Special Issue Article

Journal of Labelled Compounds and Radiopharmaceuticals

Received 22 May 2013, Revised 12 June 2013, Accepted 12 June 2013 Published online in 16 July 2013 Wiley Online Library

(wileyonlinelibrary.com) DOI: 10.1002/jlcr.3088

Synthesis of deuterated [D_{32}]oleic acid and its phospholipid derivative [D_{64}]dioleoyl-*sn*-glycero-3-phosphocholine†‡

Tamim A. Darwish,[a]* Emily Luks,[a] Greta Moraes,[a] Nageshwar R. Yepuri,[a] Peter J. Holden,[a] and Michael James[a,b,c]

Oleic acid and its phospholipid derivatives are fundamental to the structure and function of cellular membranes. As a result, there has been increasing interest in the availability of their deuterated forms for many nuclear magnetic resonance, infrared, mass spectroscopy and neutron scattering studies. Here, we present for the first time a straightforward, large-scale (gram quantities) synthesis of highly deuterated [D_{32}]oleic acid by using multiple, yet simple and high yielding reactions. The precursors for the synthesis of [D_{32}]oleic acid are [D_{14}]azelaic acid and [D_{17}]nonanoic acid, which were obtained by complete deuteration (>98% D) of their ^{1}H forms by using metal catalysed hydrothermal H/D exchange reactions. The oleic acid was produced with ca. 94% D isotopic purity and with no contamination by the *trans*-isomer (elaidic acid). The subsequent synthesis of [D_{64}]dioleoyl-*sn*-glycero-3-phosphocholine from [D_{32}]oleic acid is also described.

Keywords: deuterated oleic acid; dioleoyl-*sn*-glycero-3-phosphocholine; deuterated phospholipid; [D_{17}]nonanoic acid; [D_{14}]azelaic acid; olefinic proton

Introduction

The surface of a biological cell is of great interest because it mediates interactions and exchanges between the cell and its surroundings. Phospholipids bearing different saturated and unsaturated fatty acid chains are major components of biological membranes [Figure 1(a)] and have been the focus of a vast number of scientific studies in fields such as molecular biology, biochemistry, chemistry, biophysics and pharmacology. Oleic acid [Figure 1(c)] is a monounsaturated fatty acid, with a *cis*-configuration about its double bond and is one of the most widely distributed fatty acids in nature.

At the molecular level, oleic acid constitutes the unsaturated tail component of many phospholipid molecules such as 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and dioleoyl-*sn*-glycero-3-phosphocholine [DOPC—Figure 1(b)] that are fundamental to the structure, function, order and fluidity of cellular membranes. Oleic acid not only promotes membrane fluidity at low temperature but also allows the cell membrane to maintain a liquid crystalline state as the temperature increases.[1] Biophysical investigations into cellular membranes are of vital importance in understanding fundamental mechanisms of biomolecular transport, transcription, signalling and receptor function. In addition, numerous neurological disorders, neurodegenerative diseases (such as schizophrenia, Parkinson's, Alzheimer's and prion disease)[2–4] and the action of bacterial toxins[5–8] are closely associated with biochemical interactions at cellular membranes. Therefore, there exists substantial demand for isotopically labelled lipids and fatty acids for many studies of membrane biology, biochemistry and biophysics. Deuteration of phospholipids or other long-chain fatty acids is an essential prerequisite in many ^{1}H and ^{2}H (deuterium) nuclear magnetic resonance (NMR),[9–11] mass spectroscopy (MS),[12] and neutron scattering[13–23] studies. Although ^{1}H and ^{2}H behave similarly in chemical reactions, the composition of their nuclei results in vastly different neutron scattering properties. The use of mixtures of deuterated and hydrogenated solvents to manipulate neutron scattering length densities to enable contrast variation is widespread in techniques, such as neutron reflectometry, neutron diffraction and small angle neutron scattering. However, this approach is limited for multicomponent organic and biological systems containing molecules of similar scattering length density, and the application of molecular deuteration to solve this issue has been limited by the range of deuterated molecules that are commonly available.

In NMR spectroscopy studies, ^{2}H NMR has proven to be very effective for the elucidation of lipid structure and motion in

[a]*National Deuteration Facility, Australian Nuclear Science and Technology Organisation (ANSTO), Locked Bag 2001 Kirrawee DC NSW 2232, Australia*

[b]*School of Chemistry, The University of New South Wales, Kensington, Locked Bag 2001 NSW 2052, Australia*

[c]*Australian Synchrotron, 800 Blackburn Road, Clayton VIC 3168, Australia*

**Correspondence to: Tamim A. Darwish, Australian Nuclear Science and Technology Organisation (ANSTO), Kirrawee, DC NSW 2232, Australia. E-mail: tamim.darwish@ansto.gov.au*

† *This article is published in Journal of Labelled Compounds and Radiopharmaceuticals as a special issue on IIS 2012 Heidelberg Conference, edited by Jens Atzrodt and Volker Derdau, Isotope Chemistry and Metabolite Synthesis, DSAR-DD, Sanofi-Aventis Deutschland GmbH, Industriepark Höchst G876, 65926 Frankfurt am Main, Germany.*

‡ *Supporting information can be found in the online version of this article.*

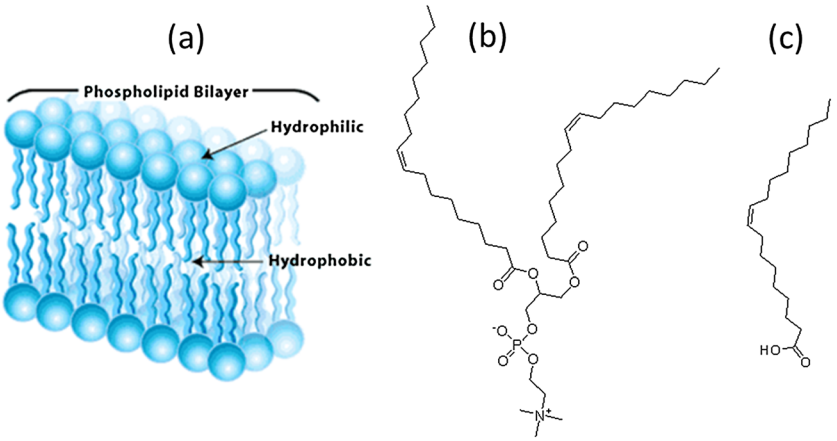
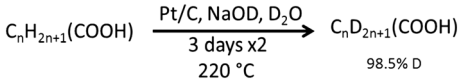


Figure 1. Chemical and schematic structure of (a) a lipid bilayer membrane, (b) dioleoyl-*sn*-glycero-3-Phosphatidylcholine and (c) oleic acid.

model and biological membranes.[9–11] However, relatively few research groups have employed this technique because of the difficulty in the synthesis of suitably deuterium labelled lipids. Although the use of commercially available deuterated phospholipids with saturated acyl chains has been prevalent, investigations of highly biologically relevant biomimetic cellular membranes[24] have been lacking because of the lack of deuterated lipids and fatty acids with unsaturated (e.g. oleoyl) chains. Previous reports in the literature have focused on site-specific deuteration of oleic acid and its subsequent lipids and derivatives.[11b,25,26] With this background in mind, it becomes evident that there is a significant need to develop a convenient synthesis of completely deuterated oleic acid, from which corresponding deuterated phospholipids can be produced. Here, we report a convenient method for the production of gram quantities of [D_{32}]oleic acid (ca. 94% D isotopic purity) and its phospholipid derivative ([D_{64}]DOPC) using a number of simple and high yielding chemical reactions. This involves the hydrothermal metal catalysed H/D exchange reactions of azelaic and nonanoic acids that are produced in high yields using upscalable quantities (12–20 g) and then conjugating the two saturated alkyl chains in a *cis*-configuration. The overall deuteration extent was measured by MS, and the deuteration level at the different methylene units was probed using NMR spectroscopy to validate the reaction process and monitor any D/H back exchange which could reduce the isotopic purity of the final products.

Results and discussions

Metal catalysed hydrothermal reactions in D_2O are known to affect H/D exchange on carbon sites in general and even on saturated aliphatic carbons (non-acidic protons) to produce completely or partially deuterated compounds (Scheme 1).[27,28] As a result of the harsh conditions of hydrothermal reactions (typically >200°C and >20 bar), undesirable side reactions, such as dehalogenation, deuterium addition to multiple bonds, hydrolysis, epimerization or the cleavage of protecting groups can take place. However, robust molecules such as saturated fatty acids are well suited to such conditions. They undergo H/D exchange when treated with alkaline D_2O solution in the presence of a metal catalyst (Pt or Pd) at high temperatures (180–240°C).[27,28]



Scheme 1. Deuteration of saturated fatty acid by using metal catalysed hydrothermal reaction.

