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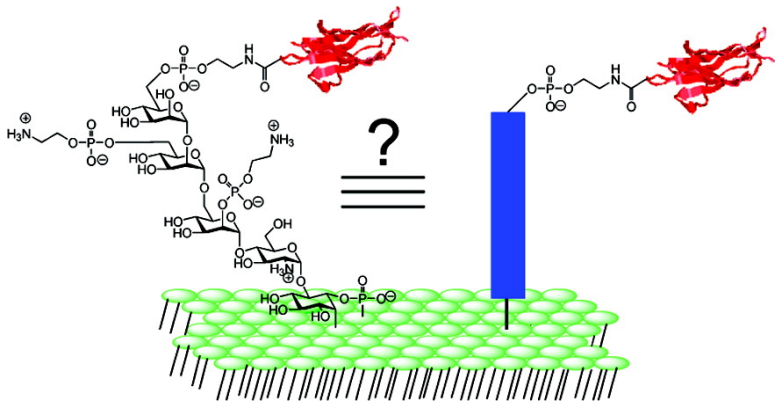

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Synthetic Analogues of Glycosylphosphatidylinositol-Anchored Proteins and Their Behavior in Supported Lipid Bilayers

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Abstract: Positioned at the C-terminus of many eukaryotic proteins, the glycosylphosphatidylinositol (GPI) anchor is a posttranslational modification that anchors the modified proteins in the outer leaflet of the plasma membrane. GPI-anchored proteins play vital roles in signal transduction, the vertebrate immune response, and the pathobiology of trypanosomal parasites. While many GPI-anchored proteins have been characterized, the biological functions of the GPI anchor have yet to be elucidated at a molecular level. We synthesized a series of GPI-protein analogues bearing modified anchor structures that were designed to dissect the contribution of various glycan components to the GPI-protein's membrane behavior. These anchor analogues were similar in length to native GPI anchors and included mimics of the native structure's three domains. A combination of expressed protein ligation and native chemical ligation was used to attach these analogues to the green fluorescent protein (GFP). These modified GFPs were incorporated in supported lipid bilayers, and their mobilities were analyzed using fluorescence correlation spectroscopy. The data from these experiments suggest that the GPI anchor is more than a simple membrane-anchoring device; it also may prevent transient interactions between the attached protein and the underlying lipid bilayer, thereby permitting rapid diffusion in the bilayer. The ability to generate chemically defined analogues of GPI-anchored proteins is an important step toward elucidating the molecular functions of this interesting post-translational modification.

Introduction

Glycosylphosphatidylinositol (GPI) anchors are glycolipid structures that are added post-translationally to a wide variety of proteins and anchor the modified proteins in the outer leaflet of the plasma membrane.[1] Proteins containing a GPI anchor play vital roles in signal transduction, immune response, cancer cell invasion and metastasis, and the pathobiology of trypanosomal parasites.[1a] The GPI anchor has been suggested to act as a targeting device, sorting proteins to the apical membrane of polarized cells or to lipid raft domains, membrane microdomains enriched in cholesterol, glycosphingolipids, and signaling proteins.[1d,2] The modification also may play a role in prion disease pathogenesis.[3] While many GPI-anchored proteins have been identified and characterized, the only confirmed biological function of the GPI anchor is to provide the attached protein with a stable membrane anchoring device.[1d,4]

The C-terminus of a GPI-anchored protein is linked through a phosphoethanolamine bridge to the conserved core glycan, Manα(1,2)Manα(1,6)Manα(1,4)GlcNα(1,6)-*myo*-inosityl-1-phospholipid (**1**, Figure 1).[1d] This glycan core can be variously modified with side chains, such as a phosphoethanolamine group

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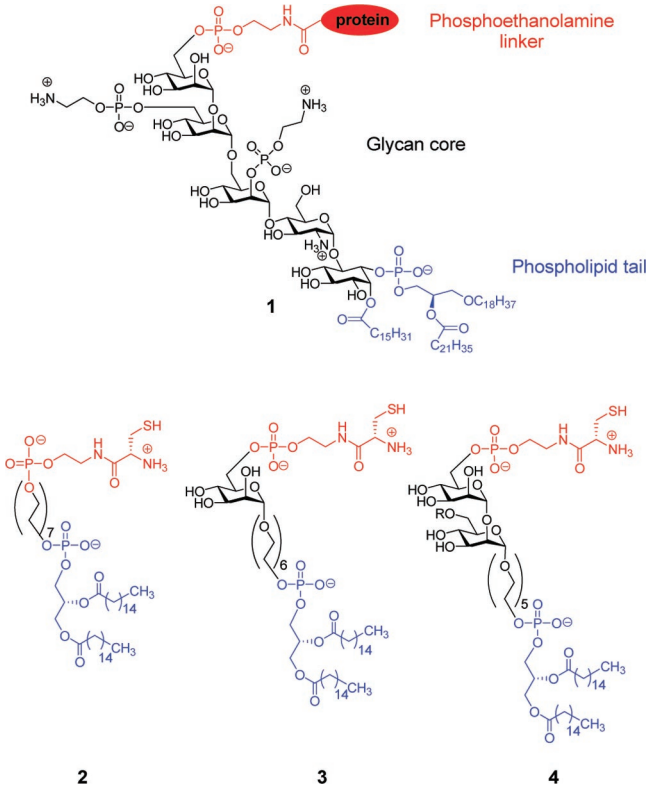

Figure 1. Structures of the native GPI anchor from human erythrocyte acetylcholinesterase (**1**) and GPI anchor analogues **2**, **3**, and **4**. These GPI anchor analogues contain mimetics of the three domains of the GPI anchor: (i) a phosphoethanolamine linker (red), (ii) the conserved glycan core (black), and (iii) a phospholipid tail (blue). R is a GPI anchor side chain, such as galactose or phosphoethanolamine.

or other sugars. The fatty acyl groups of the phospholipid tail vary in length and can be either saturated or unsaturated.

