What Happens if Cholesterol Is Made Smoother: Importance of Methyl Substituents in Cholesterol Ring Structure on Phosphatidylcholine–Sterol Interaction

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ABSTRACT Although sterols constitute one of the most important molecular species in cells, the reasons for their structure-function relationships in lipid membranes are not well understood. The main objective of this work is to elucidate the recently suggested possibility that the ordering and condensing effects of sterols on phospholipid membranes are related to the smoothness of a sterol. We focus on cholesterol, which has two methyl groups attached to its β-face, and compare its properties to those of demethylated cholesterol (Dchol), from which the two methyl groups have been removed. Atomic-scale molecular dynamics simulations of lipid membranes comprised of saturated lipids and sterols, either cholesterol or Dchol, provide compelling evidence that despite its smoother structure, the ordering and condensing effects of Dchol are less effective than those of cholesterol. The ordering capability of both cholesterol and Dchol is highly asymmetric with respect to their ring structure, but whereas cholesterol favors the α-face, Dchol favors the β-face. The origin and implications of this difference are analyzed in detail. The picture that emerges from this study supports a view that the two methyl groups at the steroid ring system of cholesterol play an important role in cholesterol-lipid interactions by reducing sterol tilt in the bilayer and hence allowing for an optimal orientation for cholesterol.

INTRODUCTION

Sterols constitute a large family of chemical species. Yet only cholesterol and ergosterol are found in substantial amounts in nature, cholesterol in animal and ergosterol in fungus membranes. Cholesterol concentration in cell membranes is usually ~30 mol %, although in red blood cells it may reach 50 mol % (1), and in ocular lens membranes even 70 mol % (2). Animal cells that are not able to synthesize cholesterol can grow in a cell culture only when the medium contains cholesterol; other sterols cannot be substituted for cholesterol (3). Of the prokaryotes, only *Mycoplasma*, a source of non-bacterial pneumonia, contains cholesterol in its plasma membrane (4). This cholesterol is, however, taken up from the host cell. To grow in a cell culture, *Mycoplasma* cells require cholesterol to be present in the medium, although cholesterol analogs such as 3β-methylcholesterol, lanosterol, and cholestanol may be substituted for cholesterol. However, these steroids require a higher concentration in the medium (5,6).

The cholesterol molecule consists of a planar tetracyclic ring system with the 3β-hydroxyl (OH-Chol) group and a short eight-carbon chain (*iso*-octyl tail) attached to C_{17} (Fig. 1). The ring system is asymmetric about the ring plane and has a flat side with no substituents (α-face) as well as a rough side with two methyl groups (β-face). The three-dimensional structure of cholesterol showing the two faces and their relative roughness is depicted in Fig. 2. As a smooth and rigid molecule, cholesterol is known to increase the order of saturated acyl chains of phospholipids (the ordering effect) (7,8) and the membrane surface density (the condensing effect) (9,10). A variety of cholesterol analogs, on the other hand, have been reported to have a much weaker effect on membrane ordering and condensation. This may sound surprising because the molecular structures of sterols such as lanosterol (11), epicholesterol (12–14), oxygenated sterols (15), cholesterol sulfate (16), desmosterol (17), or selected plant sterols (18) differ remarkably little from the structure of cholesterol.

The role of cholesterol, and that of some other sterols, has recently been discussed in the context of lipid rafts. Of the many sterols available, it is genuinely fascinating that cholesterol seems to be the sterol that drives the formation of highly ordered membrane domains: it is typically considered to be a necessary ingredient in rafts (19). It has also been shown that surprisingly small changes in the structure of cholesterol affect a sterol's ability to promote raft formation. Desmosterol, which differs from cholesterol only by an additional double bond in the *iso*-octyl tail, is a prime example of that (17). The ability to promote raft formation and a sterol's ability to enhance the ordering of lipid acyl chains have recently been related to the tilt angle that the sterol adopts in a bilayer (20). As another example, lanosterol, which has three additional methyl substituents and is the first sterol on the cholesterol biosynthetic pathway, is also not able to induce the formation of rafts (21).

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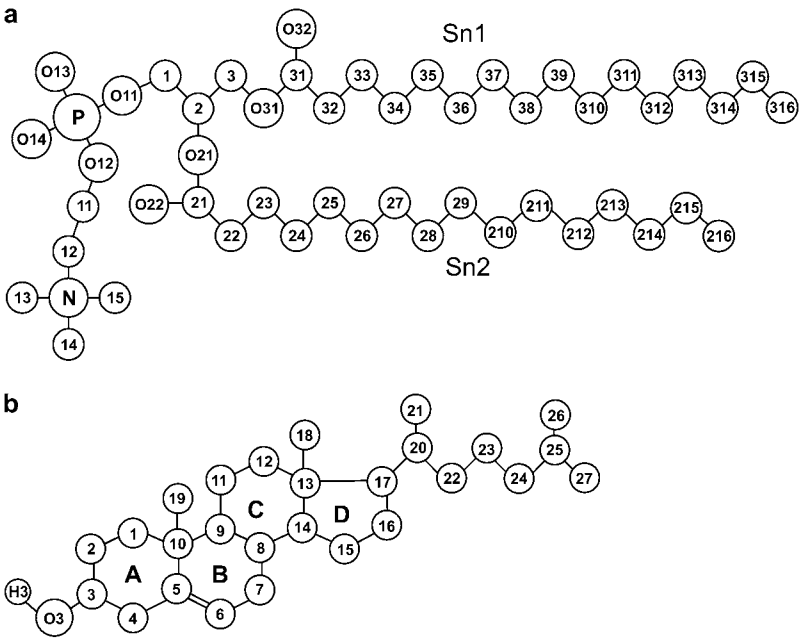

FIGURE 1 Molecular structures of (*a*) DPPC and (*b*) cholesterol molecules with numbering of atoms. The cholesterol rings are labeled A, B, C, and D. The chemical symbol for carbon atoms, C, is omitted. In DPPC, the upper acyl chain is *sn*-1, and the lower one *sn*-2. In demethylated cholesterol (Dchol), the methyl groups C_{18} and C_{19} have been removed.

The above observations suggest that during the evolutionary process the structure of cholesterol has been optimized such that its interactions with membrane lipids have become very effective. That has been achieved by the removal of methyl groups from the α-face to optimize the sterol ring structure (22–24). Thus, the relatively smooth molecular structure of cholesterol seems to be unique and optimal for its biological membrane functions (25–27).

