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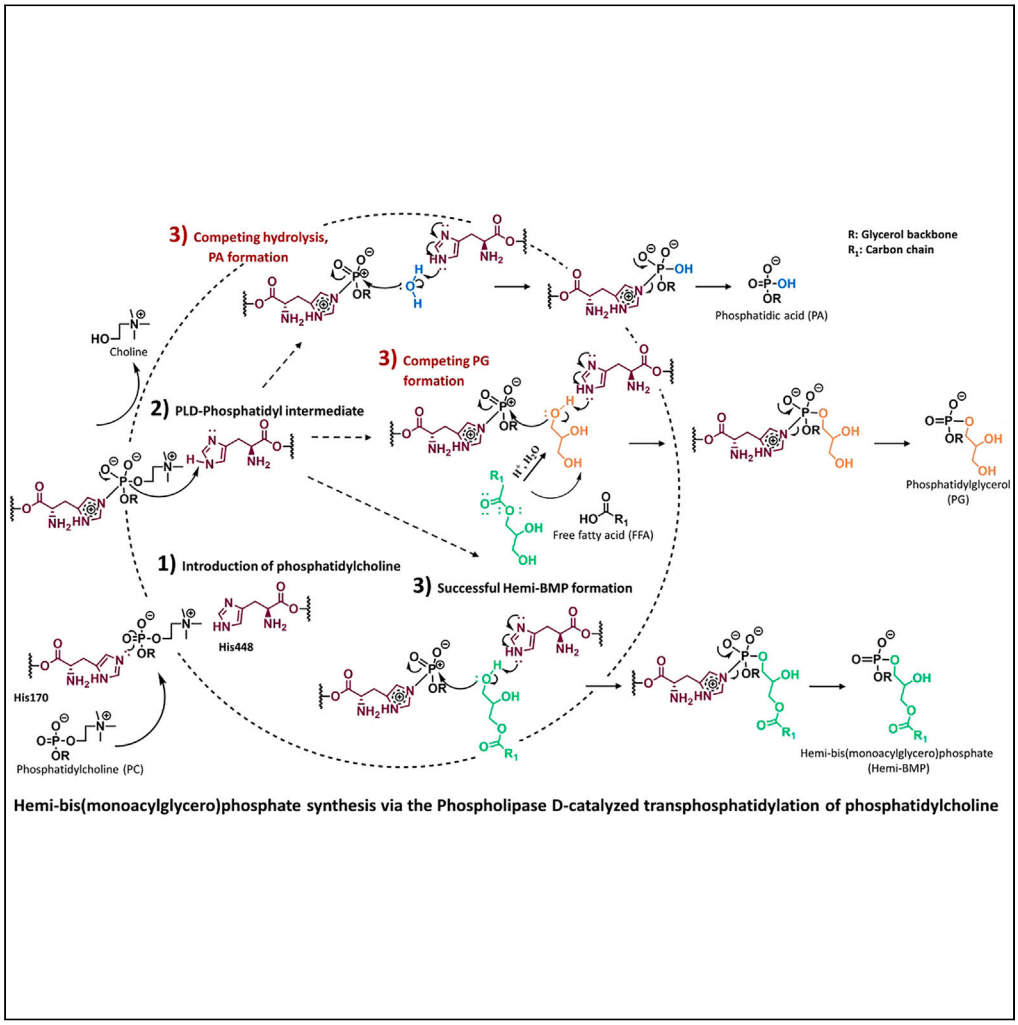

Hemi-bis(monoacylglycero)phosphate synthesis via the Phospholipase D-catalyzed transphosphatidylation of phosphatidylcholine

Oliver Bogojevic, Yan Zhang, Christian Daugaard Wolff, Jens Vinge Nygaard, Lars Wiking, Carl Arevång, Zheng Guo

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Highlights

Phospholipase D-catalyzed transphosphatidylation

Regioselective synthesis of complex phospholipid species

Hemi-bis(monoacylglycero) phosphate and bis(diacylglycero) phosphate formation

Reaction mechanism of phospholipase D

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A sustainable and regioselective synthesis of Hemi-bis(monoacylglycero)phosphates and bis(diacylglycero)phosphates

Oliver Bogojevic,[1] Yan Zhang,[1] Christian Daugaard Wolff,[1] Jens Vinge Nygaard,[1] Lars Wiking,[2] Carl Arevång,[3] and Zheng Guo[1,4,*]

SUMMARY

A sustainable and green approach was developed for the scalable synthesis of uncommon naturally occurring phospholipid species, Hemi-bis(monoacylglycero) phosphates (Hemi-BMPs) and bis(diacylglycero)phosphates (BDPs) via the phospholipase D (PLD) mediated transphosphatidylation. PLD from *Streptomyces sp.* showed great substrate promiscuity for both phospholipids from different biological sources, and alcohol donors with diverse regiochemistry; monoacylglycerols with diverse fatty acyl structures (C12-C22), affording 74–92 wt% yields in 2 h. Experimental results demonstrated that the reaction rate is rather independent of phosphatidyls but to a large extent governed by the size, shape and regiolocation of fatty acyls incorporated on the glycerol backbone, particularly for the regio-isomers of bulky diacylglycerols (*Sn*-1,3 or *Sn*-1,2), which displays great diversity. In addition, a plausible mechanism is proposed based on molecular simulations for an elaborated explanation of the reaction thermodynamic and kinetic favorability toward the synthesis of Hemi-BMPs and BDPs.

INTRODUCTION

Sustainable and environmentally friendly synthesis of phospholipids is today facilitated by numerous diverse approaches, all dependent on the target application, structural character (source), desired purity, etc.[1] Green alternatives for the recovery of isolated phospholipid species are often associated with the exclusion of harsh and toxic chemical catalysts.[2] Instead, sustainable approaches generally focus on biological alternatives, through the use of enzymatic modifications of more common phospholipid species or utilization of the natural metabolic production of phospholipids from various microorganisms.[3,4] Production of phospholipids via the cultivation of microorganisms has recently gained more attention because the new findings in process methods via genetic engineering have paved the way for easier and simpler setups. However, biological related approaches are often limited to the class of the phospholipids produced, as the main contribution of phospholipids is obtained from cellular membranes, which need a certain type of phospholipids to maintain their structural configurations and carry out their biological functions.[5,6] Modifications of more common and easily accessible phospholipid species catalyzed by enzymes offer an alternative green route for the production of an array of specific phospholipid species in high yields and quantities.[7] Phospholipases have traditionally been well utilized to carry out the transformation of either the head groups or the fatty acid residues of phospholipids species, which is a considerably greener process in comparison to traditional chemical synthesis, which often utilizes strong hazardous chemical catalysts such as DIC, DMAP, etc.[8,9]

Phospholipase D (PLD) has traditionally been used for the conversion of the naturally most abundant phospholipid class, phosphatidylcholine (PC), to other less common phospholipids classes, including phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and phosphatidic acid (PA).[10] PLD is an interfacial surface-active enzyme that operates at the interface between an aqueous solution and an organic phase, catalyzing the transphosphatidylation and the reverse hydrolysis of various phospholipid species.[11] The transphosphatidylation enables the substitution of the polar head group, allowing for the production of rare phospholipid species from more common classes. The specificity of PLD can vary depending on the sources, however, the catalytic mechanism is relatively well-conserved among species, containing the HKD-motif (HxK(x4)D(X6GSxN)) mainly responsible for the catalytic reaction.[12] The

