Articles

Conformational Study of Lipophilic Ligands in Phospholipid Model Membrane Systems by Solution NMR

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Phospholipid bicelles were employed as a membrane bilayer model in the conformational studies of two lipophilic cannabinoids, Δ^8-THC and its *O*-methyl ether analogue, Me-Δ^8-THC using conventional high-resolution NMR. A preparation of 8% (w/v) phospholipid concentration and a high DMPC/DHPC ratio ($q = 2.0$) was found to be optimal for not only effectively incorporating our ligands, but also providing a more bilayerlike environment suitable for conformational studies. While the conformational differences between the two cannabinoids could not be observed in chloroform and were barely detectable in SDS micelle solution, there is an increasing preference for the pentyl tail of Δ^8-THC to bend toward the tricyclic ring system with increasing proportions of DMPC in the bicelle preparation. Our results highlight the advantages of exploring the conformational properties of cannabinoids using bicelle preparations as a medium that more closely resembles biological membrane bilayers and eliminates the need for isotopic labeling. This approach should also be of more general value for studying the interactions of other cannabinoids and biologically active, hydrophobic or amphipathic, small molecules with membranes.

Introduction

The ability of many drugs and some endogenous ligands to reach their sites of action is believed to be associated with molecular properties governing cell membrane penetration and lateral diffusion within the membrane leaflet. Thus the conformation, orientation, and location of the ligand in the membrane is critical in understanding its ability to reach its site of action in the proper orientation and conformation so that it can interact productively with that site.[1,2] Traditionally, solid-state NMR spectroscopy and small-angle X-ray diffraction were utilized to investigate the ligand interactions with model membrane bilayers;[3,4] however, these approaches require extensive efforts in sample preparation that involve specific isotopic labeling. Alternatively, a sodium dodecyl sulfate (SDS) micellar medium[5] has been used in high-resolution NMR conformational studies to mimic the membrane environment, even though the highly curved nature of SDS micelles does not accurately represent the cellular membrane bilayers. As phospholipid bicelles have been reported to more closely resemble the bilayer structure of cellular membranes,[6,7] we became interested in exploring their suitability as model membrane systems for ligand conformational studies using high-resolution NMR.

The ligands used in this study belong to the class of classical cannabinoids, which are the tricyclic terpene analogues of (−)-Δ^9-tetrahydrocannabinol (Δ^9-THC), the key psychoactive constituent in marijuana. Many of the pharmacological effects of cannabinoids, including psychotropic activity, analgesia, and reduced intraocular pressure, have been attributed to their interactions with two cannabinoid receptors, CB1 and CB2.[8,9] As cannabinoids are generally very lipophilic, it has been postulated that they reach their receptor active sites by lateral diffusion within the cell membrane where they preferentially accumulate.[10] We have shown that using solid-state deuterium NMR and small-angle X-ray diffraction, two representative classical cannabinoids, Δ^8-THC and Me-Δ^8-THC (Figure 1), interact with DPPC multilamellar membrane bilayers quite differently even though they are closely related structurally.[11−18] These two cannabinoids are essentially water insoluble and expected to be partitioned within the lipid bilayer. We were interested in elucidating the conformations of these two ligands in a membrane bilayer environment and establishing whether the differences in the membrane interactions would be reflected in their conformational properties.

Phospholipid bicelles are typically composed of DMPC lipids with DHPC detergent, where the DMPC-rich domain has been demonstrated to be similar to those

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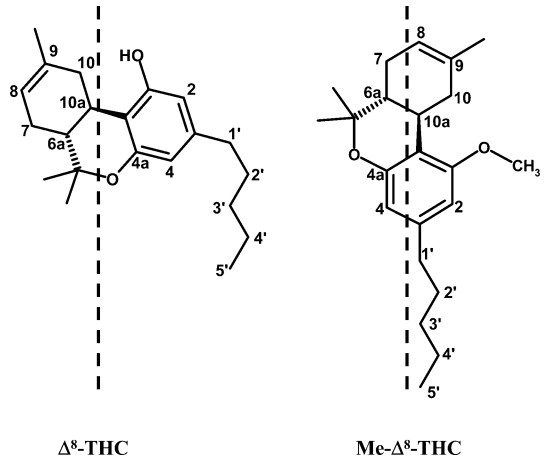

Figure 1. Chemical structures of Δ^8-THC and Me-Δ^8-THC and their proposed orientations in model membrane bilayers. The dashed lines represent the direction of the lipid acyl chains.[14]

in multilamellar membrane bilayers.[19,20] Bicelle conditions can be tuned to be either isotropic or anisotropic over wide ranges of lipid:detergent ratio (roughly 5:1−1:2) and water content (roughly 60−97%).[19,21−23] Early conformational studies using this bicelle system were able to measure anisotropic parameters of molecules that were either associated with the surface, or partially imbedded into the bicelle;[24−27] however, these approaches generally involved isotopic labeling. By decreasing the DMPC to DHPC ratio to 0.5, the system can be tuned to isotropic conditions with smaller sized bicelles that permit high-resolution NMR structural determination of membrane-associated peptides.[28−32]

It is highly desirable to obtain the conformational properties of small molecules in a bilayer environment without specific isotopic labeling. Unlike SDS micelles, which have been widely employed even though they are not ideal bilayers,[5] the bicelle system has not been fully explored as a membrane model in ligand conformational studies. In this paper, we seek to obtain an isotropic bicelle preparation with the largest possible DMPC lipid content, while high-resolution proton NMR spectra can still be acquired to ascertain ligand conformation in a bilayer environment. We further examine the NOE pattern differences observed in bicelle preparations, SDS micelles, and chloroform solution, which may reflect the conformational characteristics resulting from different solubilizing media. The methods we describe here find more general applicability with many other groups of natural hormones and neurotransmitters or their synthetic analogues that are known to interact with cellular membranes.

Results

Optimizing the Isotropic Bicelle Conditions. A series of isotropic bicelle solutions were prepared with the highest possible DMPC content for incorporating our ligands, as increased DMPC to DHPC ratio (q) will result in increased proportions of bilayerlike domain.[19,31] The ^{1}H spectra of the ligands Δ^8-THC or Me-Δ^8-THC incorporated into different bicelle preparations are shown in Figures 2 and 3. The lipid-to-detergent ratios were selected according to the temperature-composition phase diagrams of bicelles with 20% (w/v) total lipid

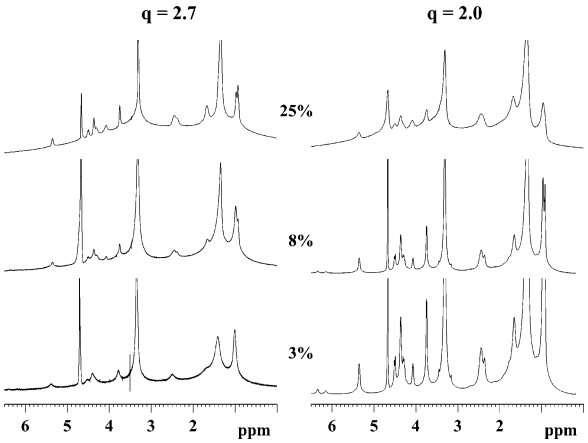

Figure 2. ^{1}H spectra of Δ^8-THC at 38°C in bicelle solutions that have DMPC:DHPC ratios as indicated of $q = 2.7$ or $q = 2.0$ and with total lipid concentrations of 25% (w/v), 8% (w/v), and 3% (w/v).

