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Design and synthesis of lipid-coupled inositol 1,2,3,4,5,6-hexakisphosphate derivatives exhibiting high-affinity binding for HIV-1 MA domain

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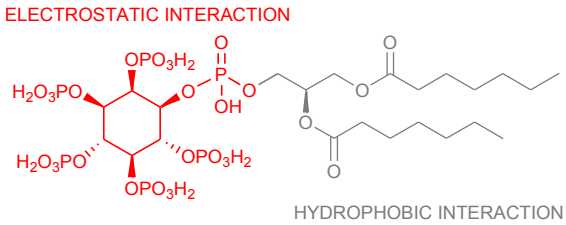


Lipid coupled IP_6: *K*d=0.25 μM for HIV-1 MA

Lipid-coupled inositol 1,2,3,4,5,6-hexakisphosphate binds to HIV-1 MA tightly through both electrostatic and hydrophobic interactions.

Abstract

Precursor of Gag protein ($Pr55^{Gag}$) of human immunodeficiency virus, the principal structural component required for virus assembly, is known to bind D-*myo*-phosphatidylinositol 4,5-bisphosphate (PIP_2). The N-terminus of $Pr55^{Gag}$, MA domain, plays a critical role in the binding of $Pr55^{Gag}$ to the plasma membrane. Herein, we designed and synthesized *myo*-phosphatidylinositol 2,3,4,5,6-pentakisphosphate (PIP_5) derivatives comprising highly phosphorylated inositol and variously modified diacylglycerol to examine the MA-binding property. The inositol moiety was synthesized starting with *myo*-inositol and assembled with a hydrophobic glycerol moiety through a phosphate linkage. The *K*d value for MA-binding of the PIP_5 derivative **2** (*K*d=0.25 μM) was the lowest (i.e., highest affinity) of all derivatives, i.e., 70-fold lower than the *K*d for the PIP_2 derivative **1** (*K*d=16.9 μM) and 100-fold lower than the *K*d for IP_6 (*K*d=25.7 μM), suggesting the possibility of the PIP_5 derivative to block the $Pr55^{Gag}$ membrane binding by competing with PIP_2 in the MA-binding.

1. Introduction

The development of anti-human immunodeficiency virus type 1 (HIV-1) drugs has achieved marked success in the past two decades as envisaged by reverse transcriptase inhibitors, protease inhibitors, entry inhibitors, and integrase inhibitors. However, because the use of these drugs has encountered limitations because of the emergence of resistant viral variants, the development of new drugs based on novel mechanisms has become urgent. This study focused on the membrane targeting of the HIV-1 precursor of Gag protein ($Pr55^{Gag}$) at the stage of virus assembly, exploiting the possibility to block the virus assembly by small molecules that compete at the membrane binding of $Pr55^{Gag}$.

HIV-1 genome-encoded $Pr55^{Gag}$ protein is the principal structural component required for virus assembly[1,2]. Following ribosomal synthesis, $Pr55^{Gag}$ is directed to the plasma membrane, where it is assembled with other components to form immature budding virions. The N-terminus of $Pr55^{Gag}$, the MA domain, plays a critical role in the binding of $Pr55^{Gag}$ to the plasma membrane[3]. Recent studies have shown that D-*myo*-phosphatidylinositol 4,5-bisphosphate (PIP_2) is the binding target of the basic patch of the MA domain[4-6].

We previously developed a highly sensitive *in vitro* assay to determine the binding affinity of $Pr55^{Gag}$/MA for phosphoinositide derivatives by employing a surface plasmon resonance (SPR) sensor in which a synthetic biotinylated inositol phosphate was immobilized[7-9]. The SPR experiments comparing the $Pr55^{Gag}$/MA affinity of IP_3 and PIP_2 suggested that both the divalent phosphate groups and the acyl chains of PIP_2 are essential for tight binding to $Pr55^{Gag}$/MA.

Because the PIP_2-binding region of the MA domain contains many basic residues that interact with acidic phosphate groups of the inositol[2,10,11], the MA-binding affinity of phosphatidylinositol derivatives would be increased by increasing the number of phosphate groups. This, together with the several previously published studies[2,10,11], would provide the basis for the molecular design of novel competitors that would block the PIP_2-$Pr55^{Gag}$ binding.

Herein, we performed SPR analysis of the MA domain binding of highly phosphorylated inositol phosphates, *myo*-inositol 1,2,3,4,5,6-hexakisphosphate (IP_6), D-*myo*-inositol 1,4,5-trisphosphate (IP_3), and a synthetic PIP_2 derivative having non-natural C8 acyl chains **1** (**Figure 1a**) and found that IP_6 bound MA strongly, demonstrating the significance of the number of the phosphate group. Further, we designed and synthesized lipid-coupled IP_6 derivatives, namely

myo-phosphatidylinositol 2,3,4,5,6-pentakisphosphate (PIP_5) derivatives, expecting their MA binding would be stronger than PIP_2 leading to the blockade of the $Pr55^{Gag}$ membrane target.

2. Results and Discussion

2.1. SPR analysis of MA-interaction of IP_3, IP_6, and PIP_2

To compare the relative MA-binding affinity of IP_6, IP_3, and the PIP_2 derivative **1** (**Figure 1a**), we performed SPR assay that we previously constructed[7]. An expression vector for MA having a FLAG tag at the C-terminus was used. Proteins were purified from transfected 293T cells using anti-FLAG agarose beads employing the FLAG tag affinity method. Purified proteins were quantified by SDS-PAGE analysis, and their concentration was estimated by comparing the band intensity with that of the protein marker. After purification, the solution in which each protein was dissolved was exchanged with flow buffer in the SPR system through dialysis. Flow buffer was supplemented with 0.5 mg/mL BSA to inhibit non-selective binding to the biotin-modified control surface, followed by 2% (v/v) glycerol to prevent protein destabilization[12]. Contrary to the previous SPR analysis[7], 5% dimethylsulfoxide was also supplemented with analysis buffer to dissolve complexes in this experiment (**Supplementary Information 2**). Association was followed for 3 min and dissociation was measured at a flow rate of 20 µl/min at 25°C, after which the surfaces were regenerated by injecting dilute NaOH solution. As shown in **Figure 1b**, the injection of 0.24, 0.48, 0.64, and 0.96 µM MA onto immobilized D-*myo*-inositol 1,3,4,5-tetrakisphosphate (IP_4) showed a concentration-dependent response unit (RU).

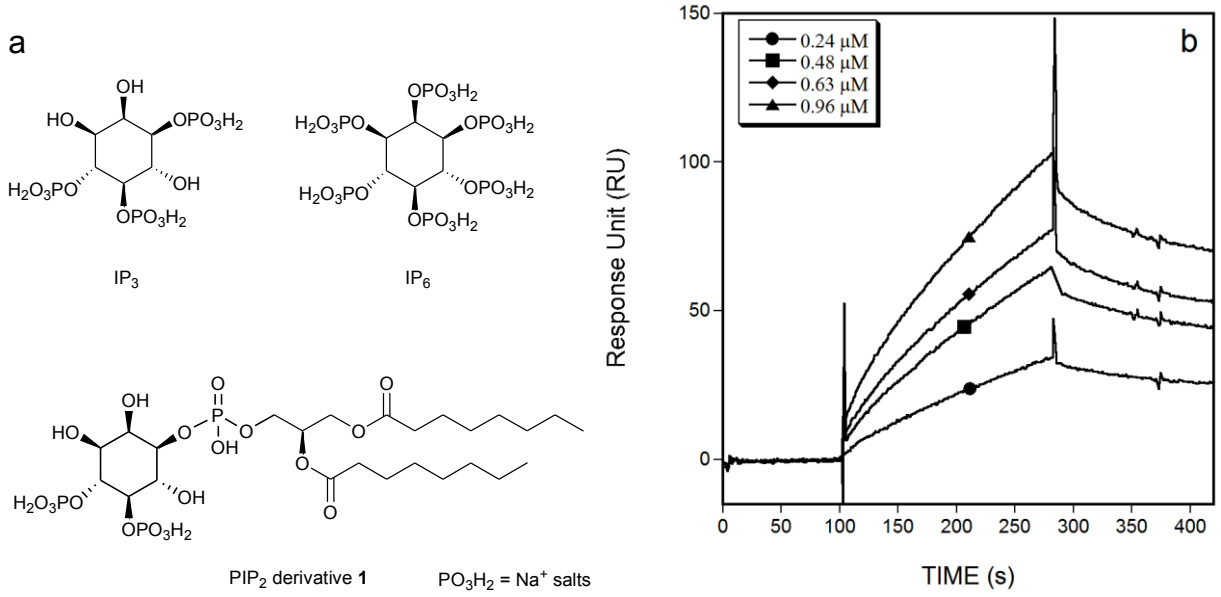
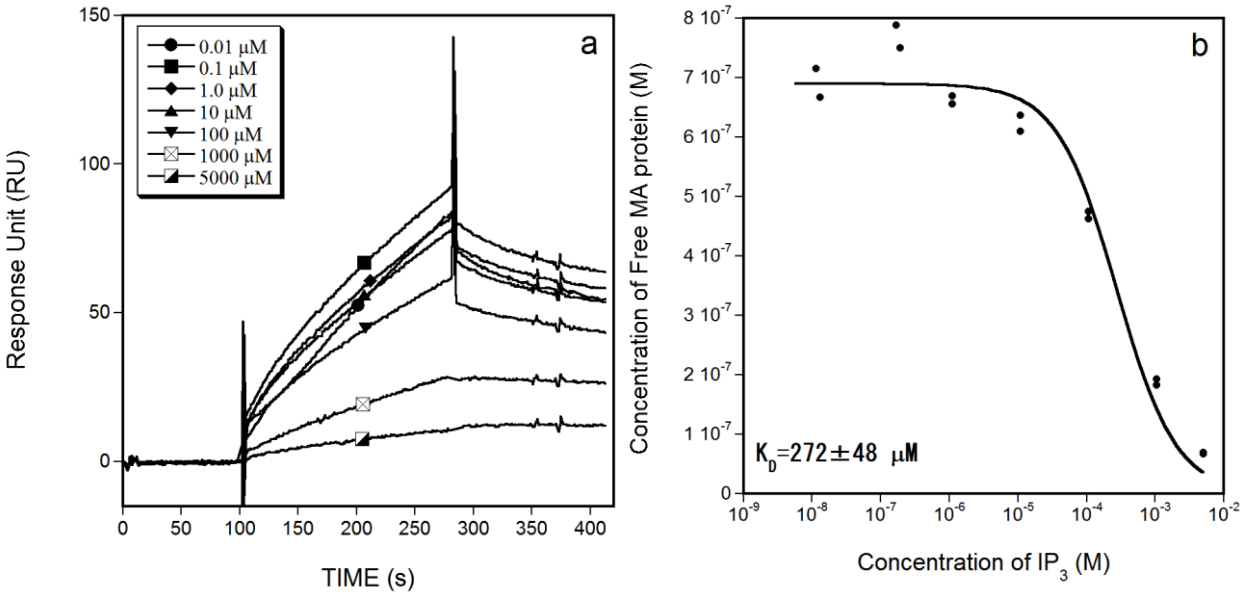


Figure 1 Structures of IP_3, IP_6, and the PIP_2 derivative **1** (**a**). Binding activity of 0.24, 0.48, 0.64 and 0.96 µM MA proteins to biotinylated IP_4. Each protein was injected over a biotinylated IP_4-immobilized sensor chip at flow rate of 20 µl/min for 180 s (**b**).

The dissociation constants (*K*d) of MA-IP_3, MA-IP_6, and MA-**1** complexes were calculated via a competition assay. Solutions containing varying concentrations of each competitor were preincubated with MA and passed over the immobilized IP_4 surface. The competition curves were obtained by setting the concentration of competitors upon the horizontal axis and the response of free MA, determined based on the concentration of MA bound to immobilized-IP_4, upon the vertical axis. The RU curves for competition between MA and the various competitors are shown in **Figure 2a**, **c**, and **e**; the corresponding *K*d values are shown in **Figure 2b**, **d**, and **f**. The *K*d value for MA in competition with IP_3 was 272 μM (**Figure 2b**), indicating IP_3 binds MA weakly. It was noteworthy that IP_6 showed *K*d (25.7 μM) (**Figure 2d**) comparable to that of **1** (16.9 μM) (**Figure 2f**), although IP_6 does not possess the diacylglycerol moiety. These findings suggested that the MA-affinity would be further increased by introducing a diacylglycerol into IP_6.



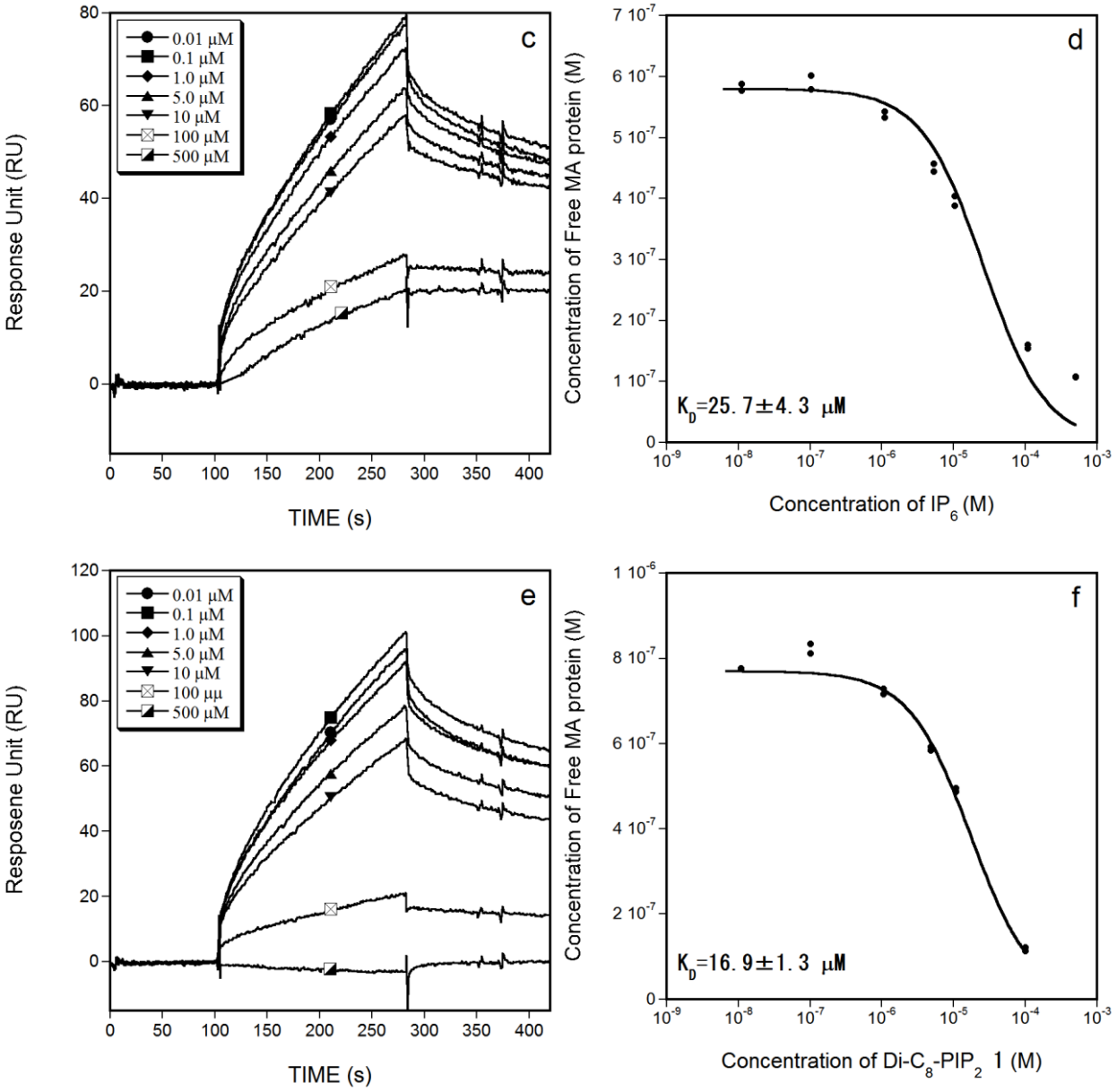


Figure 2 Competition assay and calculation of the equilibrium dissociation constants (*K*d) for MA-competitor complexes. The equilibrium mixtures of MA and competitors IP_3 (**a**), IP_6 (**c**), and the PIP_2 derivative **1** (**e**) were injected over the biotinylated IP_4-immobilized sensor chip at a flow rate of 20 μl/min for 180 s. The average response unit (RU) for the increasing concentration of each competitor was measured at 160–170 s, and each RU datum was converted to a concentration of uncompetitive MA protein used for the construction of competition curves between uncompetitive MA and IP_3 (**b**), IP_6 (**d**), and the PIP_2 derivative **1** (**f**). Calculated *K*d values are shown. Each experiment was performed in duplicate.

