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Carbohydrate Research 343 (2008) 1718–1729
Carbohydrate RESEARCH

Synthesis and biological evaluation of sperm CD52 GPI anchor and related derivatives as binding receptors of pore-forming CAMP factor

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Received 27 February 2008; received in revised form 20 March 2008; accepted 30 March 2008
Available online 4 April 2008

Abstract—Sperm CD52 GPI anchor and its derivatives containing different carbohydrate chains were prepared in a highly convergent fashion starting from the same properly protected phospholipidated pseudodisaccharide. Coupling this common key intermediate to various oligosaccharyl donors quickly afforded the framework of the synthetic targets, which was followed by global deprotection to furnish the desired structures. Preliminary studies on the biological properties of the synthetic GPI derivatives indicated that both the intact GPI anchor and the free phospholipidated pseudodisaccharide interacted strongly with CAMP factor, a pore-forming bacterial toxin.

Keywords: GPI anchor; CD52 antigen; CAMP factor; Bacterial toxin; Binding assay

1. Introduction

Glycosylphosphatidylinositols (GPIs) are a class of natural glycolipids ubiquitously expressed by eukaryotic cells.[1,2] An evident biological function of GPIs is to anchor proteins and glycoproteins onto cell surfaces by inserting their lipid chains into cell membranes.[1,3] All protein/glycoprotein-anchoring GPIs contain the α-Man*p*-(1→2)-α-Man*p*-(1→6)-α-Man*p*-(1→4)-α-Glc*p*-NH_2-(1→6)-*myo*-inositol-1-PO_4-lipid construct (**1**, Fig. 1) as their highly conserved core structure, and numerous structural variations of GPIs occur in the mannose residues.[1,2] Proteins and glycoproteins are linked to GPI anchors via the peptidic C-terminus. GPIs and GPI-anchored proteins and glycoproteins are leaflets on the cell surface and play a critical role in various important biological processes, such as cell recognition and adhesion, signal transduction, and bacterial and viral infections.[3–5]

Many bacteria produce toxins as their major virulent factors and a significant part of these toxins exhibit virulence via the formation of pores in the host cell membrane.[6–8] In this process, the toxins use host cell surface GPI anchors and/or GPI-anchored proteins and glycoproteins as binding receptors to assist the pore formation. First, the pore-forming toxins need to bind to GPI anchors. Next, the GPI-bound toxins aggregate to form porous oligomers on the host cell surface, which is facilitated by some unique properties of GPI anchors, for example, their lateral mobility in the cell membrane and their privileged presence in certain membrane domains.[9] Eventually, the resultant toxin oligomers insert into the cell membrane to form channels that can break the permeability barrier of cells and cause adverse ion exchange and other lethal reactions. It has been revealed that the pore-forming toxins mainly bind to the core

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doi:10.1016/j.carres.2008.03.033

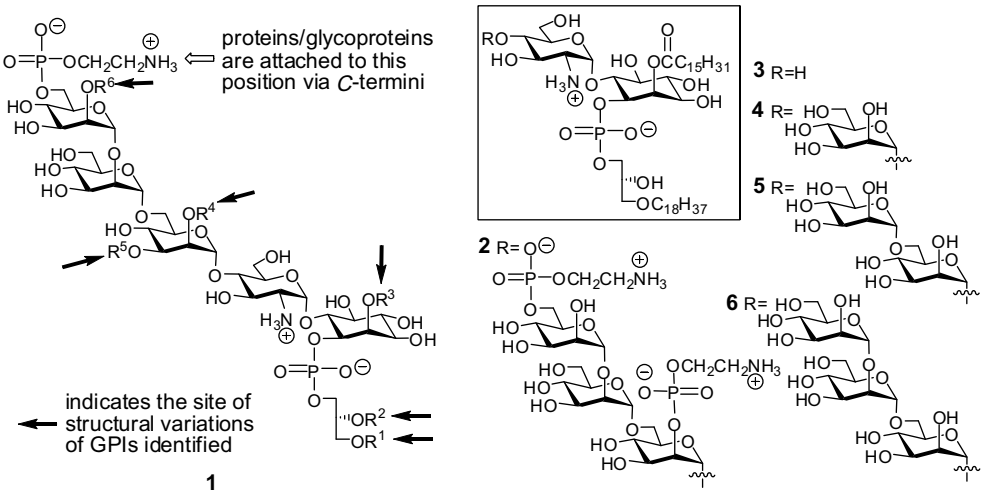


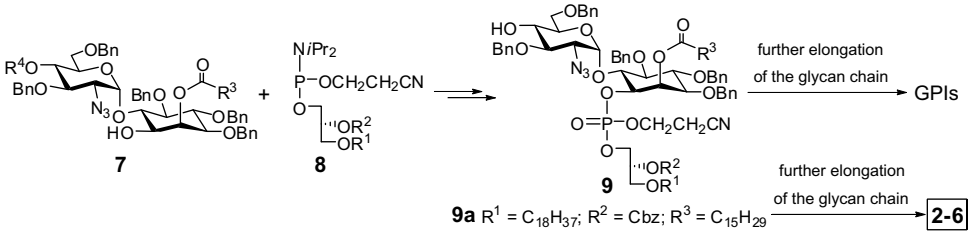
Figure 1. Structures of the conserved GPI core (**1**) and the synthetic targets including a GPI anchor of the CD52 antigen **2** and its derivatives **3**–**6**.

glycan of GPIs.[10] However, it is presently unclear how the pore-forming toxins interact with GPIs in detail and which structural components of GPIs are recognized by these toxins. To answer these questions, information concerning the structure–activity relationship (SAR) of GPI anchors as the toxin receptors is necessary, while systematic SAR studies of GPI anchors require homogeneous and structurally defined GPIs. However, the chemical synthesis of GPI anchors and related derivatives remains a significant challenge.

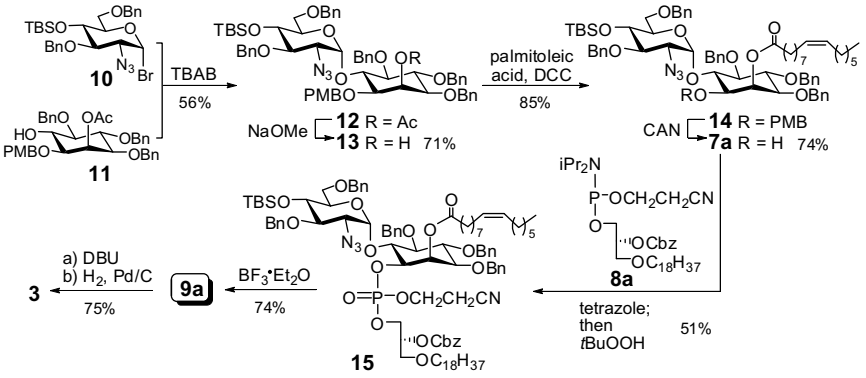
This paper describes a convergent synthesis of a GPI anchor (**2**) of sperm CD52 antigen and its derivatives **3**–**6** (Fig. 1), as well as preliminary studies on the binding between GPIs/GPI derivatives and CAMP factor, a pore-forming toxin secreted by *Streptococcus agalactiae*.[11,12] The CD52 antigen is a GPI-anchored glycopeptide, playing an important role in the human immune system and in the human reproductive system.[13,14] It has been reported that pore-forming toxins utilize the CD52 antigen as one of the binding receptors.[15] Consequently, studying the binding between CAMP factor and CD52 GPI anchor and related derivatives will provide insights into the functional mechanisms of pore-forming toxins and help to design novel antibacterial strategies.

2. Results and discussion

Most GPI syntheses reported in the literature have adopted a strategy in which the phospholipid moiety is introduced to the inositol 1-*O*-position at the final stage.[16–34] Although this strategy has been successfully used to prepare a number of GPI anchors, it is not particularly efficient for the synthesis of GPI derivatives containing different glycans. Recently, in the synthesis of CD52 GPI anchors, we have developed a strategy in which the phospholipid moiety was attached to pseudodisaccharide **7** before further elongation of the glycan (Scheme 1).[35–37] It was observed that the phospholipid moiety was not affected during subsequent deprotection and glycosylation processes. If this synthetic strategy is applied to prepare GPI derivatives having various glycans, it can have two advantages. One is that the relatively difficult reactions involved in GPI synthesis, including the glycosylation reaction to install the α-linked glucosamine unit and the phosphorylation reaction to introduce the phospholipid moiety to the inositol ring, could be realized at an early stage, which can improve the overall synthetic efficiency. The other advantage is that all syntheses could be realized using the same versatile building block, which allows various



Scheme 1.



