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Differentiation and quantification of synthetic phosphatidylethanol (PEth) homologues by ^{1}H- and ^{13}C-NMR in polar organic solvents

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Abstract Various phosphatidylethanol (PEth) derivatives, the corresponding reversed positional isomers (RPI-PEths), lyso-PEth-16:0 and penta deuterium labeled PEth analogs (d5-PEths), were synthesized by enzyme independent synthetic routes. A general solvent system consisting of a mixture of acetone-d6 and methanol-d4 (97:3; v:v) was found to provide a good solubilizing capacity and excellent ^{1}H-NMR peak resolution of various PEth homologues. Analytical differentiation of PEth from the corresponding RPI-PEth by ^{13}C-NMR was demonstrated by comparison of the ^{13}C-NMR signals of the carbonyl groups, the allylic positions and of the β-carbons. An exemplary long term room temperature stable, DMSO-d6 based and by proton sensitive quantitative nuclear magnetic resonance (^{1}H-qNMR) independently quantified calibrator comprising PEth-16:0/18:1 for liquid chromatography (tandem) mass spectrometry (LC-MS[/MS]) analytical applications was prepared by employment of sodium dodecyl sulfate (SDS) as a solubilizing additive. In summary, novel hypothetically occurring PEth derivatives, e.g. RPI-PEths, have been independently synthesized with regio- and stereochemical control. Use of polar organic solvents, e.g. mixtures of acetone-d6 and methanol-d4 or DMSO-d6, improves spectral line shapes as compared to traditional hydrophobic solvents and allow for analytical differentiation between closely related PEth derivatives, as well as LC-MS(/MS) independent concentration determination of dissolved single species, by employment of ^{1}H-qNMR.

Supplementary key words qNMR; LC-MS/MS; analytical reference; phospholipids; positional isomers; lyso-PEth

Introduction

Phosphatidylethanol (PEth) is a group of homologous phospholipids formed *in-vivo* by phospholipase D (PLD) catalyzed ethanolysis of mainly endogenous phosphatidylcholine (PC) [1]. Due to its high specificity and relatively low human clearance, PEth is attracting considerable interest and is already used as a slow eliminating bio-marker of ethanol for the objective measure of a person's historic alcohol consumption [1]. Several reports have emerged the last few years on highly sensitive and specific LC-MS/MS-based techniques that enable the quantification of individual PEth-homolouges in human blood samples [2-5]. Hence, most labs currently rely on related LC-MS/MS techniques for the analysis and quantification of PEth in clinical samples and are expected to do so in the foreseeable future. As governed by their bio-precursor PCs, the most abundant homologues carry a saturated fatty acid residue (SFA), commonly palmitoyl (16:0), at the sn-1 position and an unsaturated fatty acid residue (UFA), commonly oleoyl (18:1) or lineoyl (18:2), at the sn-2 position (Fig. 1) [6]. Accordingly, PEth-16:0/18:1 and PEth-16:0/18:2 have been found to account for a substantial part of the total amount of PEth and the levels of these homologues have thus been suggested as a representative measure thereof [7]. Despite their potential utility as analytical references and standards in LC-MS(/MS) analytical applications, only one PEth homologue simultaneously carrying one SFA and one UFA (PEth-16:0/18:1) is, to the best of our knowledge, commercially available. Furthermore, isotopically labeled variants, such as the penta deuterated PEth derivatives (d5-PEths), reversed positional PEth isomers (RPI-PEths) and lyso-PEth remain yet largely unexplored (Fig. 1).

d5-PEths have a molecular weight that is about 5 units higher than the corresponding non-labeled lipids. This difference enables use of d5-PEth as internal standard in analytical applications based on LC-MS(/MS) techniques. A recent report by Zheng *et al.* [4] describes the use of d5-PEth-16:0/18:1 and d5-PEth-16:0/18:2 as internal standards in the quantification of PEth in human blood samples by LC-MS/MS-analysis.

The RPI-PEths are expected to be present as a minor counterpart for each PEth homologue. Basis for this may be found in the reported presence of the corresponding RPI's among the bio-precursor PCs [6, 8]. Similar chromatographic and spectral properties, as well as identical molecular weights, make a RPI-PEth difficult to differentiate analytically from the corresponding PEth. Analogous to the corresponding isomeric pairs among the PCs, the relative intensities of fragments in MS/MS-applications are, however, most likely different. Hence, an unknown error in the quantification of individual PEth homologues is introduced when this quantification is based on such fragment intensities unless the isomeric distribution is accounted for.

Endogenous phospholipase activity may result in the formation of lyso-PEth metabolites by cleavage of the carboxylic acid residue at sn-2 of the parent PEth. Lyso-PEth may also be formed by biosynthesis from lyso-PC. Not only may lyso-PEth serve as an ethanol bio-marker *per-se*, but the relationship between the levels of lyso-PEth and the corresponding PEth may hold additional information relating to a person's drinking behavior.

Inspired by the potential future finding the access to above mentioned PEth derivatives and homologues would enable in the PEth research area, as well as the desire to enrich the LC-MS/MS spectroscopist's toolbox with improved and practically applicable PEth calibrators, we initiated work of which the results are summarized in this report.

Experimental

The appurtenant *Electronic Supplementary Material* comprises detailed synthetic descriptions and associated spectroscopic data and, when previously reported, CAS-registry numbers of compounds **1-4**, **5a-d**, **6c**, **7a-d**, **8**, **9**, **9-d5**, **10a'**, **10a-d**, **11a-b** and **12-14**, the structural assignment of the ^{1}H- and the ^{13}C-signals of compound **10a** and ^{1}H-, ^{13}C- and ^{31}P-NMR spectra thereof, as well as detailed descriptions on the preparation of solutions of compounds **10a** and **10a'** in DMSO-d6.

Starting materials and chemical reagents for synthesis

All starting materials, reagents and solvents were purchased from Chemtronica AB, Sweden and used as received without further purification, with exception for dichloromethane (DCM) which was dried over 4Å MS before use in reactions requiring dry solvents. Deuterated solvents and reagents were of the following quality: chloroform-d, 99.8 %d; acetone-d6, 99.8 %d; methanol-d4, 99.8 %d; DMSO-d6, 99.8 %d; ethanol-d6, 99 %d. Analytical TLC was run on Fluka 60Å Silica gel on TLC Al foils and visualized in a UV-chamber (254 nm) or by use of standard anisaldehyde-reagent. All reactions were run under nitrogen.

NMR

^{1}H-qNMR data was collected on a 500 MHz Varian Inova NMR spectrometer equipped with a 5 mm ^{1}H/^{13}C/^{15}N triple resonance gradient probe at 21 °C. ^{1}H-qNMR spectra were recorded with 120-240 transients using 30° pulse angle and a relaxation delay of 60 seconds between each transient. Data acquisition was performed during 3 seconds with a spectral width of 7250 Hz. Processing and analyses of the NMR data were performed using MestReNova 8.1.0. Prior to analyses, zero filling to 65k points, a Lorentzian function (matching filter) of 1.8-3.6 Hz and base line correction were applied.

^{1}H and ^{13}C-NMR spectra of synthetic products were collected on a 400 MHz Varian Inova NMR spectrometer equipped with a 5 mm ^{13}C selective gradient probe. Processing and analyses were performed using ACD/Labs 12.01 Academic Edition. The residual solvent signal of the major solvent was used as reference (chloroform-d: 7.270 ppm; acetone-d6: 2.050 ppm).

^{31}P-NMR of **10a** was collected on a 400 MHz Bruker Avance II spectrometer equipped with a 5mm broadband ATM probe with z-gradient operating at 21°C. Spectral reference for ^{31}P was an external sample of 85% H_3PO_4 and the experiment was performed with power-gated decoupling.

T_1 measurements and the 2D spectra COSY, HMQC and HMBC were performed on a 500 MHz Bruker Avance spectrometer using a 5 mm inverse probe with pulsed z-gradient. HMQC and HMBC spectra were recorded using J_{CH}=145 Hz and 10 Hz, respectively.

LC-MS

LC-MS analysis of synthetic products and semi-quantification of solutions of such products was performed by employment of a Agilent 1100 LC-system including degasser, auto-sampler and quaternary pump. A linear binary gradient was used starting at 100 % A: aq. NH_4OAc (10 mM, pH=6.57), acetonitrile and i-PrOH (70:15:15, v:v:v), and ending at 100 % B: aq. NH_4OAc (10 mM, pH=6.57), acetonitrile and i-PrOH (2:49:49, v:v:v), after 5 minutes. After completion of the gradient, the column was eluted wit 100 % B for another 5 minutes, before equilibration with 100 % A prior to the next run. The column used was a Waters Symmetry® C8 (3.5 um, 4.6x50 mm), which was run at a flow rate of 1.00 ml/min. The MS system was an Agilent 1100 with an electrospray ion source. The capillary voltage was set to 3000 V. The MS was scanned between 160 to 900 m/z in negative mode. In addition, an UV-detector (Agilent 1100) set at 220 nm was connected to the system.

Results and discussion

Synthesis

Known synthetic strategies for the regio-selective introduction of different carboxylic acid residues onto the sn-1 and sn-2 positions of the glycerol scaffold of PEth and similar lipids like PCs typically rely on the protection of the sn-3 hydroxyl in connection to introduction of these residues [9-11], or on PLD assisted bio-mimetic conversion [12-14] of the choline moiety of intermediate PC into the ethyl residue of PEth [4]. Several PCs, which may be converted accordingly to the corresponding PEths by PLD-assisted ethanolysis, are commercially available. It cannot, however, be ruled out that these are not completely regiochemically pure and that the PLD does not discriminate between the different regioisomers in the ethanolysis. Hence, in order to have control of the stereo- as well as the regio-chemistry, we set out a general synthetic strategy, being independent on enzymatic conversions, for the synthesis of various examples of PEth, d5-PEth, RPI-PEth and lyso-PEth (Fig. 2).

The syntheses begun with the *R*-enantiomer of *p*-methoxybenzyl (PMB) protected glycerol **1**, which may be easily synthesized from commercially available 1,2-isopropylidene-*sn*-glycerol, either by a procedure in which **1** is the product [15] or in analogy to reported procedures yielding products with other benzyl type protective groups [9, 16]. Regioselective introduction of the fatty acid residues of the 1-acylated monoesters **2**, **3** and **4** proceeded smoothly by *N,N'*-dicyclohexylcarbodiimide (DCC) mediated and 4-dimethylaminopyridine (DMAP) catalyzed condensation between diol **1** and the corresponding carboxylic acids. Minor amounts of 2-acylated and 1,2-diacylated by-products were also formed, but were easily separated from the desired 1-acylated monoester by chromatography on silica gel. Similar DCC-mediated coupling with a second carboxylic acid yielded the 1,2-diacylated derivatives **5a-d**. In our hands, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) mediated removal of the PMB-protective group of **5a-d** gave highly variable and low yields in the range of 0 to 29 % of the desired 1,2-diacylated alcohols **7a-d** due to competing formation of the corresponding 1,3-diacylated alcohols **6a-d** by 2,3-acylmigration, despite previous reports on successful use of this reagent [15-17]. Boron trichloride and dimethyl boron bromide have been used for the removal of the benzyl- [9] and the PMB [11, 18] protective groups, respectively, of similar 1,2-diacylated glycerol derivatives with no or minimal 2,3-acylmigration. We found, however, the employment of ceric ammonium nitrate (CAN) for this transformation, as developed by Abe *et al.* [10], to be the method of choice for this conversion due to its robustness, high yield and practical implementation. Hence, treatment of **5a-d** with 10 equivalents of CAN in a mixture of acetonitrile and water (9:1, v:v) at ambient temperature gave the 1,2-diacyl derivatives **7a-d** in high yield with minimal formation of rearranged **6a-d**. Two different methods were utilized for the final phosphorylation of the sn-3 hydroxyl, a direct phosphorylation, in which the phosphorous atom was entered in its pentavalent state, and an indirect method, as developed by Herbert *et al.* [11], in which the phosphorous was entered in its trivalent state. In the direct method, **7a** and **7c** were reacted with an excess of ethyl dichlorophosphate (EDCP) in the presence of triethylamine, followed by hydrolysis with aquous sodium hydrogencarbonate, to yield the PEths **10a**, **10a'** and **10c**. In the indirect method, **7b** and **7d** were reacted with crude **9** [11], followed by hydrogen peroxide oxidation to yield intermediate cyanoethyl protected derivatives of **10b** and **10d**, which were converted to **10b** and **10d** by deprotection under basic conditions by stirring in a 9:1-mixture of tetrahydrofurane (THF) and concentrated aqueous ammonia. An analogous procedure employing the deuterated variant **9-d5** was used for the synthesis of the deuterium labeled analogs **11a** and **11b**. In order to obtain reference material of a potential contaminant for comparison with PEth derivative **10c**, isomeric **8** was synthesized by the indirect method from 1,3-diacylated **6c**. The lyso-PEth **14** was synthesized from **2** in two steps by tetrahydropyranyl (THP) protection of the sn-2 hydroxyl thereof to yield **12**, followed by DDQ mediated removal of the PMB-protective group, to yield **13**, and phosphorylation by the direct method with simultaneous cleavage of the THP-group. The desired salt forms of the target lipids **10a-d**, **10a'**, **11** and **14** were easily obtained by appropriate choice of eluent system in the chromatographic purification on silica of the crude materials. In order to obtain the parent protonated form in the case of **14**, the gradient system toluene:acetone:acetic acid:water (6:4:0:0 to 55:36:7:2) was used. For retrieval of the ammonium and trimethylammonium salts in the case of **8**, **10a-d**, **11a,b** and **10a'**, the gradient systems DCM:toluene:abs. ethanol: 25 % aq. ammonia (80:9:10:1 to 74:8:17:1) and DCM:toluene:abs. ethanol: 13 % trimethylamine in THF (79:9:10:2 to 70:8:20:2), respectively, were used.

