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Synthesis and evaluation of immunostimulant plasmalogen lysophosphatidylethanolamine and analogues for natural killer T cells

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ABSTRACT

Plasmalogen lysophosphatidylethanolamine (pLPE) had been identified as a self antigen for natural killer T cells (NKT cells). It is very important in the development, maturation and activation of NKT cells in thymus. Besides, pLPE is a novel type of antigen for NKT cells. To evaluate the structure–activity relationship (SAR) of this new antigen, pLPE and its analogues referred to different aliphatic chains and linkages at the sn-1 position of the glycerol backbone were synthesized, and the biological activities of these analogues was characterized. It is discovered that the linkages between phosphate and lipid moiety are not important for the antigens' activities. The pLPE analogues **1**, **3**, **4**, **7** and **9**, which have additional double bonds on lipid parts, were identified as new NKT agonists. Moreover, the analogues **4**, **7** and **9** were discovered as potent Th2 activators for NKT cells.

1. Introduction

Natural killer T cells (NKT cells) are specific subpopulations of T lymphocytes, which express both conserved semi-invariant T cell receptors (TCR) and natural killer cell (NK) receptors.[1–3] The NKT cells regulate a broad range of immune responses, and have been recognized as potentially significant factor in the diagnosis and treatment of many human diseases.[4] The NKT cells can be stimulated by the lipid antigens which are presented by major histocompatibility complex class-I-like protein CD1d through TCR. After being activated, NKT cells secrete both T helper 1 (Th1) and Th2 types of cytokines. Th1 type cytokines such as interferon (IFN)-γ produce the proinflammatory response, and mediate protective immune functions like tumor rejection and microbial infection; whereas Th2 type cytokines such as IL-4 and IL-10, mediate regulatory immune functions to ameliorate autoimmune diseases.[5–8]

Until now, several lipid antigens involved in the activation of NKT cells have been identified (Fig. 1). Alpha-galactosylceramide (α-GalCer) is the first and most potent agonist of NKT cells, which was derived by medicinal chemistry from agelsaphin, a glycolipid found in the marine sponge Agelas mauritianus.[9,10] α-GalCer presented by CD1d will activate NKT cells by means of TCR recognition to produce Th1 and Th2 cytokines. However, the efficacy of α-GalCer has been largely limited because of the reciprocal inhibition exhibited by Th1 and Th2 cytokines.[11,12] Therefore, compounds that can increase the selectivity toward either Th1 or Th2 cytokines responses may be more advantageous. Since the discovery of α-GalCer, numerous analogues have been developed to demonstrate their structure–activity relationships (SAR).[13–22] Some of these analogues showed Th1- or Th2-biased cytokine production as compared to α-GalCer. OCH, of which the sphingosine chain is substantially shorter than that of α-GalCer (Fig. 1), stimulate NKT cells to produce many more Th2 cytokines than Th1 cytokines.[23] On the contrast, C20:2 which contains two double bonds in the acyl chain can activate NKT cells to preferentially produce Th1 cytokines and induced cellular proliferation.[24]

Recognition of self antigens is very important for the development, maturation and activation of NKT cells in thymus. Facciotti et al. extracted lipids from mouse thymus and fractionated them base on their polarities,[25,26] and then evaluated the activity on stimulating NKT cells in vitro. The active lipid fractions were identified to be 1-*O*-1′-(*Z*)-hexadecenyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine (plasmalogen C16-lysophosphatidylethanolamine, pLPE) (Fig. 1), which is a metabolic product of the peroxisomal enzyme glyceronephosphate *O*-acyltransferase (GNPAT). The mice deficient in the enzyme GNPAT which lack of pLPE had fewer total NKT cells and significant alteration of the

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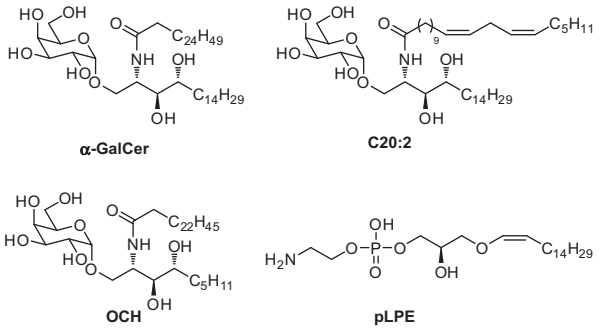


Figure 1. Structures of α-GalCer, OCH, C20:2 and pLPE.

thymic maturation of NKT cells. Thus, pLPE is a self-antigen that not only stimulates the activation of NKT cells but also is required for the generation of a full NKT cell repertoire in vivo.

Unlike α-GalCer and its analogues, which are glycosphingolipids, pLPE is a mono-alkyl glycerophosphate. To the best of our knowledge, few analogues of pLPE have been synthesized to explore its structure–activity relationship (SAR) on NKT cells. Herein, we report the synthesis of pLPE and its analogues **1**–**9** (Fig. 2) and evaluated their associated stimulatory activity on NKT cells. To explore the most efficient linkage at the sn-1 position of the glycerol backbone, we synthesized the analogues which contain different aliphatic chains at the sn-1 position. **1** contains a vinyl ether bond linking the sn-1 aliphatic chain to the glycerol backbone, while an alkynyl ether linkage contained in **2**, **3** and **4**; as well as saturated ether linkage contained in **5**–**7**; and an ester linkage contained in **8** and **9**. Moreover, the aliphatic chains of **1**, **3**, **4**, **7** and **9** were designed to contain one or more carbon–carbon double bonds, since compound C20:2 has two additional double bonds and showed excellent Th1 selectivity. The biological evaluation of these analogues would explore the SAR of linkages, and length and unsaturated bonds of lipid parts.

2. Results and discussion

2.1. Synthesis

The pLPE and all the analogues were initiated with commercially available 2,3-*O*-isopropylidene-*sn*-glycerol. The methodologies to prepare pLPE and analogues **1**–**4** are outlined in Scheme 1. Treatment of 2,3-*O*-isopropylidene-*sn*-glycerol with potassium hydride and trichloroethylene in THF followed by deacetonation with *p*-toluenesulfonic acid in MeOH gave **10** in 96% yield. The two hydroxyl groups were protected by *tert*-butyldimethylsilanyl (TBS) groups to yield **11**. Reaction of dichloroethenyl **11** with *n*-butyllithium and aliphatic iodide, and then selectively removal of the silyl protecting group on the primary hydroxyl with hydrogen fluoride–pyridine complex, provided the corresponding *O*-alkynyl ether **12a**–**c**. To prepare vinyl ether linked pLPE and analogue **1**, the alkynes **12a** and **12b** were converted to *cis*-enol ethers **13a** and **13b** by partial hydrogenation with Lindlar catalyst in hexane/EtOAc (1:1) in the presence of quinoline. Finally, the reaction of alcohols **13a** and **13b** with phosphorus oxychloride in the presence of triethylamine and pyridine in dichloromethane, followed by trapping of *N*-(monomethoxytrityl)-ethanolamine, and subsequently desilyation in the presence of hydrogen fluoride–pyridine to afford **pLPE** and analogue **1**. The preparation of alkynyl ether linked pLPE analogues **2**–**4** were achieved by directly phosphorylation of alcohols **12a**–**c** with phosphorus oxychloride and trapping with *N*-(monomethoxytrityl)-ethanolamine, and finally removal of the TBS protecting group by hydrogen fluoride–pyridine.

The analogues **5**–**9** were synthesized as shown in Scheme 2. For the alkyl ether linked analogues **5**–**7**, the 2,3-*O*-isopropylidene-*sn*-glycerol was treated with an excess of *n*-alkyl bromide under phase-transfer conditions (50% aqueous NaOH, tetrabutylammonium bromide) at 80 °C, and the isopropylidene protective group was then removed yielding **14a**–**14c**. The diols (**14a**–**14c**) were protected with TBS groups and then selectively deprotected at the primary position in the presence of hydrogen fluoride–pyridine to produce **15a**–**15c**. The reaction of alcohol **15a**–**15c** with phosphorus oxychloride in the presence of triethylamine and pyridine in dichloromethane, followed by trapping of *N*-(monomethoxytrityl)-ethanolamine, and subsequently desilyation in the presence of hydrogen fluoride–pyridine to afford analogues **5**–**7**. On another hand, the ester linked analogues **8** and **9** were generated by firstly acylated with acyl chloride (for **14d**) or acid (for **14e**) and then following the same procedures as which for analogues **5**–**7**.

