) exhibited a frequency shift with presence of cholesterol. In the 10 mol% chol/DOPC, the v(CH₂) shifted to lower frequency by 2 cm⁻¹, at 2854 cm⁻¹ (Table 1) in the presence of fluoxetine. The ordering effect of cholesterol exerts on the liquid-crystalline state of DOPC, causing a decrease in the gauche conformers as illustrated by this lower frequency of v(CH₂). No further shift was observed at higher mol% chol/DOPC liposomes.

It is interesting that the v(CH₂) observed in DOPC/Chol at all cholesterol content was centered at 2854 cm⁻¹ regardless the presence or absence of fluoxetine. This suggested that the ordering effect of cholesterol on the lipid acyl chains inhibits the
partitioning of fluoxetine into the liposomes. In addition, the presence of cholesterol in the DOPC liposomes could occupy the empty spaces, further prevents the drug molecules from deeper penetration into the lipid chains. As a consequence, no difference in the $v_\text{CH}_2$ band was obtained in the DOPC/Chol and in the fluoxetine/DOPC/Chol.

2.2. The phosphate headgroup region in fluoxetine/DOPC liposome and fluoxetine/DOPC/Chol liposomes

The frequency of the symmetric PO$_2^-$ ($v_\text{PO}_2^-$) stretching was the same, at 1086 cm$^{-1}$, for pure DOPC liposome and DOPC containing different mol% cholesterol upon the addition of 1.14 mM fluoxetine. The frequency changes of asymmetric PO$_2^-$ ($v_{\text{as} \text{PO}_2^-}$) stretching between pure DOPC liposomes and DOPC/Chol liposomes had a blue shift for both pure DOPC liposomes and DOPC/Chol liposomes. In particularly, the wavenumber of $v_{\text{as} \text{PO}_2^-}$ stretching in fluoxetine/pure DOPC liposomes increased 4 cm$^{-1}$, from 1228 cm$^{-1}$ to 1232 cm$^{-1}$ (Fig. 2, 3). The changes of the wavenumber in the interaction of fluoxetine with DOPC/10 mol% Chol and 20 mol% Chol were the same; $v_{\text{as} \text{PO}_2^-}$ stretching shifted to 4 cm$^{-1}$, from 1227 cm$^{-1}$ without drug to 1231 cm$^{-1}$ with drug (Table 1). In the presence of 28 mol% Chol, the $v_{\text{as} \text{PO}_2^-}$ frequency moved from 1226 cm$^{-1}$ to 1230 cm$^{-1}$ (Table 1). The molecular structures of DOPC and fluoxetine could be utilized in order to explain for these changes. The N$^\text{+}$(CH$_3$)$_2$ of the polar headgroup of DOPC prevents the positively charged part of fluoxetine from getting close to PO$_2^-$ region because of electrostatic repulsive force between the same charge species. This leads to the phosphate ions in DOPC becoming more freely, leading to more vibration (evidenced by an increase in the $v_{\text{as} \text{PO}_2^-}$ frequency). A possible explanation for this behavior is that fluoxetine could push cholesterol deeper in to the hydrophobic acyl chains of DOPC. This breaks down the hydrogen bond between OH group of cholesterol and PO$_2^-$ of DOPC, resulting more “free” phosphate ions (illustrated by an increase in the frequency of the $v_{\text{as} \text{PO}_2^-}$). In addition, observing the frequency changes in the effect of cholesterol in pure DOPC and fluoxetine/DOPC mixture, these changes of the phosphate headgroup region indicated the vibration induced by the presence of cholesterol. The “free” phosphate ions started decreasing with increasing the cholesterol contents, from 0 mol% to 10 mol% which was similarity to 20 mol% cholesterol continuing dropping at 28 mol% (Table 1). This supported the more pronounced ordering effect of cholesterol on the lipid acyl chain in the presence of drug as compared to ordering effect of cholesterol on the lipid acyl chain without drug.

Conclusion:

In this study, the effect of cholesterol in the interaction of fluoxetine and 1,2-dioleoyl-sn-glycerol-3-phosphocholine (DOPC) liposomes was characterized by the lipid conformational changes. Fluoxetine was shown to be able to partition into DOPC bilayer and caused the change in the lipid conformation. Furthermore, the ordering effect caused the structural changes at both the acyl chains and phosphate regions. That was found that the ordering effect by 10 mol% cholesterol introduced into DOPC liposomes reduced the partitioning of fluoxetine into the liposomes. Increasing the cholesterol content in the liposome showed no further conformational change in the DOPC liposomes. These results revealed the crucial role of cholesterol in drug partitioning into the lipid membrane.

Acknowledgement

This research is funded by Vietnam National Foundation for Science and Technology Development (NAFOSTED) under grant number 106.03-212.56.

<table>
<thead>
<tr>
<th>Lipid composition</th>
<th>Without fluoxetine</th>
<th>With fluoxetine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CH$_2$ stretching</td>
<td>phosphate regions</td>
</tr>
<tr>
<td></td>
<td>$v_\text{CH}_2$</td>
<td>$v_\text{CH}_2$</td>
</tr>
<tr>
<td></td>
<td>(cm$^{-1}$)</td>
<td>(cm$^{-1}$)</td>
</tr>
<tr>
<td>DOPC/0 mol% Chol</td>
<td>2924</td>
<td>2855</td>
</tr>
<tr>
<td>DOPC/10 mol% Chol</td>
<td>2924</td>
<td>2854</td>
</tr>
<tr>
<td>DOPC/20 mol% Chol</td>
<td>2924</td>
<td>2854</td>
</tr>
<tr>
<td>DOPC/28 mol% Chol</td>
<td>2924</td>
<td>2854</td>
</tr>
</tbody>
</table>

**Table 1.** The change in the frequency of CH$_2$ stretching and PO$_2^-$ stretching of pure DOPC and DOPC containing several different concentration of cholesterol with and without fluoxetine.
Figure 2: IR spectra of DOPC liposomes in the presence of cholesterol contents

Figure 3: IR spectra of DOPC liposomes in the presence of cholesterol contents with fluoxetine.

References
Interaction of fluoxetine and lipid membranes: effect of cholesterol

[36] Lewis RNAH, Zweyick D, Pabst G, Lohner K, McElhaney RN. Calorimetric, X-Ray Diffraction, and Spectroscopic Studies of the Thermotropic Phase Behavior and Organization of Tetramyristoyl...


