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Employing BINOL-phosphoroselenoyl chloride for selective inositol phosphorylation and chemical synthesis of glycosyl inositol phospholipid from *Entamoeba histolytica*

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Abstract: Here we describe the chemical synthesis of glycosyl inositol phospholipids from *Entamoeba histolytica*. The key feature of this synthesis is a regioselective phosphorylation reaction that occurs via desymmetrization of a *myo*-inositol derivative with phosphoroselenoyl chloride. We also developed a new protecting group strategy that utilizes allyl and alloc groups to synthesize complex glycolipids bearing unsaturated lipids. These developments provided an efficient synthetic route for various complex inositol phospholipids and their analogues. Furthermore, we have evaluated the binding affinities of the synthetic inositol phospholipids against mouse CD1d molecule and the immunostimuratory activities.

Introduction

Glycosylphosphatidylinositols (GPIs) are a class of complex glycolipids that are widely expressed in eukaryotes.[1] In protozoa, these glycoconjugates are major components of the cell surface that were originally identified as virulence factors.[2] They also have critical functions in host cell invasion[3] and in evading innate immune responses[4] during infection. Owing to their biological importance, particularly in the development of diagnostic agents or vaccines, several total syntheses of protozoan GPIs have been described.[5]

Entamoeba histolytica, an intestinal protozoan parasite, causes severe amoebiasis such as amoebic liver abscess (ALA) and accounts for 100,000 deaths worldwide annually[6]. It has recently been reported that *E. histolytica* lipopeptidophosphoglycan (*Eh*LPPG), which is a major component of the cell membrane, plays an important role in ALA development in a mouse model through the host's immune responses.[7] *Eh*LPPG has unique GPI-like structure: Gal_1Man_2GlcN-*myo*-inositol-PO_3-lipid,[8] which contains the inositol phospholipids; EhPIa and EhPIb.[7]

These structures have a characteristic long-chain unsaturated fatty acid moiety either 28 or 30 carbon atoms in length. An additional fatty acid exists at the 2-position of *myo*-inositol in EhPIb (Figure 1). The configuration of the *sn*-2 position of the glycerol moiety and the galactose-linked site were not clearly determined by spectroscopic data and were inferred based on the previously reported configurations and glycosylation site on similar types of protozoan GPI anchor, as the *sn*-1 position of the glycerol connected to a long fatty acid chain as a 2*R* configuration[7] and α-1,2 glycosidic linkage between the terminal glycan and mannose. The diacylated inositol phospholipid structure, EhPIb, showed similar immunostimulatory activity to *Eh*LPPG.[7] Although the isolated *Eh*LPPG and EhPI have been demonstrated to have intriguing biological activities including immunomodulation via CD1d binding, the effects of unique lipids and glycan structures on their biological activities remain unclear due to the heterogeneity of isolated compounds.

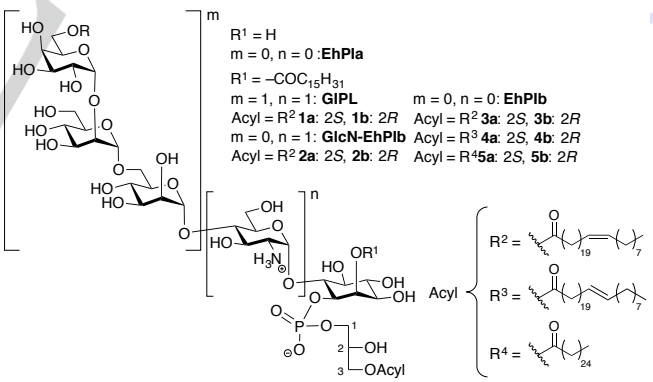

Figure 1. Proposed structure of glycosyl inositol phospholipid and inositol phospholipid from *Entamoeba histolytica.*