2.2. Immunological assay

The biological activity of pLPE analogues as NKT agonists was preliminarily assayed in vitro by measuring the production of Interleukin-2 (IL-2) of mouse NKT cell hybridoma 2H4. The analogues were presented to stimulate 2H4 cell directly by surface-bound CD1d protein in the plate-bound CD1d presenting assay. As depicted in Figure 3, analogues **1**, **3**, **4**, **7** and **9** induced obvious IL-2 production. The results illustrated that they had the ability to activate NKT cells directly and thus caused the release of IL-2 cytokines.

As a functional assay, we further cultured whole spleen cells in the presence of indicated immunostimulant (**1**, **3**, **4**, **7** and **9**) or vehicle and determined the production of Th1 and Th2 prototypic cytokines, Interferon-γ (IFN-γ) and Interleukin- 4 (IL-4) by ELISA at 72 h of culture. We found analogues **4**, **7** and **9** induced a significantly greater secretion of IL-4 than pLPE, whereas all analogues elicited slightly less IFN-γ than pLPE (Fig. 4A). When

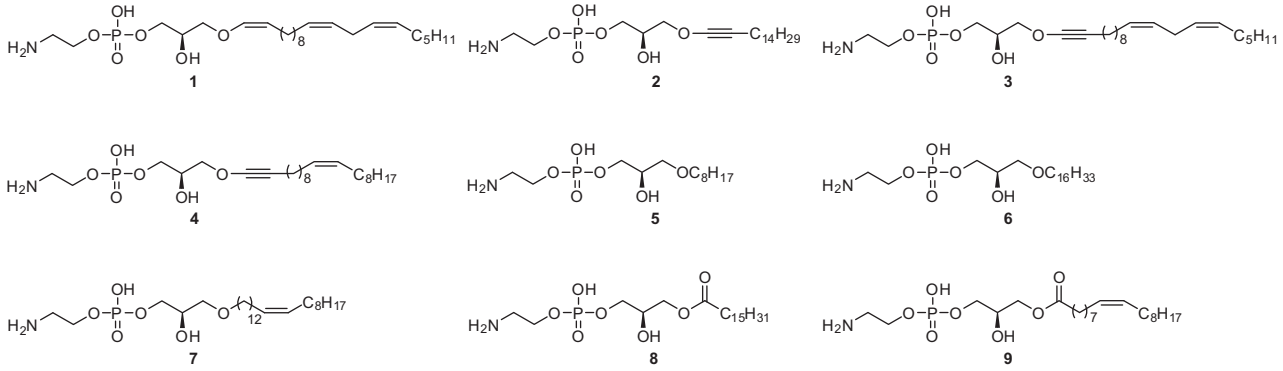
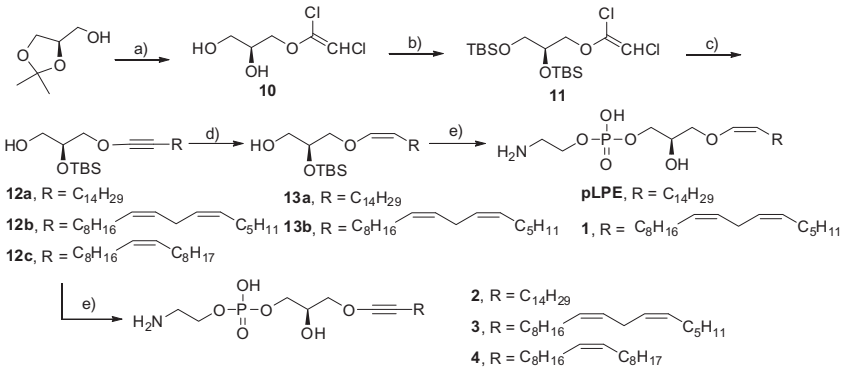
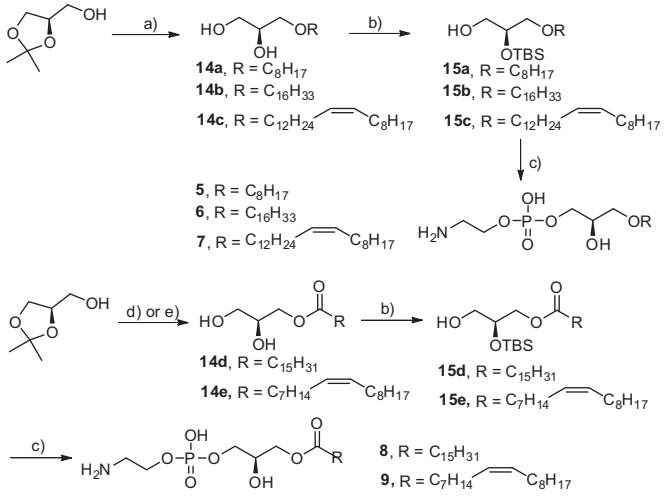


Figure 2. Structures of pLPE analogues.



Scheme 1. [a]Reagents and conditions: (a) (i) KH (30%), 0 °C, trichloroethylene, −42 °C∼rt, THF, (ii) TsOH, MeOH, 96% for two steps; (b) TBSCl, imidazole, DMAP, DMF/THF(3:1), 95%; (c) (i) *n*-BuLi(2.5 M in hexane, THF, −78 °C ∼ −42 °C, RI, HMPA, −42 °C ∼ rt, (ii) HF·pyridine, pyridine, THF, 0 °C ∼ rt 36–52% for two steps; (d) H_2, Pd/$BaSO_4$,quinoline, hexane/EtOAc (1:1), quantitative yield; (e) (i) $POCl_3$, TEA, pyridine, $MMTrNHCH_2CH_2OH$, H_2O, 0 °C ∼ rt, (ii) HF·pyridine, pyridine, THF, 47–61% for two steps.



Scheme 2. [a]Reagents and conditions: (a) (i) RBr, NaOH(50%, aq), 80 °C, (ii) AcOH, (50%, aq), 50 °C, 90–98% for two steps; (b) (i) TBSCl, imidazole, DMAP, DMF/THF(3:1), (ii) HF·pyridine, pyridine, THF, 0 °C ∼ rt; 71–78% for two steps; (c) (i) $POCl_3$, TEA, pyridine, $MMTrNHCH_2CH_2OH$, H_2O, (ii) HF·pyridine, pyridine, THF, 54–62% for two steps; (d) for **14d** (i) $C_{15}H_{31}COCl$, TEA, DMAP, DCM, (ii) AcOH, (50%, aq), 50 °C, quantative yield for two steps; (e) for **14e** (i) $C_8H_{17}CH{=}CHC_7H_{14}CO_2H$, DCC, DMAP, DCM, (ii) AcOH, (50%, aq), 50 °C, 98% for two steps.

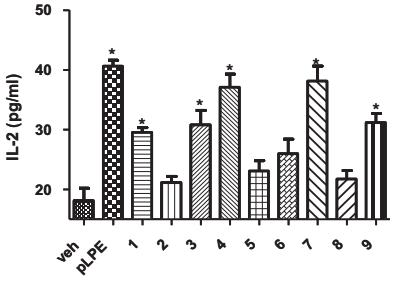


Figure 3. Reactivity of mouse NKT cell hybridoma to a series of pLPE analogues. ELISA of IL-2 released by 2H4 cells stimulated with plate-bound mCD1d loaded with equal concentrations of various compounds (100 ng/mL). Data are expressed as mean ± SD from three independent experiments. Statistical significance of the difference in secretion levels was determined by Student's *t* test. $^*P < 0.05$, compared with vehicle.

expressed as the IL-4/IFN-γ ratio (Fig. 4B), all compounds induced a Th2 biased cytokine response, and especially compound **4**, **7** and **9** induced more Th2-biased immunity than pLPE. The data strongly suggest that **4**, **7** and **9** were recognized by NKT cells stimulating their activation in a way that seems to be more biased toward Th2.