In our previous studies of phosphatidylcholine–cholesterol interactions, we have elucidated the influence of cholesterol on a variety of properties such as the extent of interlipid links at the membrane/water interface (28), ordering of hydrocarbon chains (29,30), membrane condensation (30–32), and free area and volume within a membrane (33–35). In particular, the results of these atomic-scale molecular dynamics (MD) simulation studies have shown that the order of the acyl chains neighboring the rough β-face of a cholesterol molecule is lower than the order of chains neighboring the flat α-face (29). The studies have further indicated that atomic packing around the β-face is less tight than that around the α-face (32), implying weaker van der Waals interactions of the acyl chains with the cholesterol β-face than with the α-face. This result agrees well with the experimental observation that lanosterol affects membrane order less than cholesterol (36–39). Both experimental and computational results have led to the conclusion that the presence of the methyl groups decreases interactions between the acyl chains and sterol molecules.

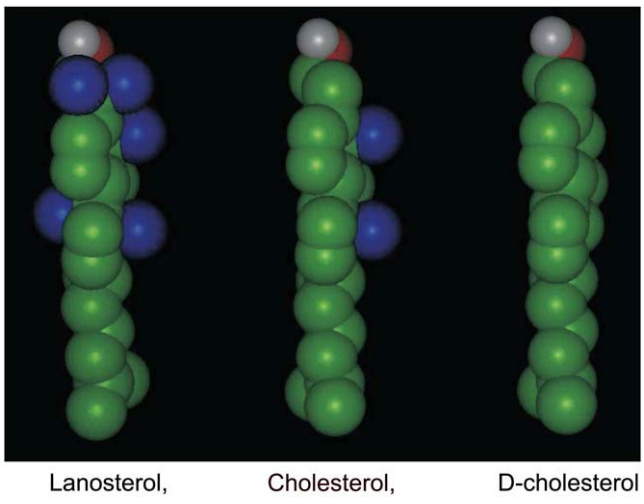

FIGURE 2 Three-dimensional structures of lanosterol, cholesterol, and demethylated cholesterol.

The above observations raise an interesting question concerning the optimal structure of cholesterol: why were the methyl groups not removed from the β-face, too? To address this question, we constructed lipid bilayers comprised of phosphatidylcholine molecules and modified cholesterol with methyl groups C_{18} and C_{19} removed (see Fig. 1 and Fig. 2). The resulting molecule is called the demethylated cholesterol (Dchol). Although such a molecule does not exist in nature, it provides us with an interesting model to clarify the role of the methyl groups in the β-face of cholesterol and hence allows us to better understand the unique properties of cholesterol.

METHODS

System description and parameters

We have performed atomic-scale MD simulations of three different membrane systems. The first bilayer was composed of 128 dipalmitoylphosphatidylcholine (DPPC) molecules, the second of 128 DPPC and 32

cholesterol (Chol) molecules, and the third of 128 DPPCs and 32 Dchols (cf. Fig. 1). All three bilayers were hydrated with 3500 water molecules. The initial structures of all bilayers were obtained by arranging the DPPC molecules in a regular array in the bilayer (x,y) plane with an initial surface area of 0.64 nm^2 per DPPC molecule. An equal number of sterol molecules were inserted into each leaflet. Before the actual MD simulations, the steepest-descent algorithm was used to minimize the energy of the initial structure (40,41). The simulations were performed using the GROMACS software package (42). The MD simulations of all bilayer systems were carried out over 100 ns. The first 20 ns was considered as an equilibration period (30), and thus only the last 80 ns of the trajectory were analyzed. The choice of this criterion is consistent with our previous studies of cholesterol (30). Fig. 1 shows the structure, the numbering of atoms, and torsion angles in DPPC and sterol molecules. Fig. 2 illustrates the three-dimensional arrangement of atoms in Dchol, including pictures of lanosterol and cholesterol as well for the purpose of comparison.

We used standard united atom force-field parameters for DPPC molecules (43), where the partial charges were taken from the underlying model description (44). For water, we employed the SPC model (45). For the sterol force field, we used the description of Holtje et al. (46) including a correction to introduce the improper torsion at the C_{10} chiral center; the missing one to four pairs in cholesterol ring A (Fig. 1) were added.

Periodic boundary conditions with the usual minimum image convention were used in all three directions. The LINCS algorithm (47) was used to preserve the bond length in sterol hydroxyl group and SETTLE algorithm was used for water (48). The time step was set to 2 fs, and the simulations were carried out at constant pressure (1 atm) and temperature (323 K), which is above the main phase transition temperature of DPPC (49). The temperature and pressure were controlled using the Berendsen method (50) with relaxation times set to 0.6 and 1.0 ps, respectively. The temperatures of the solute and solvent were controlled independently. The pressure was controlled semi-isotropically. The Lennard-Jones interactions were cut off at 1.0 nm. For the electrostatic interactions we employed the particle-mesh Ewald method (51) with a real space cutoff of 1.0 nm, β-spline interpolation (of order 5), and direct sum tolerance of 10^{-6}. The list of nonbonded pairs was updated every 10 steps. The simulation protocol used in this study has been successfully applied in various MD simulation studies of lipid bilayers (17,30,33,34,41,52).

To compare the properties of Dchol with those of cholesterol, we have reanalyzed our previous data for DPPC-Chol bilayers (33). Those simulations have been conducted under similar conditions concerning the sterol concentration, temperature, and other model parameters, thus allowing us to treat the two sterol systems on equal footing.

Analysis

In the following discussion, we consider various quantities determined from the simulation data. Surface area/DPPC was calculated by dividing the total area of the membrane by 64 (number of DPPC molecules in a single leaflet). Membrane thickness was determined from mass density profiles by considering the points where the mass densities of lipids and water merge (41). The molecular order parameter (S_{mol}), described in detail elsewhere (29), provides essentially the same information as the commonly studied NMR order parameter S_{CD} (53). For the present saturated chains of DPPC, $S_{mol} = 2|-S_{CD}|$. To characterize the orientation of sterols in a bilayer, we calculated the tilt of a sterol defined as the angle between the C_3-C_{15} vector (cf. Fig. 1 *b*) and the bilayer normal. To calculate the tilt angles for the acyl chains of DPPC, we averaged over segmental vectors ≥ 4 (the nth segmental vector links carbon atoms $n - 1$ and $n + 1$ in the acyl chain) to obtain the average segmental vector. The tilt angle for a given acyl chain is then given by $\langle arccos(sqrt(cos^2\theta))\rangle$, where θ is the angle between the bilayer normal and the average segmental vector (54).

In averaging conformational quantities in terms of *gauche* and *trans* states, only torsion angles 4–16 (see Fig. 1) were taken into account because neither in pure DMPC nor in mixed bilayers are $\beta 3$ or $\gamma 3$ in well-defined, stable conformations (*trans* or *gauche*) (29). For the torsion angles $\beta 1$, $\beta 2$, $\beta 3$ and $\gamma 1$, $\gamma 2$, $\gamma 3$ in DPPC, DPPC-Chol, and DPPC-Dchol bilayers, both sterols were found to have a negligible effect on the torsion.