2.2. Design and synthetic strategy of PIP_5 derivatives

We designed PIP_5 derivatives having modified glycerol moiety (**Figure 3**). To compare the influence of the aliphatic chain structure of the glycerol group, both acyl (**compound 2**) and alkyl ether (**compound 4**) derivatives were designed. To confirm that the 2'-acyl chain participates in PIP_2-MA binding and the 1'-acyl does not[5], 1'-*O*-methyl-2'-acyl/alkyl derivatives (**compound 3**, **compound 4**) were designed. Our synthetic strategy for the PIP_5 derivatives (**Figure 3**) was to differentiate the six hydroxyl groups of *myo*-inositol through the diacetal intermediate[13], and the suitably protected intermediate was coupled with an acyl/alkyl-glycerol moiety by a bifunctional phosphorylating agent[14]. A 1,5-dihydro-2,4,3-benzodioxaphosphepin-3-yl group was employed for the synthesis of the acyl derivatives (i.e., **12**), whereas 2-cyanoethyl group was used for phosphorylating agent of the alkyl ether derivatives (i.e., **12**).

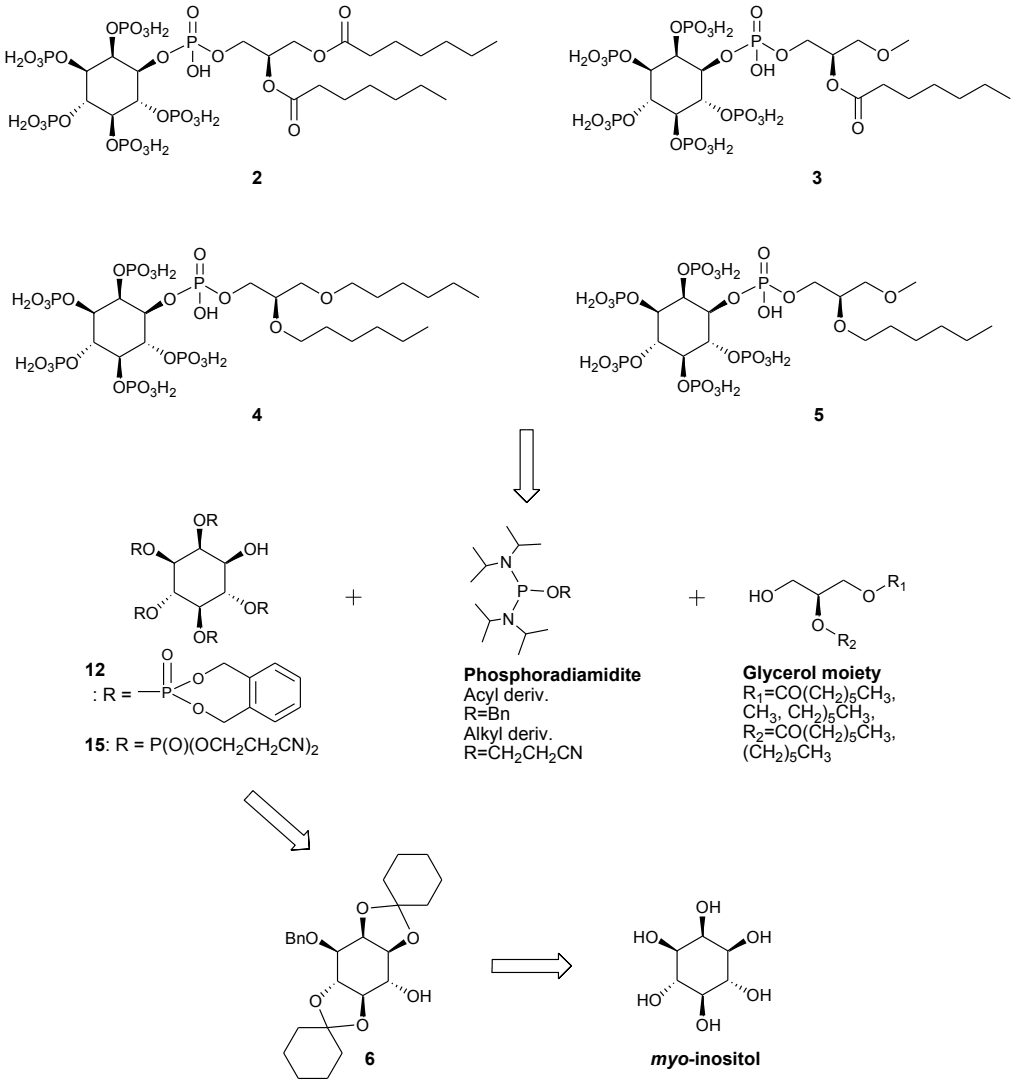
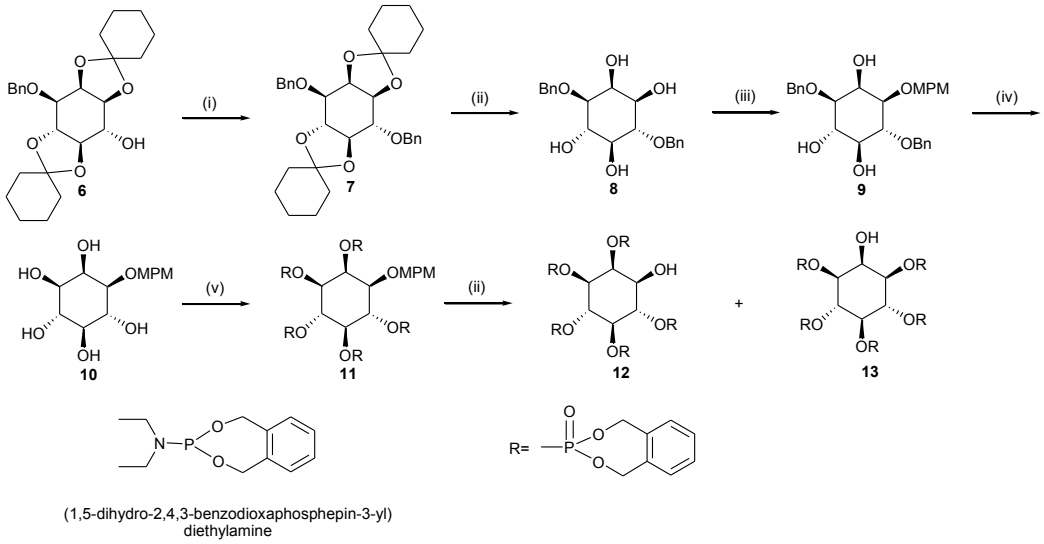


Figure 3 Design and synthetic strategy of PIP_5 derivatives

205 **2.3. Syntheses of the IP_6 moiety**

206 The syntheses of the IP_6 moiety for acyl derivatives were performed as shown in **Scheme 1**. The
207 starting material DL-3-*O*-benzyl-1,2:4,5-di-*O*-cyclohexylidene-*myo*-inositol **6** was prepared
208 according to the method of Billington *et al.*[13]. Benzylation of the alcohol **6** provided **7,** which was
209 further treated with *p*-toluenesulfonic acid and H_2O to give deacetalized **8** in 76% yield (for 2 steps).
210 The *cis*-1,2-diol of **8** was regioselectively *p*-methoxybenzylated by means of the dibutyltin oxide
211 procedure[15,16]. Thus, the tin complex of the 1,2-diol was reacted with *p*-methoxybenzyl chloride in
212 the presence of cesium fluoride to give regioselectively protected **9** in 89% yield. The selective
213 deprotection of the benzyl group of **9** by the method of Oikawa *et al.*[17] gave **10** in 45% yield. The
214 2,3,4,5,6-pentahydroxy compound **10** was converted to the corresponding pentakisphosphonate **11**
215 by treatment with (1,5-dihydro-2,4,3-benzodioxaphosphepin-3-yl)diethylamine[18] and 1*H*-tetrazole
216 and subsequent oxidation with MCPBA in 75% yield. Oxidative cleavage of *p*-methoxybenzyl
217 group with CAN[19] gave the desired IP_6 fragment **12**, accompanying a phosphate migration product
218 **13** in which the *O*-xylyl protected phosphate group at the 2-phosphate group migrated to the
219 1-phosphate allocating a stable conformation of *myo*-inositols[18]. Because compounds **12** and **13**
220 could not be separated, the mixture was used for the next coupling reaction without separation.



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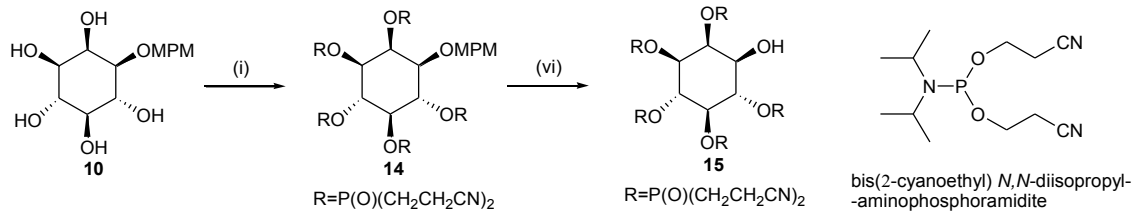
222 **Scheme 1** *Reagents and conditions*: (i) benzyl bromide, NaH, DMF, rt, overnight, 94%; (ii) TsOH,
223 THF-H_2O, reflux, 5 h, 81%; (iii) (a) Bu_2SnO, toluene, reflux, 3 h; (b) CsF, MPM-Cl, DMF, –40°C
224 then rt, 48 h, 89%; (iv) H_2/W-2 Raney-Ni, MeOH, 50°C, 3 h, 45%; (v) (a)
225 (1,5-dihydro-2,4,3-benzodioxaphosphepin-3-yl)diethylamine, 1*H*-tetrazole, CH_2Cl_2, rt, overnight;
226 (b) MCPBA, CH_2Cl_2, –40°C then rt, 1 h, 75%; (x) CAN, CH_3CN-H_2O, rt, 1 h.

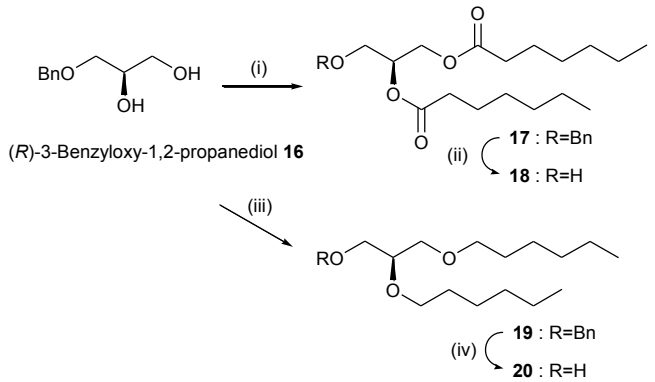
The synthesis of the IP_6 moiety for alkyl ether derivatives was performed as shown in **Scheme 2**. The 2,3,4,5,6-pentahydroxy compound **10** was converted to the corresponding pentakisphosphonate **14** by treatment with bis(2-cyanoethyl)-*N,N*-diisopropylphosphoramidite[20] and 1*H*-tetrazole and subsequent oxidation with MCPBA in 73% yield. Oxidative cleavage of *p*-methoxybenzyl group with CAN[19] gave the IP_6 fragment **15** in 68% yield.



Scheme 2 *Reagents and conditions*: (i) (a) bis(2-cyanoethyl)-*N,N*-diisopropylaminophosphoramidite, 1*H*-tetrazole, CH_2Cl_2, rt, 1.5 h; (b) MCPBA, CH_2Cl_2, −78°C then rt, 5 min, 73%; (ii) CAN, CH_3CN-H_2O, rt, 1.5 h, 68%.

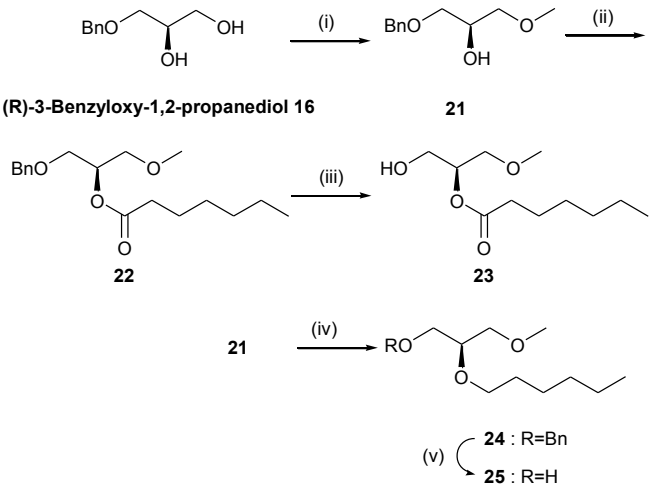
2.4. Syntheses of di/mono-acylglycerol, di/mono-alkylglycerol moiety

The syntheses of diacylglycerol and dialkylglycerol moiety were performed as shown in **Scheme 3**. The commercially available starting material (*R*)-3-benzyloxy-1,2-propanediol **16** was reacted with heptanoyl chloride under basic conditions to give compound **17** in 86 % yield. The deprotection of the benzyl group of **17** gave **18** in 96 % yield. Compound **20** was obtained by dialkylation of **16** followed by the benzyl deprotection in 59% yield (for 2 steps).



Scheme 3 *Reagents and conditions*: (i) heptanoyl chloride, DMAP, pyridine, CH_2Cl_2, overnight, 86%; (ii) H_2/Pd-C, CH_2Cl_2, overnight, 96%; (iii) hexyl bromide, NaH, DMF, rt, overnight, 70%; (iv) H_2/Pd-C, CH_2Cl_2, 24 h, 84%.

The syntheses of the monoacylglycerol and monoalkylglycerol moieties were performed as shown in **Scheme 4**. The compound **16** was regioselectively methylated by means of the dibutyltin oxide procedure. The tin complex of the 1,2-diol was reacted with methyl iodide in the presence of cesium fluoride to give **21** in 71% yield, accompanying a small amount of 2-*O*-methyl product. Acylation of the 2-hydroxyl of **21** with heptanoyl chloride gave **22** in 93% yield. The deprotection of the benzyl group of **22** gave **23** in 93% yield. Alkylation of the 2-hydroxyl of **21** with hexyl chloride gave **24** in 92% yield. Finally, compound **24** was treated with H_2/10% palladium carbon to afford the debenzylated product **25** in 89% yield.

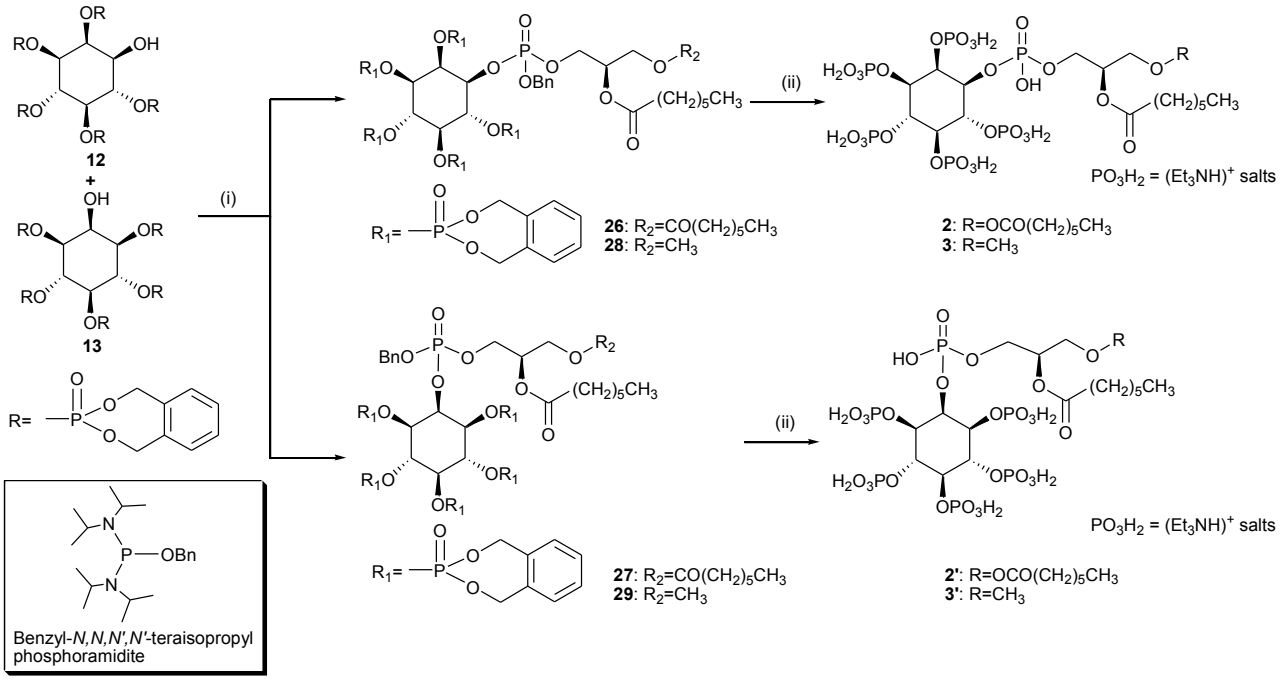


Scheme 4 *Reagents and conditions*: (i) (a) Bu_2SnO, toluene, reflux, 3 h; (b) CsF, methyl iodide, DMF, –40 °C then rt, 2 days, 71%; (ii) heptanoyl chloride, DMAP, pyridine, CH_2Cl_2, overnight, 93%; (iii) H_2/Pd-C, CH_2Cl_2, overnight, 93%; (iv) hexyl-Br, NaH, DMF, rt, overnight, 92%; (v) H_2/Pd-C, CH_2Cl_2, 24 h, 89%.

