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Authors:	Anna Leung, Hanna Wacklin-Knecht, ESS

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Abstract

The need for deuterated lipids and surfactants was reinforced by a user survey conducted by the DEUNET in 2016. Both of these classes of molecules are in high demand for neutron studies. The *lack of availability* of some known deuterated lipids and surfactants limits the number of experiments that can be performed exploiting them. The *limited range* of these molecular classes is another limiting factor. The ESS chemical deuteration laboratory aims to address both of these problems, by synthesising in-demand lipids such as deuterated oleic acid; and by establishing methods of synthesising novel surfactants such as amino acid surfactants, and novel chain-deuterated phospholipids.

Introduction

SURFACTANTS

Since traditional surfactants have potentially damaging environmental effects, an area of interest is in biodegradable surfactants such as amino acid/peptide surfactants such as N-lauroyl-L-alanine (Figure 1).

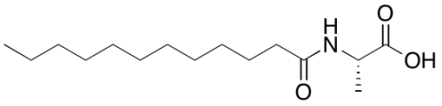


Figure 1. N-lauroyl-L-alanine, an amino acid surfactant.

Since they are a relatively new field of interest, even the unlabelled analogues are difficult or expensive to procure, and to the best of our knowledge deuterated analogues are not available from any commercial chemical supplier.

LIPIDS: SATURATED FATTY ACIDS AND DIACIDS

Perdeuterated saturated fatty acids and diacids are useful precursor molecules for a range of molecular classes, including complex lipids and surfactants. The synthesis of a number of saturated fatty acids and diacids (plus an unsaturated fatty acid, oleic acid) is performed at the ISIS deuteration laboratory (UK) but this laboratory is normally funded to provide these molecules only to UK neutron users.

LIPIDS: UNSATURATED FATTY ACIDS

Oleic acid (Figure 2) is itself an important molecule for food and drug formulation science. The deuterated analogue is useful for neutron experiments but it is not amenable to direct deuteration; rather, its long and challenging synthesis limits its availability to neutron users. Thus, the ESS lab aims to synthesise multi-gram amounts of highly deuterated oleic acid. Oleic acid is also a precursor molecule to a broad number of molecular classes such as lipids and surfactants, also highly in demand for neutron experiments.

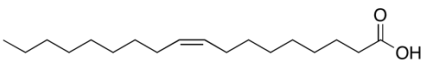


Figure 2. Oleic acid.

LIPIDS: PHOSPHOLIPIDS

Saturated and unsaturated fatty acids are important precursors to a broad number of more complex molecular classes (which cannot be directly deuterated) such as phospholipids including 1-palmitoyl-2-oleoyl-3-*sn*-glycerophosphocholine (POPC) (Figure 3a). Since the bulk of the hydrogen atoms are located in the phospholipid tails, suitable contrast for neutron experiments can often be obtained by deuterating the acyl chains only. Commercially-available tail-deuterated phospholipids are limited to phospholipids with the same, saturated chain, such as 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) (Figure 3b), while natural phospholipids typically contain a saturated chain at the first position and an unsaturated chain at the second position.