The relationship of GPI anchor structure to function is difficult to study due to the heterogeneity and limited quantities of GPI anchors from natural sources.[5] In principle, chemical synthesis can provide access to both native and novel GPI-anchored protein structures, providing valuable material for functional studies. Several total syntheses of native GPI anchors have been reported; however, these routes are complicated and not amenable to structural modification.[6] More importantly, most synthetic routes do not provide an avenue for coupling the anchor structure to a protein, the state in which they function naturally. Recently, Guo et al. attached a synthetic 12-amino acid glycopeptide from CD52, a GPI-anchored peptide, to a synthetically produced GPI anchor.[6n,o] However, almost all known GPI-anchored proteins are considerably larger than 12 amino acids and are not readily accessible by routine peptide synthesis. To circumvent the difficulty in native GPI anchor synthesis, a number of research groups have generated peptides or proteins attached to GPI anchor substitutes.[7] These GPI anchor replacements were designed to act solely as membrane-anchoring devices rather than emulating the structure of a native GPI anchor. For example, many of these GPI anchor substitutes did not have phospholipids,[7a,f] and none contained sugar residues. Therefore, the contributions of the various monosaccharides within the glycan core to the biological functions of the GPI anchor could not be assessed.

We developed a series of GPI anchor analogues where portions of the glycan core were systematically replaced with unnatural linkers of comparable length (**2**, **3**, and **4**, Figure 1). The GPI analogues **2**−**4** were designed to include mimetics of the three domains of the native structure, all with similar overall dimensions. The analogues contain no (**2**), one (**3**), or two (**4**) mannose units and replace the phosphoinositol and glucosamine units with a simple hydrophilic poly(ethylene glycol) linker. In contrast to previously designed GPI anchor substitutes,[7] all of these analogues contain a phospholipid tail and a phosphoethanolamine linker. Their syntheses are modular and allow for the installation of various side chains and different lipid tails. The phosphoethanolamine linker is attached to a cysteine moiety to allow for native chemical ligation of the GPI anchor analogue to any protein bearing a C-terminal thioester.[8] In this study, the GPI anchor analogues were attached to the green fluorescent protein (GFP), and the resulting GPI-protein analogues were incorporated into supported lipid bilayers. The effects of the glycan core modifications on protein mobility in the bilayer were investigated, revealing a possible role for the GPI glycans in preventing protein−membrane interactions.

Results and Discussion

Molecular Modeling Studies of the GPI Anchor Analogues 2, 3, and 4. Using molecular modeling (Maestro 7.5), the GPI anchor analogues **2**−**4** were designed to be similar in length to the native GPI anchor. After the structures were minimized using Macromodel (MMFF force field), the dihedral angles were manipulated to ensure that the structures of the native GPI anchor and the analogues were fully extended. The three anchor analogues were all approximately the same length (24.4 ± 0.7 Å), longer than the native GPI anchor structure by about 5 Å. However, because no solution or crystal structures of the GPI anchor have been reported, the exact conformation of the native

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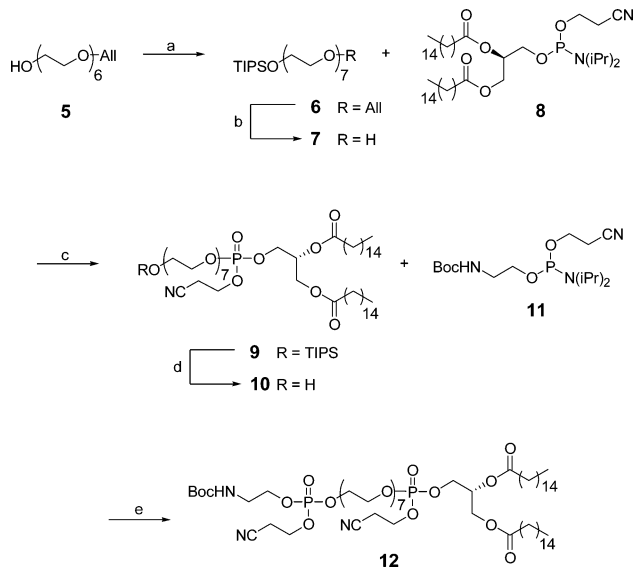
Scheme 1 [a]



[a] Reagents: (a) 2-bromoethanol triisopropylsilyl ether, NaH, DMF, 0 °C to room temperature, 18 h, 29%; (b) (i) $RhCl(PPh_3)_3$, DABCO, 9:1 $EtOH:H_2O$, 90 °C, 2 h; (ii) $KMnO_4$, NaOH, MeOH, 0 °C, 1.5 h, 63%; (c) (i) 4,5-dicyanoimidazole, CH_2Cl_2, CH_3CN, 4 h; (ii) *m*CPBA, −40 °C to room temperature, 2 h, 81%; (d) 0.5 M HCl, MeOH, CH_2Cl_2, 2 h, 89%; (e) (i) 4,5-dicyanoimidazole, CH_2Cl_2, CH_3CN, 4 h; (ii) *m*CPBA, −40 °C to room temperature, 1 h, 90%.

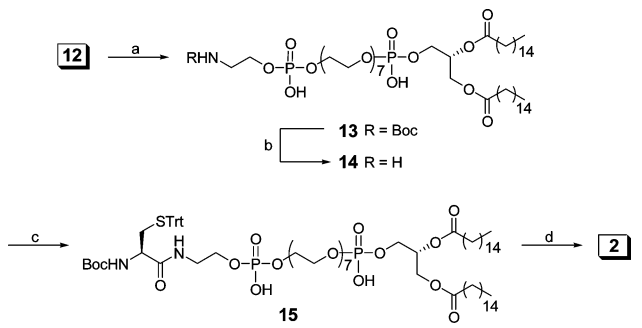
GPI anchor, likely a flexible structure, is unknown. The anchor analogues were reasonably close to the length of native GPI anchors, providing a platform to investigate the functional contributions of the various components of the GPI anchor.