2.5. Coupling of IP_6 and glycerol fragments

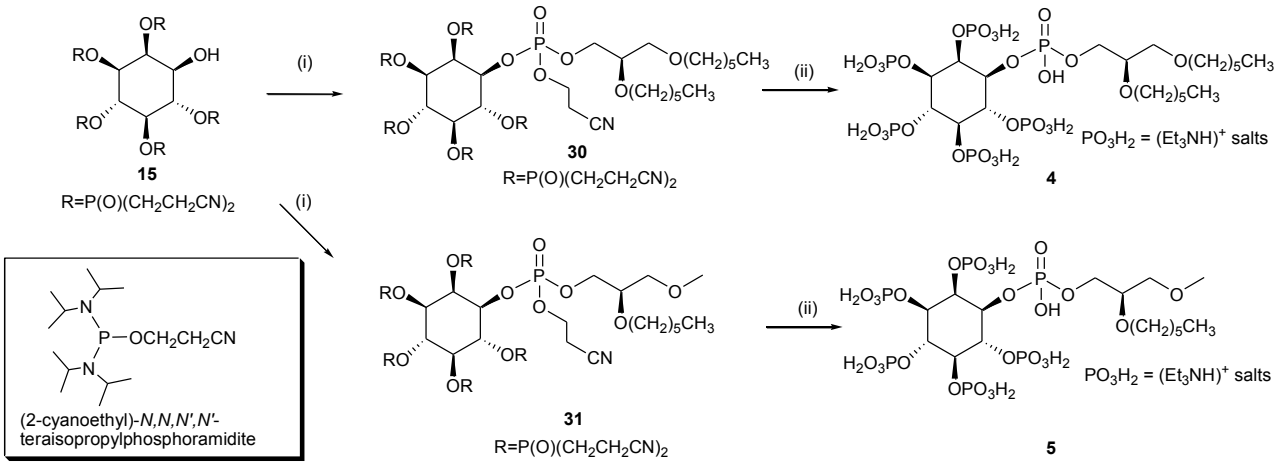
The coupling of acylated glycerol moieties and IP_6 fragments was performed as shown in **Scheme 5**. The glycerol moiety **18** was reacted with benzyl-*N, N, N', N'*- tetraisopropylphosphoramidite[14] and 1*H*-tetrazole and subsequently condensed with the IP_6 fragment mixture **12** and **13**. Oxidation with *tert*-BuOOH gave diheptanoyl glyceryl IP_6 **26** and **27** in 22% and 45% yield, respectively. Finally, the protecting groups were removed by hydrogenolysis with palladium carbon to give diheptanoyl glyceryl PIP_5 derivatives. These PIP_5 derivatives were purified by cation-exchange chromatography to give **2** and its isomer **2'** as a triethylammonium salts in 34% and 35% yield, respectively. The

monoacylglycerol derivatives, **3** and its isomer **3'** as triethylammonium salts, were synthesized by the same procedure.



Scheme 5 *Reagents and conditions*: (i) (a) Benzyl-*N, N, N', N'*-tetraisopropylphosphoramidite, 1*H*-tetrazole, CH_2Cl_2, rt, 15 min; (b) **18** or **23**, 1*H*-tetrazole, CH_2Cl_2, rt, 24 h; (c) *tert*-BuOOH, CH_2Cl_2, rt, 5 min, **26** (22%), **27** (45%), **28** (63%), **29** (11%); (ii) H_2/Pd-C, *t*BuOH-H_2O, 24 h, **2** (34%), **2'** (35%), **3** (44%), **3'** (22%).

The coupling reaction of the IP_6 fragment and the alkylated glycerol moieties was performed as shown in **Scheme 6**. The glycerol moiety **20** or **25** was reacted with bifunctional phosphorylating agent (2-cyanoethyl)-*N, N, N', N'*- tetraisopropylphosphoramidite[14] and 1*H*-tetrazole to yield a rather labile phosphoramidite. This compound was condensed with the IP_6 fragment **20** or **25** without further purification. Oxidation of the condensed product with *tert*-BuOOH gave 1,2-*O*-dihexylglyceryl or 1-*O*-methyl-2-*O*-hexyl IP_6 **30** or **31** in 41% and 63% yield, respectively. Finally, protecting groups were removed by reaction with NH_3 to give water-soluble PIP_5 derivatives that were purified by reverse phase chromatography followed by cation-exchange chromatography to give **4** and **5** as a triethylammonium salts in 64% and 31% yield, respectively.



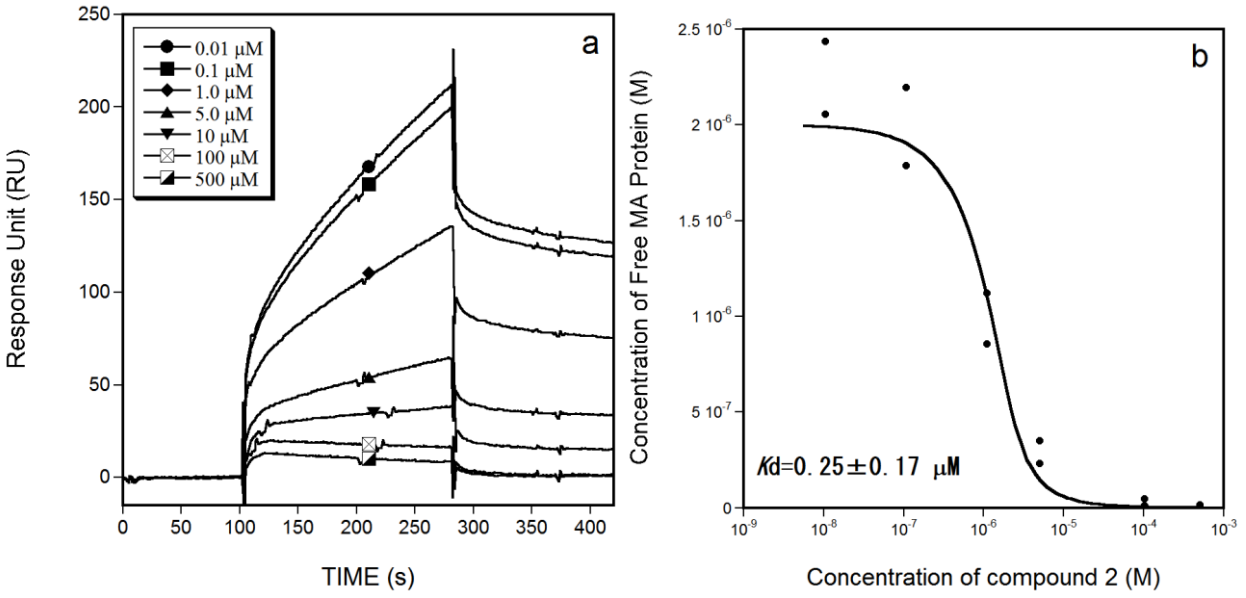
Scheme 6 *Reagents and conditions*: (i) (a) (2-cyanoethyl)-*N, N, N', N'*-tetraisopropylphosphoramidite, 1*H*-tetrazole, CH_2Cl_2, rt, 1.5 h; (b) **20** or **25**, 1*H*-tetrazole, CH_2Cl_2, rt, 2 h; (c) *tert*-BuOOH, CH_2Cl_2, rt, 5 min, **30** (41%) and **31** (63%); (ii) aq. NH_3, MeOH, 55°C, 10 h, **4** (64%) and **5** (31%).

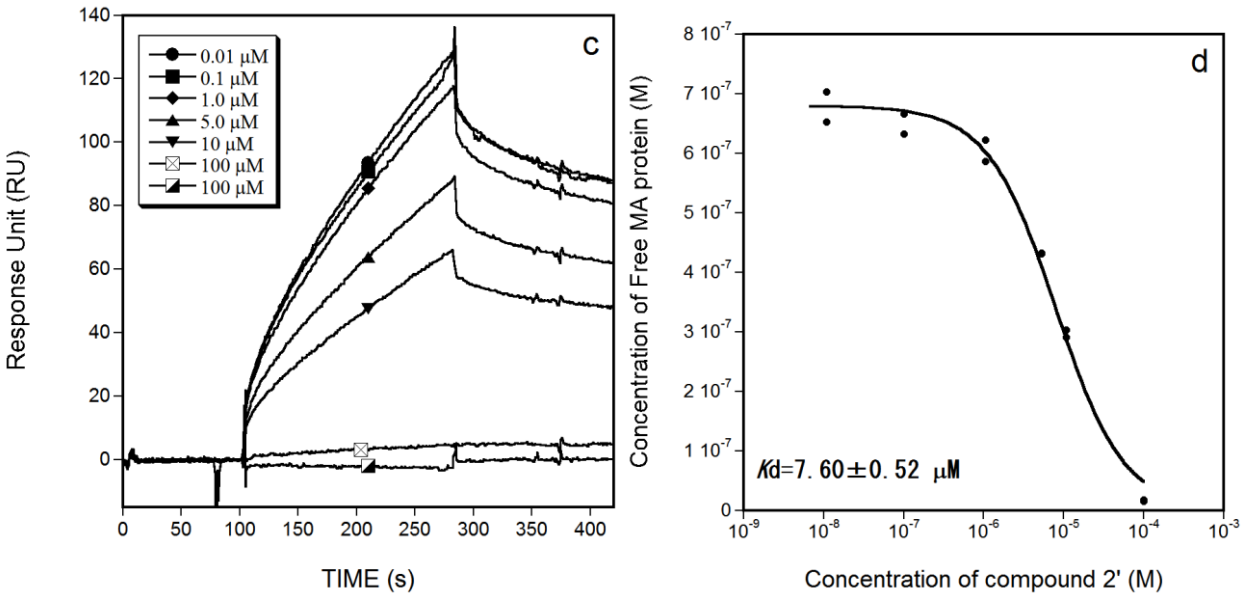
2.6. SPR analysis of MA complexes of PIP_5 derivatives

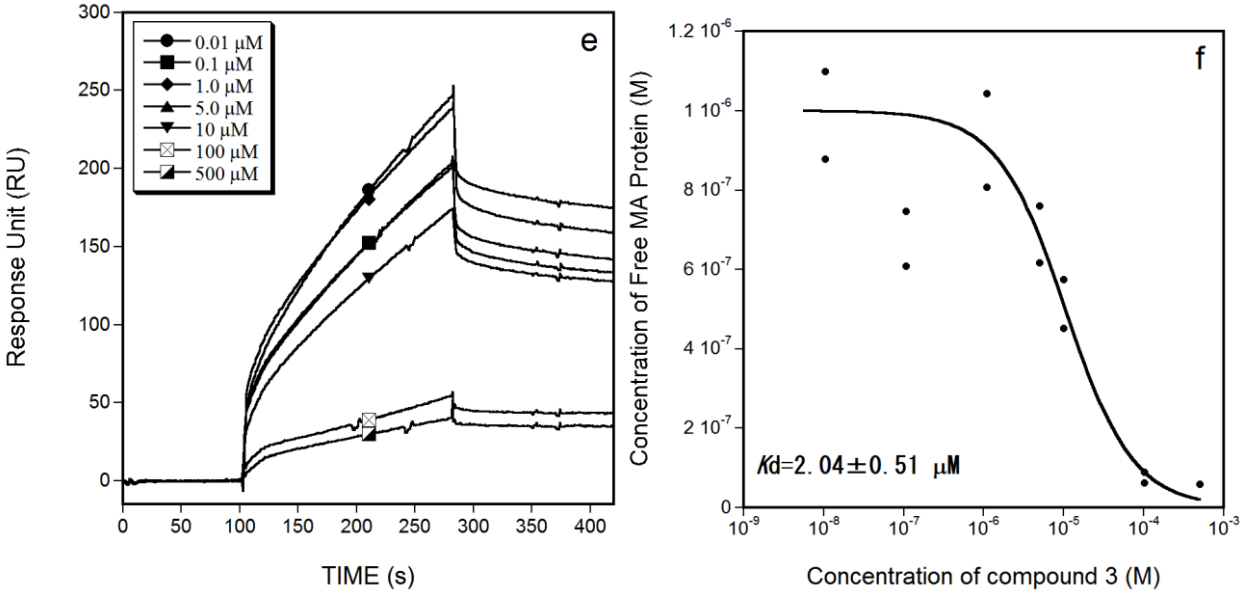
*K*d values of the MA complex of PIP_5 derivatives were calculated by the competition assay as described above. The RU curves for competition between MA and the various competitors are shown in **Figure 4a, c, e, g, i**, and **k**; the corresponding *K*d values are shown in **Figure 4b, d, f, h, j**, and **l**. As illustrated in **Figure 5**, which shows the *K*d of the MA complex of IP_3, IP_6, the PIP_2 derivative **1**, and PIP_5 derivatives with structure, the *K*d values for MA in competition with **2** (*K*d= 0.25 μM) (**Figure 4b**) was the lowest (i.e., highest affinity) of all PIP_5 derivatives, which was 70-fold lower than the *K*d for **1** (16.9 μM) and 100-fold lower than the *K*d for IP_6 (25.7 μM). Therefore, the *K*d value of the **2**-MA complex showed that PIP_5 derivatives having both IP_6 and diacylglycerol moiety interacts with MA tightly. The binding affinity of **2'** was 7.60 μM (**Figure 4d**), which was 3-fold lower than that of the **3**-MA complex (*K*d=2.04 μM) (**Figure 4f**), and almost the same as that of the **2'**-MA complex (*K*d=9.01 μM) (**Figure 4h**). These data showed that the phosphate isomers **2'** and **3'** bound MA more weakly than 1-phosphate derivatives **2** and **3**. In contrast, the MA-binding affinity of **4** having alkyl chain at glycerol moiety was 1.37 μM (**Figure 4j**), which was 18-fold lower than that of the PIP_2 derivative **1**, and was 5-fold higher than that of the diacyl derivative **2** (*K*d=0.25 μM). These data revealed that the diacyl glycerol structure is better than the dialkyl glycerol structure in MA binding. The *K*d value for the **5**-MA complex was 7.98

μM (**Figure 4l**), which was almost the same as that of **2'** and **3'**-MA complex. In SPR analyses, all PIP_5 derivatives bound MA more tightly than the PIP_2 derivative **1**, IP_6 and IP_3. The order of *K*d was **2** < **4** < **3** < **5** = **2'** < **3'** < **1** < IP_6 < IP_3. The structure activity relationship of these compounds revealed that a highly phosphorylated inositol structure and diacyl (not monoacyl) glycerol at a 1-position of inositol are important for MA domain binding.

To confirm the regiochemistry of **2** and **2'**, we synthesized **2** again by an independent route using dibenzyl *N,N*-diethylphosphoramidite that does not cause phosphate migration. In fact, compound **2** was obtained as a sole product without the accompanying isomer **2'**. The newly synthesized **2** showed a *K*d value virtually identical to that obtained before (**scheme 5**), verifying the regiochemistry of **2** (**Supplementary Information 2**).