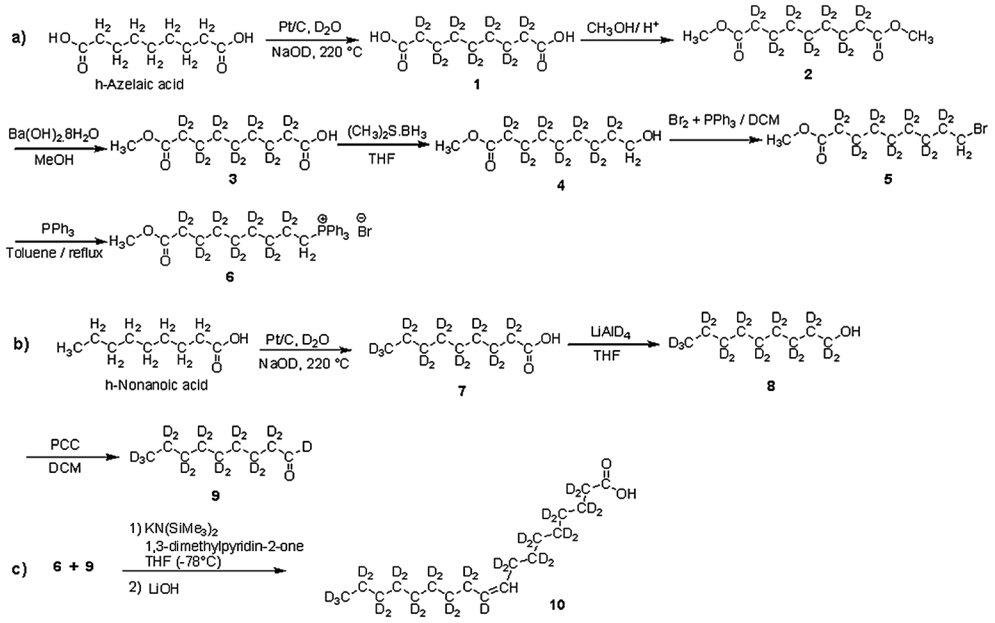
Prior to our development of a viable synthetic route to deuterate oleic acid, it was evident that hydrothermal catalytic H/D exchange of protonated oleic acid in Parr reactors reduced the double bond to a great extent and induced migration of the central double bond, scrambling the *cis*-configuration to the more stable *trans*-isomer. Thus, for the synthesis of fully deuterated oleic acid, it was necessary to start from saturated alkyl chain precursors, namely azelaic acid (diacid) and nonanoic acid, which were deuterated using hydrothermal H/D exchange reactions. Several routes for the stereospecific synthesis of unsaturated fatty acids and their derivatives have been reported,[29] with perhaps the simplest of these methods being the one reported by Bergelson *et al.*[30] in which the Wittig reaction is employed to introduce unsaturation to the molecule. The complete reaction sequence to synthesize [D_{32}]oleic acid is given in Scheme 2. Specific aspects of the chemistry and issues arising from H/D scrambling during various reaction steps are examined and discussed in the succeeding text.

Methylation of [D_{14}]azelaic acid (1) to [D_{14}]dimethyl azelate (2)

[D_{14}]Azelaic acid was methylated to protect both ends of the diacid by simply refluxing it in protonated MeOH with a catalytic amount of H_2SO_4 to give pure [D_{14}]dimethyl azelate (**2**) with 91% yield (Scheme 2a), which did not require chromatographic purification. The two protonated methyl groups on each end of the molecule were used as an internal standard to calculate the percentage deuteration at the different methylene units along the alkyl chain as well as the overall deuterium content, which was calculated to be 98.5% D for **2**. This reaction was achieved without any noticeable D/H back exchange on the methylene units including in particular the alpha carbon (C_α) positions next to the two carboxylic acid groups (~98.7% D) (Supplementary data, Figures S1 and S6).

Selective hydrolysis of [D_{14}]dimethyl azelate (2) to [D_{14}] methyl hydrogen azelate (3)

Half esters (mono-esters) have often been prepared by partial esterification[31] and direct fractional distillation of the three



Scheme 2. [D_{32}]Oleic acid synthetic steps.

products of the reaction; however, this method is laborious. Furthermore, disproportionation of the half ester occurs with higher-boiling half esters (e.g. methyl hydrogen azelate) because prolonged fractional distillation at high temperatures is needed. In this study, the deuterated diester **2** was selectively hydrolysed to yield the half ester **3** on the basis of the method of Rama Rao *et al.*[32] by using $Ba(OH)_2.8H_2O$.

The method of selective hydrolysis using $Ba(OH)_2.8H_2O$ relies on the insolubility of the mono-barium salt of the diester in MeOH. Once the mono-barium salt ester is formed by the hydrolysis of the diester **2** with the barium base, it precipitates out of the MeOH solution preventing further hydrolysis of the second ester group, and it also prevents any significant D/H back exchange in the basic solution. Any unreacted (unhydrolysed) diester **2** remains in solution and hence the precipitated mono-barium salt of the half ester was isolated by filtration, and the white product was washed with aliquots of cold MeOH to wash off the unreacted diester **2**. The diester can be recovered by dilution of the filtrates/washings with water followed by extraction. The white precipitate of the mono-barium salt of the half ester was acidified with HCl to pH 1 and extracted with EtOAc. The crude product thus obtained contained 90% of the half ester **3**, and the remainder was the diacid **1**, which was easily separated on a silica column to give 63% yield of [D_{14}] methyl hydrogen azelate (**3**). This was achieved with negligible D/H back exchange on the C_α next to the acid and the ester groups. On the basis of NMR spectroscopy, the deuteration level at C_α position decreased from 98.7% D in **2** to 97.7% D in **3** (Supplementary data, Figure S9).

Reduction of [D_{14}]methyl hydrogen azelate (3) to [D_{14}] methyl 9-hydroxynonanoate (4)

Borane complexes are known to reduce carboxylic acid groups faster than most other groups and are therefore the reagents of choice for the reduction of carboxylic acids. Commonly, ester groups remain intact during borane reduction of a carboxylic acid with almost quantitative yields. This has been demonstrated to be due to the reactivity of the acidic proton toward borane to give trialkoxyboroxin intermediates which eventually liberate the alcohol.[33,34] The rates of reduction being carboxylic acids > aldehydes > ketones > olefins, imines > nitriles, amides and epoxides > esters.[33] The reduction of the acid group to alcohol in [D_{14}]methyl hydrogen azelate (**3**) was achieved using dimethylsulfide borane (protonated version) in THF to give the corresponding alcohol ([D_{14}]methyl 9-hydroxynonanoate (**4**)) with 96% yield. This reduction step did not lead to any D/H back exchange on the deuterated methylene units and in particular on the C_α (97.5% D), see Supplementary data, Figure S12. Although the deuterated borane (BD_3) could be used to generate **4** with two deuterium atoms on the C_α, the protonated version of the borane was used in this study to generate a protonated methylene unit next to the hydroxyl group. This was deliberate to assist in characterizing the *cis*-configuration of the double bond in the final product using ^1H and ^{13}C NMR spectroscopy, as well as to allow probing of the extent of deuteration and the D/H back exchange that may occur during the subsequent reaction steps. Unlike carbon bearing deuterium, the carbon signal of carbon-bearing hydrogen will show clearly as a singlet in the ^{13}C NMR spectrum.

Synthesis of the Wittig salt [D_{14}]9-carbomethoxy-nonyl-triphenyl phosphonium bromide (6)

The bromination of the alcohol **4** using Br_2 and triphenyl phosphine in dichloromethane was a straightforward process[35] that readily produced [D_{14}]methyl 9-bromononanoate (**5**) with 71% yield, and no D/H back exchange was observed (Supplementary data, Figure S15). The synthesis of [D_{14}]9-carbomethoxy-nonyl-triphenylphosphonium bromide (**6**) was then achieved in 83% yield by refluxing **5** with triphenyl phosphine in anhydrous toluene.[36] This was achieved with a small amount of D/H back exchange at C_α position, which showed reduction in the degree of deuteration from 97.7% D to 95.3% D (Supplementary data, Figure S18).

Synthesis of [D_{18}]nonanal (9)

Reduction of [D_{17}]nonanoic acid to the [D_{19}]alcohol nonanol was achieved in 89% yield using $LiAlD_4$, (Supplementary data, Figures S21 and S22). The partial oxidation of the alcohol to the corresponding perdeuterated aldehyde **9** was then performed using pyridinium chlorochromate in dichloromethane which gave 43% yield of **9** with no back exchange or H/D scrambling (Supplementary data, Figures S23–S24).

Synthesis of methyl oleate via the Wittig reaction

Coupling [D_{18}]nonanal (**9**) to the phosphonium bromide **6** was achieved by first generating the ylide at −78°C under aprotic conditions and then adding the aldehyde at the coolest possible temperature (−70°C to −80°C). To ensure the formation of Z double bond of methyl oleate, this reaction was performed using the *Z*-specific variant of the Wittig reaction reported by Bestmann *et al.*[37], a method that has been used widely by a number of groups to obtain pure *Z*-alkene forms of related compounds, or in some cases, specifically deuterated oleic acid.[26,38–41] This involved the use of a mixture of dry THF and 1,3-dimethyl-3,4,5,6-tetrahydro-2 (1*H*)-pyrimidinone (DMPU) as solvents and potassium hexamethyldisilazide ($KN(SiMe_3)_2$) as the base. This resulted in a 60% yield of the (*Z*)-methyl oleate (*cis*-configuration at the olefinic centre) with no contamination by the (*E*)-isomer (elaidic acid methyl ester; *trans*-configuration at the olefinic centre). ^{1}H NMR of the product showed only two signals in the region of δ_H 5.0–5.5 ppm. The small signal at δ_H 5.25 ppm was assigned to the olefinic proton residue of C(6')D, whereas the signal at δ_H 5.32 ppm was assigned to the olefinic proton of C(6)H (Figure 2). Bernstein *et al.*[42] demonstrated that the *trans*-isomer (elaidic acid ester) has a signal at 0.03 ppm downfield (higher chemical shift) to that of the *cis*-isomer (methyl oleate). In Figure 2, no other proton signal of C(6)H was observed at a higher chemical shift to δ_H 5.32 ppm, demonstrating that there was no contamination by the *trans*-isomer. Further analysis of the configuration of the olefinic centre will be addressed later in the discussion.