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transphosphatidylation is carried out in two sequential steps, with the substitution initiated through the enzyme-phospholipid intermediate complex produced on the interaction with the glycerol backbone of the phospholipid.[13] In the competing hydrolysis, water will act as a nucleophile and initiate the release of the head group and in the presence of a second alcohol donor (primary or secondary alcohols) will the hydroxyl group be acting as a nucleophile and induce the substitution.[12,13] The availability and accessibility of various alcohol donors vary greatly between PLD sources and are primarily dependent on the structure and size of the alcohol donor, as large molecules, such as *myo*-inositol (facilitating the phosphatidylinositol (PI) production), are significantly harder to interact with, commonly yielding low conversions in comparison to other aforementioned species (e.g., PS, PE, or PG).[14–16]

PLD can also facilitate the production of far more rare phospholipid species, including the often overlooked bis(monoacylglycero)phosphates (BMP), hemi-bis(monoacylglycero)phosphates (Hemi-BMP) and bis(diacylglycero)phosphates (BDP). BMP analogs are naturally rare phospholipids that generally account for less than 1% of the total phospholipid content in most cells and tissues of mammalian origin.[17–20] This rare class of phospholipids is primarily identified within the late endosomal compartments of membranes, however, they are also located at higher extents in macrophages and other membrane-bound organelles (<18%).[17,18,21] Recent research suggests that the character of the BMPs plays a key role in the body's innate immune response, which aligns with the encountered higher levels in macrophages.[20] The complex nature of BMP analogs has shown promising potential in numerous different applications, which validates the importance of conducting continuous research. Potential applications for BMPs have been connected to use as biomarkers for the detection of a variety of diseases, including metastatic cancer, lysosomal storage disorders and drug-induced phospholipidosis.[20,22–24] The incorporation of monoacylglycerols (MAGs) or diacylglycerols (DAGs) directly facilitates the PLD-catalyzed transphosphatidylation producing these structural complex classes of phospholipids, which provides new insights and opportunities into the synthesis of phospholipids.[25]

The field related to phospholipid research has recently staggered as the synthetic work surrounding the production usually is challenging and complicated.[26] The amphiphilic character and diverse properties of phospholipids create multiple complex complications for the standardization and development of efficient synthetic routes. The unique ability of phospholipids to align in different secondary structures, such as micelles, liposomes and bilayer structures, creates limitations in solubilization and homogenization, commonly leading to high inaccuracy and misrepresentation on analysis and data collection. Research on efficient synthetic routes for the production of novel and rare phospholipid species is often costly and labor-intensive, as there are few universal analytical methods compatible.[27] The decreasing trend in new scientific publications related to phospholipid synthesis does not align with the increasing growth and demand for phospholipid species on the industrial markets, as phospholipids continuously are essential and vital in numerous different industries; food, pharmaceutical, cosmetic, etc.[28–30] New applications are consistently arising and the demand for isolated and pure phospholipid species with specific structural characteristics is constantly a hot topic within numerous fields, e.g. has the increased availability of neutron scattering facilities advanced the use for deuterated phospholipids species for membrane-related studies.[31]

The covered work established in this paper aims to widen the understanding of the normally challenging and complex synthetic pathway for the production of naturally occurring but rare phospholipid species, facilitating the synthesis of novel phospholipid species (Hemi-BMPs and BDPs) in high purity and medium to excellent yields through an environmentally friendly "green" process. The established procedures and findings will increase the availability of these complex phospholipid species, as well as increase the understanding and utility of the PLD-catalyzed synthesis.

RESULTS AND DISCUSSION

The reaction mechanism of phospholipase D

At the outset of our investigation was the broad substrate scope of PLD exploited for the production of a variety of less common and novel phospholipid species. MAGs with diverse fatty acid profiles (carbon chain lengths and unsaturation levels) were incorporated as alcohol donors for PLD-catalyzed transphosphatidylation (Figure 1). A proposal of the reaction mechanism for the PLD-catalyzed transphosphatidylation with the competing side reactions has been mapped out, presenting the key intermolecular interactions (Figure 1). In the initial step (1) is the phospholipid species introduced to the active site of PLD, generating an enzyme-phosphatidyl intermediate, as intermolecular interactions between the fatty acid residues and the hydrophobic regions of the PLD arise (Figure 1). In the second step (2), is the typical HKD-motif

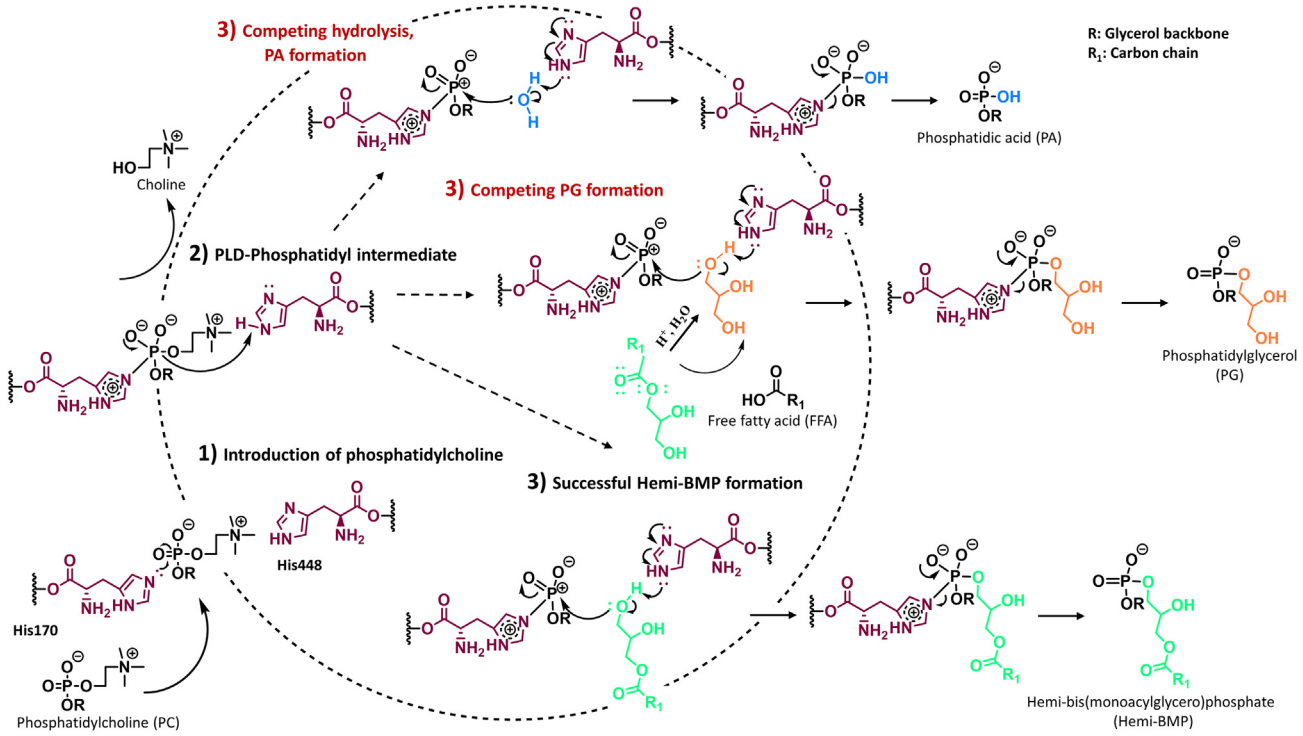

Figure 1. Overview of the mechanism behind the phospholipase D-catalyzed transphosphatidylation for the polar head group substitution