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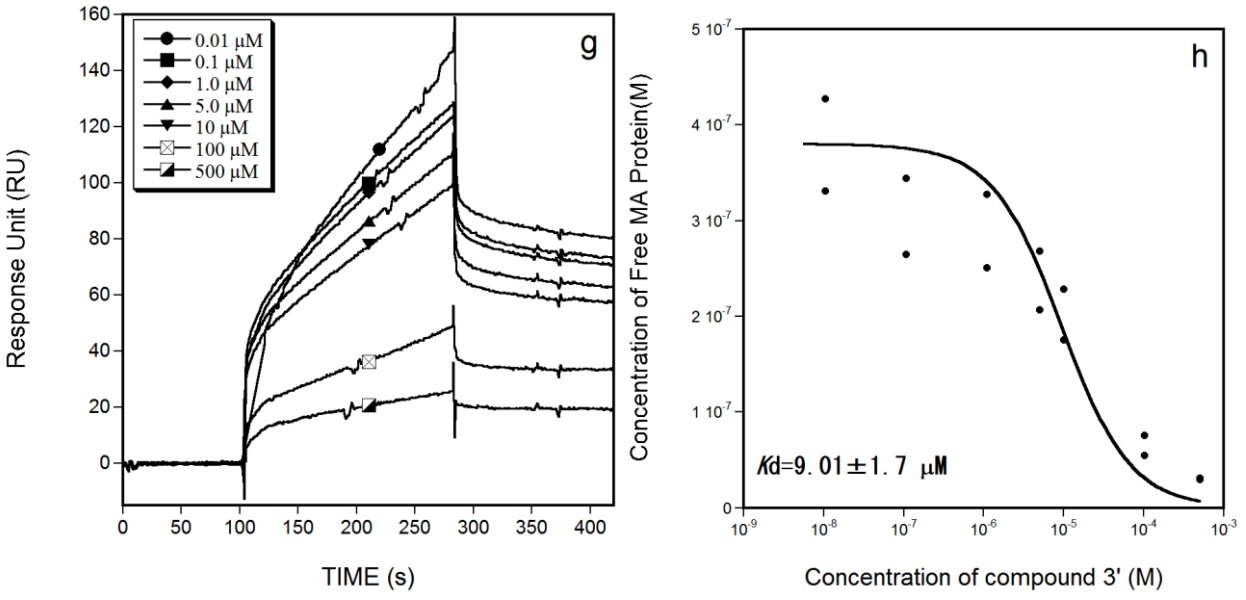

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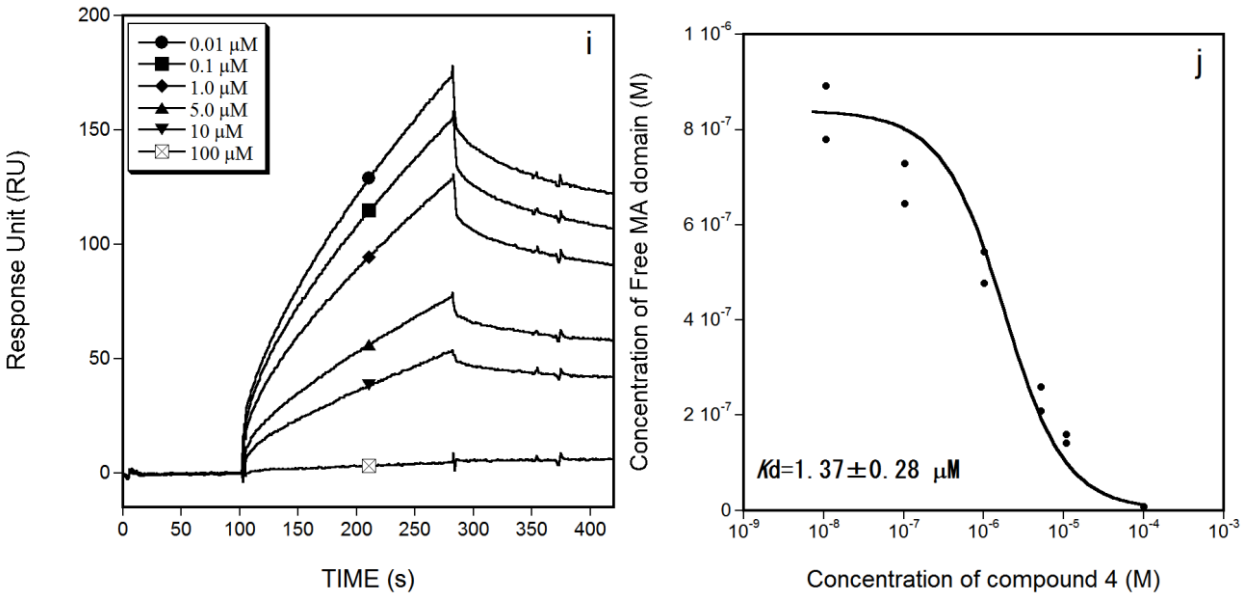

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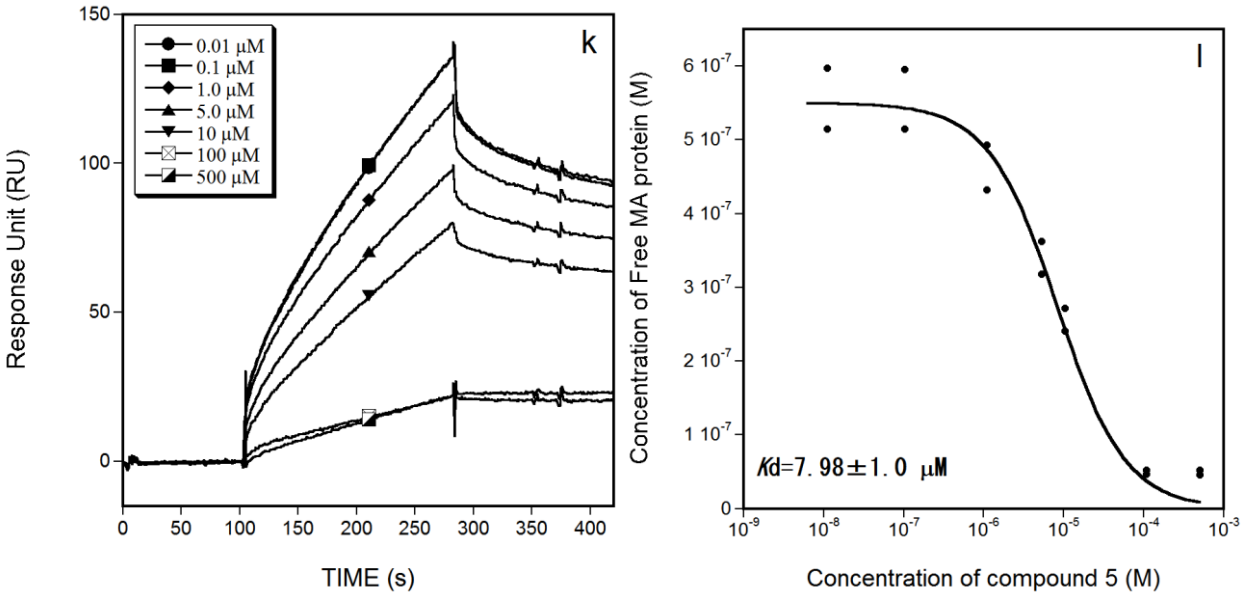
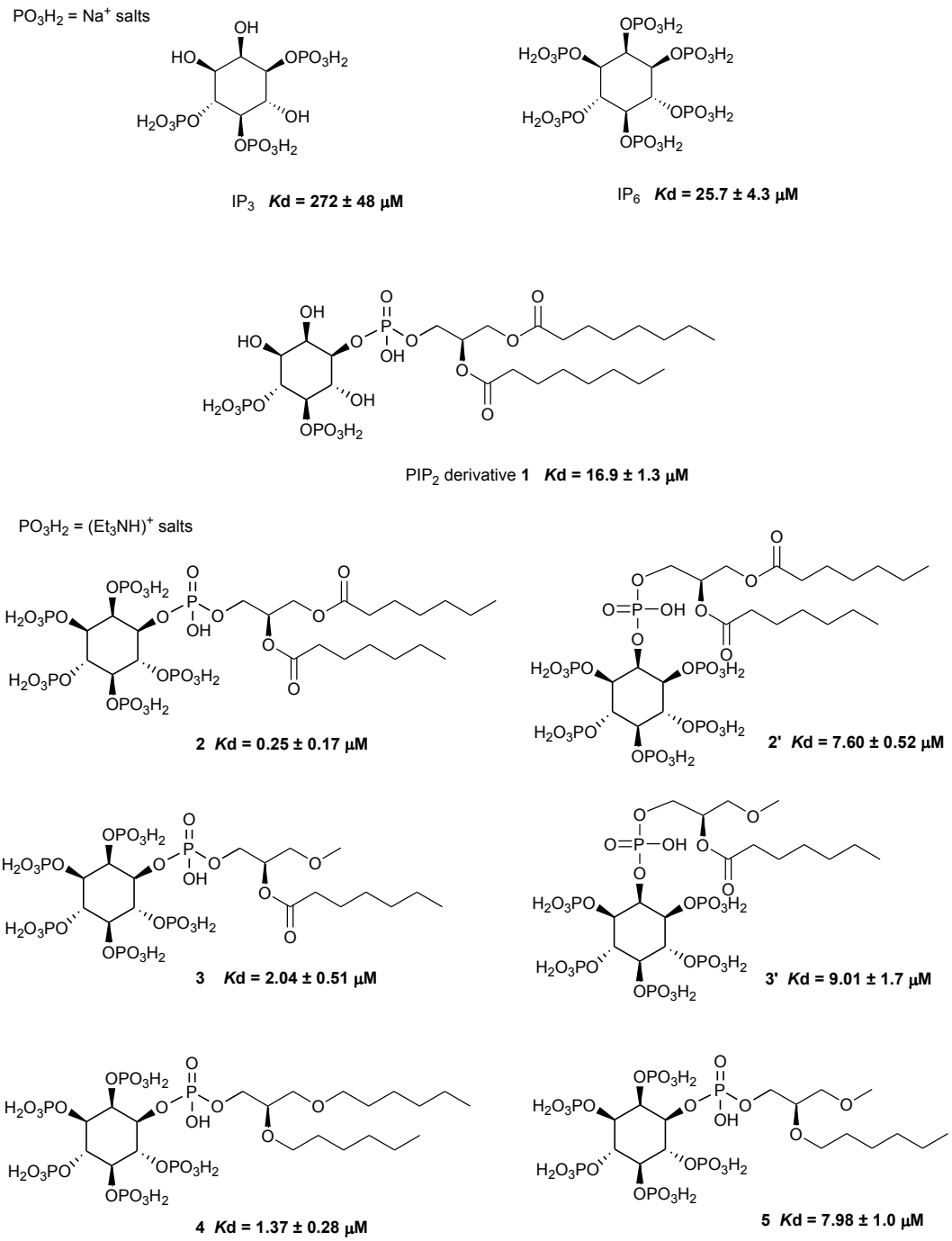


Figure 4 Competition assay and calculation of the equilibrium dissociation constants (*K*d) for MA-competitor complexes. The sensorgrams of MA and competitors, **2 (a)**, **2' (c)**, **3 (e)**, **3' (g), 4 (i)**, and **5 (k)** are shown. The competition curves between uncompetitive MA and **2 (b)**, **2' (d)**, **3 (f)**, **3' (h), 4 (j)**, and **5 (l)** are shown. Calculated *K*d values are shown. Each experiment was performed in duplicate.



335

336 **Figure 5** Dissociation constant (*K*d) of MA complexed with IPs, PI, and PIP_5 derivatives.

337

338 **2.7. Theoretical binding analysis of MA-1 or MA-2 complex**

339 Molecular docking study (MOE) was adapted to the MA-**1** and MA-**2** complexes. The structures
340 of complexes around the binding pocket are shown in **Figure 6a** and **c**, and the detailed structures
341 are shown in **Figure 6** and **d**, wherein lime green lines (ionic interaction) and light blue lines with
342 cylinder solid (H-acceptor) indicate the interaction between amino acid and **1** (or **2**) shorter than 4.0

Å, respectively. The surrounded binding pocket of the MA-**1** complex revealed that both inositol and 2'-acyl group of **1** are accommodated in the MA binding pocket. In contrast, the 1'-acyl chain is located outside the binding pocket (**Figure 6a**). Although a similar calculated result was obtained for the MA-**2** complex, the outside orientation of the 1'-acyl chain was more pronounced (**Figure 6c**). As shown in **Figure 6b**, the 1-phosphate interacts with Arg22 (2.9 Å: NH_2, ionic; 3.0, 3.7 Å: NH, H-acceptor). The 4-phosphate interacts with Lys98 (2.6, 2.9, 3.8 Å: NH_2, ionic; 2.6 Å: NH_2, H-acceptor), whereas the 5-phosphate interacts with Arg76 (3.0 Å: NH_2, 2.6, 3.6, 3.9 Å: NH, ionic; 3.0 Å: NH_2, 2.6 Å: NH, H-acceptor). In the case of **2** (**Figure 6d**), the 2'-acyl carbonyl oxygen of **2** interacts with Lys27 (2.9 Å: NH_2, H-acceptor). The 1-phosphate interacts with Arg22 (2.8, 3.0 Å: NH_2, 3.5 Å: NH_2, ionic; 3.0, 3.7 Å: NH, H-acceptor). The 2-phosphate interacts with Arg22 (2.6, 3.5 Å: NH_2, ionic; 2.6 Å: NH_2, 3.0 Å: CH_2, H-acceptor). The 3-phosphate interacts with Lys98 (2.7, 2.7 Å: NH_2, ionic; 2.7, 2.7 Å: NH_2, H-acceptor). The 4-phosphate interacts with Lys98 (2.6, 2.8 Å: NH_2, ionic; 2.6, 2.8 Å: NH_2, H-acceptor). The 5-phosphate interacts with Arg76 (2.8 Å: NH_2, 2.6, 3.4 Å: NH, ionic; 2.8 Å: NH_2, 2.6, 3.4 Å: NH, H-acceptor). The MA-**2** complex showed a greater number of amino acid interactions compared with MA-**1**, owing to the greater number of phosphates of **2**. Although 1-, 4-, and 5-phosphate of both **1** and **2** interact with Arg22, Lys98, and Arg76, respectively, 2- and 3-phosphate of **2** additionally interact with Arg22 and Lys98, respectively. In this context, judging from the results of the docking score based on the electric interaction, van der Waals attraction and strain energy of the ligand, MA-**2** complex was more stable than MA-**1** complex (–374.7 kcal and –250.2 kcal as the U_dock values, respectively). That is in agreement with SPR data (0.25 μM and 16.9 μM as the *K*d values, respectively).

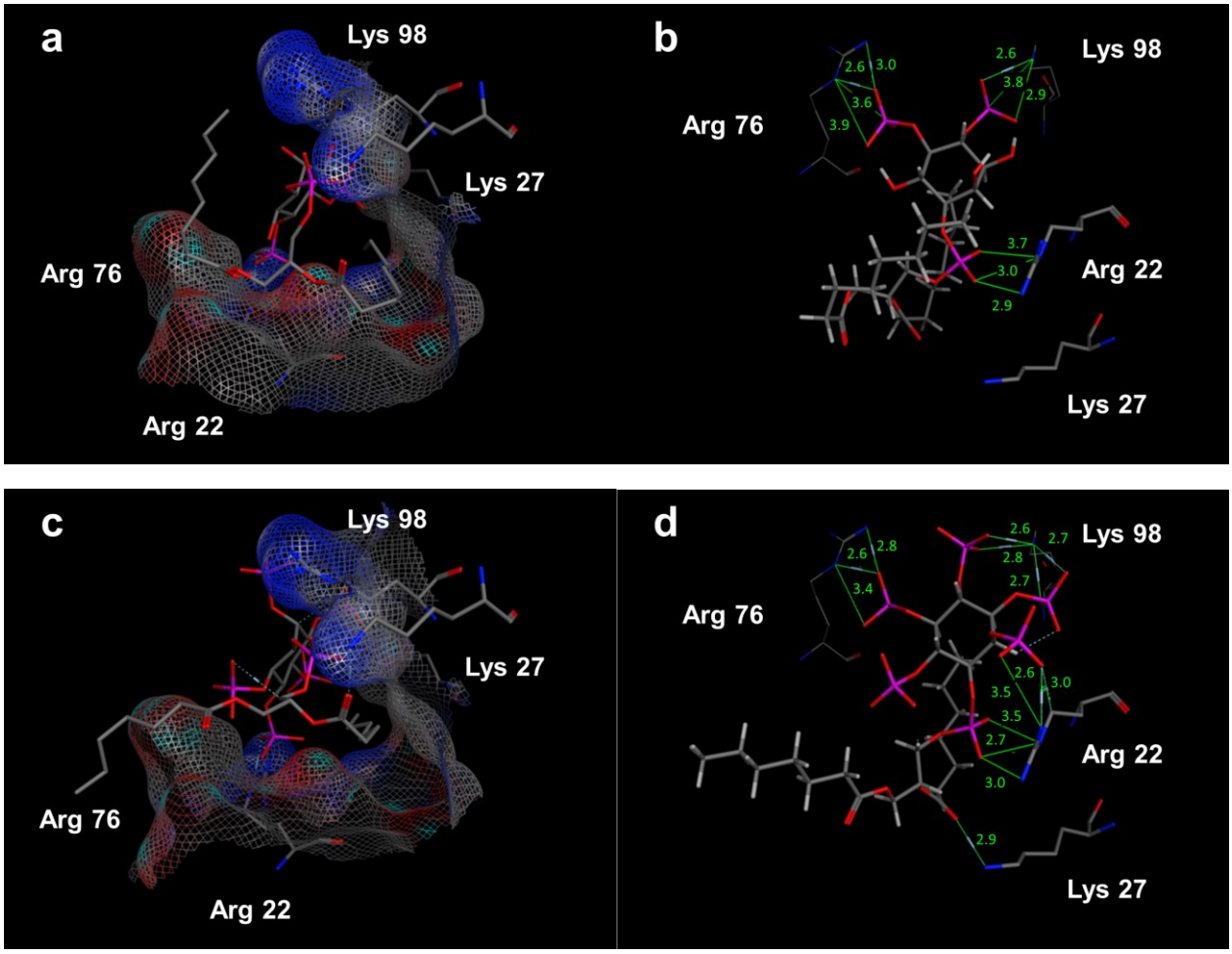


Figure 6 Docking studies of MA-**1** (**a, b**) and MA-**2** (**c, d**) complexes. The lime green lines (ionic interaction) and light blue lines with cylinder solid (H-acceptor) indicate the interaction between amino acid and **1** (**b**) or **2** (**d**) shorter than 4.0 Å, respectively.

Saad *et al*. demonstrated an "extended lipid" conformation of the MA-**1** complex, in which the glycerol 2'-acyl chain is accommodated in the MA cleft and the glycerol 1'-acyl remains buried in the membrane[5]. Thus, the 1'-acyl does not contribute to MA binding. However, in our study, although the MOE analysis of the MA-**2** complex indicates that the 1'-acyl was located outside the binding pocket, **3** (without the 1'-acyl) did not bind MA (*K*d=2.04 μM) as strongly as **2** (*K*d=0.25 μM) did, as revealed by the SPR analysis. It is hypothesized that the difference of *K*d values between **3** and **2** is caused not only by the interaction between the 2'-acyl chain and hydrophobic region of MA but also by the interaction between primordial carbons of the 1'-acyl chain of **2** and hydrophobic region of MA, which was not observed by MOE analysis.

