Enzyme-Assisted Synthesis of High-Purity, Chain-Deuterated 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine

Oliver Bogojevic and Anna E. Leung*

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ABSTRACT: 1-Palmitoyl-d₁₃-2-oleoyl-d₆₋₂-sn-glycero-3-phosphocholine (POPC-d₆₃) with the palmitoyl and oleoyl chains deuterium-labeled was produced in three steps from 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine, deuterated palmitic acid, and deuterated oleic anhydride. Esterification at the sn-2 position was achieved under standard chemical conditions, using DMAP to catalyze the reaction between the 2-lysolipid and oleic anhydride-d₆₄. Complete regioselective sn-1 acyl substitution was achieved in two steps using operationally simple, enzyme-catalyzed regioselective hydrolysis and esterification to substitute the sn-1 chain for a perdeuterated analogue. This method provides chain-deuterated POPC with high chemical purity (>96%) and complete regio purity, useful for a variety of experimental techniques. This chemoenzymatic semisynthetic approach is a general, modular method of producing highly pure, mixed-acyl phospholipids, where the advantages of both chemical synthesis (efficiency, high yields) and biocatalytic synthesis (specificity, nontoxicity) are realized.

INTRODUCTION

Phospholipids are an important component of cellular membranes, and the role they play in the structure and function of these membranes is of interest for many fields of study. Along with other techniques, they are commonly utilized in X-ray and neutron scattering experiments,¹,² where they have been used to determine structural parameters of model membranes,³ to demonstrate interactions (or a lack of) between small molecules and lipid bilayers⁴ and to locate biomolecules within bilayers.⁵ For certain NMR⁶,⁷ and vibrational spectroscopy⁸,⁹ and neutron-based experiments,¹⁰ deuterium-labeled phospholipids have advantages over their natural abundance analogues. The use of deuterium-labeled molecules in combination with these experimental techniques goes well beyond the scope of this manuscript. In the case of neutron experiments, one example can be found in the use of the contrast variation or contrast-matching technique,¹¹,¹² and a range of chain-deuterated, homoacyl phospholipids is commercially available for this purpose. However, there is a lack of availability of chain-deuterated, heteroacyl phospholipids in which the two fatty acids attached to the sn-1 and sn-2 positions of the glycerol backbone are different. Most commonly, biological phospholipids are heteroacyl in nature, possessing a saturated fatty acyl chain at the sn-1 position and an unsaturated fatty acid at the sn-2 position.¹³ The most prominent example is 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC, Figure 1).

To the best of our knowledge, perdeuterated POPC is available from a single commercial supplier but is prohibitively expensive for many experiments. Moreover, this product has an estimated regiopurity of just 90%, containing up to 10% of the regioisomer 1-oleoyl-2-palmitoyl-sn-glycero-3-phosphocholine (OPPC).¹⁴ Differentiating between the regioisomers is challenging, and quantification is more difficult still.¹⁵−¹⁷
While this purity may be sufficient for many purposes, many investigators would find it useful to know, and account for, the regioisopurity of the lipid utilized. We thus aimed to produce highly regioisopure deuterium-labeled POPC. We also acknowledged that the chain-deuterated POPC isotopologue (Figure 1) would provide suitable contrast for many neutron experiments and so this was our synthetic target.

There is a suite of methods for preparing phospholipids, but we limited our review to those that allowed for the installation of two different acyl chains. Total synthesis methods were not considered as they require lengthy synthetic sequences and suffer from low atom economy and yield. Semisynthesis from an sn-glycerol-3-phosphocholine (GPC)-based natural precursor represented the most straightforward method, primarily because the chiral center is already installed. There are several reported chemical and enzymatic methods.

Chemical semisynthesis usually reports high yields but requires protecting or directing group strategies to discriminate between the sn-1 and sn-2 positions of the glycerol backbone and typically requires toxic chemicals such as cadmium or zinc salts or tin oxides. The strength of chemical synthesis in this area is in the simplicity and efficiency with which chemical esterification can be performed. This is somewhat hampered by the tendency of chemical esterification reagents (nitrogen-containing nucleophilic catalysts, acids, bases, organic solvents) to exacerbate acyl chain migration, resulting in a product that is contaminated with the regioisomer. Many of the literature reports describing the synthesis of POPC do not report the regiopurity of the product.

