BrightnESS²

Bringing Together a Neutron Ecosystem for Sustainable Science with ESS

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Grant Agreement Number: 823867

Deliverable Report

D2.7: Report on deuteration for soft matter and life sciences: Experimental results

BrightnESS² is funded by the European Union Framework Programme for Research and Innovation Horizon 2020, under grant agreement 823867

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Authorship	Written by	Anna Leung (ESS)
	Contributors	Peixun Li (STFC) John Webster (STFC)
	Reviewed by	Arno Hiess (ESS)
	Approved	BrightnESS² Steering Board

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3. List of abbreviations and acronyms

a_w	water activity
Boc_2O	di-*tert*-butyl dicarbonate
CR	*Candida rugosa*
DCC	*N*,*N*'-dicyclohexylcarbodiimide
DCM	dichloromethane
DEMAX	Deuteration and Macromolecular Crystallisation
DEUNET	Deuteration Network
DMAP	4-dimethylaminopyridine
DOPC	1,2-dioleoyl-*sn*-glycero-3-phosphocholine
ESS	European Spallation Source
FZJ	Forschungszentrum Jülich
GC-FID	gas chromatography-flame ionisation detector
GPC	*sn*-glycero-3-phosphocholine
PLA1	phospholipase A1
PLA2	phospholipase A2
PLC	phospholipase C
PLD	phospholipase D
POnPC	1-palmitoyl-2-(9-oxononanoyl)-*sn*-glycero-3-phosphocholine
POPC	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine
POPE	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine
RM	*Rhizomucor miehei*
SINE2020	Science and Innovation with Neutrons in Europe in 2020
sn	stereospecific numbering
TFA	trifluoroacetic acid

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7. Executive summary

The aim of this pilot project was to enable better neutron experiments in the fields of soft matter and life science, by making novel deuterium-labelled molecules available to the neutron community. In these fields, a pre-requisite for performing meaningful neutron experiments is the availability of relevant and interesting samples, which are often deuterium-labelled. A lack of availability exists because the range of deuterated molecules of interest is vast, and because deuterated molecules can be fundamentally challenging to produce using the well-established methods in use today.

Lipids and lipid-based materials are in high demand within the neutron scattering community and the complexity of the desired lipids is becoming more and more pronounced. The ISIS Deuteration Facility identified heteroacyl phospholipids as one example of the type of molecules that are interesting to neutron users but that are difficult to produce in deuterated form. The ISIS Deuteration Facility recognised that the suite of potential applications for these molecules is broad, and as an example proposed utilising heteroacyl phospholipids as model skin lipids, to investigate surfactant-mediated transdermal drug delivery using the SURF neutron reflectometer at the ISIS Neutron and Muon Source. The DEMAX laboratory at ESS recently began using biocatalytic (enzymatic) methods which had the potential to simplify the production of lipids of this type and so the two laboratories worked together to identify the most interesting heteroacyl lipid(s) and to demonstrate the usefulness of the labelled lipids in neutron experiments. POPC (1-palmitoyl-2-oleoyl-*sn*-3-glycero-phosphocholine) is the most ubiquitous of the naturally-occurring heteroacyl phospholipids and for this reason it was selected as the molecular target.

An exclusively biocatalytic synthesis route was investigated but ultimately the target lipid, POPC-d_{63}, was produced using a combination of biocatalysis and traditional chemical synthesis. The approach has advantages over exclusively chemical synthesis routes; and was later applied to another lipid class, to produce POPE-d_{64} (1-palmitoyl-2-oleoyl-*sn*-3-glycero-phosphoethanolamine), demonstrating the modularity of the method.

Restrictions in place at the ISIS Neutron and Muon Source, due to the COVID-19 pandemic, have prevented the proposed neutron reflectometry experiment from taking place, but the utility of the lipids produced under this pilot programme has been demonstrated in other neutron experiments.

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8. Introduction: motivation, objectives and methods

The combination of deuterium-labelling with neutron scattering is exploited for a variety of purposes – to reduce incoherent scattering from protium (^{1}H), to observe hydrogen exchange processes, or to provide contrast between two hydrogen-containing components of a system, for example. Ready access to deuterium-labelled molecules enhances many neutron scattering experiments, but in many cases the suite of commercially-available molecules doesn't fulfil the needs of the neutron community.

One goal of the deuteration and macromolecular crystallisation platform (DEMAX) at ESS is to expand the suite of deuterated molecules available to neutron users to improve the relevance and impact of the neutron experiments performed. This goal is currently being addressed in two ways: firstly, by fostering collaboration between various deuteration laboratories within Europe and around the world via the 'Deuteration Network (DEUNET, Photo 1)'; and secondly, by adopting methods that are novel for these laboratories.