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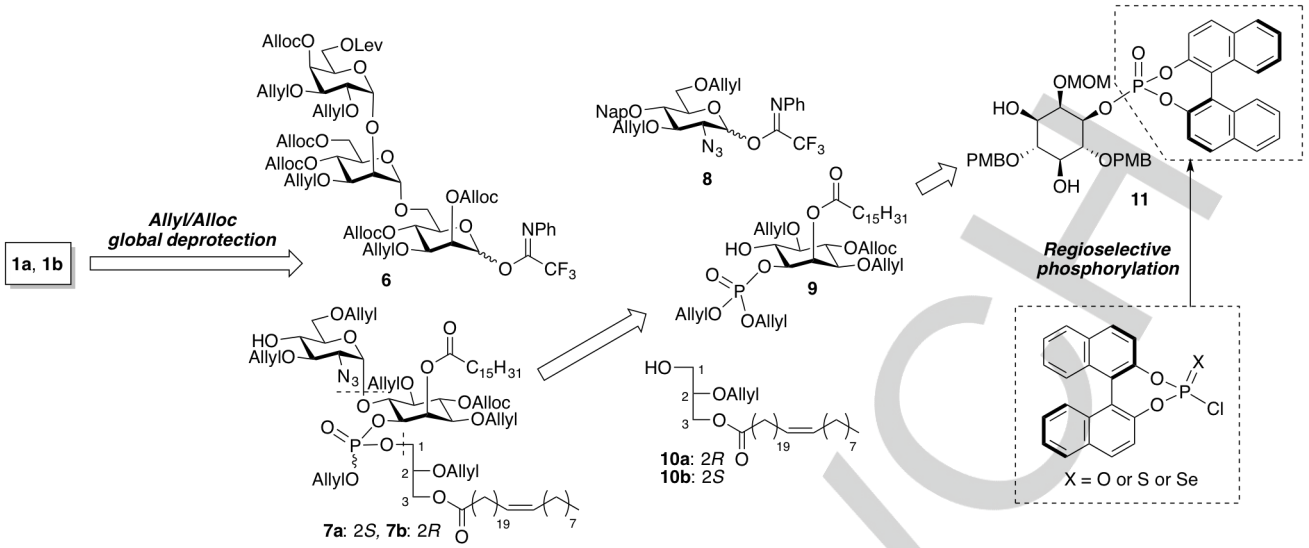


Scheme 1. Retrosynthesis of glycosylinositolphospholipids **1a**, **1b**. Lev = levulinoyl, Alloc = allyloxycarbonyl, Bn = benzyl, Ph = phenyl, Nap = naphthylmethyl, MOM = methoxymethyl,

Several synthetic studies of GPIs in recent years have focused on synthesizing GPIs that bear unsaturated lipids. This outcome is incompatible with the conventional protecting group strategy using benzyl group.[9] In 2006, Nikolaev's group demonstrated a strategy utilizing benzoyl ester for permanent protection of hydroxyl groups for the synthesis of GPI from *T. cruzi* containing unsaturated lipids.[10] More recently, strategies that employ an acid-labile protecting group such as *para*-methoxybenzyl (PMB)[11] and 2-naphthylmethyl (Nap)[12] have been developed. While these strategies have enabled the syntheses of various functionalized GPIs, there are some limitations to their use due to the undesired saponification of fatty acid ester under basic conditions and the undesirable need for the removal of acid-labile protecting groups under acidic conditions.[11b] Thus, we planned a new protecting group strategy to enable the removal of the protecting group under neutral conditions at the final stage.

