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An Effective Method for the Evaluation of the Enantiomeric Purity of 1,2-Diacyl-*sn*-glycero-3-phosphocholine-Based Lipids by NMR Analysis

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Abstract: In this article, we report a very efficient method for the determination of the enantiopurity of 1,2-diacyl-*sn*-glycero-3-phosphocholine by ^{1}H NMR analysis using a readily available chiral derivatizing boronic acid (CDA), (*R*)-(2-(((1-phenylethyl)amino)methyl)phenyl)boronic acid. After the removal of the acyl groups of 1,2-diacyl-*sn*-glycero-3-phosphocholine via methanolysis and washing fatty acid byproducts with $CHCl_3$, the obtained *sn*-glycero-3-phosphocholine (GPC) with the free diol moiety is derivatized by the chiral boronic acid and analyzed by ^{1}H NMR analysis. The choline methyl resonance of each diastereomer is observed at distinctive chemical shifts in the ^{1}H NMR spectrum. Integration of the respective resonances allows direct determination of the enantiomeric purity. The procedure was tested successfully using 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) with different enantiomeric purities and with commercially available 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC).

Keywords: glycerophospholipids; enantiomeric purity; 1,2-diacyl-*sn*-glycero-3-phosphocholine; DMPC; DPPC; DSPC; chiral derivatizing agent (CDA)

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1. Introduction

Glycerophospholipids (GPLs) are the main components of the membrane bilayer of normal cells. GPLs are chiral molecules formed by a glycerol unit which is esterified at *sn*-1 and *sn*-2 positions with fatty acids and at the *sn*-3 position with a phosphate group (Figure 1). The phosphate group can also be bonded to small molecules, usually choline. Natural phospholipids are composed of a variety of fatty acids; usually, one is a saturated fatty acid such as palmitic acid, and the other is an unsaturated fatty acid such as oleic acid [1].

However, natural phospholipids are less stable in liposome vesicle preparation due to the unsaturated nature of the hydrocarbon chain [2,3]. Conversely, synthetic saturated glycerophospholipids, such as 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), are widely used in liposome nanoparticles LNP for the delivery of mRNA in SARS-COV 2 vaccines, as well as for other drug delivery systems [4]. It is noteworthy that natural GPLs belong to L-series, and as for L-amino acids and D-carbohydrates, their chirality plays important roles in biological systems [5]. Lipid chirality has been shown to affect membrane stability and functions [6–10]. In addition, specific interactions of chiral small molecules or peptides with membranes of enantiopure phospholipids have been disclosed [11,12]. It has been demonstrated that chiral separation of racemic amino acids can be performed using a L-DPPC membrane [12]. Considering the increasing number of applications of synthetic GPLs, in recent years great efforts have been directed towards their synthesis. GPLs are usually prepared by esterification of *sn*-glycero-3-phosphocholine (L-α-GPC)

or by transformation of (*R*)-glycidol [13,14]. L-α-GPC is produced both from hydrolysis of natural sources of GPLs, which requires very complex purification procedures, or by chemical transformations of chiral starting materials, such as (*R*)-epichlorohydrin, (*R*)-glycidol, or (*R*)-3-chloro-propane-1,2-diol (3-MCPD) [13–15]. The increasing demand for synthetic GPLs for the pharmaceutical, cosmetic, and food industries poses issues related to enantiomeric purity, which can affect properties and functions [6–12]. This can be especially problematic when the synthetic pathway is not based on the use of L-α-GPC from natural sources, but rather on enantioenriched starting materials, such as (*R*)-epichlorohydrin, (*R*)-glycidol, or (*R*)-3-chloro-propane-1,2-diol (3-MCPD), which are produced by asymmetric synthesis. To our knowledge, determination of the enantiopurity of GPLs has always been considered a difficult task [16]. Itabashi reported only partial resolution on chiral stationary phase HPLC analysis [16], while VCD chiroptical methods were used to determine GPL's absolute configuration [17]. Therefore, the development of new analytical methods for determining the enantiomeric purity of GPLs is highly desirable.