Synthetic Strategy for GPI Anchor Analogues 2, 3, and 4. The syntheses of the three GPI anchor analogues **2**, **3**, and **4** were convergent, involving common building blocks and synthetic routes. GPI anchor analogue **2** is similar to some of the GPI anchor substitutes reported previously[7d,e] and provided for the direct comparison of a true GPI anchor analogue, such as **3** or **4**, to a sugar-free analogue (Figure 1). The syntheses of the GPI anchor analogues allow for the installation of different lipids to investigate their importance in GPI anchor functions. However, for our studies, we chose to synthesize **2**, **3**, and **4** with lipid tails containing two palmitoyl (C16:0) fatty acids, a common motif found in native GPI anchors.[1b,d]

Synthesis of GPI Anchor Analogue 2. To synthesize GPI anchor analogue **2**, 1-allylhexa(ethylene glycol) (**5**) was prepared from hexa(ethylene glycol) and allyl bromide and subsequently reacted with 2-bromoethanol triisopropylsilyl ether to give the hepta(ethylene glycol) derivative **6** (Scheme 1). Selective removal of the allyl group from intermediate **6** was accomplished under alkaline conditions via a published two-step procedure.[9] Standard phosphoramidite coupling conditions were then employed to generate compound **9** from compound **7** and the known phosphoramidite **8**.[10] The triisopropylsilyl (TIPS) protecting group was removed from intermediate **9** under acidic conditions, and the free alcohol of **10** was coupled to phosphoramidite **11**, under similar conditions as **7**, to afford compound **12**.

All that remained in the synthesis of compound **2** was removal of the protecting groups and coupling of the anchor analogue to cysteine. First, the 2-cyanoethyl protecting groups were removed from intermediate **12** by treatment with 1,8-diazabicyclo-[5.4.0]undec-7-ene (DBU) (Scheme 2).[6n,o] In the next step, the amine in compound **13** was deprotected (**14**) and coupled to Boc-Cys(Trt)-OPfp to generate the cysteine-containing anchor analogue **15**. Finally, the Boc and trityl protecting groups were removed from the cysteine residue in a single step, using trifluoroacetic acid (TFA), triisopropylsilane (TIS), and ethanedithiol (EDT), to give the GPI anchor analogue **2**.

Scheme 2 [a]



[a] Reagents: (a) DBU, CH_2Cl_2, 5 min, 89%; (b) 3:15:82 TIS:TFA:CH_2Cl_2, 15 min, quant.; (c) Boc-Cys(Trt)-OPfp, DIEA, 1:1 CH_2Cl_2:MeOH, 3 d, 62%; (d) 3:3:15:79 TIS:EDT:TFA:CH_2Cl_2, 15 min, 89%.

Synthesis of GPI Anchor Analogue 3. Orthogonal protecting groups for the glycan core of **3** were optimized so that the mannosyl intermediate **16** (Scheme 3) could be used in the synthesis of GPI anchor analogue **4**, containing a disaccharide moiety. Additionally, the phosphoramidite reagents **8** and **11** (Scheme 1) prepared during the synthesis of GPI anchor analogue **2** were used in the synthesis of **3** (Scheme 3).

The synthesis of the GPI anchor analogue **3** began with the glycosylation of 1-allylhexa(ethylene glycol) (**5**) with the known trichloroacetimidate donor **16**,[6l,m] using TMSOTf as an activator, to give compound **17** in 94% yield (Scheme 3). To ensure that the glycosylation of acceptor **5** with donor **16** would generate the α-mannoside, the 2-hydroxyl of compound **16** was protected with a participating acetyl group. The stereochemistry at the anomeric position of compound **17** was then verified as the α-mannoside by heteronuclear multiple quantum coherence (HMQC) NMR spectroscopy.[12] The coupling constant between H1 and C1 of compound **17** ($J_{H1,C1}$) was calculated to be 169 Hz, corresponding to the α-mannoside. The acetyl protecting group on the 2-hydroxyl of compound **17** required removal prior to installation of the phospholipid tail, which also possesses base-labile ester groups. The acetyl group was removed using potassium carbonate (K_2CO_3), and the free 2-hydroxyl was reprotected as the benzyl ether to afford compound **18**. The allyl group was then selectively removed from compound **18** using the two-step procedure developed during the synthesis of GPI anchor analogue **2**. Coupling of compound **19** to phosphoramidite **11**, followed by oxidation of the intermediate phosphite with *m*CPBA, generated compound **20**. Treatment with HCl removed the TIPS protecting group from compound **20**, and the newly unmasked alcohol of **21** was coupled to phosphoramidite **11**, under identical conditions as **10**, to afford compound **22**.

Next, the 2-cyanoethyl groups of compound **22** were removed with DBU to generate the free phosphate groups (Scheme 4).

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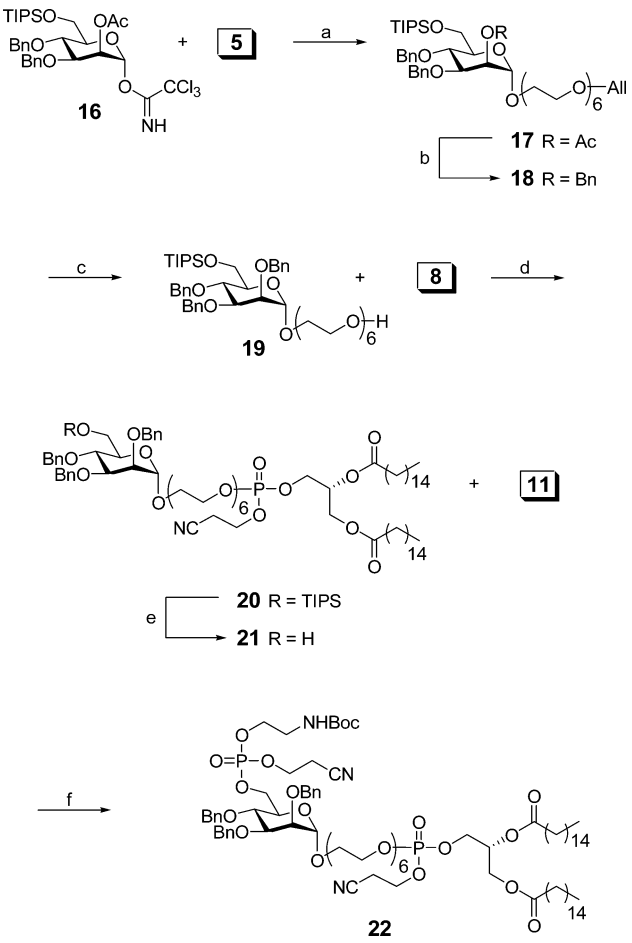
Scheme 3 [a]