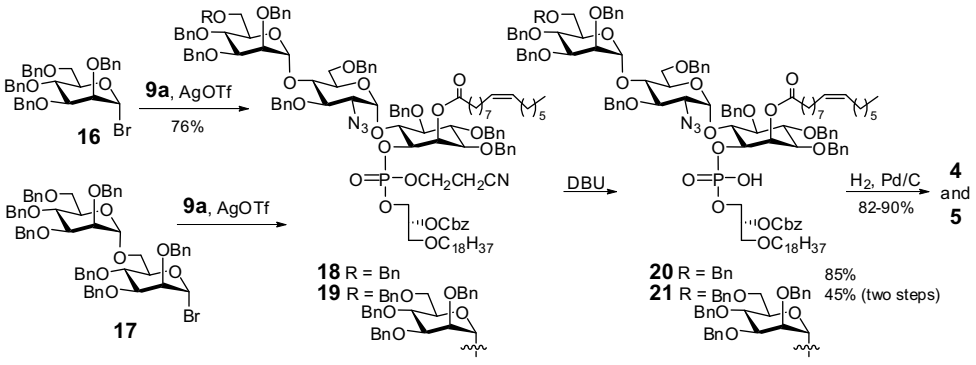
Scheme 2.

GPI derivatives to be prepared conveniently. Thus, our synthetic plan for **2**–**6** was to first prepare a properly protected phospholipidated pseudodisaccharide **9a** and then couple **9a** with oligosaccharides to obtain GPI derivatives containing different glycans (Scheme 1).

The preparation of **9a** (Scheme 2) followed our reported procedure,[37] which commenced with the stereospecific glycosylation of an inositol derivative **11**[37] by the Lemieux protocol[38] using **10**[37] as the glycosyl donor and tetrabutylammonium bromide (TBAB) as the glycosylation promoter. The α-linked pseudodisaccharide **12** was obtained in 56% yield. Here, the azido group was used as a nonparticipating latent amino group, which facilitated stereospecific α-glycosylation. Thereafter, the acetyl group at the inositol 2-*O*-position was replaced with a palmitoleoyl moiety in a two-step sequence: deacetylation with sodium methoxide (NaOMe) followed by the acylation of the resultant alcohol with palmitoleic acid and dicyclohexylcarbodiimide (DCC). The subsequent removal of the *p*-methoxybenzyl (PMB) group with ceric ammonium nitrate (CAN) provided **7a**, which was phospholipidated in a two-step one-pot fashion to afford **15** in 51% yield as a mixture of two diastereomers. The ^{31}P NMR spectrum of **15** indicated that the two diastereoisomers were formed in an almost 1:1 ratio. Because a large excess of **8a** (more than 7 equiv) was utilized in this reaction, the product purification was very difficult, and multiple column chromatography separations were necessary to obtain pure **15**. Moreover, the diastereoisomers were inseparable either by TLC or by HPLC. Consequently, the diastereomeric mixture was applied to subsequent reactions. Finally, the *t*-butyldimethylsilyl (TBS) group at the glucosamine 4-*O*-position was selectively removed with 3% $BF_3{\cdot}Et_2O$ in dichloromethane to produce **9a** (74%), which was employed to prepare all synthetic targets described in Figure 1. The key synthetic intermediate **9a** was characterized by 1D and 2D NMR spectroscopy and MS.

To prepare GPI derivative **3** (Scheme 2), the secondary alcohol **9a** was treated first with DBU for a short period (ca. 2 min) to remove the 2-cyanoethyl group that was used to protect the phosphate functionality, and subsequently with H_2 in the presence of 10% Pd–C to remove the benzyl and benzyloxycarbonyl (Cbz) groups and reduce the azido group concomitantly. The synthetic target **3** was eventually obtained in 82% yield, and its structure was affirmed by NMR spectroscopy and MS.

With access to the key intermediate **9a**, GPI derivatives **4** and **5** were synthesized according to Scheme 3. Glycosylation of **9a** with mannosyl bromides **16** and **17** in the presence of silver triflate (AgOTf) as the



Scheme 3.

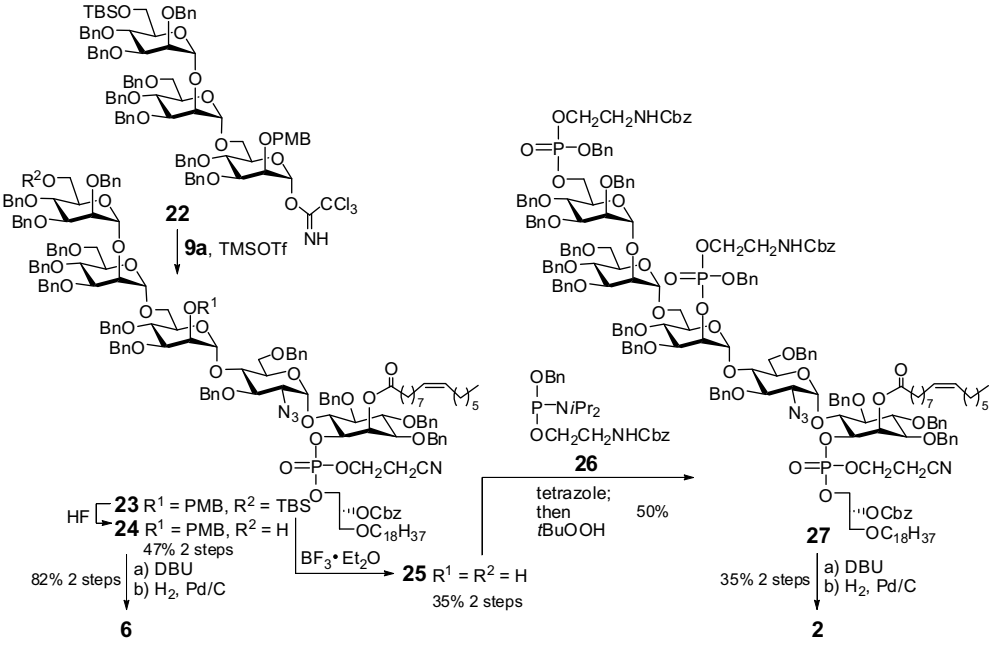
promoter and 2,6-di-*t*-butyl-6-methylpyridine (DTBMP) as an acid scavenger provided oligosaccharides **18** and **19** in 76% and ca. 65% yields, respectively. The use of excessive glycosyl donors (3 equiv) was necessary for these reactions, because the benzylated donors were particularly reactive and their decomposition was inevitable. The anomeric ^{1}H and ^{13}C NMR signals of compounds **18** and **19**, which were positively identified in their HMQC spectra, suggested α-configuration of the newly formed glycosidic linkages. The subsequent removal of the 2-cyanoethyl group in **18** with DBU followed by hydrogenolysis afforded **4** in 77% (2 steps) yield. In a similar procedure, compound **5** was obtained in 37% (3 steps) yield. The final products **4** and **5**, as well as the synthetic intermediates, were fully characterized by NMR spectroscopy and MS.

In the synthesis of compound **6**, trichloroacetimidate **22** was used as the glycosyl donor, and the glycosylation was performed with trimethylsilyl triflate (TMSOTf) as the promoter. Because an excess of **22** (3 equiv) was employed in the reaction, and its hydrolytic byproduct overlapped with the desired product **23** during column chromatography, product purification was challenging. Therefore, after the product was briefly purified by passing through a short silica gel column, it was treated with HF in a mixture of water and acetonitrile to remove the TBS group. The resulting product **24** was easily purified. Finally, **24** was subjected to global deprotection as described above to give **6** in 82% yield (Scheme 4).

On the other hand, the treatment of **23** with 3% $BF_3{\cdot}Et_2O$ in dichloromethane gave **25** that was easily purified by column chromatography, and the two diastereomers of **25** were separable. Both isomers were characterized by ^{1}H and ^{31}P NMR spectroscopy and MS. Compound **25** was then phosphorylated in the presence of **26** to introduce two phosphoethanolamine moieties. Global deprotection of the resulting product **27** in a two-step sequence, namely DBU treatment to remove the 2-cyanoethyl group followed by hydrogenolysis in the presence of H_2 and Pd–C, eventually afforded the GPI anchor **2** of the sperm CD52 antigen, which was characterized by NMR spectroscopy and MS. The NMR signals of the anomeric H and C spins, which were clearly identified in the HMQC spectra, suggested α-configuration of all glycosidic linkages.

