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Stereochemical Analysis of Glycerophospholipids by Vibrational Circular Dichroism

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Supporting Information

ABSTRACT: The stereochemistry of glycerophospholipids (GPLs) has been of interest for its roles in the evolution of life and in their biological activity. However, because of their structural complexity, no convenient method to determine their configuration has been reported. In this work, through the first systematic application of vibrational circular dichroism (VCD) spectroscopy to various diacylated GPLs, we have revealed that their chirality can be assigned by the sign of a VCD exciton couplet generated by the interaction of two carbonyl groups. This paper also presents spectroscopic evidence for the stereochemistry of GPLs isolated from bacteria, eukaryotes, and mitochondria.

Glycerophospholipids (GPLs) are generally the most abundant lipids in all mammalian membranes.[1] Not only do these molecules provide suitable membranous environments for various biomolecules to function, but also, some of them are known to act as signaling molecules called lipid mediators to regulate cell behavior.[2] GPLs consist of a polar phosphoester headgroup, a prochiral glycerol, and acyl chains that typically range from C_{14} to C_{20} with various levels of unsaturation. Phosphoesterification to glycerol was believed to occur solely at the *sn*-3 position in eukaryotic and bacterial GPLs but at the *sn*-1 position in archaeal ones (Figure 1a), which led to several hypotheses concerning the pivotal roles of their chirality in the evolution of life.[3] Interestingly, the presence of an unusual *sn*-1-phosphorylated mammalian GPL, bis(monoacylglycero)phosphate (BMP),[4] was confirmed in a recent study.[5] This finding has highlighted the need for exploration of other GPLs with unnatural configuration and verification of the previously speculated stereochemistry to obtain further insight into the evolution of life. Such stereochemical studies may also lead to the development of the biochemistry of GPLs with abnormal chirality, similar to the blossoming field of D-amino acids.[6] Nonetheless, because of the structural diversity of GPLs, no universal method to determine their chirality has been reported.

GPLs are composed of several classes of molecules that are categorized according to the structure of the headgroup. The major classes include phosphatidylcholines (PCs), phosphoethanolamines (PEs), phosphatidylserines (PSs), and cardiolipins (CLs), and there are other classes such as BMPs (Figure 1b). Conventional enzymatic stereochemical analysis necessitates a variety of enzymes, which has limited its applicability to the repertoire of GPLs. Furthermore, enzymatic results can be ambiguous when their stereospecificity is not thoroughly examined or when harsh reaction conditions are involved as a pre- or post-treatment.[4a,7] Other methods such as NMR analysis of diastereomeric derivatives[5] and chiral HPLC[7] require an array of authentic compounds.

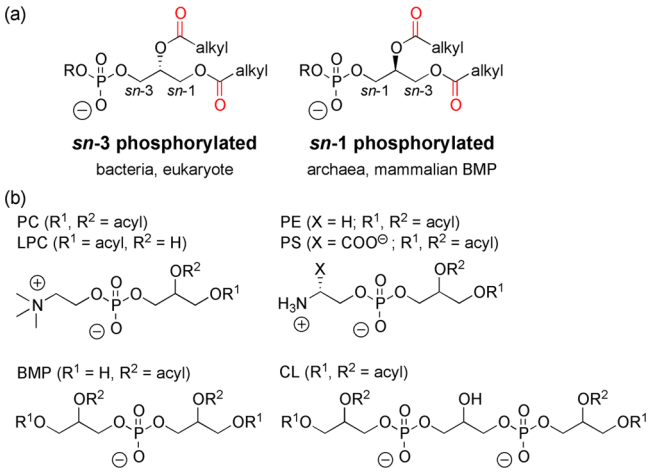

Figure 1. (a) Stereochemistry of glycerophospholipids. (b) Representative structures of the glycerophospholipids studied in this work. PC = phosphatidylcholine; LPC = lysophosphatidylcholine; PE = phosphoethanolamine; PS = phosphatidylserine; BMP = bis-(monoacylglycero)phosphate; CL = cardiolipin.