Freed *et al*. [2, 21] demonstrated the role of the MA in the HIV-1 replication and mapped the

functional domains within this protein by site-directed mutagenesis to introduce over 80 single amino acid substitutions in MA and analyzed the effects on a variety of aspects of virus life cycles. They observed that a single amino acid mutation near the terminus of MA and vicinity of residue 55 and 85 caused virus assembly defects. Furthermore, they identified that a highly basic domain between MA residues 17 and 31 (16 and 30 in the MOE number) is implicated in the membrane binding. In this MOE analysis, not only Arg22 at a highly basic region but also the amino acids which have never been investigated, Arg76 and Lys98, are implicated in MA-**1** binding.

HIV-1 is a retrovirus, which is a family of enveloped viruses that replicate in a host cell through the process of reverse transcription. Retroviruses have Gag, Pol, and Env proteins. Chan *et al.*[22] examined the possible role of PIP_2 in Gag-membrane interaction of the alpharetrovirus Rous sarcoma virus (RSV) and showed that neither membrane localization of RSV Gag-GFP nor release of virus-like particles was affected by phosphatase-mediated depletion of PIP_2 in transfected avian cells. Furthermore, Inlora *et al.*[23] determined the role of the MA-PIP_2 interaction in Gag localization and membrane binding of a deltaretrovirus, human T-lymphotropic virus type 1 (HTLV-1). They demonstrated that, unlike HIV-1 Gag, subcellular localization of Gag and virus-like particle released by HTLV-1 was minimally sensitive to polyphosphoinositide 5-phosphatase IV (5ptaseIV) overexpression. These results suggest that the interaction of HTLV-1 MA with PIP_2 is not essential for HTLV-1 particle assembly. Accordingly, MA-PIP_2 binding might be significant only in HIV-1 among retroviruses, and our findings of MA-binding of PIP_5 derivatives may be HIV-1 specific.

Although PIP_5 derivatives bind MA tightly, highly charged these derivatives would not permeabilize the cell membrane in spite of the fact that the viral assembly occurs inside the cell. We intend to use a membrane carrier or synthesize a phosphate prodrug compound to improve cell membrane permeability in the future.

3. Materials and methods

3.1. General Methods

Chemicals were purchased from Aldrich, Fluka, Kanto Chemical, Nacalai tesque, and Wako. Thin layer chromatography (TLC) was performed on precoated plates (Merck TLC sheets silica 60 F_{254}): products were visualized by spraying phosphomolybdic acid in EtOH, or basic potassium permanganate and heated at high temperature. Chromatography was carried out on Silica Gel 60 N (40-100 mesh). Reverse phase chromatography was performed using C_{18} column (Cole-Parmer, USA). Cation exchange chromatography was performed using Dowex 50WX8 (H^+, 100-200 mesh). NMR spectra (JEOL JNM-AL300) were referenced to $SiMe_4$, or (HDO). Infra-red spectra were recorded on a JASCO FT/IR-410. The samples were prepared as KBr discs, or thin films between sodium chloride discs. Microanalysis was carried out by Yanaco MT-5S. High resolution MS (HRMS) were recorded by a JEOL JMS-DX303HF by using positive and negative FAB with 3-nitrobenzyl alcohol (NBA) (containing HMPA or not) as the matrix.

3.2. DL-3,6-di-*O*-benzyl-1,2:4,5-di-*O*-cyclohexylidene-*myo*-inositol (7)

To a solution of DL-1,2:4,5-di-cyclohexylidene-*myo*-inositol **6** (2.27 g, 6.67 mmol) in DMF (10 ml) was added NaH (0.676 g, 28.1 mmol) followed by benzyl bromide (2.0 ml, 16.9 mmol), and the resulting mixture was stirred at room temperature under argon for 24h. The reaction was quenched with MeOH, and concentrated under reduced pressure, and the residue was diluted with AcOEt. The organic phase was washed with H_2O and saturated aqueous NaCl, dried over Na_2SO_4, and then concentrated under reduced pressure. The crude product was purified by silica gel column chromatography (Hexane:AcOEt=5:1) to afford **7** (3.25 g, 94%) as a white solid.

^{1}H NMR ($CDCl_3$) δ: 1.25-1.69 (20H, m, C$\underline{H}_2$ x 10), 3.33 (1H, t, *J*=9.3Hz, C$\underline{H}$), 3.62-3.67 (1H, dd, *J*=10.6, 6.6Hz, C$\underline{H}$), 3.71-3.76 (1H, dd, *J*=4.2, 10.2Hz, C$\underline{H}$), 3.98 (1H, d, *J*=9.7Hz, C$\underline{H}$), 4.02-4.06 (1H, d, *J*=5.1, 6.4Hz, C$\underline{H}$), 4.33 (1H, t, *J*=4.5Hz, C$\underline{H}$), 4.78-4.90 (4H, m, C$\underline{H}_2$ x 2), 7.22-7.43 (10H, m, $C_6\underline{H}_5$ x 2). ^{13}C NMR ($CDCl_3$) δ: 23.9, 24.2, 24.3, 24.4, 25.4, 25.5, 35.7, 36.9, 37.8, 72.0, 72.3, 75.0, 76.6, 77.2, 79.1, 80.3, 81.0, 110.8, 113.1, 127.8, 128.1, 128.4, 128.5, 128.6, 128.7, 138.5, 138.7. IR (KBr) 3030, 2935, 2860, 1500, 1165, 1110, 850, 830, 740 cm^{-1}. MS (FAB) *m/z* 521 $(M+H)^+$. Mp. 123 °C. Anal. Calcd for $C_{32}H_{40}O_6$: C, 73.82; H, 7.74. Found: C, 73.87; H, 7.98. TLC; R_f 0.42 (Hexane:AcOEt=5:1).

3.3. DL-3,6-di-*O*-benzyl-*myo*-inositol (8)

To a solution of **7** (3.95 g, 7.58 mmol) in THF-H_2O (5:1, 60ml) was added *p*-toluenesulfonic acid monohydrate (1.90 g, 10.0 mmol). The resulting mixture was refluxed for 5h, and then neutralized with Et_3N, and concentrated under reduced pressure. The crude product was washed with a heated AcOEt, and the resulting crystals were filtered. Drying the crystal under reduce pressure afforded **8** (2.22 g, 81%) as a white solid.

^{1}H NMR (DMSO) δ: 2.49 (3H, bs, OH x 3), 3.12 (2H, t, *J*=9.9Hz, CH x 2), 3.28 (1H, d, *J*=7.3Hz, CH), 3.59 (2H, t, *J*=9.5Hz, CH x 2), 3.95 (1H, s, CH), 4.53-4.79 (4H, m, CH_2), 7.21-7.42 (10H, m, C_6H_5 x 2). ^{13}C NMR ($CDCl_3$) δ: 69.8, 70.8, 71.4, 72.3, 73.4, 75.0, 79.8, 81.8, 126.9, 127.1, 127.5, 127.8, 128.0, 139.3, 139.9. IR (KBr) 3750, 3030, 2905, 1500, 1450, 1110, 900, 740 cm^{-1}. Mp. 204 °C. MS (FAB) *m/z* 360 $(M+Na)^+$. Anal. Calcd for $C_{20}H_{24}O_6$: C, 66.65; H, 6.71. Found: C, 66.40; H, 6.83. TLC; R_f 0.48 (CH_2Cl_2:MeOH=7:1).

3.4. DL-3,6-di-*O*-benzyl-1-*O*-(*p*-methoxybenzyl)-*myo*-inositol (9)

A mixture of **8** (2.10 g, 5.66 mmol) and dibutyltin oxide (1.74 g, 7.00 mmol) in toluene (100 ml) was refluxed for 3h in a Dean-Stark apparatus to remove water. The mixture was concentrated under reduced pressure. To the residue was added cesium fluoride (1.06 g, 7.00 mmol), and the mixture was suspended in heated DMF (30 ml) at 100 °C. To the resulting suspension was added *p*-methoxybenzyl chloride (0.887 ml, 6.20 mmol) at –78 °C, and the mixture was stirred at room temperature under argon for 48h. After concentration of the reaction mixture under reduced pressure, the residue was purified by silica gel column chromatography (CH_2Cl_2:MeOH=10:1) to afford **9** (2.40 g, 89%) as a white solid.

^{1}H NMR ($CDCl_3$) δ: 2.48 (1H, bs, OH), 2.65 (2H, bs, OH), 3.19-3.23 (1H, dd, *J*=2.7, 9.5Hz, CH), 3.39 (1H, t, *J*=9.3Hz, CH), 3.76-3.82 (4H, m, OCH_3, CH), 3.95 (1H, t, *J*=9.3Hz, CH), 4.16 (1H, s, CH), 4.61-4.70 (4H, m, CH_2 x 2), 4.75 (1H, d, *J*=11.2Hz, $C_6H_5CH_2$(CH)), 4.93 (1H, d, *J*=11.2Hz, $C_6H_5CH_2$(CH)), 6.85 (2H, d, *J*=8.8Hz, $CH_3OC_6H_4$(CH x 2)), 7.23-7.36 (12H, m, C_6H_5 x 2, $CH_3OC_6H_5$(CH x 2)). ^{13}C NMR ($CDCl_3$) δ: 55.2, 67.0, 71.9, 72.0, 72.2, 74.2, 75.3, 79.0, 79.4, 80.4, 113.8, 127.6, 127.9, 127.9, 128.4, 128.5, 129.5, 129.9, 137.8, 137.9, 138.7, 159.4, 162.5. IR (KBr) 3460, 2880, 1610, 1520, 1450, 1180, 1100, 810, 750 cm^{-1}. Mp. 154 °C. MS (FAB) *m/z* 503

$(M+Na)^+$. Anal. Calcd for $C_{28}H_{32}O_7$: C, 69.98; H, 6.71. Found: C, 70.02; H, 6.76. TLC; R_f 0.50 (CH_2Cl_2:MeOH=10:1).

3.5. DL-1-*O*-(*p*-methoxybenzyl)-*myo*-inositol (10)

To a solution of **9** (1.86 g, 3.87 mmol) in MeOH (25 ml) was added W-2 Raney Nickel (0.20 g, 3.03 mmol), and the resulting mixture was stirred at 50 °C under hydrogen for 3 h. The mixture was filtered through a pad of celite, and concentrated under reduced pressure. The residue was washed with heated AcOEt, and the resulting crystals were filtered. Drying of the crystals under reduced pressure afforded **10** (0.52 g, 45%) as a white solid.

^{1}H NMR (DMSO) δ: 2.91-2.94 (1H, m, C<u>H</u>), 3.03-3.06 (2H, m, C<u>H</u>), 3.33-3.36 (1H, m, C<u>H</u>), 3.48-3.52 (1H, m, C<u>H</u>), 3.73 (3H, s, C<u>H</u>), 3.91 (1H, s, C<u>H</u>), 4.36-4.57 (7H, m, O<u>H</u> x5, C<u>H$_2$</u>), 6.87 (2H, d, *J*=8.8Hz, CH_3OC_6<u>H$_5$</u>(C<u>H</u> x 2)), 7.31 (2H, d, *J*=8.4Hz, CH_3OC_6<u>H$_5$</u>(C<u>H</u> x 2)). ^{13}C NMR (DMSO) δ: 55.0, 69.3, 70.3, 71.7, 72.0, 72.4, 75.4, 79.6, 113.4, 129.0, 131.2, 158.5. IR (KBr) 3390, 2910, 1610, 1590, 1510, 1250, 1120, 890, 820 cm^{-1}. Mp. 183 °C. MS (FAB) *m/z* 299 $(M-H)^+$. Anal. Calcd for $C_{14}H_{20}O_7$: C, 55.99; H, 6.71. Found: C, 56.06; H, 6.72. TLC; R_f 0.39 (CH_2Cl_2:MeOH=3:1).

3.6. DL-1-*O*-(*p*-methoxybenzyl)-2,3,4,5,6-penta-*O*-[(1,5-dihydro-2,4,3-benzodioxaphosphepin-3-yl) phosphoryl]-*myo*-inositol (11)

To a suspension of **10** (0.050 g, 0.166 mmol) in CH_2Cl_2 (10 ml) was added MS4A, and the resulting suspension was stirred at room temperature under argon for 15 min. To the mixture was added (1,5-dihydro-2,4,3-benzodioxaphosphepin-3-yl)diethylamine (0.358 ml, 1.66 mmol) followed by 1*H*-tetrazole (0.116 g, 1.66 mmol), the resulting mixture was stirred at room temperature under argon for overnight. To the mixture was added *m*-chloroperbenzoic acid (0.336 g, 1.50 mmol) in small portions, and the resulting mixture was stirred at –40 °C to room temperature for 1hr. The mixture was purified by silica gel column chromatography (AcOEt:Hexane=15:1) to afford **11** (0.151 g, 75%) as a white yellow solid.

^{1}H NMR ($CDCl_3$) δ: 3.82 (3H, s, OC<u>H$_3$</u>), 3.92 (1H, d, *J*=8.6Hz, C<u>H</u>), 4.52 (1H, d, *J*=10.4 Hz, C<u>H</u>), 4.72-5.80 (26H, m, C<u>H$_2$</u>, C_6H_4(C<u>H$_2$</u>)$_2$ x 5, C<u>H</u> x 4), 6.90 (2H, d, *J*=8.4Hz, CH_3OC_6<u>H$_4$</u>(C<u>H</u> x 2)),

6.96 (20H, m, $C_6\underline{H_4}$ x 5), 7.46 (2H, d, *J*=8.4Hz, $CH_3OC_6\underline{H_4}(C\underline{H}$ x 2)). ^{13}C NMR ($CDCl_3$) δ: 55.1, 68.0, 68.9, 69.2, 74.4, 75.4, 76.6, 77.0, 77.2, 77.4, 113.5, 128.4, 128.5, 128.6, 128.7, 128.8, 128.8, 129.0, 129.0, 129.2, 129.4, 129.8, 134.3, 135.1, 135.2, 135.5, 135.6, 159.1. IR (KBr) 1610, 1510, 1460, 1380, 1290, 1020, 860, 730 cm^{-1}. Mp 165 °C. HRMS(FAB) *m/z* calcd for $C_{54}H_{56}O_{22}P_5$ $(M+H)^+$ 1211.2022. Found:1211.1870. Anal. Calcd for $C_{54}H_{56}O_{22}P_5$: C, 53.56; H, 4.58. Found: C, 53.21; H, 4.72. TLC; R_f 0.55 (CH_2Cl_2:MeOH=10:1).

3.7. DL-2,3,4,5,6-penta-*O*-[(1,5-dihydro-2,4,3-benzodioxaphosphepin-3-yl)phosphoryl]-*myo*-inositol (12) and DL-1,3,4,5,6-penta-*O*-[(1,5-dihydro-2,4,3-benzodioxaphosphepin-3-yl)phosphoryl]-*myo*-inositol (13)

To a solution of **11** (0.070 g, 0.0578 mmol) in CH_3CN-H_2O (9:1, 5 ml) was added diammonium cerium(IV) nitrate (0.158 g, 0.288 mmol) and the resulting mixture was stirred at room temperature for 1 hr. The mixture was concentrated under reduced pressure, and the residue was purified by silica gel column chromatography (CH_2Cl_2:MeOH=10:1) to afford the mixture of **12** and **13**. Compound **12** and **13** were used for next coupling reaction without further purification.

R_f values of compound **12** and **13** were 0.37 and 0.29, respectively (CH_2Cl_2:MeOH=10:1).

3.8. DL-1-*O*-(*p*-methoxybenzyl)-2,3,4,5,6-penta-*O*-[bis(2-cyanoethyl)phosphoryl]-*myo*-inositol (14)

To a suspension of **10** (0.050 g, 0.166 mmol) in CH_2Cl_2 (10 ml) was added MS4A, and the resulting suspension was stirred at room temperature under argon for 15 min. To the mixture was added bis(2-cyanoethyl)-*N,N*-diisopropylphosphoramidite (0.383 ml, 1.50 mmol) followed by 1*H*-tetrazole (0.105 g, 1.50 mmol), the resulting mixture was stirred at room temperature under argon for 4h. To the mixture was added *m*-chloroperbenzoic acid (0.336 g, 1.50 mmol) in small portions, and the resulting mixture was stirred at –78 °C to room temperature for 1hr. The mixture was purified by silica gel column chromatography (CH_2Cl_2:MeOH=7:1) to afford **14** (0.15 g, 73%) as a colorless oil.

^{1}H NMR (CD_3COCD_3) δ: 2.65-2.91 (20H, m, $CH_2\underline{CH_2}CN$ x 10), 3.68 (3H, s, $OC\underline{H_3}$), 3.95 (1H, d,

J=9.3Hz, C<u>H</u>), 4.11-4.51 (21H, m, <u>CH$_2$</u>CH$_2$CN x 10, C<u>H</u>), 4.65-4.80 (5H, m, C<u>H$_2$</u>, C<u>H</u> x 3), 5.36 (1H, d, *J*=9.2Hz, C<u>H</u>), 6.84 (2H, d, *J*=8.8Hz, CH$_3$OC$_6$<u>H$_5$</u>(C<u>H</u> x 2)), 7.39 (2H, d, *J*=8.63Hz, CH$_3$OC$_6$<u>H$_5$</u>(C<u>H</u> x 2)). IR (KBr) 3300, 2890, 2255, 1610, 1470, 1415, 1280, 1040, 820, 795, 765 cm^{-1}. HRMS(FAB) *m/z* calcd for $C_{44}H_{55}N_{10}O_{22}P_5$ $(M+Na)^+$ 1253.2078. Found:1253.2029. TLC; R_f 0.28 (CH_2Cl_2:MeOH=10:1).