The protonated methyl capping group ($C(5)H_3$) in this compound was used to accurately calculate the percentage deuteration on the different methine and methylene units along the alkyl chain. The percentage deuteration of the two deuterium atoms on the Cα ($C(4)D_2$) were observed to decease by ca. 44% and the olefinic proton C(6)H was observed to exchange to deuterium by ca. 59%. This indicates that H/D scrambling has occurred between these two positions (i.e. C(6)H and $C(4)D_2$) and most likely this was mediated by the strong base, hexamethyldisilazide, during the ylide formation when mixing the phosphonium bromide salt **6** and the base for 1 h before the addition of the deuterated aldehyde. The degree of exchange between the two positions was confirmed by the ^{2}H NMR spectrum of this compound, which showed an increase in the deuteration content of the olefinic position C(6)H and a decrease in the deuteration extent in the $C(4)D_2$ (Supplementary data , Figure S25). There was no indication of any H/D scrambling occurring on other sites.

Saponification of the [D_{32}]methyloleate to oleic acid (10)

The saponification of the oleic acid methyl ester by lithium hydroxide in aqueous MeOH (a mild reagent for hydrolysis that does not affect unsaturated bonds)[39,43] gave [D_{32}]oleic acid (**10**) as a colourless oil; 80% yield. In the ^{1}H NMR spectrum (Figure 3), the relative peak area of residual protons at $C(4)D_2$ and the olefinic signal C(6)H remained unaltered in comparison with the previous reaction step (Figure 2). This shows that no significant D/H back exchange has occurred during the saponification process. By considering the deuteration percentage values that were calculated from the [D_{32}]oleic acid ester in Figure 2, the overall deuteration percentage of oleic acid was calculated to be 94% D.

As expected, the ^{13}C NMR spectrum in Figure 4 (decoupling only protons, $^{13}C\{^1H\}$) showed a single resonance for the olefinic carbon C6 indicating that only a single isomer (i.e. *cis*) is present. Signals from carbons bearing only deuterium atoms are depleted in normal $^{13}C\{^1H\}$ NMR spectra because of the extensive coupling with ^{2}H atoms (spin quantum number 1). Moreover, slower relaxation rates of carbons bearing deuterium atoms in comparison with carbons bearing only protons or a mixture of protons and deuterium also lead to spectra with low

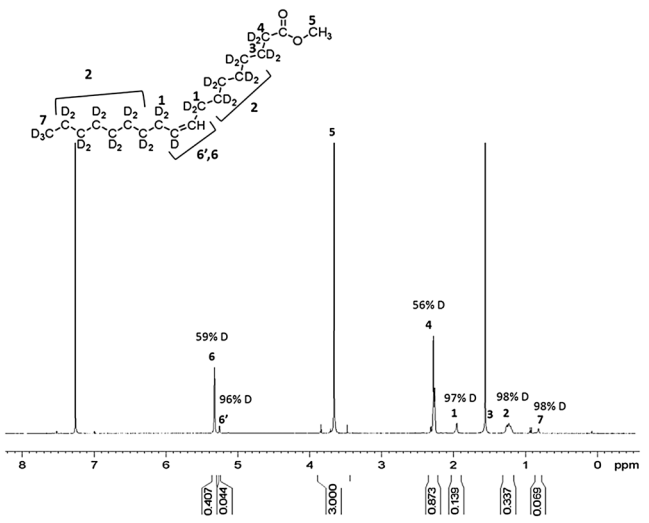


Figure 2. ^{1}H NMR ($CDCl_3$, 400 MHz) of deuterated methyl oleate showing the different resonances with their percentage deuteration, calculated using the proton signal of the methoxy-group $C(5)H_3$. Signal at δ_H 1.58 is due to H_2O in the NMR solvent.

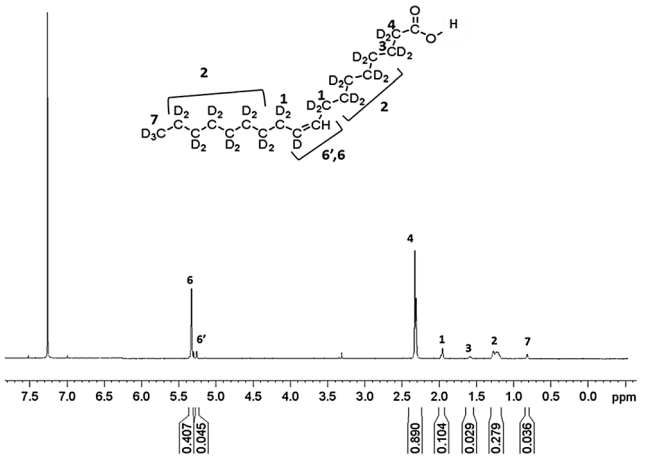


Figure 3. ^{1}H NMR (400 MHz, $CDCl_3$) of [D_{32}]oleic acid, showing the different resonances with their relative percentage deuteration. Residual dichloromethane solvent signal appears at δ_H 5.30 ppm.

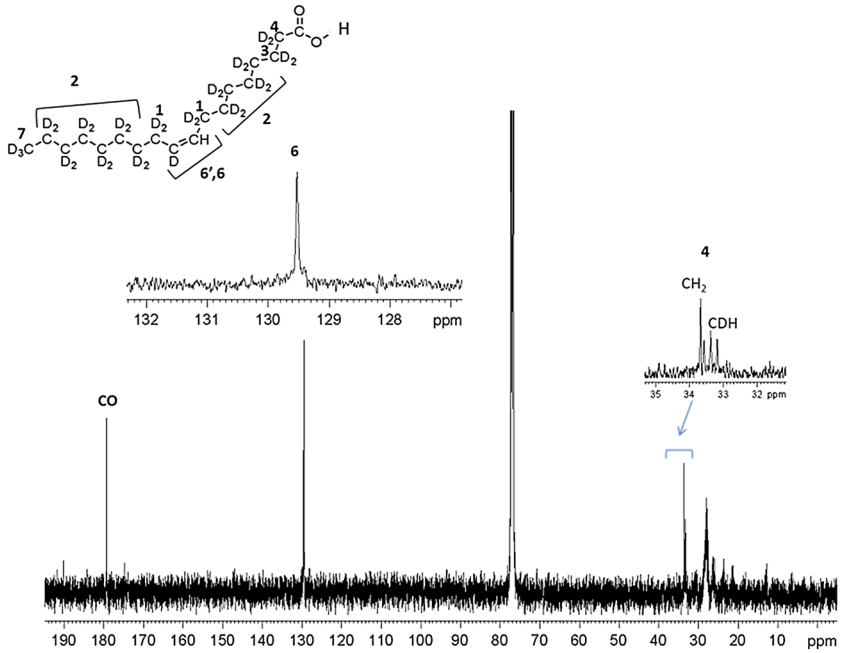


Figure 4. $^{13}C\{^{1}H\}$ NMR (400 MHz, $CDCl_3$) of [D_{32}]oleic acid showing a single resonance for the olefinic carbon of C6 and two resonances for C4, one attributed to C(4)DH (triplet) and the other to C(4)H_2 (singlet).

signal to noise ratio. H/D scrambling and back exchange at C4 was confirmed by the ^{13}C resonance of C4, which showed two isotopically shifted signals; one was attributed to C(4)DH (triplet) and the other to C(4)H_2 (singlet).

The *cis*-configuration and *trans*-configuration of the olefinic carbons in oleic acid can be distinguished by the ^{1}H-^{1}H scalar coupling, as well as by ^{1}H NMR chemical shifts of the allylic C(1)H. The ^{1}H-^{1}H scalar coupling of the olefinic proton signals in this case could not be determined because of deuteration and the extensive C-D coupling. The signals of the allylic protons in elaidic acid (*trans*-isomer) are usually shifted slightly upfield by ~0.05 ppm (lower chemical shift) compared with oleic acid (*cis*-isomer). In Figure 3, only one signal was observed for the residual allylic C(1)H. Similarly, ^{2}H NMR showed only one deuterium signal for the allylic C(1)D (Supplementary data, Figure S26). In addition, signals of the olefinic protons of elaidic acid are usually slightly downfield by ~0.03 ppm (higher chemical shift) compared with oleic acid,[44] which was not observed in Figure 3 for the olefinic proton C(6)H.

Mass spectral analysis of the deuterated oleic acid (**10**) (Figure 5) revealed the relative abundance and mass isotopic distribution of the different isotopologues, from which the overall deuteration of 93.7% D can be calculated. This value matches the value calculated from ^{1}H NMR (94% D) when considering the deuteration percentage at each carbon as previously shown.