Analytical NMR

Most ^{1}H-NMR signals specific for homologues and derivatives of PEth are found in the “finger print” region of ~3.5 to ~5.5 ppm. As result from an extensive screening effort, we found that the exact chemical shifts, the line shapes and the resolution of these signals are highly dependent on the salt-form of the PEth derivative and the solvent employed, as exemplified below in case of the commonly encountered homologue PEth-16:0/18:1 (Fig. 3). Desirable properties of a solvent for use in NMR-applications include a high solubilizing capacity of the PEth derivative and an ability to evoke high separation and resolution of the thereto belonging signals, in particular within the finger print region. With regard to the former property, the lipophilic character of PEth derivatives and other similar phosphatidyl alkyl esters are well in line with the use of the commonly used NMR-solvent chloroform-d, with or without the presence of a minor polar component, e.g. methanol-d4, as previously reported [19]. Indeed, chloroform-d alone or as the major component in mixtures with methanol-d4, was found to provide a good solubilizing power of PEth-16:0/18:1, permitting the preparation of solutions with a relatively high concentration (> ~5mM). The signal separation and resolution in the finger print region were, however, found to be very poor for chloroform-d and chloroform-d/methanol-d4-mixtures for low (< ~5mM) as well as for high (> ~5mM) concentrations of the H-form of PEth-16:0/18:1 (Fig. 3a). Two factors, the switch from the H-form to the ammonium- or trimethylammonium salt and the use of polar aprotic solvents as NMR-solvent, were pleasingly found to generally improve the signal separation and resolution in ^{1}H-NMR spectra of PEth-16:0/18:1. Although the solubility was found to generally be relatively low (< ~5mM) in these types of polar solvents, DMSO-d6 (Fig. 3b and 3c) and acetone-d6 were identified as suitable for direct practical use in low-concentration NMR-applications and for further investigations and development. Addition of minor amounts (up to ~20%) of the polar protic solvent methanol-d4 to the polar aprotic solvent was found to improve the solubility of PEth-16:0/18:1 with no or only minor decrease in signal separation or resolution. In connection to this, the mixture acetone-d6: methanol-d4 (97:3, v:v) (Fig. 3d) emerged as a practical solvent for NMR-applications due to its related high separation and resolution, as well as its high removability by evaporation. Structural assignment of the ^{1}H- and ^{13}C-signals of **10a**, as a representative PEth homologue dissolved in this solvent mixture, was conducted using standard 2D NMR techniques including COSY, HMQC and HMBC. A ^{31}P-spectrum of **10a** was also collected (see the *Electronic Supplementary Material*).

With regard to analytical separation of PEth and the corresponding RPI-PEth, we were not successful in our attempts to separate such isomers by reversed phase HPLC, nor were we able to separate the corresponding PC isomers by reversed phase ultra-performance liquid chromatography (UPLC). To the best of our knowledge, no successful chromatographic separation of either of these pairs has been reported. Furthermore, the ^{1}H-NMR spectrum at 400 MHz of the PEth homologue **10a** was found to be identical to the corresponding RPI **10c**. Only minor differences between the appearance of the signals pursuant to the fatty acid methyl groups could be identified in the case of the pair **10b** / **10d**, while these signals could well be used for differentiation across the pairs **10a** / **10c** and **10b** / **10d** (Fig. 4).

Inspired by the findings by Willmann *et al.* [20], who showed minor but significant differences in ^{13}C-shifts of the olefinic signals of PC-16:0/18:1 and PC-18:1/16:0, we next turned our attention to ^{13}C-NMR. Although no such corresponding differences could be noted in the case of either of the pairs PEth-16:0/18:1 and RPI-PEth-18:1/16:0 or PEth-16:0/18:2 and RPI-PEth-18:2/16:0 in acetone-d6:methanol-d4 (97:3, v:v), we noted significant differences in the appearance of the signals pursuant to the carbons of the carbonyl groups, in the signals of the allylic- and of the β-carbons (Fig. 5). A sample consisting of a 1:2-mixture of PEth-16:0/18:1 and RPI-PEth-18:1/16:0 displayed partial separation of the first group of signals (Fig. 5a left), no separation of the second group (Fig. 5a middle), which appeared as a non-resolved singlet, and partial separation of the third (Fig. 5a right). Taken together, these groups of signals make it possible to analytically differentiate PEth-16:0/18:1 and RPI-PEth-18:1/16:0 from each other and also, at least to a limited degree, quantify each of the components in such mixtures. With regard to the corresponding 1:2-mixture of PEth-16:0/18:2 and RPI-PEth-18:2/16:0, the carbonyl group signals did not appear as separated (Fig. 5d left), but rather as slightly broadened in comparison to the corresponding signals of either of the corresponding pure isomers (Fig. 5e left and 5f left). The most pronounced differentiating feature in the ^{13}C-NMR spectra of PEth-16:0/18:2 and RPI-PEth-18:2/16:0 relates to the β-carbon signals, which were overlapping into one singlet in the former case (Fig. 5e right) and separated into two resolved singlets in the latter (Fig. 5f right).

LC-MS/MS calibrators

NMR spectroscopy is readily available for highly precise quantification of organic compounds in solution, commonly referred to as qNMR. By taking necessary precautions in experimental setup, as thoroughly investigated by Griffiths and Irvine [21], qNMR may be performed with a standard deviation below 1%. In this work, we employed dimethyl sulfone (DMSO2) as the internal standard in all qNMR measurements due to its advantageous signal separation from all signals of all investigated PEth homologues, its availability as a verified qNMR-standard and its proximity (~3.0 ppm) to the interesting signals in the “finger print” region (~3.5 to ~5.5 ppm). DMSO2 has previously been described by Wells *et al.* [22] as a useful reference standard in qNMR-applications. Quantitative ^{1}H-NMR (^{1}H-qNMR) has previously been employed for the quantification of lipids in D_2O [23]. With the knowledge gained through this work of well resolved ^{1}H-NMR spectra in other polar solvents, we envisioned calibrator solutions of various PEth homologues for LC-MS(/MS) applications, in which the concentration thereof is independently determined by ^{1}H-qNMR. For this purpose we chose DMSO-d6 as a suitable polar solvent due to the readily availability, relatively low cost and, in particular, its low volatility. In order to overcome the relatively low solubility of PEth homologues in DMSO-d6 we applied the use of a solubilizing agent, equivalent to the use of sodium cholate for solubilization of lipids in aqueous solutions [23,24]. Hence, we analogously screened the alternative detergents sodium dodecyl sulfate (SDS), dioctyl sodium sulfosuccinate (Na-AOT), Tween 20® (CAS-no.: 9005-64-5) and Triton-X 100® (CAS-no.: 9002-93-1) as additives in a “precipitation model” (see the *Electronic Supplementary Material*). The utilized precipitation model was designed to give an estimate of the thermodynamically limited solubility of PEth, as examplified by **10a’**. In short, an excess of **10a’** dissolved in DCM was treated with a solution of the detergent and the internal qNMR-standard DMSO2 in DMSO-d6 under reduced pressure at a rotary evaporator, until only trace amounts of the DCM remained. The remaining solution was thereafter decanted from precipitated **10a’** before quantification of dissolved **10a’** employing ^{1}H-qNMR by comparison of the areas of the signals derived from **10a’** in the finger print region of ~3.5 to ~5.5 ppm, with the area of the singlet at 2.99 ppm of the DMSO2-standard. All detergents were found to significantly increase the solubility of **10a’** from about 8 mM, in the absence of any detergent, to as much as 24 mM, in the presence of Triton-X 100 at a concentration of 46 mg/ml (Fig. 6).

A corresponding “dissolution model” was employed for the estimation of the kinetically limited solubility, i.e. straight forward dissolution of neat **10a’** in DMSO-d6. In short, solid **10a’** was treated with DMSO-d6 before decantation, filtration and quantification by ^{1}H-qNMR (see the *Electronic Supplementary Material*). The kinetically limited solubility of **10a’**, estimated by use of this model, was found to be highly dependent on experimental factors, but invariably lower than the corresponding thermodynamically limited solubility estimate.

SDS was selected as a suitable solubility enhancing detergent in the preparation of calibrators for LC-MS(/MS) due to its minimal interference with the signals in the finger print region of ~3.5 to ~5.5 ppm. Hence, a DMSO-d6 based calibrator comprising **10a’**, SDS (50 mg/ml) and the radical scavenger 2,6-di-tert-butyl-4-methylphenol (BHT, 1.2 mg/ml) was prepared in accordance with the dissolution model (see the *Electronic Supplementary Material*). This calibrator was blindly evaluated in an independent LC-MS/MS system at the Lund University Hospital, Sweden, used for routine quantification of PEth in clinical samples. The thereby determined concentration of **10a’** (1.16 umol/g) was found to be in accordance with the concentration independently determined by ^{1}H-qNMR (1.20 ±2.1% µmol/g [P=0.95]). No significant negative effect on the performance of the chromatography and other aspects of the LC-MS/MS system could be observed. The ^{1}H-qNMR determination was performed by comparison of the averaged integral values of the H_{C2}-multiplets and the $H_{C1''}$–double doublets at 5.1 and 4.1 ppm, respectively, with the DMSO2 internal standard singlet at 2.99 ppm, in five independently prepared samples. Although in general suitable for integration due to e.g. its advantageous line-shape, the $H_{C1'}$–double doublet at 4.3 ppm was excluded as a representative signal in the quantification due to its interference by partial overlap with signals of decomposition products, as observed upon long term storage of the calibrator at ambient temperature in the dark.

The relaxation delay used in any ^{1}H-qNMR determination experiment has to be set to a value several times larger than the nuclear relaxation time T_1 for the particular sample studied, in order to achieve a full recovery of signal intensity between each repetition in the measurement, as required for a quantitative comparison of NMR signals. We measured the T_1 of **10a'** and DMSO2 using a standard inversion recovery experiment. All signals were found to have a T_1 of < 3 s. Employment of a relaxation delay 6 times T_1 will give a recovery of nuclear intensity of > 99.75 %. In order to account for variations between samples we used a 60 s relaxation delay, which is three times longer than the estimated need of 18 s. As a separate confirmative control experiment, we collected integral values for the H_{C2}-, the $H_{C1''}$-, the $H_{C1'}$– and the DMSO2 signals in experiments with different relaxation delays T_1. Accordingly, it was found that the integrals varied significantly between different relaxation delays for $T_1 < \sim 15$ s, but remained constant for $T_1 > \sim 15$ s. Phasing and baseline adjustment of all ^{1}H-qNMR spectra, as well as integration, were performed manually rather than by use of automatic algorithms. As expected, the result of control experiments revealed that the former method resulted in determinations with a better precision as compared to the latter.

A ^{1}H-qNMR based quantification, similar to the one described above but performed after 9 month of storage at ambient temperature in the dark, indicated ~10 % decrease of the concentration of **10a'** in the calibrator and the appearance of new minor yet unidentified signals in the finger print region of ~3.5 to ~5.5 ppm. General ^{1}H-NMR monitoring of another aliquot of the calibrator comprising DMSO2, stored under more demanding condition (exposure to sunlight in an NMR-tube stored at a window ledge) during a similar time period, revealed the appearance of a minor singlet at 2.08 ppm. Based on the action of molybdenum oxotransferases such as DMSO-reductase, an endogenous enzyme among various bacteria [25], we interpret this singlet as potentially due to the reduction product dimethyl sulfide, which may be formed from DMSO2 and/or DMSO in the presence of such bacteria growing in the calibrator.

With regard to the usability of the $H_{C1''}$–double doublet at 4.1 ppm in ^{1}H-qNMR determination of PEth in calibrators as described herein, we recommend careful chromatographic purification of any synthetically prepared PEth homologue (e.g. **10a-d** and **10a'**) to ensure absence of isomeric **8** or corresponding potential contaminants. By experiments involving the spiking of the calibrator with isomeric **8**, it was found that ^{1}H-NMR signals of the latter overlaps with the $H_{C1''}$–double doublet at 4.1 ppm of the former. Isomeric **8** or corresponding compounds are, however, easily separated as a faster eluting fraction in the purification of PEth homologues by chromatography on silica gel, by employment of the herein described mobile phases. Furthermore, based on the present experimentally supported assumptions, that reducing bacteria may grow in the composition upon long term storage, we recommend the inclusion of a minor amount of a suitable antimicrobial agent in calibrators of this type to minimize this type of potential degradation of constituting components. We have initiated experiments with 2-methyl-4-isothiazolin-3-one as such a suitable antimicrobial agent and future results will be reported in due course (see the *Electronic Supplementary Material*).

Of relevance for the practical usability of the calibrator, we were pleased to find that aqueous dilution of the calibrator by a factor of 30 to 50, followed by quantitative analysis by LC-MS analysis, indicated miscibility with water in any practically useful proportion without the precipitation or any other loss of **10a'**. Hence, initial or final dilution to furnish a solution comprising of a mixture of water and DMSO-d6 in the range from 1:1 to 98:2 by volume, respectively, resulted in final solutions that displayed, with exception for experimental and instrumental imprecision, the same concentration of **10a'**.

A comparison between ^{1}H-NMR spectra of **10a'** in DMSO-d6 revealed that the shift and appearance of the apparent H_{C3}-broad triplet and the H_{et}-quintet thereof is dependent on the presence of SDS as well as the method of preparation of the composition (Fig. 7). In the presence of SDS and the absence of e.g. traces of DCM, the H_{C3}-broad triplet and the H_{et}-quintet are overlaid onto the α-methylene signals of SDS and the side-bands thereof, whereby integration of the former signals for ^{1}H-qNMR determinations are practically impossible (compare Figs. 7a and 7b). When the solution was made in accordance with the precipitation model, which leaves a trace of DCM therein, a significant downfield shift of these signals were interestingly observed (compare Figs. 7c with 7a and 7b). Interestingly, the corresponding downfield shift was less pronounced in the case of the corresponding ammonium salt **10a**. We speculate that dissolved complex species in which DCM is closely associated with the phosphorous containing part of **10a'** is responsible for this downfield shift. This is supported by the fact that the type of counter ion (trimethylammonium or ammonium) modulates the amplitude of the effect.

Conclusions

This work has shown that ^{1}H-NMR spectra of various PEth homologues may be recorded with significantly improved line shapes and resolution of individual signals by employment of solvents with hydrophilic character, as compared to traditionally used lipophilic solvents like e.g. chloroform-d. A mixture of acetone-d6 and methanol-d4 (97:3, v:v) was identified as a practically useful hydrophilic solvent in NMR applications for the analysis of PEth. This mixture provides an advantageous balance between high signal resolution, acceptable solubilizing capability and readily removability by evaporation. We recommend it for use in NMR-analysis of PEth and can foresee a potential high applicability in the corresponding analyses of at least related lipids which, however, needs to be confirmed by further investigations.