3.9. DL-2,3,4,5,6-penta-*O*-[bis(2-cyanoethyl)phosphoryl]-*myo*-inositol (15)

To a solution of **14** (0.073 g, 0.059 mmol) in CH_3CN-H_2O (9:1, 10 ml) was added diammonium cerium(IV) nitrate (0.208 g, 0.379 mmol) and the resulting mixture was stirred at room temperature for 1.5h. The mixture was concentrated under reduced pressure, and the residue was purified by silica gel column chromatography (CH_2Cl_2:MeOH=7:1 to 3:1) to afford **15** (0.055 g, 68%) as a colorless oil.

^{1}H NMR (CD_3COCD_3 + D_2O) δ: 2.93-3.02 (20H, m, CH$_2$<u>CH$_2$</u>CN x 10), 4.22 (1H, s, C<u>H</u>), 4.41-4.53 (20H, m, <u>CH$_2$</u>CH$_2$CN x 10), 4.64-4.94 (4H, m, C<u>H</u> x 4), 5.20 (1H, d, *J*=9.0Hz, C<u>H</u>). ^{13}C NMR (CD_3COCD_3) δ: 19.8, 19.9, 19.9, 20.0, 20.0, 63.9, 64.0, 64.1, 64.2, 64.3, 64.3, 64.6, 68.8, 74.5, 76.1, 76.8, 79.0, 79.2, 79.2, 118.3, 118.4, 118.6. IR (film) 3020, 2910, 2255, 1635, 1470, 1415, 1340, 1280, 1040 cm^{-1}. HRMS(FAB) *m/z* calcd for $C_{36}H_{47}N_{10}O_{21}P_5$ $(M+Na)^+$ 1133.1503. Found:1133.1545. R_f 0.25 (CH_2Cl_2:MeOH=7:1).

3.10. (R)-1-benzyloxy-2,3-bis(heptanoyl)propane (17)

A mixture of (*R*)-3-benzyloxy-1,2-propandiol (**16**) (0.10 g, 0.549 mmol) in CH_2Cl_2 (5 ml) was added pyridine (0.11 ml, 1.37 mmol) followed by dimethylaminopyridine (0.0036 g, 0.27 mmol) and the resulting mixture was cooled to 0 °C. To the mixture was added heptanoyl chloride (0.20 ml, 1.26 mmol) and the resulting mixture was stirred at room temperature under argon for overnight. The reaction was quenched with H_2O (25 ml), and the resulting water phase was extracted with CH_2Cl_2. The organic layer was washed with 2 M aqueous hydrogen chloride (20 ml) and H_2O (25 ml). The resulting organic phase was further washed Brine (30 ml) and dried over Na_2SO_4, and then concentrated under reduced pressure. The crude product was purified by silica gel column chromatography (Hexane:AcOEt=9:1) to afford **17** (0.193 g, 86%) as a colorless oil.

^{1}H NMR ($CDCl_3$) δ: 0.86-0.90 (6H, m, C<u>H$_3$</u> x 2), 1.28-1.36 (12H, m, C<u>H$_2$</u> x 6), 1.54-1.66 (4H, m,

562 C$\underline{H_2}$ x 2), 2.25-2.34 (4H, m, C$\underline{H_2}$ x 2), 3.59 (2H, d, *J*=5.1Hz, C$\underline{H_2}$OCH_2C$_6$H$_5$), 4.15-4.22 (1H, dd,
563 *J*=6.2, 11.7Hz, C$\underline{H_2}$OCO), 4.32-4.37 (1H, dd, *J*=3.8, 11.9Hz, C$\underline{H_2}$OCO), 4.49-4.58 (2H, dd, *J*=12.1,
564 15.2Hz, C$_6$H$_5$C$\underline{H_2}$), 5.20-5.27 (1H, ddt, *J*=3.9, 5.1, 6.2Hz, CH$_2$C$\underline{H}$CH$_2$), 7.26-7.37 (5H, m, C$_6\underline{H_5}$).
565 ^{13}C NMR (CDCl$_3$)
566 δ: 14.0, 22.4, 24.8, 24.9, 28.7, 28.8, 31.4, 34.1, 34.3, 62.6, 68.3, 70.0, 73.3, 127.6, 127.7, 128.4, 137
567 .7, 173.1, 173.4. IR (KBr) 2820, 1740, 1460, 1160, 1100, 740, 700 cm^{-1}. HRMS(FAB) *m/z* calcd for
568 $C_{24}H_{39}O_5$ $(M+H)^+$ 407.2797. Found: 407.2760. Anal. Calcd for $C_{24}H_{39}O_5$: C, 70.90; H, 9.42. Found:
569 C, 70.61; H, 9.62. TLC; R_f 0.35 (Hexane:AcOEt=9:1).
570
571 **3.11. 1,2-*O*-diheptanoyl-*sn*-glycerol (18)**
572 To a solution of **17** (0.193 g, 0.475 mmol) in CH_2Cl_2 (10 ml) was added 10% Pd-C (0.126 g, 0.119
573 mmol), and the resulting mixture was stirred at room temperature under hydrogen for overnight.
574 The mixture was filtered through a pad of celite, and the resulting filtrate was concentrated under
575 reduced pressure. The crude product was purified by silica gel column chromatography
576 (Hexane:AcOEt=2:1) to afford **18** (0.144 g, 96%) as a colorless oil.
577 ^{1}H NMR (CDCl$_3$) δ: 0.89 (6H, t, *J*=6.8Hz, C$\underline{H_3}$ x 2), 1.21-1.37 (12H, m, C$\underline{H_2}$ x 6), 1.50-1.68 (4H, m,
578 C$\underline{H_2}$ x 2), 2.12 (1H, bs, O$\underline{H}$), 2.30-2.37 (4H, dd, *J*=7.1, 14.5 Hz, C$\underline{H_2}$ x 2), 3.38 (2H, bs, HOC$\underline{H_2}$),
579 4.20-4.26 (1H, dd, *J*=5.7, 11.9Hz, OCOC$\underline{H}$H), 4.30-4.35 (1H, dd, *J* =4.6, 11.9Hz, OCOCH$\underline{H}$),
580 5.00-5.12 (1H, m, C$\underline{H}$). ^{13}C NMR (CDCl$_3$)
581 δ: 14.0, 22.4, 22.5, 24.8, 24.9, 28.7, 28.8, 31.4, 34.1, 34.3, 61.5, 62.0, 173.4, 173.6. IR (KBr) 3590,
582 3140, 2930, 2860, 1740, 1160, 1100 cm^{-1}. HRMS(FAB) *m/z* calcd for $C_{17}H_{32}O_5$ $(M+Na)^+$ 339.2147.
583 Found: 339.2154. Anal. Calcd for $C_{17}H_{32}O_5$: C, 64.53; H, 10.19. Found: C, 64.33; H, 10.22. TLC;
584 R_f 0.45 (Hexane:AcOEt=2:1).
585
586 **3.12. (R)-1-benzyloxy-2,3-bis(hexyloxy)propane (19)**
587 A mixture of **16** (0.366 g, 2.03 mmol) in DMF (10 ml) was added NaH (0.406 g, 16.9 mmol)
588 followed by bromohexane (0.708 ml, 5.0 mmol), and the resulting mixture was stirred at room
589 temperature under argon for 24h. The reaction was quenched with MeOH, and concentrated under
590 reduced pressure, and then the residue was diluted with AcOEt. The organic phase was washed with
591 H_2O and saturated aqueous NaCl, dried over Na_2SO_4, and then concentrated under reduced pressure.

The crude product was purified by silica gel column chromatography (Hexane:AcOEt=5:1) to afford **19** (0.506 g, 70%) as a colorless oil.

^{1}H NMR ($CDCl_3$) δ: 0.88 (6H, m, C$\underline{H_3}$ x 2), 1.29 (12H, bs, C$\underline{H_2}$ x 6), 1.52-1.59 (2H, m, C$\underline{H_2}$ x 2), 3.40-3.59 (9H, m, C$\underline{H_2}$OCH$_2$ x 3, $\underline{CH_2}OCH_2C_6H_5$, $CH_2C\underline{H}CH_2$), 4.55 (2H, s, $C_6H_5C\underline{H_2}$), 7.25-7.34 (5H, m, $C_6\underline{H_5}$). ^{13}C NMR ($CDCl_3$) δ: 14.0, 22.5, 25.7, 25.7, 29.5, 30.0, 31.6, 70.2, 70.5, 70.6, 71.6, 73.2, 77.8, 127.4, 127.5, 128.2, 138.3. IR (KBr) 3070, 3030, 2970, 2850, 1600, 1455, 1380, 1270, 1115, 730, 700 cm^{-1}. MS (FAB) *m/z* 351 $(M+H)^+$. HRMS(FAB) *m/z* calcd for $C_{22}H_{39}O_3$ $(M+H)^+$ 351.2889. Found:351.2892. TLC; R_f 0.58 (Hexane:AcOEt=5:1).

3.13. 1,2-*O*-dihexyl-*sn*-glycerol (20)

19 (0.406 g, 1.13 mmol) was allowed to react under the same condition as described for the preparation of **18** to give **20** (0.285 g, 84%) as a colorless oil.

^{1}H NMR ($CDCl_3$) δ: 0.87 (3H, t, *J*=6.7Hz, C$\underline{H_3}$ x 2), 1.30 (12H, m, C$\underline{H_2}$ x 6), 1.54-1.57 (4H, m, C$\underline{H_2}$ x 2), 2.30 (1H, bs, O$\underline{H}$), 3.42-3.71 (9H, m, C$\underline{H_2}$OCH$_2$ x 3, $\underline{CH_2}OCH_2C_6H_5$, $CH_2C\underline{H}CH_2$). ^{13}C NMR ($CDCl_3$) δ:14.0, 22.6, 25.7, 29.5, 30.0, 31.6, 31.6, 63.0, 70.3, 70.9, 71.8, 78.2. IR (KBr) 3440, 2960, 2930, 1465, 1380, 1120 cm^{-1}. MS (FAB) *m/z* 261 $(M+H)^+$. HRMS(FAB) *m/z* calcd for $C_{15}H_{33}O_3Na$ $(M+Na)^+$ 283.2249. Found:283.2252. TLC; R_f 0.53 (Hexane:AcOEt=2:1).

3.14. (R)-1-benzyloxy-3-methoxypropan-2-ol (21)

A mixture of **16** (0.50 g, 2.74 mmol) and dibutyltin oxide (0.697 g, 2.80 mmol) in toluene (50 ml) was refluxed for 3h in a Dean-Stark apparatus to remove water. The mixture was concentrated under reduced pressure. To the residue was added cesium fluoride (0.759 g, 5.0 mmol), and the mixture was suspended in heated DMF (30 ml) at 100 °C. To the resulting suspension was added methyl iodide (0.311 ml, 10.0 mmol) at –78 °C, and the mixture was stirred at room temperature under argon with light shielding for 48h. After concentration of the reaction mixture under reduced pressure, the residue was purified by silica gel column chromatography (Hexane:AcOEt=1:2) to afford **21** (0.386 g, 71%) as a colorless oil.

^{1}H NMR ($CDCl_3$) δ: 2.71 (1H, bs, O$\underline{H}$), 3.36 (3H, s, OC$\underline{H_3}$), 3.38-3.56 (4H, m, $CH_3OC\underline{H_2}$, C$\underline{H_2}$OH), 3.98 (1H, d, *J*=4.4Hz, $CH_2C\underline{H}CH_2$), 4.54 (2H, s, $C_6H_5C\underline{H_2}$), 7.25-7.32 (5H, m, $C_6\underline{H_5}$). ^{13}C NMR ($CDCl_3$) δ: 59.0, 69.2, 71.2, 73.3, 73.7, 127.6, 128.3, 137.8. IR (KBr) 3450, 3060, 3030, 2890, 1500,

1450, 1360, 1330, 1200, 1100, 970, 740, 700 cm^{-1}. HRMS(FAB) *m/z* calcd for $C_{11}H_{16}O_3$ $(M+Na)^+$ 219.0997. Found:219.1012. TLC; R_f 0.58 (Hexane:AcOEt=1:2).

3.15. (R)-1-benzyloxy-2-heptanoyl-3-methoxypropane (22)

21 (0.119 g, 0.608 mmol) was allowed to react under the same condition as described for the preparation of **17** to give **22** (0.175 g, 93%) as a colorless oil.

^{1}H NMR ($CDCl_3$) δ: 0.85-0.90 (3H, m, $C\underline{H}_3$), 1.25-1.36 (6H, m, $C\underline{H}_2$ x 3), 1.57-1.67 (2H, m, $C\underline{H}_2$), 2.34 (2H, t, *J*=7.5Hz, $C\underline{H}_2$), 3.35 (3H, s, $OC\underline{H}_3$), 3.55-3.57 (2H, d, *J*=5.1Hz, $CH_3OC\underline{H}_2$), 3.61-3.62 (2H, d, *J*=5.0Hz, $C_6H_5CH_2OC\underline{H}_2$), 4.50-4.59 (2H, dd, *J*=12.1, 12.3Hz, $C_6H_5C\underline{H}_2$), 5.16-5.22 (1H, m, $CH_2C\underline{H}CH_2$), 7.25-7.37 (5H, m, $C_6\underline{H}_5$). ^{13}C NMR ($CDCl_3$) δ:14.0, 22.4, 24.9, 28.7, 31.4, 34.3, 59.2, 68.6, 71.0, 71.3, 73.2, 127.6, 127.6, 128.3, 138.0, 173.4. IR (KBr) 3290, 2990, 2850, 1740, 1500, 1460, 1370, 1100, 740, 700 cm^{-1}. HRMS(FAB) *m/z* calcd for $C_{18}H_{29}O_4$ $(M+H)^+$ 309.2066. Found: 309.2068. TLC; R_f 0.23 (Hexane:AcOEt=9:1).

3.16. 2-*O*-heptanoyl-1-*O*-methyl-*sn*-glycerol (23)

22 (0.390 g, 1.27 mmol) was allowed to react under the same condition as described for the preparation of **17** to give **18** (0.258 g, 93%) as a colorless oil.

^{1}H NMR ($CDCl_3$) δ: 0.88 (3H, t, *J*=6.8Hz, $C\underline{H}_3$), 1.26-1.35 (6H, m, $C\underline{H}_2$ x 3), 1.59-1.65 (2H, m, $C\underline{H}_2$), 2.32-2.39 (3H, m, O$\underline{H}$, $C\underline{H}_2$), 3.38 (3H, s, $OC\underline{H}_3$), 3.55-3.60 (1H, dd, *J*=4.8, 10.6Hz, $CH_3OCH_2(C\underline{H})$), 3.59-3.64 (1H, dd, *J*=4.9, 10.4Hz, $CH_3OCH_2(C\underline{H})$), 3.79 (2H, d, *J* =4.4Hz, $C\underline{H}_2OH$), 5.00-5.03 (1H, m, C$\underline{H}$). ^{13}C NMR ($CDCl_3$) δ: 14.0, 22.4, 24.9, 28.7, 31.4, 34.3, 59.3, 62.5, 71.6, 72.7, 173.7. IR (KBr) 3630, 3240, 2810, 1735, 1460, 1110 cm^{-1}. HRMS(FAB) *m/z* calcd for $C_{11}H_{23}O_6$ $(M+H)^+$ 219.1596. Found: 219.1590. TLC; R_f 0.44 (Hexane:AcOEt=1:1).

3.17. (R)-1-benzyloxy-2-hexyloxy-3-methoxypropane (24)

21 (0.120 g, 0.611 mmol) was allowed to react under the same condition as described for the preparation of **19** to give **24** (0.157 g, 92%) as a colorless oil.