To analyze hydrogen bonding, water bridging, and charge pairing, we employed the same geometric definitions as in our previous articles (28,55,56). Charge pairing, which essentially describes the electrostatic interaction between a positively charged molecular moiety (such as a methyl group in PC choline) and a negatively charged one (such as an oxygen atom in the sterol OH-group), complements our studies for atomic-scale interaction mechanisms and is most useful in describing interactions in the head group region.

Standard errors given for all numerical values in the text below were estimated using block analysis described by Pasenkiewicz-Gierula et al. (57).

RESULTS

Area per molecule and membrane thickness

Time development of the potential energy (Fig. 3 *a*), temperature (Fig. 3 *b*), and surface area/DPPC (Fig. 3 *c*) of the DPPC-Dchol system was monitored throughout the 100-ns simulation. From the time profile of the surface area/DPPC

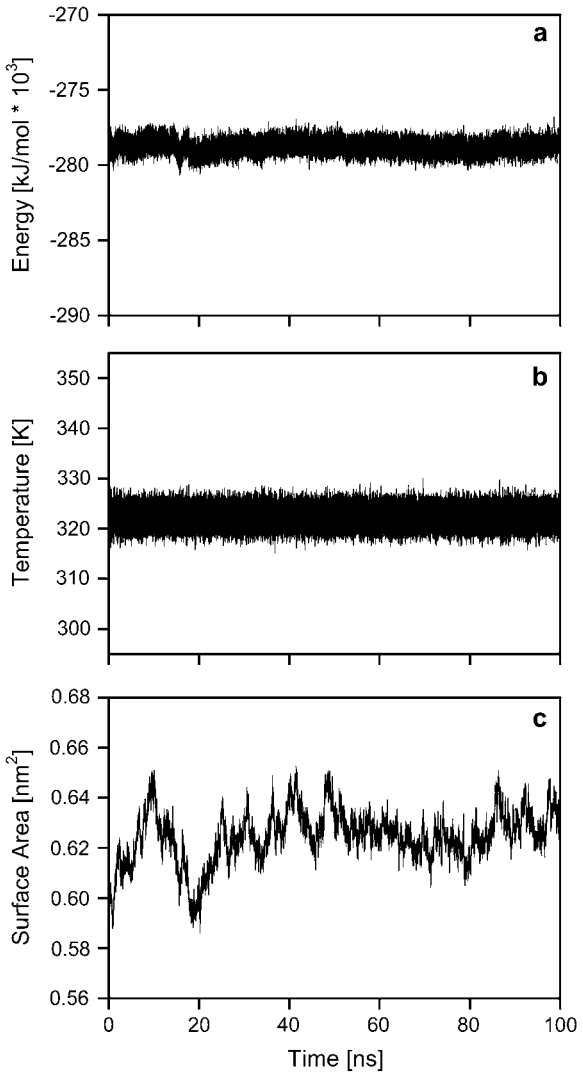

FIGURE 3 (*a*) potential energy, (*b*) temperature, and (*c*) surface area per DPPC in the DPPC-Dchol bilayer as a function of time.

(Fig. 3) and low value of drift comparing with standard deviation (2.7×10^{-7} versus 0.01 nm^2), it was reasoned that the system does not evolve and is stable over an 80-ns fragment of analyzed trajectory.

The surface area per lipid is easy to calculate in a single-component bilayer by dividing the total area of the bilayer by the number of lipids in a single leaflet. For binary mixtures and many-component systems, this is no longer obvious, as has been discussed in recent works (32,58). One approach used in binary mixtures of PC and sterol molecules is to compute the average area per PC by extracting the sterol area from the total area. However, that approach is based on an assumption that the average area of a sterol is fixed and does not depend on sterol concentration, the PC under study, and so forth. Furthermore, even if the close-packed cross-sectional area of a sterol could be estimated, it remains unclear how to partition the free area among the different molecules in a bilayer. With this in mind, it is not too surprising to find that there are varying estimates for the surface area occupied by cholesterol, values ranging from 0.39 to 0.41 nm^2 obtained through monolayer studies (59,60) to 0.22 nm^2 (61) and 0.27–0.29 nm^2 (58) deduced from MD simulations of mixed bilayers. For this reason, in this work we prefer to avoid this subtle issue by considering the total area divided by the number of DPPC molecules only (Table 1). For our purposes this is completely reasonable because our main objective is to compare the influence of Dchol and cholesterol on the membrane system. The surface areas given in Table 1 then show that the presence of either sterol leads to membrane condensation and that the effect of cholesterol is stronger than that of its demethylated analog. Decrease of surface area is associated with an increase of membrane thickness (Table 1) (see Fig. 4 *b* for an example). As Fig. 4 and Table 1 illustrate, the effect of cholesterol is stronger than that of Dchol.

Location and orientation of sterols in the bilayer

Fig. 4 shows the most relevant mass density profiles for the systems studied: whole DPPC molecules, the DPPC head group and glycerol backbone, the *sn*-1 and *sn*-2 chains, and water together with the partial density profiles of cholesterol and Dchol. For clarity, the density profiles of the sterol ring and tail atoms along the bilayer normal are shown separately for both bilayer leaflets in Fig. 4 *c*.

The density profiles indicate that cholesterol and Dchol have different influences on the ordering of lipid acyl chains (see below), which is reflected in differences in membrane thickness. On the other hand, the shapes of the profiles suggest that the orientations of cholesterol and Dchol are different as well. The average tilt of a sterol with respect to membrane normal shows that this is indeed the case: for cholesterol it was found to be 20°, whereas for Dchol it was considerably larger, ~25° (Table 1), the errors in both cases being less than 0.2°.

Although the orientations of cholesterol and Dchol differ from each other, Fig. 5 shows that they both reside at the membrane-water interface in a similar manner. This illustrates the density profiles of sterol oxygen and PC phosphate oxygen atoms (Op) along the bilayer normal. We find that, when the profiles are displayed in such a way that the different membrane thicknesses are accounted for, the OH groups of Chol and Dchol are at the same distance from the phosphate oxygen atoms.