Another major finding that followed is that by use of SDS as a solubility enhancing detergent and BHT as a radical scavenger, DMSO-d6 could be used as a hydrophilic solvent to provide room temperature long term stable PEth solutions (~10 % decrease in concentration over 9 month) that are fully miscible with water and simultaneously allow accurate determination of the intimate PEth homologue by ^{1}H-qNMR with good precision (~2 % error, n=5, P=0.95). Such “calibrators” may advantageously by used as analytical standards for the calibration of analytical instruments, e.g. LC-MS/MS instruments, as their concentration rely on qNMR, an independent analytical method based on first principle assumptions.

Synthetic routes, starting from the common *R*-enantiomer of PMB protected glycerol **1** with regio- and stereochemical control throughout**,** leading to a great variety of PEth homologues and related derivatives including PEths, d5-PEths, RPI-PEths and lyso-PEths, were developed. To the best of our knowledge, the thereby exemplary synthesized compounds **14** (lyso-PEth) and **10a,c** (RPI-PEths) are novel and may serve as highly useful reference materials for further investigations related to human PEth metabolism and regiochemical distribution.

We further conclude that it is possible to, at least qualitatively, differentiate between pure homologues and mixtures of at least comparable amounts of PEth-16:0/18:1, RPI-PEth-18:1/16:0, PEth-16:0/18:2 and RPI-PEth-18:2/16:0 by comparison of the ^{13}C-signals (acetone-d6:methanol-d4; 97:3, v:v) pursuant to the carbons of the carbonyl groups, the signals of the allylic- and of the β-carbons. Further investigations related to qualitative and quantitative differentiation of in particular PEth and the corresponding RPI-PEth homologues by NMR, e.g. ^{31}P-NMR, is warranted.

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Figures

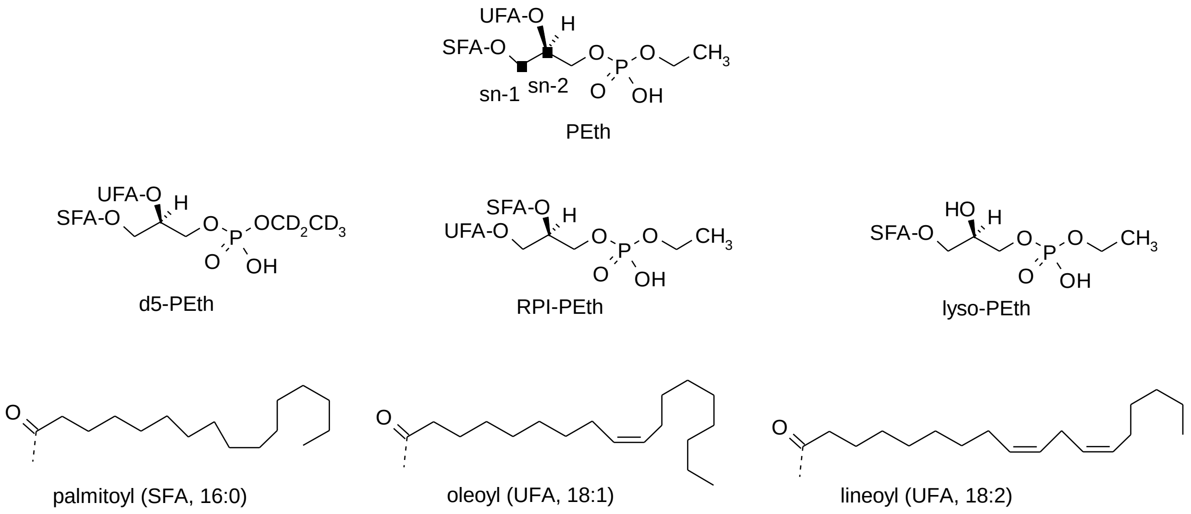

Fig. 1 The structures of PEth with indicated sn-1 and sn-2 positions, d5-PEth with the deuterium labeling at the ethyl moiety, RPI-PEth, the potential metabolite or bio-marker lyso-PEth and the commonly encountered carboxylic acid residues palmitoyl (SFA), oleoyl (UFA) and lineoyl (UFA)

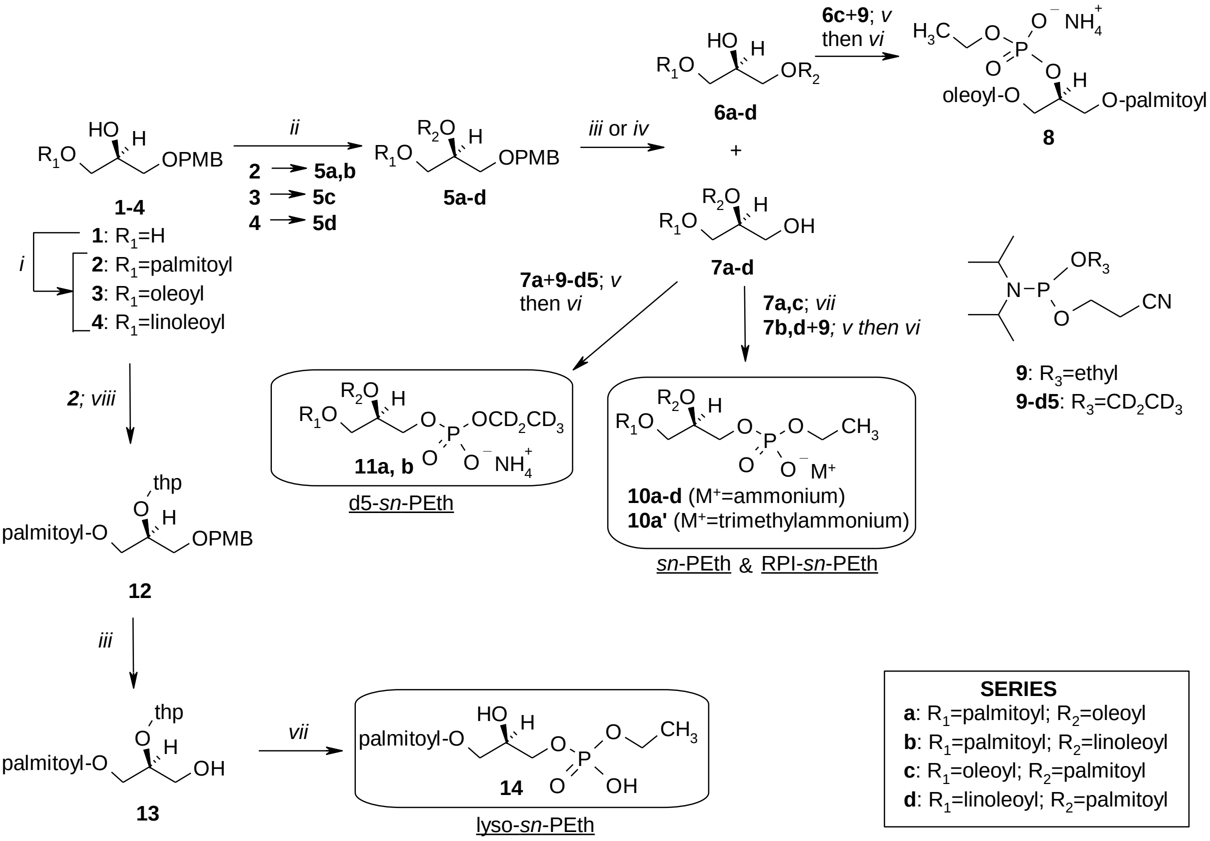

Fig. 2 Synthesis of representative PEth, RPI-PEth, d5-PEth and lyso-PEth homologues, reagents and conditions – (*i*) SFA or UFA (1.05 eq.), DCC (1.05 eq.), DMAP (0.05 eq.), DCM, 0°C to rt (35 to 45 %), (*ii*) SFA or UFA (1.4 eq.), DCC (1.4 eq.), DMAP (0.1 eq.), DCM, rt (91 to 99.6 %), (*iii*) DDQ (1.05 eq.), DCM:H_2O (96:4, v:v), rt (29 % of **7a**, 77 % of **6c**), (*iv*) CAN (10 eq.), acetonitrile:H_2O (9:1, v:v), rt (13 to 89 % of **7b-d**), (*v*) **9** (for **8**, **10b** and **10d**) or **9-d5** (for **11**) (2.0 eq.), tetrazole (2.2 eq), acetonitrile:THF (1:2.6, v:v), rt, then H_2O_2 (30% aq., 8 eq.), (*vi*) THF:25 % aq. ammonia (9:1, v:v), rt (25 to 58 %, two steps), (*vii*) EDCP (2.5 eq.), triethylamine (5.2 eq.), DCM, 0°C to rt (16 to 22 %), (*viii*) 3,4-dihydro-*2H*-pyran (2.0 eq.), pyridinium *p*-toluenesulfonate (0.2 eq.), DCM, rt (quant.)

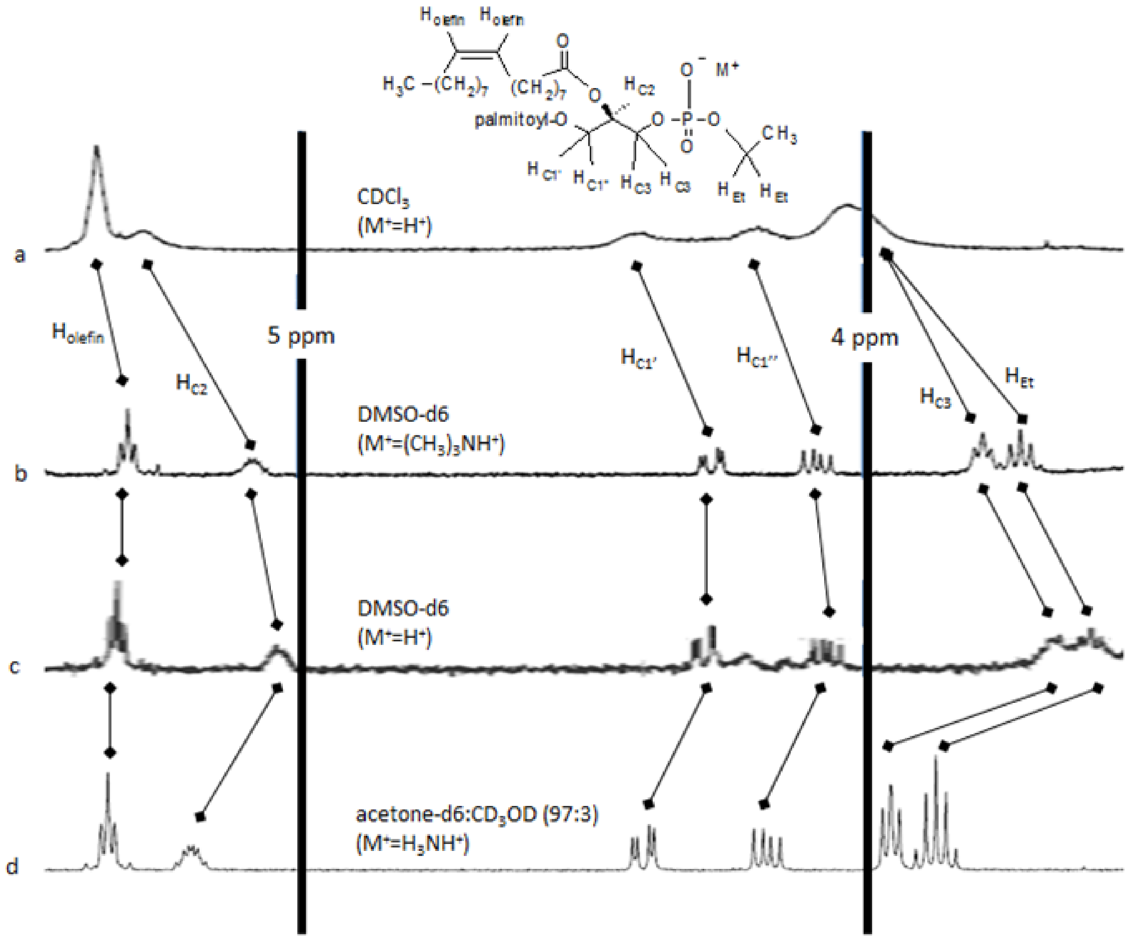

Fig. 3 ^{1}H-NMR finger print regions of various salt forms of PEth-16:0/18:1 in various solvents *a*: the H-form in chloroform-d; *b*: the trimethylammonium salt in DMSO-d6; *c*: the H-form in DMSO-d6; and *d*: the ammonium salt in a mixture of acetone-d6 and methanol-d4 [97:3, v:v]

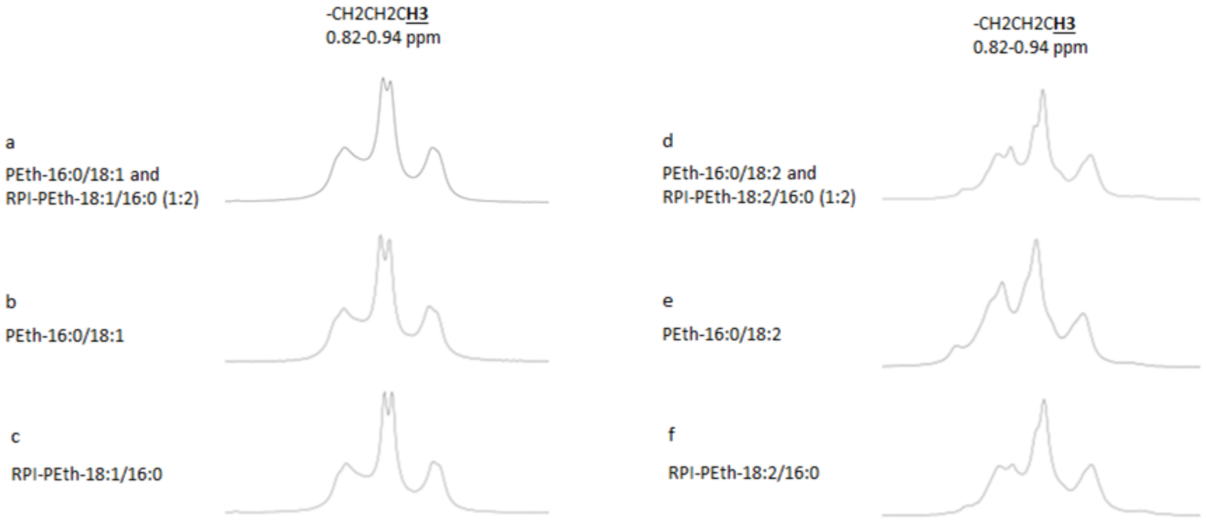

Fig. 4 Signal appearance of ^{1}H-NMR spectra in the region of 0.82 to 0.94 ppm of *a*: a 1:2-mixture of PEth-16:0/18:1 and RPI-PEth-18:1/16:0, *b*: pure PEth-16:0/18:1, *c*: pure RPI-PEth-18:1/16:0, *d*: a 1:2-mixture of PEth-16:0/18:2 and RPI-PEth-18:2/16:0, *e*: pure PEth-16:0/18:2 and *f*: pure RPI-PEth-18:2/16:0. The appearance of the signal in this region allows the analytical differentiation between PEth-16:0/18:1 or RPI-PEth-18:1/16:0 and PEth-16:0/18:2 or RPI-PEth-18:2/16:0. A vague difference in the appearances of the signals of PEth-16:0/18:2 and RPI-PEth-18:2/16:0 may be noted