After the designed GPI derivatives were obtained, their ability to bind CAMP factor was studied by means of quartz crystal microbalance (QCM).[39] For this purpose, GPI derivatives were deposited onto QCM electrodes using a procedure described by Stine and Pishko.[40] In short, after the surface of a gold QCM electrode was modified with a monolayer of octanethiol (OT) and hexadecanethiol (HDT) (1:1), it was exposed to the liposomal solution of a GPI derivative mixed with sphingomyelin (SM) (in a 1:2 ratio) to attach a layer of GPIs onto the sensor surface (Fig. 2). The electrode was then ready for the analysis of the binding between GPIs and CAMP factor.

For the binding assays, *Erythrina cristagalli* lectin (ECL), a galactose-specific legume lectin,[41] was used as a negative control to eliminate any nonspecific protein binding to GPI-modified QCM sensors. As shown in Figure 3, when ECL was added to QCM equipped with the **2** and **6**-modified sensors, no obvious QCM



Scheme 4.

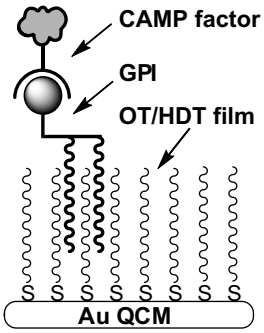


Figure 2. Lipid-modified gold QCM electrode used to deposit GPI derivatives for the analysis of binding between GPIs and CAMP factor.

frequency change was observed, which clearly suggested that nonspecific interaction between the GPI derivatives and the carbohydrate-binding protein was negligible. In contrast, CAMP factor induced significant frequency changes in both GPI-modified QCM sensors. For example, after two portions of CAMP factor were added to QCM equipped with the **2**-modified sensor, which formed 0.8 and 1.6 μM CAMP factor solutions, the QCM frequency changed by ca. 35 and 42 Hz (Fig. 3a), respectively. Under the same conditions, CAMP factor induced a frequency change of 75 and 84 Hz, respectively, with the **6**-modified QCM sensor (Fig. 3b). These results demonstrated that CAMP factor exhibited specific binding to both the intact GPI anchor and a partial sequence of the GPI core. At this point, the reason why the GPI segment **6** gave a larger signal than the intact GPI **2** is unclear. We speculate that the structural difference between the two GPI derivatives might have affected their alignment on the QCM sensor surface. Compared to **6**, GPI anchor **2** is larger in size, so its density on the sensor surface might be lower. Alternatively, **2** and **6** might have similar densities on the sensor surface, but the intact GPI anchor **2** was more sterically hindered, which would affect the binding. In either case, the available binding sites for the **2**-modified sensor would be less than that for the **6**-modified sensor. To understand this phenomenon, more detailed investigations using different concentrations of GPI derivatives to modify QCM sensors are underway.

Nevertheless, to precisely characterize the interaction between GPI derivatives and CAMP factor, we analyzed the binding thermodynamics. To obtain an accurate affinity constant, we needed to examine the energy loss that occurred in the oscillatory system due to (1) a viscoelastic porous structure that is strained during oscillation; (2) trapped liquid that moves between or in and out of pores caused by the deformation of the film; (3) the load from the bulk liquid, which increases the strain of film. Thus, all the experiments were performed using a QCM impedance analyzer, which allowed simultaneous measurements of the frequency and the energy dissipation in the binding process.[42,43] By obtaining the damping resistance through fitting the Butworth-van Dyk Circuit,[44] the QCM impedance analyzer can verify the validity of the Sauerbrey equation and the dissipative properties, if the modified layer shows viscoelastic properties. The change of damping resistance in our cases was $|\Delta R_q|/R_q \leqslant 1.1\%$, which suggested that the GPI-modified films behaved rigidly in our experiments; therefore, the Sauerbrey equation was valid for directly correlating mass with the frequency change.

Based on the Langmuir adsorption isotherm,[45] the association (K_a) and dissociation (K_d) constants for the GPI–CAMP factor interaction shown in Eq. 1 should have the correlation described by Eq. 2:

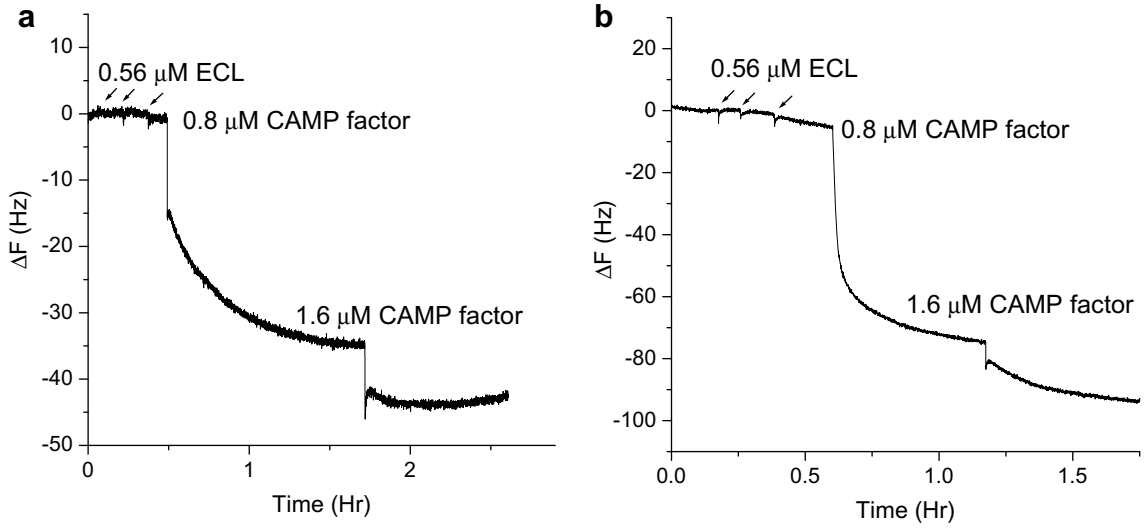


Figure 3. QCM results when ECL and CAMP factor were added sequentially to (a) GPI anchor **2**-modified sensor and (b) GPI segment **6**-modified sensor.

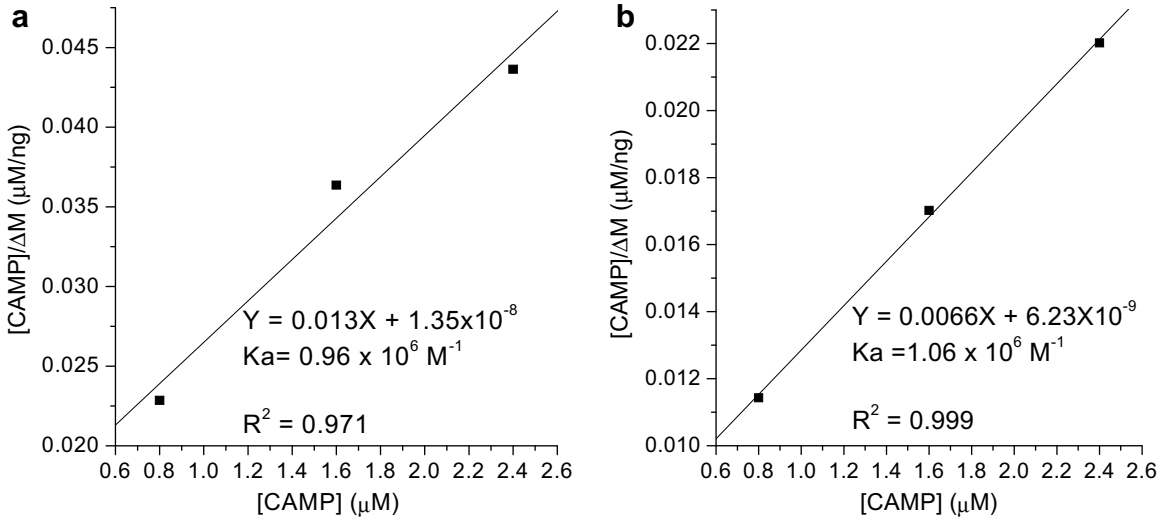


Figure 4. [CAMP]/Δ*M* versus [CAMP] plotting of the QCM results observed for the binding between CAMP factor and (a) intact GPI anchor **2** or (b) GPI segment **6**.