Schematic representation of the structural mechanism of phospholipase D-catalyzed transphosphatidylation of phosphatidylcholine in the formation of Hemi-bis(monoacylglycero)phosphate products from monoacylglycerols.

at the active site assisted by two Histidine residues (His170 and His448), which facilitates the nucleophilic attack responsible for the breakage of the phosphodiester bond and associated protonation, allowing the head group to be released; thereby a phosphatidyl-cation intermediate is generated accordingly (Figure 1). Depending on the character and presence of additional alcohol donors the respective transphosphatidylation will occur, in the presence of MAGs are the two hydroxyl groups of the glycerol backbone available as alcohol donors, facilitating the sequential esterification and thereby formation of Hemi-BMPs (3) (Figure 1). At low levels or in the absence of any additional primary or secondary alcohol donor can water instead act as a nucleophile facilitating the competing hydrolysis producing phosphatidic acid (PA), which can be observed as low levels of PA were identified for most reactions. It is also possible that MAGs can be further hydrolyzed to glycerol via a nucleophilic attack facilitated by a water molecule in the slightly acidic environment of pH 5.6 (Figure 1). The produced glycerol can then be reintroduced to the cycle and yield the production of PG. Low levels of PG can be observed for the optimized process (<5%, wt), as the hydrolysis of the MAGs is thought to be suppressed and far less frequently occurring. To minimize the PA and PG production is it therefore of interest to reduce the required amounts of water, and optimize the presence of the additional alcohol donor (MAG). PLD has previously been reported to have a high affinity and preference for short-chained primary alcohols (1000-folds), which promotes the transphosphatidylation reaction over the hydrolysis.[12]

Homogenization, lipid aggregation, and experimental setup

The amphiphilic properties of phospholipids could yield challenges for representative sample collection and accurate analysis as a consequence of limitations in solubility and homogenization. In the facilitated biphasic reaction system (ethyl acetate and sodium acetate buffer) is PC initially well-dispersed and homogenously distributed, however, in line with the progression of the polar head group substitution and the product formation are larger lipid assemblies observed (Figure 2A). After 30 min, the formation of larger lipid aggregates could be observed for all transphosphatidylation reactions, which raises concern regarding the collection of representable samples and potentially entrapping unreacted PC in lipid clusters limiting and blocking its access to the enzymes (Figure 2A). The polarity of the formed products directly

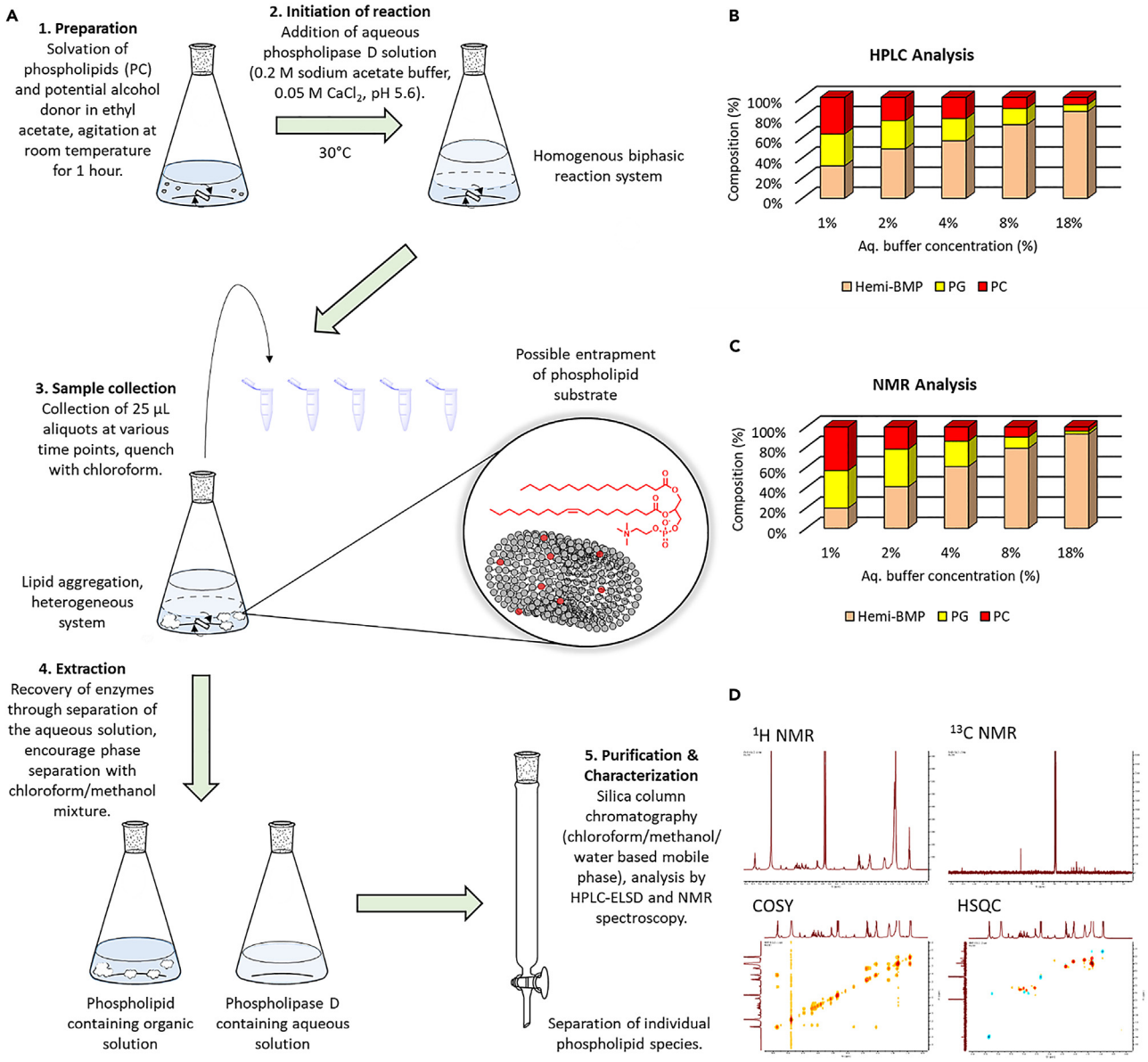

Figure 2. Experimental setup overview

(A) Illustrates the A: experimental setup in the PLD-catalyzed transphosphatidylation/hydrolysis of phosphatidylcholine, highlighting the possible entrapment of starting lipid species.

(B) Displays the average determination of phospholipid composition after the transphosphatidylation of phosphatidylcholine and monoolein with various aqueous concentrations analyzed with HPLC with data are represented as mean ± 5% SEM.