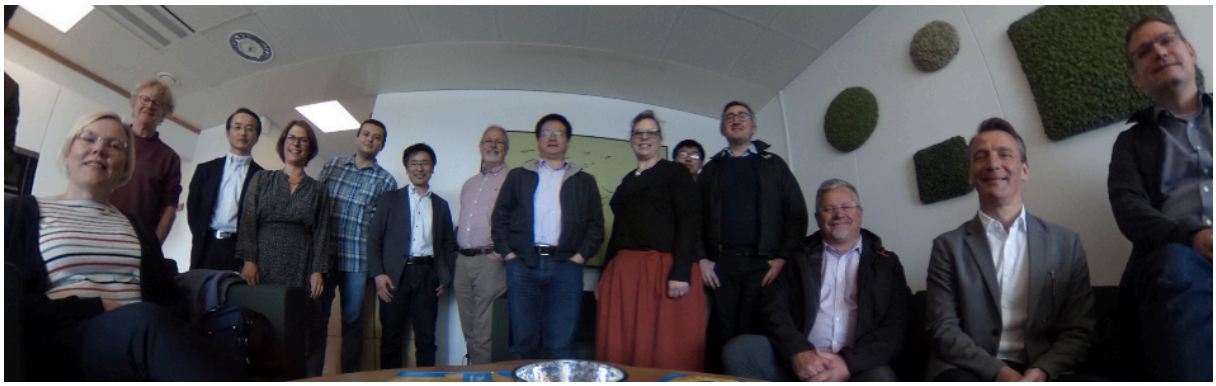

Photo 1: Some of the members of DEUNET

Previous collaborations within the DEUNET have demonstrated the usefulness of collaboration between deuteration laboratories with different specialisations, enabling the production of molecules that a single laboratory alone could not produce. And an earlier project in the ESS laboratory (under the European Union programme Science and Innovation with Neutrons in Europe [SINE2020])[1] had demonstrated the utility of biocatalysis in place of traditional chemical synthesis, using enzymes to perform reactions in a *stereoselective* manner – something chemical catalysts are not able to reproduce. With this in mind, we designed a project that would allow us to produce novel, high-value deuterated molecules using ***biocatalysis*** and the ***expertise*** of two deuteration laboratories.

9. Background

9.1. Phospholipids for soft matter and life science

Phospholipids are a key component of cellular membranes and are of interest for a broad range of research fields such as biology, pharmacology and formulation science. They are commonly studied using X-rays and neutrons for instance to determine structure and functional characteristics of cell membranes and interactions between cell membranes and bioactive compounds.

Scattering experiments often utilise natural-abundance phospholipids (i.e. almost all hydrogen in the form of protium, ^{1}H) but for certain neutron-based experiments, deuterated analogues (with some or all of the hydrogen atoms in the form of deuterium, ^{2}H or D) are advantageous. The majority of the

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hydrogen atoms present in phospholipids resides on the 'tails' with a minority on the 'heads' (Figure 1) and so it is these portions of the molecules that represent the most obvious target for hydrogen/deuterium (H/D) exchange. Some tail-deuterated phospholipids are commercially-available (such as DPPC, Figure 1), but they tend to consist of two of the same tails, whereas in nature phospholipids tend to contain two different tails – one saturated and one unsaturated (see POPC, Figure 1), with these differences having significant impacts on physical properties such as membrane fluidity, and related functions.

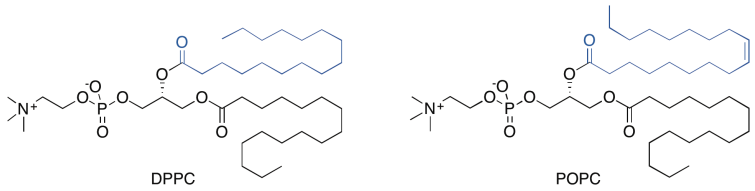
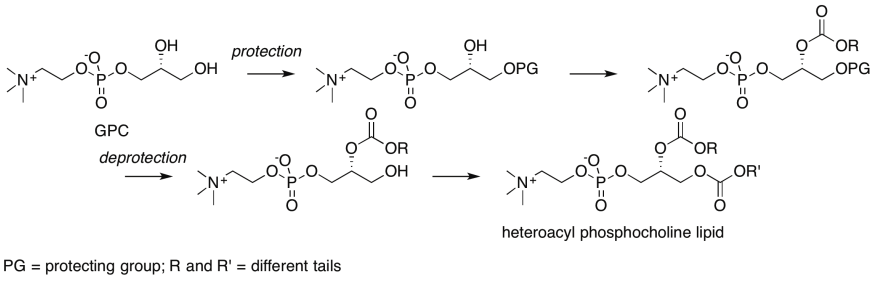


Figure 1: DPPC, a homoacyl phospholipid with two of the same saturated tail; and POPC, a heteroacyl phospholipid with one saturated and one unsaturated tail.

9.2. Existing methods of sample preparation

Unnatural phospholipids, such as those possessing atomic labels, are produced via chemical synthesis. The most efficient methods use chemical *semisynthesis*, beginning from a biologically-derived *sn*-glycero-3-phosphocholine (GPC)-based precursor (Scheme 1). This method requires no enantioselectivity because the only chiral centre in the phospholipid is present in the starting material and retained throughout the synthesis. The method still requires regioselectivity, however, or a strategy to circumvent this requirement, such as a protection-deprotection strategy to temporarily mask reactive functionality.



Scheme 1: Typical synthetic route to an unnatural, heteroacyl phosphocholine lipid, starting from GPC.

The drawbacks to this method are:

1) poor efficiency due to the need for protecting group installation and removal;
2) use of toxic chemicals such as cadmium or zinc salts or tin oxides;
3) contamination of the product with a regioisomer, with the tails in the opposite positions (typically 10% impurity).[2]

10. Method development

10.1. Lipase and phospholipase enzymes as biocatalysts

Enzymes are non-toxic, operate under mild conditions, and show extraordinary specificity. Some enzymes are relatively simple to produce, isolate and utilise; others are more challenging – these differences are reflected in their commercial availability and cost and the breadth of associated literature.

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The phospholipase enzymes are the natural catalysts of phospholipid hydrolysis, and show remarkable specificity for the region of the phospholipid molecule at which they are active, such as phospholipase A1, active at the 1-position, or phospholipase A2, active at the 2-position (Figure 2). Lipase enzymes, which have glycerides as their natural substrates, are also known to act on a broad range of lipid substrates including phospholipids. Compared to phospholipases, lipases are more readily available, cheaper and more well studied and so we decided to use lipase enzymes in place of phospholipases where possible.

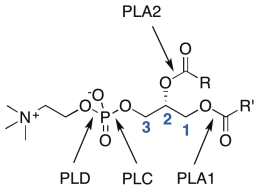
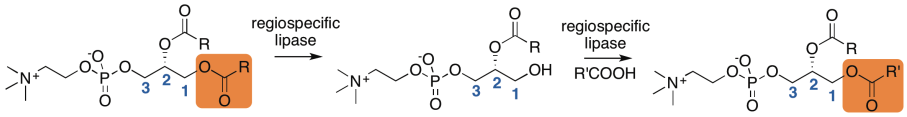


Figure 2: Regiospecificity of the phospholipase enzymes PLA1, PLA2, PLC and PLD.