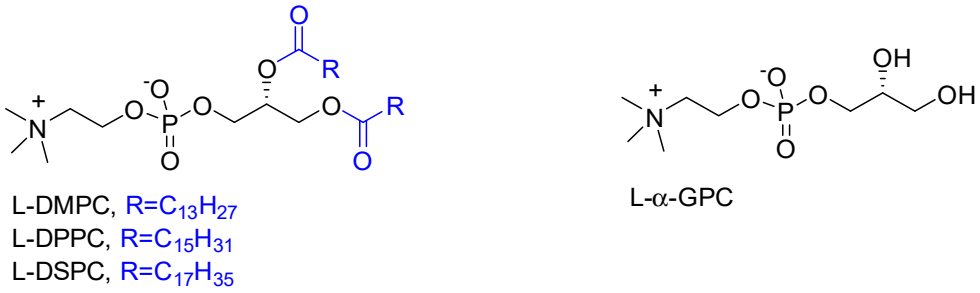


Figure 1. Structures of common synthetic 1,2-diacyl-*sn*-glycero-3-phosphocholine (L-DMPC: 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; L-DPPC: 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; L-DSPC: 1,2-distearoyl-*sn*-glycero-3-phosphocholine); and *sn*-glycero-3-phosphocholine (L-α-GPC).

Recently, we have reported a protocol for the determination of the enantiopurity of L-α-GPC [15], a drug that also has many applications [18,19], by NMR analysis. This protocol is based on the use of a readily available chiral boronic acid, (*R*)-(2-(((1-phenylethyl)amino)methyl)phenyl)boronic acid, as a chiral derivatizing agent (CDA) [15,20,21], which reacts with the 1,2-diol moiety of GPC to form diastereomeric cyclic boronate esters. ^{1}H NMR spectroscopic analysis of the reaction mixture allowed the determination of the enantiomeric purity by the integration of well-resolved diastereomeric resonances of the choline group [15]. Based on these previous findings, we believed that this method could be easily applied to the determination of the enantiopurity of 1,2-diacyl-*sn*-glycero-3-phosphocholine. Within our interest in the analysis of the composition and structure of modified biomolecules by NMR spectroscopy [15,22], herein we describe a very efficient method for the determination of the enantiopurity of 1,2-diacyl-*sn*-glycero-3-phosphocholine.

2. Materials and Methods

2.1. General Information

Enantiopure phospholipids (DMPC, DPPC, and DSPC), racemic DPPC, $NaOCH_3$ 25% wt, 2-formylbenzeneboronic acid, (*R*)-α-methylbenzyl amine, $NaBH(OAc)_3$, 1,2-dichloroethane, and DMSO-d_6 were purchased by Merck. (*R*)-(2-(((1-phenylethyl)amino)methyl)phenyl)boronic acid was prepared by modification of methodologies already reported in the literature (see Section 2.2) [15,20,21]. All the reactions were performed under a nitrogen atmosphere. The NMR spectra were recorded on Bruker DRX 600 or 400 MHz spectrometers, equipped with PA BBO 400 and 600 S1 BBF-H-D05 Z PLUS probes, respectively. NMR spectra were recorded at 298 ± 0.1 K (temperature control BCU 05, BVT 3000). DMSO-d_6 was used for internal lock purposes; chemical shifts (δ) are expressed in parts per million (ppm), and a DMSO residual signal was used as a reference (δ = 2.50 ppm). High-resolution

mass spectrum (HRMS) was acquired by using a Bruker SolariX XR Fourier transform ion cyclotron resonance mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) equipped with a 7T refrigerated actively-shielded superconducting magnet. For ionization of the samples, electrospray ionization (ESI) or MALDI was applied.

2.2. Synthesis of (R)-(2-(((1-Phenylethyl)amino)methyl)phenyl)boronic Acid ((R)-CDA) [20,21]

First, 2-Formylbenzeneboronic acid (1.50 mmol, 225 mg) was dissolved in anhydrous 1,2-dichloroethane (6.0 mL) under nitrogen in the presence of activated 4Å molecular sieves (0.5 g). After the slow addition of (*R*)-α-methylbenzyl amine (1.50 mmol, 182 mg), the solution was stirred at r.t. for 16 hr. AcOH (1.50 mmol, 90 mg, 90 µL) was added, followed by $NaBH(OAc)_3$ (2.36 mmol, 500 mg), and the solution was stirred for a further 6 h. The solvent was removed under vacuum and the residue was diluted with CH_2Cl_2 (25 mL) before the precipitate was removed with vacuum filtration. The filtrate was concentrated and the residue was purified on neutral alumina column chromatography (0–2% CH_3OH in CH_2Cl_2) to afford the pure product 75% yield (287 mg).