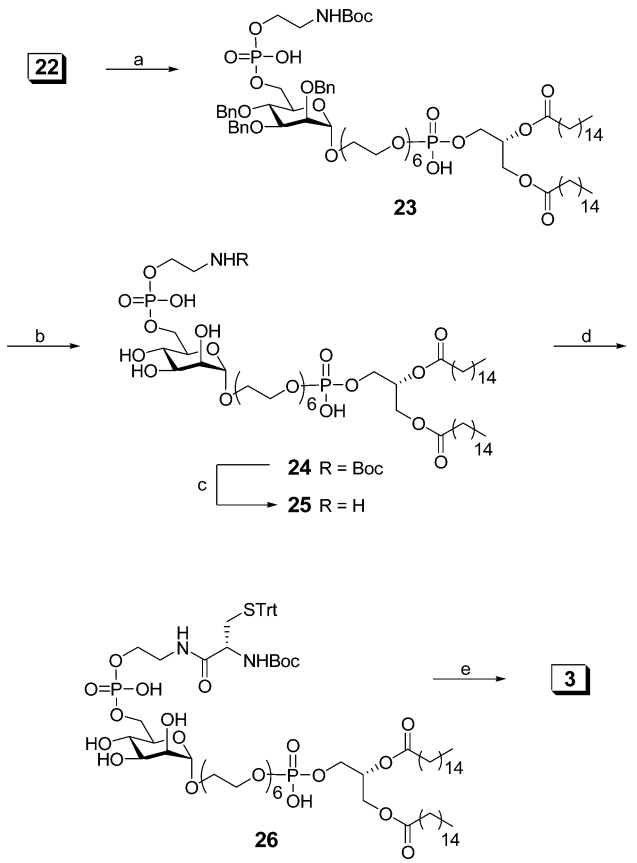


[a] Reagents: (a) TMSOTf, Et_2O, 4 Å MS, −78 °C to room temperature, 3 h, 92%; (b) (i) K_2CO_3, 2:1 MeOH:H_2O, 45 °C, 4 h; (ii) BnBr, NaH, DMF, 0 °C to room temperature, 17 h, 93%; (c) (i) $RhCl(PPh_3)_3$, DABCO, 9:1 EtOH:H_2O, 90 °C, 4 h; (ii) $KMnO_4$, NaOH, MeOH, 0 °C, 20 min, 62%; (d) (i) 4,5-dicyanoimidazole, CH_2Cl_2, CH_3CN, 4 h; (ii) *m*CPBA, −40 °C to room temperature, 1 h, 82%; (e) 0.5 M HCl, MeOH, CH_2Cl_2, 5 h, 86%; (f) (i) 4,5-dicyanoimidazole, CH_2Cl_2, CH_3CN, 3.5 h; (ii) *m*CPBA, −40 °C to room temperature, 1 h, 80%.

Hydrogenolysis of the benzyl ethers from compound **23** was achieved using 30% Pd/C and hydrogen in a methanol−methylene chloride mixture to help solubilize compounds **23** and **24**. The product from this reaction was immediately used in the next step, removal of the Boc protecting group to produce compound **25**. The free amine of compound **25** was coupled to Boc-Cys(Trt)-OPfp using a mixture of 3:1 chloroform:methanol to help solubilize **25**. Finally, the last two protecting groups were removed from compound **26** with TFA, TIS, and EDT to provide the GPI anchor analogue **3**.

Synthesis of GPI Anchor Analogue 4. The synthesis of GPI anchor analogue **4** used intermediates prepared during the syntheses of compounds **2** and **3**, such as the phosphoramidite reagents **8** and **11** and the mannosyl trichloroacetimidate **16**. Additionally, the 6′-hydroxyl of the glycan core of **4** was converted to the TIPS ether, whereas the 6-hydroxyl was converted to the *tert*-butyldiphenylsilyl (TBDPS) ether, as it is more acid stable than the TIPS protecting group and can only be removed upon treatment with fluoride ion (Scheme 5). In native GPI anchors, the 6-hydroxyl is often modified with a galactosyl or phosphoethanolamine side chain. Thus, the use of an orthogonal protecting group at that site allows for future

Scheme 4 [a]



[a] Reagents: (a) DBU, CH_2Cl_2, 5 min, 96%; (b) 30% Pd/C, H_2, MeOH, trace CH_2Cl_2, 15 h, 81%; (c) 3:15:82 TIS:TFA:CH_2Cl_2, 15 min, quant.; (d) Boc-Cys(Trt)-OPfp, DIEA, 3:1 $CHCl_3$:MeOH, 24 h, 59%; (e) 3:3:15:79 TIS:EDT:TFA:CH_2Cl_2, 15 min, quant.

incorporation of these side chains and the investigation of their possible contributions to the functions of the GPI anchor. The synthesis of GPI anchor analogue **4** was designed so that the addition of a side chain at the 6-hydroxyl could occur at a late stage, thereby reducing the number of synthetic steps required to generate multiple GPI anchor analogues with various side chains.

The synthesis of compound **4** began with the TBDPS-protected trichloroacetimidate **27**,[13] which was coupled to the penta(ethylene glycol) derivative **28**, using TMSOTf as an activator, in excellent yield (91%) to produce compound **29** (Scheme 5). Removal of the acetyl protecting group at the 2-hydroxyl position, followed by glycosylation with the previously synthesized TIPS-protected trichloroacetimidate **16**, yielded disaccharide **31**. The stereochemistries at the anomeric positions of this compound required verification by HMQC analysis. The two coupling constants, $J_{H1,C1}$ and $J_{H1',C1'}$, were calculated to be 176 and 173 Hz, respectively, corresponding to the expected α anomers. As in the synthesis of GPI anchor analogue **3**, the acetyl protecting group at the 2-hydroxyl of compound **31** required removal prior to installation of the phospholipid tail. After deprotection, the 2-hydroxyl was reprotected as the benzyl ether to give compound **32**. Finally, the allyl group was selectively removed from this disaccharide to generate **33**, which was poised for coupling to phosphoramidite **8**.