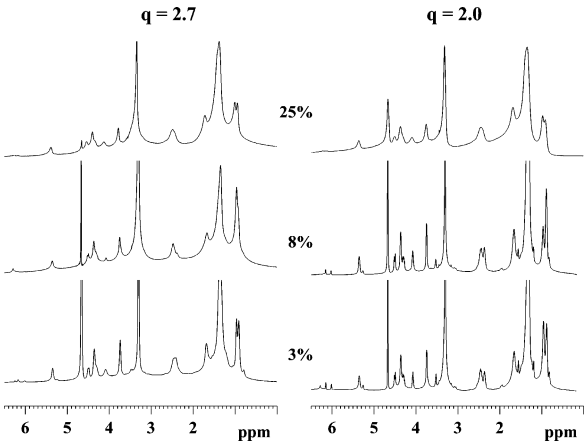

Figure 3. ^{1}H spectra of Me-Δ^8-THC at 38°C in bicelle solutions that have DMPC:DHPC ratios as indicated of $q = 2.7$ or $q = 2.0$ and with total lipid concentrations of 25% (w/v), 8% (w/v), and 3% (w/v).

concentration.[21] To maintain isotropic conditions at higher values of q, it is necessary to reduce the overall lipid concentration which allows faster tumbling of the bicelles. In addition, our preparations were free of KCl, which allowed us to use high values of q while maintaining isotropic conditions.[21]

At $q = 2.7$, we estimated that the system conditions border the isotropic bicelle domain based on the temperature-phase diagrams. At a lipid concentration of 25% (w/v), lipid proton resonances are very broad, indicating a relatively high degree of orientation and a very slow tumbling rate of the bicelles. Similar broadening of the ligand proton resonances is consistent with the association of the ligands with the bicelles so that they assume a high degree of ordering. Reducing the lipid concentration does not adequately result in isotropic conditions. At the lower lipid concentrations of 8% and 3%, proton resonances are similarly broad, suggesting that the bicelles still retain a certain degree of orientation relative to the magnetic field. Two representative ^{31}P spectra that are of the 25% and 8% (w/v) preparations, with incorporation of Me-Δ^8-THC, are shown in Figure 4. The upfield phosphorus resonance indicates that at this lipid-to-detergent ratio, bicelles are partially oriented in the external magnetic field and are not in a fully isotropic state.

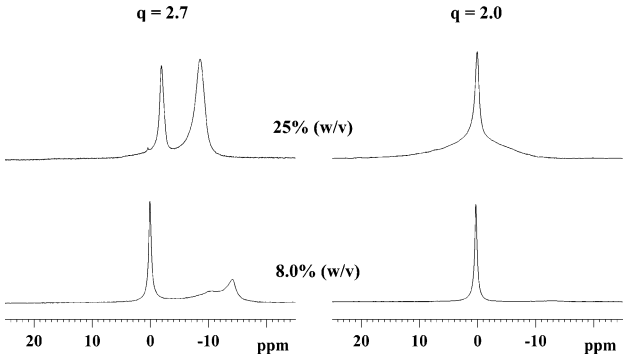

Figure 4. ^{31}P spectra of Me-Δ^8-THC in bicelle preparations with 25% and 8% (w/v) lipids and $q = 2.7$ and $q = 2.0$.

Solutions with $q = 2.0$ are more within the isotropic bicelle condition as shown by the observed single resonance in the ^{31}P spectrum (Figure 4). The phosphorus spectrum of the 25% solution, however, is quite broad, possibly as a result of long correlation times. This preparation does not yield sharp ligand proton resonances as shown in Figures 2 and 3. When the lipid concentration is reduced to 8% and 3%, there is a dramatic resolution increase in both ^{1}H and ^{31}P spectra. Well-resolved lines reflect the isotropic nature of these media, which results from the decrease of the total lipid concentration and a slightly reduced value of q. The isotropic state under these conditions may be due to a decrease in diameter of the bicelle disks combined with an increase in the spacing between them that results in faster motional averaging.[28,31,33]

A DMPC:DHPC ratio of 2.0 is near the upper limit that may practically be employed as an isotropic model membrane preparation. A certain degree of broadening in the ^{1}H spectrum still exists, which prevents accurate measurements of J-couplings and residual dipolar couplings between the multiple proton pairs. However, the resolution is adequate for conventional 2D-NMR experiments such as DQF−COSY and NOESY from which ligand conformational properties can be ascertained. In addition, no precipitation of either the ligand or lipid was observed from the 25% and 8% preparations over the course of several days. The 3% (w/v) sample, however, was very sensitive to temperature and lipid precipitation was typically observed after several hours. Therefore, a total lipid concentration of 8% (w/v) and $q = 2.0$ was chosen as an optimized bicelle preparation for the following ligand conformational study using two-dimensional NMR experiments.

Assignments of the Ligand Resonances. The DQF−COSY spectra, in conjunction with the NOESY spectra, were used for the assignment of Me-Δ^8-THC resonances and partial assignment of Δ^8-THC resonances in 8% (w/v), $q = 2.0$ bicelle preparations that are shown in Figure 5 (the lipid resonances were referenced according to Lee and Griffin [34]). In general, the DQF−COSY spectra of Δ^8-THC were not as well resolved as those for Me-Δ^8-THC due to the slightly larger line widths of Δ^8-THC resonances (Figure 6). Cross-peaks are observed that allow assignment of the pentyl-tail protons unambiguously; however, there are few cross-peaks that reveal the positions of ring protons due to the increased line widths. There is a larger number of cross-peaks observed for Me-Δ^8-THC, which allows a complete assignment of all the resonances.

Assignment of the aromatic protons of each ligand was confirmed by observed NOEs (discussed below) and carbon−proton heteronuclear multiple quantum coherence (HMQC) experiments, of which a representative spectrum is shown in Figure 7. The carbon chemical shift of C2 is upfield of C4 in $CDCl_3$ solution (data not shown) due to the proximity to the phenolic hydroxyl or the arylmethoxy group. For Me-Δ^8-THC there are no significant chemical shift changes in going from a chloroform solution to the bicelle preparation. For Δ^8-THC, however, the aromatic H2 proton is shifted downfield of the H4 resonance, while there is a corresponding downfield shift in the C2 carbon.