The site-selective introduction of a phosphate to *myo*-inositol was required to synthesize inositol lipids. Typical methods for preparing chiral inositol building blocks are chiral resolution[13] and chiral pool synthesis.[14] Recently, several approaches for generating chiral inositol phosphate directly from *meso*-inositol have also been reported. Miller and co-workers reported an enantioselective phosphorylation via desymmetrization of a *meso*-inositol derivative using peptide catalysts,[15] and Jessen and co-workers reported a diastereoselective phosphorylation using a chiral phosphoramidite reagent.[16] We have demonstrated a more effective diastereoselective phosphorylation with utilizing BINOL derived phosphoramidite reagent.[17] The method enabled us to obtain the desired isomer with relatively higher selectivity and was also advantageous for separating diastereomers. On the other hand, our previous method using BINOL-phosphoramidite required an excess of the phosphatizing reagent due to the lower reactivity of BINOL-phosphoramidite compared with BINOL-phosphorochloridate. Therefore, in this study, we aimed to develop an efficient diastereoselective phosphorylation of *meso*-inositol based on our previous results.

Here, we report an efficient strategy for synthesizing glycosyl inositol phospholipids from *E. histolytica* bearing unsaturated lipids. For the structure-activity relationship (SAR) study of the *sn*-2 position of glycerol and glycan structures, we aimed to synthesize the tetrasaccharide derivatives (GIPL) of both stereoisomers of glycerol (**1a** and **1b**) and monosaccharide derivatives (GlcN-EhPIb) (**2a** and **2b**). The key features of our strategy include the use of allyl and allyloxycarbonyl (alloc) groups to protect hydroxy groups permanently, and the regioselective phosphorylation of *myo*-inositol using BINOL-derived phosphoryl chlorides, such as phosphoroselenoyl chloride, to ensure greater reactivity and selectivity. Furthermore we evaluated the binding affinity between synthesized compounds and mouse CD1d (mCD1d) molecule and also their immunostimulatory activities.

Results and Discussion

The retrosynthetic analysis of **1a** and **1b** is shown in Scheme 1. Considering the unique acyl modification at the *myo*-inositol, unsaturated lipid structures at the acylglycerol, and also the effect for the selective phosphorylation, we designed the protecting group strategy. We planned to use allyl and alloc groups for permanent protection of hydroxyl groups. In the final step, these protecting groups were removed by a transition-metal-catalyzed deallylation reaction. The compounds **7a** and **7b** were delivered from α-selective glycosylation using azido-glucose donor **8** and
inositol **9** and subsequent coupling with acylglycerols **10a** and **10b** bearing long-chain unsaturated lipids, which were prepared by a Ni-catalyzed alkyl-alkyl cross coupling reaction.[18] The phosphate group of inositol **9** was introduced during an early-stage in this synthesis through regioselective phosphorylation with BINOL-derived phosphoryl chlorides having O, S or Se.

We initially investigated the regioselective introduction of phosphate group to *meso*-inositol **13**,[17] by using chiral phosphatizing reagents **14a**, **14b** or **14c** (Table 1). In the case of

phosphoryl chloride **14a** (X = O)**,** as we have previously observed,[17] we did not achieve regioselectivity under the present conditions either (entry 1 and 2). Specifically, the reaction of **13** with (*R*)-BINOL-derived phosphoryl chloride (**14a**) gave the desired product **11** at very low yield and with no selectivity (**11**:**12** = 50:50, entry 1). Although using two equivalents of **14a** increased the yield, the regioselectivity was not improved (entry 2). We next examined phosphatizing reagents having sulfur or selenium atoms on phosphorus (**14b** (X = S), **14c** (X = Se)). These reagents have been developed as chiral derivatizing agents for racemic alcohols and amines.[19] Either the sulfur or selenium atom of these reagents can be easily converted to oxygen by the subsequent addition of an oxidant such as *m*CPBA. The use of phosphorothioyl chloride **14b** provided the desired product but at a very low yield. However, the selectivity was slightly increased (**11**:**12** = 67:33) (entry 3). Changing the reagent to phosphoroselenoyl chloride **14c** improved the yield to 79% with good regioselectivity (**11**:**12** = 86:14) (entry 4). These results indicate that as atomic radius increases (O<S<Se), diastereoselectivity improves. Conducting the reaction on a larger scale (ca. 10 g) also gave a similar yield of the desired product with similar selectivity (74%, **11**:**12** = 88:12) (entry 5). Switching the reaction solvent from CH_2Cl_2 to either THF or toluene decreased both the yield and selectivity (entry 6, 7). The diastereomixture of **11** and **12** was separated by the reported procedure to isolate the desired isomer **11**. This method using the phosphoroselenoyl chloride will be also applicable for the various types of substrates.