Order and conformation of acyl chains

The subtle difference in the structure of Chol and Dchol leads to a rather profound difference in the ordering of DPPC acyl chains. This is illustrated by the molecular order parameter, S_{mol}, whose profiles along the *sn*-1 and *sn*-2 chains of DPPC are shown in Fig. 6. Mean values (averages over segments 4–16) of S_{mol} for the *sn*-1 and *sn*-2 chains are given in Table 1. Fig. 6 and Table 1 clearly highlight the stronger ordering

TABLE 1 Ordering and condensing effects of sterols

Membrane		DPPC	DPPC-Chol	DPPC-Dchol
Smol	*sn*-2	0.28 ± 0.01	0.55 ± 0.01	0.48 ± 0.01
	sn-1	0.29 ± 0.01	0.58 ± 0.01	0.51 ± 0.01
Tilt (°)	*sn*-2	23.8 ± 0.2	15.7 ± 0.2	17.0 ± 0.2
	sn-1	23.6 ± 0.2	16.0 ± 0.2	17.3 ± 0.2
	sterol	-	19.8 ± 0.2	25.3 ± 0.2
No. *gauche*	*sn*-2	3.0 ± 0.05	2.3 ± 0.05	2.5 ± 0.05
	sn-1	3.0 ± 0.05	2.3 ± 0.05	2.5 ± 0.05
Lifetime (ps)	*sn*-2	84 ± 4	115 ± 4	107 ± 4
	sn-1	87 ± 4	119 ± 4	111 ± 4
No. of neighbors	*sn*-2	32.4 ± 0.05	36.8 ± 0.05	37.4 ± 0.05
	sn-1	33.0 ± 0.05	37.2 ± 0.05	37.8 ± 0.05
Area/DPPC [nm^2]		0.660 ± 0.04	0.600 ± 0.04	0.624 ± 0.04
Thickness [nm]		3.92 ± 0.06	4.69 ± 0.06	4.39 ± 0.06

Average values of the molecular order parameter, S_{mol}, chain tilt angle, number of *gauche* per acyl chain, and lifetimes of *trans* conformations. All results are given separately for the *sn*-1 and *sn*-2 chains of DPPC. Also given here are the average surface area per DPPC and the membrane thickness of DPPC, DPPC-Chol, and DPPC-Dchol bilayers.

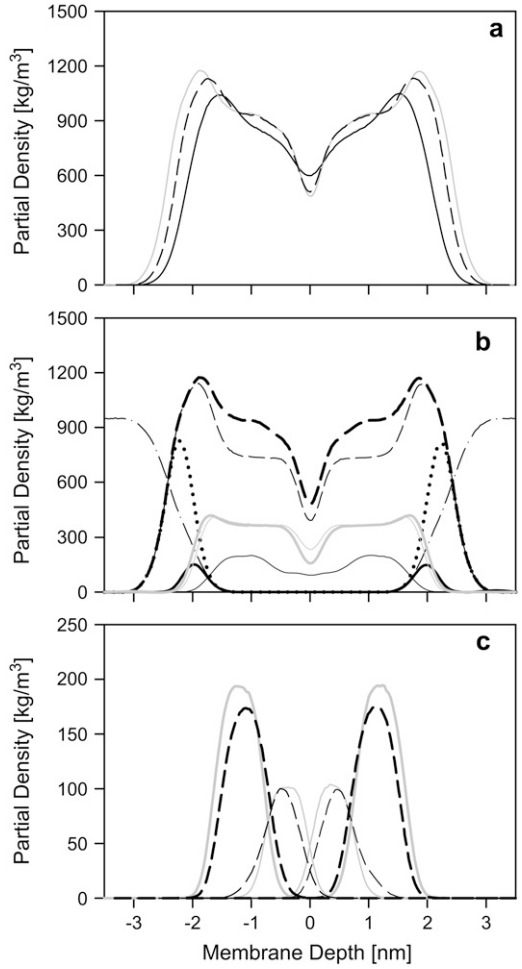

FIGURE 4 Partial density profiles along the bilayer normal. (*a*) All bilayer atoms in DPPC (*black line*), DPPC-Chol (*gray line*), and DPPC-Dchol (*dashed line*) bilayer. (*b*) In the DPPC-Chol bilayer: all bilayer atoms, DPPC (*dashed line*), DPPC head groups (*dotted line*), DPPC *sn*-2 (*gray-thin line*) and *sn*-1 chains (*gray line*), glycerol backbone (*black-thick line*), cholesterol (*black line*), and water (*dash-dot line*). (*c*) Sterol ring (*thick line*) and tail (*thin line*) atoms in DPPC-Chol (*gray line*) and DPPC-Dchol (*dashed line*) bilayers, separately for upper and lower leaflets. The coordinate $z = 0$ corresponds to membrane center.

effect of cholesterol, the difference to Dchol being ~14%. Nevertheless, both sterols increase S_{mol} of DPPC acyl chains at all depths in the membrane.

Distributions of the tilt angle of the *sn*-1 and *sn*-2 chains of DPPC are shown in Fig. 7, and the corresponding average values are given in Table 1. It is evident that cholesterol decreases the average tilt of both the *sn*-1 and *sn*-2 chains by ~8°. A similar effect is seen for Dchol with a reduction of ~7°.

As for isomerization and its dependence on membrane composition, we found that the differences between the average numbers of *gauche* states per chain in DPPC, DPPC-Chol, and DPPC-Dchol bilayers are small (Table 1). In all systems, the probability of being in the *gauche* state is ~0.30 and slightly larger at the end of the chain. The lifetime profiles of *gauche* and *trans* conformations, however, differ rather

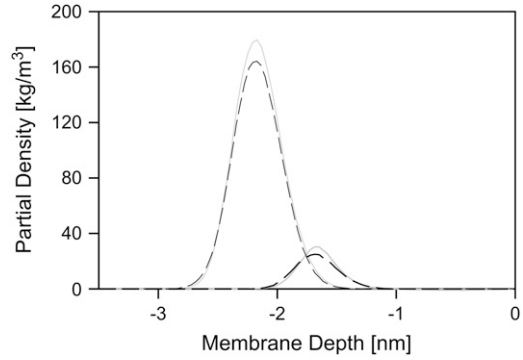

FIGURE 5 Profiles of the atom density of Op (*thin line*), and sterol OH group (*thick line*) in DPPC-Chol (*gray line*) and DPPC-Dchol (*dashed line*) bilayers.

markedly between the different systems (see Fig. 8 for *trans* conformation profiles and Table 1 for average lifetimes of the *trans* conformation). From those data, we can conclude that cholesterol is more effective in stabilizing the *trans* conformation than Dchol. The observed average lifetimes of *trans* and *gauche* conformations are shorter than in our previous studies (29), but this difference is likely to be related to the 13 K higher temperature in this work.