Enzymatic semisynthesis demonstrates regiospecificity and thus requires no protecting/directing group strategies. It typically employs mild conditions without the need for hazardous chemicals. However, while hydrolase enzymes are unsurprisingly efficient at catalyzing hydrolysis reactions, their activity is known to be affected by temperature, pH, and duration, for instance. Fatty acid anhydrides, typical reagents used for the chemical acylation of 2-lyso phospholipids, are easily prepared from the corresponding acids.

The main advantage offered by enzymatic catalysts here, regiospecificity, is not required in this step due to the identity of the starting material. We also considered that enzymatic (PLA2) esterification reports high yields, by virtue of the conflicting requirements of low water activity to encourage the equilibrium to favor esterification, and higher water activity (>0.2) to ensure PLA2 activity. To obtain optimal yields, the sn-2 enzymatic esterification requires a very high excess of fatty acid, which in this case is deuterated oleic acid, the synthesis of which is non-trivial. We also considered that many of the sources of PLA2 reported for this application in the literature (including porcine pancreas PLA2, the most commonly used enzyme for this purpose) were difficult to procure and additionally required us to immobilize the enzyme catalyst prior to the synthesis.

Modification at the sn-1 Position. The use of a regiospecific biocatalyst was required for the hydrolysis step, since there are no known chemical conditions able to perform regioselective hydrolysis of a phospholipid. For this purpose, an sn-1,3 specific lipase from Rhizomucor miehei (RM) was selected. This lipase is known to accept phospholipids as substrates and is commercially available in the immobilized form (acyl resin as solid support). The hydrolysis reaction is fast, efficient, and highly regioselective. A 1-lysolipid is much more prone to acyl migration than a 2-lysolipid, so we selected reaction, purification, and analysis conditions that would limit this phenomenon.

An enzyme catalyst was chosen for the second esterification reaction, as the 1-lysolipid is prone to acyl migration. If acyl...
migration were to occur to form the 2-lysolipid, the use of sn-1,3-specific RM lipase ensures that this will not react further (Scheme 2, red route); so while it would reduce the yield of the product, it would not compromise regio-purity. Furthermore, the catalysts for sn-1 esterification reactions, lipase enzymes, exhibit activity even in nearly anhydrous systems, which favors the synthesis over the hydrolysis reaction. Although deuterated palmitic acid is required in excess, it is relatively simple and inexpensive to produce directly via platinum-catalyzed H/D exchange.

**RESULTS AND DISCUSSION**

We began our synthesis with commercially available 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine and esterified at the sn-2 position using oleic anhydride-D₆₄ (Scheme 2, black route). Oleic anhydride-D₆₄ was synthesized according to published methods or modifications of them.

The diacylated lipid PO-D₃₂PC 1a was obtained in a 72% yield after purification by flash column chromatography on silica gel. The oleic acid-D₃₂ byproduct was also easily recovered. We acknowledged that some of the regioisomer, O-D₃₂PPC 1b, might be present (produced via the blue route, Scheme 2), but the challenge of differentiating between the lipid regioisomers prevented us from confirming this. Instead, we assessed 1a for chemical purity using ¹H NMR spectroscopy and used it in the subsequent reaction.

Immobilized RM lipase was used to catalyze the hydrolysis of the chain at the sn-1 position of 1a to produce 2a (Scheme 2, black route) using ethanol (96%). A high enzyme amount was utilized in order to minimize reaction time and to counter the anticipated inactivation of the enzyme by ethanol. The diacylphospholipid was completely consumed within hours, and the enzyme was removed from the reaction immediately via filtration. It is difficult to quantitate mixtures of isomers of lysophospholipids without causing isomerization, since acyl migration is first-order in acid or base and a slow acyl migration also occurs in organic solvents. We thus aimed to limit acyl migration of the 1-lysolipid 2a by using short-duration (2 min) ¹H NMR spectroscopy experiments for analysis. Using this method, we did not observe any of the 2-lysolipid (unlike diacyl regioisomers, 1- and 2-lysolipids can easily be distinguished in the ¹H NMR spectrum by the sn-2-CH signal, which appears at 4.9 ppm for the 1-lysolipid and 3.9 ppm for the 2-lysolipid). If the reaction was allowed to continue after consumption of the diacyl lipid, a small amount (2–3%) of an additional component was identified as GPC (2c in Scheme 2) by ¹H NMR spectroscopy. If the enzyme was immediately removed from the reaction mixture, the formation of 2c was seemingly suppressed, though we acknowledged that 2c would likely have low solubility in the NMR spectroscopy solvent (methanol-D₄). We also acknowledged that this would not be separated from 2a using the chosen purification method of cold diethyl ether extraction of the fatty acid byproduct (selected in favor of chromatographic purification to minimize further acyl migration).