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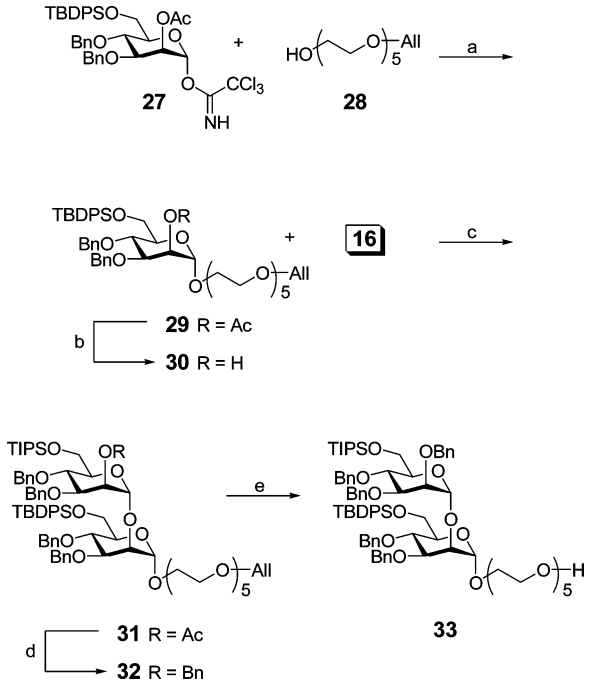
Scheme 5 [a]



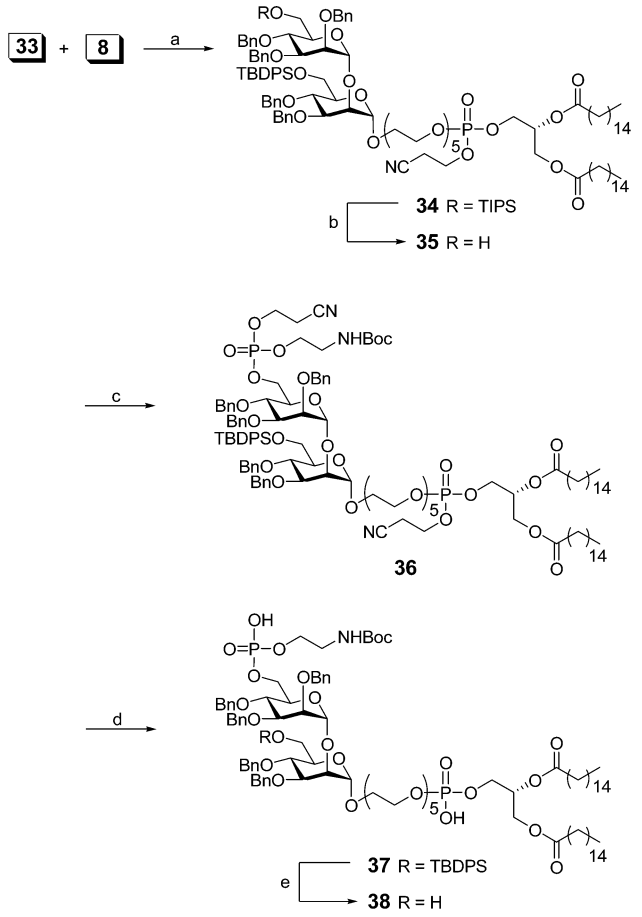
[a] Reagents: (a) TMSOTf, Et_2O, 4 Å MS, −78 °C to room temperature, 5 h, 91%; (b) K_2CO_3, 3:1 MeOH:H_2O, 50 °C, 2 h, 90%; (c) TMSOTf, CH_2Cl_2, 4 Å MS, −78 °C to room temperature, 6 h, 84%; (d) (i) K_2CO_3, 4:1 MeOH:H_2O, 50 °C, 2.5 h; (ii) BnBr, NaH, DMF, 0 °C to room temperature, 14 h, 70%; (e) (i) $RhCl(PPh_3)_3$, DABCO, 9:1 EtOH:H_2O, 90 °C, 1.5 h; (ii) $KMnO_4$, NaOH, MeOH, 0 °C, 1 h, 68%.

Standard phosphoramidite coupling conditions were employed to generate the lipid-modified disaccharide **34** (Scheme 6). Although removal of the TIPS protecting group was accomplished using HCl during the synthesis of **3**, these conditions unexpectedly cleaved the lipid esters from compound **34**, resulting in a poor yield of disaccharide **35**. A higher yield of **35** was obtained by treating compound **34** with $BF_3{\cdot}OEt_2$ to remove the TIPS protecting group.[14] Coupling of the free alcohol of **35** to phosphoramidite **11** proceeded smoothly to give compound **36** in 90% yield. Although a side chain was not added in this synthesis, the TBDPS group could be removed from the 6-hydroxyl group and coupled to a suitably protected GPI anchor side chain, such as phosphoethanolamine or galactose. Instead, the 2-cyanoethyl groups were removed from the phosphates of compound **36** to generate disaccharide **37** in excellent yield. Attempts to remove the TBDPS group using TBAF were unsuccessful. However, the TBDPS group was readily removed using HF-pyridine to give disaccharide **38**.

The final steps of the synthesis of GPI anchor analogue **4** began with removal of the five benzyl groups from compound **38** using 30% Pd/C and H_2 (Scheme 7). Removal of the Boc group and coupling to Boc-Cys(Trt)-OPfp gave the cysteine-containing disaccharide **41**. Finally, the last two protecting groups were removed from compound **41** in a single step to generate the GPI anchor analogue **4**.

Coupling of GPI Anchor Analogues to GFP. Because of its convenient spectroscopic properties, GFP was chosen as a model protein to couple to the GPI anchor analogues. GFP can be produced in large quantities by overexpression in *Escherichia coli* (*E. coli*), and its fluorescent properties allow for the facile assessment of physical location and diffusion kinetics following incorporation in supported lipid bilayers.[15]

Scheme 6 [a]



[a] Reagents: (a) (i) 4,5-dicyanoimidazole, CH_2Cl_2, CH_3CN, 5.5 h; (ii) *m*CPBA, −40 °C to room temperature, 1 h, 90%; (b) $BF_3{\cdot}OEt_2$, CH_2Cl_2, 50 min, 66%; (c) (i) **11**, 4,5-dicyanoimidazole, CH_2Cl_2, CH_3CN, 5.5 h; (ii) *m*CPBA, −40 °C to room temperature, 1.5 h, 88%; (d) DBU, CH_2Cl_2, 5 min, 98%; (e) HF-pyridine, pyridine, THF, 0 °C to room temperature, 16 h, 74%.