^{1}H NMR ($CDCl_3$) δ: 0.88 (3H, m, $C\underline{H}_3$), 1.29 (6H, bs, $C\underline{H}_2$ x 3), 1.53-1.60 (2H, m, $C\underline{H}_2$), 3.35 (3H, s, $OC\underline{H}_3$), 3.45-3.62 (7H, m, $CH_3OC\underline{H}_2$, $C\underline{H}_2OCH_2C_6H_5$, $CH_2C\underline{H}CH_2$, $OC\underline{H}_2$), 4.55 (2H, s,

652 $C_6H_5C\underline{H_2}$), 7.25-7.34 (5H, m, $C_6\underline{H_5}$). ^{13}C NMR ($CDCl_3$) δ: 14.0, 22.6, 25.7, 30.0, 31.6, 59.1, 70.0,
653 70.5, 72.7, 73.3, 77.7, 127.4, 127.5, 128.2, 138.3. IR (KBr) 3285, 3065, 2960, 1600, 1455, 1270,
654 1200, 1100, 700 cm^{-1}. MS (FAB) *m/z* 281 $(M+H)^+$. Anal. Calcd for $C_{11}H_{16}O_3$: C, 72.82; H, 10.06.
655 Found: C, 72.67; H, 10.28. TLC; R_f 0.58 (Hexane:AcOEt=5:1).
656
657 **3.18. 2-*O*-hexyl-1-*O*-methyl-*sn*-glycerol (25)**
658 **24** (0.153 g, 0.54 mmol) was allowed to react under the same condition as described for the
659 preparation of **20** to give **25** (0.092 g, 89%) as a colorless oil.
660 ^{1}H NMR ($CDCl_3$) δ: 0.89 (3H, t, *J*=6.8Hz, $C\underline{H_3}$), 1.30-1.37 (6H, m, $C\underline{H_2}$ x 3), 1.54-1.61 (2H, m,
661 $C\underline{H_2}$), 2.35 (1H, bs, O<u>H</u>), 3.37 (3H, s, $OC\underline{H_3}$), 3.46-3.70 (7H, m, $CH_3OC\underline{H_2}$, $C\underline{H_2}OH$, $CH_2C\underline{H}CH_2$,
662 $OC\underline{H_2}$). ^{13}C NMR ($CDCl_3$) δ: 14.0, 22.5, 25.7, 30.0, 31.6, 59.2, 62.6, 70.3, 72.6, 78.3. IR (KBr)
663 3310, 2935, 1455, 1104 cm^{-1}. MS (FAB) *m/z* 281 $(M+H)^+$. HRMS(FAB) *m/z* calcd for $C_{11}H_{22}O_3Na$
664 $(M+Na)^+$ 213.1467. Found:213.1466. TLC; R_f 0.58 (Hexane:AcOEt=1:2).
665
666 **3.19. DL-2, 3,4,5,6-penta-*O*-[(1,5-dihydro-2,4,3-benzodioxaphosphepin-3-yl)**
667 **phosphoryl]-*myo*-inositol 1-{[1,2-*O*-diheptanoyl-*sn*-glyceryl](benzyl)phosphate} (26)**
668 **DL-1,**
669 **3,4,5,6-penta-*O*-[(1,5-dihydro-2,4,3-benzodioxaphosphepin-3-yl)phosphoryl]-*myo*-inositol**
670 **2-{[1,2-*O*-diheptanoyl-*sn*-glyceryl](benzyl)phosphate} (27)**
671 To a mixture of **18** (0.117 g, 0.54 mmol) in CH_2Cl_2 (5 ml) was added Benzyl-*N, N, N', N'*-tetraisopropylphosphoramidite (0.20 ml, 0.54 mmol) followed by MS4A (0.20 g), and the
672 resulting mixture was stirred at room temperature under argon for 15min. To the mixture was added
673 1*H*-tetrazole (0.038 g, 0.54 mmol), and the resulting mixture was stirred at room temperature under
674 argon for 10min. To the mixture was added completely dissolved a mixture of compound **12** and **13**
675 (0.118 g, 0.108 mmol) in CH_2Cl_2 (10 ml) with MS4A, followed by adding 1*H*-tetrazole (0.076 g,
676 1.08 mmol), and the resulting mixture was stirred at room temperature for further 24h. To the
677 mixture was added *tert*-butylhydroperoxide (0.082 ml, 0.818 mmol), and stirred at room
678 temperature for further 5min. The mixture was purified by silica gel column chromatography
679 (CH_2Cl_2:MeOH=20:1) to afford compound **26** (0.056 g, 22%) as a white solid and compound **27**
680 (0.092 g, 45%) as a white solid.
681

Compound **26**

^{1}H NMR ($CDCl_3$) δ: 0.70-0.80 (6H, m, C$\underline{H}_3$ x 2), 1.01-1.18 (12H, m, C$\underline{H}_2$ x 6), 1.35-1.40 (4H, m, C$\underline{H}_2$ x 2), 1.91-2.14 (4H, m, C$\underline{H}_2$ x 2), 3.97-4.03 (2H, dd, *J*=5.1, 5.7Hz, C$\underline{H}_2$OP), 4.16-4.33 (3H, m, C$\underline{H}$, C$\underline{H}_2$OCO), 4.68-5.69 (28H, m, C$\underline{H}$ x 5, $C_6H_5C\underline{H}_2$, $CH_2C\underline{H}CH_2$, $C_6H_4(C\underline{H}_2)_2$ x 5), 6.91-7.53 (25H, m, $C_6\underline{H}_4$ x 5, $C_6\underline{H}_5$). ^{13}C NMR ($CDCl_3$) δ: 13.9, 22.3, 24.5, 28.6, 31.3, 33.9, 61.7, 66.5, 68.4, 68.9, 69.0, 69.1, 69.2, 69.3, 69.4, 69.5, 70.0, 70.2, 73.8, 76.2, 76.7, 76.9, 77.0, 77.2, 77.3, 127.7, 128.3, 128.4, 128.7, 128.8, 128.9, 129.0, 129.1, 129.2, 129.3, 129.4, 134.9, 135.1, 135.4, 135.6, 135.7, 172.6, 173.1. IR (KBr) 2930, 1740, 1460, 1380, 1300, 1160, 1020, 860, 770, 730 cm^{-1}. HRMS(FAB) *m/z* calcd for $C_{70}H_{84}O_{28}P_6Na$ 1581.3473. Found: 1581.3435 $(M+Na)^+$. Mp 98 °C. Anal. Calcd for $C_{11}H_{16}O_3$: C, 5.57; H, 53.92. Found: C, 5.57; H, 54.37. R_f 0.46 (CH_2Cl_2:MeOH=10:1).

Compound **27**

^{1}H NMR ($CDCl_3$) δ: 0.75-0.82 (6H, m, C$\underline{H}_3$ x 2), 1.12-1.19 (12H, m, C$\underline{H}_2$ x 6), 1.40-1.58 (4H, m, C$\underline{H}_2$ x 2), 2.17-2.25 (4H, m, C$\underline{H}_2$ x 2), 3.99-4.37 (6H, m, C$\underline{H}$ x 2, C$\underline{H}_2$OP, C$\underline{H}_2$OCO), 4.48-4.64 (2H, m, C$\underline{H}$ x 2), 4.70-5.77 (25H, m, C$\underline{H}$ x 2, $CH_2C\underline{H}CH_2$, $C_6H_4(C\underline{H}_2)_2$ x 5, $C\underline{H}_2C_6H_5$), 7.17-7.44 (25H, m, $C_6\underline{H}_4$ x 5, $C_6\underline{H}_5$). ^{13}C NMR ($CDCl_3$) δ: 14.1, 22.6, 24.8, 28.9, 31.6, 34.1, 61.8, 61.9, 65.8, 67.1, 67.2, 69.3, 69.6, 69.7, 69.8, 70.4, 70.9, 71.0, 73.5, 76.3, 76.7, 77.4, 128.0, 128.3, 128.4, 128.5, 128.6, 128.9, 129.0, 129.1, 129.2, 129.3, 129.4, 129.6, 129.7, 129.8, 134.7, 135.0, 135.1, 135.6, 135.7, 135.8, 135.9, 136.0, 173.0, 173.4.IR (KBr) 2930, 1740, 1460, 1300, 1020, 860, 770, 730 cm^{-1}. HRMS(FAB) *m/z* calcd for $C_{70}H_{84}O_{28}P_6Na$ 1581.3473. Found: 1581.3490 $(M+Na)^+$. R_f 0.67 (CH_2Cl_2:MeOH=10:1).

3.20. DL-1-*O*-(1,2-*O*-diheptanoyl-*sn*-glyceryl) hydrogen phosphoryl]-*myo*-inositol 2,3,4,5,6-pentakis(hydrogenphosphate): 2

To a solution of **26** (0.030 g, 0.019 mmol) in *t*BuOH (8 ml) and H_2O (1.5 ml) was added 10% Pd-C (0.15 g, 0.14 mmol), and the resulting mixture was stirred at room temperature under hydrogen for 24 h. The mixture was filtered through a pad of celite, and then washed the celite pad with H_2O. The resulting filtrate was lyophilized. The residue was dissolved H_2O (2 ml), and filtered through the cation-exchange resin. To the resulting filtrate (0.009 g, 0.009 mmol) was added triethylamine (0.014 ml, 0.10 mmol), and concentrated under reduced pressure. The resulting residue was

dissolved in H_2O, and lyophilized to afford **2** (0.010 g, 34% from compound **26**) as a white solid. ^{1}H NMR (D_2O) δ: 0.70 (6H, bs, C$\underline{H}_3$ x 2), 1.12 (12H, bs, C$\underline{H}_2$ x 6), 1.42 (4H, bs, C$\underline{H}_2$ x 2), 2.06-2.30 (4H, m, C$\underline{H}_2$ x 2), 3.96-4.47 (10H, m, C$\underline{H}$ x 6, C$\underline{H}_2$OP, C$\underline{H}_2$OCO), 5.22 (1H, bs, $CH_2C\underline{H}CH_2$). HRMS(FAB) *m/z* calcd for $C_{23}H_{47}O_{28}P_6$ 957.0680. Found: 957.0623 $(M-H)^+$.

3.21. DL-2-*O*-(1,2-*O*-diheptanoyl-*sn*-glyceryl) hydrogen phosphoryl]-*myo*-inositol 1,3,4,5,6-pentakis(hydrogenphosphate): 2'

27 (0.045 g, 0.029 mmol) was allowed to react under the same condition as described for the preparation of **2** to give **2'** (0.008 g, 39% from an acid form of **2'**) as a white solid.

^{1}H NMR (D_2O) δ: 0.70 (6H, bs, C$\underline{H}_3$ x 2), 0.98-1.22 (12H, m, C$\underline{H}_2$ x 6), 1.43 (4H, bs, C$\underline{H}_2$ x 2), 2.23-2.28 (4H, m, C$\underline{H}_2$ x 2), 3.34 (1H, bs, C$\underline{H}$), 3.60 (1H, bs, C$\underline{H}$), 3.77 (1H, bs, C$\underline{H}$), 4.05-4.30 (7H, m, C$\underline{H}$ x 3, C$\underline{H}_2$OP, C$\underline{H}_2$OCO), 5.20 (1H, bs, $CH_2C\underline{H}CH_2$).

^{1}H NMR (D_2O) δ: 0.66-0.68 (6H, m, C$\underline{H}_3$ x 2), 1.03-1.24 (111H, m, C$\underline{H}_2$ x 6, $NCH_2C\underline{H}_3$ x 33), 1.28-1.43 (4H, m, C$\underline{H}_2$ x 2), 1.90-2.28 (4H, m, C$\underline{H}_2$ x 2), 2.86-3.05 (66H, m, $NC\underline{H}_2CH_3$ x 33), 3.57-3.59 (1H, m, C$\underline{H}$), 3.82 (1H, t, *J*=5.6Hz, C$\underline{H}$), 3.99 (2H, bs, C$\underline{H}$ x 2), 4.08-4.16 (4H, m, C$\underline{H}$ x 2, C$\underline{H}_2$OCO), 4.26-4.40 (2H, m, C$\underline{H}_2$OP), 5.13 (1H, bs, $CH_2C\underline{H}CH_2$). HRMS(FAB) *m/z* calcd for $C_{23}H_{47}O_{28}P_6$ 957.0680. Found: 957.0756 $(M-H)^+$.

3.22. DL-2, 3,4,5,6-penta-*O*-[(1,5-dihydro-2,4,3-benzodioxaphosphepin-3-yl)phosphoryl]-*myo*-inositol 1-{[2-*O*-heptanoyl-1-*O*-methyl-*sn*-glyceryl] (benzyl)phosphate} (28) DL-1, 3,4,5,6-penta-*O*-[(1,5-dihydro-2,4,3-benzodioxaphosphepin-3-yl)phosphoryl]-*myo*-inositol 2-{[2-*O*-heptanoyl-1-*O*-methyl-*sn*-glyceryl] (benzyl)phosphate} (29)

22 (0.117 g, 0.54 mmol) was allowed to react under the same condition as described for the preparation of **27** to give **28** (0.098 g, 63%) as a white solid and compound **29** (0.018 g, 11%) as a white solid.

Compound **28**

^{1}H NMR ($CDCl_3$) δ: 0.77-0.88 (3H, m, C$\underline{H}_3$), 1.19-1.28 (6H, m, C$\underline{H}_2$ x 3), 1.42-1.63 (2H, m, C$\underline{H}_2$), 2.21-2.27 (2H, m, C$\underline{H}_2$), 3.17-3.31 (5H, m, OC$\underline{H}_3$, CHC$\underline{H}_2$), 3.45-4.53 (2H, m, C$\underline{H}_2$CH), 4.25-4.38

(2H, m, C<u>H</u> x 2), 4.88-5.75 (29H, m, C<u>H</u> x 4, C<u>H</u>$_2$C$_6$H$_5$, C<u>H</u>$_2$OP, CH$_2$C<u>H</u>CH$_2$, (C<u>H</u>$_2$)$_2$C$_6$<u>H</u>$_5$ x 5), 7.14-7.48 (25H, m, C$_6$<u>H</u>$_4$ x 5, C$_6$<u>H</u>$_5$). IR (KBr) 2930, 1740, 1460, 1380, 1290, 1230, 860, 730, 700 cm^{-1}. HRMS(FAB) *m/z* calcd for $C_{64}H_{74}O_{27}P_6Na$ 1483.2741. Found: 1483.2659 $(M+Na)^+$. R_f 0.63 (AcOEt CH_2Cl_2:MeOH=15:5:1).

Compound **29**

^{1}H NMR ($CDCl_3$) δ: 0.80-0.86 (3H, m, C<u>H</u>$_3$), 1.20-1.30 (6H, m, C<u>H</u>$_2$ x 3), 1.49-1.74 (2H, m, C<u>H</u>$_2$), 2.24-2.33 (2H, m, C<u>H</u>$_2$), 3.28-3.37 (5H, m, OC<u>H</u>$_3$, CHC<u>H</u>$_2$), 3.45-4.59 (2H, m, C<u>H</u>$_2$CH), 4.17-4.37 (2H, m, C<u>H</u> x 2), 4.90-5.66 (27H, m, C<u>H</u> x 4, C<u>H</u>$_2$C$_6$H$_5$, C<u>H</u>$_2$OP, CH$_2$C<u>H</u>CH$_2$, (C<u>H</u>$_2$)$_2$C$_6$<u>H</u>$_5$ x 5), 7.16-7.52 (25H, m, C$_6$<u>H</u>$_4$ x 5, C$_6$<u>H</u>$_5$). IR (KBr) 3000, 2880, 1740, 1460, 1300, 1020, 860, 730 cm^{-1}. HRMS(FAB) *m/z* calcd for $C_{64}H_{74}O_{27}P_6Na$ 1483.2741. Found: 1483.2697 $(M+Na)^+$. R_f 0.72 (AcOEt CH_2Cl_2:MeOH=15:5:1).

3.23. DL-1-*O*-[(2-*O*-heptanoyl-1-*O*-methyl-*sn*-glyceryl) hydrogen phosphoryl]-*myo*-inositol 2,3,4,5,6-pentakis(hydrogenphosphate): 3

28 (0.098 g, 0.0671 mmol) was allowed to react under the same condition as described for the preparation of **2** to give **3** (58.2 mg, 44%) as a white solid.

^{1}H NMR (D_2O) δ: 0.22 (3H, t, *J*=6.4Hz, C<u>H</u>$_3$), 0.42-0.66 (60H, m, C<u>H</u>$_2$ x 3, NCH$_2$C<u>H</u>$_3$ x 18), 0.98 (2H, t, *J*=6.8Hz, C<u>H</u>$_2$), 1.80 (2H, m, C<u>H</u>$_2$), 2.55-2.57 (36H, m, NC<u>H</u>$_2$CH$_3$ x 18), 2.74 (3H, s, OC<u>H</u>$_3$), 3.06 (2H, t, *J*=6.0Hz, CHC<u>H</u>$_2$), 3.33 (2H, *J*=5.5Hz, C<u>H</u>$_2$CH), 3.44-3.54 (1H, m, C<u>H</u>), 3.61-3.70 (3H, m, C<u>H</u> x 3), 3.81-3.94 (2H, m, C<u>H</u> x 2), 4.55-4.64 (1H, bs, CH$_2$C<u>H</u>CH$_2$). HRMS(FAB) *m/z* calcd for $C_{16}H_{37}O_{26}P_6$ 858.9948. Found: 859.0034 $(M-H)^+$.

3.24. DL-2-*O*-[(2-*O*-heptanoyl-1-*O*-methyl-*sn*-glyceryl) hydrogen phosphoryl]-*myo*-inositol 1,3,4,5,6-pentakis(hydrogenphosphate): 3'

29 (0.018 g, 0.0121 mmol) was allowed to react under the same condition as described for the preparation of **2** to give **3'** (0.0051 g, 22%) as a white solid.