$$\text{GPI} + \text{CAMP factor} \underset{K_{\text{off}}}{\overset{K_{\text{on}}}{\rightleftharpoons}} \text{GPI–CAMP factor complex} \quad (1)$$

$$\frac{[\text{CAMP}]}{\Delta \text{M}} = \frac{[\text{CAMP}]}{\Delta \text{M}_{\text{max}}} + \frac{1}{\Delta M K_{\text{a}}} \quad (2)$$

where Δ*M* (nanogram) is the amount of CAMP factor bound to the QCM sensor at equilibrium, which can be obtained from the QCM frequency decrease; ΔM_{max} is the maximum amount of CAMP factor that can be bound; [CAMP] is the original concentration of CAMP factor. Δ*M* is a function of the CAMP factor concentration, but it will not change with time. Plotting [CAMP]/Δ*M* versus [CAMP] of the QCM results can produce the association constant K_{a}, which, according to Eq. 2, should be the ratio of the slope to the interception. As shown in Figure 4, the association constants for GPI anchor **2** and GPI segment **6** to bind to CAMP factor were 0.96×10^6 and $1.06 \times 10^6\ \text{M}^{-1}$, respectively. These results indicated that intact GPI anchor **2** and GPI segment **6** have similar binding affinities to CAMP factor and that both have high binding affinities.

In conclusion, a highly convergent strategy was successfully applied to synthesize GPI derivatives. This synthesis is highlighted by the use of a common key intermediate, starting from which various GPI derivatives containing different carbohydrate chains can be assembled efficiently and rapidly. The synthetic GPI derivatives were used to investigate the interactions between pore-forming toxins and their GPI receptors. Our preliminary results suggest that the segments of GPI anchor may also bind to the pore-forming CAMP factor effectively, an observation that has never been reported before. Although there have been reported studies on the interactions between GPIs and pore-forming toxins,[10,15,46–48] the conclusions were indefinite and sometimes contradictory, because complex systems or undefined GPI derivatives were used previously. The use of structurally well defined GPIs and derivatives to study this problem may provide more conclusive results, and the SAR from these studies may provide insights into the understanding of this important process and facilitate novel antibacterial designs. Currently, our lab is focused on studying the interactions of all the synthetic GPI derivatives with various pore-forming toxins. The results will be reported in due time.

3. Experimental

3.1. General methods

^{1}H NMR spectra were recorded at 400 or 500 MHz with chemical shifts reported in ppm (δ) downfield from tetramethylsilane (TMS) if not specified otherwise. ^{13}C NMR spectra were recorded at 100 or 125 MHz with chemical shifts reported in ppm (δ) in reference to the solvent peak of $CDCl_3$ (δ 77.16). The coupling constants (*J*) are reported in Hertz (Hz). Thin layer chromatography (TLC) was performed on Silica Gel GF_{254} plates with detection by charring with phosphomolybdic acid in EtOH or 5% H_2SO_4 in EtOH solutions. Molecular sieves 4 Å (4 Å MS) used were dried in high vacuum at 170–180 °C for 6–10 h immediately before use. Anhydrous solvents were obtained from a solvent purification system, while commercial anhydrous reagents were used without further purification.

3.2. 2-*O*-Acetyl-6-*O*-[2-azido-3,6-di-*O*-benzyl-4-*O*-*tert*-butyldimethylsilyl-2-deoxy-α-D-glucopyranosyl]-3,4,5-tri-*O*-benzyl-1-*O*-(4-methoxybenzyl)-*myo*-inositol (12)

After a mixture of **11** (1.02 g, 1.67 mmol), 4 Å MS (790 mg) and TBAB (710 mg, 1.75 mmol) in 15 mL of

dry CH_2Cl_2 was stirred for 4 h at rt, the freshly prepared glycosyl bromide **10** (1.7 g, 2.51 mmol) was added to the mixture under an argon atmosphere. After being stirred for 3 days in the dark, the reaction mixture was diluted with Et_2O and the organic layer was isolated and washed with satd aq $NaHCO_3$ and brine solutions. The organic phase was dried over Na_2SO_4 and concentrated, and the residue was separated by column chromatography to afford **12** as colorless syrup (1.02 g, 56%); $[\alpha]_D$ +50 (*c* 1.0, $CHCl_3$); $R_f = 0.46$ (EtOAc–hexane 1:4); ^1H NMR ($CDCl_3$, 400 MHz): δ 7.23–7.47 (m, 27H), 6.92 (d, $J = 8.4$ Hz, 1H), 5.98 (t, $J = 2.4$ Hz, 1H), 5.79 (d, $J = 3.6$ Hz, 1H), 5.04 (t, $J = 11.2$ Hz, 1H), 4.91–4.98 (m, 2H), 4.88 (d, $J = 11.2$ Hz, 1H), 4.82 (d, $J = 11.2$ Hz, 1H), 4.61 (d, $J = 11.6$ Hz, 1H), 4.45–4.50 (m, 2H), 4.17 (t, $J = 9.6$ Hz, 1H), 4.08 (d, $J = 1.2$ Hz, 1H), 3.99 (t, $J = 9.6$ Hz, 1H), 3.83–3.86 (m, 5H), 3.64 (dd, $J = 2.4$, 6 Hz, 1H), 3.20–3.52 (m, 2H), 3.44 (m, 1H), 3.22 (m, 1H) 2.22 (s, 3H), 0.81 (s, 9H), −0.01 (d, $J = 8$ Hz, 6H). FABMS *m*/*z*: $[M+Na]^+$ calcd for $C_{63}H_{75}N_3NaO_{12}Si$, 1116.5; found, 1117.0.

3.3. 6-*O*-[2-Azido-3,6-di-*O*-benzyl-4-*O*-*tert*-butyldimethylsilyl-2-deoxy-α-D-glucopyranosyl]-3,4,5-tri-*O*-benzyl-1-*O*-(4-methoxybenzyl)-*myo*-inositol (13)

To a solution of **12** (1.02 g, 0.93 mmol) in 10 mL of dry MeOH was added 0.1 mL of sodium methoxide solution in MeOH (1 M, 0.1 mmol). After being stirred at rt for 3 days, the mixture was neutralized with Amberlyst resin to pH 7–8. After the resin was filtered off, the filtrate was concentrated, and the residue was separated by column chromatography to give **13** as colorless syrup (753 mg, 71%); $[\alpha]_D$ +44 (*c* 1.0, $CHCl_3$); $R_f = 0.16$ (EtOAc–hexane 1:4); ^1H NMR ($CDCl_3$, 400 MHz): δ 7.19–7.45 (m, 27H), 6.95 (d, $J = 8.4$ Hz, 2H), 5.80 (d, $J = 3.6$ Hz), 4.90–5.02 (m, 3H), 4.88 (t, $J = 11.2$ Hz, 2H), 4.76–4.78 (m, 3H), 4.60–4.63 (d, $J = 12.4$ Hz, 2H), 4.50 (s, 2H), 4.28 (t, $J = 9.2$ Hz, 2H), 4.03 (q, $J = 9.2$, 14.2 Hz, 2H), 3.79–3.90 (m, 5H), 3.59 (dd, $J = 2.4$, 6 Hz, 1H), 3.49 (t, $J = 9.6$ Hz, 1H), 3.42 (d, $J = 2.4$ Hz, 2H), 3.29 (dd, $J = 3.6$, 6.8 Hz, 1H), 0.80 (s, 9H), 0.01 (d, $J = 8$ Hz, 6H). FABMS *m*/*z*: $[M+Na]^+$ calcd for $C_{61}H_{73}N_3NaO_{11}Si$, 1074.5; found, 1074.9.