A combination of expressed protein ligation (EPL) and native chemical ligation (NCL) was chosen for the coupling of GFP to the GPI anchor analogues.[8] EPL and NCL are now widely used technologies for coupling large peptides and proteins under mild aqueous reaction conditions. Numerous proteins have been prepared using EPL and NCL, as well as some glycosylated and lipid-modified proteins.[7d,8,16] The deconstruction of GPI-anchored proteins into two components, cysteine-bearing GPI analogues and protein thioesters, represents a flexible route for preparing a wide range of C-terminally GPI-anchored proteins. In our case, expression of GFP as an intein fusion, using EPL technology, was employed to generate GFP containing a C-terminal thioester (GFP-MESNa). This activated form of GFP was then coupled to the GPI anchor analogues by NCL (Figure 2).

GFP was expressed in *E. coli* as a fusion protein with an intein domain and a chitin-binding domain fused to its C-terminus.[17] The soluble fraction of the *E. coli* lysate was purified on chitin resin and treated with 2-mercaptoethanesulfonic acid

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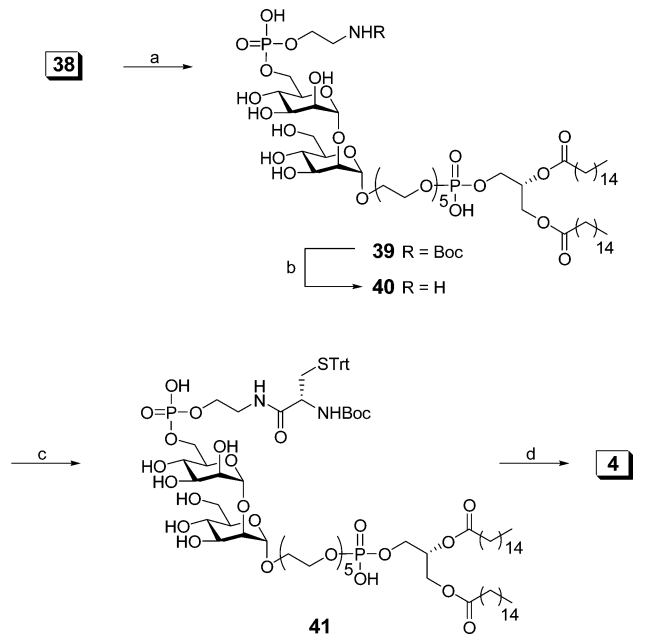
Scheme 7 [a]



[a] Reagents: (a) 30% Pd/C, H_2, MeOH, trace CH_2Cl_2, 1.5 d, 83%; (b) 3:15:82 TIS:TFA:CH_2Cl_2, 15 min, quant.; (c) Boc-Cys(Trt)-OPfp, DIEA, 3:1 CH_2Cl_2:MeOH, 20 h, 66%; (d) 3:3:15:79 TIS:EDT:TFA:CH_2Cl_2, 15 min, quant.

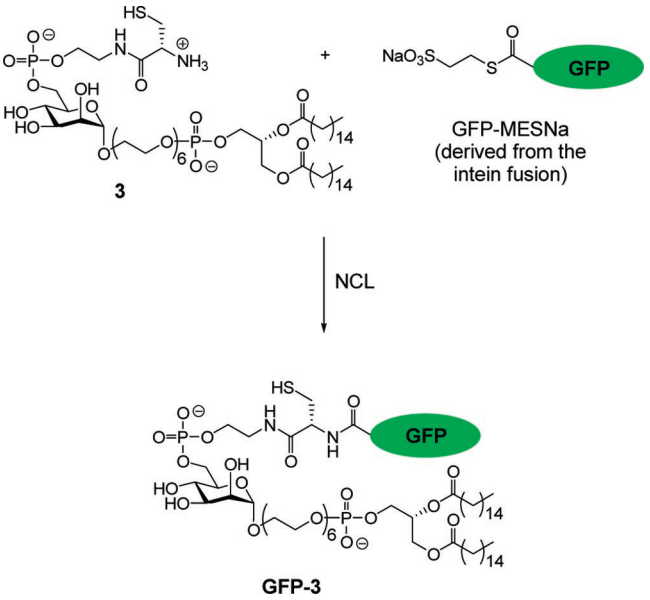


Figure 2. Synthesis of GFP-GPI anchor analogues by a combination of EPL and NCL. In this example, GPI anchor analogue **3** is coupled to GFP containing a C-terminal MESNa thioester to generate GFP-**3**.

(MESNa) to release pure GFP-MESNa as indicated by SDS-PAGE. The protein was then concentrated and used immediately for NCL to the GPI anchor analogues.

To prepare the GFP conjugates, a 1% (w/v) solution of compounds **2**, **3**, or **4** solubilized with 1% (w/v) β-octylglucoside was mixed with the purified GFP-MESNa solution containing 3% (w/v) MESNa for 24 h at 23 °C. After the ligation reactions, the modified GFPs were dialyzed into PBS (phosphate-buffered saline) containing 0.05% (w/v) β-octylglucoside and then partitioned by the detergent Triton X-114.[18] At 37 °C, a solution of Triton X-114 separates into an aqueous phase and a detergent phase. Hydrophilic proteins selectively partition into the aqueous

Table 1. Mean Fluorescence Intensities (MFI)[a] of Detergent and Aqueous Phases from Triton X-114 Partitioning of GFP-Cys, GFP-**2**, GFP-**3**, and GFP-**4**, and Estimated Yields of NCL Reactions

	total fluorescence[b] (MFI)	aqueous phase fluorescence (MFI)	detergent phase fluorescence (MFI)	estimated yield of ligation
GFP-Cys	56 035 ± 4051	49 264 ± 1449	5924 ± 667	n.d.[c]
GFP-**2**	61 128 ± 1894	24 366 ± 1615	36 824 ± 1013	60%
GFP-**3**	58 629 ± 3917	17 745 ± 2058	43 739 ± 870	75%
GFP-**4**	55 617 ± 2887	19 536 ± 6056	32 744 ± 11039	59%

[a] Fluorescence measurements are an average of three separate Triton X-114 partitionings, and error values indicate the standard deviation. [b] Total fluorescence values were determined from equal amounts of protein not subjected to detergent partitioning. [c] n.d. is not determined.