To more meaningfully address the biological activity of the analogues and physiological consequences, we resorted to in vivo study. We administered 1 μg of the analogues intraperitoneally and measured the levels of IFN-γ and IL-4 in serum at 2 and 24 h. Similarly to splenocyte cultures, all compounds induced obvious production of IL-4 upon in vivo administration as detected in the serum of treated mice 2 h later, and analogues **4**, **7** and **9** induced a significantly greater secretion of IL-4 than pLPE (Fig. 5), while IFN-γ induction in vivo was weak barely over background levels at 2 and 24 h in any treatment (Fig. 5).

2.3. Discussion

In this study, we designed and synthesized a series of monoaliphatic chain modified pLPE analogues, and evaluated their biological activity. According to the reactivity of mouse NKT cell hybridomas to a series of pLPE analogues presented by CD1d protein, in which assay the affects from the antigen presenting cells were excluded, we found analogues **1**, **3**, **4**, **7**, and **9** could also stimulate NKT cells activation significantly while the activity of **2**, **5**, **6**, and **8** were weak. There is a 16-carbon chain with a vinyl ether bond linking to the sn-1 position of the glycerol of pLPE. **2**, **6** and **8** had the same 16-carbon chain as pLPE but with different linkage at the sn-1 position: an alkynyl ether linkage, a saturated ether linkage, or an ester linkage respectively. It indicated that only the change of the vinyl ether bond in pLPE would impair its activity. Meanwhile, the activity of **5** which had an 8-carbon chain with a saturated ether linkage at the sn-1 position like **6** also became weaker. Interestingly, we found that analogues **3**, **4**, **7** and **9** which did not contain a vinyl ether bond could still stimulate NKT cells obviously. It is notably that the aliphatic chains of **3**, **4**, **7** and **9** contained one or more carbon–carbon double bonds. It suggested that carbon–carbon double bonds in the aliphatic chains made a great improvement on the immunostimulatory activity in comparison with **2**, **6** and **8** which did not contain a vinyl ether bond. Unlike **3**, **4**, **7** and **9**, we noticed the activity of **1**, which contained carbon–carbon double bonds in the aliphatic chains with a vinyl ether bond linkage at the sn-1 position, was not higher than that of pLPE. It indicated that carbon–carbon double bonds in the aliphatic chains could not enhance the activity when there was a vinyl ether bond linkage at the sn-1 position of the glycerol. In other words, pLPE analogues could activate NKT cells obviously if they contained a saturated aliphatic chain with a vinyl ether bond linkage at the sn-1 position, or an unsaturated aliphatic chain with an alkynyl ether or saturated ether or ester linkage, which was in agreement with a report LPC that contained an unsaturated aliphatic chain with an ester linkage could also stimulate NKT cells

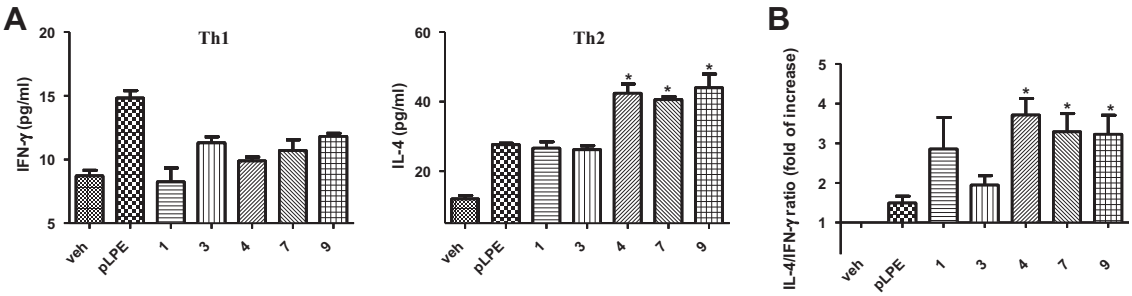


Figure 4. Effect of pLPE analogues on Th1/Th2 cytokine production by mouse splenocytes. Splenocytes (5×10^5) from C57BL/6 mice were cultured for72 h with 100 ng/mL of pLPE analogues or DMSO vehicle. Cytokines in the supernatants were measured by ELISA. (A) Induction of IFN-γ and IL-4. (B) Ratio of IL-4 over IFN-γ, normalized DMSO control. Assays were performed in triplicate, and data are given as mean ± SD. *P <0.05, compared with pLPE.

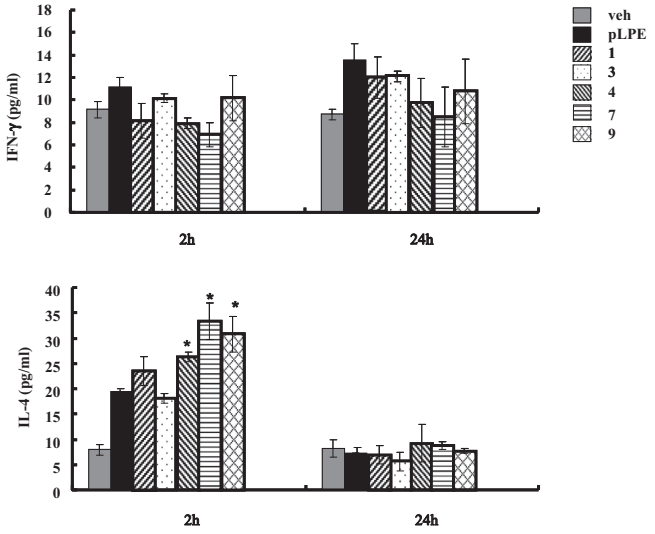


Figure 5. In vivo cytokine induction in mice. Amounts of 1 μg of the analogues and pLPE were i.p. injected into C57BL/6 mice, and IFN-γ and IL-4 in serum were measured after 2 h and 24 h. Data are given as mean ± SD. *P <0.05, compared with pLPE.

activation.[27] Besides, the observation of immunostimulatory activity of **7** and **9** will benefit the further SAR study on pLPE, because it was much more economic and easier to synthesize saturated ether or ester linkage than a vinyl ether bond linkage.

Although the modification of the mono-aliphatic chain of pLPE by changing the vinyl ether bond linkage did not elicit greater IL-2 release of NKT cell hybridoma, we found **4**, **7** and **9** polarized the release of cytokines more obviously. As we know, α-GalCer induces both Th1 and Th2 cytokines, and the two types of cytokines inhibit each other. There were no clinical responses in a phase I study of α-GalCer in patients with solid tumors because the effects of Th1 cytokines were hindered by Th2 cytokines.[12] As a result, the efficacy of α-GalCer has been limited. Therefore, the selectivity toward either Th1 or Th2 cytokines responses is more critical. The five immunostimulants (**1**, **3**, **4**, **7** and **9**) were further evaluated for their in vitro and in vivo immune-modulating effects. We found they all induced a Th2-biased cytokine response, as deduced from the high level of IL-4 in the splenocyte cultures and in vivo assays compared with the very weak IFN-γ induction. Especially, compounds **4**, **7** and **9**, induced more Th2-biased immunity than pLPE. Thus, **4**, **7** and **9** have an efficacy as potent Th2 response inducer but do not activate Th1 cytokines, so avoiding one main problem in the therapeutical application of α-GalCer, the simultaneous and potent induction of contradictory responses. These results demonstrate that even relatively minor changes in key structure on NKT cell antigen can result in large affinity differences among binding partners and have a surprising degree of influence on cytokine-inducing selectivity.