The same type of immobilized RM lipase was then used to re-esterify at the sn-1 position using deuterated palmitic acid-D₃₁ to provide POPC-D₆₃ (Scheme 2, black route). Unlike PLA₂ enzymes, RM lipase is active at very low water activity and so there is no trade-off between conditions to encourage the synthetic reaction and to ensure enzyme activity. The effect of water activity on lipase-catalyzed synthetic reactions has been well studied. Some water is required in order for the enzyme to be active (initial experiments with anhydrous toluene were unsuccessful), and reaction rates increase with increased water activity, but simultaneously the yields decrease...
because of competing hydrolysis reactions.\textsuperscript{39} To balance these considerations, we chose to use reagent-grade (rather than anhydrous) toluene as the solvent, with the water activity maintained at or below 0.11, which was achieved by gas-phase equilibration (placing the open reaction vessel inside a closed vessel containing a saturated solution of LiCl).\textsuperscript{26} This system limits the water activity to 0.11 throughout the reaction as the saturated solution can absorb the additional water produced in the reaction. The enzyme pleasedly accepted the labeled fatty acid as the substrate to provide the desired lipid in modest yield, and the excess fatty acid could be recovered quantitatively during purification.

The use of sn-1,3-specific RM lipase in the final step ensured that none of the regioisomer, OPPC-d\textsubscript{6}\textsubscript{2}, could be produced (Scheme 2, red route). However, in addition to POPC-d\textsubscript{6}\textsubscript{2}, two isotopologues of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC-d\textsubscript{6}\textsubscript{4} and DPPC-d\textsubscript{6}\textsubscript{2}) could be identified in the final product by mass spectrometry. DPPC-d\textsubscript{6}\textsubscript{4} is attributed to the acyl migration that occurs prior to the first, chemical esterification (Scheme 2, blue route), while DPPC-d\textsubscript{6}\textsubscript{2} arises from the presence of GPC in the hydrolysis mixture (Scheme 2, green route).

The deuterium-labeling of the lipids precluded quantification of their relative ratio by \textsuperscript{1}H NMR spectroscopy. Instead, we calculated the relative amounts of the palmitoyl and oleoyl chains using GC-FID after transesterification of the lipid mixture in anhydrous methanolic hydrochloric acid to produce their corresponding fatty acid methyl esters (FAMES). This is a simple, well-known method available to most synthetic laboratories and is applicable to deuterated molecules as deuterium-labeling does not change the relative molar density (SLD) for neutron experimentation.\textsuperscript{12} However, we found no need to separate the POPC-d\textsubscript{6}\textsubscript{3} from DPPC isotopologues, although this can be done using HPLC with far less specialized columns than those required to separate regioisomers such as POPC and OPPC.\textsuperscript{16}

The yield of chain-deuterated POPC obtained over three steps was 23%, and the final lipid mixture (96.4% POPC-d\textsubscript{6}\textsubscript{3}) was obtained on a 57 mg scale, an amount suitable for neutron reflectometry and small-angle neutron scattering investigations.

\section*{CONCLUSIONS}

This method combines chemical and enzymatic synthesis to exploit the benefits of each method to produce high-purity, mixed-acyl, chain-deuterated POPC suitable for use in neutron experiments exploiting the contrast-match technique. Efficient chemical esterification is used to install a high-value perdeuterated fatty acid synthon, where regiospecificity is not required, while RM lipase is exploited where regiospecificity is required—to selectively substitute the sn-1 chain without the need for protecting or directing groups. This is an operationally simple method for synthetic chemists with limited knowledge of enzyme structure or function to perform. To the best of our knowledge this is the first reported use of lipase enzymes to effect condensation reactions of a lyso phospholipid using a deuterated acyl donor. Phospholipids with high chemical purity and complete regioisopurity are produced, facilitating the use of readily accessible analytical and purification techniques. The modular nature of the method should also allow for a suite of analogues to be produced.