Seeking a direct analytical method that obviates the need for authentic samples, we thought of applying vibrational circular dichroism (VCD) spectroscopy. The VCD technique combined with density functional theory (DFT) calculations has become one of the most reliable methods to determine the chirality of small molecules.[8] However, GPLs may not be amenable to normal computation because of their amphiphilicity, flexibility, and structural complexity. These properties have also deterred a systematic VCD study of biological lipids, in stark contrast to widely studied biomolecules such as proteins, nucleic acids, and glycoconjugates.[9] Recently, inspired by the electronic CD (ECD) exciton chirality method by Harada and Nakanishi,[10] we found that two C═O groups in

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close vicinity exhibit an intense bisignate VCD signal whose sign reflects the chirality of the molecule.[11] This result led us to hypothesize that VCD signals originating from the carbonyls in diacylated GPLs could be indicative of their stereochemistry. In the present work, through the first systematic VCD measurements of GPLs, we demonstrate that the chirality of various GPLs can be determined using a VCD exciton approach.

A fundamental challenge to our hypothesis is the possible fluctuation of the orientation of the C═O groups in their acyclic structures, unlike the rigid cyclic molecules we studied previously.[11] Therefore, it was necessary to examine whether two carbonyl groups exhibit a meaningful VCD signal and whether the sign of the couplet is consistent for different classes of GPLs. To this end, we first measured the VCD spectra of phosphatidylcholines, a representative class of GPLs. Both *sn*-3 and *sn*-1 phosphatidylcholines were chemically synthesized (see the Supporting Information, (SI)), and their VCD spectra were measured in $CDCl_3$. Figure 2a shows the VCD results for

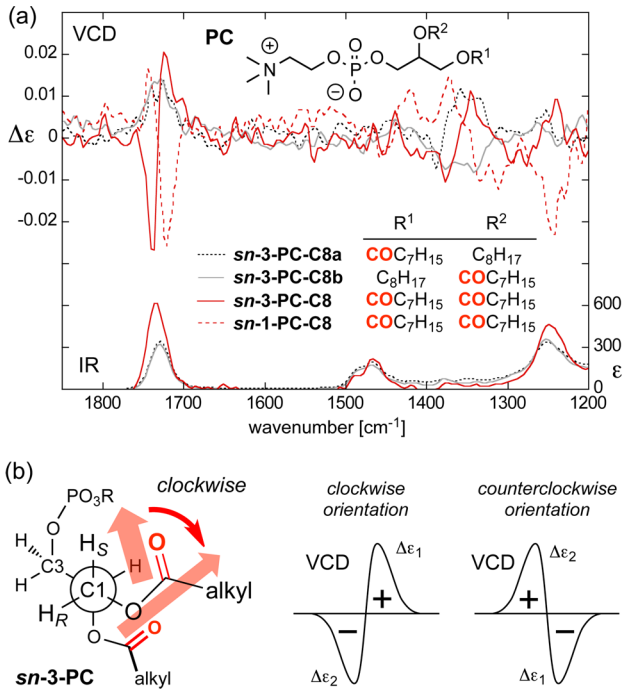

Figure 2. (a) VCD and IR spectra of phosphatidylcholines. Each spectrum was measured in $CDCl_3$ (l = 100 μm) at a concentration of 0.1 M (***sn*-3-PC-C8a** and ***sn*-3-PC-C8b**) or 0.08 M (***sn*-3-PC-C8** and ***sn*-1-PC-C8**). (b) Schematic orientation of the two carbonyl groups of ***sn*-3-PC** (left) and the relationship between the arrangement of the electric transition moments (red arrows parallel to the C═O bonds) and the sign of the VCD couplet (right). The carbonyl orientation is depicted on the basis of the predicted stable conformers of ***sn*-3-PC-C4**, where the C═O group at C1 prefers a *syn* orientation with regard to C1−H_S (see Figure S2).