Significant ^{13}C chemical shift changes of Δ^8-THC are also observed for resonances from the pentyl tail region of the HMQC spectra. The C1′ resonance shifts upfield by ∼1.7 ppm and the C2′ resonance also shifts upfield by ∼0.6 ppm. These chemical shift changes can be explained by a gauche C3−C1′−C2′−C3′ dihedral angle.[35] In the case of Me-Δ^8-THC, however, the ^{13}C chemical shifts are not significantly affected when compared to spectra acquired in chloroform, where the pentyl tail generally assumes a trans conformation.

Assignment of the ring proton resonances for Me-Δ^8-THC was further confirmed based on observed NOEs. For example, H10α can be distinguished from H10a due to a weak NOE between the $9CH_3$ protons (1.55 ppm) and H10α (3.14 ppm) and the expected close proximity of $9CH_3$ to H10α. All NOEs are unambiguous except for

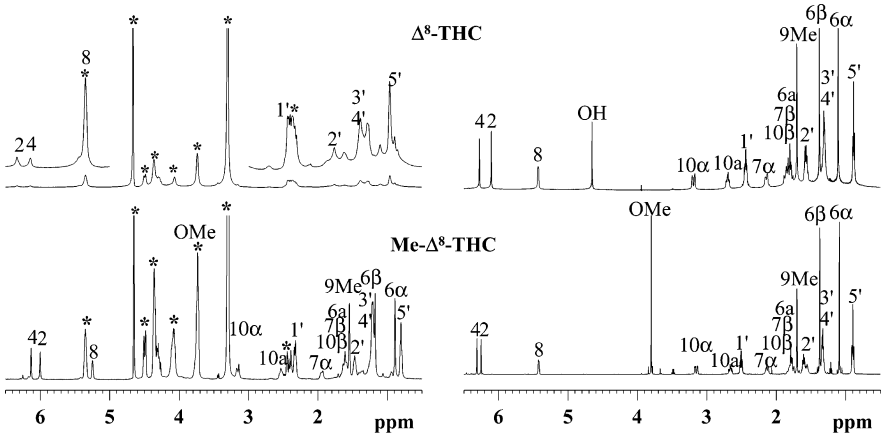

Figure 5. A comparison of the ^{1}H NMR spectra of Δ^8-THC and Me-Δ^8-THC in an 8% (w/v) and $q = 2.0$ bicelle preparation (left panel) and in $CDCl_3$, (right panel). Resonances from the phospholipid and water are labeled with asterisk.

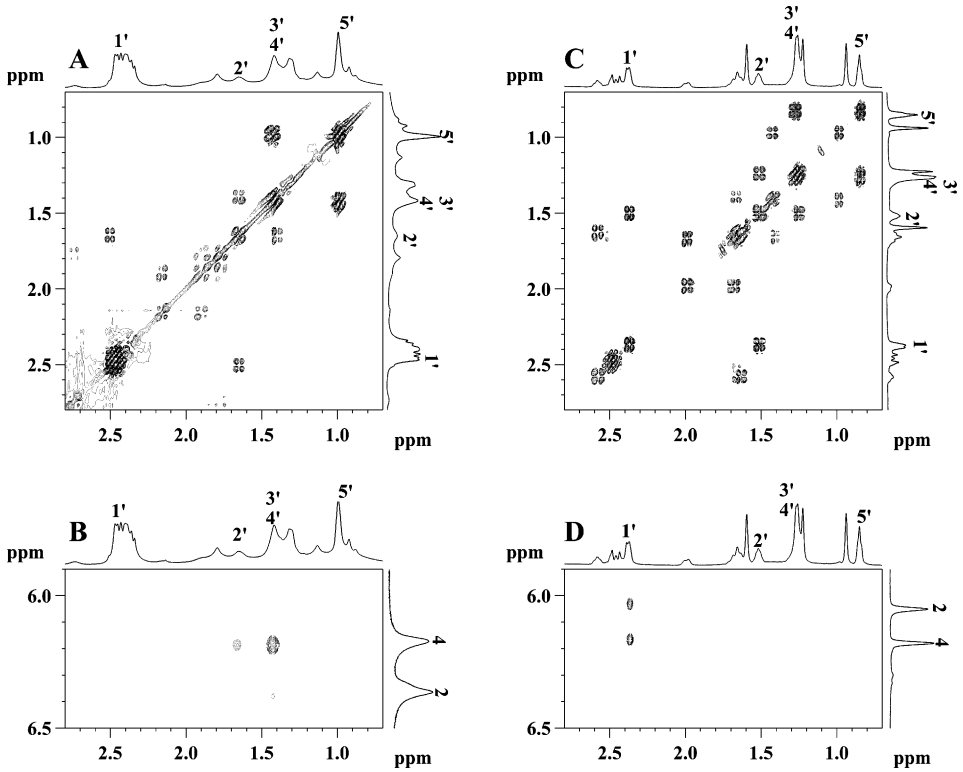

Figure 6. Selected regions from DQF−COSY and 100 ms NOESY spectra of the ligands in a preparation with a total deuterated lipid concentration of 8% (w/v) and a DMPC/DHPC ratio of 2.0:1. Panel A is an expansion of the DQF−COSY spectrum of Δ^8-THC focusing on the upfield aliphatic region. Panel B is an expansion of the NOESY spectrum, correlated to panel A, showing NOEs between the aromatic protons and protons of the pentyl-tail. Likewise, Panel C and D are correlated expansions of the DQF−COSY and NOESY spectra of Me-Δ^8-THC.

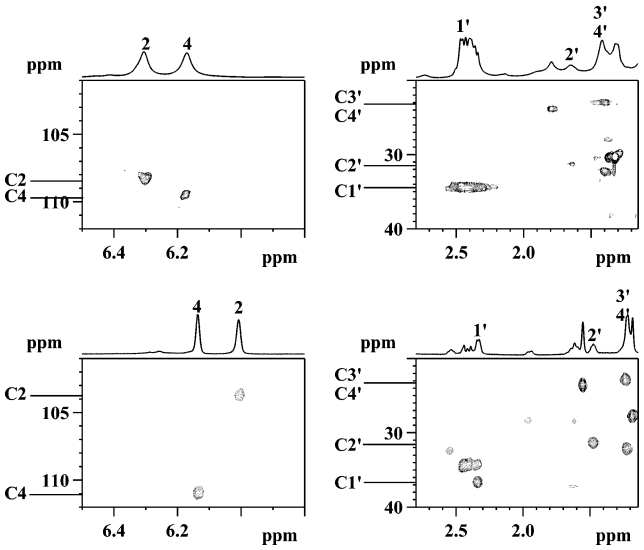

Figure 7. Expansions of the HMQC spectra of Δ^8-THC (top panels) and Me-Δ^8-THC (bottom panels) highlighting the assignment of the aromatic resonances (left panels) as well as the resonances from the pentyl tail (right panels).

two cross-peaks to the overlapped ligand OCH_3 and lipid choline 2-CH_2 resonances to 3.73 ppm. One of these is an extremely weak NOE to the overlapped H3′ and H4′ resonances of the ligand at 1.21 ppm. The second of these NOEs is to the aromatic resonance at 6.00 ppm. Taking into account the ortho position of the H2 relative to the arylmethoxy group and lack of NOEs to other protons of the lipid headgroup, this is most likely an intramolecular cross-peak between H2 (6.00 ppm) and the OCH_3 protons (3.73 ppm) of the ligand. No NOEs are observed between the overlapped ligand OCH_3 and lipid headgroup 2-CH_2 resonance at 3.73 ppm with the other aromatic proton (H4) at 6.13 ppm, which is congruent with the assignments of the ligand H2 and H4 aromatic proton resonances and the idea that the aromatic ring is not just associated with the surface but is within the bilayer.