3.4. 6-*O*-[2-Azido-3,6-di-*O*-benzyl-4-*O*-*tert*-butyldimethylsilyl-2-deoxy-α-D-glucopyranosyl]-3,4,5-tri-*O*-benzyl-1-*O*-(4-methoxybenzyl)-2-*O*-palmitoleoyl-*myo*-inositol (14)

To a solution of **13** (650 mg, 0.62 mmol) in 10 mL of dry CH_2Cl_2 was added palmitoleic acid (206 mg, 0.81 mmol), DCC (167 mg, 0.81 mmol) and a catalytic amount of DMAP (8 mg, 0.06 mmol). After being stirred at rt for 3 days, the reaction mixture was concentrated under reduced pressure, and the resultant residue was separated by column chromatography to afford **14** as colorless syrup (677 mg, 85%); $[\alpha]_D$ +22 (*c* 1.0, $CHCl_3$); $R_f = 0.66$ (EtOAc–hexane 1:4); ^1H NMR ($CDCl_3$, 400 MHz): δ 7.16–7.37 (m, 27H), 6.83 (d, $J = 11.2$ Hz, 2H), 5.93 (t, $J = 2.4$ Hz, 1H), 5.70 (d, $J = 4.8$ Hz, 1H), 5.30–5.36 (m, 2H), 4.94–5.00 (m, 2H), 4.90 (s, 1H), 4.86 (d, $J = 3.2$ Hz, 1H) 4.82 (s, 1H), 4.77 (d, $J = 6.4$ Hz, 1H), 4.71 (d, $J = 8.8$ Hz, 1H), 4.62–4.67 (m, 1H), 4.53 (d, $J = 15.4$ Hz, 1H), 4.37–4.55 (m, 3H), 4.08 (t, $J = 9.2$ Hz, 1H), 4.00 (dd, $J = 2.4$, 6 Hz, 1H), 3.89 (t, $J = 9.6$ Hz, 1H), 3.75–3.79 (m, 5H), 3.18 (dd, $J = 2.4$, 6 Hz, 1H), 3.40–3.54 (m, 2H), 3.55 (t, $J = 9.6$ Hz, 1H), 3.16 (m, 1H), 2.52 (t, $J = 7.6$ Hz, 2H), 2.08 (m, 4H), 1.55 (t, $J = 12.4$ Hz, 2H), 1.30 (m, 18H), 0.88 (t, $J = 12.4$ Hz, 3H), 0.74 (s, 9H), −0.06 (d, $J = 8$ Hz, 6H). ESIMS *m*/*z*: $[M+K]^+$ calcd for $C_{77}H_{101}N_3KO_{12}Si$, 1326.8; found, 1327.0.

3.5. 6-*O*-[2-Azido-3,6-di-*O*-benzyl-4-*O*-*tert*-butyldimethylsilyl-2-deoxy-α-D-glucopyranosyl]-3,4,5-tri-*O*-benzyl-2-*O*-palmitoleoyl-*myo*-inositol (7a)

To a solution of **14** (780 mg, 0.65 mmol) in 10 mL of CH_3CN and H_2O (9:1) was added CAN (206 mg, 1.6 mmol). After the mixture was stirred at rt for 2 h, it was diluted with EtOAc. The organic phase was washed with satd aq $NaHCO_3$ and brine solutions. After the organic phase was dried over Na_2SO_4 and concentrated, the residue was separated by column chromatography to afford **7a** as colorless syrup (530 mg, 74%); $[\alpha]_D$ +34 (*c* 1.0, $CHCl_3$); $R_f = 0.16$ (EtOAc–hexane 1:4); ^1H NMR ($CDCl_3$, 400 MHz): δ 7.23–7.39 (m, 25H), 5.78 (t, $J = 2.4$ Hz, 1H), 5.34–5.38 (m, 2H), 5.06 (d, $J = 10.4$ Hz, 1H), 4.94 (d, $J = 10.4$ Hz, 1H), 4.93 (s, 2H), 4.81 (t, $J = 5.2$ Hz, 2H), 4.76 (d, $J = 10.4$ Hz, 1H), 4.53 (d, $J = 11.2$ Hz, 1H), 4.31 (s, 2H), 3.93 (t, $J = 9.2$ Hz, 1H), 3.75–3.85 (m, 5H), 3.47–3.60 (m, 3H), 3.37 (dd, $J = 2.8$, 7 Hz, 1H), 3.12 (d, $J = 9.6$ Hz, 1H), 2.42 (t, $J = 7.2$ Hz, 2H), 2.02 (t, $J = 5.2$ Hz, 4H), 1.69 (t, $J = 5.2$ Hz, 2H), 1.24–1.37 (m, 18H), 1.88 (s, 9H), 0.01 (s, 6H). ESIMS *m*/*z*: calcd for $C_{69}H_{93}N_3O_{11}Si$, 1167.6; found, 1190.5 $[M+Na]^+$, 1206.5 $[M+K]^+$.

3.6. 6-*O*-[2-Azido-3,6-di-*O*-benzyl-4-*O*-*tert*-butyldimethylsilyl-2-deoxy-α-D-glucopyranosyl]-3,4,5-tri-*O*-benzyl-1-*O*-[(2-cyanoethyoxy)-(2-*O*-benzyloxycarbonyl-3-*O*-octadecyl-*sn*-glycerol)-phosphono]-2-*O*-palmitoleoyl-*myo*-inositol (15)

To a solution of **7a** (110 mg, 0.94 mmol) in dry CH_2Cl_2 (10 mL) was added a tetrazole solution in dry CH_3CN (1.95 mL, 0.45 M, 0.88 mmol), which was followed by the addition of freshly prepared **8a** (550 mg, 0.8 mmol) at rt. After the mixture was stirred at rt for 10 min, TLC showed that compound **7a** was completely consumed. Then, the reaction vessel was cooled down to −20 °C, and a solution of *t*-BuOOH in decane

(0.22 mL, 5.5 M, 1.2 mmol) was added dropwise. After being warmed to rt in 30 min, the reaction mixture was continuously stirred for another 1 h. EtOAc was added to dilute the reaction mixture, and the organic phase was isolated and washed with satd aq $NaHCO_3$ and brine solutions, dried over Na_2SO_4 and then concentrated under vacuum. The residue was separated by column chromatography to afford an inseparable mixture of two isomers (1:1.1) of **15** as colorless syrup (85 mg, 51%); $R_f = 0.56$ (acetone–hexane 1:2); ^1H NMR ($CDCl_3$, 400 MHz): δ (the major isomer) 5.95 (t, $J = 2.4$ Hz, 1H), 5.55 (d, $J = 3.6$ Hz, 1H), 5.39 (m, 3H), 2.76 (t, $J = 6.0$ Hz, 2H), 2.44 (q, $J = 7.6$ Hz, 2H), 2.05 (t, $J = 6$ Hz, 4H), 0.95 (t, $J = 6.8$ Hz, 6H), 0.82 (s, 9H), 0 (d, $J = 3.6$ Hz, 6H); δ (the minor isomer) 5.98 (t, $J = 2.4$ Hz, 1H), 5.55 (d, $J = 3.6$ Hz, 1H), 5.39 (m, 3H), 2.70 (t, $J = 6.0$ Hz, 2H), 2.44 (q, $J = 7.6$ Hz, 2H), 2.05 (t, $J = 6$ Hz, 4H), 0.95 (t, $J = 6.8$ Hz, 6H), 0.82 (s, 9H), 0.0 (d, $J = 3.6$ Hz, 6H); ^{31}P NMR ($CDCl_3$, 162 MHz): δ −1.18, −1.05. ESIMS m/z: $[M+Na]^+$ calcd for $C_{101}H_{145}N_4NaO_{18}PSi$, 1785.0; found, 1784.7.

3.7. 6-*O*-[2-Azido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl]-3,4,5-tri-*O*-benzyl-1-*O*-[(2-cyanoethyoxy)-(2-*O*-benzyloxycarbonyl-3-*O*-octadecyl-*sn*-glycerol)-phosphono]-2-*O*-palmitoleoyl-*myo*-inositol (9a)

At 0 °C, **15** (120 mg, 0.68 mmol) was added to 5 mL of 3% $BF_3{\cdot}Et_2O$ in CH_2Cl_2, and the solution was stirred for 3 h. After the reaction mixture was diluted with CH_2Cl_2, the organic phase was isolated and washed with satd aq $NaHCO_3$ and brine solutions, dried over Na_2SO_4 and then concentrated under vacuum. Column chromatography of the residue afforded an isomeric mixture (1:1.2) of **9a** (83 mg, 74%) as colorless syrup; $R_f = 0.53$ (acetone–hexane 1:2); ^1H NMR ($CDCl_3$, 400 MHz): δ (the major isomer) 5.81 (t, $J = 2.4$ Hz, 1H), 5.35 (d, $J = 3.6$ Hz, 1H), 5.26 (m, 3H), 2.63 (t, $J = 6.0$ Hz, 2H), 2.28 (q, $J = 7.6$ Hz, 2H), 1.92 (t, $J = 6$ Hz, 4H), 0.77 (t, $J = 6.8$ Hz, 6H); δ (the minor isomer) 5.84 (t, $J = 2.4$ Hz, 1H), 5.35 (d, $J = 3.6$ Hz, 1H), 5.26 (m, 3H), 2.58 (t, $J = 6.0$ Hz, 2H), 2.22 (q, $J = 7.6$ Hz, 2H), 1.92 (t, $J = 6$ Hz, 4H), 0.77 (t, $J = 6.8$ Hz, 6H); ^{31}P NMR ($CDCl_3$, 162 MHz): δ −0.91, −1.04. ESIMS m/z: $[M+K]^+$ calcd for $C_{95}H_{131}N_4KO_{18}P$, 1686.0; found, 1686.4.