3. Conclusion

In summary, we described total synthesis of pLPE, an endogenous antigen for NKT cells, and its analogues. We identified pLPE analogues **1**, **3**, **4**, **7** and **9** as new NKT agonists and **4**, **7** and **9** become more potent Th2 activators for NKT cells which have superior properties for the treatment of autoimmune and inflammatory diseases. It will provide a new strategy and theoretical basis for the designation and clinical application of NKT cell antigens.

4. Experimental

4.1. General

All the reagents were obtained from commercial suppliers and used without further purification unless stated otherwise. THF (tetrahydrofuran) was distilled from sodium/benzophenone, DCM (dichloromethane) was distilled from CaH_2, acetone was distilled from anhydrous $CaSO_4$, pyridine was dried by boiling with CaH_2 prior to distillation and stored over KOH, DMF(*N*,*N*-Dimethylformamide) was stored over 4 Å molecular sieves, TEA (triethylamine) was distilled from $Ca(OH)_2$ and stored over KOH. All the reactions were performed in oven-dried or flame-dried apparatus under argon atmosphere. Reactions were monitored by thin-layer chromatography (TLC), performed on 0.2 mm silica gel GF254 plates. Visualization on TLC was achieved using UV light, iodine vapor and/or phosphomolybdic acid reagent. Chromatographic purification of products was accomplished using forced-flow chromatography on 300–400 mesh silica gel. NMR spectra were recorded on Bruker 400, Bruker 500, Bruker 600 or Avance 600. High-resolution mass spectra (HRMS) were performed on Waters AutoSpec Premier P776 or Agilent G6230.

4.1.1. 1-*O*-(1′,2′-Dichloroethenyl)-*sn*-glycerol (10)

To a suspension of KH (8.1 g, 30%, 60.7 mmol) in THF (250 mL) was added 2,3-*O*-isopropylidene-*sn*-glycerol (5.0 mL, 40.5 mmol) dropwise at 0 °C and stirred for 1 h. The reaction mixture was cooled to −42 °C and trichloroethylene (4.0 mL, 44.6 mmol) was added dropwise. The resulting mixture was warmed to room temperature and stirred for 1 h. Saturated NH_4Cl solution was added to quench the reaction and the solution was extracted with EtOAc. The combined organic layers were washed with brine, dried over Na_2SO_4, concentrated and treated with TsOH (500 mg) in methanol (100 mL). After stirring overnight, the mixture was neutralized by NH_4OH, concentrated and purified by column chromatography (petroleum ether/acetone 3:1) to give 7.23 g (96%) diol **10** as light

yellow oil: ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.58 (d, J = 8.7 Hz, 1H), 4.15–3.95 (m, 3H), 3.80 (dd, J = 11.6, 3.2 Hz, 1H), 3.70 (dd, J = 11.6, 5.2 Hz, 1H), 2.87 (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 143.59, 98.72, 72.83, 70.07, 63.32; HRMS (EI+) calcd for $C_5H_8O_3Cl_2$ $[M]^+$ 185.9850, found 185.9855.

4.1.2. 2,3-*O*-bis-*tert*-Butyldimethylsilanyl-1-*O*-(1′,2′-dichloroethenyl)-*sn*-glycerol (11)

To a solution of diol **10** (207.1 mg, 1.1 mmol) in DMF (9 mL) and THF (3 mL), was added imidazole (238.3 mg, 3.5 mmol) and DMAP (24.7 mg, 0.22 mmol), followed by *tert*-butyldimethylsilyl chloride (497.4 mg, 3.3 mmol). The resulting reaction mixture was stirred overnight. The reaction mixture was diluted with Et_2O and was washed with saturated NH_4Cl, saturated $NaHCO_3$, brine, and then dried over Na_2SO_4. The solvents were removed under reduced pressure and the crude product was purified by flash chromatography on silica gel (petroleum ether). The desired bis-TBS (431.6 mg, 95%) was obtained as colorless oil: ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.44 (s, 1H), 4.08 (dd, J = 9.9, 4.1 Hz, 1H), 4.02–3.83 (m, 2H), 3.77–3.50 (m, 2H), 0.97–0.86 (m, 18H), 0.19–0.02 (m, 12H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 144.15, 96.95, 72.94, 71.75, 64.37, 26.04, 25.90, 18.44, 18.24, −4.56, −4.67, −5.26, −5.30; HRMS (EI+) calcd for $C_{17}H_{36}O_3Si_2Cl_2$ $[M]^+$ 414.1580, found 414.1588.

4.1.3. General procedure for the synthesis of compounds 12a–12c

To a solution of dichloroethenyl (**11**, 1 equiv) in THF was added *n*-BuLi (2.5 M in hexane, 2 equiv) dropwise at −78 °C. After 1 h, the reaction mixture was warmed to −42 °C. RI (1.1 equiv) in HMPA was added. The solution was warmed to room temperature and stirred for 24 h. Saturated NH_4Cl solution was added and the mixture was extracted with Et_2O three times, the combined organic layers were washed with water and brine, dried over Na_2SO_4, filtered and concentrated. The residue was purified by flash chromatography on silica gel (petroleum ether) to afforded the desired *O*-alkynyl ether as colorless oil. A stirring solution of the bis-TBS-protected alcohol prepared above in THF at 0 °C was treated with HF-pyridine in pyridine/THF (prepared by mixing 1 volume of HF-pyridine complex with 4 volume of pyridine and diluted with 4 volume of THF) dropwise via addition funnel. The reaction was allowed to slowly warm to rt and stirred for 1 h. The reaction mixture was cooled to 0 °C and quenched by the dropwise addition of saturated $NaHCO_3$ and stirring continued until no gas evolution was observed. The solution was transferred into a separatory funnel and the two layers were allowed to separate. The organic layer was collected and the aqueous layer extracted with EtOAc three times, the combined organic layers were washed with water and brine, dried over Na_2SO_4, filtered and concentrated. The residue was purified by flash chromatography on silica gel (petroleum ether/EtOAc 100:1) to afforded the desired primary alcohol (**12a**–**c**) as colorless oil.

4.1.3.1. 2-*O*-*tert*-Butyldimethylsilanyl-1-*O*-1′-hexadecynyl-2-hydroxy-*sn*-glycerol (12a). 44% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.03 (dd, J = 10.8, 4.7 Hz, 1H), 3.97 (dd, J = 10.2, 5.2 Hz, 1H), 3.90 (dd, J = 10.2, 6.4 Hz, 1H), 3.65 (dd, J = 11.3, 3.7 Hz, 1H), 3.60–3.47 (m, 1H), 2.08 (t, J = 6.9 Hz, 2H), 1.92 (br s, 1H), 1.42 (dd, J = 14.2, 7.1 Hz, 2H), 1.37–1.12 (m, 22H), 0.93–0.84 (m, 12H), 0.18–0.07 (m, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 89.63, 78.68, 70.41, 63.76, 37.53, 32.07, 29.84, 29.81, 29.77, 29.75, 29.51, 29.36, 29.01, 26.24, 25.88, 22.84, 18.21, 17.24, 14.27, −4.55, −4.78; HRMS (EI+) calcd for $C_{25}H_{50}O_3Si$ $[M]^+$ 426.3529, found 426.3528.

4.1.3.2. 2-*O*-*tert*-Butyldimethylsilanyl-1-*O*-11′, 14′-(*Z*,*Z*)-icosadien-1′-ynyl-*sn*-glycerol (12b). 52% yield; ^{1}H NMR (600 MHz, $CDCl_3$) δ 5.48–5.14 (m, 4H), 4.05–3.99 (m, 1H), 3.99–3.94 (m, 1H), 3.90 (dd, J = 10.2, 6.3 Hz, 1H), 3.70–3.61 (m, 1H), 3.61–3.48 (m, 1H), 2.76 (t, J = 7.0 Hz, 2H), 2.13–1.87 (m, 6H), 1.46–1.19 (m, 18H), 0.94–0.81 (m, 12H), 0.13–0.08 (m, 6H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 130.34, 130.28, 128.11, 128.07, 89.67, 78.72, 70.45, 63.78, 31.67, 29.82, 29.77, 29.62, 29.49, 29.45, 29.32, 29.00, 27.37, 27.34, 26.24, 26.21, 25.92, 25.89, 25.77, 22.72, 17.24, 14.22, −4.54, −4.77; HRMS (EI+) calcd for $C_{29}H_{54}O_3Si$ $[M]^+$ 478.3842, found 478.3864.