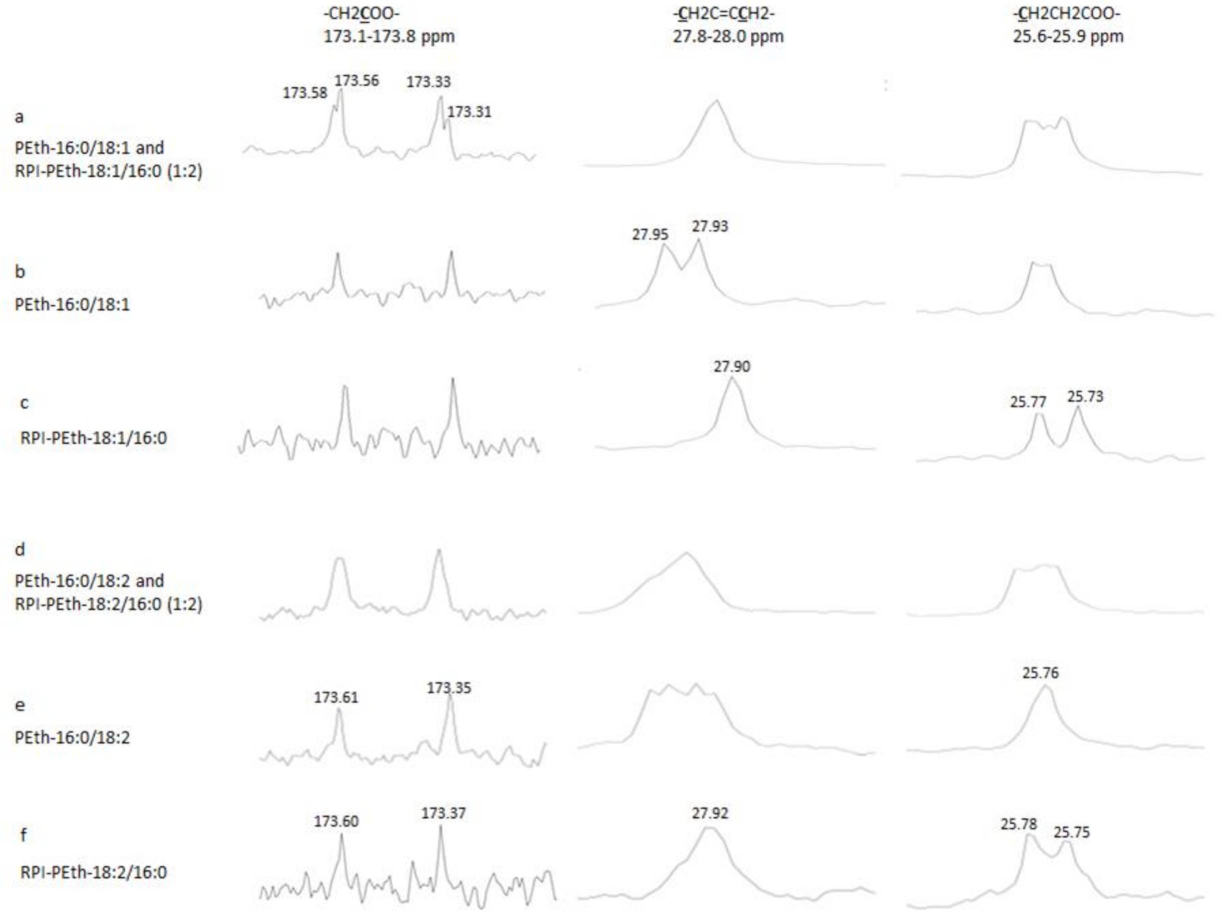

Fig. 5 Regions with differentiating signal appearance of ^{13}C-NMR spectra of *a*: a 1:2-mixture of PEth-16:0/18:1 and RPI-PEth-18:1/16:0, *b*: pure PEth-16:0/18:1, *c*: pure RPI-PEth-18:1/16:0, *d*: a 1:2-mixture of PEth-16:0/18:2 and RPI-PEth-18:2/16:0, *e*: pure PEth-16:0/18:2 and *f*: pure RPI-PEth-18:2/16:0. The chemical shifts (ppm) of selected peak maxima are depicted above the respective peaks

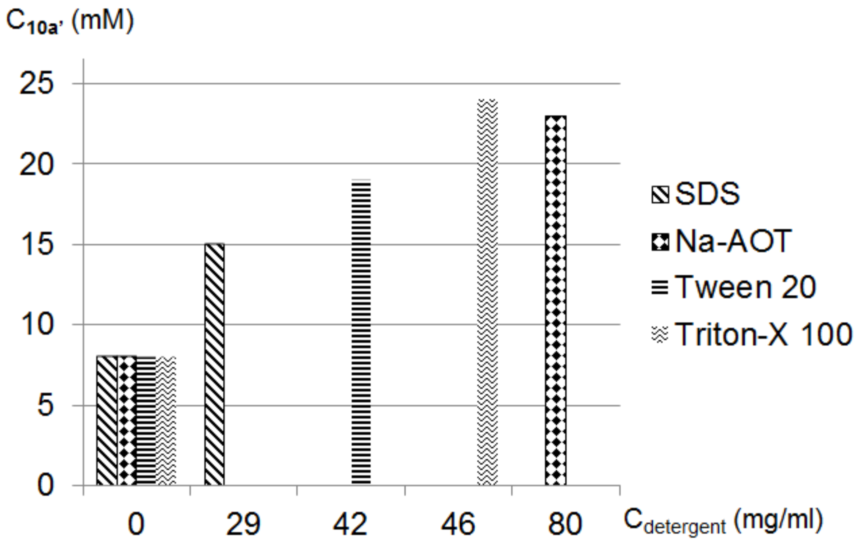

Fig. 6 The solubility of **10a’** in DMSO-d6 in the presence of various detergents as determined by qNMR by employment of DMSO2 as internal reference

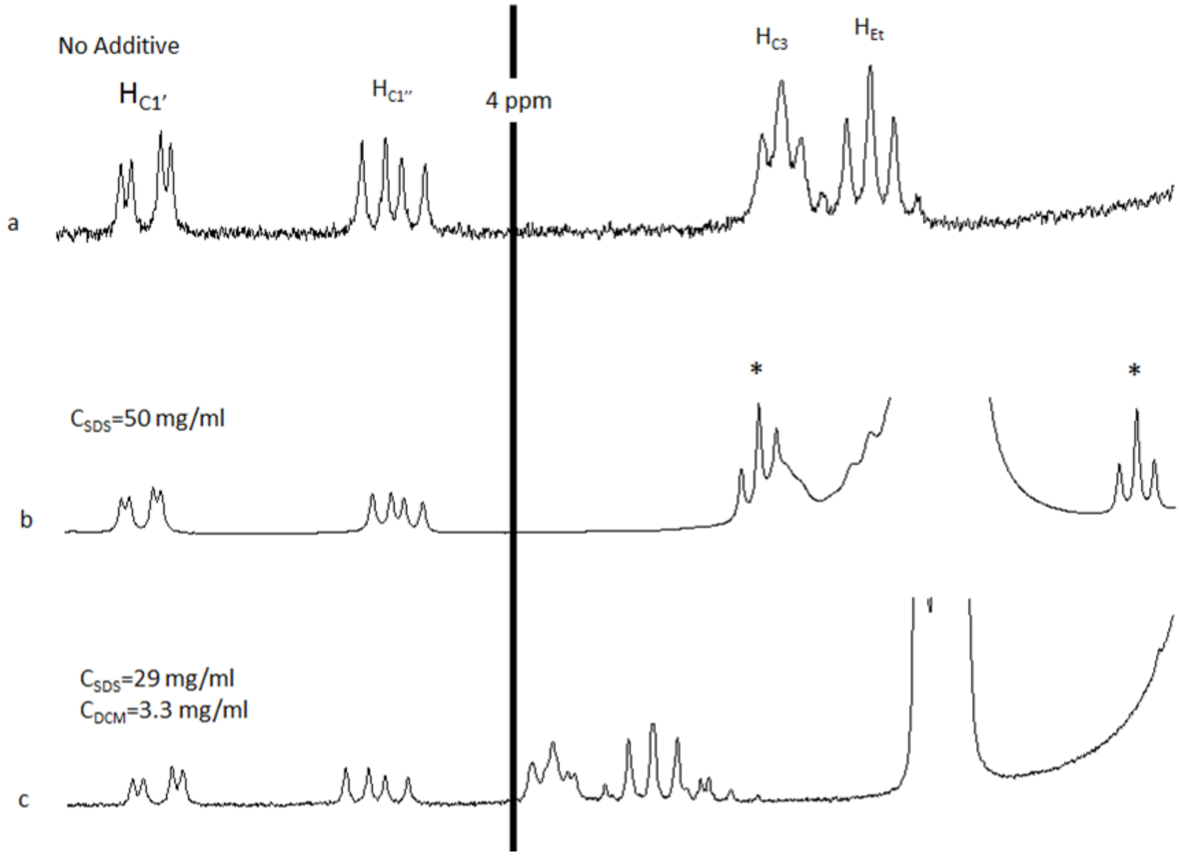

Fig. 7 ^{1}H-NMR spectra of **10a'** in DMSO-d6 in the region of 3.5 to 4.35 ppm in the presence of *a*: no additional additives, *b*: SDS (50 mg/ml), and *c*: SDS (29 mg/ml) together with trace amounts of residual DCM (3.3 mg/ml). Marked triplets (*) are due to SDS-^{13}C-satelites

Differentiation and quantification of synthetic phosphatidylethanol (PEth) homologues by ^{1}H- and ^{13}C-NMR in polar organic solvents

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Supplemental Material

Previously known or reported compounds are indicated with their respective CAS-number in the preparative descriptions below [CAS: “CAS-number”].

Synthesis of compounds **1-4**, **5a-d**, **6c**, **7a-d**, **8**, **9**, **9-d5**, **10a’**, **10a-d**, **11a-b** and **12-14**

*3-(4-Methoxybenzyl)-*sn*-glycerol (**1**) [CAS: 109786-74-5]*

To a stirred mixture of sodium hydride (57-63 % in mineral oil, 2.44 g, 58-64 mmol) in dry THF (100 ml) at ambient temperature was added, in consecutive order, 1,2-isopropylidene-*sn*-glycerol (6.61 g, 50.0 mmol), tetrabutylammonium iodide (1.93 g, 5.2 mmol) and 4-methoxybenzyl chloride (9.0 g, 57.5 mmol). After reflux over night the reaction was allowed to attain ambient temperature and cautiously quenched by addition of aqueous phosphate buffer (pH 7, 300 ml). The obtained mixture was extracted with ethyl acetate (200 + 3x100 ml), followed by washing of the combined organics with brine and drying (sodium sulfate), before concentration and purification of the obtained crude material by chromatography on silica gel (gradient: *n*-heptane to *n*-heptane:ethyl acetate 8:2) to yield 12.7 g (quantitative yield) of 1,2-isopropylidene-3-(4-methoxybenzyl)-*sn*-glycerol as a colorless oil. This oil was refluxed in aqueous acetic acid (10 vol-%, 50 ml) for 30 min. followed by stirring at ambient temperature for 48 h. Water (50 ml) was subsequently added and the obtained mixture was extracted with ethyl acetate (100 + 3x50 ml), followed by washing of the combined organics with brine and drying (sodium sulfate), before concentration and purification of the obtained crude material by chromatography on silica gel (*n*-heptane:ethyl acetate 1:1) to yield 7.59 g (71% yield, two steps) of the title compound as a colorless waxy solid. ^{1}H-NMR (400 MHz, chloroform-d) δ ppm 1.95-2.85 (br. d, 2H), 3.50-3.59 (m, 2H), 3.60-3.74 (m, 2H), 3.82 (s, 3H), 3.85-3.92 (m, 1H), 4.49 (s, 2H), 6.90 (d, *J*=8.6Hz, 2H), 7.25 (d, *J*=8.6Hz, 2H).