To differentiate the overlapped resonances between the Me-Δ^8-THC methoxy protons and lipid choline 2-CH_2 protons, an HMQC experiment was performed on Me-Δ^8-THC with a ^{13}C-enriched methoxy group (Figure 8). This resolved the overlapping by splitting the methoxy proton resonances of 143 Hz. No intermolecular NOEs were observed from any of the lipid headgroup protons to the ligand, which supports that the ligand is partitioned within the bilayer rather than associated with the surface.

NOE and Dihedral Angle Restraints. Long-range NOEs observed in the 100 ms NOESY spectra were classified as medium, weak, or very weak using the H8−H7α NOE as a standard for a strong NOE, which provides internuclear distance restraints in the subsequent molecular modeling. Three long-range NOEs were observed in the case of Δ^8-THC, that could be assigned unambiguously (Table 1) and for Me-Δ^8-THC, seven NOEs were identified between proton pairs not adjacent to each other (Table 2).

Of greatest interest are the NOEs between the aromatic ring protons and protons on the pentyl-tail (Figure 6B and 6D). For Me-Δ^8-THC, the only NOEs observed are those between the H1′ protons and the H2/H4 protons of the aromatic ring. For Δ^8-THC, however, additional NOEs are observed between the aromatic H4

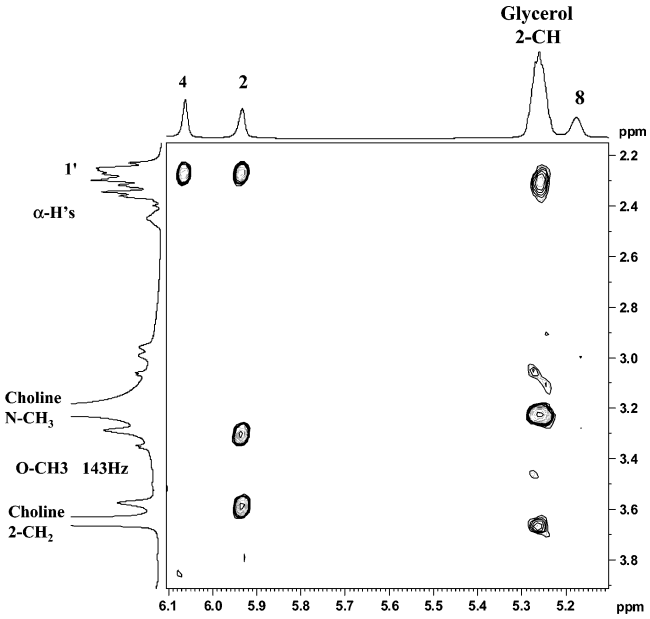

Figure 8. Expansion of a 100ms NOESY spectrum of ^{13}C-labeled Me-Δ^8-THC in a $q = 2.0$, 8% w/v bicelle preparation.

Table 1. Intramolecular NOEs Observed from Δ^8-THC in $q = 2.0$ Bicelle

proton A	proton B	intensity	distance Å
4	3′, 4′	medium	2.5−4.0
4	2′	weak	3.5−5.0
2	3′, 4′	very weak	4.0−6.5
8	7α	strong	1.5−2.5

Table 2. Intramolecular NOEs Observed from Me-Δ^8-THC in $q = 2.0$ Bicelle

proton A	proton B	intensity	distance Å
2	1′	medium	2.5−4.0
2	-OCH_3	medium	2.5−4.0
4	1′	medium	2.5−4.0
8	7α	strong	1.5−2.5
8	9Me	medium	2.5−4.0
10α	9Me	weak	3.5−5.0
10a	6α	medium	2.5−4.0
OCH_3	3′, 4′	very weak	4.0−6.5

proton and H3′/H4′ and H2′ protons of the pentyl-tail, while there is another very weak NOE from the H2 proton to the H3′/H4′ protons.

For the tricyclic ring proton resonances, an NOE between the axial C6 methyl protons (0.89 ppm) and H10a was observed from Me-Δ^8-THC while there was no NOE between the equatorial C6 methyl protons (1.18 ppm) and H10a. This suggests that the tricyclic ring system is quite rigid for Me-Δ^8-THC with the axial C6 methyl group is on the same side of the molecule (α-side) as H10a. However, there were no well-resolved NOEs for Δ^8-THC due to the increased line widths.

The methoxy group orientation with respect to the aromatic ring of the Me-Δ^8-THC was elucidated from its ^{13}C chemical shift (55.0 ppm) in the HMQC experiment. It has been demonstrated [36] that the ^{13}C chemical shift of the methoxy group can be utilized to predict whether the O−CH_3 bond is coplanar with the phenyl ring. The ^{13}C chemical shift of the arylmethoxy group would be very similar to that of unsubstituted anisole (55.1 ppm) for a coplanar conformation whereas a chemical shift of 5−7 ppm downfield from that of anisole is expected for an out-of-plane conformation. The OCH_3 chemical shift of Me-Δ^8-THC in our bicelle preparation provided clear evidence that the O−CH_3 bond is coplanar with the phenyl ring. The dihedral angle C2−C1−O−CH_3 of Me-Δ^8-THC was then set to between −10.0° and +10.0° and used as a restraint in the subsequent molecular modeling studies.

Ligand Conformations. Two families of Δ^8-THC conformations were found from the NMR constrained molecular modeling calculations. In each of these families, the pentyl tail is generally perpendicular to the long axis of the tricyclic ring system (Figure 9A). The difference between these two families arises from the orientation of the pentyl-tail relative to the plane of the tricyclic ring system. In one set of the conformers, the ligand pentyl-tail is oriented toward the α-face of the tricyclic ring at a maximum of ∼30° out of the plane. The second conformer is oriented toward the *β*-face of the ring, also to a maximum of ∼30° out of the plane. In the case of Me-Δ^8-THC (Figure 9B), the most favorable conformations are those where the pentyl-tail extends away from the tricyclic ring system. As for the O−CH_3 conformation, the dihedral angle C2−C1−O−CH_3 is found to be within −5.7° and 3.8° out of a total of 100 conformers recorded from the simulation, illustrating that the energetically favorable conformations place the O−CH_3 bond coplanar with the aromatic ring.