Packing of chains

Intermolecular two-dimensional radial distribution functions (RDFs) calculated for the centers of mass of the DPPC acyl

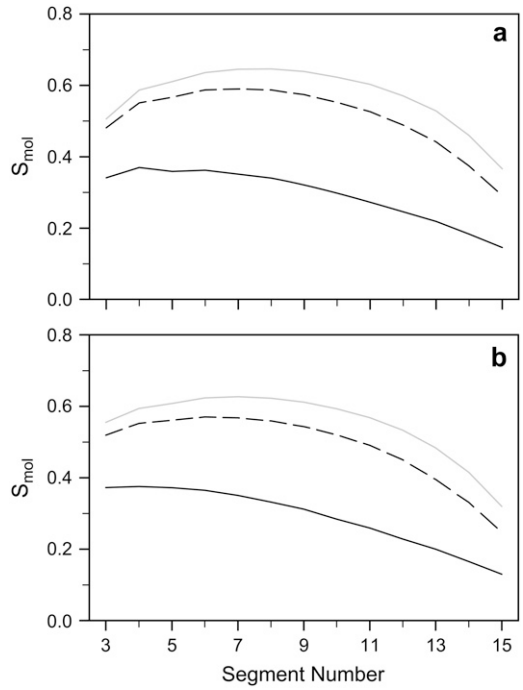

FIGURE 6 Profiles of the molecular order parameter (S_{mol}) calculated for (*a*) the DPPC *sn*-2 and (*b*) *sn*-1 chains in DPPC (*black line*), DPPC-Chol (*gray line*), and DPPC-Dchol (*dashed line*) bilayer. Small segment numbers correspond to carbons close to the glycerol group.

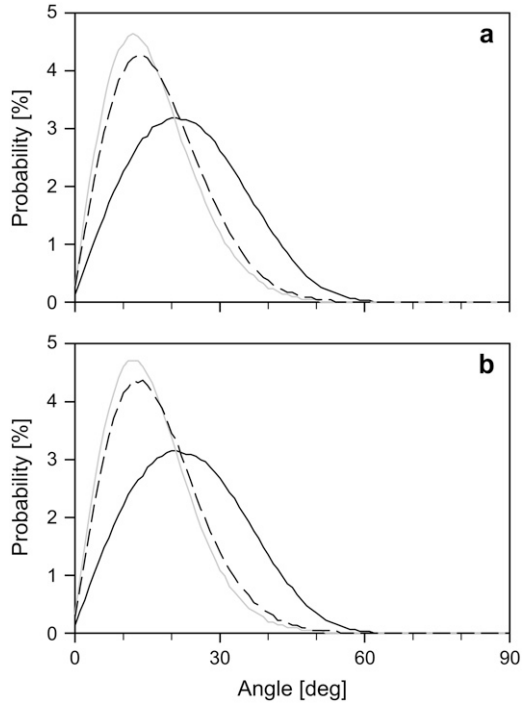

FIGURE 7 Distribution of tilt angles for (*a*) *sn*-2 and (*b*) *sn*-1 chains in DPPC (*black line*), DPPC-Chol (*gray line*), and DPPC-Dchol (*dashed line*) bilayers.

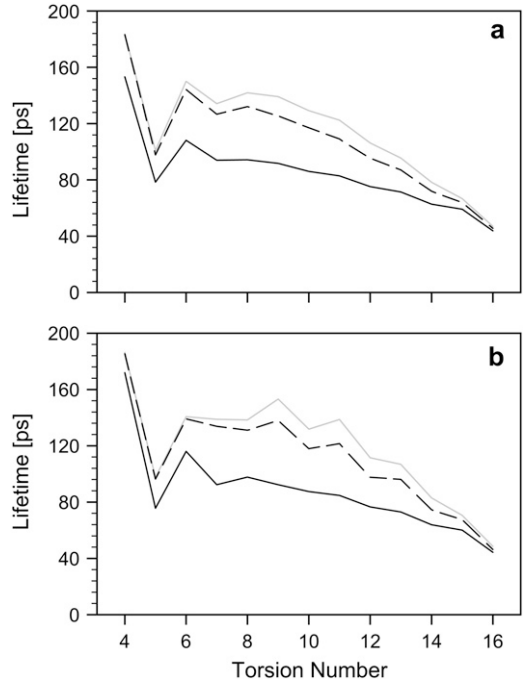

FIGURE 8 Profiles of lifetimes of the *trans* conformations along (*a*) the *sn*-2 chain and (*b*) the *sn*-1 chain in DPPC (*black line*), DPPC-Chol (*gray line*), and DPPC-Dchol (*dashed line*) bilayers.

chains and sterols are shown in Fig. 9. Overall, the effect of Dchol and cholesterol is clear: they both enhance the ordering of the relative lipid arrangement in the bilayer plane. Placing Dchol or cholesterol in a membrane results in the removal of the soft core at short distances, intensifies the peaks and reduces their widths, and extends the range of correlations. Nevertheless, in all respects cholesterol is more capable of promoting ordering than Dchol.

Although in most of the cases the behavior of Dchol and cholesterol is similar, Fig. 9 *d* illustrates one clear difference. The additional structure observed for Dchol around 0.7 nm indicates the possibility of direct Dchol interactions, which was not observed for cholesterol molecules. A similar shape of the RDF for cholesterol-cholesterol pairs was recently observed for a binary mixture of cholesterol and DPPC in higher cholesterol concentrations (62).

Packing of atoms relative to acyl chain atoms

The packing of atoms in the bilayer core can be estimated by calculating the number of neighbors using the method described by Róg and Pasenkiewicz-Gierula (31). The neighbor for an arbitrarily chosen carbon atom in the hydrophobic core of the bilayer is defined to be an atom belonging to a different molecule and located no further than 0.7 nm (the position of the first minimum in the RDF) from the carbon atom in question. The average number of neighbors is 32.7 in DPPC, 37.0 in DPPC-Chol, and 37.6 in DPPC-Dchol bilayers (the errors were less than 0.05). Profiles of the number of neighbors along the *sn*-1 and *sn*-2 chains are shown in Fig. 10. Although we find the number of neighbors to increase along the chains, there is no essential difference between Dchol and cholesterol. However, here the analysis does not differentiate between the α- and β-faces of the sterols, which actually makes a difference (see below).

Packing of atoms relative to sterol ring atoms

As discussed in the Introduction, it seems evident that cholesterol inherently orders surrounding lipids differently depending on their relative position with respect to its α- and β-faces. Obviously, this is because of the structure of cholesterol and should in some way change as cholesterol is replaced with Dchol. To quantify this, the RDF of the carbon atoms of DPPC acyl chains relative to selected atoms belonging to cholesterol molecules was decomposed into two components: the first component was calculated for the atoms located on the side of the α-face of the cholesterol ring and the second one for atoms located on the β-face side. To establish whether a carbon atom C is located on the α- or β-face side, the angle between the C_{10}-C_{19} bond (which should be perpendicular to the cholesterol ring faces, cf. Fig. 2), and the C_{10}-C vector was calculated. In Dchol molecules, the position of the substituent equivalent to C_{19} was calculated using tetrahedral geometry. For atoms located on the β-face,