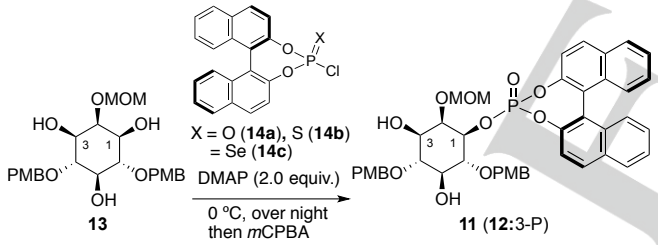


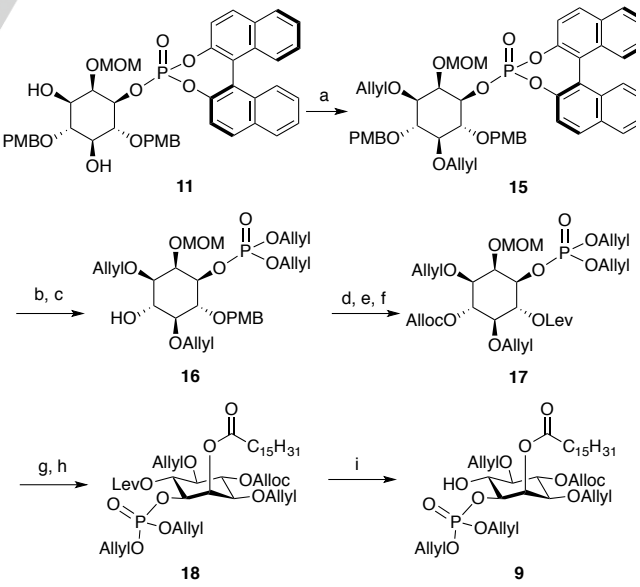
Table 1. Regioselective phosphorylation of **13**

Entry	X (equiv.)	Solvent	Yield (%)[a]	d.r. (**11**:**12**) [b]
1 [c]	O (1.0)	CH_2Cl_2	2	50:50
2 [c]	O (2.0)	CH_2Cl_2	56	50:50
3	S (2.0)	CH_2Cl_2	4 [d]	67:33
4 [e]	Se (2.0)	CH_2Cl_2	79	86:14
5 [f]	Se (2.0)	CH_2Cl_2	74	88:12
6	Se (2.0)	THF	16	76:24
7	Se (2.0)	Toluene	3	61:39

[a] Isolated yield. [b] Determined by ^{1}H NMR analysis. [c] Without *m*CPBA. [d] Yield was determined by ^{1}H NMR analysis. [e] 0.21 mmol of **14c** was used. [f] 20 mmol of **14c** was used. d.r. = diastereomeric ratio, DMAP = 4-dimethylaminopyridine, *m*CPBA = *meta*-chloroperoxybenzoic acid