(*R*)-CDA: ^{1}H NMR (400 MHz, CD_3OD) 7.53–7.39 (m, 6H), 7.23–7.12 (m, 2H), 7.00 (d, J = 7.0 Hz, 1H), 4.23 (q, J = 6.8 Hz, 1H), 3.81 (d, J = 13.6 Hz, 1H), 3.67 (d, J = 13.2 Hz, 1H), 1.68 (d, J = 6.8Hz, 3H). HRMS calculated by $(M + H)^+$ calc 256.1509 found 256.1514.

2.3. Procedure for Phospholipid Methanolysis

First, 100 mg of L-DPPC was dissolved in 1 mL of dry methanol. Then, 100 µL of $NaOCH_3$ 25% wt in methanol was added until pH = 12. Stirring was continued for 2 h. Then, pH was adjusted to 6 first by the addition of 1.0 M H_2SO_4 (0.235 mL) and then with 0.10 M H_2SO_4 (0.230 mL). A white solid precipitated and the filtrate was concentrated by rotavapor, giving a mixture of GPC and methyl esters of palmitic acid. After drying under vacuum, the mixture was washed twice with chloroform to remove the methyl esters, obtaining L-GPC (25 mg).

2.4. Procedure for GPC-Chiral Boronic Acid Reaction

The obtained GPC was completely dissolved in a known amount of MeOH and an aliquot containing 5 mg was taken and dried under vacuum before setting up the reaction. Under a nitrogen atmosphere, 5 mg (1 equiv.) of GPC were solubilized in anhydrous DMSO-d_6 (0.750 mL) and the mixture was stirred for 30 min at 65 °C. Then, 350 mg of activated 4Å molecular sieves were added, and stirring (rpm 50) was continued for 1 h. Temperature was reduced to 50 °C and the chiral boronic acid (2 equiv.) was added, keeping the reaction for 24 h (rpm 50). Then, the solution was transferred into an NMR tube, further diluted with 350 µL of anhydrous DMSO-d_6 and analyzed by ^{1}H NMR.

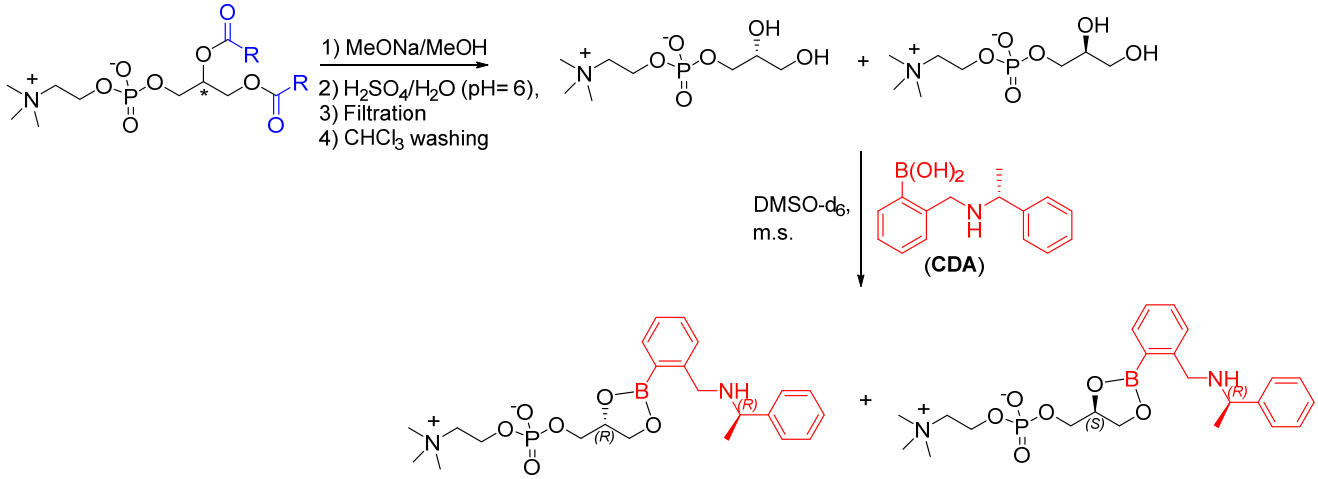
2.5. Procedure for Analysis of GPC-Chiral Boronic Acid Adduct by ^{1}H NMR

All ^{1}H NMR experiments were carried out using pulse sequences supplied by the spectrometer manufacturer (BRUKER—TOPSPIN 2.1). Prior to Fourier transformation, a Lorentz-Gauss transform (GFP function) was applied to the free induction decays (FIDs), with line broadening parameter LB= −1.60 Hz and Gaussian parameter GB = 0.3 (GB determines the position of the Gaussian within the acquisition time window, $0 < GB < 1$). The resulting spectra were automatically phased and baseline corrected.