(C and D) Displays the average determination of phospholipid composition after the transphosphatidylation of phosphatidylcholine and monoolein with various aqueous concentrations analyzed with P^{31} NMR with standard deviation under 5% for all tested samples; and D: general NMR characterization of the Hemi-bis(monoacylglycero)phosphate product after the transphosphatidylation of phosphatidylcholine and monoolein (^{1}H NMR, ^{13}C NMR, COSY and HSQC).

affects the solubility in the given system, which potentially could lead to lower yields and product inhibition. The phospholipid's ability to align in supramolecular structures, micelles, liposomes or bilayers, is highly dependent on its chemical structure and the given solvent system.[32] The polarity and amphiphilic properties of the acyl donor had a direct correlation on the aggregation status, as more hydrophobic products tended to produce larger aggregates (saturated and longer carbon chains of the fatty acid residues). The production of larger and less soluble lipid aggregates caused limitations in the selected method of analysis. NMR spectroscopy was efficiently used instead of the developed normal phase-based

HPLC-ELSD system for transphosphatidylation assays with expected highly hydrophobic compounds, such as DAGs and monodocosanoin (Figure 2D). The further possible entrapment of unreacted lipid substrates in the formed lipid aggregates has not been shown to have any significant effect on the diffusion and access to the active site of the enzyme, because previous results show no substantial variation in homogeneous and heterogeneous produced systems.[33] The fact that the experimental setup was carried out on a smaller scale in comparison to the previously developed system by Bogojevic et al., 2022.,[33] and still illustrates the same tendencies in regard to lipid aggregation and formation of heterogeneous systems, further verifies the preference for creating homogeneous systems.[1]

The analytical tools should be able to facilitate the identification and separation of multiple different phospholipid species, as there are many possible outcomes (Hemi-BMP, lyso-PC, PC, PA, PG, lyso-PA, etc.). Based on the findings from the previously published work is it possible to ensure that both the samples collected were representative and that the analytical methods were accurate, the samples were re-dissolved and analyzed on both NMR and HPLC-ELSD and can be seen in the adopted figures (Figures 2B and 2C).[33] A comparison of the analysis results indicates a small variance between the systems with the isolation of individual phospholipid species, presenting an average error margin of 5%, which verifies and accredits the accuracy and reliability of the two analytical methods (Figure 2). Previously reported studies have also utilized High-Performance-TLC (HPTLC) as an analytical tool for BMP detection, which should be of consideration as an alternative.[18] Figures 2B and 2C clearly depicted the dependency of the composition of the products mixture on the hydration state (ratio of aqueous phase) of the reaction system, indicating that sufficient hydration (>18%) resulted in the high conversion of PC (>95%), with high yields of Hemi-BMP (>90%) and small inhibition from hydrolysis of PC (<5%).

Substrate affinity, phospholipid source & alcohol-donor contribution

The substrates accepted by PLD can vary dependent on the specificity of the enzyme, however, also commonly defined by the size and structural character of substrates.[12] The transphosphatidylation requires both a phosphatidyl-containing lipid species and an additional alcohol donor, PLD has previously been shown to accept a wide range of different phosphatidyl-containing substrates (diverse head groups and acyl chains), which facilitates the production of a variety of phospholipid classes.[15] Of interest, the PLD is not able to perform any reactions with lysophosphatidylcholine (lyso-PC) or glycerophosphatidylcholine (GPC) even though they are significantly less sterically hindered, which is suggesting that both the fatty acyls are required and involved in the binding mechanisms (data not shown). The structural character of the acyl chains does although seem to be of less important magnitude as PC from various natural sources (marine, egg and soybean), containing a wide variety of fatty acid composition, all show high affinity (Figures 3A–3C). The presence of two acyl chains allows the phospholipid to align in an orientation that fits the active site of the enzyme, at the same time as the acyl chains potentially are involved in reconfiguration of the PLD substrate (lid-opening), allowing the reaction to take place.[12] The fatty acid composition of PC from three common sources of phospholipids (marine, soybean and egg) was analyzed by GC-FID after respective hydrolysis and methylation, and the related transphosphatidylation suggest an even production of Hemi-BMP products regardless of the fatty acid profiles (Figures 3B and 3C). Polyunsaturated chains, such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), are of far more bulky volume than palmitic acid or oleic acid and are usually associated with large restraints in the formation of enzyme-substrate complex because of their sterical hindrance.[34–36] Of interest, the potential limitations in sterical hindrance did not significantly influence the catalytic activity of PLD; from egg PC to marine PC, the yield of Hemi-BMP only yielded little deduction from 87% to 80% (Table 1). This suggests that the PC-PLD binding step is not the rate-limiting step for the transphosphatidylation reaction (Figure 1).

Instead, higher unsaturation levels of the fatty acids were found to facilitate the homogenization and solubilization of PC in the reaction system. PC containing high levels of unsaturated fatty acids, from marine PC and to some extent soybean, was more in a semi-liquid state to reduce the formation of lipid aggregates. The reduction in the creation of larger lipid aggregates allows phospholipids to stay in a "soluble" state, which increases the rate of transphosphatidylation and eases the sample collection.

Regioselective study

The PLD-catalyzed transphosphatidylation carried out with regioisomerically pure *sn-1* and *sn-2* MAGs showed different preference in yields for the naturally more stable and abundant *sn-1* configuration for all the MAGs tested, e.g. showing total Hemi-BMP yields of 91% with 1-monomyristin and 78% with