phosphatidylcholines with C_8 acyl chains (**PC-C8**). Despite our concern, a characteristic bisignate signal was observed in the carbonyl stretching region: positive−negative (from lower to higher frequency) for the *sn*-3 enantiomer and negative−positive for the *sn*-1 enantiomer. On the other hand, only a broad positive band was seen for the monoacyl monoalkyl derivatives **PC-C8a** and **PC-C8b**. This comparison suggested that the observed VCD couplet of **PC-C8** was produced by the intramolecular interaction of the two carbonyl groups.[12] The weak intensities of their VCD signals compared with those observed for cyclic molecules[11] are probably due to the conformational flexibility of GPLs. Importantly, the sign of the exciton signals was not affected by biologically abundant longer saturated and unsaturated acyl chains (**PC-C18** and **PC-C18Δ^9**) (Table 1 and Figure S1). These results proposed that the stereochemistry of phosphatidylcholines can be assigned simply by observing their carbonyl stretching VCD, irrespective of the types of their acyl chains.

According to the coupled oscillator model,[13] the positive−negative couplet in *sn*-3 phosphatidylcholines corresponds to a clockwise carbonyl orientation. To better understand the tolerance of the couplet to acyl chains, a preliminary conformational calculation was performed for a phosphatidylcholine with C_4 acyl chains (***sn*-3-PC-C4**) to see the geometry around the glycerol core (see the SI for details of the calculation procedure and the choice of the model compound).[14,15] Molecular mechanics force field (MMFF) and subsequent DFT calculations yielded multiple stable conformers, in accordance with its expected flexibility. Among them, the conformers with a clockwise carbonyl twist are predominant over those with a counterclockwise twist (Figures 2b and S2). The linear fatty acid chains point away from the glycerol moiety and thus are less likely to interfere with the orientation of the C═O groups, which corroborates the validity of the VCD analysis of the chirality of various phosphatidylcholines.

The applicability to other types of headgroup was then confirmed by a study of a phosphatidylserine and BMP. As summarized in Table 1, the L-serine in **PS-C8** did not alter the sign of the VCD couplet. A similar positive−negative pattern was also observed for a dimeric *sn*-3,3′ BMP derivative that has four acyl chains (**BMP-C8c**). Meanwhile, **BMP-C8** did not show a couplet in spite of the presence of two carbonyl groups (Figure 3). These results indicated that the C═O groups at the *sn*-1 and -2 positions do not cause any significant excitonic interaction with those at the *sn*-1′ and -2′ positions. Such independence of each glycerol moiety is advantageous in applying this VCD method to GPLs with other headgroups.

Having verified the compatibility of the VCD exciton approach with various GPL structures, we applied this method to a synthetic BMP with exceptional *sn*-1 stereochemistry (***sn*-1,1′-BMP-C8**) and a lysophosphatidylcholine (LPC), a lipid mediator, isolated from avian egg (**LPC-C16**). In the case of ***sn*-1,1′-BMP-C8**, which did not show an exciton VCD in its intact state, two more octanoyl groups were introduced using Yamaguchi reagent in a manner similar to a reported procedure.[16] The resultant ***sn*-1,1′-BMP-C8c** yielded a negative−positive couplet, consistent with its *sn*-1,1′ configuration (Figure 3a). Similarly, installation of a C_{16} fatty acid into **LPC-16** led to a positive−negative VCD couplet, which unambiguously confirmed its eukaryotic *sn*-3 configuration (Figure 3b).