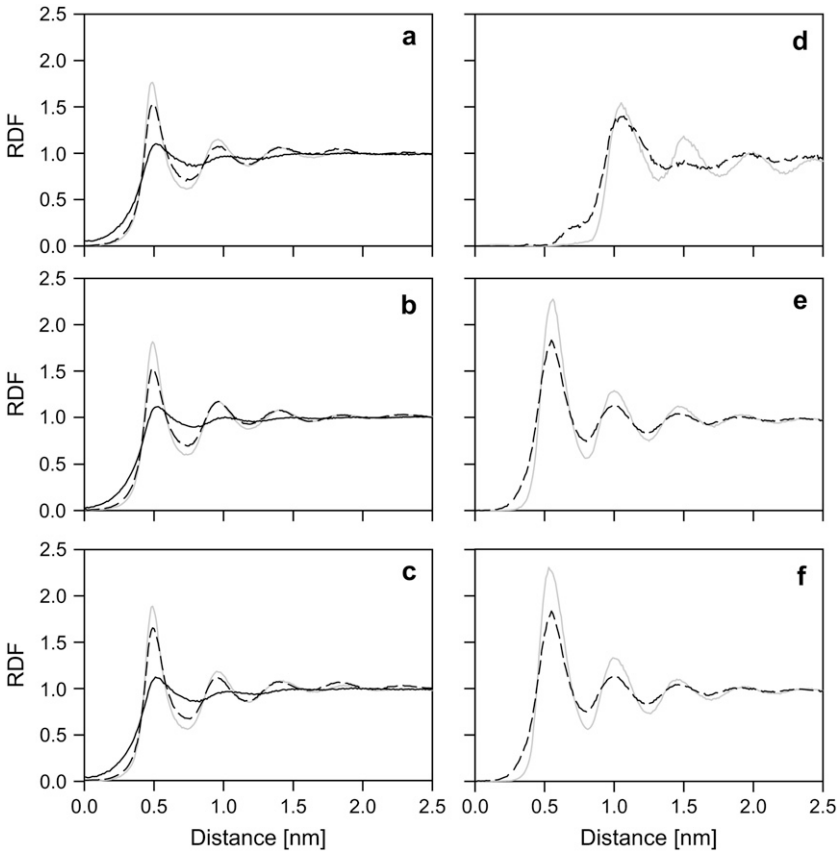

FIGURE 9 Two-dimensional RDFs for pairs of particles described by their center of mass positions. (*a*) *sn*-2 chain relative to *sn*-2 chain. (*b*) *sn*-2 chain relative to *sn*-1 chain. (*c*) *sn*-1 chain relative to *sn*-1 chain. (*d*) Sterol relative to sterol. (*e*) Sterol relative to *sn*-2 chain. (*f*) Sterol relative to *sn*-1 chain. All cases are shown in DPPC (*black line*), DPPC-Chol (*gray line*), and DPPC-Dchol (*dashed line*) bilayers.

the angle is less than or equal to 90°, and for atoms located on the α-face, the angle is greater than 90°.

In Fig. 11 the two components for selected sterol ring atoms (C_1, C_7, and C_{16}) are shown. Let us first concentrate on the α-face component of cholesterol (*gray lines* in Fig. 11, *a, c, e*). We find that these RDFs are regular in the sense that the peak heights decrease monotonously and individual peaks are readily observable. The β-face component of cholesterol, however, is distinctly different because the first peak is usually not the most significant one and because the RDF has a lot of fine structure within a distance of 1.5 nm. This is in line with previous findings where it was concluded that the packing of acyl chain atoms relative to the cholesterol β-face is less regular than with respect to the α-face (32).

In the bilayer containing Dchol, the situation is different, as the regular and nonregular regimes have changed their positions relative to cholesterol: whereas the RDFs in the β-face component of Dchol are regular, the radial distribution functions in the α-face become less regular on approaching C_{17} (Fig. 11 *e*). That indicates that packing becomes less tight.

As for the average number of neighbors of the cholesterol ring (cholesterol methyl groups were not included), we found 37.8, of which 21.1 are located on the α-face, and 16.7 on the β-face. For the Dchol ring this number turned out to be 38.2, of which 17.8 are located on the α-face and 20.4 on the β-face. The errors in all cases were less than 0.05. Hence, here we also find a similar division in which the α- and β-faces of cholesterol and Dchol behave differently. This is clearly illustrated in Fig. 12, where we show the number of neighbors of sterol ring carbon atoms. We find that for carbons close to the OH-group, the number of neighbors is essentially the same in cholesterol and Dchol (carbons 1–4, see Fig. 1 *b*). For carbons 5–9 and 14–17, which form the core of rings B and D (see Fig. 1 *b*), the number of neighbors in the smooth α-face is clearly larger for cholesterol. In the remaining region for carbons 10–13 (close to the methyl groups C_{18} and C_{19} in cholesterol), the number of neighbors in the α-face of Dchol is larger than that in cholesterol. In the case of Dchol the situation is complementary, i.e., for carbons 10–13 the number of neighbors is smaller, and for carbons 5–9 and 14–17 the number of neighbors is larger than in cholesterol. The overall effect is a redistribution of material from the α- to the β-face. This redistribution may be facilitated by hydrogen bonding between the β-OH group of Dchol and phospholipid carbonyl groups leading to tighter packing of atoms on the β-face.

Membrane/water interface

To elucidate the effect of Dchol on the bilayer/water interface, we analyzed the atomic-level interactions of the Dchol hydroxyl group (OH-Dchol) with PC head groups and water molecules. In particular, we considered the role of OH-Dchol on the formation of hydrogen bonds, water bridges,

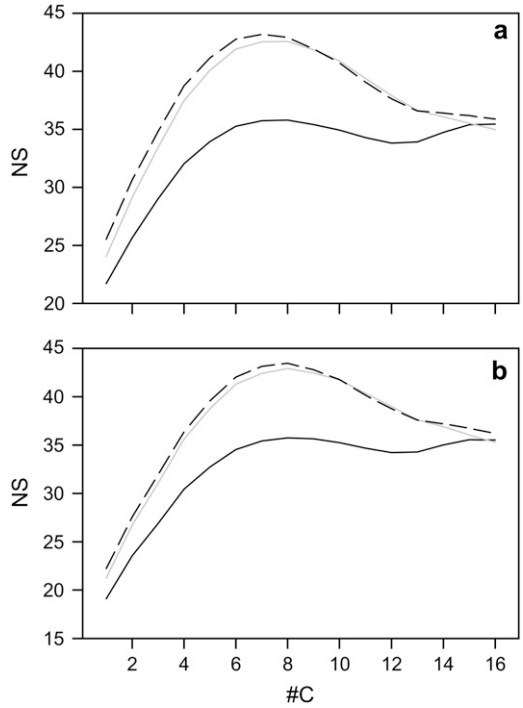

FIGURE 10 Profiles of the number of neighbors (NS) along (*a*) the *sn*-2 chain and (*b*) the *sn*-1 chain in DPPC (*black line*), DPPC-Chol (*gray line*), and DPPC-Dchol (*dashed line*) bilayers. Small carbon numbers correspond to those close to the glycerol group.

and charge pairs (55,56) and compared these results to those induced by the cholesterol hydroxyl group (OH-Chol). The presence of charge pairs was confirmed by following the approach in our previous studies (28,55,56).