4.1.3.3. 2-*O*-*tert*-Butyldimethylsilanyl-1-*O*-11′-(*Z*)-icosen-1′-ynyl-*sn*-glycerol (12c). 36% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.49–5.16 (m, 2H), 4.05–4.00 (m, 1H), 3.97 (dd, J = 10.2, 5.2 Hz, 1H), 3.94–3.84 (m, 1H), 3.68–3.61 (m, 1H), 3.61–3.51 (m, 1H), 2.09 (t, J = 7.0 Hz, 2H), 2.05–1.90 (m, 4H), 1.86 (dd, J = 7.7, 5.2 Hz, 1H), 1.47–1.37 (m, 2H), 1.38–1.13 (m, 22H), 0.91–0.87 (m, 12H), 0.20–0.08 (m, 6H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 130.09, 130.00, 87.07, 78.73, 70.47, 63.81, 37.54, 32.06, 29.93, 29.79, 29.68, 29.64, 29.47, 29.33, 29.02, 27.36, 26.26, 26.00, 25.90, 22.83, 18.22, 17.26, 14.26, −4.53, −4.76; HRMS (EI+) calcd for $C_{29}H_{56}O_3Si$ $[M]^+$ 480.3999, found 480.4008.

4.1.4. General procedure for the synthesis of compounds 13a and 13b

To a solution of the *O*-alkynyl ether (**12a** and **12b**) in hexane/EtOAc (1:1) were added $Pd/BaSO_4$ and quinoline. The resulting suspension was degassed by H_2 and then a H_2 balloon was applied. After stirring overnight, the mixture was filtered through a plug of Celite, washed with EtOAc and concentrated. The residue was purified by flash chromatography on silica gel (petroleum ether/EtOAc 100:1) to afforded the desired *cis*-enol ether (**13a** and **13b**) as colorless oil.

4.1.4.1. 2-*O*-*tert*-Butyldimethylsilanyl-1-*O*-1′-(*Z*)-hexadecenyl-2-hydroxy-*sn*-glycerol (13a). Quantative yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.90 (d, J = 6.2 Hz, 1H), 4.33 (dd, J = 13.6, 7.2 Hz, 1H), 3.95–3.88 (m, 1H), 3.77–3.62 (m, 3H), 3.62–3.52 (m, 1H), 2.12–2.00 (m, 2H), 1.91 (dd, J = 7.4, 5.4 Hz, 1H), 1.50–1.14 (m, 24H), 0.93–0.88 (m, 12H), 0.20–0.09 (m, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 145.02, 107.48, 73.39, 71.50, 64.33, 32.08, 29.94, 29.86, 29.82, 29.69, 29.52, 29.50, 25.93, 24.17, 22.85, 18.23, 14.27, −4.48, −4.74; HRMS (EI+) calcd for $C_{25}H_{52}O_3Si$ $[M]^+$ 428.3686, found 428.3703.

4.1.4.2. 2-*O*-*tert*-Butyldimethylsilanyl-1-*O*-1′,11′,14′-(*Z*,*Z*,*Z*)-icosatrienyl-sn-glycerol (13b). Quantative yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.90 (d, J = 6.2 Hz, 1H), 5.63–5.12 (m, 4H), 4.46–4.20 (m, 1H), 4.13–3.98 (m, 1H), 3.95–3.83 (m, 1H), 3.71–3.67 (m, 1H), 3.66–3.62 (m, 1H), 3.60–3.53 (m, 1H), 2.77 (t, J = 6.4 Hz, 2H), 2.15–1.98 (m, 6H), 1.38–1.23 (m, 18H), 0.91–0.89 (m, 12H), 0.17–0.09 (m, 6H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 145.03, 130.34, 128.10, 128.08, 107.42, 79.40, 73.38, 71.48, 70.26, 64.31, 63.65, 31.68, 29.92, 29.84, 29.69, 29.65, 29.48, 27.40, 27.35, 26.25, 25.92, 25.88, 25.78, 24.16, 22.72, 19.31, 14.22, −3.89, −4.48; HRMS (EI+) calcd for $C_{29}H_{56}O_3Si$ $[M]^+$ 480.3999, found 480.3987.

4.1.5. General procedure for the synthesis of pLPE, 1–4

To a solution of the $POCl_3$ (1.05 equiv) in DCM was added TEA (20 equiv) and pyridine (1 equiv) dropwise at 0 °C. After 0.5 h, alcohol (**12a**–**12c**, **13a**–**13b**, 1 equiv) in DCM was added dropwise. After 1 h, *N*-(monomethoxytrityl)-ethanolamine(2.5 equiv) in DCM was added dropwise and the mixture was stirred 1 h before H_2O (0.5 mL) was added. After another 1 h stirring, the mixture was treated with HF-pyridine (2 equiv) and stirred overnight. The resulting mixture was dissolved with DCM and washed with

H_2O. The organic layer was concentrated, and the residue was purified by flash chromatography on silica gel ($CHCl_3$/MeOH/H_2O 70:10:1) to afforded the compounds (pLPE, **1**–**5**) as white solid.

4.1.5.1. 1-*O*-1′-(*Z*)-Hexadecenyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine (pLPE). 61% yield; ^{1}H NMR (500 MHz, $CDCl_3$/MeOD) δ 8.31 (br s, 2H), 5.93 (d, J = 6.1 Hz, 1H), 4.37–4.30 (m, 1H), 4.26–4.06 (m, 2H), 4.06–3.91 (m, 2H), 3.90–3.80 (m, 1H), 3.79–3.65 (m, 2H), 3.29–3.15 (m, 2H), 2.09–1.86 (m, 2H), 1.25 (s, 24H), 0.88 (t, J = 6.9 Hz, 3H); ^{13}C NMR (150 MHz, $CDCl_3$/MeOD) δ 144.80, 107.76, 72.43, 69.55, 67.48, 62.19, 45.94, 32.02, 29.91, 29.85, 29.82, 29.81, 29.76, 29.63, 29.49, 29.46, 24.05, 22.77, 14.18, 8.60; ^{31}P NMR (162 MHz, $CDCl_3$/MeOD) δ 0.61; HRMS (EI+) calcd for $C_{21}H_{44}NO_6P$ $[M]^+$ 437.2906, found 437.2915.

4.1.5.2. 1-*O*-1′,11′,14′-(*Z*,*Z*,*Z*)-icosatrienyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine (1). 47% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.10–5.76 (m, 1H), 5.57–5.16 (m, 4H), 4.32 (dd, J = 13.2, 6.4 Hz, 1H), 4.24–3.59 (m, 5H), 3.53–3.05 (m, 4H), 2.77 (t, J = 6.4 Hz, 2H), 2.30–2.03 (m, 6H), 1.68–1.06 (m, 18H), 0.89 (t, J = 6.8 Hz, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 145.02, 130.34, 130.28, 128.11, 128.08, 109.57, 71.97, 69.66, 62.45, 45.98, 40.64, 32.06, 31.67, 29.89, 29.85, 29.79, 29.58, 29.50, 29.47, 27.42, 27.35, 26.23, 25.78, 24.15, 22.84, 22.73, 14.24, 8.76; ^{31}P NMR (162 MHz, $CDCl_3$) δ −0.24; HRMS (EI+) calcd for $C_{25}H_{48}NO_6P$ $[M]^+$ 489.3219, found 489.3229.