For the tricyclic ring conformation, even though there were no NOE restraints for Δ^8-THC, our calculations nevertheless yielded very similar results for both Δ^8-THC and Me-Δ^8-THC. Out of all derived conformers of Me-Δ^8-THC, the distance between H10a−C6α is 2.95−3.04 Å with a much larger distance between H10a and C6*β* (4.41−4.45 Å). This is also the case for Δ^8-THC and is probably due to the fact that the calculations seek to minimize the steric clash between H6a and the 6α/*β*-CH_3 groups. This observation is consistent with a previous investigation[37] of Δ^9-THC in $CDCl_3$ solution where the H10a and 6α−CH_3 carbon are oriented 1,3 diaxial to each other with a distance of 2.9 Å and an optimized C10a−C6a−C6−O dihedral angle of 56°.

Comparison of Ligands in Chloroform, Micelles, and Bicelles. NOESY spectra were also acquired from ligands dissolved in chloroform, SDS micelles, and a bicelle preparation with $q = 0.5$. Figure 10 shows that the NOE patterns observed between the ortho aromatic ring protons and protons of the pentyl tail of Δ^8-THC are closely related to the choice of solubilizing media. In chloroform, only two NOEs were observed between the aromatic protons H2 and H4 and the pentyl tail H1′ protons. In membrane mimetic media, additional NOEs were observed between the aromatic H4 resonance and resonances that arise from further down the pentyl-tail, where the relative intensities of these NOEs varied depending on the membrane preparations. In SDS micelles, the NOESY spectrum from Δ^8-THC is comparable to that observed in chloroform where strong NOEs were observed between the aromatic protons and the H1′ protons in the pentyl tail. Two weak NOEs were also observed between H4 and the pentyl tail protons H2′, and the overlapped H3′ and H4′ protons, which shows that there is a conformational preference for orientation of the pentyl tail toward the H4 side of the

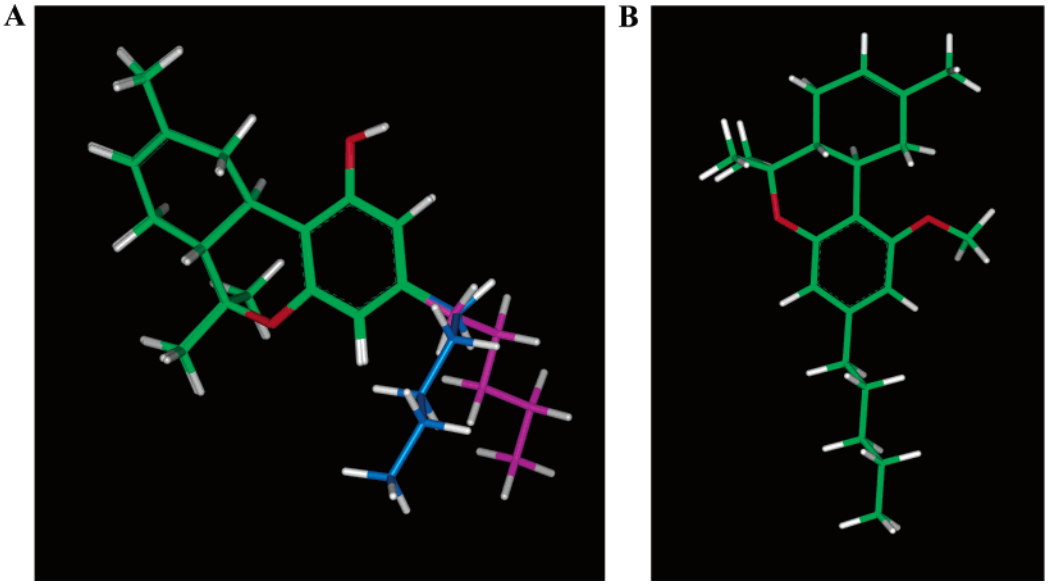

Figure 9. (A) Two representative structures from each of the two families of Δ^8-THC conformers. The structures have been superimposed and the pentyl tail carbons of the conformer in which the tail is toward the α-face of the tricyclic ring system are colored pink; while the pentyl tail carbons of the conformer in which the tail is toward the *β*-face are colored blue. (B) A typical structure from the derived set of conformations of Me-Δ^8-THC.

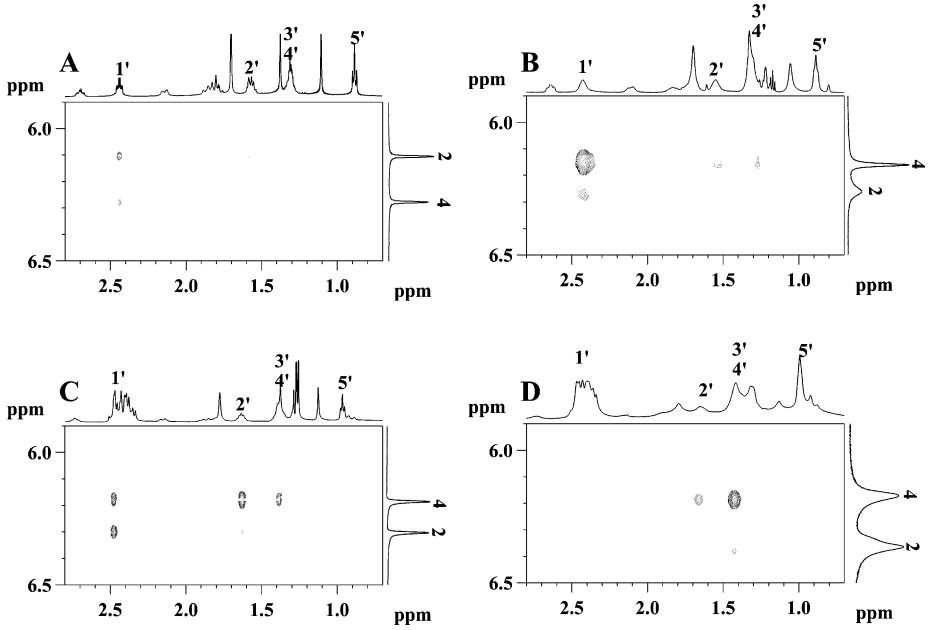

Figure 10. Expansions of NOESY spectra showing NOEs between the aromatic ring and the pentyl-tail of Δ^8-THC in (A) $CDCl_3$, (B) SDS micelles, (C) $q = 0.5$ bicelle, and (D) $q = 2.0$ bicelle.

tricyclic ring system. In the $q = 0.5$ bicelle solution, the H4−H2′ and H4−H3′/H4′ NOEs are similar in intensity to the H4−H1′, H2−H1′ NOEs, while in the $q = 2.0$ solutions, the former set of NOEs are significantly stronger than the latter. The above observation shows that with increasing proportions of DMPC in the bicelle, there is an increasing preference for Δ^8-THC where the pentyl tail is oriented toward the H4 side of the tricyclic ring system.

For Me-Δ^8-THC, however, the only NOEs observed in membrane mimetic media are those between the H1′ protons and the H2/H4 protons of the aromatic ring, which are similar to those observed in chloroform (data not shown) except that the NOEs are positive from the chloroform solution due to a much shorter correlation time. The NOEs observed in chloroform may reflect rapid transitions between all possible conformations and that NOEs with protons in the tail that are further away from the aromatic ring are not observed. Alternatively, the observation of a similar pattern of negative NOEs in membrane mimetic media may reflect a predominantly extended conformation of the tail with respect to the tricyclic ring system.