3. Results and Discussion

In the first part of the work, we developed a procedure to easily obtain GPC from the respective GPLs on a relatively small scale, which can then be analyzed by reaction with the chiral boronic acid. To this purpose, 100 mg of commercially available 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (L-DPPC) was treated with MeONa solution in MeOH under conditions already reported for L-α-GPC production from a mixture of natural phospholipids contained in soy lecithin [23]. Attempts to perform ^{1}H NMR analysis after the chiral boronic acid derivatization directly on the reaction crude were unsuccessful because of the

complexity of the reaction mixture, which did not lead to well-resolved spectra. Therefore, we thought that the purification and isolation of L-α-GPC was a necessary step. According to the literature, the purification and isolation of L-α-GPC obtained from soy lecithin is usually carried out by multiple ionic exchange chromatography using water as an eluent, followed by crystallization [24]. However, we envisioned the possibility of avoiding such a long and tedious procedure, considering the different natures of the polar L-α-GPC and the apolar methyl esters of fatty acids obtained during transesterification. Therefore, after performing the methanolysis of DPPC, simply washing the crude product with chloroform was sufficient to remove the methyl esters of the palmitoyl fatty acid, leaving the L-α-GPC in the flask in good yield and good purity, as confirmed by ^{1}H NMR analysis (Scheme 1). Then, the recovered L-α-GPC was subjected to a reaction with the chiral boronic acid (enantiomeric purity >99.9% ee) [15,20,21] in deuterated DMSO-d$_6$ in the presence of activated 4Å molecular sieves.



Scheme 1. Sequence of reactions and operations for the chiral derivatization of 1,2-diacyl-*sn*-glycero-3-phosphocholine (GPL).

The ^{1}H NMR spectrum of the reaction mixture was compared with those obtained from the reaction of commercially available *rac*-GPC with the chiral boronic acid (Figure 2a). This comparison highlighted a very high enantiomeric purity of the analyzed L-DPPC. No residual peak was observed at 3.08 ppm corresponding to the resonance of the choline moiety of the (*S*,*R*) diastereomer derived from the D-α-GPC with (*S*) absolute configuration. As demonstrated in previous work [15], the peak at 3.13 ppm belongs to unreacted L-α-GPC, but the presence of unreacted starting material does not affect the analysis since kinetic resolution is not detected in this chiral derivatization [15]. High-resolution mass spectrum analysis (HRMS) was also performed directly from the reaction mixture in DMSO-d$_6$, confirming the formation of the boronate ester (see Supplementary Materials for details).

In order to highlight the resonances of the choline moiety of the two boronate diastereomers and to further validate the protocol, different samples of DPPC at 90:10, 95:5, and 99.5:0.5 enantiomeric ratio were prepared mixing enantiopure L-DPPC and *rac*-DPPC and then subjected to the process described in Scheme 1. All the ^{1}H NMR analyses were performed with a 400 MHz instrument. To increase the resolution of the peaks, prior to Fourier transformation, free induction decays (FIDs) were multiplied by an exponential with a positive exponent and by a Gaussian with a negative exponent (see Section 2.4 for further details). This procedure does not significantly distort the spectrum, as can be inferred by the close values of integrated areas obtained by non-linear least squares of the spectrum as a sum of Lorentzians (Figure 3) [25]. Only for the analysis of the mixture at 99.5:0.5 er we used the 600 MHz NMR instrument to further increase the sensitivity and resolution of the spectrum. In all the cases, the presence of the diastereomeric resonance corresponding to the D-enantiomer of the GPC was clear at 3.08 ppm, and the integrals

were in agreement with the expected enantiomeric composition (Figure 3b-d). Nicely, in the sample at 99.5:0.5 er, prepared mixing of 99 mg of L-DPPC and 1 mg of *rac*-DPPC gave a good agreement (Figure 2d). As can be observed, the peak corresponding to the minor diastereomer is well resolved and evident, while in the presence of commercially available L-DPPC, no residual peak of the (*S*,*R*) diastereomer was observed (Figure 2a).