H-bonds formed by sterols

The OH-group in Dchol, like the OH-group in cholesterol, participates in hydrogen (H) bonding with water and PC oxygen atoms. The average numbers of OH-Dchol and OH-Chol H-bonds with water and PC phosphate oxygen atoms are given in Table 2. The H-bond pattern is almost the same for both sterols: they make H-bonds predominantly with the ester group of the *sn*-2 chain (56% of all H bonds). It is worth stressing that the low level of hydration (0.38 H bonds per Chol molecule although three are possible) observed for hydroxyl group of sterol agrees well with experimental measurements (63).

PC-sterol water bridges

The numbers of PC-sterol water bridges are 0.32 per sterol for both cholesterol and Dchol. More than half (65%) of the water bridges are formed with the ester group of the *sn*-2 chain (O_{22} and O_{21}).

PC-sterol charge pairs

The negatively charged oxygen atom of the sterol hydroxyl group can interact with the positively charged methyl group of the PC choline moiety ($N\text{-}CH_3$) to form charge pairs. In DPPC-Chol and DPPC-Dchol bilayers the average number of $O\text{–}N\text{-}CH_3$ charge pairs per sterol molecule are 1.15 and 1.10, respectively (Table 2).

Summarizing, the interaction patterns of the hydroxyl groups of Dchol and cholesterol are essentially similar at the membrane-water interface.

DISCUSSION

In this work, we have compared the effects of cholesterol and demethylated cholesterol, cholesterol in which methyl groups C_{18} and C_{19} have been removed from the β-face. The inspiration for this study came from the observation that during cholesterol biosynthesis starting from lanosterol, methyl groups are removed from the cholesterol α-face. The biosynthetic pathway of cholesterol is likely to reflect the evolutionary process that selected the cholesterol structure as the optimal for biological function. Konrad Bloch suggested (22) that removal of a methyl group from the α-face of the sterol ring during sterol evolution optimized van der Waals attraction between the sterol and phospholipid chains. This speculation has been supported by a higher microviscosity of a membrane containing cholesterol than its methylated precursors on the biosynthetic pathway (5,6), as well as by the weaker condensing and ordering effects of lanosterol than of cholesterol (64).

Our previous MD simulation studies of DMPC-Chol bilayers also supported this hypothesis: we observed higher ordering of acyl chains neighboring the α-face than the β-face as well as better packing of hydrocarbon chain atoms around the α-face. This indicates that van der Waals interactions between the DMPC chains and the smooth α-face are stronger than those between the DMPC acyl chains and the methylated rough β-face of the cholesterol ring. These observations seem to suggest that the removal of the remaining methyl groups from the β-face of the cholesterol ring system could further optimize the sterol action in lipid bilayers, mainly by increasing the degree of condensing and ordering effects.

Contrary to naive expectations, however, the results of our simulations have shown that the removal of the methyl groups has the opposite effect, as both the ordering and condensing effects of Dchol are lower than those of cholesterol.

A detailed analysis showed that the properties of cholesterol and Dchol close to the membrane-water interface are essentially identical and therefore not responsible for this difference. We also did not observe any effect of methyl group removal on sterol ring flexibility and conformation. Instead, the root of the issue is in the packing close to the steroid ring structure. Cholesterol strongly favors the packing of nearby lipids on its smooth α-face. Removal of the methyl groups attached to the cholesterol ring leads to a situation in which the α- and β-faces compete for acyl chains, as both have a smooth structure. Dchol favors packing of chains on its β-face. This preference can be explain by an additional interaction taking place on the Dchol β-face, i.e.,

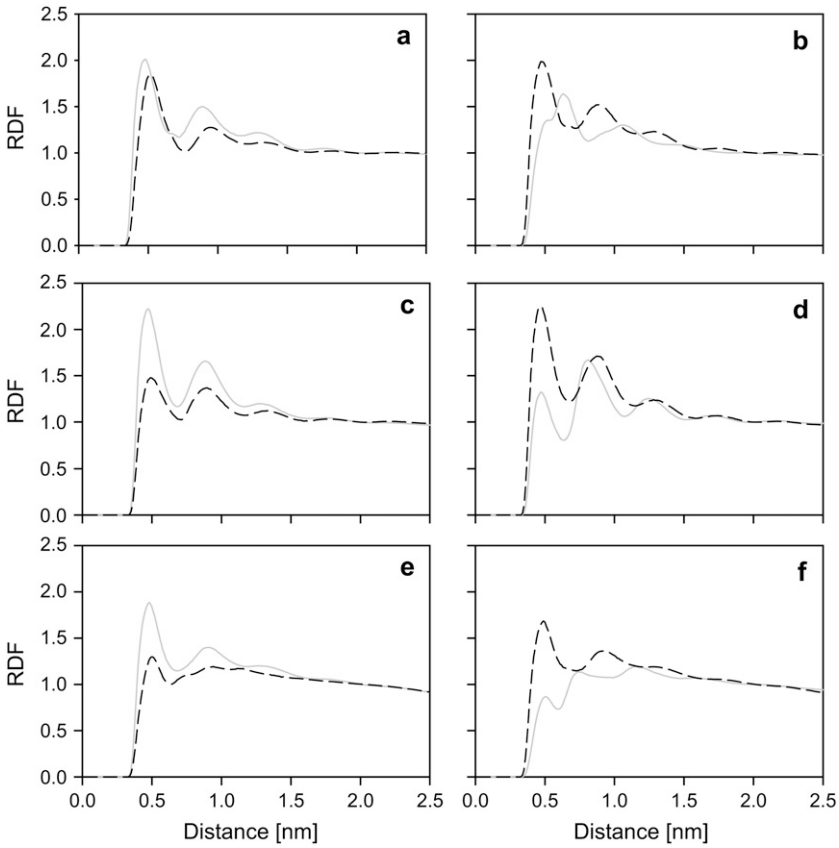

FIGURE 11 α-Face (*a, c*, and *e*) and β-face (*b, d*, and *f*) components of three-dimensional RDFs of the carbon atoms in the bilayer core with respect to the sterol atoms C_1 (*a* and *b*), C_7 (*c* and *d*), and C_{16} (*e* and *f*), in DPPC-Chol (*gray line*) and DPPC-Dchol (*dashed line*) bilayers.