It is also worthy to note that in both the SDS micelle and the $q = 0.5$ bicelle preparations, Δ^8-THC was observed to precipitate over 3−6 h whereas it was still well solubilized in the $q = 2.0$ bicelle solution for days. These results suggest that the choice of lipid−detergent composition can greatly influence the degree of ligand solubilization. Our earlier investigation has shown that DPPC multilamellar model membrane bilayers can effectively incorporate Δ^8-THC at a ratio as high as

40%.[12] The fact that our ligands can be well incorporated into the $q = 2.0$ bicelle at 10% suggests that our bicelle preparation provides a more bilayerlike environment compared to the SDS micelle and the $q = 0.5$ bicelle systems.

Discussion

Comparing the isotropic solutions in Figures 2 and 3 (i.e. 8% w/v and 3% w/v at $q = 2.0$), it is evident that there are significant differences between the interactions of Δ^8-THC and Me-Δ^8-THC with the bicelles. The ^{1}H signals observed for Me-Δ^8-THC at 8% and 3% w/v are much narrower than those observed for Δ^8-THC, which can be easily seen from the aromatic resonances near 6.0 ppm highlighted in Figure 5. Given that there is no corresponding change in the line widths of the lipid resonances, we cannot attribute the decrease in ligand line widths to differences in the overall dynamic properties of the bicelle disks; however, the explanation may lie in the orientations adopted by these molecules in membrane bilayers.[12,13,17] The orientation of Me-Δ^8-THC where the long axis of the molecule is generally parallel to the DMPC acyl chains in bilayers (Figure 1) may result in a greater degree of motional averaging within the bilayer relative to Δ^8-THC. The "awkward" orientation of Δ^8-THC where the long axis of the molecule is perpendicular to DMPC acyl chains is not as easily accommodated among the acyl chains and so is more restricted.

The downfield shift of the aromatic H2 proton of Δ^8-THC in the $q = 2.0$ bicelles compared to the chloroform solution may be attributed to a polar environment. It has been reported that protons situated ortho to hydroxyl groups in phenolic systems experience stronger deshielding effects in polar solvents such as pyridine.[37] The downfield shift of the ortho H2 proton reflects that the aromatic hydroxyl group resides in the lipid/water interface region. The interaction between the Δ^8-THC phenolic OH and lipid polar headgroup results in an anchoring effect that may further restrict the molecule. Such a strong anchoring effect may orient the psychoactive Δ^8-THC with the long axis of its tricyclic ring system almost parallel to the bilayer surface, which is consistent with our previous orientational study using DPPC multilamellar model membrane.[12,13,17]

The conformational differences observed between Δ^8-THC and Me-Δ^8-THC are clearly a reflection of the conditions imposed by a bilayerlike environment on each ligand. The NOEs observed between the aromatic protons of Δ^8-THC and protons in the pentyl-tail confirms the postulation in our previous publications that the pentyl-tail of Δ^8-THC tends to align generally parallel to the lipid acyl chains and is in this way accommodated among the lipid acyl chains.[15,17] Therefore, the pentyl-tail tends to bend toward the tricyclic ring system and adopt a gauche C3−C1′−C2′−C3′ dihedral angle, which is demonstrated by the upfield ^{13}C chemical shift change of the pentyl tail carbons.[35] For Me-Δ^8-THC, however, the observed pattern of NOEs favors a conformation with the pentyl-tail extended away toward the center of the bilayer, which is also consistent with our earlier orientational studies within DPPC model membrane bilayers.[12,17]

The conformational properties of lipophilic ligands may be greatly influenced by the choice of its solubilizing membrane mimetic media. As can be seen from the observations of Δ^8-THC, NOEs indicative of the conformation favored in bilayers[14,15,17] (H4−H2′, H4−H3′/4′) are barely observable in SDS micelles, suggesting that the tail has a greater degree of conformational freedom in the micellar environment. In going from the $q = 0.5$ to the $q = 2.0$ bicelle preparation, NOEs indicative of a bent conformation become more intense, and this may be a reflection of an environment that more closely resembles a bilayer. A fluorescent study[31] has shown that at conditions of $q < 0.5$, the size of the planar bilayer domain is directly proportional to the ratio of DMPC/DHPC. Despite the fact that the morphology of these fast-tumbling isotropic bicelle preparations may in fact be quite complex,[21,23] it is very unlikely that the observed NOEs arise from ligands partitioned into the DHPC domain, as in this case, a pattern more similar to that observed from SDS micelles would be expected. This notion is consistent with a recent study that the structural difference between bicelles and spherical micelles influence the conformation of peptides that are solubilized by them.[7]

Our results obtained from higher proportions of DMPC ($q = 2.0$) bicelle preparation are congruent with the previous studies using DPPC multilamellar membrane bilayers,[12,13,17] which suggests that the $q = 2.0$ bicelle provides an ideal membrane model for ligand conformational analysis. These bicelle disks can be tumbling isotropically in such a dilute solution (8% w/v) as a result of faster motional averaging combined with a decrease in bicelle size.[28,31,33] Nevertheless, at the experimental temperature (38 °C), the bilayer domain of each bicelle disk is still in the liquid crystalline (L_α) phase where the DMPC acyl chains undergo rapid trans-gauche isomerization. At such isotropic conditions, conventional high-resolution NMR methods can be employed to ascertain ligand conformation within a membrane bilayer environment.

Conclusions

The conformations of Δ^8-THC and Me-Δ^8-THC were determined, and while there are no observable conformational differences in $CDCl_3$ solution, they are found to differ within membrane mimetic media due to the more amphipathic-like properties imparted by the phenolic hydroxy group of Δ^8-THC compared to Me-Δ^8-THC. The solubilizing medium influences the variations in the conformation of the pentyl tail. While differences between the two ligands are barely detectable in SDS micelles, there is an increasing preference for Δ^8-THC where the pentyl tail bends toward the tricyclic ring system with increasing proportions of DMPC in the bicelle preparation. This is most likely a reflection of the more bilayerlike morphology of the lipid bicelles compared to SDS micelles. The congruency between observations from the bicelle preparations and multilamellar DPPC model membrane systems indicates that the $q = 2.0$ preparations provide a bilayer environment capable of incorporating our lipophilic cannabinoids in a manner similar to DPPC model membrane bilayers.

Without the need for isotopic labeling of the ligands, our bicelle preparations provide an avenue for obtaining ligand conformation in a lipid bilayer environment using conventional solution NMR spectrometers. Thus, this

technique is complementary to solid-state NMR methods such as REDOR[3] that requires specific isotopic labeling. To further define the interactions between our cannabinoid ligands and the lipid membrane, work is currently in progress to measure the anisotropic parameters, which allows us to determine the orientation of the molecules within the bilayer. This can be accomplished by using specifically deuterated ligands and by modifying the bicelle preparations to yield oriented bicelles.