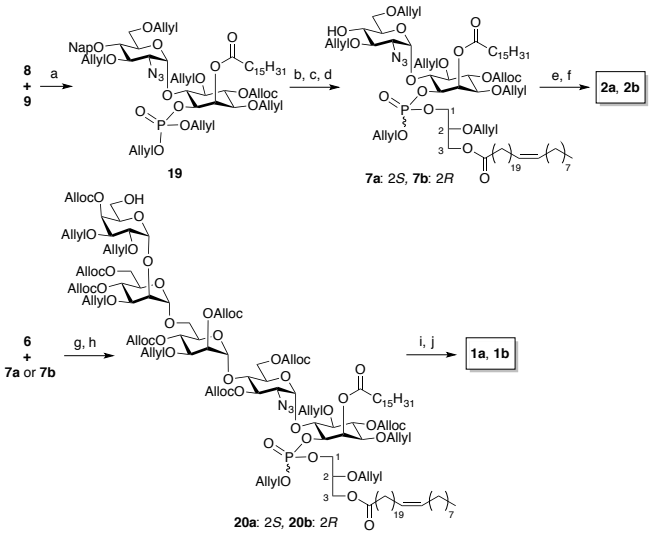
The inositol building block **9** for the synthesis of inositol phospholipids **1a** and **1b** was prepared from compound **11** (Scheme 2). *O*-Allyl protection of **11** with allyl alcohol and catalytic $[CpRu(C_3H_5)(C_9H_6NCO_2)]PF_6$ in 1,2-dichloroethane[20] gave the desired compound **15** in 55% yield. Selective cleavage of 4-*O*-PMB in **15** with DDQ generated the corresponding alcohol in 57% yield (71% brsm). The sterically hindered environment of 6-*O*-PMB by BINOL presumably caused this regioselectivity. Transesterification to allyl ester gave the inositol **16**. After protecting group manipulations and introduction of a palmitoyl group, we obtained the desired inositol acceptor **9**.

With the inositol building block **9** in hand, we advanced the synthesis of inositol phospholipids **1a**, **1b**, **2a** and **2b** (Scheme 7). Glycosylation between **9** and azido-glucose donor **8** using TMSOTf in Et_2O/CH_2Cl_2 provided **19** in 81% yield (α/β = 4:1). The pseudodisaccharides **7a** and **7b** were obtained by Mitsunobu coupling[21] of mono-acylglycerol **10a** or **10b** and removal of the Nap group. The deallylation reaction with $Pd(PPh_3)_4$ in a high polarity solvent[22] proceeded smoothly and was followed by reduction of the azide group with Zn-Cu. Subsequent purification with a Sephadex LH-20 column produced GlcN-EhPIb **2a** and **2b** at 75% and 66% for the two steps, respectively. The glycosylation of trisaccharide **6** (see Supporting Information) and inositol **7a** and **7b** followed by cleavage of the Lev group generated **20a** and **20b**. Finally, global deprotection in a similar manner led to **1a** and **1b** (58% for **1a**, 60% for **1b** in two steps). Structures of the target compounds were characterized with ^{1}H, ^{13}C, ^{31}P and 2D NMRspectroscopy and ESI-Q-TOF mass spectrometry.



Scheme 2. Synthesis of inositol building block **9**. Reagents and conditions: a) $[CpRu(C_3H_5)(C_9H_6NCO_2)]PF_6$, allyl alcohol, 1,2-dichloroethane, reflux, 55%; b) DDQ, CH_2Cl_2/pH 7 phosphate buffer, 0 ºC, 57% (71% brsm); c) allyl alcohol, NaH, THF, 60%; d) AllocOBt, DMAP, CH_2Cl_2, 97%; e) DDQ, CH_2Cl_2/pH 7

phosphate buffer, 96%; f) levulinic acid, EDCI•HCl, DMAP, CH_2Cl_2, 80%; g) 50% TFA in CH_2Cl_2, 0 ºC; h) palmitic acid, DCC, DMAP, CH_2Cl_2, 76% for two steps; i) H_2NNH_2·AcOH, CH_2Cl_2/MeOH, 97%. THF = tetrahydrofuran, DDQ = 2,3-dichloro-5,6-dicyano-*p*-benzoquinone, Bt = benzotriazole, TFA = trifluoracetic acid, EDCI = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide.