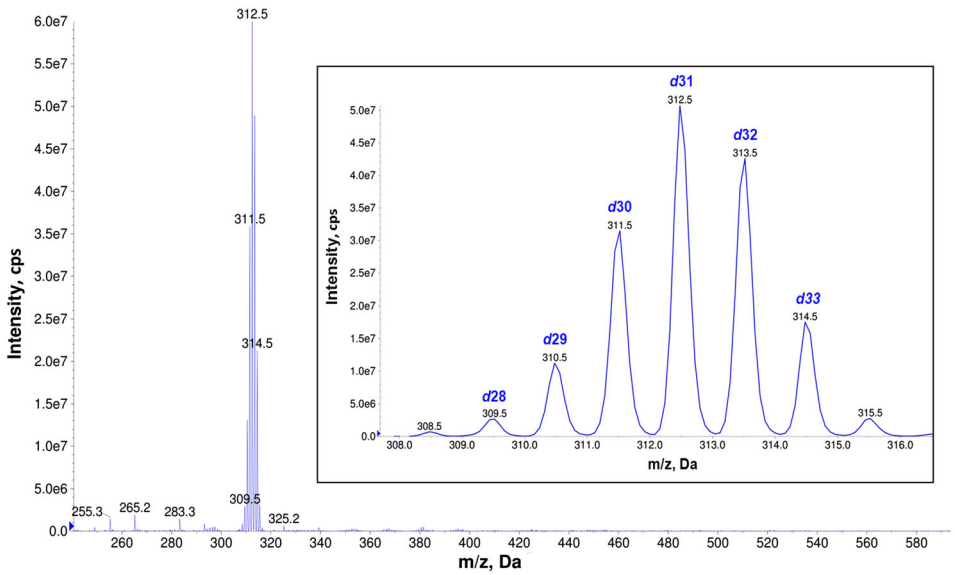
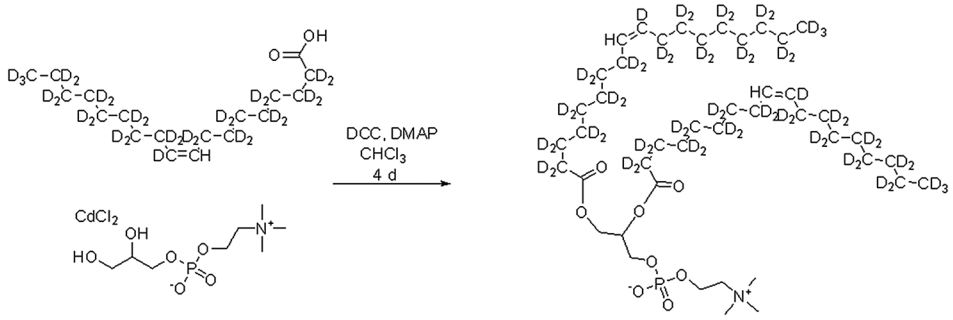


Figure 5. Electrospray ionization mass spectra in negative mode of [D_{32}]oleic acid showing the mass distribution of the different isotopologues, which range from d_{28}–d_{33}. The distribution of the isotopologues is as follows: 5.9%, d_{33}; 25.3%, d_{32}; 34.8%, d_{31}; 23.4%, d_{30}; 8.3%, d_{29}; 2%, d_{28}.



Scheme 3. Reaction for synthesizing deuterated [D_{64}]dioleoyl-*sn*-glycero-3-phosphocholine from [D_{32}]oleic acid.

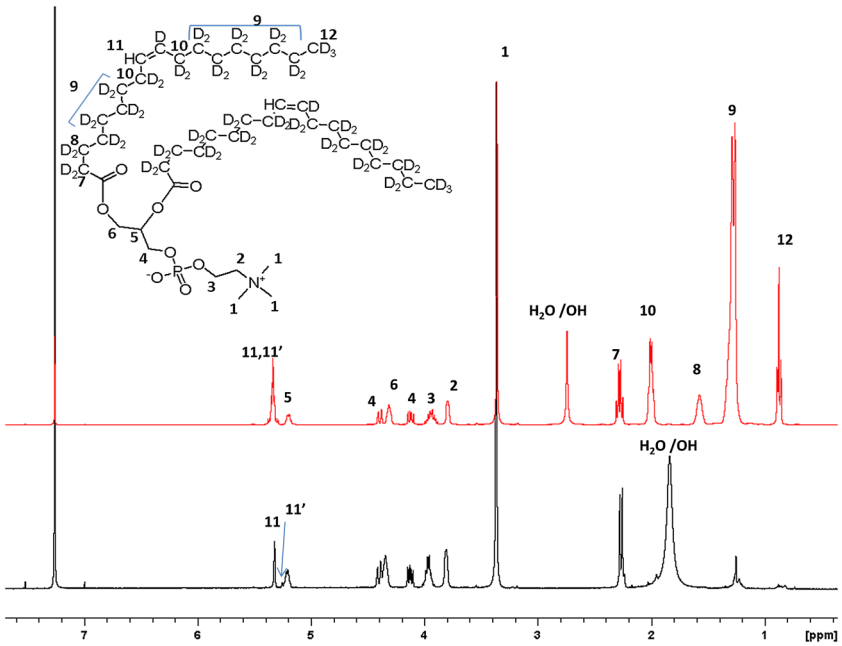


Figure 6. ^{1}H NMR ($CDCl_3$, 400 MHz) overlay spectra of protonated dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) (commercial source, Avanti) and [D_{64}]DOPC produced in this study. Methylene units at C(7) (C_α) showed significant proton resonance in the [D_{64}]DOPC suggesting considerable D/H back exchange. C(11')D is the proton residue of the deuterated olefinic carbon.

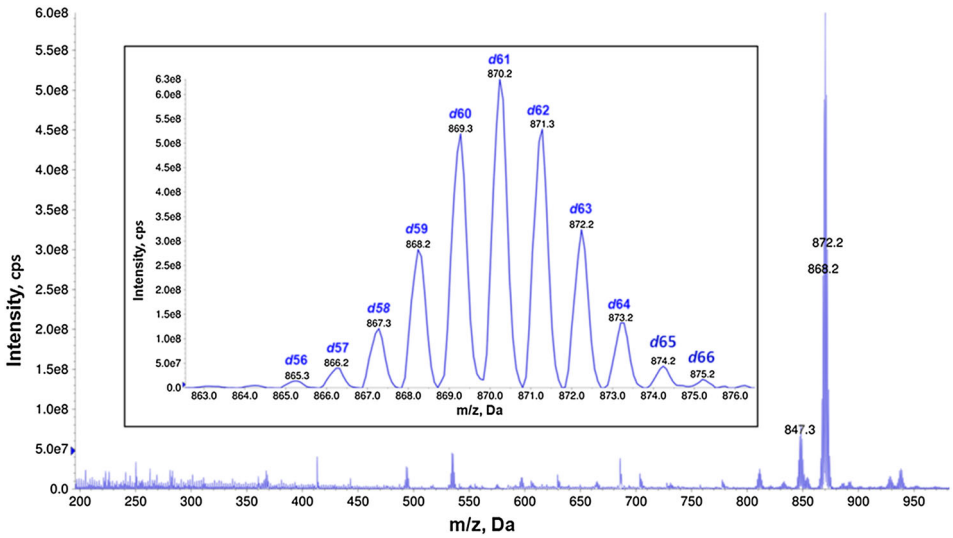


Figure 7. Electrospray ionization mass spectra in positive mode of [D_{64}]dioleoyl-*sn*-glycero-3-phosphocholine showing the mass distribution of the different isotopologues, which ranges from d_{56}–d_{66}. The distribution of the isotopologues is as follows ($M^+ + Na^+ + 1$): 0.7%, d_{66}; 0.5%, d_{65}; 1.8%, d_{64}; 7.2%, d_{63}; 16.7%, d_{62}; 24.1%, d_{61}; 23.7%, d_{60}; 14.8%, d_{59}, 6.7%, d_{58}; 2.6%, d_{57}; 1.2%, d_{56}.

Synthesis of [D_{64}]dioleoyl-*sn*-glycero-3-phosphocholine

[D_{32}]Oleic acid produced using the aforementioned method was subsequently used to synthesize deuterated [D_{64}]DOPC. This involved a single step, Steglich esterification, using a commercially available compound (*sn*-glycero-3-phosphocholine-$CdCl_2$, GPC), following the method of Sing[45] and is summarized in Scheme 3. This resulted in the corresponding deuterated DOPC with 60% yield, and ca. 91.5% deuteration of the oleoyl tails.

The ^{1}H NMR spectrum of [D_{64}]DOPC (Figure 6) was found to contain resonances with the same structure and chemical shift as observed for a commercially available protonated sample (Avanti Polar Lipids Inc. Alabama, USA). The only significant difference between these two spectra arises from the presence of D atoms at specific sites along the oleoyl tails. By integrating the unlabelled trimethylamine subunit and comparing the values with the two methylene units at the C_α positions (C(7) D_2), it was evident that the percentage deuteration at this position had decreased to 21% D (Supplementary data, Figure S27). This was also confirmed by ^{2}H NMR, which showed a diminished deuterium signal for the same methylene units (Supplementary data, Figure S29). This is attributed to the basic conditions of the coupling reaction which can lead to back exchange at these relatively acidic sites. No exchange was observed to occur at any other carbon in this molecule. By considering the new values of the percentage deuteration of the oleoyl chains, the overall percentage deuteration of deuterated alkyl chains of DOPC can be calculated to be 91.9% D by NMR. This value agrees with the percentage deuteration obtained from the MS of [D_{64}]DOPC by calculating the relative abundance and mass isotopic distribution of the different isotopologues, which showed an overall value of 91.5% D (Figure 7). The ^{31}P NMR spectrum of [D_{64}]DOPC was found to be identical to that of the commercially available protonated analogue (Supplementary data, Figure S28).