Experimental Section

The lipid molecules for the bicelle preparations were 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dihexanoic-*sn*-glycero-3-phosphocholine (DHPC), and the acyl-chain perdeuterated D_{54}-DMPC and D_{22}-DHPC (Avanti Polar Lipids, Inc. Alabaster, AL). Deuterated sodium dodecyl sulfate (D_{25}−SDS) was purchased from Cambridge Isotope Laboratory (Andover, MA). (−)-Δ^8-tetrahydrocannabinol (Δ^8-THC), (−)-*O*-methyl-Δ^8-tetrahydrocannabinol (Me-Δ^8-THC), and ^{13}C-labeled Me-Δ^8-THC were synthesized in our laboratory. The ligand, Δ^8-THC or Me-Δ^8-THC, was first dissolved along with DMPC and DHPC in chloroform that was then evaporated using an N_2 stream. The sample was vacuum-dried overnight followed by adding an appropriate amount of D_2O (Aldrich Chemical Co., Milwaukee, WI). To improve the stability of the bicelle and prevent phase separation in dilute solutions,[38] a 0.5 mM deuterated SDS/D_2O solution, rather than pure D_2O, was added, which yielded an SDS:DMPC ratio of 1:60 for the 3% (w/v) solution and 1:160 for the 8% (w/v) bicelle system. The preparation then underwent a combination of mechanical blending, heating, and cooling until a clear and homogeneous system was obtained. Bicelle solutions were prepared in which the molar ratio of DMPC to DHPC was either 2.7:1, 2.0:1, or 0.5:1 and total lipid concentrations were 25% (w/v), 8% (w/v), or 3% (w/v), where the ligand molar concentration relative to the long acyl-chain DMPC in each solution was 10%. SDS micelle solutions containing 10% ligand were prepared by first dissolving an appropriate amount of ligand in chloroform, evaporating by an N_2 stream, and leaving a film coating at the bottom of a vial. A 12% (w/v) SDS solution was then added, and the mixture was sonicated using a Branson probe sonicator for 15 min.

All NMR experiments were carried out on a Bruker DMX-500 high-resolution spectrometer. One- and two-dimensional NMR spectra from all the bicelle preparations were acquired at 38 °C, while the samples were allowed to equilibrate at least 30 min in the magnet. ^{31}P spectra were recorded using a phase-cycled Hahn-echo pulse sequence with gated proton decoupling,[39] and the chemical shifts were externally referenced to 1 M H_3PO_4. The NMR spectra for ligands in $CDCl_3$ and SDS micelle solutions were recorded at 25 °C.

NMR constrained molecular modeling of the conformation of Δ^8-THC and Me-Δ^8-THC was achieved using the Biosym InsightII/Discover molecular modeling package on an SGI Indigo workstation. The Biosym integrated CVFF force field was employed in the calculation with a dielectric constant $\epsilon = 2$, which has been generally accepted for the lipid hydrocarbon region within a membrane bilayer.[40] A data file containing the NMR restraints was created by including the NOE-derived internuclear distances as well as dihedral angles derived from chemical shift observations. Initial energy minimization by molecular mechanics was performed to relieve any overly strained bonds. The resulting structures underwent NMR-constrained molecular dynamics, performed by heating to 1000 K and recording 100 atomic coordinate trajectories every 200 fs. The 100 frames recorded during the dynamics run were retrieved and minimized with a two-step energy minimization, using the steepest descent method for the first 100 iterations and then conjugate gradient method until the maximum derivative was less than 0.001 kcal/mol.

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References

(1) Herbette, L. G.; Chester, D. W.; Rhodes, D. G. Structural Analysis of Drug Molecules in Biological Membranes. *Biophys. J.* **1986**, *49*, 91−93.
(2) Makriyannis, A. The role of cell membranes in cannabinoid activity. *Cannabinoid Receptors*; Academic Press: London, UK, 1995; pp 87−115.
(3) Gullion, T.; Schaefer, J. Rotational-Echo Double-Resonance NMR. *J. Magn. Reson.* **1989**, *81*, 196−200.
(4) Mavromoustakos, T.; Yang, D. P.; Broderick, W.; Fournier, D.; Makriyannis, A. Small-angle X-ray diffraction studies on the topography of cannabinoids in synaptic plasma membranes. *Pharmacol. Biochem. Behav.* **1991**, *40*, 547−552.
(5) Henry, G.; Sykes, B. Strategies for the use of NMR spectroscopy in biological macromolecules and assemblies. *Bull. Can. Biochem. Soc.* **1987**, *24*, 21−26.
(6) Sanders, C. R.; Hare, B. J.; Howard, K. P.; Prestegard, J. H. Magnetically oriented phospholipid micelles as a tool for the study of membrane-associated molecules. *Prog. Nucl. Magn. Reson. Spectrosc.* **1994**, *26*, 421−444.
(7) Chou, J. J.; Kaufman, J. D.; Stahl, S. J.; Wingfield, P. T.; Bax, A. Micelle-induced curvature in a water-insoluble HIV-1 Env peptide revealed by NMR dipolar coupling measurement in stretched polyacrylamide gel. *J. Am. Chem. Soc.* **2002**, *124*, 2450−2451.
(8) Matsuda, L. A.; Lolait, S. J.; Brownstein, M. J.; Young, A. C.; Bonner, T. I. Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature* **1990**, *346*, 561−564.
(9) Munro, S.; Thomas, K. L.; Abu-Shaar, M. Molecular characterization of a peripheral receptor for cannabinoids. *Nature* **1993**, *365*, 61−65.
(10) Xie, X. Q.; Melvin, L. S.; Makriyannis, A. The conformational properties of the highly selective cannabinoid receptor ligand CP-55,940. *J. Biol. Chem.* **1996**, *271*, 10640−10647.
(11) Makriyannis, A.; Yang, D. P.; Mavromoustakos, T. Combined use of solid-state nuclear magnetic resonance spectroscopy, small-angle X-ray diffraction, and differential scanning calorimetry in studies of cannabinoid-membrane interactions. *NIDA Res. Monogr.* **1991**, *112*, 106−128.
(12) Makriyannis, A.; Yang, D. P. How to Study Drug: Membrane Interactions Using Differential Scanning Calorimetery, Solid State NMR and Small-Angle X-ray Diffraction. *Recent Advances in the Study of Neurotransmitter Receptors*; Central Drug Research Institute: Lucknow, India, 1994; pp 329−348.
(13) Yang, D. P.; Banijamali, A.; Charalambous, A.; Marciniak, G.; Makriyannis, A. Solid-state deuterium-NMR as a method for determining the orientation of cannabinoid analogues in membranes. *Pharmacol. Biochem. Behav.* **1991**, *40*, 553−557.
(14) Yang, D. P.; Mavromoustakos, T.; Beshah, K.; Makriyannis, A. Amphipathic interactions of cannabinoids with membranes. A comparison between Δ^8-THC and its O-methyl analogue using differential scanning calorimetry, X-ray diffraction and solid-state deuterium. *Biochim. Biophys. Acta* **1992**, *1103*, 25−36.
(15) Yang, D. P.; Mavromoustakos, T.; Makriyannis, A. Small-angle X-ray diffraction studies of (−)-Δ^8-tetrahydrocannabinol and its O-methyl analogue in membranes. *Life Sci.* **1993**, *53*, 117−122.
(16) Mavromoustakos, T.; Yang, D. P.; Charalambous, A.; Herbette, L. G.; Makriyannis, A. Study of the topography of cannabinoids in model membranes using X-ray diffraction. *Biochim. Biophys. Acta* **1990**, *1024*, 336−344.
(17) Mavromoustakos, T.; Yang, D. P.; Makriyannis, A. Small-angle X-ray diffraction and differential scanning calorimetric studies on O-methyl-(−)-Δ^8-tetrahydrocannabinol and its 5′ iodinated derivative in membrane bilayers. *Biochim. Biophys. Acta* **1995**, *1237*, 183−188.
(18) Mavromoustakos, T.; Yang, D. P.; Makriyannis, A. Topography and thermotropic properties of cannabinoids in brain sphingomyelin bilayers. *Life Sci.* **1996**, *59*, 1969−1979.
(19) Sanders, C. R.; Schwonek, J. P. Characterization of Magnetically Orientable Bilayers in Mixtures of Dihexanoylphosphatidylcholine and Dimyristoylphosphatidylcholine by Solid-State NMR. *Biochemistry* **1992**, *31*, 8898−8905.
(20) Sanders, C. Solid state 13C NMR of unlabeled phosphatidylcholine bilayers: spectral assignments and measurement of carbon−phosphorus dipolar couplings and 13C chemical shift anisotropy. *Biophys. J.* **1993**, *64*, 171−181.
(21) Raffard, G.; Steinbruckner, S.; Arnold, A.; Davis, J. H.; Dufourc, E. J. Temperature-Composition Diagram of dimyristoylphosphatidylcholine dicaproylphosphatidylcholine "Bicelles" Self-Orienting in the Magnetic Field. A Solid State ^{2}H and ^{31}P NMR Study. *Langmuir* **2000**, *16*, 7655−7662.