3.8. 6-*O*-(2-Amino-2-deoxy-α-D-glucopyranosyl)-1-*O*-[(3-*O*-octadecyl-*sn*-glycerol)-phosphono]-2-*O*-hexadecanoyl-*myo*-inositol (3)

At room temperature, a drop of DBU was added into a solution of **9a** (9 mg, 5.46 μmol) in 2 mL of dry CH_2Cl_2. After 2 min, TLC indicated the completion of reaction and then a drop of AcOH was introduced to quench the reaction. After the removal of the solvent, the resulted residue was subjected to preparative TLC to provide the partially deprotected intermediate (7 mg, 4.39 μmol). ^1H NMR (10% CD_3OD–$CDCl_3$, 400 MHz): δ 5.92 (br, 1H), 5.62 (br, 1H), 5.26 (m, 2H), 2.27 (t, $J = 6.8$ Hz, 2H), 1.92 (dd, $J = 9.2$, 6.4 Hz, 4H); HMQC NMR ($CDCl_3$, ^{13}C 100 MHz): δ 70.0/5.92 (Insitol-2), 96.4/5.62 (Glc-1); ^{31}P NMR ($CDCl_3$): δ 0.58. ESI (negative mode) m/z: $[M-H]^-$ calcd for $C_{92}H_{127}N_3O_{18}P$, 1592.9; found, 1593.1. A mixture of this product and 10% Pd–C (10 mg) in $CHCl_3$–MeOH–H_2O (4 mL, 10:10:3) was stirred under an H_2 atmosphere for 3 days. The reaction mixture was filtered off through a pad of Celite, and the Celite was washed with a 4 mL mixture of $CHCl_3$, MeOH, and H_2O (10:10:3). The filtrates were combined and concentrated to give **3** (4 mg, 4.05 μmol, 75% two steps). Selected ^1H NMR ($CDCl_3$–CD_3OD–D_2O 3:3:1, 500 MHz): δ 5.53 (br, 1H), 2.41 (br, 1H), 1.57 (m, 4H), 0.86 (br, 6H). MALDI TOFMS (negative mode) m/z: $[M-H]^-$ calcd for $C_{49}H_{95}NO_{16}P$, 984.6; found, 984.4.

3.9. 6-*O*-[(2,3,4,6-Tetra-*O*-benzyl-α-D-mannopyranosyl)-(1→4)-(2-azido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl)]-1-*O*-[(2-cyanoethyoxy)-(2-*O*-benzyloxycarbonyl-3-*O*-octadecyl-*sn*-glycerol)-phosphono]-2-*O*-palmitoleoyl-3,4,5-tri-*O*-benzyl-*myo*-inositol (18)

To a mixture of **9a** (10 mg, 5.49 μmol) and 4 Å molecular sieves (10 mg) in 2 mL of dry CH_2Cl_2 was added DTBMP (2.4 mg, 11.7 μmol). The mixture was stirred for 2 h at rt. Then, freshly prepared **16** (11 mg, 18.3 μmol) was introduced at −20 °C. After 0.5 h of stirring, a solution of AgOTf (4.6 mg, 17.9 μmol) in dry toluene was injected into the reaction mixture which was stirred overnight. The reaction mixture was diluted with ether, and the organic layer was isolated and then washed with satd aq $NaHCO_3$ and brine solutions. The solvent was removed under reduced pressure, and the resultant mixture was subjected to preparative TLC to provide **18** (10 mg, 76%). ^1H NMR ($CDCl_3$, 400 MHz): δ (the major isomer) 5.88 (br, 1H), 5.47 (m, 1H), 5.33 (m, 2H), 2.69 (t, $J = 6.4$ Hz, 2H), 2.36 (m, 2H), 1.98 (t, $J = 6.4$ Hz, 4H), 1.61 (t, $J = 7.2$ Hz, 2H), 0.88 (t, $J = 6.8$ Hz, 6H). MALDI TOFMS m/z: $[M+Na]^+$ calcd for $C_{129}H_{165}N_4NaO_{23}P$, 2192.3; found, 2193.6.

3.10. 6-*O*-[(2,3,4,6-Tetra-*O*-benzyl-α-D-mannopyranosyl)-(1→4)-(2-azido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl)]-1-*O*-[(2-*O*-benzyloxycarbonyl-3-*O*-octadecyl-*sn*-glycerol)-phosphono]-2-*O*-palmitoleoyl-3,4,5-tri-*O*-benzyl-*myo*-inositol (20)

A drop of DBU was added into a solution of **18** (10 mg, 5.04 μmol) in 2 mL of dry CH_2Cl_2. Two minutes later, TLC indicated the completion of reaction, and then a drop of HOAc was added to quench the reaction. After

the removal of the solvent under reduced pressure, the resultant residue was subjected to preparative TLC to provide **20** (9 mg, 85%). ^{1}H NMR (CD_3OD and $CDCl_3$ 1:9, 400 MHz): δ 5.93 (br, 1H), 5.70 (br, 1H), 5.26 (m, 3H), 2.26 (br, 2H), 1.93 (m, 4H), 0.84 (br, 6H); ^{1}H–^{13}C HMQC NMR (CD_3OD–$CDCl_3$ 1:9, ^{13}C 100 MHz/^{1}H 400 MHz): 69.1/5.93 (Inos-2), 97.5/5.70 ($GlcN_3$-1), 101.2/5.26 (Man-1); ^{31}P NMR ($CDCl_3$): δ 0.58. ESIMS (negative mode) m/z $[M-H]^-$ calcd for $C_{126}H_{161}N_3O_{23}P$, 2115.1; found, 2115.2.

3.11. 6-*O*-[(α-D-Mannopyranosyl)-(1→4)-(2-amino-2-deoxy-α-D-glucopyranosyl)-1-*O*-[(3-*O*-octadecyl-*sn*-glycerol)-phosphono]-2-*O*-hexadecanoyl-*myo*-inositol (4)

A mixture of **20** (9 mg, 4.24 μmol) and 10% Pd–C (10 mg) in $CHCl_3$–MeOH–H_2O (10:10:3, 4 mL) was stirred in an H_2 atmosphere for 3 days. The reaction mixture was filtered through a pad of Celite, and the Celite was washed with a mixture of $CHCl_3$–MeOH–H_2O (10:10:3, 4 mL). The filtrates were combined and concentrated to afford **4** (4 mg, 90%). ^{1}H NMR ($CDCl_3$–CD_3OD–D_2O 3:3:1, 500 MHz): δ 5.52 (br, 1H), 5.25 (br, 1H), 2.41 (br, 1H), 0.87 (t, $J = 6.8$ Hz, 6H). MALDI TOFMS (negative mode) m/z: $[M-H]^-$ calcd for $C_{55}H_{105}NO_{21}P$, 1146.7; found, 1146.5.