^{1}H NMR (D_2O) δ: 0.74 (3H, t, *J*=6.2Hz, C<u>H</u>$_3$), 1.05-1.18 (114H, m, C<u>H</u>$_2$ x 3, NCH$_2$C<u>H</u>$_3$ x 36), 1.50 (2H, t, *J*=7.3Hz, C<u>H</u>$_2$), 2.29-2.35 (2H, m, C<u>H</u>$_2$), 2.93-3.19 (72H, m, NC<u>H</u>$_2$CH$_3$ x 36), 3.22-3.33 (5H, s, OC<u>H</u>$_3$, C<u>H</u>$_2$CH), 3.56-3.57 (2H, m, C<u>H</u>$_2$CH), 3.86-3.89 (1H, m, C<u>H</u>), 4.22-4.48 (5H, m, C<u>H</u> x5), 5.03-5.13 (1H, m, CH$_2$C<u>H</u>CH$_2$). HRMS(FAB) *m/z* calcd for $C_{16}H_{37}O_{26}P_6$ 858.9948. Found:

858.9951 $(M-H)^+$.

3.25. DL-2, 3,4,5,6-penta-*O*-[bis(2-cyanoethyl)phosphoryl]-*myo*-inositol 1-{[1,2-*O*-dihexyl-*sn*-glyceryl] (2-cyanoethyl)phosphate} (30)

To a solution of **20** (0.098 g, 0.378 mmol) in CH_2Cl_2 (5ml) was added (2-cyanoethyl)-*N, N, N', N'*-tetraisopropylphosphoramidite (0.150 ml, 0.473 mmol) followed by MS4A (0.10 g), and the resulting mixture was stirred at room temperature under argon for 15min. To the mixture was added 1*H*-tetrazole (0.026 g, 0.378 mmol), and the resulting mixture was stirred at room temperature under argon for 10min. To the mixture was added completely dissolved compound **12** (0.061 g, 0.0549 mmol) in CH_2Cl_2 (10 ml) and CH_3CN (5 ml) with MS4A, followed by adding 1*H*-tetrazole (0.035 g, 0.50 mmol), and the resulting mixture was stirred at room temperature for further 24h. To the mixture was added *tert*-butylhydroperoxide (0.058 ml, 0.40 mmol), and stirred at room temperature for further 5min. The mixture was purified by silica gel column chromatography (CH_2Cl_2:MeOH=7:1 to 5:1) to afford crude compound **30** (0.025 g, 31%) as a colorless oil.

^{1}H NMR (CD_3OD) δ: 0.79-0.84 (6H, m, C<u>H</u>$_3$ x 2), 1.10-1.23 (12H, m, C<u>H</u>$_2$ x 6), 1.47 (4H, bs, C<u>H</u>$_2$ x 2), 2.51-2.89 (22H, m, CH_2C<u>H</u>$_2$CN x 11), 3.34-3.71 (7H, m, C<u>H</u>$_2$ x 3, C<u>H</u>), 4.22-4.68 (27H, m, C<u>H</u>$_2$$CH_2$CN x 11, CH x 5), 4.68-4.84 (2H, m, C<u>H</u>$_2$), 5.33 (1H, bs, C<u>H</u>). HRMS(FAB) *m/z* calcd for $C_{54}H_{81}N_{11}O_{26}P_6$ 1508.3678. Found: 1508.3728. $(M+Na)^+$. TLC; R_f 0.46 (CH_2Cl_2:MeOH=7:1).

3.26. DL-1-*O*-(1,2-*O*-dihexyl-*sn*-glyceryl) hydrogen phosphoryl]-*myo*-inositol 2,3,4,5,6-pentakis(hydrogenphosphate): 4

To a solution of **30** (0.025 g, 0.0168 mmol) in MeOH (5 ml) was added 25% NH_4OH (5 ml, 66.4 mmol), and the resulting mixture was stirred at 55 °C for 12h. The mixture was concentrated under reduced pressure, and the residue was adapted to reverse phase chromatography (C_{18} column, 5 g, 50% CH_3CN to 100% CH_3CN). The resulting eluted fraction was concentrated under reduced pressure. The residue was dissolved H_2O (2 ml), and filtered through the cation-exchange resin. To the resulting filtrate was added triethylamine (0.0460 ml, 0.337 mmol), and concentrated under reduced pressure. The resulting residue was dissolved in H_2O, and lyophilized to afford **4** (0.016 g, 64%) as a colorless oil.

^{1}H NMR (D_2O) δ: 0.76 (6H, bs, C<u>H</u>$_3$), 1.14-1.19 (66H, m, C<u>H</u>$_2$ x 6, NCH_2C<u>H</u>$_3$ x 18), 1.38-1.47 (4H,

m, C$\underline{H}_2$ x 2), 3.05-3.12 (36H, m, NC$\underline{H}_2$CH$_3$ x 18), 3.41-3.67 (7H, m, C$\underline{H}_2$ x 3, C$\underline{H}$), 3.92-4.19 (5H, m, C$\underline{H}$ x 5), 4.43-4.88 (3H, m, C$\underline{H}_2$OP, C$\underline{H}$). HRMS(FAB) *m/z* calcd for $C_{21}H_{47}O_{26}P_6$ 901.0781. Found: 901.0793 $(M-H)^+$.

3.27. DL-2, 3,4,5,6-penta-*O*-[bis(2-cyanoethyl)phosphoryl]-*myo*-inositol 1-{[2-*O*-hexyl-1-*O*-methyl-*sn*-glyceryl] (2-cyanoethyl)phosphate} (31)

25 (0.090 g, 0.473 mmol) was allowed to react under the same condition as described for the preparation of **30** to give **31** (0.023 g, 41%) as a colorless oil.

^{1}H NMR (CD_3OD) δ: 0.87 (3H, bs, C$\underline{H}_3$), 1.10-1.30 (6H, m, C$\underline{H}_2$ x 3), 1.56 (2H, bs, C$\underline{H}_2$), 2.99 (22H, bs, CH$_2$C$\underline{H}_2$CN x 11), 3.29-3.74 (10H, m, OC$\underline{H}_3$, C$\underline{H}_2$ x 3, C$\underline{H}$), 4.30-4.49 (22H, m, C$\underline{H}_2$CH$_2$CN x 11), 4.74-4.97 (5H, m, C$\underline{H}$ x 5), 5.41 (1H, s, C$\underline{H}$). HRMS(FAB) *m/z* calcd for $C_{49}H_{71}N_{11}O_{26}P_6$ 1438.2895. Found: 1438.2861. $(M+Na)^+$. TLC; R_f 0.35 (CH_2Cl_2:MeOH=7:1).

3.28. DL-1-*O*-[(2-*O*-hexyl-1-*O*-methyl-*sn*-glyceryl) hydrogen phosphoryl]-*myo*-inositol 2,3,4,5,6-pentakis(hydrogenphosphate): 5

31 (0.023 g, 0.0164 mmol) was allowed to react under the same condition as described for the preparation of **4** to give **5** (0.0147 g, 63%) as a colorless oil.

^{1}H NMR (D_2O) δ: 0.72 (3H, bs, C$\underline{H}_3$), 1.11-1.16 (60H, m, C$\underline{H}_2$ x 3, NCH$_2$C$\underline{H}_3$ x 18), 1.44 (2H, bs, C$\underline{H}_2$), 3.01-3.09 (36H, m, NC$\underline{H}_2$CH$_3$ x 18), 3.25 (3H, s, OC$\underline{H}_3$), 3.43-3.65 (5H, m, C$\underline{H}_2$ x 2, C$\underline{H}$), 3.97-4.09 (5H, m, C$\underline{H}$ x 5), 4.36-4.72 (3H, m, C$\underline{H}_2$OP, C$\underline{H}$). HRMS(FAB) *m/z* calcd for $C_{16}H_{37}O_{26}P_6$ 830.9999. Found: 830.9959 $(M-H)^+$.

3.29. Plasmids, cells, and transfection

The designated pEF-Gag (p17) cFLAG was used for expression vectors of MA domain. 293T cells[24] were cultured in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated FBS. The calcium phosphate coprecipitation method[25] was used for the transfection of 293T cells. Transfected cells were cultured at 37 °C for 48 h before use in protein purification.

3.30. Protein purification

Vector-transfected 293T cells were lysed with TNE buffer (10 mM Tris-HCl, 150 mM NaCl, 1 mM

EDTA, 1%NP-40, and 10 μg/mL aprotinin, pH 7.8) containing 1 mM dithiothreitol (DTT). After centrifugation (12000 rpm, 4 °C, 5 min), the supernatant was mixed with Sepharose CL-4B (Sigma-Aldrich, St. Louis, MO), and the resulting suspension was incubated for 2 h at 4 °C. This incubation was repeated twice, and the final supernatant was treated with mouse anti-FLAG M2 affinity gel (Sigma-Aldrich, St. Louis, MO) and 0.5 ng/mL 1× FLAG peptide (Sigma-Aldrich, St. Louis, MO), to remove nonspecific components interacting with the FLAG antibody, and incubated for 8 h at 4 °C. The beads were washed five times with TNE buffer plus 1 mM DTT. A solution of 150 μg/mL 3× FLAG peptide (Sigma-Aldrich, St. Louis, MO) in TBS buffer (50 mM Tris-HCl and 150mM NaCl, pH7.4) with 1 mM DTT was loaded onto the beads and incubated for 30 min at 4 °C. Following centrifugation, the resulting supernatant was used for the SPR assay.

3.31. Protein quantification

The cFLAG proteins were resolved by SDS-PAGE followed by Coomassie Brilliant Blue (CBB) staining. Each gel band was quantified using ImageJ (version 1.38×) software, and protein concentrations were determined by comparing the intensity of protein bands with the intensity of a protein marker.

3.32. SPR studies

A BIACORE2000 (GE Healthcare, BIACORE AB, Uppsala, Sweden) was used as the surface plasmon resonance biosensor. To prepare the IP_4 immobilized sensor chip surface for the BIACORE, biotinylated IP_4[9] in HEPES buffer (50 mM HEPES, 500 mM NaCl, 3.4 mM EDTA, and 0.005% Tween 20, pH 7.4) was injected over streptavidin covalently immobilized upon the sensor chip surface (Sensor Chip SA, GE Healthcare, BIACORE AB, Uppsala, Sweden) until a suitable level was achieved. The flow buffer contained 10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.005% Tween 20, 2% (v/v) glycerol, and 0.5 mg/mL BSA (pH 7.8). Purified proteins were dialyzed against flow buffer and injected over the immobilized IP_4 sensor chip. Association was followed for 3 min, and dissociation was measured at a flow rate of 20 μL/min at 25 °C. The surfaces were regenerated by injecting three 15 s pulses of 50 mM NaOH in 1 M NaCl, three 15 s pulses of 50 mM NaOH, and then a single 15 s pulse of 10 μM IP_4. The resulting surfaces were post conditioned by injecting three 15 s pulses of 10 mM NaOH. Analysis of the response was performed using evaluation

862 software supplied with the instrument (BIAevaluation version 3.1). To eliminate small bulk
863 refractive change differences at the beginning and end of each injection, binding responses were
864 referenced by subtracting the response generated across a surface modified with biotin.
865
866 **3.33. Equilibrium-binding measurement**
867 To determine *K*d values, 1.96 μM MA was mixed with various concentrations of inositol
868 phosphates, phosphatidylinositols. After reaching equilibrium (less than 30 min in all cases at
869 25 °C), 60 μL of each mixture was injected over the IP_4 surface at 20 μL/min to quantify the free
870 MA remaining in the equilibrium mixture. The *K*d was obtained by fitting the data to a solution
871 affinity model using BIAevaluation 3.1: $A_{free}=0.5(B - A - Kd) + (0.25(A +B + Kd)^2 -AB)^{0.5}$, where
872 A=initial concentration of proteins, A_{free}=concentration of unbound proteins remaining in the
873 equilibrium mixture, and B=initial concentration of IP_4.
874
875 **3.34. Molecular docking methodology**
876 Docking studies were performed using MOE 2012.10. Crystal structure of myr-MA (PDB code:
877 1UPH)[26] was obtained from the Protein Data Bank to prepare protein for docking studies. Docking
878 procedure was followed using the standard protocol implemented in MOE 2012.10. To the structure
879 was added hydrogen atom and electric charge by Protonate 3D, and the resulting structure was
880 optimized by Amber12: EHT, and then the dummy atoms were disposed in the docking site by Site
881 finder (Alpha Site Setting; Probe Radius 1: 1.4 Å, Probe Radius 2: 1.8 Å, Isolated Donor/Acceptor:
882 3 Å, Connection Distance: 2.5 Å, Minimum size: 3 Å, and Radius: 2 Å). The docking simulation
883 was carried out by ASEDock. The targeting ligands were assigned in ASEDock, and the
884 conformations were integrated by LowModeMD based on the algorithm of conformation analysis
885 (Step1; cutoff: 4.5 Å, RMS (root mean square) gradient: 10 kcal/mol/Å, energy threshold: 500
886 kcal/mol, Step2; optimize 5 lowest energy or 5 best score conformation, cutoff: 8 Å, RMS gradient:
887 0.1 kcal/mol/Å).
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891

4. Conclusion

In this study, lipid-coupled *myo*-inositol 1,2,3,4,5,6-hexakisphopshate (IP_6) derivatives having both IP_6 and diacylglycerol moiety that could interact with the HIV-1 MA domain, were designed and synthesized. These compounds, in fact, bound to MA domain more tightly than the PIP_2 derivative **1** or IP_6 does and may provide the structural basis of the molecular design of novel anti-HIV agents that block the membrane localization of $Pr55^{Gag}$.

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6. References

1 J. W. Wills, R. C. Craven, *AIDS* 1991, **5**, 639-654.

2 E. O. Freed, *Virology* 1998, **251**, 1-15.

3 M. R. Conte, S. Matthews, *Virology* 1998, **246**, 191-198.

4 A. Ono, S.D. Ablan, S. J. Lockett, K. Nagashima, and E. O. Freed. *Proc. Natl. Acad. Sci. USA* 2004, **101**, 14889-14894.

5 J. S. Saad, J. Miller, J. Tai, A. Kim, R. H. Ghanam, and M. F. Summers, *Proc. Natl. Acad. Sci. USA* 2006, **103**, 11364-11369.

6 N. Shkriabai, S. A. Datta, Z. Zhao, S. Hess, A. Rein, and M. Kvaratskhelia, *Biochemistry* 2006, **45**, 4077-4083.

7 K. Anraku, R. Fukuda, N. Takamune, S. Misumi, Y. Okamoto, M. Otsuka, and M. Fujita, *Biochemistry* 2010, **49**, 5110-5116.

8 K. Anraku, T. Inoue, K. Sugimoto, Y. Okamoto, T. Morii, Y. Mori, M. Otsuka. *Org. Biomol. Chem.* 2008, **6**, 1822-1830.

9 K. Anraku, T. Inoue, K. Sugimoto, K. Kudo, Y. Okamoto, T. Morii, Y. Mori, M. Otsuka. *Bioorg. Med. Chem.* 2011, **19**, 6833-6841.

10 E. O. Freed, J. M. Orenstein, A. J. Buckler-White, M. A. Martin, *J. Virol.*1994, **68**, 5311-5320.

11 W. Zhou, L. J. Parent, J. W. Wills, M. D. Resh, *J. Virol.*1994, **68**, 2556-2569.

12 C. G. Ferguson, R. D. James, C. S. Bigman, D. A. Shepard, Y. Abdiche, P. S. Katsamba, D. G. Myszka, and G. D. Prestwich, *Bioconjug. Chem.* 2005, **16**, 1475-1483.

13 D. C. Billington, R. Baker, J. J. Kulagowski, I. M. Mawer, *J. Chem. Soc. Chem. Commun.* 1987, **4**, 314-316.

14 W. Bannwarth, A. Trzeciak, *Helv. Chim. Acta* 1987, **70**, 175-186.

15 N. Nagashima, M. Ohno, *Chem. Lett.* 1987, 141-144.

16 C. Liu, B. L. Potter, *J. Org. Chem.* 1997, **62**, 8335-8340.

17 Y. Oikawa, T. Tanaka, K. Horita, O. Yonemitsu, *Tetrahedron Lett.* 1984, **25**, 5397-5400.

18 S. Ozaki, Y. Kondo, N. Shiotani, T. Ogasawara, Y. Watanabe, *J. Chem. Soc., Perkin Trans. 1* 1992, 729-737.

19 B. Classon, P. Garegg, B. Samuelsson, *Acta chemica Scandinavica. Series B. Organic chemistry and biochemistry* 1984**, 38**, 419-422.

20 E. Uhlmann, J. Engels, *Tetrahedron Lett.* 1986, **27**, 1023-1026.

21 E. O. Freed, J. M. Orenstein, A. J. Buckler-White, M. A. Martin, *J. Virol.* 1994, **68,** 5311-5320.

22 J. Chan, R. Dick, V. M. Vogt, *J. Virol.* 2011, **85**, 10851-10860.

23 J. Inlora, V. Chukkapalli, D. Derse, A. Ono, *J. Virol.* 2011, **85**, 3802-3810.

24 J. S. Lebkowski, S. Clancy, M. P. Calos, *Nature* 1985, **317**, 169–171.

25 A. Adachi, H. E. Gendelman, S. Koenig, T. Folks, R. Willy, A. Rabson, M. A. Martin, *J. Virol.* 1986, **59**, 284-291.

26 C. Tang, E. Loeliger, P. Luncsfold, I. Kinde, D. Beckett, M. F. Summers, *Proc. Natl. Acad. Sci. USA* 2004, **101**, 517-522.

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