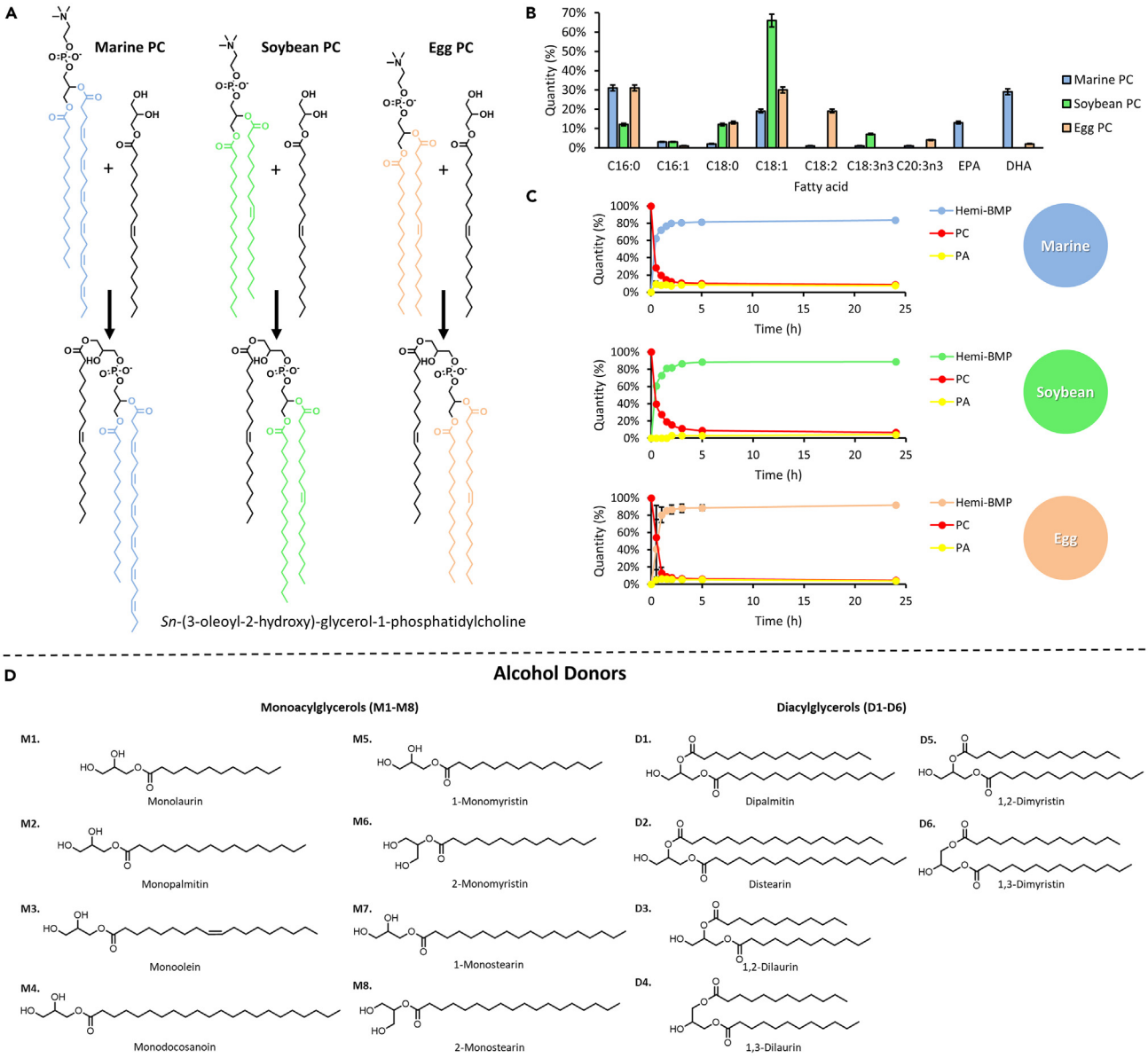

Figure 3. Substrate screening of phosphatidylcholine of various sources

(A) Illustrates A: the model reaction scheme for the PLD-catalyzed transphosphatidylation of PC from various sources (marine-blue, soybean-green and egg-orange) with monoolein.

(B) The associated fatty acid composition of PC from the various sources (marine-blue, soybean-green and egg- orange) analyzed through sequential hydrolysis of fatty acids and associated methylation prior to GC-FID analysis (data are represented as mean ± 5% SEM).

(C and D) NMR analysis of the PLD-catalyzed transphosphatidylation of PC from various sources (marine-blue, soybean-green and egg- orange) with monoolein, and D: substrate screening scope with the structures of the respective alcohol donors, with the division of monoacylglycerols (M1-M8) and diacylglycerols (D1-D6).

2-monomyristin respectively (Table 1). The results align well with the previously observed yields for the racemic mixtures, as MAGs are expected to prominently exist in the *sn-1* configuration (naturally abundant to a ratio of 1:9 at equilibrium).[37,38] The increased affinity for more open *sn-1* isomers follows the logical reasoning surrounding the less sterically hindrance and open access toward the free hydroxyl chains on the glycerol backbone. Of interest, the transphosphatidylation carried out with *sn-2* regioisomerically pure MAGs also displayed significant levels of Hemi-BMPs with fatty acids located on the *sn-1* position of the glycerol backbone, revealed as doubled signals on the phosphorus NMR analysis (supplemental

Table 1. Hemi-bis(monoacylglycero)phosphate and Bis(diacylglycero)phosphate production

Entry	Phospholipid species	Phospholipid source	Alcohol Donor	Product	Yield (%)
1	PC	Marine	Monoolein (Racemic mixture)	Hemi-BMP	80
2	PC	Soybean	Monoolein (Racemic mixture)	Hemi-BMP	82
3	PC	Egg	Monoolein (Racemic mixture)	Hemi-BMP	87
4	PC	Egg	Monolaurin (Racemic mixture)	Hemi-BMP	92
5	PC	Egg	Monopalmitin (Racemic mixture)	Hemi-BMP	91
6	PC	Egg	Monodocosaoin (Racemic mixture)	Hemi-BMP	74
7	PC	Egg	Monomyristin (*Sn-1* enantiomer)	Hemi-BMP	91
8	PC	Egg	Monomyristin (*Sn-2* enantiomer)	Hemi-BMP	78
9	PC	Egg	Monostearin (*Sn-1* enantiomer)	Hemi-BMP	89
10	PC	Egg	Monostearin (*Sn-2* enantiomer)	Hemi-BMP	81
11	PC	Egg	Dipalmitin (Racemic mixture)	BDP	13
12	PC	Egg	Distearin (Racemic mixture)	BDP	8
13	PC	Egg	Dilaurin (*Sn-1,2* enantiomer)	BDP	33
14	PC	Egg	Dilaurin (*Sn-1,3* enantiomer)	BDP	0
15	PC	Egg	Dimyristin (*Sn-1,2* enantiomer)	BDP	40
16	PC	Egg	Dimyristin (*Sn-1,3* enantiomer)	BDP	0

Presents the results from the transphosphatidylation with varied alcohol donors and phospholipid species from various sources. All transphosphatidylation was run for 2 h and carried out at temperatures of 30°C, substrate ratio of PC:alcohol donor (1:5), enzyme loading (5U) and aqueous concentration (18% v/v). Data are represented as mean ± 5% SEM.

information, Figure S10). The acyl chains of MAGs and DAGs primarily located on the second position of the glycerol backbone can spontaneously migrate to the more stable and robust endpoints of the glycerol backbone, hence producing the observed mixture of the two isomers of Hemi-BMP. The quantities of 1′-Hemi-BMPs and 2′-Hemi-BMPs observed for the transphosphatidylation with 2-MAGs are consistent at 54% 1′-Hemi-BMPs and 46% 2′-Hemi-BMPs respectively regardless of the character of the selected MAGs (2-monomyristin or 2-monostearin), which are in accordance with the theory regarding the acyl migration rate of MAGs with similar carbon chain lengths (supplemental information, Figure S10).[38]

The transphosphatidylation with regioisomerically pure DAGs further confirmed the proposed reaction mechanism, with PLD solely showing affinity toward *sn-1,2* specific DAGs (33% BDP formation with 1,2-dilaurin and 40% BDP formation with 1,2-dimyristin) (Table 1). The positions of the acyl chains on the glycerol backbone of DAGs have a significantly larger impact on the availability and accessibility of the free hydroxyl group in comparison to MAGs, which clearly are unveiled as the PLD lack affinity toward any tested 1,3-DAGs (Table 1). The transphosphatidylation of the racemic mixtures of DAGs presents a significantly lower amount of BDP products, as the composition of the regioisomers primarily is constituted of 1,3-DAG isomers (66%).[39] The results align well with previous findings and further conclude that the carbon chain length of the acyl chains residues is of minor impact in relation to the position on the glycerol backbone. The DAG-facilitated transphosphatidylation also presents elevated levels of PA and Hemi-BMPs, which further confirms and supports the proposed reaction mechanism (Figure 1). A lower substrate affinity enables water to act as a nucleophile and hence promote the formation of PA, which could be observed