As they are catalytic, enzymes can be recycled so long as they can practically be separated from a reaction mixture. For this reason, they are often attached to a solid support such as a polymer resin and used as heterogenous, rather than homogenous catalysts, allowing them to be removed from a liquid medium via filtration, and re-used. A variety of lipase enzymes are commercially-available in immobilised form.

10.2. Enzymatic modification of phospholipids: lipases

There are well-established methods for the use of regiospecific lipase enzymes to modify phospholipids at the 1-position (Scheme 2).[3, 4] Though their natural function is to *hydrolyse* esters, these enzymes are even able to conduct the reverse function, *esterification*, under certain conditions.[4] There are no reports of lipases showing regiospecificity for the 2-position.



Scheme 2: Use of lipase enzymes to modify phospholipids regiospecifically at the 1-position without affecting the 2-position

We selected a *sn*-1 specific lipase from a species of fungus, *Rhizomucor miehei*, and applied a two-step process to a homoacyl phospholipid, DOPC, to produce a heteroacyl phospholipid, POPC-d_{31} (Scheme 3). The first step uses the lipase to perform its natural function, hydrolysis of the ester at the 1-position to produce a 1-lysolipid. Next, the same lipase is subjected to different conditions, under which it catalyses the reverse reaction, esterification between the hydroxyl group of the 1-lysolipid and deuterated palmitic acid (the synthesis of saturated fatty acids is reported in the literature)[5, 6]. This enzyme is commercially-available in immobilised form (attached to an acrylic resin), allowing it to easily be removed from the reaction mixture, simplifying purification.

Reaction conditions were optimised by varying reaction duration, temperature, catalyst loading and agitation method; the optimised yield over two steps was 40%.

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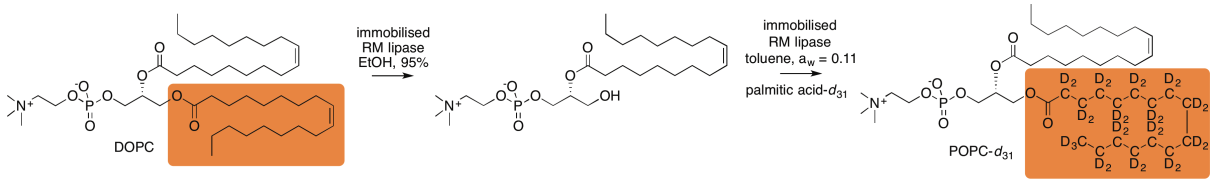
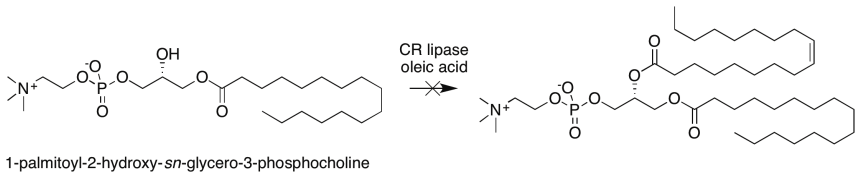


Scheme 3: Two-step modification of a homoacyl phospholipid, DOPC, to produce a heteroacyl, partially-deuterated phospholipid, POPC-d31.

Since there are no known reports of lipases that are specific for the 2-position, we could not use the same two-step modification method for the other phospholipid position. We attempted to start with a 2-lysolipid rather than an intact (diacyl) phospholipid; this meant that the 2-position could be altered to include a deuterium-labelled tail in a single step. A non-specific lipase from *Candida rugosa* fungus was used to esterify at the 2-position but no reaction was observed (Scheme 4).



Scheme 4: Attempts to catalyse an esterification reaction between a 2-lysolipid and oleic acid using CR lipase were unsuccessful.

10.3. Enzymatic modification of phospholipids: phospholipases

Since attempts to use a non-specific lipase enzyme were unsuccessful, we turned to using phospholipase enzymes that are specific for the 2-position. These enzymes are sourced from snake or bee venom or porcine or bovine pancreas rather than fungus, as for the lipase enzymes. They are difficult to obtain commercially and are vastly more expensive. We selected PLA2 from porcine pancreas but severe delays in delivery of the enzyme hindered our efforts in this area.

10.4. Chemo-enzymatic synthesis of POPC: a combined approach

To address delays in delivery of the PLA2 enzyme, we investigated a combined chemical-enzymatic approach to the synthesis of tail-deuterated POPC. We started with a 2-lysolipid and used standard chemical esterification methodology to install a deuterated tail at the 2-position, with the established 2-step enzymatic modification of the 1-position subsequently providing the tail-deuterated heteroacyl lipid.

Starting with the 2-lysolipid 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine, a deuterated oleoyl tail was installed using a chemical esterification reaction with deuterated oleoyl anhydride and 4-dimethylaminopyridine as catalyst (Scheme 5). Deuterated oleoyl anhydride was synthesised from deuterated oleic acid (which was synthesised in-house according to a literature procedure[6]). Then we applied the method of enzymatic *sn*-1 modification to replace the palmitoyl chain with the deuterated analogue (Scheme 5).