*1-Palmitoyl-3-(4-methoxybenzyl)-*sn*-glycerol (**2**) [CAS: 139100-83-7]*

To a cool (0 °C) mixture of 3-(4-methoxybenzyl)-*sn*-glycerol **1** (4.245 g, 20.0 mmol), palmitic acid (5.385 g, 21.0 mmol) and DMAP (122 mg, 1.0 mmol) in dry DCM (100 ml) was added while stirring over 14 h, by employment of a syringe pump, a solution of DCC in dry DCM (0.874 M, 24.0 ml, 21.0 mmol). The reaction mixture was then allowed to attain ambient temperature over 12 h and the formed precipitate was removed by filtration. The filtrate was washed with DCM (3 X 50 ml) before concentration of the combined DCM portions and purification of the obtained crude material by chromatography on silica gel (gradient from *n*-heptane to *n*-heptane:ethyl acetate:*i*-PrOH 67.5:30:2.5) to yield 4.096 g (45% yield) of the title compound as a colorless waxy solid. ^{1}H-NMR (400 MHz, chloroform-d) δ ppm 0.89 (t, J=6.8Hz, 3H), 1.24-1.36 (m, 24H), 1.62 (pent, J=7.3Hz, 2H), 2.33 (t, J=7.6Hz, 2H), 2.43-2.49 (br.s, 1H), 3.47 (dd, J=9.6, 6.2, 1H), 3.54 (dd, J=9.6, 4.3, 1H), 3.82 (s, 3 H), 3.98 - 4.06 (m, 1 H), 4.13 (dd, J=11.5, 6.0Hz, 1H), 4.18 (dd, J=11.5, 4.4Hz, 1H), 4.50 (s, 1H), 6.87-6.92 (m, 2H), 7.24-7.28 (m, 2H). A slower eluting fraction (2.49 g, 28% yield) comprising a 9:1-mixture of the title compound and the byproduct 2-palmitoyl-3-(4-methoxybenzyl)-*sn*-glycerol, as evident by the presence of a separate pentet at 5.04 ppm and a separate multiplet at 3.59 to 3.68 ppm (^{1}H-NMR, 400 MHz, chloroform-d), was also obtained.

*1-Oleoyl-3-(4-methoxybenzyl)-*sn*-glycerol (**3**)*

The title compound was prepared according to the procedure for the preparation of 1-palmitoyl-3-(4-methoxybenzyl)-*sn*-glycerol **2** from 3-(4-methoxybenzyl)-*sn*-glycerol **1** (5.00 g, 23.56 mmol), oleic acid (6.32 g, 22.38 mmol), DMAP (280 mg, 2.36 mmol) and DCC in dry DCM (1.031 M, 24.0 ml, 24.74 mmol) to yield 5.08 g (48% yield) of the title compound as a colorless waxy solid. ^{1}H-NMR (400 MHz, chloroform-d) δ ppm 0.89 (t, J=6.8Hz, 3H), 1.24-1.39 (m, 20H), 1.62 (pent, J=7.2Hz, 2H), 2.02 (q, J=6.0Hz, 4H), 2.33 (t, J=7.6Hz, 2H), 2.50 (d, J=4.8Hz, 1H), 3.47 (dd, J=9.6, 6.2, 1H), 3.53 (dd, J=9.6, 4.3, 1H), 3.82 (s, 3 H), 3.98 - 4.06 (m, 1 H), 4.13 (dd, J=11.5, 6.0Hz, 1H), 4.18 (dd, J=11.5, 4.4Hz, 1H), 4.49 (s, 1H), 5.30-5.41 (m, 2H), 6.87-6.92 (m, 2H), 7.24-7.28 (m, 2H).

*1-Lineoyl-3-(4-methoxybenzyl)-*sn*-glycerol (**4**) [CAS: 1225479-75-3]*

The title compound was prepared according to the procedure for the preparation of 1-palmitoyl-3-(4-methoxybenzyl)-*sn*-glycerol **2** from 3-(4-methoxybenzyl)-*sn*-glycerol **1** (5.00 g, 23.56 mmol), linoleic acid (6.28 g, 22.38 mmol), DMAP (280 mg, 2.36 mmol) and DCC in dry DCM (1.031 M, 24.0 ml, 24.74 mmol) to yield 3.72 g (35% yield) of the title compound as a colorless transparent oil. ^{1}H-NMR (400 MHz, chloroform-d) δ ppm 0.90 (t, J=6.8Hz, 3H), 1.24-1.39 (m, 14H), 1.62 (pent, J=7.2Hz, 2H), 1.96-2.12 (m, 4H), 2.33 (t, J=7.6Hz, 2H), 2.43-2.51 (br.s, 1H), 2.78 (t, J=6.5Hz, 2H), 3.47 (dd, J=9.6, 6.2, 1H), 3.54 (dd, J=9.6, 4.3, 1H), 3.82 (s, 3 H), 5.09 (p, J=5.0Hz, 1H), 4.13 (dd, J=11.5, 6.0Hz, 1H), 4.18 (dd, J=11.5, 4.4Hz, 1H), 4.50 (s, 1H), 5.29-5.44 (m, 4H), 6.87-6.92 (m, 2H), 7.24-7.28 (m, 2H).

*1-Palmitoyl-2-oleoyl-3-(4-methoxybenzyl)-*sn*-glycerol (**5a**)*

1-Palmitoyl-3-(4-methoxybenzyl)-*sn*-glycerol **2** (2.398 g, 5.32 mmol), oleic acid (1.65 g, 5.85 mmol), DCC (1.208 g, 5.85 mmol) and DMAP (65 mg, 0.53 mmol) was stirred in DCM (100 ml) at ambient temperature for 16 h. Another portion of each of oleic acid (451 mg, 1.60 mmol) and DCC (329 mg, 1.60 mmol) was then added, followed by stirring for another 16 h before removal of the formed precipitate by filtration. The filtrate was the washed with DCM (3 X 50 ml) before concentration of the combined DCM portions and purification of the obtained crude material by chromatography on silica gel (gradient from *n*-heptane to *n*-heptane:ethyl acetate 9:1) to yield 3.465 g (91% yield) of the title compound as a colorless syrup that slowly crystallized. ^{1}H-NMR (400 MHz, chloroform-d) δ ppm 0.89 (t, J=6.8Hz, 6H), 1.24-1.38 (m, 44H), 1.55-1.67 (m, 4H), 2.02 (q, J=6.4Hz, 4H), 2.30 (dt, J=16.4, 7.6Hz, 4H) , 3.56 (dd, J=5.2, 1.1Hz, 2H) , 3.81 (s, 3 H), 4.18 (dd, J=11.8, 6.4Hz, 1H), 4.34 (dd, J=11.8, 3.8Hz, 1H), 4.47 (dd, J=18.4, 11.7Hz, 3H), 5.20 - 5.26 (m, 1 H), 5.30-5.40 (m, 2H), 6.86-6.90 (m, 2H), 7.22-7.26 (m, 2H).

*1-Palmitoyl-2-linoleoyl-3-(4-methoxybenzyl)-*sn*-glycerol (**5b**)*

The title compound was prepared according to the procedure for the preparation of 1-palmitoyl-2-oleoyl-3-(4-methoxybenzyl)-sn-glycerol **5a** from 1-palmitoyl-3-(4-methoxybenzyl)-*sn*-glycerol **2** (1.20 g, 2.66 mmol) and linoleic acid (820 mg, 2.92mmol) to yield 1.89 g (99.6 % yield) of the title compound as a colorless syrup that slowly crystallized. ^{1}H-NMR (400 MHz, chloroform-d) δ ppm 0.86-0.93 (m, 6H), 1.24-1.41 (m, 38H), 1.55-1.67 (m, 4H), 1.94-2.12 (m, 4H), 2.30 (dt, J=16.4, 7.6Hz, 4H), 2.78 (t, J=6.5Hz, 2H), 3.56 (dd, J=5.2, 1.1Hz, 2H) , 3.81 (s, 3 H), 4.18 (dd, J=11.8, 6.4 Hz, 1H), 4.34 (dd, J=11.8, 3.8Hz, 1H), 4.47 (dd, J=18.4, 11.7Hz, 3H), 5.20 - 5.26 (m, 1 H), 5.29-5.43 (m, 4H), 6.86-6.90 (m, 2H), 7.22-7.26 (m, 2H).

*1-Oleoyl-2-palmitoyl-3-(4-methoxybenzyl)-*sn-*glycerol (**5c**)*

The title compound was prepared according to the procedure for the preparation of 1-palmitoyl-2-oleoyl-3-(4-methoxybenzyl)-sn-glycerol **5a** from 1-oleoyl-3-(4-methoxybenzyl)-*sn*-glycerol **3** (2.89 g, 6.22 mmol) and palmitic acid (1.75 g, 6.84 mmol) to yield 4.42 g (99.3 % yield) of the title compound as a colorless syrup that slowly crystallized. ^{1}H-NMR (400 MHz, chloroform-d) δ ppm 0.89 (t, J=6.8Hz, 6H), 1.24-1.38 (m, 44H), 1.61 (sex, J=7.1Hz, 4H), 2.02 (q, J=6.4Hz, 4H), 2.30 (dt, J=15.7, 7.6Hz, 4H), 3.56 (dd, J=5.2, 1.1Hz, 2H) , 3.81 (s, 3 H), 4.18 (dd, J=11.8, 6.4Hz, 1H), 4.34 (dd, J=11.8, 3.8Hz, 1H), 4.47 (dd, J=18.4, 11.7Hz, 3H), 5.20 - 5.26 (m, 1 H), 5.30-5.40 (m, 2H), 6.86-6.90 (m, 2H), 7.22-7.26 (m, 2H).

*1-linoleoyl-2-palmitoyl-3-(4-methoxybenzyl)-*sn-*glycerol (**5d**)*

The title compound was prepared according to the procedure for the preparation of 1-palmitoyl-2-oleoyl-3-(4-methoxybenzyl)-sn-glycerol **5a** from 1-linoleoyl-3-(4-methoxybenzyl)-*sn*-glycerol **4** (2.16 g, 4.55 mmol) and palmitic acid (1.28 g, 5.00 mmol) to yield 3.18 g (98 % yield) of the title compound as a colorless syrup that slowly crystallized.^{1}H-NMR (400 MHz, chloroform-d) δ ppm 0.86-0.93 (m, 6H), 1.24-1.41 (m, 38H), 1.61 (sex, J=7.1Hz, 4H), 1.94-2.12 (m, 4H), 2.30 (dt, J=15.7, 7.6Hz, 4H) , 2.78 (t, J=6.5Hz, 2H), 3.56 (dd, J=5.2, 1.1Hz, 2H) , 3.81 (s, 3 H), 4.18 (dd, J=11.8, 6.4Hz, 1H), 4.34 (dd, J=11.8, 3.8Hz, 1H), 4.47 (dd, J=18.4, 11.7Hz, 3H), 5.20 - 5.26 (m, 1 H), 5.29-5.43 (m, 4H), 6.86-6.90 (m, 2H), 7.22-7.26 (m, 2H).

*1-Oleoyl-3-palmitoyl-*sn-*glycerol (**6c**) [CAS: 36805-77-3]*

To a stirred mixture of 1-oleoyl-2-palmitoyl-3-(4-methoxybenzyl)-*sn*-glycerol **5c** (3.29 g, 4.60 mmol) and water (3.0 ml) in DCM (75 ml) was added 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (1.09 g, 4.83 mmol) in one portion. After stirring at ambient temperature for 7 h, the reaction mixture was filtered through a short plug of celite® and concentrated. The obtained residue was purified by chromatography on silica gel (gradient from DCM to DCM:i-PrOH 97.5:2.5). Besides slightly more slowly eluting fractions, which contained various amounts of the title compound and 1-oleoyl-2-palmitoyl-*sn*-glycerol **7c**, 2.107 g (77 % yield) of the title compound was obtained in a pure form as a white solid. ^{1}H-NMR (400 MHz, chloroform-d) δ ppm 0.89 (t, J=6.8Hz, 6H), 1.24-1.38 (m, 44H), 1.64 (pent, J=7.1Hz, 4H), 2.02 (q, J=5.9Hz, 4H), 2.36 (t, J=7.6Hz, 4H), 4.05-4.23 (m, 5H), 5.31-5.41 (m, 2H)

*1-Palmitoyl-2-oleoyl-*sn-*glycerol (**7a**) [CAS: 29541-66-0]*

To a stirred mixture of 1-palmitoyl-2-oleoyl-3-(4-methoxybenzyl)-*sn*-glycerol **5a** (3.35 g, 4.684 mmol) and water (2.0 ml) in DCM (50 ml) was added 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (1.117 g, 4.92 mmol) in one portion. After stirring at ambient temperature for 16 h, the reaction mixture was filtered through a short plug of celite® and concentrated. The obtained residue was purified by chromatography on silica gel twice using two different eluent systems, first a gradient from DCM to DCM:i-PrOH 97.5:2.5 to remove faster eluting UV-active byproducts, and then a gradient from *n*-heptane to *n*-heptane:ethyl acetate 9:2 to separate the desired material from the slightly faster eluting by-product, in which the palmitoyl group had migrated from the 2- to the 1-position of the glycerol skeleton. Beside fractions which contained various amounts of the title compound and this byproduct, 796 mg (29 % yield) of the title compound was obtained in a pure form as a colorless oil. ^{1}H-NMR (400 MHz, chloroform-d) δ ppm 0.89 (t, J=6.8Hz, 6H), 1.2 -1.38 (m, 44H), 1.63 (sex, J=7.1Hz, 4H), 2.02

(q, J=5.9Hz, 4H), 2.30-2.39 (m, 4H), 3.74 (d, J=4.8Hz, 2H), 4.25 (dd, J=12.0, 5.6Hz, 1H), 4.33 (dd, J=12.0, 4.6Hz, 1 H), 5.09 (p, J=5.0Hz, 1H), 5.30-5.40 (m, 2H)

*1-Palmitoyl-2-linoleoyl-*sn*-glycerol (**7b**) [CAS: 51621-26-2]*

To a rapidly stirred mixture of 1-palmitoyl-2-linoleoyl-3-(4-methoxybenzyl)-*sn*-glycerol **5b** (668 mg, 0.936 mmol) and water (1.0 ml) in acetonitrile (9.0 ml) was added CAN (5.13 g, 9.36 mmol). Stirring was continued at ambient temperature for 3 h before pouring onto brine (50 ml) and extraction with DCM (50 + 3x20 ml). The combined organics were dried over sodium sulphate and concentrated to furnish the crude material. Purification by chromatography on silica gel (*n*-heptane:ethyl acetate 9:1) gave 115 mg (21 % yield) of the title compound as a colorless oil. ^{1}H-NMR (400 MHz, chloroform-d) δ ppm 0.86-0.93 (m, 6H), 1.24-1.40 (m, 38H), 1.63 (sex, J=7.1Hz, 4H), 1.94-2.14 (m, 4H), 2.30-2.39 (m, 4H), 2.78 (t, J=6.5Hz, 2H), 3.74 (d, J=4.8Hz, 2H), 4.24 (dd, J=12.0, 5.6Hz, 1H), 4.33 (dd, J=12.0, 4.6Hz, 1 H), 5.09 (p, J=5.0Hz, 1H), 5.29-5.43 (m, 4H)