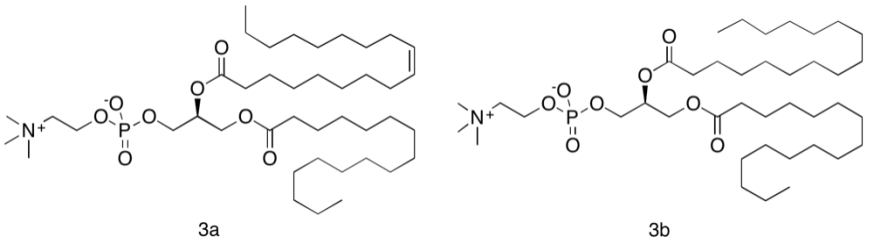
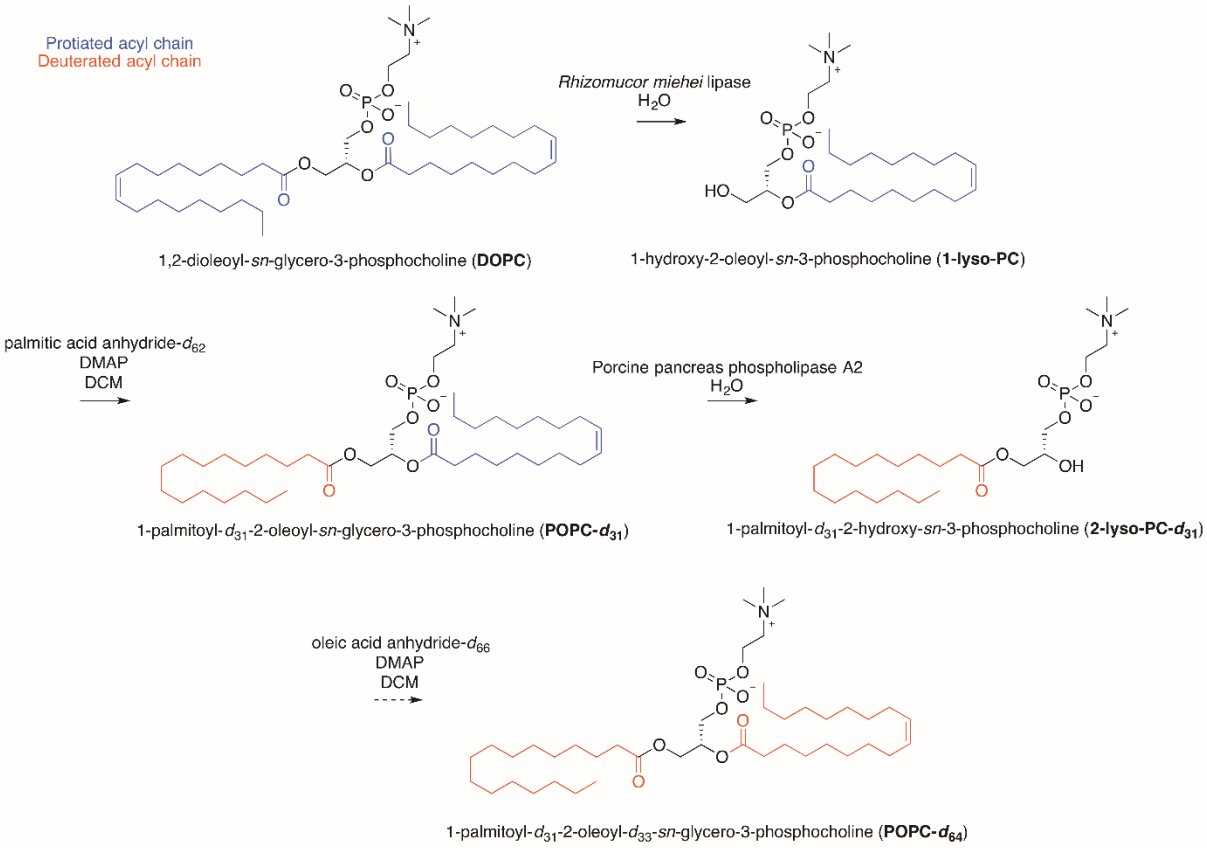


Figure 3a. POPC and 3b. DPPC.

For this class of molecules, ESS proposed using a combined biochemical-chemical approach to modify unlabelled phospholipids, using site-specific enzymes to hydrolyse the tails at the 1- and 2-positions respectively, and standard chemical esterification conditions to install deuterated tails synthesised from saturated and unsaturated fatty acids such as palmitic and oleic acids (Scheme 1).

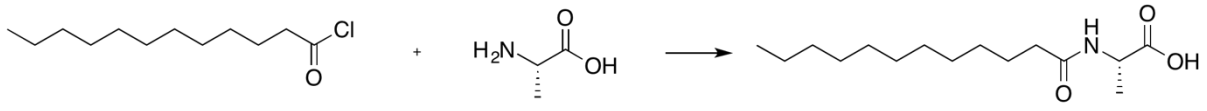


Scheme 1. Proposed synthesis of tail-deuterated POPC from unlabelled DOPC in four synthetic steps.

Synthesis

SURFACTANTS

Work began with the identification of useful amino acid surfactants: the existence of twenty standard amino acids (positively charged, negatively charged and neutral; chiral and achiral) broadens the options significantly. This is further complicated by the ability to synthesise surfactants with a range of chain lengths. Guided by a neutron scattering user with an interest in these surfactants, an initial amino acid surfactant was synthesised at the ESS chemistry laboratory in order to perform initial solubility testing (without deuterium labelling) (Scheme 2). This was comprised of a medium-chain (C12) fatty acid chain and a natural, chiral amino acid, L-alanine:

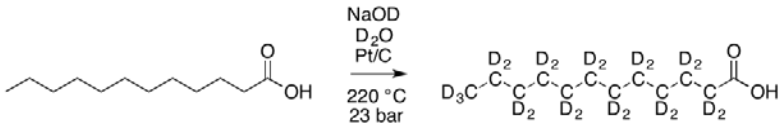


Scheme 2. Synthesis of N-lauroyl-L-alanine, a chiral amino acid surfactant.