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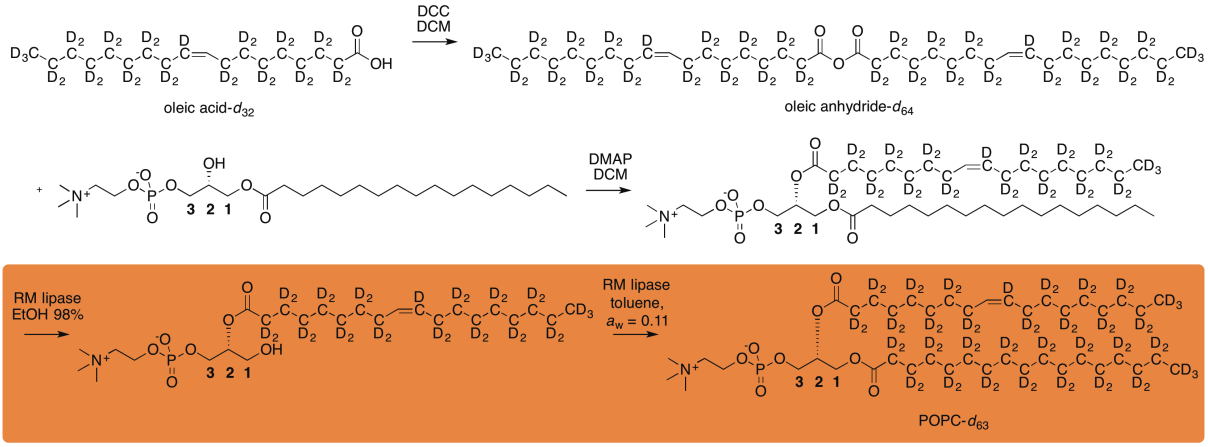
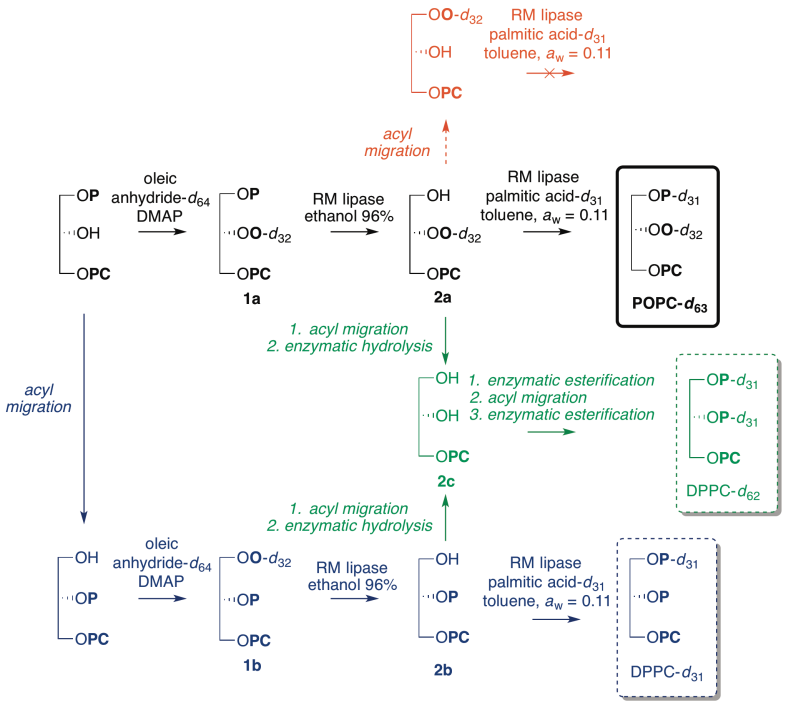


Scheme 5: Chemo-enzymatic synthesis of tail-deuterated POPC-d63. (Enzymatic modification in orange.)

The yield over four steps was 27% and produced tail-deuterated POPC on a 50 mg scale, sufficient amounts for neutron reflectometry experiments. The use of a regiospecific enzyme catalyst in the two-step modification of the 1-position assured complete regiopurity. The chemical purity was assessed using a simple, well-established technique to transesterify the fatty acid tails to their methyl ester analogues and analyse these by gas chromatography with a flame ionisation detector (GC-FID), an analytical instrument available in most chemistry laboratories. Using this method, the product was determined to consist of 97% POPC-d_{63}. The remaining 3% consists of DPPC-d_{31} and DPPC-d_{62} which are present as a result of a small degree of acyl migration during the synthesis (Scheme 6). Separation of this minor impurity was not necessary for the intended neutron experiments. The synthetic method is published, see appendix 1.



P = palmitoyl; **O** = oleoyl; **PC** = phosphocholine. Intermediate compounds in blue and green were not observed but are proposed to justify the route to the obtained product mixture.

Scheme 6: Synthesis of POPC-d63, 97% chemical purity. Residual 3% consists of DPPC-d62 and DPPC d31, produced as a result of acyl migration during the synthesis.

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10.5. Neutron experiments

Ready access to deuterium-labelled POPC and other biologically-relevant lipids enables more realistic neutron experiments to be performed, where currently less relevant labelled lipids are used due to their availability. Tail-deuterated POPC made at the ESS chemical deuteration laboratory will be used as a model skin lipid to investigate improvements in transdermal drug delivery via neutron reflectometry using the SURF instrument at ISIS. Here, deuteration provides contrast between two hydrogen-containing components (POPC as a skin surface lipid mimic and a transdermal drug); it would not be possible to discriminate between these components without deuterium labelling.

The COVID-19 pandemic has resulted in some restrictions coming into force at the ISIS neutron and muon source, preventing the planned experiment from taking place. It is hoped the experiment can be performed when restrictions can be eased.

Tail-deuterated POPC has been supplied to two additional research groups for use in neutron reflectometry and small-angle neutron scattering experiments, via the 2019 and 2020 ESS DEMAX user programme.

Tail-deuterated POPC continues to be requested for neutron reflectometry experiments and has also been requested for use in small-angle neutron scattering and neutron spin-echo experiments. These latter types of experiments typically demand higher amounts of sample (100-200 mg lipid). Future work will focus on completing this synthesis on a larger scale. The main roadblock to producing larger amounts of tail-deuterated POPC is accessing sufficient amounts of deuterated oleic acid, which requires a long synthetic sequence and is time-consuming. Via the DEUNET, ESS has an agreement to procure deuterated oleic acid from the National Deuteration Facility (NDF) at the Australian Nuclear Science and Technology Organisation (ANSTO), where a larger team of chemists synthesise it routinely.