*1-Oleoyl-2-palmitoyl-*sn*-glycerol (**7c**) [CAS: 64199-61-7]*

The title compound was prepared according to the procedure for the preparation of 1-palmitoyl-2-linoleoyl-*sn*-glycerol **7b** from 1-oleoyl-2-palmitoyl-3-(4-methoxybenzyl)-*sn*-glycerol **5c** (0.902 g, 1.26 mmol)to yield 670 mg(89 % yield) of the title compound as a colorless oil. ^{1}H-NMR (400 MHz, chloroform-d) δ ppm 0.89 (t, J=6.8Hz, 6H), 1.24-1.38 (m, 44H), 1.58-1.68 (m, 4H), 2.02 (q, J=5.9Hz, 4H), 2.34 (q, J=7.8Hz, 4H), 3.74 (d, J=4.8Hz, 2H), 4.24 (dd, J=12.0, 5.6Hz, 1H), 4.33 (dd, J=12.0, 4.6Hz, 1 H), 5.09 (p, J=5.0Hz, 1H), 5.30-5.40 (m, 2H)

*1-Linoleoyl-2-palmitoyl-*sn*-glycerol (**7d**)*

The title compound was prepared according to the procedure for the preparation of 1-palmitoyl-2-linoleoyl-*sn*-glycerol **7b** from 1-linoleoyl-2-palmitoyl-3-(4-methoxybenzyl)-*sn*-glycerol **5d** (1.409 g, 1.976 mmol)to yield 150 mg (13 % yield) of the title compound as a colorless oil. ^{1}H-NMR (400 MHz, chloroform-d) δ ppm 0.86-0.93 (m, 6H), 1.24-1.38 (m, 38H), 1.58-1.68 (m, 4H), 1.93-2.11 (m, 4H), 2.34 (q, J=7.8Hz, 4H), 2.78 (t, J=6.5Hz, 2H), 3.74 (d, J=4.8Hz, 2H), 4.25 (dd, J=12.0, 5.6Hz, 1H), 4.33 (dd, J=12.0, 4.6Hz, 1 H), 5.09 (p, J=5.0Hz, 1H), 5.29-5.43 (m, 4H)

*2-Cyanoethoxy-(ethoxy)-(*N,N*-diisopropylamino)phosphine (**9**) [CAS: 140210-65-7]*

To a cool (0 °C) and stirred solution of 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (892 µL, 4.0 mmol) in dry DCM (20 ml) was added, in consecutive order, triethylamine (586 µL, 4.2 mmol) and abs. ethanol (245 µL, 4.2 mmol). The reaction mixture was then allowed to attain ambient temperature and stirred for another 50 min. before concentration *in vacuo*. The residue was treated with diethyl ether (10 + 2x5 ml) followed by filtration to remove solids through a syringe filter. The filtered solution was concentrated by a slow stream of nitrogen to furnish 911 mg (92 % yield) of the crude title compound as a yellowish oil. This oil was dissolved in dry THF (15.2 ml) to provide a solution (60 mg/ml) which was used directly as reagent in further reactions described herein.

*2-Cyanoethoxy-(1,1,2,2,2-petadeuteroethoxy)-(*N,N*-diisopropylamino)phosphine (**9-d5**)*

The title compound was prepared according to the procedure used for the preparation of 2-cyanoethoxy-(ethoxy)-(*N,N*-diisopropylamino)phosphine **9**, with the only exception that dry deuturated ethanol (ethanol-d5) was used instead of abs. ethanol, to furnish 945 mg (94 % yield) the title compound as a yellowish oil. ^{1}H-NMR (400 MHz, chloroform-d) δ ppm 1.19 (dd, J=6.7, 4.2Hz, 12H), 2.65 (t, J=6.6Hz, 2H), 3.54-3.68 (m, 2H), 3.76-3.92 (m, 2H).

*1-Oleoyl-2- ethoxyhydroxyphosphinyl-3-palmitoyl-*sn*-glycerol ammonium salt (**8**)*

To 1-oleoyl-3-palmitoyl-*sn*-glycerol **6c** (297 mg, 0.50 mmol) was added a solution of 2-cyanoethoxy-(ethoxy)-(*N,N*-diisopropylamino)phosphine **9** in dry THF (60 mg/ml, 4.1 ml, 1.0 mmol) while stirring at ambient temperature. A solution of tetrazole in acetonitrile (0.45 M, 2.55 ml, 1.15 mmol) was then added and stirring was continued for another 2 h before addition of aqueous hydrogen peroxide (35 wt%, 387 µL, 4.5 mmol). The reaction mixture was then stirred for another 30 min. before partitioning between water (50 ml) and DCM (25 ml). The aqueous phase was extracted with DCM (3x20 ml), the combined organics washed with saturated aqueous sodium hydrogen carbonate and dried over sodium sulphate before concentration *in vacuo*, to provide 422 mg of the crude cyanoethyl protected intermediate as colorless oil. This oil was then stirred in a mixture of THF (4.5 ml) and aquous ammonia (25 wt%, 0.5 ml) at ambient temperature for 24 h before concentration *in vacuo* and purification of the obtained residue by chromatography on silica gel (DCM:toluene:abs. ethanol: aqueous ammonia 25 wt%, gradient from 80:9:10:1 to 74:8:17:1). The title compound (R_f=0.4 using DCM:toluene:methanol:aqueos ammonia 25 wt%, 75:12:12:1, as analytical eluent) was obtained as a white solid (208 mg, 58 % yield). ^{1}H-NMR (400MHz, acetone-d6:methanol-d4 97:3) δ ppm 0.85-0.92 (m, 6H), 1.21 (t, J=7.1Hz, 3H), 1.24-1.42 (m, 44H), 1.61 (p, J=7.0Hz, 4H), 2.05-2.08 (m, 4H), 2.34 (t, J=7.4Hz, 4H), 3.88 (q, J=7.0Hz, 2H), 4.23 (d, J=5.1Hz, 4H), 4.46-4.55 (m, 1H), 5.30-5.40 (m, 2H); ; MS (LCMS-ESI$^-$) m/z 701.5 (M-H).

*1-Palmitoyl -2-oleoyl-3-ethoxyhydroxyphosphinyl-*sn*-glycerol trimethylammonium salt (**10a'**)*

To a cool (0 °C) solution of ethyl dichlorophosphate (401 mg, 2.46 mmol) in dry DCM was added triethylamine (0.512 g, 5.12 mmol) while stirring. A solution of 1-palmitoyl-2-oleoyl-*sn*-glycerol **7a** (586 mg, 0.984 mmol) in dry DCM (2.0 ml) was then added over a period of 5 min. The reaction mixture was then allowed to attain ambient temperature and stirred for 1 h before quench by pouring onto saturated aqueous sodium hydrogen carbonate (10 ml). Extraction with DCM (10 + 3x5 ml) and concentration *in vacuo* then followed to provide a crude product that was purified by chromatography on silica gel (DCM:toluene:abs. ethanol: 13 wt%, trimethylamine in THF, gradient from 79:9:10:2 to 70:8:20:2). The title compound (R_f=0.3 using DCM:toluene:methanol:aqueos ammonia 25 wt%, 75:12:12:1, as analytical eluent) was obtained as a white solid (134 mg, 18 % yield). ^{1}H-NMR (400MHz, acetone-d6:methanol-d4 97:3) δ ppm 0.85-0.92 (m, 6H), 1.23 (t, J=7.1Hz, 3H), 1.24-1.42 (m, 44H), 1.61 (sex, J=7.0Hz, 4H), 2.05-2.08 (m, 4H), 2.32 (q, J=7.6Hz, 4H), 3.92 (q, J=7.2Hz, 2H), 4.00 (dd, J=6.5, 5.4Hz, 2H), 4.18 (dd, J=12.0, 6.8Hz, 1H), 4.40 (dd, J=12.0, 3.4Hz, 1H), 5.18-5.26 (m, 1H), 5.30-5.40 (m, 2H), 5.77 (s, 9H); ^{13}C NMR (acetone-d6:methanol-d4 97:3) δ ppm 14.43, 16.92, 16.98, 23.28, 23.42, 23.59, 25.75, 25.76, 27.90, 27.91, 29.97, 30.02, 30.14, 30.17, 30.32, 30.46, 30.48, 30.51, 30.58, 32.72, 32.74, 34.61, 34.81, 44.57, , 47.11, 47.28, 47.46, 47.62, 47.80, 52.19, 61.88, 61.93, 63.27, 64.29, 64.33, 71.46, 71.52, 130.61, 130.69, 173.35, 173.61; MS (LCMS-ESI$^-$) m/z 701.5 (M-H).

*1-Palmitoyl -2-oleoyl-3-ethoxyhydroxyphosphinyl-*sn-*glycerol ammonium salt (**10a**)*

The title compound was prepared according to the procedure used for the preparation of 1-palmitoyl -2-oleoyl-3-ethoxyhydroxyphosphinyl-*sn*-glycerol trimethylammonium salt **10a'** from 1-palmitoyl-2-oleoyl-*sn*-glycerol **7a** (197 mg, 0.331 mmol), with the only exception that a gradient from 80:9:10:1 to 74:8:17:1 of DCM:toluene:abs. ethanol: aqueous ammonia 25 wt% was used as gradient in the purification on silica gel, to furnish 38 mg (16 % yield) the title compound as a white waxy solid. ^{1}H-NMR (400MHz, acetone-d6:methanol-d4 97:3) δ ppm 0.85-0.92 (m, 6H), 1.21 (t, J=7.1Hz, 3H), 1.24-1.42 (m, 44H), 1.61 (sex, J=7.0Hz, 4H), 2.05-2.08 (m, 4H), 2.32 (q, J=7.6Hz, 4H), 3.88 (q, J=7.2Hz, 2H), 3.96 (dd, J=6.5, 5.4Hz, 2H), 4.18 (dd, J=12.0, 6.8Hz, 1H), 4.40 (dd, J=12.0, 3.4Hz, 1H), 5.17-5.24 (m, 1H), 5.30-5.40 (m, 2H); ^{13}C NMR (acetone-d6:methanol-d4 97:3) δ ppm 14.46, 16.90, 16.97, 23.44, 25.76, 25.78, 27.93, 27.95, 29.97, 39.03, 30.17, 30.20, 30.24, 30.35, 30.42, 30.55, 30.6, 30.62, 32.76, 34.63, 34.81, 44.89, 48.65, 48.87, 49.08, 49.29, 62.00, 62.06, 63.29, 64.3, 64.35, 71.38, 71.46, 130.61, 130.68, 173.36, 173.62; MS (LCMS-ESI$^-$) m/z 701.5 (M-H).

*1-Palmitoyl -2-linoleoyl-3-ethoxyhydroxyphosphinyl-*sn-*glycerol ammonium salt (**10b**)*

The title compound was prepared according to the procedure used for the preparation of 1-oleoyl-2-ethoxyhydroxyphosphinyl -3-palmitoyl-*sn*-glycerol ammonium salt **8** from 1-palmitoyl-2-linoleoyl-*sn*-glycerol **7b** (55.6 mg, 0.0938 mmol), to furnish 38 mg (57 % yield) the title compound as a white solid. ^{1}H-NMR (400MHz, acetone-d6:methanol-d4 97:3) δ ppm 0.85-0.92 (m, 6H), 1.22 (t, J=7.1Hz, 3H), 1.24-1.42 (m, 38H), 1.61 (sex, J=7.0Hz, 4H), 2.05-2.12 (m, 4H), 2.32 (q, J=7.6Hz, 4H), 2.80 (t, J=6.4Hz, 2H), 3.88 (q, J=7.2Hz, 2H), 3.96 (t, J=5.8Hz, 2H), 4.18 (dd, J=12.0, 6.8Hz, 1H), 4.42 (dd, J=12.0, 3.4Hz, 1H), 5.18-5.26 (m, 1H), 5.28-5.43 (m, 4H); ^{13}C NMR (acetone-d6:methanol-d4 97:3) δ ppm 14.46, 16.95, 17.02, 23.34, 23.44, 25.76, 26.33, 27.91, 27.94, 30.02, 30.17, 30.19, 30.22, 30.40, 30.54, 30.60, 32.35, 32.75, 34.62, 34.81, 48.42, 48.63, 48.84, 49.05, 49.27, 61.82, 61.88, 63.31, 64.21, 64.27, 71.45, 71.53, 128.87, 128.91, 130.61, 130.69, 130.74, 130.80, 173.35, 173.61; MS (LCMS-ESI$^-$) m/z 699.5 (M-H).

*1-Oleoyl-2-palmitoyl-3-ethoxyhydroxyphosphinyl-*sn-*glycerol ammonium salt (**10c**)*

The title compound was prepared according to the procedure used for the preparation of 1-palmitoyl -2-oleoyl-3-ethoxyhydroxyphosphinyl-*sn*-glycerol trimethylammonium salt **10a'** from 1-oleoyl-2-palmitoyl-*sn*-glycerol **7c** (381.7 mg, 0.6415 mmol), with the only exception that a gradient from 80:9:10:1 to 74:8:17:1 of DCM:toluene:abs. ethanol: aqueous ammonia 25 wt% was used as gradient in the purification on silica gel, to furnish 83 mg (18 % yield) the title compound as a yellowish thick syrup. ^{1}H-NMR (400MHz, acetone-d6:methanol-d4 97:3) δ ppm 0.85-0.92 (m, 6H), 1.21 (t, J=7.1Hz, 3H), 1.24-1.42 (m, 44H), 1.61 (sex, J=7.0Hz, 4H), 2.05-2.08 (m, 4H), 2.32 (q, J=7.6Hz, 4H), 3.88 (q, J=7.2Hz, 2H), 3.96 (dd, J=6.5, 5.4Hz, 2H), 4.18 (dd, J=12.0, 6.8Hz, 1H), 4.40 (dd, J=12.0, 3.4Hz, 1H), 5.17-5.24 (m, 1H), 5.30-5.40 (m, 2H); ^{13}C NMR (acetone-d6:methanol-d4 97:3) δ ppm 14.45, 16.92, 16.89, 23.42, 25.73, 25.77, 27.90, 29.97, 30.07, 30.15, 30.18, 30.21, 30.38, 30.48, 30.53, 30.57, 32.73, 32.74, 34.58, 34.81, 46.44, 48.65, 48.86, 49.08, 49.28, 61.78, 61.84, 63.27, 64.21, 64.26, 71.43, 71.51, 130.60, 130.67, 17.30, 173.55; MS (LCMS-ESI$^-$) m/z 701.5 (M-H).