4.1.5.3. 1-*O*-1′-Hexadecynyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine (2). 63% yield; ^{1}H NMR (600 MHz, $CDCl_3$/MeOD) δ 4.25–3.92 (m, 6H), 3.91–3.83 (m, 1H), 3.29–3.11 (m, 2H), 2.07 (t, J = 7.2 Hz, 2H), 1.49–1.38 (m, 2H), 1.29–1.20 (m, 22H), 0.88 (t, J = 7.0 Hz, 3H); ^{13}C NMR(150 MHz, $CDCl_3$/MeOD) δ 89.49, 78.32, 68.53, 67.11, 62.54, 40.56, 37.56, 34.27, 32.10, 29.95, 29.93, 29.87, 29.56, 25.09, 22.86, 17.35, 14.27, 0.14; ^{31}P NMR (162 MHz, $CDCl_3$/MeOD) δ 0.41; HRMS (EI+) calcd for $C_{21}H_{42}NO_6P$ $[M]^+$ 435.2750, found 435.2739.

4.1.5.4. 2-Hydroxy-1-*O*-11′,14′-(*Z*,*Z*)-icosadien-1′-ynyl-*sn*-glycero-3-phosphoethanolamine (3). 52% yield; ^{1}H NMR (600 MHz, $CDCl_3$/MeOD) δ 5.39–5.16 (m, 4H), 4.11–3.72 (m, 7H), 3.18–2.96 (s, 2H), 2.68 (t, J = 6.8 Hz, 1H), 2.30–2.13 (m, 2H), 2.11–1.76 (m, 4H), 1.64–1.47 (m, 2H), 1.36–1.17 (m, 18H), 0.79 (t, J = 6.0 Hz, 3H); ^{13}C NMR (150 MHz, $CDCl_3$/MeOD) δ 130.16, 130.09, 127.94, 127.89, 89.34, 72.86, 71.47, 64.79, 61.83, 37.20, 34.05, 31.86, 31.48, 29.64, 29.47, 29.30, 29.16, 28.91, 27.19, 27.15, 25.57, 24.82, 22.52, 17.03, 13.96, −0.16; ^{31}P NMR (162 MHz, $CDCl_3$/MeOD) δ 0.55; HRMS (EI+) calcd for $C_{25}H_{46}NO_6P$ $[M]^+$ 487.3063, found 487.3068.

4.1.5.5. 1-*O*-11′-(*Z*)-icosen-1′-ynyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine (4). 49% yield; ^{1}H NMR (400 MHz, $CDCl_3$/MeOD) δ 8.26 (br s, 2H), 5.56–5.06 (m, 2H), 4.68–4.32 (m, 1H), 4.31–3.88 (m, 4H), 3.88–3.56 (m, 2H), 3.17–2.82 (m, 2H), 2.30 (d, J = 7.2 Hz, 1H), 2.10–1.86 (m, 6H), 1.66–1.05 (m, 24H), 0.88 (t, J = 6.6 Hz, 3H); ^{13}C NMR (150 MHz, $CDCl_3$/MeOD) δ 129.99, 129.87, 89.35, 72.02, 68.71, 64.85, 62.12, 40.32, 34.13, 32.65, 31.94, 29.83, 29.80, 29.75, 29.70, 29.59, 29.56, 29.53, 29.35, 29.24, 29.01, 27.24, 24.91, 22.72, 17.13, 14.12, 8.53, −0.02; ^{31}P NMR (162 MHz, $CDCl_3$/MeOD) δ −0.61; HRMS (EI+) calcd for $C_{25}H_{48}NO_6P$ $[M]^+$ 489.3219, found 489.3221.

4.1.6. General procedure for the synthesis of compounds 14a–14c[28]

2,3-*O*-Isopropylidene-*sn*-glycerol (500.0 mg, 3.8 mmol) was added to a 50% aqueous solution of NaOH (8 mL). The reaction mixture was heated at 80 °C, before the addition of the *n*-alkyl bromide (15.2 mmol) and Bu_4NBr (245.0 mg, 0.76 mmol). After disappearance of the starting materials (2 h), the reaction mixture was cooled and extracted with CH_2Cl_2. The organic solution was washed with water, brine, dried over Na_2SO_4, concentrated and purified by column chromatography (petroleum ether, then 20:1 petroleum ether/ethyl acetate) to give the 1-*O*-*n*-alkyl-2,3-*O*-isopropylidene-*sn*-glycerol. The 1-*O*-*n*-alkyl-2,3-*O*-isopropylidene-*sn*-glycerol was dissolved in 80% aqueous AcOH (1 mmol/5 mL). The mixture was stirred overnight at room temperature and then evaporated to dryness in vacuo. The residue was purified by column chromatography (3:1 petroleum ether/acetone) to afford the 1-*O*-*n*-alkyl-*sn*-glycerol.

4.1.6.1. 1-*O*-*n*-Octyl-*sn*-glycerol (14a)[29]. White solid; 761.2 mg; 98% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.01–3.80 (m, 1H), 3.80–3.56 (m, 2H), 3.56–3.37 (m, 4H), 2.97 (d, J = 4.8 Hz, 1H), 2.63 (t, J = 5.8 Hz, 1H), 1.55 (dd, J = 14.0, 6.9 Hz, 2H), 1.51–1.13 (m, 10H), 0.87 (t, J = 6.8 Hz, 3H).

4.1.6.2. 1-*O*-*n*-Hexadecyl-*sn*-glycerol (14b)[30]. White solid; 1.17 g; 98% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.85 (d, J = 4.5 Hz, 1H), 3.71 (dd, J = 11.4, 3.5 Hz, 1H), 3.64 (dd, J = 11.4, 5.2 Hz, 1H), 3.58–3.35 (m, 4H), 2.68 (s, 1H), 2.26 (s, 1H), 1.62–1.52 (m, 2H), 1.25 (s, 26H), 0.87 (t, J = 6.7 Hz, 3H).

4.1.6.3. 1-*O*-*cis*-Docos-13-enyl-*sn*-glycerol (14c). Colorless oil; 1.37 g; 90% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.57–5.06 (m, 2H), 3.85 (d, J = 3.9 Hz, 1H), 3.71 (dd, J = 11.3, 3.5 Hz, 1H), 3.64 (dd, J = 11.4, 5.3 Hz, 1H), 3.56–3.41 (m, 4H), 2.75 (s, 1H), 2.36 (s, 1H), 2.12–1.90 (m, 4H), 1.66–1.49 (m, 2H), 1.34–1.22 (m, 30H), 0.87 (t, J = 6.8 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 130.03, 130.01, 72.56, 71.98, 70.66, 64.35, 32.03, 29.90, 29.83, 29.78, 29.74, 29.72, 29.70, 29.65, 29.59, 29.45, 27.33, 26.20, 22.80, 14.23; HRMS (EI+) calcd for $C_{25}H_{50}O_3$ $[M]^+$ 398.3760, found 398.3751.

4.1.7. 1-*O*-Hexadecanoyl-*sn*-glycerol (14d)[31]

To an ice-cold solution of 2,3-*O*-isopropylidene-*sn*-glycerol (1 g, 6 mmol) and DMAP(17.0 mg, 0.15 mmol) in DCM (10 mL) and triethylamine (3 mL) was added palmitoyl chloride (2.5 g, 9.0 mmol) in DCM (3 mL) dropwise. The reaction mixture was warmed to room temperature and stirred overnight. The mixture was filtered, and the filtrate was washed with 1 N HCl, saturated $NaHCO_3$, brine, and then dried over Na_2SO_4. The solvents were removed under reduced pressure and the crude product dissolved in 80% aqueous AcOH (27 mL). The mixture was stirred overnight at room temperature and then evaporated to dryness in vacuo. The residue was purified by column chromatography (3:1 petroleum ether/acetone) to afford 2.51 g 1-*O*-hexadecanoyl-*sn*-glycerol as a white solid (quantitative yield): ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.26–4.02 (m, 2H), 4.00–3.86 (m, 1H), 3.69 (dd, J = 11.5, 3.5 Hz, 1H), 3.59 (dd, J = 11.4, 5.9 Hz, 1H), 2.91 (br s, 1H), 2.57 (br s, 1H), 2.34 (t, J = 7.5 Hz, 2H), 1.70–1.53 (m, 2H), 1.24 (s, 24H), 0.87 (t, J = 6.4 Hz, 3H).