Conclusions

We have developed a straightforward and effective method for the production of highly deuterated [D_{32}]oleic acid in multiple gram quantities, starting from deuterated fatty acids that can be readily produced by hydrothermal exchange reactions on a large scale. The overall deuteration level of the final product is very high (ca. 94% D), making this a very effective molecule for use in chemical or biomolecular studies using Fourier transform infrared spectroscopy, ^{2}H NMR or neutron scattering techniques. It is worth noting that the difference in scattering length density values (SLD) of protonated oleic acid ($SLD = 0.08 \times 10^{-6}$ A^{-2}) versus the deuterated [D_{32}]oleic acid produced in this study ($SLD = 7.07 \times 10^{-6}$ A^{-2}) is quite significant, which makes this deuterated form very suitable for contrast variation techniques in neutron scattering studies of oleic acid. Characterisation by ^{1}H, ^{2}H and ^{13}C NMR spectroscopies confirms the presence of only the *cis*-isomer and reveals that certain sites within the molecule were susceptible to exchange during the different synthetic steps. Specifically, one of the olefinic protons, which was selectively protonated, was observed to exchange and gain deuterium to 59% D content, whereas the degree of deuteration on the C_α next to the carboxylic acid group decreased to 56% D. Subsequently, the synthesis of [D_{64}]DOPC from [D_{32}]oleic acid has been achieved, which demonstrates that similarly labelled lipid species of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (sodium salt) (DOPS), 1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt) (DOPG) and 1,2-dioleoyl-sn-glycero-3-phosphate (sodium salt) acid (DOPA) may also be readily derived for the first time given the current availability of deuterated oleic acid that is produced using the method reported in this study.

Experimental section

General

Chemicals and reagents of the highest grade were purchased from Sigma-Aldrich (Sydney, Australia) and were used without further purification. Solvents were purchased from Sigma-Aldrich and Merck and were purified by established methods.[46] NMR solvents were purchased from Cambridge Isotope Laboratories Inc. (MA, USA) and Sigma-Aldrich and were used without further purification. D_2O (99.8%) was supplied by AECL, Canada. Thin-layer chromatography (TLC) was performed on Fluka analytical silica gel aluminium sheets (25 F254) (product of Sigma-Aldrich). Davisil® silica gel (LC60 Å 40–63 μm) (product of Sigma-Aldrich) was used for bench-top column chromatography. Hydrothermal reactions were performed in D_2O at high temperatures by mixing the appropriate fatty acid with NaOD and Pt/C (10% w/w) in a Mini Benchtop 4560 Parr reactor (600 mL vessel capacity, 3000 psi maximum pressure, 350°C maximum temperature) (Moline, USA). This was followed by filtering the catalyst, acidifying the solution and then extracting the aqueous phase with EtOAc. Thin layer chromatography was used (referenced with the protonated compound) to estimate the purity and to develop separation protocols. ^{1}H NMR (400 MHz), ^{13}C NMR (100.6 MHz) and ^{2}H NMR (61.4 MHz) spectra were recorded on a Bruker 400 MHz spectrometer at 298 K (Sydney, Australia). Chemical shifts, in parts per million, were referenced to the residual signal of the corresponding NMR solvent. Deuterium NMR was performed using the probe's lock channel for direct observation. In ^{1}H NMR measurements, the protonated group within the molecule was used as an internal standard to calculate the percentage deuteration of the other deuterated sites within the molecule. This was achieved by comparing the relative areas of the ^{1}H peaks of the protonated group and the proton residue of the deuterated groups. Alternatively, in some cases, methylsulfonylmethane or dichloromethane was used as internal standard to quantify the extent of deuteration. The overall deuterium content (percentage) of a compound was calculated from the individual determinations at the different deuterated positions. This was performed by taking the sum of the percentage deuteration of each position multiplied by the number of protons/deuterium atoms at each of these positions and then dividing the answer by the total number of protons/deuterium in the compound (excluding any exchangeable protons, e.g. carboxylic acid and alcohol protons).

Electrospray ionization mass spectra (ESI-MS) (MA, USA) were recorded on a 4000 QTrap AB Sciex spectrometer. The overall percentage deuteration of the molecules was calculated by MS using the isotope distribution analysis of the different isotopologues. This was calculated taking into consideration the ^{13}C natural abundance, whose contribution was subtracted from the peak area of each M + 1 isotopologue to allow for accurate estimation of the percentage deuteration of each isotopologue.

Synthesis of the deuterated materials

Perdeuteration of azelaic acid

A mixture of azelaic acid (12 g, 63.7 mmol), Pt/activated carbon (10% Pt) (0.38 g, 0.195 mmol) and 40% w/w NaOD (13 g, 127 mmol) in D_2O (120 mL) was loaded into the Parr pressure reactor. The contents of the reactor were degassed by purging with N_2 gas and then sealed and heated to 220°C (23 bar), with constant stirring for 3 days. The reactor was cooled to room temperature, and the contents were filtered through a short plug of Celite to remove the catalyst, which was further washed with H_2O (100 mL). The aqueous filtrate was acidified to pH 2 using 1 M HCl. The white solid (azelaic acid) that was formed upon acidification was extracted from the aqueous solution using EtOAc (3 × 100 mL). The

organic layers were combined and dried with anhydrous Na_2SO_4, and the solvent was removed under vacuum to give 11.5 g of white solid of deuterated [D_{14}]azelaic acid (92% D). The aforementioned quantity of acid (11.5 g) was reloaded into the reactor, and the aforementioned method was repeated using fresh reagents to give 10.5 g of a mixture of products which was shown to contain (ca. 1 g) of deuterated octanoic acid (98% D) in addition to deuterated [D_{14}]azelaic acid (**1**). The mixture was washed with light petroleum to remove completely the deuterated octanoic acid leaving behind the target compound, deuterated azelaic acid in a pure form (9.5 g, 74% yield on the basis of the original amount of the protonated azelaic acid used; 98.3% D). ^{1}H NMR (400 MHz, MeOD-d_4, methylsulfonylmethane as internal standard): δ_H 1.29 (0.322 H, br s, residual 3 × CH_2,), 1.55 (0.158 H, br s, residual 2 × CH_2,), 2.24 (0.161 H, br s, residual 2 × CH_2-COOH), 3.00 (6.000 H, s, 2 × CH_3, internal standard). ^{2}H NMR (61.4 MHz, MeOH-d_4): δ_D 1.28 (6.0D s, 3 × CD_2,), 1.53 (3.9D, s, 2 × CD_2,), 2.23 (4.0D s, 2 × CD_2). (Supplementary data, Figures S1–S3).

Perdeuteration of nonanoic acid

[D_{17}]Nonanoic acid was prepared according to the method described previously for [D_{14}]azelaic acid and on a similar scale (i.e. 12 g of protonated reagent). In this case, the by-product produced as a result of decarboxylation was deuterated octane, which was removed under high vacuum to give pure [D_{17}]nonanoic acid (10 g, 75% yield on the basis of the original amount of the protonated nonanoic acid; 98.3% D). ^{1}H NMR (400 MHz, $CDCl_3$, dichloromethane as internal standard): δ_H 0.82 (0.058 H, br s, residual CH_3), 1.23 (0.192 H, br m, residual 5 × CH_2), 1.58 (0.038 H, br s, residual CH_2), 2.30 (0.038 H, br s, CH_2-COOH), 5.29 (2.000 H, s, CH_2, internal standard). ^{2}H NMR (61.4 MHz, $CDCl_3$): δ_D 0.81 (3.0D, s, CD_3), 1.21 (10.2D, m, 5 × CD_2), 1.56 (2.0D, s, CD_2), 2.29 (2.0D, s, CD_2-COOH,). (Supplementary data, Figures S4 and S5).

Preparation of compound 2

[D_{14}]Azelaic acid (30 g, 148 mmol), anhydrous MeOH (150 mL) and concentrated H_2SO_4 (3 mL) was refluxed under N_2 for 48 h. The solvent was evaporated until a small amount of MeOH remained. This was then poured on ice and water and extracted three times with diethyl ether. The organic layer was washed with 10% $NaHCO_3$, followed by brine and dried over anhydrous sodium sulfate. Evaporation of the solvent gave [D_{14}]dimethyl azelate (**2**) (31 g, 91%), which was used without further purification. Integration of the two methyl groups and comparing them with the residual proton signals of the deuterated sites gave an overall isotopic purity of 98.5% D. ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 1.24 (0.096 H, br s, residual 3 × CH_2), 1.54 (0.055 H, br s, residual 2 × CH_2), 2.24 (0.051 H, br s, residual 2 × CH_2), 3.64 (6.000 H, s, 2 × CH_3). ^{2}H NMR (61.4 MHz, $CDCl_3$): δ_D 1.22 (5.9D, s, 3 × CD_2), 1.54 (4.0D, s, 2 × CD_2), 2.22 (4.0D , s, 2 × CD_2). ^{13}C{^{1}H} NMR (100.6 MHz, $CDCl_3$) δ_C 23.7 (m, 3 × CD_2), 27.5 (m, 2 × CD_2), 33.2 (m, 2 × CD_2), 51.3 (s, 2 × CH_3), 174.3 (s, 2 × CO carbonyl). (Supplementary data, Figures S6–S8).