11. Method extension

11.1. POPE (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine)

We decided to apply our method of phospholipid modification to another class of phospholipids – phosphoethanolamines, and produce tail-deuterated 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (Scheme 7). Unlike phosphocholine lipids like POPC, phosphoethanolamine lipids contain a reactive primary amine which must be protected (masked) before any chemical modification of the tails takes place. At the end of the synthesis the protecting group is removed. The use of this protecting group strategy is reported.[7]

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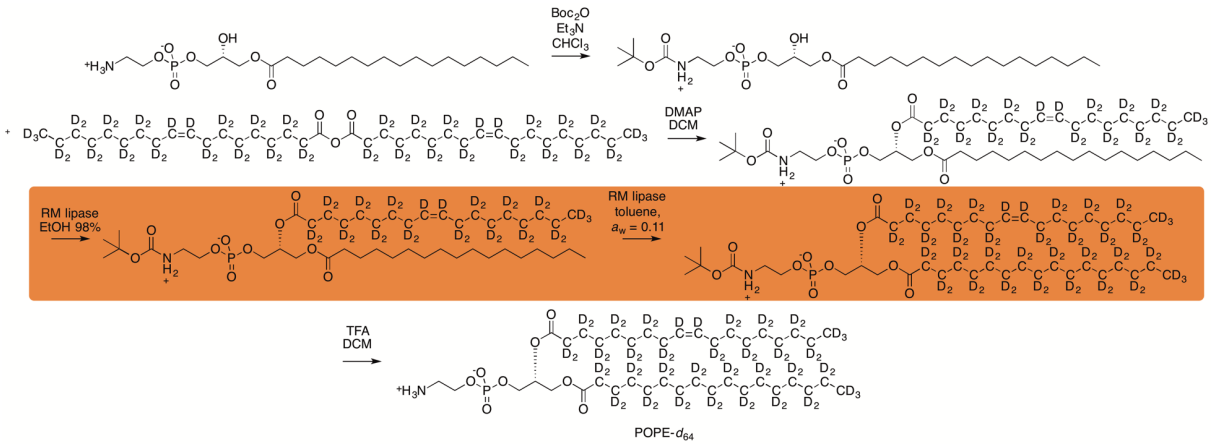

Scheme 7: Chemo-enzymatic synthesis of tail-deuterated POPE-d64. (Enzymatic modification in orange.)

Along with POPC, POPE was supplied to a proposer as a result of the DEMAX 2019 proposal call.

11.2. Oxidised lipids: POnPC (1-palmitoyl-2-(9-oxononanoyl)-*sn*-glycero-3-phosphocholine)

Oxidative damage to unsaturated lipids like POPC results in lipids such as POnPC (1-palmitoyl-2-(9-oxononanoyl)-*sn*-glycero-3-phosphocholine. Such oxidative damage is linked to a range of adverse health effects related to ageing, cell apoptosis and cancer. The oxidation of unsaturated lipid tails is thought to cause structural changes to the lipid mono/bilayers wherein the oxidised tail undergoes chain reversal. Neutron reflectometry is a useful tool for investigating the structure of lipid mono/bilayers, and selective deuteration is a tool which facilitates discrimination between the two phospholipid tails. A proposal received by DEMAX through the 2019 round requested two isotopologues of POnPC, one with the chain at the 1-position deuterium-labelled (Pd_{31}OnPC, Figure 3), and the other with the chain at the 2-position deuterium-labelled (POnd_{14}PC, Figure 3).

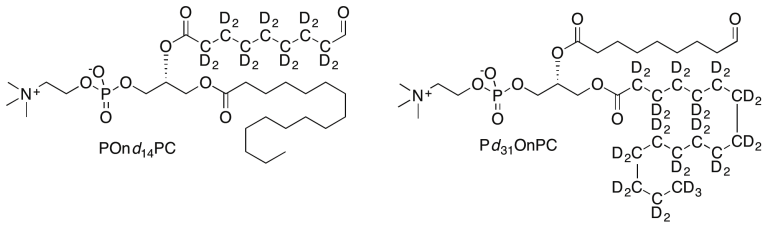

Figure 3: Two isotopologues of the oxidised lipid POnPC (1-palmitoyl-2-(9-oxononanoyl)-sn-glycero-3-phosphocholine.

We planned to use the same combination of chemical synthesis and enzymatic modification established previously to synthesise Pd_{31}OnPC. POnd_{14}PC could be produced using chemical methods only.[6, 8, 9] Due to the complexity of the synthesis, only one isotopologue was provided to the proposers (POnd_{14}PC, Scheme 8), though the synthesis of Pd_{31}OnPC is of interest for the future.

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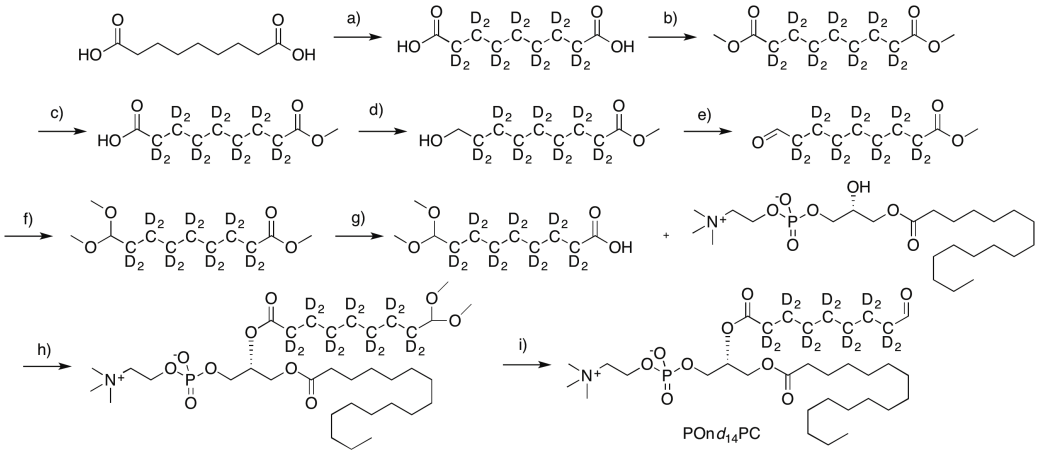

a) Pt/C, D_2O, NaOD, 220 °C, 23 bar (2 cycles); b) H^+, MeOH; c) $Ba(OH)_2 \cdot 8H_2O$, MeOH; d) $(CH_3)_2S \cdot BH_3$, THF; e) PCC, DCM; f) TMOF, NH_4NO MeOH; g) NaOH, H_2O/MeOH/THF; h) DCC, DMAP, $CHCl_3$; i) Amberlyst-15 resin, acetone-d_6/D_2O.