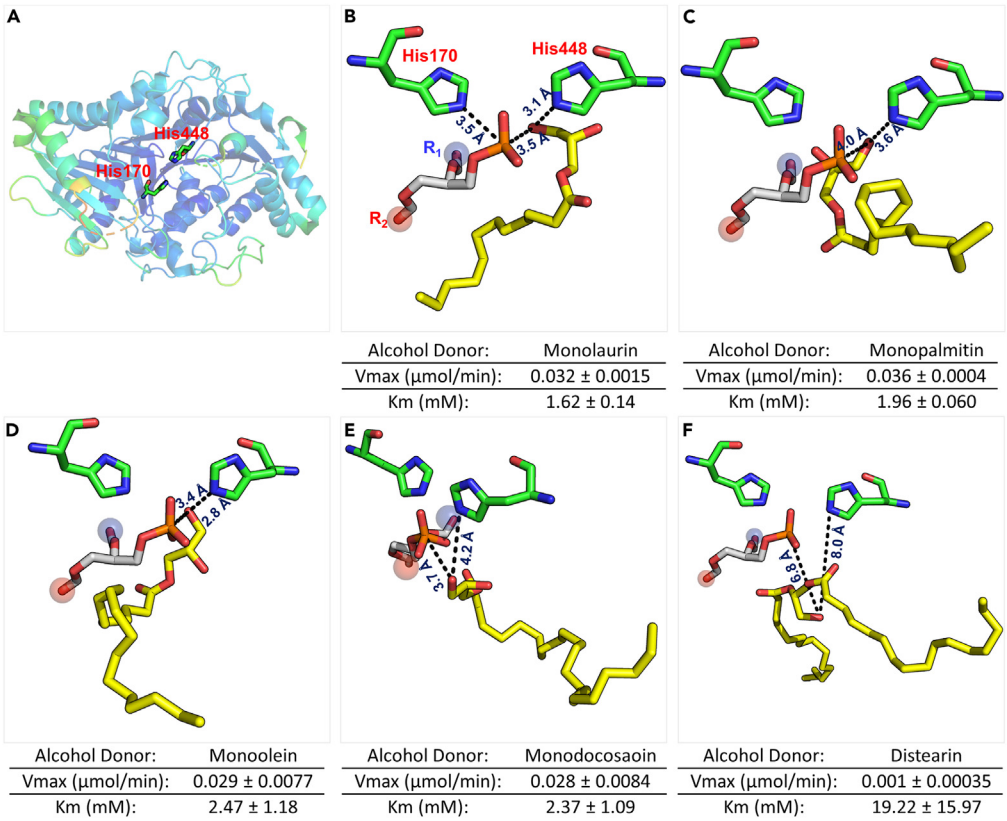

Figure 4. Overview substrate affinity docking models

Illustrates the substrate affinity and docking models of phospholipase D for 1-palmitoyl-2-oleoyl-*sn*-glycero-phosphatidylcholine (POPC) and various alcohol donors (racemic mixtures), with the side chains of the POPC excluded, R_1 (blue) and R_2 (red). Vmax and Km are presented with the binding distances between the phosphate and the specific alcohol donor, in regard to the catalytic active histidine groups (His170 and His 448).

(A) Overall ribbon structure of phospholipase D (Streptomyces *sp.*).

(B) Monolaurin.

(C) Monopalmitin.

(D) Monoolein.

(E and F) Monodocosaoin and F: Distearin.

for all DAGs-facilitated reactions. Based on the same principles associated with the PG formation can the PLD-catalyzed transphosphatidylation with DAGs promote the formation of Hemi-BMPs, as the slightly acidic pH of 5.6 facilitates the hydrolysis of the acyl chains of DAGs producing MAGs, which sequentially enables the formation of Hemi-BMPs (supplemental information, Figure S12).

Model docking and enzyme kinetics

The synthesis of Hemi-BMP analogs was carried out through enzymatic incorporation of MAGs with diverse fatty acid profiles. Similar to variations in the fatty acid composition of the phospholipid-containing residue is the character of the acyl chain length of the MAGs of minor significance (Table 1). By varying the acyl chain length from C12-C22 and by alternating the unsaturation levels could similar results be observed for the Hemi-BMP production, with yields between 74 and 92% (Table 1). Monodocosaoin (M4) presented the lowest yields (74%) of all the MAGs from regioisomer mixtures and regioisomerically pure isomer mixtures, even though the Km value were similar to the other MAGs (Figure 4 and Table 1). The similar Km values propose that there are no significant differences in the affinity of PLD for the various MAGs, suggesting that even longer hydrophobic carbon chains (C22) still provide no significant sterical hindrance. The transphosphatidylation with monodocosaoin still presents a slight decrease in Hemi-BMP levels, which could be correlated with its significantly lower solubility. Longer saturated carbon chains enable additional intramolecular van der Waals interactions between multiple chains, which not only create more rigid secondary configurations but also decrease its

aqueous solubility. Monodocosaoin was not completely dissolved which could explain the lower yields, instead slightly higher concentrations of PA were observed as the water was acting as a nucleophile in the competing hydrolysis, similar to what was observed for the transphosphatidylation with DAGs.

More challenge was met for the attempts to incorporate DAGs, the yield of the respective BDPs was far lower, especially for the regioisomser mixtures (Table 1). The respective Km-values were also higher compared to the MAGs, suggesting that substrates containing two acyl chains have a much lower affinity which is more likely because of the stronger steric hindrance of substrate diglycerides (Figure 4). The interfacial surface activation mechanism of PLD favors a maximum intersurface area in the biphasic system, which could be achieved under the optimized conditions. The lower affinity for DAGs favors the competing hydrolysis and thereby requires reduced levels of the aqueous solution (data not shown). A much greater Vmax for MAGs compared to DAGs also suggestively indicates that PLD-catalyzed transphosphatidylation might not be an appropriate method for the synthesis of BDP. The enzymatic kinetics were analyzed and carried out on a racemic mixture of the MAGs and DAGs, in which the most abundant configuration naturally will be *sn-1* MAGs and *sn-1,3* DAGs respectively. It should be safe to say that the Vmax and Km-values most certainly would present significantly more favorable values with higher activities for DAGs with the *sn-1,2* isomerization, however, these assays are significantly harder to monitor as the acyl migration spontaneously will facilitate the re-isomerization into the more stable configuration. The kinetic models in combination with the substrate screening still facilitate the impact of the acyl chain lengths on the alcohol donors, further confirming the proposed reaction mechanism.

The docking models of the different substrates plausibly emphasize and explain the experimental results (Figure 4). The distance between the phosphatidyl-cation intermediate and the incorporated alcohol donor in MAG (3.4–4.0 Å), and the distance between PLD catalytic site His-448 and alcohol donor (2.8–4.2 Å) (Figures 4B–4E) suggests only small variations and low dependencies on the character of the fatty acyl in MAG alcohol donors. However, the distance for DAG distearin (racemic mixture) to phosphatidyl-cation intermediate (6.8 Å) and to His-448 (8.0 Å) is significantly larger (Figure 4F). The observed results illustrate and emphasize the thermodynamic favorability for the synthesis of Hemi-BMPs with diverse fatty acid compositions and unfavorability for the PLD-catalyzed synthesis of BDPs.