4.1.8. 1-*O*-*cis*-Octadec-9-enoyl-*sn*-glycerol (14e)[32]

DCC (1.18 g, 5.7 mmol) and DMAP (213.1 mg, 1.9 mmol) were added to a solution of 2,3-*O*-isopropylidene-*sn*-glycerol (0.50 g, 3.8 mmol) and oleic acid(1.8 mL, 5.7 mmol) in 50 mL of DCM. After the mixture was stirred at room temperature overnight, the solvent was removed under vacuum. The crude product was dissolved in 80% aqueous AcOH (17 mL) and stirred overnight. The mixture was concentrated in vacuo and the residue was purified by column chromatography (3:1 petroleum ether/acetone) to afford 1.33 g (98%) 1-*O*-*cis*-octadec-9-enoyl-*sn*-glycerol as a white solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.57–5.12 (m, 2H), 4.39–4.02 (m, 2H), 3.99–3.87 (m, 1H), 3.70 (dd, J = 11.4, 3.8 Hz, 1H), 3.60 (dd,

J = 11.4, 5.8 Hz, 1H), 2.57 (br s, 1H), 2.35 (t, J = 7.6 Hz, 2H), 2.14 (br s, 1H), 2.01 (dd, J = 14.3, 8.5 Hz, 4H), 1.66–1.60 (m, 2H), 1.40–1.23 (m, 20H), 0.88 (t, J = 6.7 Hz, 3H).

4.1.9. General procedure for the synthesis of compounds 15a–15e

To a solution of diol (**14a**–**14e**, 1 equiv) in DMF and THF, was added imidazole (10 equiv) and DMAP (0.2 equiv), followed by tert-butyldimethylsilyl chloride (3 equiv). The resulting reaction mixture was stirred overnight. The reaction mixture was diluted with Et_2O and was washed with saturated NH_4Cl, saturated $NaHCO_3$, brine, and then dried over Na_2SO_4. The solvents were removed under reduced pressure and the crude product was purified by flash chromatography on silica gel (petroleum ether) to afford the desired 2, 3-*O*-bis-*tert*-butyldimethylsilanyl-*sn*-glycerol as a colorless oil. A stirring solution of the bis-TBS-protected prepared above in THF at 0 °C was treated with HF-pyridine in pyridine/THF (prepared by mixing 1 volume of HF-pyridine complex with 4 volume of pyridine and diluted with 4 volume of THF) dropwise via addition funnel. The reaction was allowed to slowly warm to rt and stirred for 1 h. The reaction mixture was cooled to 0 °C and quenched by the dropwise addition of saturated $NaHCO_3$ and stirring continued until no gas evolution was observed. The solution was transferred into a separatory funnel and the two layers were allowed to separate. The organic layer was collected and the aqueous layer extracted with EtOAc three times, the combined organic layers were washed with water and brine, dried over Na_2SO_4, filtered and concentrated. The residue was purified by flash chromatography on silica gel (petroleum ether/EtOAc 100:1) to afforded the desired primary alcohol(**15a**–**e**) as colorless oil.

4.1.9.1. 2-*O*-*tert*-Butyldimethylsilanyl-1-*O*-*n*-octyl-*sn*-glycerol (15a). 78% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.93–3.82 (m, 1H), 3.70–3.60 (m, 1H), 3.60–3.53 (m, 1H), 3.49–3.35 (m, 4H), 2.23–1.97 (m, 1H), 1.56–1.48 (m, 2H), 1.37–1.25 (m, 10H), 0.90–0.84 (m, 12H), 0.10 (s, 6H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 72.92, 71.95, 71.29, 65.28, 31.96, 29.80, 29.57, 29.39, 26.25, 26.02, 25.95, 22.80, 18.27, 14.25, −4.45, −4.72; HRMS (EI+) calcd for $C_{17}H_{38}O_3Si$ $[M]^+$ 318.2590, found 318.2592.

4.1.9.2. 2-*O*-*tert*-Butyldimethylsilanyl-1-*O*-*n*-hexadecyl-*sn*-glycerol (15b). 75% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.95–3.82 (m, 1H), 3.71–3.52 (m, 2H), 3.49–3.27 (m, 4H), 2.13 (dd, J = 7.4, 5.3 Hz, 1H), 1.55–1.48 (m, 2H), 1.25 (s, 26H), 0.91–0.85 (m, 12H), 0.10 (d, J = 0.9 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 72.93, 71.96, 71.31, 65.28, 32.08, 29.85, 29.81, 29.76, 29.62, 29.51, 26.26, 25.95, 22.84, 18.27, 14.27, −4.44, −4.72; HRMS (EI+) calcd for $C_{25}H_{54}O_3Si$ $[M]^+$ 430.3842, found 430.3855.

4.1.9.3. 2-*O*-*tert*-Butyldimethylsilanyl-1-*O*-*cis*-docos-13-enyl-*sn*-glycerol (15c). 72% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.49–5.18 (m, 2H), 3.93–3.82 (m, 1H), 3.73–3.51 (m, 2H), 3.48–3.27 (m, 4H), 2.14 (dd, J = 7.3, 5.3 Hz, 1H), 2.01 (dd, J = 11.6, 6.0 Hz, 4H), 1.54 (dd, J = 13.7, 6.8 Hz, 2H), 1.26 (s, 30H), 0.92–0.80 (m, 12H), 0.10 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 130.05, 72.93, 71.95, 71.31, 65.27, 32.06, 29.93, 29.81, 29.74, 29.68, 29.63, 29.48, 27.36, 26.26, 25.95, 22.83, 18.26, 14.26, −4.45, −4.72; HRMS (EI+) calcd for $C_{31}H_{64}O_3Si$ $[M]^+$ 512.4625, found 512.4631.

4.1.9.4. 2-*O*-*tert*-Butyldimethylsilanyl-1-*O*-hexadecanoyl-*sn*-glycerol (15d). 76% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.17–4.01 (m, 2H), 3.99–3.87 (m, 1H), 3.68–3.45 (m, 2H), 2.31 (t, J = 7.5 Hz, 2H), 1.96 (dd, J = 7.2, 5.8 Hz, 1H), 1.70–1.59 (m, 2H), 1.42–1.12 (m, 24H), 0.98–0.78 (m, 12H), 0.11 (d, J = 1.9 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.91, 70.74, 64.95, 64.06, 34.37, 32.07, 29.84, 29.80, 29.75, 29.60, 29.51, 29.41, 29.30, 25.87, 25.05, 22.84, 18.21, 14.26, −4.50, −4.68; HRMS (EI+) calcd for $C_{25}H_{52}O_4Si$ $[M]^+$ 444.3635, found 444.3633.

4.1.9.5. 2-*O*-*tert*-Butyldimethylsilanyl-1-*O*-*cis*-octadec-9-enoyl-*sn*-glycerol (15e). 71% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.48–5.18 (m, 2H), 4.16–4.02 (m, 2H), 3.99–3.86 (m, 1H), 3.68–3.48 (m, 2H), 2.31 (t, J = 7.6 Hz, 2H), 2.10–1.82 (m, 4H), 1.67–1.58 (m, 2H), 1.38–1.23 (m, 20H), 0.95–0.83 (m, 12H), 0.11 (d, J = 1.9 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.88, 130.17, 129.89, 70.74, 64.95, 64.05, 34.36, 32.06, 29.92, 29.84, 29.67, 29.47, 29.31, 29.27, 29.24, 27.37, 27.32, 25.88, 25.04, 22.83, 18.21, 14.26, −4.50, −4.68; HRMS (EI+) calcd for $C_{27}H_{54}O_4Si$ $[M]^+$ 470.3791, found 470.3788.