Scheme 8: The synthesis of POnd14PC used chemical methods only, without the need for enzymatic modification .[6, 8, 9]

12. Conclusion

This pilot programme reinforces that method development is a valuable use of time for highly-specialised laboratories such as those focusing on deuteration for neutron scattering. The range and complexity of deuterium-labelled molecules required for neutron experiments is ever expanding, and deuteration laboratories ought to spend time establishing robust and/or modular methods which increase the efficiency of synthesis. The methods established during this project produced three deuterium-labelled lipids which are not commercially available and delivered samples of these to four research groups to enable neutron scattering experiments in the fields of biology, pharmacology and human health.

This pilot further highlights the benefits of finding synergies between deuteration laboratories, and of collaborating when the value of a partnership is greater than the sum of the separate efforts of isolated laboratories. Each of the deuteration laboratories in the DEUNET have areas of specialisation and expertise that can be utilised by the other laboratories, removing the need to duplicate efforts in these areas. Long-term cooperation within the DEUNET offers the possibility to dissociate the locations of sample preparation and neutron experimentation, to enable high-impact science by using the most appropriate samples and instrumentation, and to contribute to addressing the most pressing scientific questions.

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13. Appendices

13.1. Appendix 1: "Enzyme-assisted synthesis of high-purity, chain-deuterated POPC"

ACS OMEGA

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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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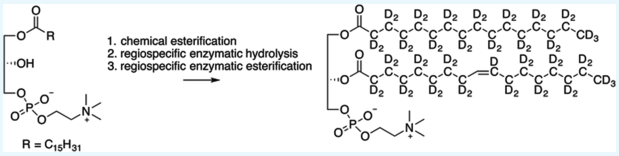

ABSTRACT: 1-Palmitoyl-d_{31}-2-oleoyl-d_{32}-*sn*-glycero-3-phosphocholine (POPC-d_{63}) with the palmitoyl and oleoyl chains deuterium-labeled was produced in three steps from 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine, deuterated palmitic acid, and deuterated oleic anhydride. Esterification at the *sn*-2 position was achieved under standard chemical conditions, using DMAP to catalyze the reaction between the 2-lysolipid and oleic anhydride-d_{64}. 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 regiopurity, 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 bilayers[4] and to locate biomolecules within bilayers.[5] For certain NMR[6,7] and vibrational spectroscopy[8,9] 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).

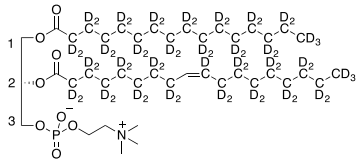

Figure 1. Chain-deuterated isotopologue of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC-d_{63}), with stereospecific numbering indicated.

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]

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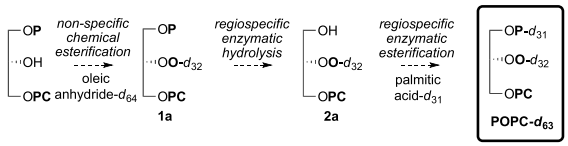
Scheme 1. Proposed Chemoenzymatic Synthesis of Chain-Deuterated POPC



P = palmitoyl; **O** = oleoyl; **PC** = phosphocholine.

While this purity may be sufficient for many purposes, many investigators would find it useful to know, and account for, the regiopurity of the lipid utilized. We thus aimed to produce highly regiopure 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,[18] 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.[19,20] Semisynthesis from an *sn*-glycero-3-phosphocholine (GPC)-based natural precursor represented the most straightforward method, primarily because the chiral center is already installed;[18] 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[21] and typically requires toxic chemicals such as cadmium or zinc salts or tin oxides.[22,23] 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)[24] to exacerbate acyl chain migration, resulting in a product that is contaminated with the regioisomer.[25] Many of the literature reports describing the synthesis of POPC do not report the regiopurity of the product.[19,20]

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 success in the reverse reaction differs widely, since it requires low water activity (a_w), which is incompatible with the requirements of some enzymes for activity.[26,27] It also requires large excesses of fatty acids,[26,27] which is particularly relevant to us, since we aim to install two deuterium-labeled chains, one of which is challenging to produce. Some enzymes, including lipases used to modify phospholipids at the *sn*-1 position, are cheap, readily available, and even available in the immobilized form. Others, such as the phospholipase A_2 (PLA_2) enzymes typically used to modify phospholipids at the *sn*-2 position, suffer from the disadvantages of high cost, limited availability, and the requirement for the enzymes to be immobilized in-house. Immobilization strategies require knowledge of the enzyme structure, function and stability, solid supports and principles of attachment, and effects of immobilization on enzyme activity, which are usually unfamiliar to synthetic chemists.

We decided upon a mixed chemoenzymatic synthesis of chain-deuterated POPC balancing considerations of efficiency, purity, and operational simplicity. Reported two-step processes starting with regiospecific enzymatic esterification of GPC suffer from the insolubility of GPC in nonpolar solvents,[28−30] the formation of byproducts,[29−31] and the requirement of chromatographic separation of the lysolipid intermediate,[30] which may itself compromise regiopurity. Instead, a three-step process starting from a lysolipid was preferred, using chemical (Steglich-type) conditions for the condensation reaction at the *sn*-2 position, and regiospecific enzymatic catalysis for the hydrolysis and esterification reactions at the 1-position (Scheme 1).