The utility of this method for other complex lipids was examined in the analysis of cardiolipins from *Escherichia coli* (**CL-*EC***) and bovine heart (**CL-Mt**, where **Mt** refers to mitochondria, as the biosynthesis of cardiolipins in eukaryotic cells occurs exclusively in mitochondria). **CL-*EC*** and **CL-Mt** not only belong to one of the most structurally complex classes of GPL but also are composed of mixtures of isomers differing in acyl chains (see Figure S3), which poses difficulties in the analysis of their chirality by other methods. Nevertheless, both cardiolipins yielded positive−negative couplets that were clearly

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Table 1. VCD Couplets of Glycerophospholipids[a]

GPL	R^1 [b]	R^2 [b]	$\Delta\varepsilon_1$ [c] (ν [cm^{-1}])	$\Delta\varepsilon_2$ [c] (ν [cm^{-1}])
***sn*-3-PC-C8a**	COC_7H_{15}	C_8H_{17}	+0.014 (1724)[d]	
***sn*-3-PC-C8b**	C_8H_{17}	COC_7H_{15}	+0.014 (1728)[d]	
***sn*-3-PC-C8**	COC_7H_{15}	COC_7H_{15}	+0.020 (1724)	−0.027 (1740)
***sn*-3-PC-C18**	$COC_{17}H_{35}$	$COC_{17}H_{35}$	+0.010 (1724)	−0.013 (1740)
***sn*-3-PC-C18Δ^9**	$COC_{17}H_{33}$	$COC_{17}H_{33}$	+0.012 (1720)	−0.021 (1740)
***sn*-1-PC-C8**	COC_7H_{15}	COC_7H_{15}	−0.026 (1724)	+0.017 (1744)
***sn*-1-PC-C18**	$COC_{17}H_{35}$	$COC_{17}H_{35}$	−0.014 (1724)	+0.011 (1740)
***sn*-1-PC-C18Δ^9**	$COC_{17}H_{33}$	$COC_{17}H_{33}$	−0.019 (1720)	+0.011 (1736)
***sn*-3-PS-C8**[e]	COC_7H_{15}	COC_7H_{15}	+0.027 (1717)	−0.011 (1736)
***sn*-1,1′-BMP-C8**[e]	H	COC_7H_{15}	ND[f]	
***sn*-1,1′-BMP-C8c**[e]	COC_7H_{15}	COC_7H_{15}	−0.029 (1713)	+0.038 (1740)
***sn*-3,3′-BMP-C8c**[e]	COC_7H_{15}	COC_7H_{15}	+0.028 (1720)	−0.027 (1740)
PC-C16[g]	$COC_{15}H_{31}$	$COC_{15}H_{31}$	+0.010 (1724)	−0.013 (1740)
CL-*EC*[e,h]	mixtures ranging from $COC_{13}H_{27}$ to $COC_{18}H_{37}$[i]		+0.018 (1724)	−0.038 (1736)
CL-Mt[e,j]	mostly $COC_{17}H_{31}$[i]		+0.035 (1717)	−0.025 (1740)

[a]Measured in $CDCl_3$ (l = 100 μm, c = 0.03−0.1 M). [b]See Figure 1 for the parent structure of each GPL. [c]Intensities at the extrema of each VCD band, in M^{-1} cm^{-1}. [d]Only a monosignate band was observed. [e]Sodium salt. [f]No significant band was detected. [g]Derived from avian egg **LPC-C16**. [h]From *E. coli*. [i]See Figure S3. [j]From bovine heart.