Preparation of compound 3

This compound was prepared following a modified literature procedure by Rao *et al.*[32] A solution of $Ba(OH)_2.8H_2O$ (20.8 g, 66 mmol) in anhydrous MeOH (500 mL) was added to a solution of **2** (30 g, 130 mmol) in anhydrous MeOH (50 mL) and left stirring at room temperature for 24 h. The resulting white precipitate of the barium salt of the half ester was collected by suction filtration. The filtrate was concentrated and refiltered to give a second crop. Both crops were washed with *cold* MeOH (2 × 50 mL), and the MeOH washings and the filtrate were combined and diluted with water and extracted with dichloromethane (DCM) to recover unreacted starting material **2**. The white solid (combined) was suspended in H_2O and 50% HCl was added to bring it to ca. pH 1, before extracting with diethyl ether. The combined diethyl ether layers were washed with brine, dried over anhydrous Na_2SO_4 and the solvent removed in vacuo. The crude material was purified on a silica column (eluent: dichloromethane/EtOAc) giving the desired compound **3** as a waxy white solid (17.5 g, 63% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 1.25 (0.089 H, br s, residual 3 × CH_2), 1.56 (0.065 H, br s, residual 2 × CH_2), 2.26 (0.047 H, br s, residual CH_2), 2.30 (0.044 H, br s, residual CH_2), 3.66 (3.000 H, s, CH_3). ^{2}H NMR (61.4 MHz, $CDCl_3$): δ_D 1.25 (6.3D, s, 3 × CD_2), 1.55 (4.0D, s, 2 × CD_2), 2.25–2.28 (4.0D, br s , 2 × CD_2). ^{13}C{^{1}H} NMR (100.6 MHz, $CDCl_3$,): δ_C 23.5 (m, 3 × CD_2), 27.5 (m, 2 × CD_2), 33.1 (m, 2 × CD_2), 51.4 (s, CH_3), 174.3 (s, CO ester), 179.8 (s, CO acid) (Supplementary data, Figures S9–S11).

Preparation of compound 4

Borane-methyl sulfide complex (ca. 10 M) (100 mmol, 10 mL) was added dropwise to a stirred solution of **3** (17.5 g, 81 mmol) in anhydrous THF (100 mL) at 0°C under an inert atmosphere. The solution was maintained at 0°C for 2 h and then allowed to warm up gradually to room temperature overnight (18 h). MeOH (12 mL) was added dropwise and the mixture was stirred continuously for a further 2 h. The solution was then sparged gently with N_2 and the solvent removed in vacuo. Water and diethyl ether were added to the residue; the diethyl ether layer and the aqueous layer were extracted twice more with diethyl ether. The combined organic layers were washed sequentially with $NaHCO_3$, brine and H_2O, dried over anhydrous Na_2SO_4 and the solvent removed in vacuo. The resulting clear liquid required no further purification (15.7 g, 96% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 1.25 (0.115 H, br s, residual 4 × CH_2), 1.33 (1.066 H, br s, OH), 1.51–1.56 (0.091 H, m, residual 2 × CH_2), 2.26 (0.050 H, br s, residual CH_2), 3.61 (2.157 H, s, CH_2-OH,), 3.65 (3.000 H, s, CH_3). ^{2}H NMR (61.4 MHz, $CDCl_3$): δ_D 1.24 (8.0D, s, 4 × CD_2), 1.53 (4.0D, br m, 2 × CD_2), 2.25 (1.8D, s, CD_2). ^{13}C {^{1}H} NMR (100.6 MHz, $CDCl_3$): δ_C 24.1 (m, 4 × CD_2), 27.7 (m, 2 × CD_2), 33.1 (m, CD_2), 51.4 (s, CH_3), 62.8 (s, CH_2-OH), 174.3 (s, CO). (Supplementary data, Figures S12–S14).

Preparation of compound 5

Triphenylphosphine (30.2 g, 115 mmol) was dissolved in dry DCM (140 mL) under a N_2 atmosphere. The solution was cooled to 0°C, and bromine (5.8 mL, 113 mmol) was added dropwise, ensuring that the solution remained cold. After stirring for 20 min at 0°C, pyridine (10.6 mL, 131 mmol) was added and the reaction stirred for a further 5 min. Compound **4** (15.7 g, 77 mmol) was dissolved in dry DCM (40 mL) and added by syringe to the cold solution. The mixture was left stirring at 0°C for 2 h, before allowing it to warm to room temperature and then stirring in air for a further 30 min. The salts were removed by vacuum filtration and the solvent from the filtrate removed *in vacuo* to yield the crude product. Crude material was adsorbed on silica and purified by flash chromatography (eluent: light petroleum/dichloromethane) to yield a colourless liquid (14.6 g, 71% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 1.25 (0.117 H, br s, residual 3 × CH_2), 1.36 (0.037 H, br s, residual CH_2), 1.56 (masked by the H_2O signal, residual CH_2 integration not available), 1.81 (0.045 H, br s, residual CH_2), 2.26 (0.046 H, br s, residual CH_2), 3.38 (1.980 H, s, CH_2-Br), δ 3.66 (3.000 H, s, CH_3). ^{2}H NMR (61.4 MHz, $CDCl_3$): δ_D 1.24 (6.0D, s, 3 × CD_2), 1.35 (2.3D, s, CD_2), 1.55 (2.1D, s, CD_2), 1.79 (2.2D, s, CD_2,), 2.25 (2.0D, s, CD_2). ^{13}C{^{1}H} NMR (100.6 MHz, $CDCl_3$): δ_C 23.0–32.0 (m, all CD_2), 33.7 (s, CH_2-Br), 51.4 (s, OCH_3), δ 174.3 (s, C = O). (Supplementary data, Figures S15–S17).

Preparation of compound 6

Triphenylphosphine (44.1 g, 168 mmol) was added to a flame dried Schlenk flask and then gently put under vacuum to dry any residual moisture. Under a N_2 atmosphere, dry toluene (250 mL) was added and the solution stirred to ensure that it was completely dissolved. Compound **5** (14.6 g, 55 mmol) was dissolved in dry toluene (2 × 50 mL) and added to the aforementioned solution. The resulting mixture was refluxed for 68 h at 120°C under a N_2 atmosphere. On cooling, an oily layer was formed, which solidified upon standing. The toluene was decanted off and the solid washed with toluene (2 mL) with gentle heating and then cooling before decanting the toluene. The crude

material was purified on a silica column (eluent: dichloromethane/MeOH) to give a pale yellow oil (25.0 g, 83% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 1.17 (0.108 H, br s, residual 4 × CH_2), 1.56 (0.094 H, br s, residual 2 × CH_2), 2.20 (0.093 H, br s, residual CH_2), 3.62 (3.000 H, s, CH_3), 3.69 (2.013 H, d, $J_{H\text{-}P}$ = 12.9 Hz, CH_2-P), 7.70–7.81 (15.897 H, m, PPh_3). ^{2}H NMR (61.4 MHz, $CDCl_3$): δ_D 1.10–1.52 (12.0D, br m, 6 × CD_2), 2.19 (2.1D, br m, CD_2). ^{13}C{^{1}H} NMR (100.6 MHz, $CDCl_3$,): δ_C 22.5 (d, $J_{C\text{-}P}$ = 50 Hz, CH_2-P), 27.0–28.0 (m, all CD_2), 51.4 (s, CH_3), 118.4 (d, $J_{C\text{-}P}$ = 85.6 Hz, Ph), 130.5 (d, $J_{C\text{-}P}$ = 12.3 Hz, Ph), 133.7 (d, $J_{C\text{-}P}$ = 9.98 Hz, Ph), δ 135.0 (d, $J_{C\text{-}P}$ = 3 Hz, Ph), δ 174.4 (s, CO). (Supplementary data, Figures S18–S20).

Preparation of compound 8

Anhydrous THF (ca. 220 mL) was added under a dry and inert atmosphere into a flask containing lithium aluminium deuteride (6.5 g, 15 mmol) cooled in an ice bath. Nonanoic acid-d_{17} (17.1 g, 98 mmol) in ca. 50 mL of anhydrous THF was added slowly, and the resulting mixture was refluxed overnight with vigorous stirring. Water (ca. 50 ml) was added very slowly to decompose the excess lithium aluminium deuteride, followed by addition of dilute sulfuric acid (1 M) to dissolve the resulting precipitate. The mixture was extracted with diethyl ether, the extract washed with $NaHCO_3$ and dried with anhydrous $MgSO_4$. The solvent was removed *in vacuo*, and the crude material was purified on a silica column (eluent: dichloromethane/EtOAc, 9:1) to give a colourless oil (14.3 g, 89% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 0.87 (br s, residual CH_3), 1.26 (br s, residual CH_2), 1.41 (s, OH), 1.52 (br s, residual CH_2), 3.60 (br s, residual CH_2-OH). ^{2}H NMR (61.4 MHz, $CDCl_3$): δ_D 0.81 (3.0D, br s, CD_3), 1.20 (12.3D, br s, 6 × CD_2), 1.50 (2.2D, br s, CD_2), 3.59 (1.8D, br s, CD_2-OH). (Supplementary data, Figures S21 and S22).