Micelle formation of the surfactant will be probed in an upcoming neutron scattering experiment, which will direct the synthesis of deuterated analogues of amino acids surfactants with different chain length and amino acid headgroups which will enable more detailed structural and functional investigations using neutron techniques.

LIPIDS: SATURATED FATTY ACIDS AND DIACIDS

The perdeuteration of saturated fatty acids and diacids occurs under hydrothermal conditions, in a Parr reactor, capable of operating under high temperature and pressure. In order to perform this task, the ESS chemical deuteration laboratory procured a Parr reactor (installed July 2018) capable of operating at 350 °C and 200 bar. Lauric acid, nonanoic acid, palmitic acid and azelaic acid were perdeuterated using D_2O as deuterium source and Pt/C as C-H activation catalyst (Scheme 3 for lauric acid):



Scheme 3. H/D exchange of lauric acid under metal-catalysed, hydrothermal conditions.

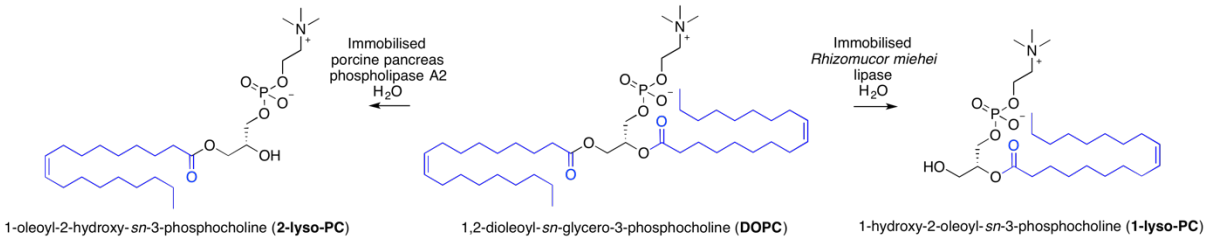
To achieve deuterium incorporation (non-exchangeable positions) of 98%, these molecules are subjected to H/D exchange two or three times (depending on the number of moles of hydrogen per molecule), replacing the reagents each time.

LIPIDS: UNSATURATED FATTY ACIDS

The ESS produced 2.2 g of highly deuterated methyl oleate-d_{32}, the precursor to oleic acid-d_{32} (Figure 2), according to a literature procedure. This will be made available to neutron users during the first round of proposals for the ESS Deuteration and Macromolecular Crystallisation (DEMAX) Platform.

LIPID: PHOSPHOLIPIDS

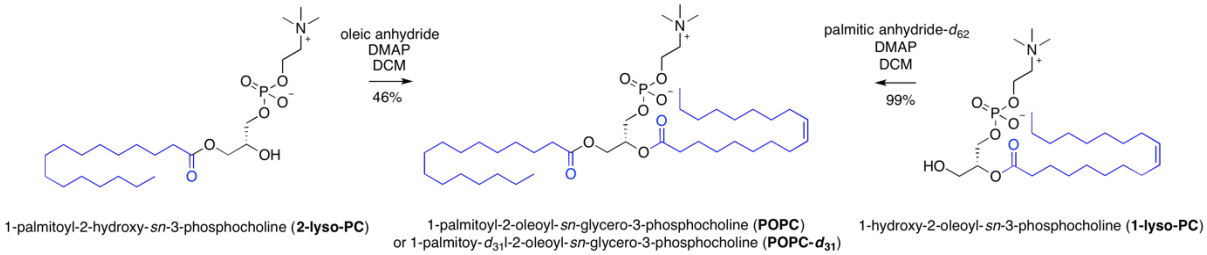
Selective hydrolysis of the acyl chains at the 1- and 2-positions of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), using a lipase from *Rhizomucor miehei* fungus and a phospholipase A2 from porcine pancreas, respectively, provided 1- and 2-lyso PC (Scheme 2). In these hydrolysis reactions where two chains are present on the substrate, these regiospecific enzymes offer a clear advantage: they hydrolyse only the chains at one position on the molecule (either the 1- or 2-position). (Though the chemical hydrolysis of one of these chains is theoretically more favourable than the other, no chemical reagents show specificity to hydrolyse only one.)