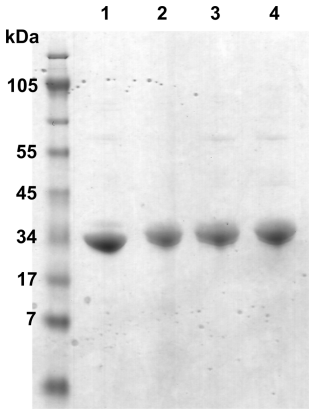


Figure 3. Coomassie-blue stained 12% Bis-Tris PAGE gel of GFP-MESNa, GFP-**2**, GFP-**3**, and GFP-**4**. Lane 1: GFP-MESNa. Lane 2: GFP-**2**. Lane 3: GFP-**3**. Lane 4: GFP-**4**.

phase, and amphiphilic proteins, such as GPI-anchored proteins and integral membrane proteins, partition into the detergent phase. Aliquots of GFP-**2**, GFP-**3**, and GFP-**4**, as well as unmodified GFP containing a free cysteine at the C-terminus (GFP-Cys), were subjected to a Triton X-114 partitioning. As expected, the majority of GFP fluorescence was found in the aqueous phase of the GFP-Cys solution. For GFP-**2**, GFP-**3**, and GFP-**4** solutions, the majority of GFP fluorescence was found in the detergent phases (Table 1). Fluorescence in the aqueous phases of the modified GFP solutions was most likely due to incomplete reaction of GFP-MESNa with the GPI anchor analogues **2**, **3**, and **4** or to hydrolysis of GFP-MESNa. From these data, the ligation yields were estimated to be 60% for GFP-**2**, 75% for GFP-**3**, and 59% for GFP-**4**.

Characterization of GFP-2, GFP-3, and GFP-4. To assess the purities of GFP-**2**, GFP-**3**, and GFP-**4**, aliquots of these proteins were subjected to polyacrylamide gel electrophoresis (PAGE) and visualized by Coomassie blue (Figure 3). The modified GFPs were estimated to be >95% pure by this analysis.

The modified GFPs were also analyzed by mass spectrometry (MS). Preparation of protein samples for electrospray MS (ESI-MS) or MALDI-TOF MS analysis required removal of the excess GPI anchor analogues (compounds **2**, **3**, and **4**) used in the coupling reactions. Although the molecular weights of these anchor analogues were much lower than those of the modified GFPs, compounds **2**, **3**, and **4** were amphiphilic and probably formed micelles in aqueous solution. Therefore, dialysis did not

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Table 2. Calculated and Observed Masses of GFP-**2**, GFP-**3**, and GFP-**4**

	calculated mass (Da)	observed mass (Da)
GFP-**2**	28 128.2	28 128.2 ± 1.9
GFP-**3**	28 246.2	28 248 ± 50[a]
GFP-**4**	28 364.2	28 365.1 ± 1.9

[a] MALDI-TOF MS analysis. The standard deviation associated with MALDI-TOF MS is much greater than that for ESI-MS, which is generally within ±2 *m/z*.

remove these anchor analogues from the protein solutions. Instead, a chloroform/methanol/water precipitation procedure was employed to separate GFP-**2**, GFP-**3**, and GFP-**4** from compounds **2**, **3**, and **4**, respectively.[19] In this procedure, the excess anchor analogues were solubilized in the chloroform/methanol mixture, which was separated from the aqueous solution. The modified GFPs were precipitated from the organic solution, redissolved in a small volume of neat formic acid, and immediately subjected to reverse-phase high performance liquid chromatography (HPLC) to remove salts and to separate the modified GFPs from GFP-MESNa and GFP-COOH. The purified analogues were analyzed by ESI-MS or MALDI-TOF MS. Although GFP-**2** and GFP-**4** were separated from the contaminating GFP-MESNa and GFP-COOH by HPLC, the GFP-**3** sample retained these byproducts. The ESI-MS of GFP-**3** was overwhelmed by the GFP-COOH, which ionized more efficiently than the modified GFP-**3**. However, MALDI-TOF MS analysis of GFP-**3** generated an *m/z* value corresponding to the modified GFP. For all three modified GFPs, the MS results confirmed that the ligations had yielded the correct products (Table 2).

Incorporation of Modified GFPs into Supported Lipid Bilayers. Native GPI-anchored proteins diffuse more rapidly in supported lipid bilayers than transmembrane proteins, presumably because the lipid tail of the GPI anchor does not completely extend through the lipid bilayer.[20] Indeed, it has been speculated that conferring high relative mobility in the cell membrane is an explicit function of the GPI anchor. We were therefore interested in how the deletion of various monosaccharides of the GPI anchor affected membrane mobility. To address this question experimentally, we used supported lipid bilayers as models of the cell membrane.[21] The supported lipid bilayers, composed largely of phosphatidylcholine and doped with 3% phosphatidylethanolamine, were formed on SiO_2 coverslips using standard vesicle-fusion techniques.[22] The modified GFPs or the unmodified GFP-Cys were incubated with the supported lipid bilayers (1 μg protein/mL) for 12 h at 23 °C in the dark, after which the bilayers were washed with PBS and analyzed by fluorescence microscopy to confirm the presence of the protein. Bilayers incubated with GFP-Cys showed no significant GFP fluorescence, confirming that the lipid modification is required for integration into supported lipid bilayers.

The diffusion properties of GFP-**2**, GFP-**3**, and GFP-**4** in supported lipid bilayers were investigated using fluorescence correlation spectroscopy (FCS). From these FCS measurements, the diffusion coefficient (*D*), a physical measure of protein mobility, can be calculated.[23] A faster moving protein has a higher diffusion coefficient. The data from the FCS measurements were used to determine the characteristic correlation times (τ_D) and to calculate *D* values for GFP-**2**, GFP-**3**, and GFP-**4** in supported lipid bilayers (Figure 4).