\section*{EXPERIMENTAL SECTION}

\subsection*{General Procedures.}

Lipozyme RM (lipase from \textit{Rhizomucor miehei}, immobilized on acrylic resin, 275 IUN/g) was a generous gift from Novozymes A/S, Bagsværd, Denmark. 1-Palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine was from Avanti Polar Lipids (product code: 855675P) via Sigma-Aldrich/Merck. Palmitic acid-d\textsubscript{14} and oleic acid-d\textsubscript{18} were prepared based upon literature methods.\textsuperscript{34} One of the olefinic hydrogens in the prepared deuterated oleic acid-d\textsubscript{12} exists as \textsuperscript{3}H by virtue of the synthetic method. Additionally, some back-exchange (D/H) occurs at the methylene adjacent to the carboxylic acid during the synthesis;\textsuperscript{44} this is retained in the prepared phospholipids. Other chemicals, including deuterium oxide (99.8%), and solvents of the appropriate grade were used as received from Sigma-Aldrich. NMR spectra were obtained on a Varian Unity INOVA spectrometer operating at 400 MHz for \textsuperscript{1}H and 100 MHz for \textsuperscript{13}C isotopes and referenced to residual solvent signals. MS analysis was performed either on an Agilent 1260 Infinity II system coupled with an Agilent InfinityLab LC/MSD XT or on a Waters QTOF XEVO-G2. Deuterium incorporation was calculated from mass spectra using a comparison of the isotopologues, taking into consideration the contribution of \textsuperscript{13}C species, using a method developed at the National Deuteration Facility, Australian Nuclear Science and Technology Organisation, Australia. Gas chromatographic analysis was carried out on a Shimadzu GC 2010 Plus gas chromatograph equipped with a flame ionization detector and a BPX70 capillary column (70% cyanopropyl polysilphenylene-siloxane; 25 m x 0.22 mm x 0.25 µm; SGE Analytical Science, Trajan Scientific Australia). GC standard ME 32 was from Larodan AB, Sweden. Analytical data (\textsuperscript{1}H and \textsuperscript{13}C NMR spectra, GC traces and mass spectra) are provided in the Supporting Information.

\textbf{Oleic Anhydride-d\textsubscript{66}.} A solution of dicyclohexyl carbonodimide (1.0 M in DCM, 1.08 mL, 1.08 mmol) was added to a solution of oleic acid-d\textsubscript{32} (676 mg, 2.15 mmol) in anhydrous DCM (5 mL). The vessel was protected from light, and the mixture was stirred at room temperature under a nitrogen atmosphere overnight (monitored by TLC using 20% EtOAc in hexane as solvent and bromocresol green to visualize) and then filtered through a frit. The solid residue was washed with DCM (20 mL), and the combined filtrate was concentrated under reduced pressure. The resulting oil was used without further purification (618 mg, 94%). \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}) \textsuperscript{6} 2.40–2.42 (br complex, 2H), 5.32 (br s, 1H).

*Contributions from residual protons not reported.

\textbf{1-Palmitoyl-2-oleoyl-d\textsubscript{2}sn-glycero-3-phosphocholine 1a.} Oleic anhydride-d\textsubscript{66} (618 mg, 1.00 mmol) and 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (151 mg, 0.30 mmol) were dissolved in anhydrous DCM (18 mL) under vigorous stirring. 4-Dimethylaminopyridine (81 mg, 0.66 mmol) was added to the solution. The reaction was stirred vigorously at room temperature under a slight positive pressure of nitrogen, protected from light, until all the starting material had been consumed (47 h; monitored by TLC). Solvent residues were removed under reduced pressure. Flash column chromatography (gradient elution: 65/25/4 CHCl\textsubscript{3}/CH\textsubscript{3}OH/H\textsubscript{2}O, using bromocresol green and Hanessian’s stains separately to visualize) provided the pure title ester as a white solid (174
mg, 72%). 1H NMR (CD3OD, 400 MHz) δ 0.91 (m, 3H), 1.30 (s, 24H), 1.60 (m, 2H), 2.32 (t, J = 7.4 Hz, 3H*), 3.23 (s, 9H), 3.64 (m, 2H), 4.00 (m, 2H), 4.18 (m, 2H), 4.27 (m, 2H), 4.44 (dd, J = 12.2, 3.4 Hz, 1H), 5.24 (m, 1H), 5.34 (s, 0.62H*). Contributions from residual protium from oleoyl chain.