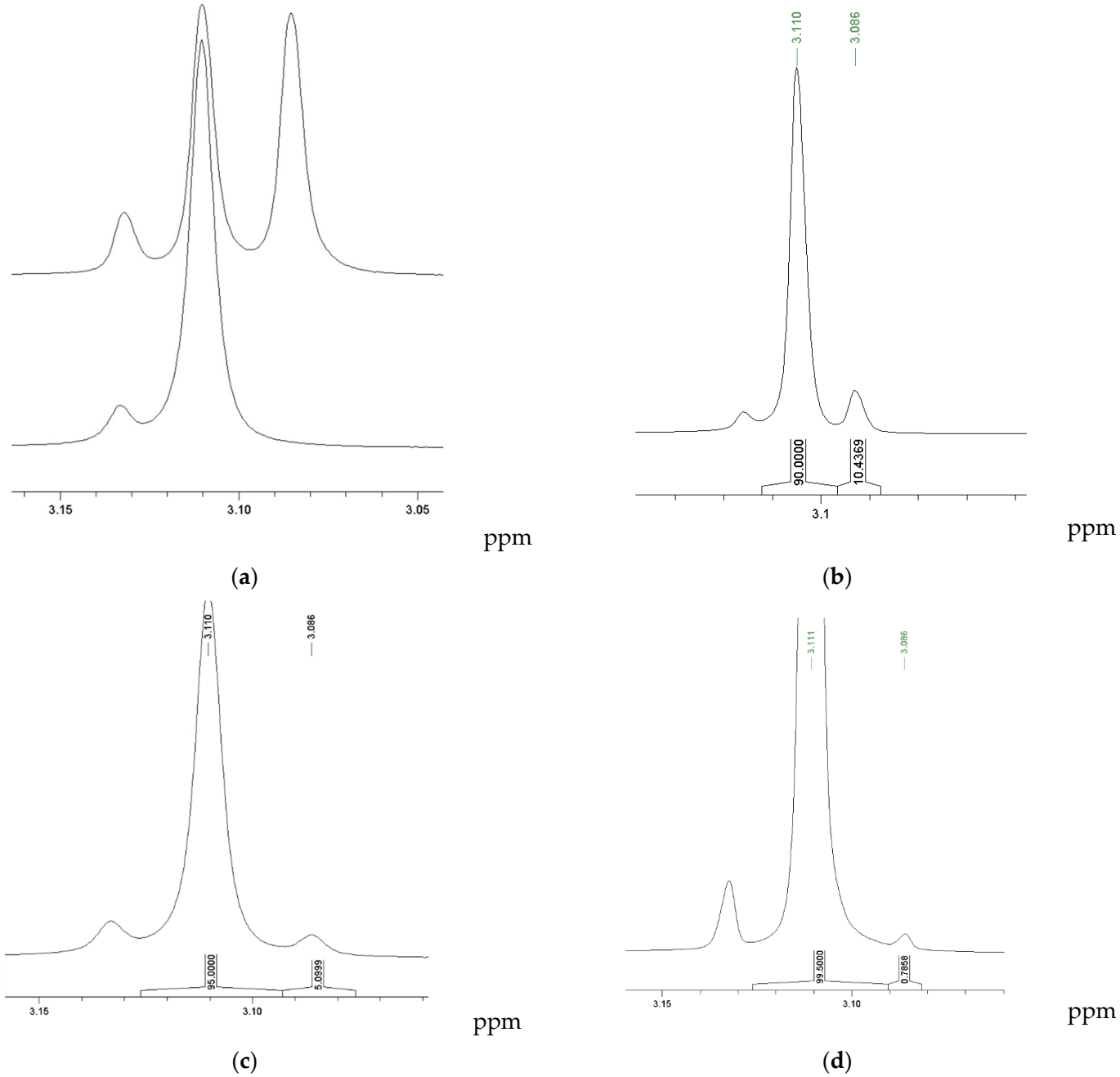


Figure 2. Portion of ^{1}H NMR resolution enhanced spectra (ppm in abscissa) displaying choline resonance of GPC-boronate product. (**a**) Superimposed spectra obtained from *rac*-GPC (upper) and commercially available L-DPPC (lower). Analysis of DPPC samples at: (**b**) 90/10 er, (**c**) 95/5 er, (**d**) 99.5/0.5 er.