The work covered in this article presented a sustainable and efficient approach for the synthesis of a variety of rare Hemi-BMP and BDP analogs using phosphatidylcholines from various sources, highlighting the limitations and challenging aspects of the synthesis. A scalable synthesis of this kind of naturally occurring but hardly obtainable rare phospholipids will create an extraordinary opportunity for diagnostic, clinical or medical study by a facile and sustainable production using the method developed in this study.

Limitations of the study

The main drawbacks and limitations of the designed system are aligned with the compatibility between the structural unique compounds and the analytical tools. The solubility of lipid species with longer hydrophobic carbon chains is significantly lower in the designed biphasic system, which puts limitations on both the continuous analysis and achievable yields. In addition, the docking models of phospholipase D in various combinations in the performed transphosphatidylation are crude, because of the lack of accurate crystallographic data.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
- METHOD DETAILS
  - Transphosphatidylation of phosphatidylcholine
  - Quantitative analysis of Hemi-BMP products by high-performance liquid chromatography (HPLC)
  - Structural characterization and quantitative analysis of Hemi-BMP and BDP products with nuclear magnetic resonance spectroscopy (NMR)

- Purification of Hemi-BMP products
- Purification of marine-based phosphatidylcholine
- Fatty acid compositional analysis via respective hydrolysis and methylation prior to gas chromatography flame ionization detector (GC-FID) analysis
- Molecular docking and structural analysis
- Enzyme kinetics

- QUANTIFICATION AND STATISTICAL ANALYSIS
  - Statistical analysis

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.107075.

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AUTHOR CONTRIBUTIONS

O.B.: Conceptualization, Methodology, Validation, Visualization, Writing- original draft, Writing – review & editing. Y.Z.: Formal analysis, Validation, Visualization, Writing – original draft. C.D.W.: Investigation. J.V.N.: Supervision. L.W.: Supervision. C.A.: Funding acquisition, Supervision. Z.G.: Conceptualization, Methodology, Project administration, Supervision, Writing – review and editing.

DECLARATION OF INTERESTS

There are no conflicts to declare.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Phospholipase D from *Streptomyces* sp.	Nagase & CO., LTD, Japan.	EC No.: 232-639-8 (9001-87-0)
Egg L-α-phosphatidylcholine (>98%)	Larodan	Cas # 97281-44-2
Soybean L-α-phosphatidylcholine (>98%)	Larodan	Cas # 97281-47-5
Marine-based phosphatidylcholine	TripleNine	Cat # 999MPL40
1-Monomyristin	Larodan	Cas # 589-68-4
2-Monomyristin	Larodan	Cas # 3443-83-2
1-Monostearin	Larodan	Cas # 123-94-4
2-Monostearin	Larodan	Cas # 621-61-4
1,2-Dilaurin	Larodan	Cas # 17598-94-6
1,3-Dilaurin	Larodan	Cas # 539-93-5
1,2-Dimyristin	Larodan	Cas # 20255-94-1
1,3-Dimyristin	Larodan	Cas # 7770-09-4
Monolaurin (Racemic mixture)	Dupont Danisco	Cas # 27215-38-9
Monopalmitin (Racemic mixture)	Dupont Danisco	Cas # 26657-96-5
Monoolein (Racemic mixture)	Dupont Danisco	Cas # 25496-72-4
Distearin (Racemic mixture)	Dupont Danisco	Cas # 1323-83-7
Monodocosanoin- Compritol®888	Gattefosse	Cas # 30233-64-8
1,2-dipalmitoyl-sn-glycero-3-phosphatidic acid disodium salt	BACHEM	Cas # 169051-60-9
(*sn-1,2/sn-1,3*) dipalmitin	Sigma Aldrich	Cas # 26657-95-4
1,2-palmitoyl-sn-glycero-3-phosphor-rac-(1-glycerol) ammonium salt	Sigma Aldrich	Cas # 200880-41-7
Supelco 37 component FAME mix	Sigma Aldrich	Cas # 75-09-2
Chloroform	Sigma Aldrich	Cas # 67-66-3
Methanol	Sigma Aldrich	Cas # 67-56-1
Ethanol	Sigma Aldrich	Cas # 64-17-5
Acetic acid	Sigma Aldrich	Cas # 64-19-7
Ethyl acetate	Sigma Aldrich	Cas # 141-78-6
Sodium acetate	Sigma Aldrich	Cas # 127-09-3
Heptane	Sigma Aldrich	Cas # 142-82-5
Methanolic sodium hydroxide	Sigma Aldrich	Cas # 1310-73-2
Boron triflouride	Sigma Aldrich	Cas # 7637-07-2
Hydroquinone	Sigma Aldrich	Cas # 123-31-9
d1-Chloroform	Sigma Aldrich	Cas # 865-49-6
d4-Methanol	Sigma Aldrich	Cas # 811-98-3
Sodium chloride	Sigma Aldrich	Cas # 7440-23-5
Calcium chloride	Sigma Aldrich	Cas # 7440-70-2
Potassium carbonate	Sigma Aldrich	Cas # 584-08-7
TIBP	Sigma Aldrich	Cas # 126-71-6
Cesium hydroxide monohydrate	Thermo Fisher -Scientific	Cas # 35103-70-8
Trans-1,2-Cyclohexanediaminetetraacetic acid monohydrate	Thermo Fisher - Scientific	Cas # 125572-95-4

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
Pymol	LeDock Program	Phospholipase D - PDB ID: 1V0S
ChemDraw Professional 19.1	PerkinElmer	https://www.perkinelmer.com/category/chemdraw
Other		
Scion436-GC	Bruker	https://www.bruker.com/en.html
Bruker Avance III Spectrometer	Bruker	https://www.bruker.com/en.html
Agilent HPLC- 1260 infinity) equipped with an evaporative light scattering detector (ELSD) (Varian 385-LC)	Agilent Technologies	https://www.agilent.com/en/product/liquid-chromatography/hplc-systems/analytical-hplc-systems

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Zheng Guo, (guo@bce.au.dk).

Materials availability

All the phospholipids species produced in this study are available directly from the authors.

This study did not produce any additional unique reagents.

Data and code availability

- The published article includes all datasets generated or analyzed during this study.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

This study does not use experimental models.

METHOD DETAILS

Transphosphatidylation of phosphatidylcholine

The transphosphatidylation were developed and carried out with slight modifications from a previously established method.[33] 0.031 mmol of PC (egg yolk, soybean, and marine oil) and 0.163 mmol of respective alcohol donors (monoacylglycerols and diacylglycerols) were dissolved in 1.125 ml of ethyl acetate at room temperature. The enzyme loading was defined in terms of Unit (U), in which one U will liberate 1.0 μmol of choline from L-α-phosphatidylcholine per hour at pH 5.6 and at 30°C. 0,25 ml of sodium acetate buffer (0.2 M, 0.05 M $CaCl_2$, pH 5.6) was used to dissolve phospholipase D, yielding a total content of 5 U. The reaction was then initiated by mixing the aqueous buffer with the organic solution at 600 RPM in a water bath at 30°C. The reaction was quenched after 2 hours by the addition of 3 ml chloroform and followed by drying in nitrogen prior to HPLC and NMR analysis. In the case of time-course evaluation for HPLC analysis, aliquots of 0,25 μL were collected and quenched through the addition of 25 μL chloroform prior to the analysis.