13C NMR (CD3OD, 100 MHz) δ 14.5, 23.7, 36.0, 30.2, 30.47, 30.50, 30.66, 30.78, 30.81, 31.3, 34.89*, 34.94, 54.7 (t, J = 3.8 Hz), 60.5 (d, J = 5.0 Hz), 63.7, 64.9 (d, J = 5.3 Hz), 67.5 (m), 71.8 (d, δ J = 8.1 Hz), 130.6, 174.6, 174.9. Contribution from residual protiated carbon atoms from oleoyl chain.

1-Hydroxy-2-oleoyl-d15-sn-glycero-3-phosphocholine 2a. According to a literature method for an analogous compound, 1-palmitoyl-2-oleoyl-d15-sn-glycero-3-phosphocholine 1a (174 mg, 219 μmol) was dissolved in ethanol (10 mL, 96%). Lipase from Rhizomucor melei (immobilized on acrylic resin, 275 IUN/g, 1.40 g) was added to the lipid solution and the reaction was vigorously stirred at room temperature until no further consumption of the starting material could be observed by TLC (2 h; monitored by TLC, solvent conditions: 65/25/4 CHCl3/CH3OH/H2O, using Hanessian’s stain to visualize). The resins were removed via filtration and washed with toluene, methanol, and ethanol. The combined organic extracts were concentrated under reduced pressure and the resulting solid was washed with diethyl ether (7 x 15 mL) and re-dried under reduced pressure to provide 1-hydroxy-2-oleoyl-d15-sn-glycero-3-phosphocholine 2a as a white solid (93.5 mg, 75%), which required no further purification and which was used directly in the next reaction.

1H NMR (CD3OD, 400 MHz) δ 0.90 (s, 0.17H*), 1.29 (s, 1.56H*), 2.34 (s, 1.32H*), 3.23 (s, 9H), 3.64 (m, 2H), 3.70 (m, 2H), 4.01 (m, 2H), 4.28 (m, 2H), 4.57 (br s, 1H), 5.03 (m, 1H), 5.34 (s, 0.51H*). Contributions from residual protium from oleoyl chain.

1-Palmitoyl-d15,2-oleoyl-d13,sn-glycero-3-phosphocholine. Palmitic acid-d15 (2.40 g, 8.36 mmol) and 1-hydroxy-2-oleoyl-d13-sn-glycero-3-phosphocholine 2a (93.5 mg, 167 μmol) were dissolved in toluene (17 mL) under vigorous stirring and the resulting solution was added to a solution of H2SO4 (3 mL, 2.5% v/v) and POPC-d31 \([\text{M + Na}^+]\) as 845.9; found: 845.9. Isotope contributions: 1.5% d15, 0.9% d15, 1.5% d31, 1.5% d45, 1.8% d55, 3.0% d65, 4.6% d75, 6.7% d85, 13.1% d95, 18.8% d105, 22.5% d115, 15.8% d125, 8.2% d135.

Three additional adducts were observed: (1) calculated for C40H30D31NO8P (POPC-d42) \([\text{M + Na}^+]\) as 879.0; found: 879.0. Isotope contributions: 1.0% d49, 1.8% d15, 1.9% d31, 2.4% d33, 1.9% d41, 2.3% d43, 3.0% d45, 3.9% d51, 3.9% d53, 5.8% d61, 7.7% d63, 10.1% d65, 12.1% d67, 15.9% d71, 16.9% d81, 12.6% d83, (2) calculated for C40H30D31NO8P (DPPC-d42) \([\text{M + Na}^+]\) as 765.8; found: 765.7. Isotope contributions: 0.7% d41, 0.7% d51, 0.7% d53, 1.4% d55, 1.4% d61, 1.4% d63, 3.5% d65, 4.2% d71, 8.3% d73, 16.0% d81, 27.1% d83, 35.4% d91.

Fatty acid methyl ester composition by GC: 53.6% palmitoyl; 46.4% oleoyl (96.4% POPC;3.6% DPPC).