Preparation of compound 9

Pyridinium chlorochromate (13.8 g, 64 mmol) and celite (14 g) were added in one portion to a stirred solution of compound **8** (7.1 g, 43.5 mmol) in dry DCM (110 mL). The suspension was stirred vigorously under N_2 atmosphere for 18 h, and then the dark mixture was filtered through a plug of florisil. Diethyl ether (400 mL) was run through the plug. The ether washing was evaporated under vacuum to give a pale orange oil (7.5 g). The oil was distilled under reduced pressure (11 mbar) and fractions boiling between 70°C and 90°C were collected to give the aldehyde **9** as clear oil (3 g, 43% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 0.82 (br s, residual CH_3), 1.23 (br m, residual 5 × CH_2), 1.57 (br s, residual CH_2 masked by H_2O), 2.37 (br s, residual CH_2). ^{2}H NMR (61.4 MHz, $CDCl_3$): δ_D 0.81 (3.0D, br s, CD_3), 1.21 (9.8D, br s, 5 × CD_2), 1.56 (2.0D, br s, CD_2), 2.35 (1.7D, br s, CD_2), 9.79 (0.8D, br s, CDO). (Supplementary data, Figures S23 and S24).

Preparation of compound 10

Phosphonium salt **6** (9.6 g, 18.3 mmol) was dissolved in dry 1,3-Dimethyl-3,4,5,6-tetrahydro-2(1*H*)-pyrimidinone (60 mL) under a dry and inert atmosphere and then dry THF (160 mL) was added. The solution was cooled to −78°C and a 0.5 M solution of potassium hexamethyldisilazide (40 mL, 20 mmol) was added dropwise over a period of 30 min, when a deep orange colour developed. The reaction mixture was stirred for 1 h at −78°C. Aldehyde **9** (3.0 g, 0.0187 mol) was then added (neat) dropwise over 30 min. The reaction mixture was stirred at −78°C for 2 h, and then allowed to come to room temperature over night while stirring. The reaction was then quenched with water (30 mL) and extracted with diethyl ether (4 × 30 mL). The combined organic layers were washed sequentially with 0.5 M HCl, saturated $NaHCO_3$ and brine, and dried over anhydrous $MgSO_4$. The solvent was evaporated under reduced pressure to give a pale yellow oil which was purified on a silica column (eluent: dichloromethane/hexane, 1:1) to give a colourless oil (3.6 g, 60% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 0.82 (0.069 H, br s, residual CH_3), 1.23 (0.337 H, br m, residual 10 × CH_2), 1.57 (masked by H_2O signal), 1.95 (0.139 H, br s, residual 2 × CH_2-C = C,), 2.27 (0.873 H, br m, residual CH_2COO-), 3.66 (3.000 H, s, O-CH_3), 5.25 (0.044 H, br s, residual CH = *CD*) and 5.32 (0.407 H, br s, *CH* = CD). ^{2}H NMR (61.4 MHz, $CDCl_3$): δ_D 0.81 (3.5D, br s, CD_3), 1.21 (23.8D, br s, 10 × CD_2), 1.55 (1.64D, br s, CD_2), 1.94 (3.8D, br s, 2 × CD_2-C = C), 2.25 (1.2D, br s, CD_2COO,) 5.36 (1.6D, br s, CH = *CD*). (Figures 2 and S25 in the Supplementary data).

The aforementioned methyl ester (3.6 g, 10 mmol) was dissolved in N_2-purged MeOH (250 mL). Lithium hydroxide monohydrate (3.95 g, 94 mmol) dissolved in N_2-purged water (100 mL) was added to the methyl ester solution with stirring. Initially, the reaction mixture was cloudy but eventually the solution became clear. The hydrolysis reaction was monitored by TLC by using protonated oleic acid as reference. The reaction reached completion after 24 h. HCl (1 M) was added to the reaction mixture to acidify it to pH 1, where the solution became turbid again indicating the liberation of the free acid. The turbid aqueous solution was extracted three times with diethyl ether. The organic phase was washed once with brine and then dried over anhydrous Na_2SO_4. The solvent was evaporated under reduced pressure to give pale yellow oil. The oil was purified on a silica column [eluant DCM : EtOAc (10:4)] to give a colourless oil of deuterated oleic acid (**10**) (2.5 g, 80% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 0.82 (0.036 H, br s, residual CH_3), 1.23 (0.279 H, br m , residual 10 × CH_2,), 1.58 (0.029, br s, residual CH_2), 1.95 (0.104 H, br s, residual 2 × CH_2-C = C), 2.32 (0.890 H, br m, residual CH_2COO-), 5.25 (0.045 H, br s, residual CH = *CD*) and 5.32 (0.407 H, br s, *CH* = CD). ^{2}H NMR (61.4 MHz, $CDCl_3$): δ_D 0.81 (4.5D, br s, CD_3), 1.21 (23.8D, br s, 10 × CD_2), 1.57 (1.9D, br s, CD_2), 1.94 (4.5D, br s, 2 × CD_2-C = C), 2.30 (1.2D, br s, CD_2COO-) 5.36 (1.4D, br s, CH = *CD*). ^{13}C{^{1}H} NMR (100.6 MHz, $CDCl_3$): δ_C 12.9 (m), 21.5 (m), 23.7 (m), 26.2 (m), 28.0 (br m), 30.6 (m), 33.3 (m), 129.5 (s), 179.2 (s). ESI-MS: 5.9%, d_{33}; 25.3%, d_{32}; 34.8%, d_{31}; 23.4%, d_{30}; 8.3%, d_{29}; 2%, d_{28}. (Figures 3–5 and Figure S26 in Supplementary data).

Synthesis of [D_{64}]dioleoyl-*sn*-glycero-3-phosphocholine (11) from deuterated oleic acid (10)

[D_{64}]Dioleoyl-*sn*-glycero-3-phosphocholine was synthesised according to a modified literature procedure[45], where the oleoyl chains are deuterated. In a 50-mL single-necked round-bottomed flask containing *sn*-glycero-phosphocholine-$CdCl_2$ complex (60 mg, 0.136 mmol) was added 105 mg (0.335 mmol) of **10** dissolved in 10 ml of alcohol free chloroform (anhydrous). The resulting suspension was vigorously stirred and 4-dimethylamino pyridine (48 mg, 0.393 mmol) was added followed by dicyclohexylcarbodiimide (81 mg, 0.393 mmol). The contents of the flask were then degassed with N_2, stoppered, protected from light and stirred for 4 days at room temperature. The progress of the reaction was monitored by TLC on silica gel $CHCl_3$-CH_3OH-H_2O 65:25:4. When the reaction was complete, chloroform was added, and the mixture was filtered through a Celite pad (product of Sigma-Aldrich), which was then washed with 15 mL more of chloroform. The chloroform was removed under reduced pressure at room temperature, and the residue was dissolved in 5 mL of $CHCl_3$/CH_3OH 1:1 and passed through a silica column, which was preconditioned with $CHCl_3$. The fractions containing phospholipids were combined, and the solvent was removed in vacuo at 25°C. This vacuum-dried fraction was then dissolved in a minimal volume of $CHCl_3$/CH_3OH/H_2O 65:25:4 and further purified on a column of silica gel by using the same solvent system. Fractions were analyzed by TLC, and those fractions containing a product with an R_f identical to that of commercially available protonated DOPC were combined and the solvent was removed in vacuo at 25°C. The phospholipid thus obtained (70 mg, 60%) gave ^{31}P NMR spectra identical to that of the commercially sourced protonated sample. ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 0.83 (0.073 H, br m, residual CH_3), 1.23 (1.170 H, br m, residual 10 × CH_2), 1.55 (0.046 H, br m, residual CH_2), 1.95 (br m, masked by the H_2O/OH signal), 2.26 (3.175 H, br m, residual CH_2COO-), 3.37 (9.000 H, s, $N(CH_3)_3$), 3.81 (1.946 H, b m, CH_2-CH_2-N), 3.96 (1.994 H, m, CH_2-CH_2-N), 4.12 (1.032H, m, -O-CH_2-C(O)H-CH*H*-O-P), 4.34 (1.776 H, m, -O-CH_2-C(O)H-CH_2-O-P), 4.39 (1.090 H, m, -O-CH_2-CH-*CH*H-O-P), 5.21 (0.978H, br m, -O-CH_2-*C(O)H*-CH_2-O-P), 5.25 (0.081 H, b s, residual -CH = *CD*-) and 5.32 (0.822 H, br s, *CH* = CD). ^{2}H NMR (61.4 MHz, $CDCl_3$): δ_D 0.81 (3.0D, br s, CD_3), 1.20 (17.3D, br s, 10 × CD_2), 1.52 (1.6D, br s, CD_2), 1.94 (3.1D, br s, 2 × CD_2-C = C), 2.33 (0.2D, br m, CD_2COO) 5.37 (1.0D, br s, CH = *CD*). ^{31}P NMR (161.9 MHz,

$CDCl_3$) δ_P 0.498 (s). ESI-MS: 0.7%, d_{66}; 0.5%, d_{65}; 1.8%, d_{64}; 7.2%, d_{63}; 16.7%, d_{62}; 24.1%, d_{61}; 23.7%, d_{60}; 14.8%, d_{59}, 6.7%, d_{58}; 2.6%, d_{57}; 1.2%, d_{56}. (Figures S27–S29 in Supplementary data)

Acknowledgments

The authors acknowledge the National Deuteration Facility at the Australian Nuclear Science and Technology Organization (ANSTO); the operation of which was partially funded by the National Collaborative Research Infrastructure Strategy (NCRIS). The assistance of Ms Marie Gillon in preparing the deuterated fatty acid precursors for this work is gratefully acknowledged.