4.1.10. General procedure for the synthesis of compounds 5–9

To a solution of the $POCl_3$ (1.05 equiv) in DCM was added TEA (20 equiv) and pyridine (1 equiv) dropwise at 0 °C. After 0.5 h, alcohol (**15a**–**15e**, 1 equiv) in DCM was added dropwise. After 1 h, *N*-(monomethoxytrityl)-ethanolamine (2.5 equiv) in DCM was added dropwise and the mixture was stirred 1 h before H_2O (0.5 mL) was added. After another 1 h stirring, the mixture was treated with HF-pyridine (2 equiv) and stirred overnight. The resulting mixture was dissolved with DCM and washed with H_2O. The organic layer was concentrated, and the residue was purified by flash chromatography on silica gel ($CHCl_3$/MeOH/H_2O 70:10:1) to afford the compounds (**5**–**9**) as white solid.

4.1.10.1. 1-*O*-*n*-Octyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine (5). 62% yield; ^{1}H NMR (400 MHz, $CDCl_3$/MeOD) δ 3.91–3.79 (m, 2H), 3.79–3.65 (m, 2H), 3.66–3.55 (m, 1H), 3.28–3.15 (m, 4H), 2.97–2.82 (m, 2H), 1.31 (dd, J = 13.8, 6.9 Hz, 2H), 1.13–0.94 (m, 10H), 0.63 (t, J = 6.8 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$/MeOD) δ 71.49, 71.19, 69.48, 67.27, 61.35, 40.21, 31.50, 29.21, 29.11, 28.92, 25.68, 22.28, 13.52; ^{31}P NMR (162 MHz, $CDCl_3$/MeOD) δ 0.70; HRMS (ESI+) calcd for $C_{13}H_{31}NO_6P$ ($[M+H]^+$) 328.1889, found 328.1891.

4.1.10.2. 1-*O*-*n*-Hexadecyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine (6). 57% yield; H NMR (600 MHz, $CDCl_3$/MeOD) δ 3.98–3.85 (m, 2H), 3.85–3.72 (m, 2H), 3.70–3.65 (m, 1H), 3.30–3.28 (m, 2H), 3.20–3.15 (m, 2H), 2.98–2.89 (m, 2H), 1.52–1.24 (m, 2H), 1.23–1.02 (m, 26H), 0.70 (t, J = 7.0 Hz, 3H); ^{13}C NMR (150 MHz, $CDCl_3$/MeOD) δ 71.65, 71.29, 69.61, 67.44, 61.39, 40.28, 31.74, 29.50, 29.46, 29.44, 29.38, 29.34, 29.17, 25.84, 22.48, 13.77; ^{31}P NMR (162 MHz, $CDCl_3$/MeOD) δ 4.77; HRMS (ESI+) calcd for $C_{21}H_{46}NNaO_6P$ ($[M+Na]^+$) 462.2960, found 462.2966.

4.1.10.3. 1-*O*-*cis*-Docos-13-enyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine (7). 59% yield; ^{1}H NMR (400 MHz, $CDCl_3$/MeOD) δ 5.28–5.07 (m, 2H), 3.98–3.84 (m, 2H), 3.82–3.70 (m, 2H), 3.70–3.63 (m, 1H), 3.27 (t, J = 6.8 Hz, 4H), 3.19–3.12 (m, 2H), 1.97–1.70 (m, 4H), 1.47–1.26 (m, 2H), 1.08 (s, 30H), 0.70 (t, J = 6.0 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$/MeOD) δ 129.67, 71.65, 71.30, 69.51, 67.44, 39.46, 31.70, 29.55, 29.46, 29.37, 29.30, 29.11, 26.97, 25.83, 22.45, 13.74; ^{31}P NMR (162 MHz, $CDCl_3$/MeOD) δ 0.96; HRMS (ESI+) calcd for $C_{27}H_{56}NNaO_6P$ ($[M+Na]^+$) 544.3743, found 544.3747.

4.1.10.4. 1-*O*-Hexadecanoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine (8). 62%; ^{1}H NMR (400 MHz, $CDCl_3$/MeOD) δ 3.93–3.86 (m, 2H), 3.86–3.75 (m, 2H), 3.76–3.67 (m, 1H), 3.66–3.54 (m, 2H), 2.98–2.92 (m, 1H), 2.89–2.82 (m, 1H), 2.18 (t, J = 7.6 Hz, 2H), 1.54–1.30 (m, 2H), 1.23–0.97 (m, 24H), 0.72 (t, J = 6.8 Hz, 3H); ^{13}C NMR (150 MHz, $CDCl_3$/MeOD) δ 174.28, 64.72, 61.42, 57.60, 53.27, 41.54, 33.98, 31.80, 29.57, 29.53, 29.50, 29.37, 29.23, 29.17, 29.04, 24.74, 22.55, 13.88; ^{31}P NMR

(162 MHz, $CDCl_3$/MeOD) δ 0.81; HRMS (ESI−) calcd for $C_{21}H_{43}NO_7P$ ($[M-H]^-$) 452.2777, found 452.2785.

4.1.10.5. 1-*O*-*cis*-Octadec-9-enoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine (9). 54% yield; ^1H NMR (400 MHz, $CDCl_3$) δ 8.34 (brs, 2H), 5.46–5.33 (m, 2H), 4.26–4.03 (m, 4H), 4.02–3.93 (m, 2H), 3.88–3.80 (m, 1H), 3.40–3.11 (m, 2H), 2.93–2.60 (m, 2H), 2.31 (t, J = 7.5 Hz, 2H), 2.17–1.85 (m, 4H), 1.73–1.49 (m, 2H), 1.43–1.16 (m, 20H), 0.88 (t, J = 5.3 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.98, 130.15, 129.83, 68.99, 67.65, 64.90, 61.96, 34.24, 32.06, 31.67, 29.92, 29.69, 29.47, 29.37, 27.37, 25.78, 25.04, 22.83, 14.26; ^{31}P NMR (162 MHz, $CDCl_3$) δ 0.54; HRMS (ESI+) calcd for $C_{23}H_{46}NNaO_7P$ ($[M+Na]^+$) 502.2910, found 502.2906.

4.2. Methods for measurement of biological activity

4.2.1. Cell culture

The 2H4 mouse NKT hybridoma was kindly provided by Mitchell Kronenberg (La Jolla Institute for Allergy and Immunology, La Jolla, CA). Cell lines were maintained in RPM1640 medium containing 10% fetal calf serum (FCS), 2 mM sodium pyruvate, 2 mM L-glutamine, and 50 μM 2-mercaptoethanol.

4.2.2. Plate-bound CD1d presenting assay

The stimulation of mouse NKT cell hybridomas in a cell-free antigen presentation assay was performed as described by published protocol[33], with some modification. 1 μg mCD1d protein was coated in 96-well flat-bottom plate at 4 °C overnight. The plates were washed with PBS and blocked by PBS and 10% FCS for 1 h. After washing, the pLPE and anologs at indicated concentration were added to each well and incubated for 24 h at 37 °C. After washing, 2H4 hybridoma cells (5×10^4) were added to each well. IL-2 release was measured after 24 h of culture by ELISA using commercially available ELISA kits (Shanghai ExCell Biology, Inc., Shanghai, China).

4.2.3. Mouse primary splenocytes assay

Splenocytes (5×10^5) from C57BL/6 mice were cultured for 72 h with 100 ng/mL of pLPE and analogues or DMSO vehicle in 96-well U-bottom plates containing RPMI1640 supplemented with 10% FCS, L-glutamine, 2-mercaptoethanol, penicillin and streptomycin in humidified 5% CO_2 at 37 °C. For cytokine determination, cell-free supernatants were collected and IFN-γ and IL-4 levels were measured by ELISA.

4.2.4. In vivo assays

1 μg of the pLPE and analogues in 200 μL of PBS were injected intraperitoneally to C57BL/6 mice. Sera were collected at 2 time points (2 and 24 h) and both IFN-γ and IL-4 were measured by ELISA.

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