Quantitative analysis of Hemi-BMP products by high-performance liquid chromatography (HPLC)

The quantitative analysis of the various synthesized Hemi-BMP analogs was carried out by the use of high-performance liquid chromatography (HPLC) (Agilent Technologies, 1260 infinity) equipped with an evaporative light scattering detector (ELSD) (Varian 385-LC). The separation was carried out in a normal

phase-based system, using a silica gel column (Zorbax RX-SIL, 4.6 × 250 mm, 5 μm). The designed system used two different eluents, eluent A (90/10- chloroform/methanol, v/v) and eluent B (65/25/4- chloroform/methanol/water, v/v/v). The chromatographic separation was conducted with a linear gradient: 0–10 min eluent A with a decrease in flow rate from 1 ml/min to 0.5 ml/min (which was constant for the remaining separation), 10–11 min eluent A-B, 11–25 min eluent B, 25–26 eluent B-A and 26–35 re-equilibration. The conditions of the ELSD were set at; evaporation temperature (50°C), nebulizer temperature (50°C), and gas flow (1.05 SLM). Egg PC and the purified Hemi-BMP (monoolein with egg PC) were used to construct calibration curves, which were further used for the quantitative assessment of the different products (mol/L%). All samples were performed in duplicates and reported as mean values. The transphosphatidylation performed with the different monoacylglycerols (except monodocosaoin) was analyzed with HPLC, while the other samples were confirmed with phosphorous NMR due to solubility issues.

Structural characterization and quantitative analysis of Hemi-BMP and BDP products with nuclear magnetic resonance spectroscopy (NMR)

The structural characterization of the Hemi-BMP and BDP products were carried out by ^{31}P NMR analysis, identifying the chemical shift of the unique phospholipid specie. Purified and characterized Hemi-BMP product (PC from egg yolk and monoolein) was characterized and utilized as standards for the products with various monoacylglycerols. The chemical shift of the BDPs was in alignment with the expected chemical shifts observed from the additional acyl chains, (similar to GPC, lyso-PC and PC). The NMR analysis was carried out on a Bruker Avance III Spectrometer operating at 400 MHz for ^{1}H, 100 MHz for ^{13}C, and 162 MHz for ^{31}P isotopes and referenced to respective residual solvent signals. All experiments were carried out at either 298 K or 300 K, with various numbers of transients per increment depending on the concentration of the sample (1–20 mg/ml). The characterization with ^{1}H, ^{13}C, COSY and HSQC were all performed in d6-methanol while respective ^{31}P measurements used a solvent mixture of d3-chloroform/methanol/CsCDTA (0.5/0.4/0.1, v/v/v) with triisobutyl phosphate (TIBP) used as an internal standard. Data interpretation and processing were further carried out through the use of the MestReNova software.

Purification of Hemi-BMP products

The produced Hemi-BMP products were purified using silica column chromatography with a developing solvent consisting of chloroform/methanol/water (65/25/4, v/v/v) and Hanessian's stain as the visualizing agent. The Hemi-BMP fractions were combined and the solvents were removed through evaporation under reduced pressure (rotary evaporator), followed by extensive characterization on NMR. 28.5 mg of the Hemi-BMP product from the model reaction was recovered with a purity of <95%.

Purification of marine-based phosphatidylcholine

32 g of phospholipid-concentrated fish oil and 400 ml of ethanol were stirred in an incubator for 3 h at 160 RPM. The solution was then transferred into 50 ml centrifugation tubes and centrifuged at 4000 RPM for 10 minutes at 15°C. The supernatant was recovered and the ethanol was evaporated through the use of a rotary evaporator. The dried lipid fraction was in multiple fractions dissolved in a chloroform/methanol/acetic acid (60/15/6, v/v/v) solution before being separated by silica column chromatography. The aforementioned solvent solution was used as the developing solvent and the column dimensions were set at: a height of 7 cm (wet loading) and a width of 5 cm. The purification was monitored through TLC using Hanessian's stain as the visualizing agent. The purified fractions were further dried under reduced pressure and the products were weighted and analyzed with NMR. 2.28 g of the marine PC was recovered with a purity of >95%.

Fatty acid compositional analysis via respective hydrolysis and methylation prior to gas chromatography flame ionization detector (GC-FID) analysis

The fatty acid compositional analysis was carried out with slight modifications of the American Oil Chemists' Society's (AOCS) Official Methods – Ce 2–66 (Reapp. 1997) and Ce 1e-91 (Rev. 2001). The analysis was carried out with a Scion436-GC (Bruker, Billerica, MA) system equipped with a fused-silica capillary column: SP-2380 (60 m × 0.25 mm x 0.2μm film thickness) with a constant column flow at a pressure of 22 (Psi) and helium as the carrier gas. Approximately 30 mg of phosphatidylcholine from soybean, egg yolk and purified phosphatidylcholine content from marine oil were dissolved with 0.7 ml of (0.5 M) methanolic sodium hydroxide and heated at 80°C for 5 minutes. 0.7 ml of 20% boron trifluoride was then additionally added together with 0.1 ml of 0.1% hydroquinone and once again heated to 80°C for 2 min. 1 ml of heptane

was then added and the solution was vigorously shaken, followed by the addition of 0.5 ml of saturated NaCl + K_2CO_3 solution, yielding the produced Fatty Acid Methyl Esters (FAME). 0.4 ml of the FAME solution (upper layer) was then utilized for the GC-FID analysis. The GC-FID followed the AOCS official method Ce 2–66, with analysis of 100 μL prepared solutions, the FID detector was set to 250°C, with a start oven temperature of 80°C and a hold temperature of 1 min. The heat rate was then set at 20°C/min until reached 125°C with 1 min hold time. The following increase was done at 5°C/min until a temperature of 240°C was achieved. All the FAMEs were eluted after 40 min. The yielding signals were integrated and aligned to the Supelco 37-component FAME standard mix and pure FAME of cis-4-7-10-13-16-19 docosahexaenoic acid (DHA) standard.

Molecular docking and structural analysis

Ligands were docked into Phospholipase D by using LeDock program with the default parameters.[40,41] The central coordinates of the grid box were identified by using GetBox Plugin in PyMOL. The docking modes were also visualized and analyzed in PyMOL. According to the binding affinities and conformations, the best binding mode was picked from 20 resulting docking modes.

Enzyme kinetics

1,30 μmol of PC from egg yolk was mixed with varying concentrations of monolaurin, monopalmitin, monoolein, monodocosaoin and distearin (all in racemic mixtures). The Michaelis Menten kinetic model was utilized to establish Lineweaver Burk-plots for estimations of Vmax (μmol/min) and Km (mM). All measurements were carried out in duplicates.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis

Throughout the entire manuscript were all experiments replicated and conducted in duplicates, if not stated otherwise. The Standard Error of Mean (SEM) and Standard Deviation (SD) were calculated and accounted for during all the assays, with a p value set at 0.05. The figures show individual replicate signals as mean values, with associated error bars. All statistical computational tasks were carried out using Excel.