Scheme 3. Synthesis of GlcN-EhPIb **2a**, **2b** and GIPL **1a**, **1b**. Reagents and conditions: a) TMSOTf, MS4A, CH_2Cl_2/Et_2O, –20 ºC to rt, 81% (a/b = 4/1); b) LiBr, acetone, reflux; c) **10a** (for **7a**) or **10b** (for **7b**), DEAD, PPh_3, THF; d) DDQ, CH_2Cl_2/pH 7 phosphate buffer, **7a**: 27%, **7b**: 32% for three steps; e) $Pd(PPh_3)_4$, 1,3-DMBA, CH_2Cl_2/MeOH/H_2O, 40 ºC; f) Zn-Cu, AcOH, THF/MeOH/H_2O, **2a**: 75%, **2b**: 66% for two steps; g) TMSOTf, MS4A, CH_2Cl_2/Et_2O, –20 ºC to rt; h) H_2NNH_2·AcOH, CH_2Cl_2/MeOH, **20a**: 38%, **20b**: 22% for two steps; i) $Pd(PPh_3)_4$, 1,3-DMBA, CH_2Cl_2/MeOH/H_2O, 40 ºC; j) Zn-Cu, AcOH, THF/MeOH/H_2O, **1a**: 60%, **1b**: 58% for 2 steps. DEAD = diethyl azodicarboxylate, 1,3-DMBA = 1, 3-dimethylbarbituric acid.

We next investigated the binding affinity between mCD1d and the synthesized glycosyl inositol phospholipids possessing *R*-configuration at glycerol, tetrasaccharide **1b** and GlcN-EhPIb **2b**, by competitive binding ELISA based on reported protocols[23]. We used protein G coated plate for capturing mCD1d-Fc fusion protein and 18:1 biotinylated phosphatidylethanolamine (PE) as an indicator, which was shown to bind mCD1d molecule (IC_{50} value of non-labeled PE: 1.33 μM.[23]) In this competition assay, **1b** and **2b** indicated direct binding to mCD1d and showed lower affinity than the known CD1d ligand, α-galactosylceramide (α-GalCer; 0.257 μM), and **2b** (1.62 μM) displayed slightly lower IC_{50} value than **1b** (3.89 μM). The data demonstrated that this type of inositol phospholipids directly bind to mCD1d, for the first time. We also analyzed the immunostimulatory activities of the synthesized glycosyl inositol phospholipids compared to the isolated natural *Eh*LPPG. The cytokine induction of mouse spleen cells was analyzed by enzyme-linked immunosorbent assay (ELISA) (Figure 2B). The GlcN-EhPIb **2b** showed higher IFN-γ inducing activities than tetrasaccharide **1b** and *Eh*LPPG in the cytokine induction assay. The *Eh*LPPG has been shown to activate NKT cells by the presence of CD1d and simultaneously TLR receptor signaling.[7] The synthesized fragment structures of *Eh*LPPG will lead to further investigations of the immunomodulatory mechanism of the *Eh*LPPG.