Modification at the *sn*-2 Position. Chemical esterification was selected over enzymatic esterification at the *sn*-2 position because this reaction is high yielding and because the susceptibility of 2-lysolipids to undergoing intramolecular acyl migration to form 1-lysolipids is low,[21,26] meaning that acyl migration could be minimized under the right conditions (of temperature, pH, and duration, for instance). Fatty acid anhydrides, typical reagents used for the chemical acylation of 2-lysophospholipids, are easily prepared from the corresponding acids.[32]

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 (PLA_2) esterification reports low yields, by virtue of the conflicting requirements of low water activity to encourage the equilibrium to favor esterification,[27] and higher water activity (>0.2)[33] to ensure PLA_2 activity. To obtain optimal yields, the *sn*-2 enzymatic esterification requires a very high excess of fatty acid,[27] which in this case is deuterated oleic acid, the synthesis of which is non-trivial.[34] We also considered that many of the sources of PLA_2 reported for this application in the literature (including porcine pancreas PLA_2, the most commonly used enzyme for this purpose)[33] were difficult to procure[35] 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 meihei* (RM) was selected. This lipase is known to accept phospholipids as substrates and is commercially available in the immobilized form (acrylic resin as solid support). The hydrolysis reaction is fast, efficient, and highly regiopecific.[36] A 1-lysolipid is much more prone to acyl migration than a 2-lysolipid,[21] 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.[24] If acyl

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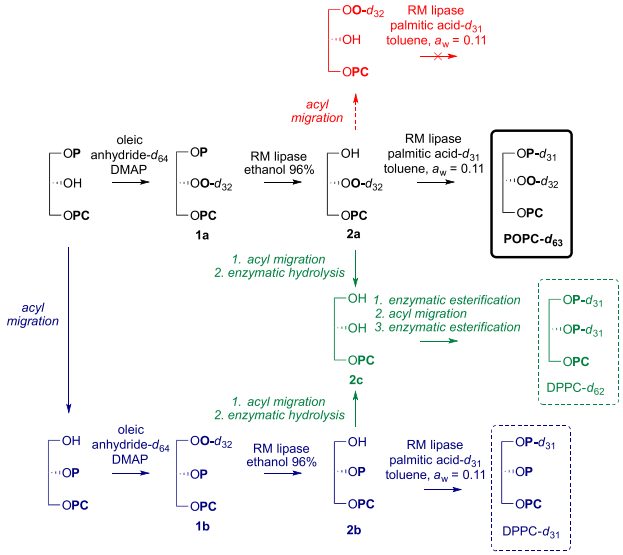
Scheme 2. Synthesis of POPC-d_{63} and Rationalization for the Presence of Isotopologues of DPPC



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 regiopurity. Furthermore, the catalysts for *sn*-1 esterification reactions, lipase enzymes, exhibit activity even in nearly anhydrous systems,[33] 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.[34]

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-d_{64} (Scheme 2, black route). Oleic anhydride-d_{64} was synthesized according to published methods or modifications of them.[32,34,37]

The diacylated lipid PO-d_{32}PC **1a** was obtained in a 72% yield after purification by flash column chromatography on silica gel. The oleic acid-d_{32} byproduct was also easily recovered. We acknowledged that some of the regioisomer, O-d_{32}PPC **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 ^{1}H 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.[24] We thus aimed to limit acyl migration of the 1-lysolipid **2a** by using short-duration (2 min) ^{1}H 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 ^{1}H 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).[38] 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 ^{1}H 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-d_4). 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).[13,18]

The same type of immobilized RM lipase was then used to re-esterify at the *sn*-1 position using deuterated palmitic acid-d_{31} to provide POPC-d_{63} (Scheme 2, black route). Unlike PLA_2 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.[26] 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

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because of competing hydrolysis reactions.[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).[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 pleasingly 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_{63}, could be produced (Scheme 2, red route). However, in addition to POPC-d_{63}, two isotopologues of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC-d_{31} and DPPC-d_{62}) could be identified in the final product by mass spectrometry. DPPC-d_{31} is attributed to the acyl migration that occurs prior to the first, chemical esterification (Scheme 2, blue route), while DPPC-d_{62} 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 ^{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 flame ionization detection response (RMR).[40] Using this method, we determined that the lipids in the product mixture were in a POPC-d_{63}:DPPC-d_{31}/d_{62} ratio of 96.4%:3.6%.

It was helpful to quantify both the chemical and isotopic purity as these are applied to calculations of scattering length density (SLD) for neutron experimentation.[12] However, we found no need to separate the POPC-d_{63} 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.[16]

The yield of chain-deuterated POPC obtained over three steps was 23%, and the final lipid mixture (96.4% POPC-d_{63}) was obtained on a 57 mg scale, an amount suitable for neutron reflectometry and small-angle neutron scattering investigations.

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 lysophospholipid using a deuterated acyl donor. Phospholipids with high chemical purity and complete regiopurity 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.

EXPERIMENTAL SECTION

General Procedures. Lipozyme RM (lipase from *Rhizomucor meihei*, 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_{31} and oleic acid-d_{32} were prepared based upon literature methods.[34] One of the olefinic hydrogens in the prepared deuterated oleic acid-d_{32} exists as ^{1}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;[34] 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 ^{1}H and 100 MHz for ^{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 ^{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 (^{1}H and ^{13}C NMR spectra, GC traces and mass spectra) are provided in the Supporting Information.

Oleic Anhydride-d_{64}. A solution of dicyclohexylcarbodiimide (1.0 M in DCM, 1.08 mL, 1.08 mmol) was added to a solution of oleic acid-d_{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%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.40−2.42 (br complex, 2H), 5.32 (br s, 1H). *Contributions from residual protons not reported.

1-Palmitoyl-2-oleoyl-d_{32}-sn-glycero-3-phosphocholine ***1a.*** Oleic anhydride-d_{64} (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 $CHCl_3$/CH_3OH/H_2O, using bromocresol green and Hanessian's stains separately to visualize) provided the pure title ester as a white solid (174

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mg, 72%). ^{1}H NMR (CD_3OD, 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, 1H), 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. ^{13}C NMR (CD_3OD, 100 MHz) δ 14.5, 23.7, 26.0, 30.2, 30.47, 30.50, 30.66, 30.78, 30.81, 33.1, 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, J = 8.1 Hz), 130.6, 174.6, 174.9. *Contribution from residual protiated carbon atoms from oleoyl chain.