Conflict of Interest

The authors did not report any conflict of interest.

References

[1] M. R. Michaud, D. L. Denlinger, *J. Insect Physiol.* **2006**, *52*, 1073–1082.
[2] R. M. Adibhatla, J. F. Hatcher, *Fut. Lipidol.* **2007**, *2*, 403–422.
[3] G. Valincius, F. Heinrich, R. Budvytyte, D. J. Vanderah, D. J. McGillivray, Y. Sokolov, J. E. Hall, M. Lösche, *Biophys. J.* **2008**, *95*, 4845–4861.
[4] M. P. Boland, C. R. Hatty, F. Separovic, A. F. Hill, D. J. Tew, K. J. Barnham, C. L. Haigh, M. James, C. L. Masters, S. J. Collins, J. Biol. *Chem.* **2010**, *285*, 32282–32292.
[5] A. Chenal, L. Prongidi-Fix, A. Perier, C. Aisenbrey, G. Vernier, S. Lambotte, G. Fragneto, B. Bechinger, D. Gillet, V. Forge, M. Ferrand, J. Mol. *Biol.* **2009**, *391*, 872–883.
[6] C. E. Miller, J. Majewski, K. Kjær, M. Weygand, R. Faller, S. Satija, T. L. Kuhl, *Colloid Surface B* **2005**, *40*, 159–163.
[7] M. Nöllmann, R. Gilbert, T. Mitchell, M. Sferrazza, O. Byron, *Biophys. J.* **2004**, *86*, 3141–3151.
[8] D. J. McGillivray, G. Valincius, F. Heinrich, J. W. F. Robertson, D. J. Vanderah, W. Febo-Ayala, I. Ignatjev, M. Lösche, J. J. Kasianowicz, *Biophys. J.* **2009**, *96*, 1547–1553.
[9] B. Steinbauer, T. Mehnert, K. Beyer, *Biophys. J.* **2003**, *85*, 1013–1024.
[10] J. D. Gehman, M. A. Sani, F. Separovic, Solid-state NMR of membrane-acting antimicrobial peptides. *In Biomolecular NMR Spectroscopy*, eds. A. Dingley and S. Pascal, IOS Press, Amsterdam, The Netherlands, **2011**, Chapter 8, pp 137–161.
[11] a) J. Seelig, P. M. Macdonald, *Accounts Chem. Res.* **1987**, *20*, 221–228; b) A. P. Tulloch, *Chem. Phys. Lipids* **1979**, *24*, 391–406; c) P. B. Kingsley, G. W. Feigenson, *Chem. Phys. Lipids* **1979**, *24*, 135–147; d) Y. Han, T. E. Alexander, G. P. Tochtrop, *Mol. BioSys.* **2008**, *4*, 551–557.
[12] a) A. J. Aasen, W. M. Lauer, R. T. Holman, *Lipids* **1970**, *5*, 869; b) K. K. Sun, H. W. Hayes, R. T. Holman, *Org. Mass. Spectrom.* **1970**, *3*, 1035; c) B. A. Andersson, F. Dinger, Nguyen Dinh-Nguyen, *Chem. Scr.* **1976**, *9*, 155.
[13] a) D. I. Fernandez, A. P. Le Brun, T. C. Whitwell, M.-A. Sani, M. James, F. Separovic, *Phys. Chem. Chem. Phys.* **2012**, *14*, 15739–15751; b) A. P. Le Brun, T. A. Darwish, M. James, *Journal of Chemical and Biological Interfaces* **2013**, *1*, In Press (references there in). DOI:10.1166/jcbi.2013.1005
[14] D. Fernandez, A. Brun, T.-H. Lee, P. Bansal, M.-I. Aguilar, M. James, F. Separovic, *Eur. Biophys. J.* **2013**, *42*, 47–59.
[15] H.-H. Shen, P. G. Hartley, M. James, A. Nelson, H. Defendi, K. M. McLean, *Soft Matter* **2011**, *7*, 8041–8049.
[16] G. Fragneto, F. Graner, T. Charitat, P. Dubos, Bellet-Amalric, E. *Langmuir* **2000**, *16*, 4581–4588.
[17] L. A. Clifton, M. Sanders, C. Kinane, T. Arnold, K. J. Edler, C. Neylon, R. J. Green, R. A. Frazier, *Phys. Chem. Chem. Phys.* **2012**, *14*, 13569–13579.
[18] H. P. Wacklin, *Langmuir* **2011**, *27*, 7698–7707.
[19] X. Han, K. Hristova, *J. Membrane Biol.* **2009**, *227*, 123–131.
[20] M. Mihailescu, O. Soubias, D. Worcester, S. White, K. Gawrisch, *J. Membrane Biol.* **2011**, *239*, 63–71.
[21] J. Lemmich, K. Mortensen, J. H. Ipsen, T. Hønger, R. Bauer, O. G. Mouritsen, *Phys. Rev. E* **1996**, *53*, 5169–5180.
[22] J. F. Hunt, P. D. McCrea, G. Zaccaï, D. M. Engelman, *J. Mol. Biol.* **1997**, *273*, 1004–1019.
[23] J. Lemmich, K. Mortensen, J. H. Ipsen, T. Hønger, R. Bauer, O. G. Mouritsen, *Phys. Rev. Lett.* **1995**, *75*, 3958–3961.
[24] P. R. Cullis, B. De Kruijfi, Biochim. *Biophys. Acta* **1979**, *559*, 399–420.
[25] H. Chen, E. Plettner, J. Labelled Compd. *Rad.* **2012**, *55*, 66–70.
[26] S. B.; Farren, E. Sommerman, P. R. Cullis, *Chem. Phys. Lipids* **1984**, *34*, 279–286.
[27] V. Heyningen, W. Rittenberg, R. Schoenheimer, J. Biol. *Chem.* **1938**, *125*, 495–500.
[28] C. Y. Y. Hsiao, C. A. Ottaway, D. B. Wetlaufer, *Lipids* **1974**, *9*, 913–915.
[29] F. D. Gunstone, I. A. Ismail, Chem. Phys. *Lipids* **1967**, *1*, 209–224.
[30] L. D. Bergelson, M. M. Shemyakin, *Angew. Chem. Int. Edit.* **1964**, *3*, 250–260.
[31] S. Swann, R. Oehler, R. J. Buswell, *Org. Syntheses Coll.* **1943**, *2*, 276.
[32] A. V. R. Rao, S. V. Mysorekar, J. S. Yadav, *Synth. Commun.* **1987**, *17*, 1339–1347.
[33] E. R. Burkhardt, K. Matos, *Chem. Rev.* **2006**, *106*, 2617–2650.
[34] N. M. Yoon, C. S. Pak, C. B. Herbert, S. Krishnamurthy, T. P. Stocky, J. Org. *Chem.* **1973**, *38*, 2786–2792.
[35] G. A. Wiley, R. L. Hershkowitz, B. M. Rein, B. C. Chung, *J. Am. Chem. Soc.* **1964**, *86*, 964–965.
[36] H. J. Bestmann, K. H. Koschatzky, W. Schätzke, J. Süß, O. Vostrowsky, Lieb. *Anna. Chemie* **1981**, 1981, 1705–1720.
[37] H. J. Bestmann, W. Stransky, O. Vostrowsky, *Chem. Berichte* **1976**, *109*, 1694–1700.
[38] L. Gehring, D. Haase, K. Habben, C. Kerkhoff, H. H. Meyer, V. Kaever, *J. Lipid Res.* **1998**, *39*, 1118–1126.
[39] C. Jacquot, C. M. McGinley, E. Plata, T. R. Holman, W. A. van der Donk, *Org. Biomol. Chem.* **2008**, *6*, 4242–4252.
[40] G. Brunow, R. Stick, K. Syrjanen, D. Tilbrook, S. Williams, *Aus. J. Chem.* **1995**, *48*, 1893–1897.
[41] W. P. Tucker, S. B. Tove, C. R. Kepler, *J. Labelled Compd.* 1971, *7*, 11–15.
[42] K. Schaumburg, H. J. Bernstein, *Lipids* **1968**, *3*, 193–198.
[43] B. Dayal, G. Salen, B. Toome, G. S. Tint, S. Shefer, J. Padia, *Steroids* **1990**, *55*, 233–237.
[44] D. J. Frost, F. D. Gunstone, Chem. Phys. *Lipids* **1975**, *15*, 53–85.
[45] A. Singh, *J. Lipid Res.* **1990**, *31*, 1522–1525.
[46] D. D. Perrin, W. L. Armarego, D. R. Perrin. *Purification of Laboratory Chemicals*, 2nd ed.; Pergamon: New York, **1980**.