(22) Luchette, P. A.; Vetman, T. N.; Prosser, R. S.; Hancock, R. E.; Nieh, M. P. et al. Morphology of fast-tumbling bicelles: a small angle neutron scattering and NMR study. *Biochim. Biophys. Acta* **2001**, *1513*, 83−94.
(23) Sternin, E.; Nizza, D.; Gawrisch, K. Temperature Dependence of DMPC/DHPC Mixing in a Bicellar Solution and Its Structural Implications. *Langmuir* **2001**, *17*, 2610−2616.
(24) Sanders, C. R.; Landis, G. C. Reconstitution of Membrane Proteins into Lipid-Rich Bilayered Mixed Micelles for NMR Studies. *Biochemistry* **1995**, *34*, 4030−4040.
(25) Howard, K. P.; Prestegard, J. H. Conformation and Dynamics of Membrane-Bound Digalactosyldiacylglycerol. *J. Am. Chem. Soc.* **1996**, *118*, 3345−3353.
(26) Losonczi, J. A.; Prestegard, J. H. Nuclear Magnetic Resonance Characterization of the Myristoylated, N−Terminal Fragment of ADP−Ribosylation Factor 1 in a Magneticaly Oriented Memebrane Array. *Biochemistry* **1998**, *37*, 706−716.
(27) Sanders, C. R.; Landis, G. C. Facile Acquisition and Assignment of Oriented Sample NMR Spectra for Bilayer Surface-Associated Proteins. *J. Am. Chem. Soc.* **1994**, *116*, 6470−6471.
(28) Vold, R. R.; Prosser, R. S.; Deese, A. J. Isotropic Solutions of Phospholipid Bicelles: A New Membrane Mimetic for high-Resolution NMR Studies of Polypeptide. *J. Biomol. NMR* **1997**, *9*, 329−335.
(29) Whiles, J. A.; Brasseur, R.; Glover, K. J.; Melacini, G.; Komives, E. A. et al. Orientation and effects of mastoparan X on phospholipid bicelles. *Biophys. J.* **2001**, *80*, 280−293.
(30) Glover, K. J.; Whiles, J. A.; Wood, M. J.; Melacini, G.; Komives, E. A. et al. Conformational Dimorphism and Transmembrane Orientation of Prion Protein Residues 110−136 in Bicelles. *Biochemistry* **2001**, *40*, 13137−13142.
(31) Glover, K. J.; Whiles, J. A.; Wu, G.; Yu, N.-J.; Deems, R. et al. Structural evaluation of phospholipid bicelles for solution-state studies of membrane-associated biomolecules. *Biophys. J.* **2001**, *81*, 2163−2171.
(32) Glover, K. J.; Whiles, J. A.; Vold, R. R.; Melacini, G. Position of residues in transmembrane peptides with respect to the lipid bilayer: A combined lipid NOEs and water chemical exchange approach in phospholipid bicelles. *J. Biomol. NMR* **2002**, *22*, 57−64.
(33) Tjandra, N.; Bax, A. Direct Measurement of Distances and Angles in Biomolecules by NMR in a Dilute Liquid Crystalline Medium. *Science* **1997**, *278*, 1111−1114.
(34) Lee, C. W. B.; Griffin, R. G. Two-dimensional proton/C13 heternuclear chemical shift correlation spectroscopy of lipid bilayers. *Biophys. J.* **1989**, *55*, 355−358.
(35) Grant, D. M.; Cheney, B. V. Carbon-13 Magnetic Resonance. VII Steric Perturbation of the Carbon-13 Chemical Shift. *J. Am. Chem. Soc.* **1967**, *89*, 5315−5318.
(36) Makriyannis, A.; Fesik, S. Methoxy Group Conformations of Phenyl Methyl Ethers in Solution. *J. Am. Chem. Soc.* **1982**, *104*, 6462−6463.
(37) Archer, R. A.; Boyd, D. B.; Demarco, P. V.; Tyminski, I. J.; Allinger, N. L. Structural Studies of Cannabinoids. A Theoretical and Proton Magnetic Resonance Analysis. *J. Am. Chem. Soc.* **1970**, *92*, 5200−5206.
(38) Losonczi, J. A.; Prestegard, J. H. Improved Dilute Bicelle Solutions for High-Resolution NMR of Biological Macromolecules. *J. Biomol. NMR* **1998**, *12*, 447−451.
(39) Rance, M.; Byrd, R. A. Obtaining high-fidelity spin-1/2 powder spectra in anisotropic media: phase-cycled Hahn echo spectroscopy. *J. Magn. Reson.* **1983**, *52*, 221−240.
(40) Marsh, D. Polarity and permeation profiles in lipid membranes. *Proc. Natl. Acad. Sci.* **2001**, *98*, 7777−7782.

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