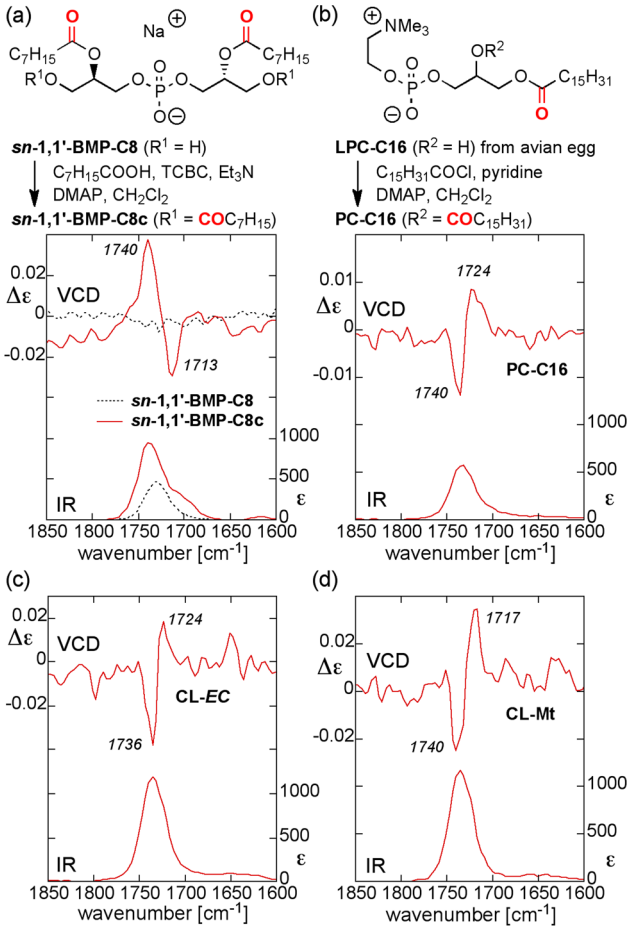

Figure 3. VCD and IR spectra of (a) a synthetic BMP and its derivative, (b) phosphatidylcholine derived from avian egg lysophosphatidylcholine, (c) cardiolipin from *E. coli*, and (d) cardiolipin from bovine heart. Each spectrum was measured in $CDCl_3$ (l = 100 μm) at a concentration of 0.1 M (**PC-C16**) or 0.05 M (others). The concentrations of **CL-*EC*** and **CL-Mt** were calculated on the basis of their average molecular weights. Wavenumbers at the VCD extrema are labeled in italics. The derivatization schemes are shown in (a) and (b). TCBC = 2,4,6-trichlorobenzoyl chloride.

recognized even with a relatively small amount of sample (Figure 3c,d). Thus, simple observation of VCD exciton signals allowed spectroscopic verification of their *sn*-3 stereochemistry. Further development of more sensitive VCD spectrometers should allow chiral analysis of even smaller amounts of GPLs in the future.[8c,17]

This analytical method relies on the conformational preference of GPLs in $CDCl_3$.[18] Therefore, GPLs not soluble enough to $CDCl_3$ may require chemical modification to increase the solubility, as often needed in computation-based VCD protocols.[19] On the other hand, extensive VCD studies on GPL conformations in liposomes or micelles may be of interest, especially in relation to their functions in cell membranes.

Lastly, this work has shown the effectiveness of the VCD exciton approach for the stereochemical analysis of acyclic molecules. Considering the success of the ECD exciton chirality method for acyclic systems,[10b,20] the VCD method also should be of help in solving stereochemical problems of various acyclic molecules that are abundant in nature.[21]

In summary, we have established a convenient method to assign the stereochemistry of GPLs based on the sign of bisignate VCD signals in the C═O stretching region. Bacterial and eukaryotic *sn*-3 GPLs exhibited a positive−negative couplet, whereas the *sn*-1 configuration, which is characteristic of archaeal GPLs and mammalian BMPs, showed a negative−positive couplet. The applicability of this method to structurally complex GPLs such as BMPs and isomeric mixtures of cardiolipins was demonstrated. This work has also presented spectroscopic evidence of the chirality of GPLs originating from bacteria, eukaryotes, and mitochondria. Through the elucidation of the chirality of GPLs, this VCD approach should provide further insight into the evolution of life and advance the biochemistry of GPLs with irregular stereochemistry.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b05832.

Experimental details and additional VCD, DLS, and structural data (PDF)

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Notes

The authors declare no competing financial interest.

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