H-bonding between the DPPC headgroup and OH-Dchol, which is located on the β-face (other minor factors are also possible). In general, the van der Waals attraction between the lipid acyl chains and the Dchol β-face, as well as the whole ring, is higher than in the case of cholesterol.

The key factor that seems to be related to the effects of different sterols on bilayer properties is the sterol orientation in the bilayer (17,20). Studies indicate that the tilt of a sterol in a membrane correlates with its ordering and condensing ability. It should be kept in mind, though, that the microscopic origin of the tilt is in the atomic-level interactions, and thus the tilt of a sterol is a manifestation of its interactions with other molecules in the membrane. Nevertheless, the tilt angle seems to provide a physically meaningful and experimentally measurable quantity that can be used to compare the ordering properties of various sterols in membranes. As an example, in this work we have found that the tilt of the Dchol ring is 25°, whereas that for cholesterol is 20°. Similarly, the higher tilt of cholesterol observed in unsaturated bilayers has been found to be correlated with its reduced ability to modify the properties of unsaturated bilayers (65). A similar relationship between the sterol ordering effect and the sterol tilt was observed in our previous MD simulation studies of cholesterol's immediate precursor, desmosterol (17), which differs from cholesterol only in the tail structure, having an additional double bond between C_{24} and C_{25}. The higher desmosterol tilt of 27° was correlated with its lower ability to increase acyl chain order and condensation. The reduced ability to modify the ordering of acyl chains seems to be greater in the case of desmosterol than of Dchol. This difference can be related to better packing of acyl chain atoms near both Dchol faces and stronger van der Waals interactions compared with desmosterol.

Comparative studies concerning the effect of cholesterol and epicholesterol on bilayer properties have shown that modification of the conformation of the sterol hydroxyl group also decreases the sterol's ability to modify acyl chain order and condensation. In this case a correlation between the sterol's vertical location, the sterol–phosphatidylcholine H-bonding pattern, and membrane properties was established (12). In the case of Dchol, we did not observe any differences in the hydroxyl group location (Fig. 5) or its polar interaction with DPPC (Table 2) compared to cholesterol.

Despite stronger van der Waals attraction between saturated acyl chains and Dchol than cholesterol, the ordering and condensing effects of Dchol are lower than those of cholesterol. Thus, the methyl groups in the cholesterol ring are crucial for its optimal effect on the membrane. Therefore, one can speculate that less efficient packing of saturated PC chains on the cholesterol β-face results in stronger chain-chain interactions in a PC-Chol bilayer compared to a pure bilayer (31). Following this reasoning, the removal of methyl groups

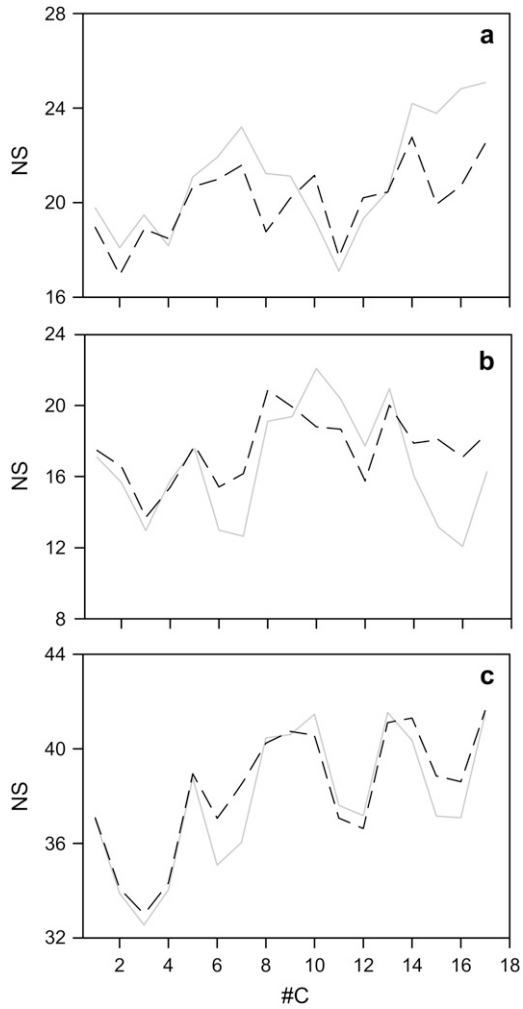

FIGURE 12 Number of neighbors of sterol ring carbon atoms located on (*a*) the α-face, (*b*) the β-face, and (*c*) the total number of neighbors, in DPPC-Chol (*gray line*) and DPPC-Dchol (*dashed line*) bilayers. Small carbon numbers correspond to those close to the hydroxyl group.

from the sterol β-face, i.e., conversion of cholesterol into Dchol, results in stronger Dchol-chain interactions but weaker chain-chain interactions. As a consequence, Dchol has less vertical orientation in the bilayer and is less effective in influencing bilayer properties. To show directly that the Chol β-face promotes better packing among saturated PC chains necessitates detailed analysis and will be carried out at a later stage.

Finally, we would like to mention that although Dchol does not exist in nature and has not, to our knowledge, been synthesized yet, there is no fundamental obstacle to doing that (confirmed by a number of synthetic chemists, private communications). We are aware that the synthesis would be difficult because DChol is highly stereospecific. Interestingly, de novo synthesis of an artificial sterol, *ent*-cholesterol, has been described in literature (66, 67). That indicates that there is both interest and ability to synthesize new sterols.

TABLE 2 Interactions in the membrane/water interface

	DPPC-Chol	DPPC-Dchol
Sterol-water H bonds	0.38	0.39
Sterol-PC H bonds	0.82	0.80
Op	0.08	0.10
O_{22}/O_{21}	0.38/0.20	0.36/0.19
O_{32}/O_{31}	0.08/0.08	0.08/0.08
Sterol-PC water bridges	0.32	0.32
Op	0.08	0.09
O_{22}/O_{21}	0.14/0.08	0.13/0.06
O_{32}/O_{31}	0.04/0.01	0.04/0.01
Sterol-PC charge pairs	1.15	1.10

Sterol-water and sterol-DPPC H-bonds, water bridges, and charge pairs in DPPC, DPPC-Chol, and DPPC-Dchol bilayers. Errors are smaller than 0.02.

To summarize, we have presented a detailed study that clarifies the structure-function relationship of cholesterol in a membrane. Our findings also provide atomic-level support to Konrad Bloch's suggestion (22) that removal of a methyl group from the α-face of the sterol ring optimized the van der Waals attraction between the sterol and phospholipids. Our results show that the two methyl groups in the steroid ring system of cholesterol play an important role in cholesterol-lipid interactions by reducing sterol tilt in the bilayer and hence allowing for an optimal orientation for cholesterol.

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