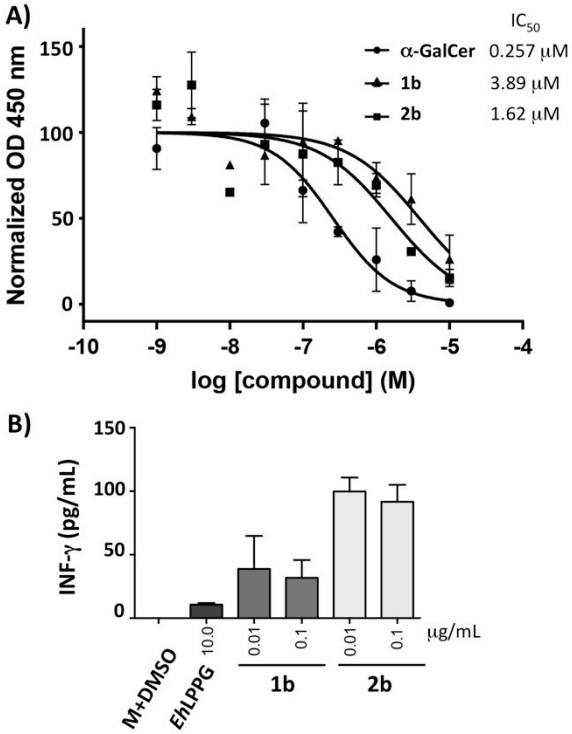


Figure 2. A) The binding affinity of synthesized compounds **1b**, **2b** and α-GalCer against mCD1d with the competitive ELISA. The assays were done in duplicate and the error bars indicate Standard Deviation (SD). B) Induction of IFN-γ in murine spleen cells by synthetic inositol phospholipids 1b and 2b in comparison with the isolated *Eh*LPPG. The assays were done in duplicate and the error bars indicate Standard Deviation (SD).

Conclusions

In conclusion, we have developed a regioselective phosphorylation of *myo*-inositol using phosphoroselenoyl chloride, and successfully implemented the protecting group strategy utilizing allyl/alloc groups for permanent protection of hydroxyl groups. Using these methods, we successfully accomplished the chemical synthesis of glycosyl inositol phospholipids **1a**, **1b**, **2a**, and **2b** from *E. histolytica*. The synthetic method we presented in this report has the potential for synthesizing various inositol phospholipids bearing reduction-sensitive, acid or base-labile functionalities. We evaluated the binding affinity of the synthesized **1b** and **2b** against mCD1d, and obtained the direct binding against mCD1d. We also analyzed the immunostimulatory activities of synthesized compounds, revealing that **2b** induced higher levels of IFN-γ

secretion than *Eh*LPPG. The further investigation of their biological activities including **1a** and **2a** is now underway.

Experimental Section

Detailed synthetic procedures and their characterization can be found in the Supporting Information.

Enzyme-linked immunosorbent assay (ELISA)-based competitive binding assay

Pierce protein G coated 96 well plates (Thermo Fisher Scientific) were washed with 200 µL of PBST (PBS containing 0.05% Tween-20) three times. 50 µL of mouse CD1d:Ig fusion protein (BD bioscience) (10 µg/mL in PBS) for 1h at room temperature. 25 µL of Serially diluted inositol phospholipids and α-galactosylceramide (GalCer) with PBS were added to the plates and then added 25 µL of 18:1 biotinyl PE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(Biotinyl), Avanti Polar Lipids) (8 µg/mL in PBS) and incubated for overnight at 37 ºC. After washing the plates with PBST three times, mCD1d:Ig-biotinyl PE complex was detected with HRP-labeled Avidin (Biolegend). Inhibition curves were constructed using GraphPad Prism software (ver. 7.02, GraphPad).

Mouse spleen cell stimulation assay

Spleen cells were isolated from C57BL/6 mice, seeded at a concentration of $2x10^5$/well and incubated with isolated *Eh*LPPG or synthetic inositol phospholipids at indicated concentrations for 48 h. IFN-γ induction in the supernatant was determined by ELISA.

Acknowledgements

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Keywords: inositol phospholipid • phosphorylation • synthetic strategy • *Entamoeba histolytica* • CD1d

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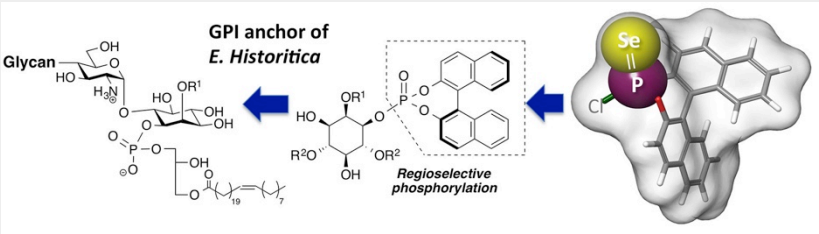

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