Scheme 2. Regiospecific hydrolysis of the chains at the 1- and 2-positions of DOPC, using enzymes.

Rhizomucor miehei lipase was a generous gift from Novozymes. Phospholipase A2 (PLA2) from porcine pancreas, was purchased from Sigma-Aldrich. In order to facilitate recycling of the PLA2 it was immobilised in-house.

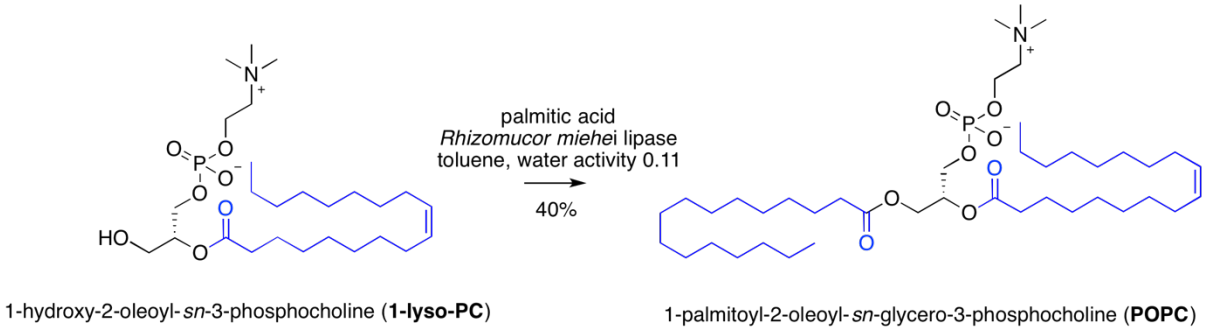
The subsequent esterification requires no specificity, since there is only one position on the molecule where esterification is possible. We thus established esterification conditions of 1- and 2-lyso PC, using standard chemical esterification conditions, using an acid anhydride (prepared prior to the esterification reaction) and an acid catalyst (4-dimethylaminopyridine, DMAP) (Scheme 3). The work was performed using both protiated and deuterated acid anhydrides.



Scheme 3. Chemical esterification of 1- and 2-lyso PC using acid anhydrides and DMAP.

A review of the literature suggested that the conditions used for the chemical esterification, particularly the use of DMAP, an acid catalyst, was likely to promote a side reaction in our system, which might compromise the purity of the phospholipid products. Current work focuses on establishing a suitable method of analysis for the products to determine whether this side reaction occurs, and if so, to what extent. Attempts to do so have been hampered by unknown amounts of the side product being present in commercially available POPC, which we aim to use as our standard. The most promising method is the use of tandem mass spectrometry which has the ability to discriminate between molecules on the basis of fragmentation patterns at a given voltage. We have engaged the Swedish Metabolomics Centre to perform this analysis for us, which showed promising initial results.

Meanwhile, we are also investigating the possibility of using *Rhizomucor miehei* lipase and porcine pancreas phospholipase A2 enzymes to perform the esterification reactions in addition to the hydrolysis reactions (though regiospecificity is not required here, the enzymatic reactions are beneficial since they occur at neutral pH, which should suppress the undesired side reaction). To date, 1-lyso PC has been esterified under enzymatic conditions (Scheme 4). Yields are typically lower than those for the chemical esterification, but if the purity of the product is higher than that produced chemically, this method may still prove more desirable.



Scheme 4. Enzymatic esterification of 1-lyso PC.

Conclusions and Future work

Work at ESS, using standard chemical techniques and novel biochemical methods, has enabled the synthesis of a wide range of labeled and unlabeled lipids and surfactants, and has allowed ESS to offer these molecules to the neutron scattering community via the ESS DEMAX call for proposals. ESS have begun to routinely synthesise perdeuterated saturated fatty acids and diacids, as well as unsaturated oleic acid. We have established the synthesis of chiral amino acid surfactants, and have made significant progress towards the synthesis of chain-deuterated phospholipids such as POPC.

Current and future work focuses on completing the synthesis of chain-deuterated phospholipids. Analysis of the products produced via chemical synthesis will either direct the method towards an entirely biochemical route, or encourage us to continue with a combined chemical-biochemical method. We will then extend the method to deuterated systems, to produce chain-deuterated phospholipids.