Analysis of Fatty Acid Composition by Gas Chromatography (GC). Methyl palmitate-d33 and methyl oleate-d33 were prepared as follows: Palmitic acid-d33 (128 mg, 445 μmol) was dissolved in a methanolic solution of H2SO4 (5 mL, 2.5% v/v) and heated to 100 °C for 45 min in a sealed tube. The mixture was cooled and water (3 mL) was added. The mixture was extracted into heptane (3 x 3 mL). The combined organic extracts were dried to afford methyl palmitate-d33. Methyl oleate-d33 was produced using the same method with oleic acid-d32 (28 mg, 89 μmol) in a methanolic solution of H2SO4 (3 mL, 2.5% v/v). Solutions of these (0.05 mg/mL) were used to identify retention times for the deuterated FAME. A standard mixture (ME 32) containing C16:0, C18:0, C18:1(9Z), C18:2(9Z,12Z), and C18:3(9Z,12Z,15Z) was used to identify the retention time of protiated (natural abundance) methyl palmitate and to calculate a relative response factor for palmitoyl and oleoyl methyl esters. Since organic molecules show no change in the relative molar flame ionization detection response (RMR) when deuterium is substituted for hydrogen, the protiated analogues are suitable proxies for the deuterated chains, and the raw peak areas for deuterated and protiated methyl palmitate were added together. This value was used in a molar comparison with deuterated methyl oleate, applying a relative response factor calculated previously to account for differences in effective carbon number and saturation (the molar mass used was for palmitic acid-d33 since this was the major isotopologue). Samples of POPC-d63 were prepared as follows: A methanolic solution of H2SO4 (3 mL, 2.5% v/v) and POPC-d63 (0.54 mg) in a sealed tube was heated to 100 °C for 1 h and then allowed to cool to room temperature. Water (3 mL) was added, and then the mixture was extracted into heptane (4 x 0.5 mL) and dried under a stream of nitrogen at room temperature. The mixture was then re-dissolved in 4000 μL of heptane, and 5 μL of samples were injected into a gas chromatography system coupled to a flame ionization detector. Analysis was carried out using helium as the carrier gas (1.04 mL/min). Samples and standards were injected at 250 °C with a split ratio of 80. The column temperature was increased from 155 to 180 °C (2 °C/min). The detector temperature was 260 °C. Measurements were performed in triplicate, and the averages of these were used in our calculations.
**ASSOCIATED CONTENT**

1. **Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c02823.

- NMR spectra, mass spectra, and GC traces (Figures S1–S17) (PDF)

**AUTHOR INFORMATION**

**Corresponding Author**

Anna E. Leung — Scientific Activities Division, European Spallation Source ERIC, Lund 221 00, Sweden; orcid.org/0000-0002-8196-9774; Email: Anna.Leung@ess.eu

**Author**

Oliver Bogojевич — Scientific Activities Division, European Spallation Source ERIC, Lund 221 00, Sweden

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.0c02823

**Author Contributions**

O.B. and A.E.L. designed the project. A.E.L. synthesized palmitic acid-\( \text{d}_{13} \) and oleic acid-\( \text{d}_{18} \), and from these O.B. prepared POPC-\( \text{d}_{33} \). A.E.L. wrote the manuscript with assistance from O.B.

**Notes**

The authors declare no competing financial interest.

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(15) The chemical and spectroscopic similarity between regioisomers POPC and OPPC makes discriminating between them, and thus quantification of the regiopurity, challenging requiring specialized methods of analysis which are not available in our laboratory, or many other laboratories. \(^{13}\)C NMR spectroscopy can be used for identification as the carbonyl signals of the regioisomers appear at different chemical shifts but this is usually not relied upon for quantification due to the low abundance of the \(^{13}\)C isotope. Using highly-specialised HPLC columns, coupled with ESI-MS techniques based on the difference in fragmentation energies required to fragment sn-1 and sn-2 fatty acids, one can approximate the POPC to OPPC ratio, but pure standards of the regioisomers are required, the deuterated analogues of which are not available. Digestion of the lipid using sn-1 or sn-2-selective hydrodases, followed by analysis of the resultant fatty acids, can also be used but this relies upon the assumption that the enzyme is completely regio- and substrate-specific; that its activity is the same for artificial lipids as for natural analogues and that its activity is not influenced by the assay conditions employed. See references 16 and 17 for further discussion.

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