The isolated yields of GPC after methanolysis were not quantitative, usually in the range of 70–75%. Probably, part of the GPC is lost during pH adjustment at the end of the transesterification, which leads to salt precipitation and subsequent filtration. Since only a

part of the obtained product (5 mg) is necessary for reaction with the chiral boronic acid, the GPC was completely dissolved in MeOH, and only an aliquot was taken to prepare a homogeneous sample of about 5 mg. According to the spectra shown in Figure 2b–d, the analysis of non-enantiopure samples guarantees that enantio-enrichment or kinetic resolution is not occurring during the treatment.

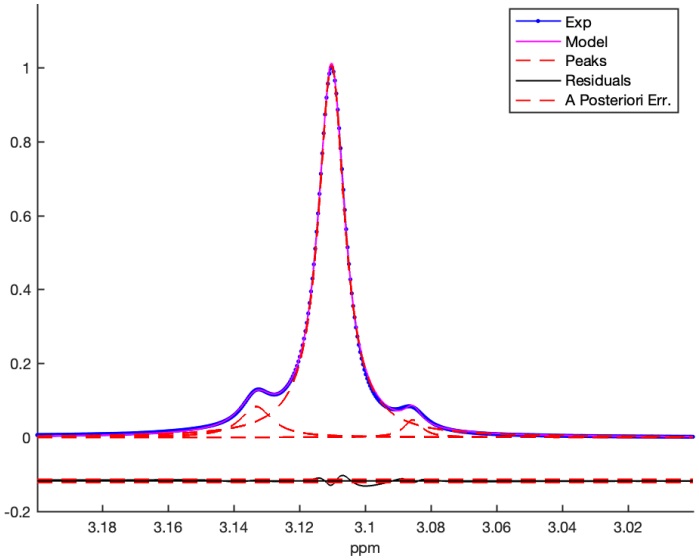


Figure 3. Portion of the ^{1}H NMR spectrum of the prepared DPPC sample with 95/5 er. The experimental spectrum (blue dots) is fitted as a sum of 3 Lorentzian functions. Residuals are shown below, together with the standard error of the fit (dashed red line). Normalizing the integral of the highest peak to 95, integrated areas of the 3 peaks are, from left to right, 9.42(16), 95.00(15), and 2.98(11), where the digits in parentheses are standard deviations obtained by linearized least squares.

Commercially available 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) were also analyzed according to the procedure shown in Scheme 1. In both cases, we were able to easily isolate the L-α-GPC in good yields after methanolysis. Then, the treatment of the obtained L-α-GPC with the chiral boronic acid in DMSO-d_6 and subsequent ^{1}H NMR analysis also showed high enantiomeric purity for both, demonstrating that the method can be extended to all the synthetic 1,2-diacyl-*sn*-glycero-3-phosphocholine with high precision and reproducibility (see Figure S1 of Supplementary Materials).

4. Conclusions

In this work, we report a very efficient method for the analysis of enantiopurity of readily available synthetic 1,2-diacyl-*sn*-glycero-3-phosphocholine (DMPC, DPPC, and DSPC) by ^{1}H NMR analysis after chiral derivatization with a chiral derivatizing boronic acid (CDA), (*R*)-(2-(((1-phenylethyl)amino)methyl)phenyl)boronic acid. In particular, the treatment of the glycerophospholipids with MeONa in MeOH, followed by washing the crude with chloroform, led to the isolation of *sn*-glycero-3-phosphocholine (L-α-GPC), which was then analyzed by derivatization with a readily available chiral boronic acid. The ^{1}H NMR analysis of the prepared enantioenriched mixture of DPPC in DMSO-d_6 at different enantiomeric ratios led to the identification of two distinct resonances belonging to the choline moiety of the two diastereomeric boronates, whose integrals were in agreement with the expected enantiomeric purity of the samples.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/sym16050624/s1, copies of ^{1}H NMR and HRMS spectra.

Author Contributions: A.M. conceived the project; All authors contributed to the experiment designs; A.D.M. carried out the experiments; A.M. directed the research. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data underlying this study are available in the published article and its Supporting Information.

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Conflicts of Interest: The authors declare no competing interests.

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