*1-Linoleoyl-2-palmitoyl-3-ethoxyhydroxyphosphinyl-*sn-*glycerol ammonium salt (**10d**)*

The title compound was prepared according to the procedure used for the preparation of 1-oleoyl-2-ethoxyhydroxyphosphinyl -3-palmitoyl-sn-glycerol ammonium salt **8** from 1-linoleoyl-2-palmitoyl-*sn*-glycerol **7d** (75.5 mg, 0.1273 mmol), to furnish 40.4 mg (39.5 % yield) the title compound as a white solid. ^{1}H-NMR (400MHz, acetone-d6:methanol-d4 97:3) δ ppm 0.85-0.92 (m, 6H), 1.21 (t, J=7.1Hz, 3H), 1.24-1.42 (m, 38H), 1.61 (sex, J=7.0Hz, 4H), 2.05-2.12 (m, 4H), 2.32 (q, J=7.6Hz, 4H), 2.80 (t, J=6.4Hz, 2H), 3.88 (q, J=7.2Hz, 2H), 3.96 (t, J=5.8Hz, 2H), 4.18 (dd, J=12.0, 6.8Hz, 1H), 4.41 (dd, J=12.0, 3.4Hz, 1H), 5.17-5.24 (m, 1H), 5.28-5.43

(m, 4H) ^{13}C NMR (acetone-d6:methanol-d4 97:3) δ ppm 14.45, 16.93, 17.00, 23.43, 25.75, 25.78, 27.92, 29.97, 30.17, 30.19, 30.23, 30.40, 30.54, 30.59, 32.33, 32.75, 34.61, 34.82, 48.42, 48.63, 48.84, 49.05, 49.27, 61.17, 61.83, 63.31, 64.18, 64.23, 71.44, 71.53, 130.61, 130.69, 173.37, 173.60; MS (LCMS-ESI$^-$) m/z 699.5 (M-H).

*1-Palmitoyl-2-oleoyl-3-(1,1,2,2,2-pentadeuteroethoxy)hydroxyphosphinyl-*sn*-glycerol ammonium salt (**11a**)*

The title compound was prepared according to the procedure used for the preparation of 1-oleoyl-2-ethoxyhydroxyphosphinyl-3-palmitoyl-sn-glycerol ammonium salt **8** from 1-palmitoyl-2-oleoyl-*sn*-glycerol **7a** (188.7 mg, 0.3172 mmol), by employment of 2-cyanoethoxy-(1,1,2,2,2-petadeuteroethoxy)-(*N*,*N*-diisopropylamino)phosphine **9-d5** as phosphorylating reagent, to furnish 148.3 mg (65.5 % yield) the title compound as a white solid. ^{1}H-NMR (400MHz, acetone-d6:methanol-d4 97:3) δ ppm 0.85-0.92 (m, 6H), 1.24-1.42 (m, 44H), 1.61 (sex, J=7.0Hz, 4H), 2.05-2.08 (m, 4H), 2.32 (q, J=7.6Hz, 4H), 3.96 (t, J=5.8Hz, 2H), 4.18 (dd, J=12.0, 6.8Hz, 1H), 4.41 (dd, J=12.0, 3.4Hz, 1H), 5.17-5.24 (m, 1H), 5.30-5.40 (m, 2H); MS (LCMS-ESI$^-$) m/z 706.5 (M-H).

*1-Palmitoyl-2-lineoyl-3-(1,1,2,2,2-pentadeuteroethoxy)hydroxyphosphinyl-*sn*-glycerol ammonium salt (**11b**)*

The title compound was prepared according to the procedure used for the preparation of 1-oleoyl-2-ethoxyhydroxyphosphinyl-3-palmitoyl-*sn*-glycerol ammonium salt **8** from 1-palmitoyl-2-linoleoyl-*sn*-glycerol **7b** (55.7 mg, 0.09394 mmol), by employment of 2-cyanoethoxy-(1,1,2,2,2-petadeuteroethoxy)-(N,N-diisopropylamino)phosphine **9-d5** as phosphorylating reagent, to furnish 20 mg (25 % yield) the title compound as a white solid. ^{1}H-NMR (400MHz, acetone-d6:methanol-d4 97:3) δ ppm 0.85-0.92 (m, 6H), 1.26-1.42 (m, 38H), 1.61 (sex, J=7.0Hz, 4H), 2.05-2.12 (m, 4H), 2.32 (q, J=7.6Hz, 4H), 2.80 (t, J=6.4Hz, 2H), 3.96 (t, J=5.8Hz, 2H), 4.18 (dd, J=12.0, 6.8Hz, 1H), 4.41 (dd, J=12.0, 3.4Hz, 1H), 5.17-5.24 (m, 1H), 5.28-5.43 (m, 4H); MS (LCMS-ESI$^-$) m/z 704.5 (M-H).

*1-Palmitoyl-2-(2-tetrahydropyranyl)-3-(4-methoxybenzyl)-*sn*-glycerol (**12**)*

A mixture of 1-palmitoyl-3-(4-methoxybenzyl)-*sn*-glycerol **2** (0.901 g, 2.0 mmol), 3,4-dihydro-*2H*-pyran (0.336 g, 4.0 mmol) and pyridinium *p*-toluenesulfonate (0.100 g, 0.40 mmol) was stirred in dry DCM (10 ml) at ambient temperature for 14 h. Volatile matter was then removed by a stream of nitrogen and the obtained residue was purified by chromatography on silica gel (gradient from *n*-heptane to *n*-heptane:ethyl acetate: *i*-PrOH 80:18:2) to yield 1.05 g (98 % yield) the title compound as a colorless oil. ^{1}H-NMR (400 MHz, chloroform-d) δ ppm 0.89 (t, J=6.8Hz, 3H), 1.24-1.35 (m, 24H), 1.48-1.90 (m, 6H), 2.30 (td, J=7.5, 4.5Hz, 2H), 3.45-3.65 (m, 3H), 3.81 (d, J=0.9Hz, 3H), 3.87-3.96 (m, 1H), 4.01-4.15 (m, 2H), 4.18-4.28 (m, 2H), 4.35 (dd, J=11.5, 3.9Hz, 1H), 4.49 (d, J=10.1Hz, 2H), 4.77-4.83 (m, 1H), 6.85-6.91 (m, 2H), 7.23-7.28 (m, 2H).

*1-Palmitoyl-2-(2-tetrahydropyranyl)-*sn*-glycerol (**13**)*

The title compound was prepared according to the procedure used for the preparation of 1-oleoyl-3-palmitoyl-sn-glycerol **6c** from 1-palmitoyl-2-(2-tetrahydropyranyl)-3-(4-methoxybenzyl)-*sn*-glycerol **12** (1.03 g, 1.926 mmol), with the only exception that a gradient from *n*-heptane to *n*-heptane:ethyl acetate: *i*-PrOH 80:18:2 was used in the purification on silica gel, to furnish 0.511 g (62 % yield) the title compound as a white solid. ^{1}H-NMR (400 MHz, chloroform-d) δ ppm 0.89 (t, J=6.8Hz, 3H), 1.24-1.35 (m, 24H), 1.48-1.90 (m, 7H), 2.30-2.38 (m, 2H), 3.50-3.74 (m, 3H), 3.85-4.04 (m, 2H), 4.06-4.23 (m, 3H), 4.34 (dd, J=11.5, 5.3Hz, 1H), 4.57-4.61 (m, 0.5H), 4.77-4.80 (m, 0.5H).

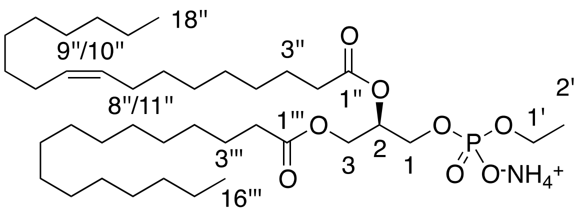
*1-Palmitoyl-3-ethoxyhydroxyphosphinyl-*sn-*glycerol (**14**)*

The title compound was prepared according to the procedure used for the preparation of 1-palmitoyl -2-oleoyl-3-ethoxyhydroxyphosphinyl-*sn*-glycerol trimethylammonium salt **10a'** from 1-palmitoyl-2-(2-tetrahydropyranyl)-*sn*-glycerol **13** (266 mg, 0.642mmol), with the only exception that a gradient from toluene:acetone 6:4 to toluene:acetone:acetic acid:water 55:36:7:2 was used in the purification on silica gel, to furnish 63 mg (22 % yield) the title compound as a glas.^{1}H-NMR (400 MHz, chloroform-d) δ ppm 0.88 (t, J=6.8Hz, 3H), 1.23-1.34 (m, 27H), 1.59 (br. s, 2H), 2.25-2.38 (m, 2H), 3.45-3.68 (m, 7H); MS (LCMS-ESI$^-$) m/z 437.3 (M-H).

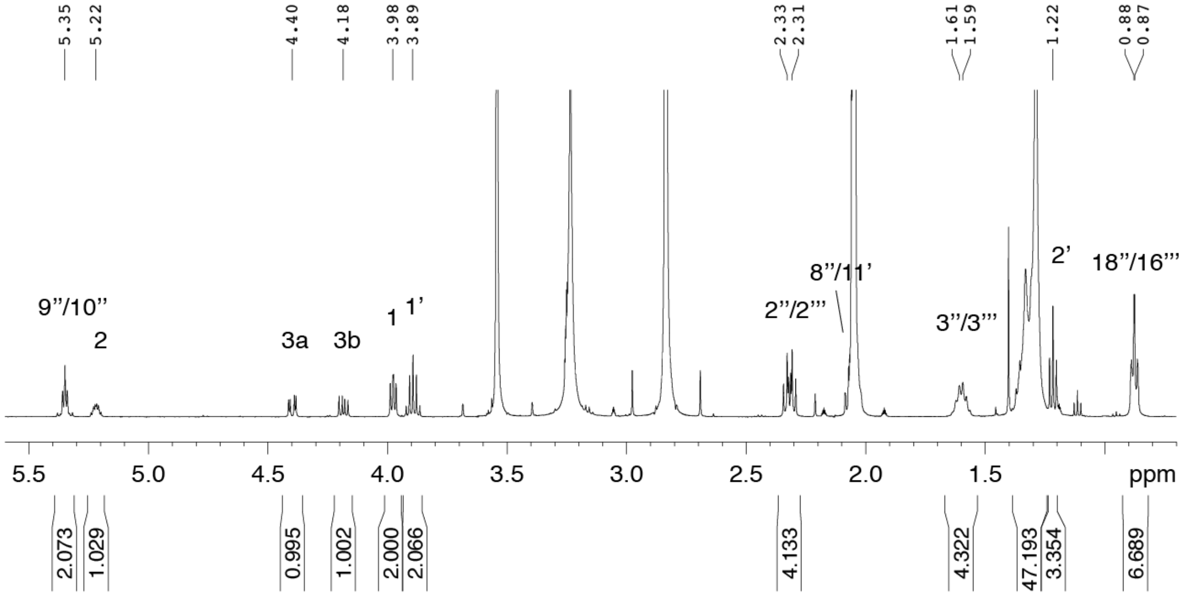
*Structural assignment of ^{1}H- and ^{13}C-signals and ^{31}P-spectrum of **10a***

A solution of **10a** in acetone-d6:methanol-d4 97:3 (10 mg/ml) was subjected to 1D- (^{1}H, ^{13}C and ^{31}P) and 2D- (cosy, HMBC, HSQC) NMR experiments. The structure of **10a** with by numerals indicated atoms/positions relating to various NMR-signals (A), ^{1}H-NMR spectrum of **10a** (B), ^{13}C-NMR spectrum of **10a** (C), chemical shift data of **10a** (D), ^{31}P-NMR spectrum of **10a** (E), COSY-spectrum of **10a** (F), HMQC-spectrum of **10a** (G) and HMBC-spectrum of **10a** (H) are shown below.