3.12. 6-*O*-[(2,3,4,6-Tetra-*O*-benzyl-α-D-mannopyranosyl)-(1→6)-(2,3,4-tri-*O*-benzyl-α-D-mannopyranosyl)-(1→4)-(2-azido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl)]-1-*O*-[(2-cyanoethyoxy)-(2-*O*-benzyloxycarbonyl-3-*O*-octadecyl-*sn*-glycerol)-phosphono]-2-*O*-palmitoleoyl-3,4,5-tri-*O*-benzyl-*myo*-inositol (21)

To a mixture of **9a** (10 mg, 5.49 μmol) and 4 Å molecular sieves (10 mg) in 2 mL of dry CH_2Cl_2 was added DTBMP (2.82 mg, 13.7 μmol), and the mixture was stirred at rt for 2 h. Then, freshly prepared **17** (22 mg, 21.3 μmol) was introduced at −20 °C; 30 min later, a solution of AgOTf (5.59 mg, 21.8 μmol) in dry toluene was injected, and the mixture was stirred overnight. The reaction mixture was diluted with ether, and the organic layer was washed with satd aq $NaHCO_3$ and brine solutions. Under reduced pressure, the solvent was removed to give **19**, which was directly applied to the next step. After **19** was dissolved in 2 mL of CH_2Cl_2, a drop of DBU was added at rt. Two minutes later, TLC showed the completion of reaction, and then a drop of HOAc was added to quench the reaction. The solvent was removed under reduced pressure, and the residue was subjected to preparative TLC to give **21** (7 mg, 45%). ^{1}H NMR (CD_3OD–$CDCl_3$ 1:9, 400 MHz): δ 5.93 (br, 1H), 5.67 (br, 1H), 5.26 (br, 3H), 2.28 (t, $J = 6.4$ Hz, 2H), 1.91 (m, 4H), 0.83 (br, 6H); ^{1}H–^{13}C HMQC NMR (CD_3OD–$CDCl_3$ 1:9, ^{13}C 100 MHz/^{1}H 400 MHz): 69.1/5.93 (Inos-2), 97.5/5.67 ($GlcN_3$-1), 101.6/5.13 (Man-1), 98.8/5.05 (Man-1); ^{31}P NMR ($CDCl_3$): δ −1.56. ESIMS (negative mode) m/z: $[M-H]^-$ calcd for $C_{153}H_{189}N_3O_{28}P$, 2547.3; found, 2547.5.

3.13. 6-*O*-[(α-D-mannopyranosyl)-(1→6)-(α-D-mannopyranosyl)-(1→4)-(2-amino-2-deoxy-α-D-glucopyranosyl)]-1-*O*-[(3-*O*-octadecyl-*sn*-glycerol)-phosphono]-2-*O*-hexadecanoyl-*myo*-inositol (5)

A mixture of **21** (9 mg, 4.25 μmol) and 10% Pd–C (10 mg) in $CHCl_3$–MeOH–H_2O (10:10:3, 4 mL) was stirred in an H_2 atmosphere for 3 days. The mixture was filtered through a pad of Celite, and the Celite was washed with a mixture of $CHCl_3$–MeOH–H_2O (10:10:3, 4 mL). The filtrates were combined and concentrated to afford **5** (4 mg, 82%). ^{1}H NMR ($CDCl_3$–CD_3OD–D_2O 3:3:1, 500 MHz): δ 5.58 (br, 2H), 4.93 (s, 1H), 2.40 (br, 2H), 1.58 (br, 4H), 0.94 (br, 6H). MALDI TOFMS m/z: $[M+H]^+$ calcd for $C_{61}H_{117}NO_{26}P$, 1310.8; found, 1310.9.

3.14. 6-*O*-[(2,3,4-tri-*O*-Benzyl-α-D-mannopyranosyl)-(1→2)-(3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl)-(1→6)-(3,4-di-*O*-benzyl-2-*O*-*p*-methoxybenzyl-α-D-mannopyranosyl)-(1→4)-(2-azido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl)]-3,4,5-tri-*O*-benzyl-1-*O*-[(2-cyanoethyoxy)-(2-*O*-benzyloxycarbonyl-3-*O*-octadecyl-*sn*-glycerol)-phosphono]-2-*O*-palmitoleoyl-*myo*-inositol (24)

To a mixture of **9a** (70 mg, 0.04 mmol) and freshly prepared **22** (0.17 g, 0.11 mmol) in dry CH_2Cl_2 (20 mL) was added 2 μL of TMSOTf at −20 °C. After the mixture was stirred for 10 min, Et_3N (2 drops) was added to quench the reaction, and then, the solvent was removed under vacuum. The resultant residue was subjected to column chromatography to afford **23** as colorless syrup. After the product was dissolved in 5 mL of CH_3CN, 0.25 mL of aqueous HF solution (48%) was added. After 2 h of stirring, TLC showed that all starting material was consumed, and the reaction mixture was diluted with CH_2Cl_2. The organic layer was washed with satd aq $NaHCO_3$ solution and dried over $MgSO_4$. After concentration in vacuum, the residue was subjected to column purification to give **24** (50 mg, 47% two steps). ^{1}H NMR ($CDCl_3$, 400 MHz): δ (the major isomer) 5.91 (br, 1H), 5.47 (br, 1H), 2.69 (t, $J = 5.6$ Hz, 1H), 2.34 (m, 4H), 0.86 (m, 15H), 0.02 (t, $J = 4.8$ Hz, 6H). MALDI TOFMS m/z: $[M+Na]^+$ calcd for $C_{177}H_{217}N_4NaO_{34}P$, 2997.5; found, 2997.8.

3.15. 6-*O*-[(α-D-mannopyranosyl)-(1→2)-(α-D-mannopyranosyl)-(1→6)-(α-D-mannopyranosyl)-(1→4)-(2-amino-2-deoxy-α-D-glucopyranosyl)]-1-*O*-[(3-*O*-octadecyl-*sn*-glycerol)-phosphono]-2-*O*-hexadecanoyl-*myo*-inositol (6)

Two drops DBU was added into a solution of **24** (10 mg, 5.04 μmol) in 2 mL of dry CH_2Cl_2. After 2 min of

stirring, TLC indicated the completion of reaction, and a drop of HOAc was added to quench the reaction. The solvent was removed, and the resultant residue was subjected to preparative TLC to give the partially deprotected product. It was then dissolved in $CHCl_3$–MeOH–H_2O (10:10:3, 4 mL), and the solution was mixed with 10% Pd–C (20 mg). The mixture was stirred in an H_2 atmosphere for 3 days. The reaction mixture was filtered off through a pad of Celite, and the Celite pad washed with a 4 mL mixture of $CHCl_3$–MeOH–H_2O (10:10:3). The filtrates were combined and then concentrated to afford **3** (22 mg, 82% two steps). ^{1}H NMR ($CDCl_3$–CD_3OD–D_2O 3:3:1, 500 MHz): δ 5.52 (br, 2H), 5.20 (br, 1H), 5.10 (br, 1H), 5.00 (br, 1H), 2.42 (br, 2H), 2.31 (br, 2H), 1.58 (br, 4H), 0.94 (br, 6H). MALDI TOFMS (negative mode): *m*/*z*: $[M-H]^-$ calcd for $C_{67}H_{125}NO_{31}P$: 1470.8; found, 1470.5.

3.16. 6-*O*-[(2,3,4-tri-*O*-Benzyl-α-D-mannopyranosyl)-(1→2)-(3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl)-(1→6)-(3,4-di-*O*-benzyl-α-D-mannopyranosyl)-(1→4)-(2-azido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl)]-3,4,5-tri-*O*-benzyl-1-*O*-[(2-cyanoethyoxy)-(2-*O*-benzyloxycarbonyl-3-*O*-octadecyl-*sn*-glycerol)-phosphono]-2-*O*-palmitoleoyl-*myo*-inositol (25)