1-Hydroxy-2-oleoyl-d_{32}-sn-glycero-3-phosphocholine **2a.** According to a literature method for an analogous compound,[36] 1-palmitoyl-2-oleoyl-d_{32}-*sn*-glycero-3-phosphocholine **1a** (174 mg, 219 μmol) was dissolved in ethanol (10 mL, 96%). Lipase from *Rhizomucor meihei* (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 between measurements (2 h; monitored by TLC, solvent conditions: 65/25/4 $CHCl_3/CH_3OH/H_2O$, 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-d_{32}-*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. ^{1}H NMR (CD_3OD, 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-d_{31}-2-oleoyl-d_{32}-sn-glycero-3-phosphocholine. Palmitic acid-d_{31} (2.40 g, 8.36 mmol) and 1-hydroxy-2-oleoyl-d_{32}-*sn*-glycero-3-phosphocholine **2a** (93.5 mg, 167 μmol) were dissolved in toluene (17 mL) under vigorous stirring. The mixture was transferred into a glass beaker together with lipase from *Rhizomucor meihei* (immobilized on acrylic resin, 275 IUN/g, 1.26 g) and placed inside a closed vessel containing a saturated solution of LiCl (a_w of 0.11). The closed vessel was gently agitated at 25 °C until no further consumption of the starting material could be observed between measurements (45 h; monitored by TLC). The resins were removed via filtration and washed with toluene, methanol, and water; the combined extracts were concentrated under reduced pressure. Flash column chromatography (gradient elution: 65/25/4 $CHCl_3/CH_3OH/H_2O$, using bromocresol green and Hanessian's stains separately to visualize) provided the pure title lipid as a white solid (57 mg, 42%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.90 (s, 0.26H*), 1.22−1.29 (complex, 3.08H*), 2.31−2.32 (complex, 1.21H*), 3.23 (s, 9H), 3.64 (m, 2H), 4.00 (t, J = 6.0 Hz, 2H), 4.17 (dd, J = 12.0, 7.0 Hz, 1H), 4.27 (m, 2H), 4.44 (dd, J = 12.0, 3.2 Hz, 1H), 4.57 (br s, 1H), 5.24 (m, 1H), 5.33 (s, 0.56H*). *Contributions from residual protium from oleoyl chain. ^{13}C NMR (CD_3OD, 400 MHz) δ 30.8*, 34.9*, 54.7 (t, J = 3.8 Hz), 60.5 (d, J = 5.0 Hz), 63.7, 64.9 (d, J = 5.4 Hz), 67.5 (m), 71.7 (d, J = 8.4 Hz), 130.6*, 174.7, 175.0. *Contributions from residual protiated carbon atoms from oleoyl chain. MS (ESI+) calculated for $C_{42}H_{20}D_{63}NO_8P$ (POPC-d_{63}) $[M + H]^+$ as 823.98; found: 823.90. Isotope contributions: 0.2% d_{48}, 0.2% d_{49}, 0.3% d_{50}, 0.4% d_{51}, 0.6% d_{52}, 0.8% d_{53}, 1.2% d_{54}, 1.9% d_{55}, 2.7% d_{56}, 4.5% d_{57}, 6.9% d_{58}, 13.2% d_{59}, 19.0% d_{60}, 22.6% d_{61}, 16.9% d_{62}, 8.5% d_{63}.

Three additional adducts were observed: (1) calculated for $C_{42}H_{19}D_{63}NaNO_8P$ (POPC-d_{63}) $[M + Na]^+$ as 845.9; found: 845.9. Isotope contributions: 1.5% d_{51}, 0.9% d_{52}, 1.5% d_{53}, 1.5% d_{54}, 1.8% d_{55}, 3.0% d_{56}, 4.6% d_{57}, 6.7% d_{58}, 13.1% d_{59}, 18.8% d_{60}, 22.5% d_{61}, 15.8% d_{62}, 8.2% d_{63}.

(2) Calculated for $C_{40}H_{19}D_{62}NO_8P$ (DPPC-d_{62}) $[M + H]^+$ as 797.0; found: 796.9. Isotope contributions: 1.0% d_{49}, 1.4% d_{50}, 1.9% d_{51}, 2.4% d_{52}, 3.9% d_{53}, 3.9% d_{54}, 4.3% d_{55}, 5.8% d_{56}, 7.7% d_{57}, 10.1% d_{58}, 12.1% d_{59}, 15.9% d_{60}, 16.9% d_{61}, 12.6% d_{62}.

(3) Calculated for $C_{40}H_{50}D_{31}NO_8P$ (DPPC-d_{31}) $[M + H]^+$ as 765.8; found: 765.7. Isotope contributions: 0.7% d_{21}, 0.7% d_{22}, 1.4% d_{23}, 1.4% d_{24}, 1.4% d_{25}, 3.5% d_{26}, 4.2% d_{27}, 8.3% d_{28}, 16.0% d_{29}, 27.1% d_{30}, 35.4% d_{31}.

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-d_{31} and methyl oleate-d_{32} were prepared as follows: Palmitic acid-d_{31} (128 mg, 445 μmol) was dissolved in a methanolic solution of H_2SO_4 (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-d_{31}. Methyl oleate-d_{32} was produced using the same method with oleic acid-d_{32} (28 mg, 89 μmol) in a methanolic solution of H_2SO_4 (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,[40] 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-d_{31} since this was the major isotopologue).[41] Samples of POPC-d_{63} were prepared as follows: A methanolic solution of H_2SO_4 (3 mL, 2.5% v/v) and POPC-d_{63} (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 in performed in triplicate, and the averages of these were used in our calculations.

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■ ASSOCIATED CONTENT

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 Bogojevic − *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-d_{31} and oleic acid-d_{32}, and from these O.B. prepared POPC-d_{63}. A.E.L. wrote the manuscript with assistance from O.B.

Notes

The authors declare no competing financial interest.

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