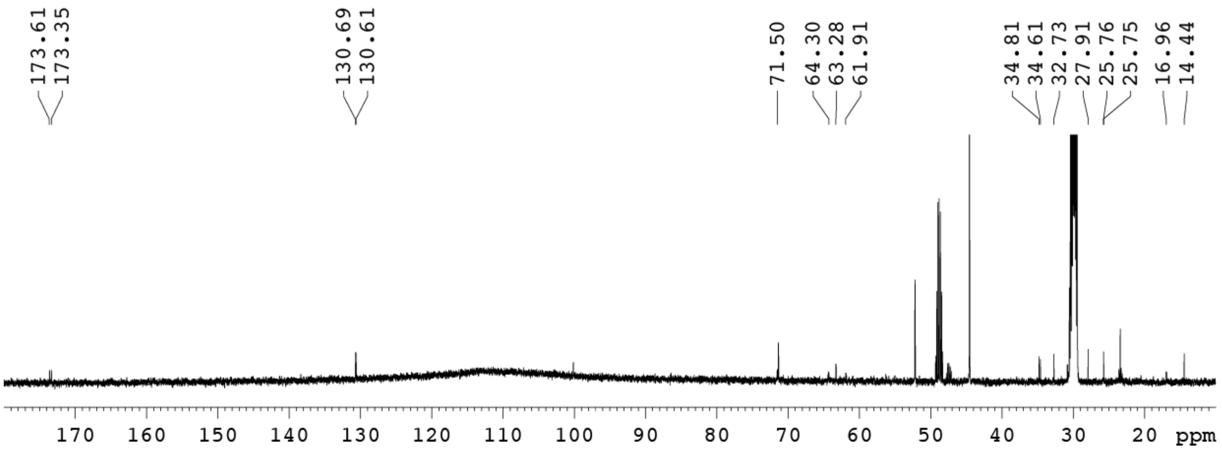
A


18''
9''/10''
3''
O
1''
O
2'
8''/11''
1'''
O
1'
3'''
2
3
1
O
P
O
O
O
O-NH4+
16'''

B


5.35
5.22
4.40
4.18
3.98
3.89
2.33
2.31
1.61
1.59
1.22
0.88
0.87
9''/10''
2
3a
3b
1
1'
8''/11'
2''/2'''
3''/3'''
2'
18''/16'''
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
ppm
2.073
1.029
0.995
1.002
2.000
2.066
4.133
4.322
47.193
3.354
6.689

C

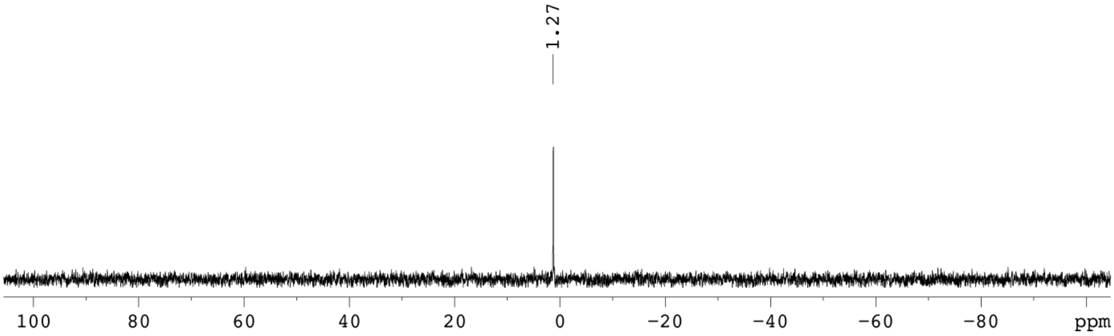

173.61
173.35
130.69
130.61
71.50
64.30
63.28
61.91
34.81
34.61
32.73
27.91
25.76
25.75
16.96
14.44
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
ppm

D

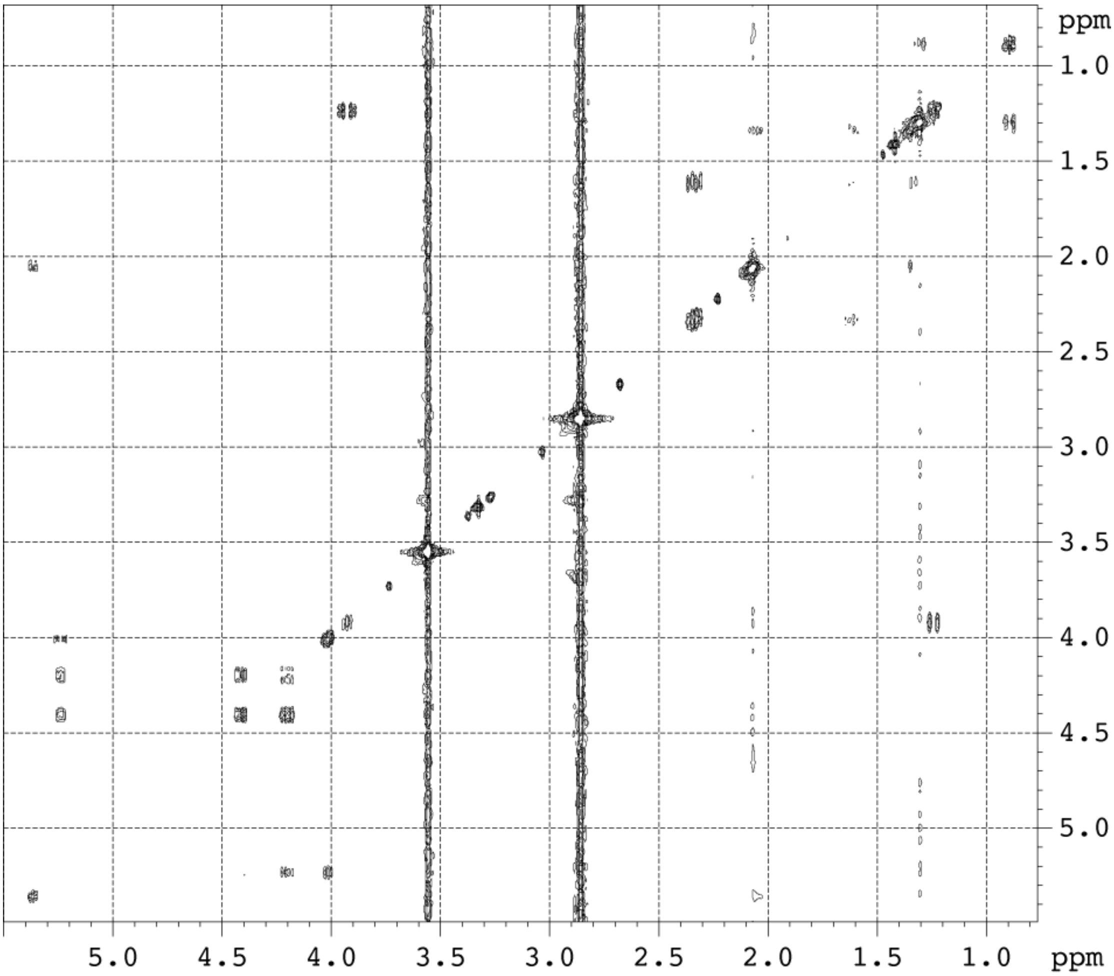
	13C [ppm]	1H [ppm]	mult	Int	J [Hz]
1	64.3	3.98			
2	71.5	5.22	dd	2	5.4, 6.7
3	63.3	4.40	dtd	1	3.4, 5.4, 6.8
			dd	1	3.4, 12.0
		4.18	dd	1	6.8, 12.0
1'	61.9	3.89	dq	2	7.1, 7.1
2'	17.0	1.22	t	3	7.1
1''/1'''	173.6/173.3				
2''/2'''	34.8/34.6	2.33/2.31	t/t	4	7.5
3''/3'''	25.8/25.7	1.59/1.61	m/m	4	
9''/10''	130.7/130.6	5.35	m	2	

31P 1.27 ppm

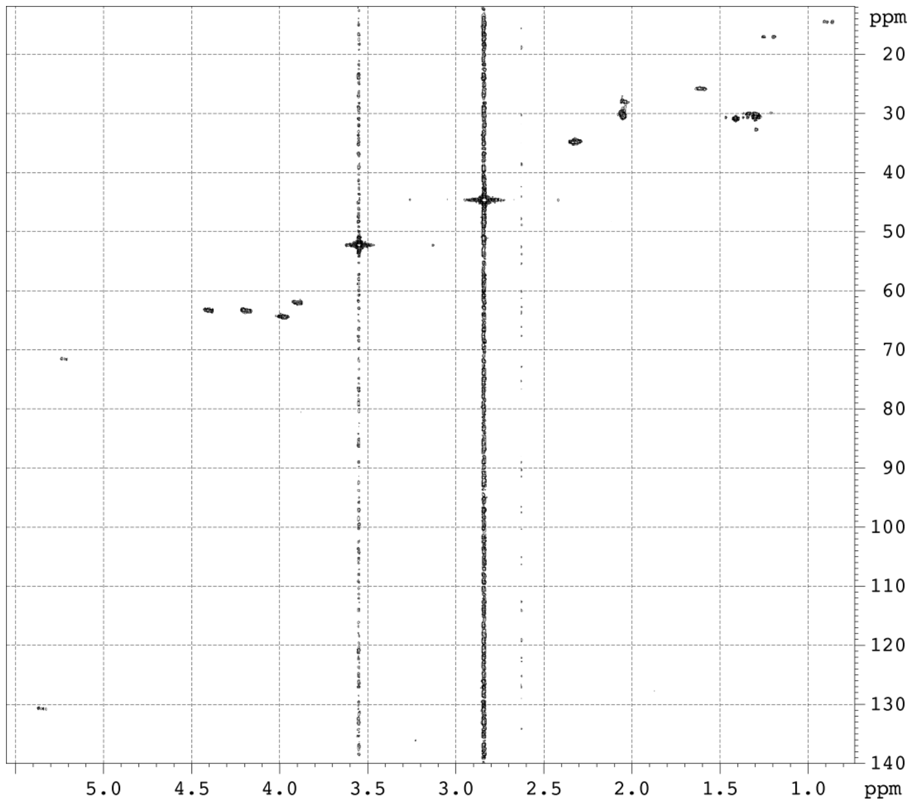
E



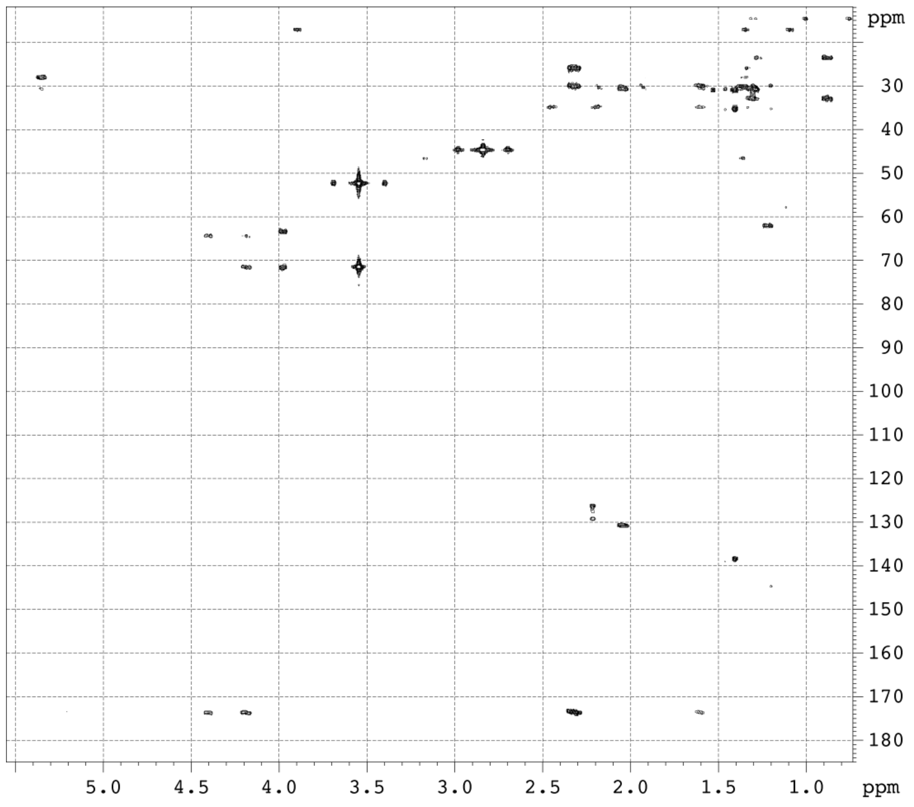
F



G


ppm
20
30
40
50
60
70
80
90
100
110
120
130
140
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
ppm

H


ppm
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40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
ppm

Preparation of solutions of **10a** and **10a’** in DMSO-d6

*In accordance with the “precipitation model” for estimation of the dependence of the thermodynamically limited solubility of **10a’** on the presence of various detergents*

A 10-ml flask with an arrow-shaped bottom was charged with **10a’** (7.5 mg), DMSO2 (1.30 mg), the detergent (SDS, Na-AOT, Tween 20 or Triton-X 100; amount as calculated on the basis of the volume of DMSO-d6), followed by addition of DCM (1.5 ml). After shaking the mixture until complete dissolution, DMSO-d6 (0.75 ml) was added and the flask was applied to a rotary evaporator operating at 200 rpm in vacuo, at 22 °C over night. The obtained solution was transferred to an NMR-tube by decantation, followed by ^{1}H-NMR recording. The concentration of **10a’** was calculated by comparison of the integral of the $H_{C1'}$ signal thereof with the DMSO2-singlet at 2.99 ppm to furnish the following results, as presented in Fig. 5 (Detergent / $C_{Detergent}$ [mg/ml] / $C_{10a'}$ [mM]): no detergent / 0 / 8; SDS / 29 / 15; Tween 20 / 42 / 19; Triton-X 100 / 46 / 24; Na-AOT / 80 / 23.

*In accordance with the “dissolution model” for the preparation of a calibrator solution of **10a’***

To a 15-ml flask charged with SDS (505.6 mg) was added DMSO-d6 (10.0 ml), followed by dissolution by shaking (total weight: 11.7894 g). **10a’** (30 mg) and BHT (1.7 mg) was then added, followed by closing of the flask and gently shaking for 4 h at ambient temperature (22 °C). The resulting solution was decanted from not dissolved **10a’** and filtered through a cotton-plug to remove other potential heterogeneous matter. Five aliquots (in the range of 460.3 to 475.4 mg) were taken and individually mixed with five individually prepared solutions of DMSO2 in DMSO-d6 (238.5 to 239.1 mg; 0.523 to 0.544 mg/g). ^{1}H-qNMR spectra were collected for each of the resulting mixtures. The concentration of **10a’** was found to be 1.20 ±2.1% µmol/g (P=0.95) by calculation on basis of the averaged integral values of the H_{C2}-multiplets and the $H_{C1''}$–double doublets at 5.1 and 4.1 ppm, respectively, with the DMSO2 internal standard singlets at 2.99 ppm.

*In accordance with the “precipitation model” for the preparation of a calibrator solution of **10a***

To a 15-ml flask charged with **10a** (5 mg), SDS (125 mg), BHT (5.0 mg) and 2-methyl-4-isothiazolin-3-one (1.4 mg) was added DCM (5.0 ml). After shaking the mixture until complete dissolution, DMSO-d6 (5.0 ml) was added and the flask was applied to a rotary evaporator operating at 200 rpm in vacuo, at 22 °C over night. The resulting solution was decanted and filtered through a cotton-plug to remove potential heterogeneous matter. Three aliquots (in the range of 460.9 to 466 mg) were taken and individually mixed with three individually prepared solutions (in the range of 238.5 to 240.2 mg) of DMSO2 in DMSO-d6 (1.263 to 1.474 mg; 0.537 to 0.541 mg/g). ^{1}H-qNMR spectra were collected for each of the resulting mixtures. The concentration of **10a** was found to be 1.37 ±8.6% µmol/g (P=0.95) by calculation on basis of the averaged integral values of the H_{C2}-multiplets and the $H_{C1''}$–double doublets at 5.1 and 4.1 ppm, respectively, with the DMSO2 internal standard singlets at 2.99 ppm.