To a mixture of **9a** (70 mg, 0.04 mmol) and freshly prepared trichloroacetimidate **22** (0.17 g, 0.11 mmol) in dry CH_2Cl_2 (20 mL) was added 2 drops of TMSOTf at −20 °C. After 10 min of stirring, Et_3N (4 drops) was added to quench the reaction, and the solvent was removed in vacuum. The residue was subjected to column chromatography to obtain **23** as colorless syrup, which was directly dissolved in a solution of 3% $BF_3{\cdot}Et_2O$ in dry CH_2Cl_2 (5 mL) at 0 °C. After 0.5 h of stirring, the reaction mixture was diluted with CH_2Cl_2, and the organic layer was washed with satd aq $NaHCO_3$ and brine solutions. The organic phase was dried over $MgSO_4$ and concentrated in vacuum, and the residue was carefully purified by column chromatography to give two isomers **25a** (8 mg, 10%) and **25a** (13 mg, 15%), as well as a mixture of the two isomers (8 mg, 10%). Some of substrate **9a** (20 mg, 0.01 mmol) was also recovered. Compound **25a**: $R_f = 0.46$ (acetone–hexane 1:2). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.06–7.35 (m, 70H), 5.88 (s, 1H), 5.32–5.48 (m, 4H), 5.00–5.22 (m, 6H), 4.18–4.95 (m, 26H), 4.02–4.4.10 (m, 3H), 3.25–3.86 (m, 27H), 2.70 (t, $J = 6.4$ Hz, 2H), 2.37 (t, $J = 7.2$ Hz, 2H), 1.98 (t, $J = 6.4$ Hz, 4H), 1.50 (m, 8H), 1.25 (m, 38H), 0.87 (t, $J = 6.4$ Hz, 6H); ^{31}P NMR ($CDCl_3$, 162 MHz): δ −0.98. Compound **25b**: $R_f = 0.45$ (acetone–hexane 1:2); ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.06–7.35 (m, 70H), 5.92 (s, 1H), 5.31 (m, 2H), 4.99–5.16 (m, 6H), 4.11–4.92 (m, 36H), 4.11 (m, 2H), 3.25–3.86 (m, 36H), 2.63 (m, 2H), 2.35 (t, $J = 7.2$ Hz, 2H), 1.98 (t, $J = 6.4$ Hz, 4H), 1.50–1.60 (m, 8H), 1.25 (m, 38H), 0.88 (t, $J = 6.4$ Hz, 6H); ^{31}P NMR ($CDCl_3$, 162 MHz): δ −1.14. MALDI TOFMS *m*/*z*: $[M+Na]^+$ calcd for $C_{169}H_{209}N_4NaO_{33}P$, 2876.5; found, 2876.9.

3.17. 6-*O*-[(2,3,4-Tri-*O*-benzyl-6-*O*-[benzyl-[2-(benzylcarbonylamino)ethyl]-phospono]-α-D-mannopyranosyl)-(1→2)-(3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl)-(1→6)-(3,4-di-*O*-benzyl-2-*O*-[benzyl-[2-(benzylcarbonylamino)ethyl]-phospono]-α-D-mannopyranosyl)-(1→4)-(2-azido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl)]-3,4,5-tri-*O*-benzyl-1-*O*-[(2-cyanoethyoxy)-(2-*O*-benzyloxycarbonyl-3-*O*-octadecyl-*sn*-glycerol)-phosphono]-2-*O*-palmitoleoyl-*myo*-inositol (27)

To a solution of **25b** (13 mg, 4.6 μmol) in 2 mL of dry CH_2Cl_2 was added tetrazole in dry CH_3CN solution (0.3 mL, 0.45 M, 0.14 mmol). After the mixture was stirred for 15 min, freshly prepared **26** (39.3 mg, 0.09 mmol) was added under an argon atmosphere at rt. After 3 h of stirring, the reaction mixture was cooled to −20 °C, and then *t*-BuOOH in decane (20 μL, 5.5 M, 0.11 mmol) was added. The mixture was warmed to rt in 30 min and was stirred for another 2 h, and it was then diluted with CH_2Cl_2 and washed with satd aq $NaHCO_3$ solution. The aqueous phase was extracted with CH_2Cl_2 twice and the combined organic phase was dried over $MgSO_4$ and concentrated in vacuum. Column chromatography of the residue afforded **27** (8 mg, 50%) as colorless syrup. $R_f = 0.44$ (acetone–hexane, 1:2); ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.10–7.41 (m, 90H), 5.90 (s, 1H), 5.32–5.40 (m, 4H), 2.54 (d, $J = 6.4$ Hz, 2H), 2.35 (t, $J = 5.2$ Hz, 2H), 1.98 (t, $J = 6.4$ Hz, 4H), 1.47–1.61 (m, 8H), 1.22 (m, 38H), 0.87 (t, $J = 6.4$ Hz, 6H). MALDI TOFMS *m*/*z*: calcd for $C_{203}H_{245}N_6O_{43}P_3$, 3547.6; found, 3570.4 $[M+Na]^+$, 3586.4 $[M+K]^+$.

3.18. 6-*O*-[[6-*O*-(2-Aminoethyl)phosphono-α-D-mannopyranosyl]-(1→2)-(α-D-mannopyranosyl)-(1→6)-[2-*O*-(2-aminoethyl)phosphono-α-D-mannopyranosyl]-(1→4)-(2-amino-2-deoxy-α-D-glucopyranosyl)]-1-*O*-[(3-*O*-octadecyl-*sn*-glycerol)-phosphono]-2-*O*-hexadecanoyl-*myo*-inositol (1)

To the solution of **27** (8 mg, 2.2 μmol) in 2 mL of dry CH_2Cl_2 was added 2 drops of DBU. About 2 min later, 2 drops of HOAc was added to quench the reaction. The reaction mixture was concentrated in vacuum, and the residue was subjected to column chromatography to give the partially deprotected product as colorless syrup (6.2 mg, 78%). $R_f = 0.61$ (MeOH–CH_2Cl_2 1:10); ^{1}H NMR ($CDCl_3$, 700 MHz): δ 7.05–7.52 (m, 90H), 5.88 (s, 1H), 5.32–5.49 (m, 4H); HMQC NMR ($CDCl_3$, ^{13}C at 125 MHz and ^{1}H at 500 MHz): 104.2/5.15 (Man-1), 99.2/5.10 (Man-1), 98.8/4.79 (Man-1), 98.5/5.49 ($GlcN_3$-1), 68.5/5.88 (Ino-2). ESIMS *m*/*z*: $[M+H]^+$ calcd for $C_{200}H_{243}N_5O_{43}P_3$, 3495.6; found, 3496.0. The prod-

uct (4 mg, 1.3 μmol) was dissolved in $CHCl_3$–MeOH–H_2O (10:10:3, 3 mL) and then mixed with 10% Pd–C (15 mg). The mixture was stirred in an H_2 atmosphere for 3 days. The reaction mixture was filtered through a pad of Celite, and the Celite pad was washed with a mixture of $CHCl_3$–MeOH–H_2O (10:10:3). The filtrate was combined and concentrated in vacuum to afford the synthetic target **2** (1.6 mg, 82%) as a white powder. ^{1}H NMR (CD_3OD–$CDCl_3$–D_2O 3:3:1, 500 MHz): δ 5.44 (m, 4H), 5.12 (s, 1H), 4.76 (s, 1H), 2.35 (br, 2H), 1.51 (br, 4H), 0.75 (t, $J = 6.0$ Hz, 6H); ^{13}C NMR (CD_3OD–$CDCl_3$–D_2O 3:3:1, 125 MHz): δ 174.11 (C═O), 101.38 (C-1, Man), 100.10 (C-1, Man), 100.07 (C-1, Man), 95.45 (C-1, GlcNH_2). MALDI TOFMS *m*/*z*: $[M+2H]^{2+}$ calcd for $C_{71}H_{137}N_3O_{37}P_3Na_3$, 892.9; found, 893.3.

3.19. Assays of the interaction between GPI derivatives and CAMP factor

A freshly washed gold QCM electrode was immersed in a 4 mM solution of octanethiol (OT) and hexadecanethiol (HDT) (1:1) in ethanol for 3 h to form a hydrophobic monolayer. The OT/HDT modified QCM surface was subsequently exposed to the liposome solution of a GPI derivative (1 mM) and sphingomyelin (2 mM) in MeOH and $CHCl_3$ (1:1) for 4 h to attach the GPI derivative onto the sensor surface. The electrode was then installed to the QCM instrument in a chamber containing 1 mL of PBS buffer (pH 7.2) with 1 mM Mn^{2+} and 1 mM Ca^{2+}. First, 10 μL of ECL (18.7 μM in PBS buffer) was introduced in three portions, and its final concentration reached 0.56 μM. Thereafter, 10 μL of CAMP factor (80 μM) in PBS buffer was added on separate portions. Solutions were stirred constantly during sample addition and subsequent measurement to ensure that the mass transfer process was not a rate-limiting step. The QCM frequencies were recorded real-time under various conditions.

Acknowledgments

This work was supported in part by NSF and Mitzutani Foundation for Glycoscience. X. Wu thanks Dr. Jie Xue for many helpful discussions. We also appreciate Dr. B. Shay and Dr. L. Hryhorczuk for the MS measurements and Dr. B. Ksebati for his help with some NMR measurements.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2008.03.033.

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