A

Sample	τ_D (s)	D ($\mu m^2/s$)
GFP-**2**	6.0 ± 0.2	2.5 ± 0.1
GFP-**3**	5.3 ± 0.2	2.8 ± 0.1
GFP-**4**	4.8 ± 0.2	3.2 ± 0.1

B

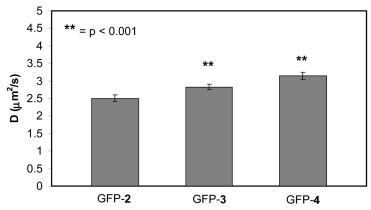

Figure 4. GFP-**2**, GFP-**3**, and GFP-**4** are mobile in a supported lipid bilayers. (A) Mean values for the correlation time, τ_D, and the diffusion coefficient, *D*, for GFP-**2**, GFP-**3**, and GFP-**4** in supported lipid bilayers at 25 °C. The mean values and the standard deviations (SD) representing the error were calculated using all measurements for each particular GFP construct. (B) Graphical representation of the data from (A). The τ_D and *D* values represent the mean τ_D ± SD or mean *D* ± SD, respectively.

The *D* values calculated for GFP-**2**, GFP-**3**, and GFP-**4** were similar to the *D* value calculated for the phosphatidylcholine lipids ($D = 3.3 \pm 0.1\ \mu m^2/s$), indicating that all three chemically modified GFPs are mobile and diffuse rapidly in the supported lipid bilayers. Interestingly, the *D* value calculated for GFP-**2** was significantly lower than the *D* value for GFP-**3** ($p < 0.001$), which, in turn, was significantly lower than the *D* value for GFP-**4** ($p < 0.001$) (Figure 4).

Conclusion

In conclusion, the data described here suggest that the monosaccharides of the GPI anchor glycan core may affect the mobility of the protein in lipid bilayers. GFP-**4**, which contains two monosaccharides in its modified GPI anchor, diffused more rapidly in the supported lipid bilayers than GFP-**3** or GFP-**2**, which contain one or no mannose residues, respectively. The more flexible linkers of GFP-**2** and GFP-**3** may permit greater movement of the attached protein, thus allowing for increased interaction of the protein with the lipid bilayer. These transient interactions could decrease the speed at which a protein moves laterally through the lipid bilayer. Furthermore, these results imply that a protein attached to a native GPI anchor, containing four monosaccharides in addition to the rigid *myo*-inositol moiety, should move even more rapidly through the lipid bilayer. The extra sugars may sufficiently rigidify the native GPI anchor so as to limit the interactions of the attached protein with the lipid bilayer, accounting for the increased mobility of the protein.

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Therefore, the GPI anchor may be more than just a membrane anchor; it may also serve to prevent transient interactions between the attached protein and the lipid bilayer, thus permitting rapid diffusion in the membrane. The versatile approach described herein allows for the synthesis of a variety of GPI analogues that differ in their monosaccharide units, side chain modifications, and lipid tails, thus accelerating the further investigation of GPI anchor structure−function relationships.

Experimental Section

The EGFP/pTXB1 plasmid containing the EGFP-GyrA intein-chitin binding domain gene was kindly donated by T. W. Muir.[17] Chitin resin was obtained from New England Biolabs. Protease inhibitor cocktail tablets were purchased from Roche Diagnostics. β-Octylglucoside and Triton X-114 were obtained from Anatrace. Tris(2-carboxyethyl)-phosphine hydrochloride and L-cysteine were purchased from Fluka. Methionine and bovine serum albumin (BSA) were obtained from Sigma-Aldrich. Criterion 12% Bis-Tris gels and MES buffer were purchased from Bio-Rad Laboratories. The 1,2-dioleoyol-*sn*-glycero-3-phosphocholine (DOPC) was obtained from Avanti Polar Lipids, and the Marina Blue 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (Marina Blue DHPE) was obtained from Invitrogen. Fluorescence measurements were acquired on a Molecular Devices Spectra Max Gemini XS fluorimeter. *E. coli* cells were lysed with an Avestin EmulsiFlex-C5 homogenizer. For MS analysis of GFP-**2**, GFP-**3**, and GFP-**4**, proteins were desalted by microbore rHPLC (Michrom) on a PLRP300 column using a water−acetonitrile−0.1% trifluoroacetic acid gradient. ESI-MS spectra of GFP-**2** and GFP-**4** were obtained by flow-injection at 1 μL/min on a Bruker-Agilent 3000 ion trap mass spectrometer. The MALDI-TOF spectrum of GFP-**3** was acquired on a Bruker Reflex III mass spectrometer using sinapinic acid as the matrix.

GFP-MESNa Expression. The EGFP-GyrA-intein-chitin binding domain fusion protein was expressed in *E. coli* BL21 cells. The cells were pelleted, resuspended in 150 mL of lysis buffer (20 mM Tris-HCl, 200 mM NaCl, pH 7.5, protease inhibitor cocktail), and passed through a homogenizer (2 × 8000 psi) to lyse the cells. The lysed cells were centrifuged; the supernatant was applied to 10 mL of chitin beads and rocked at 4 °C for 2.5 h. The chitin beads were isolated, washed (10 × 25 mL of lysis buffer, 5 × 25 mL of ligation buffer), and incubated with 20 mL of ligation buffer (100 mM HEPES, 200 mM NaCl, pH 8.0) and 100 mM MESNa (2-mercaptoethane sulfonic acid) at room temperature for 24 h. The beads were filtered off and rinsed (5 × 15 mL of ligation buffer). The GFP-MESNa solution was then rocked for 1 h at 4 °C with 10 mL of clean chitin beads to remove any GFP-GyrA-intein chitin binding domain fusion protein from the GFP-MESNa. The GFP-MESNa solution was then concentrated 10-fold by Amicon Ultra-15 centrifugal dialysis (Millipore). This GFP-MESNa solution in ligation buffer was used immediately for ligation to **2**, **3**, and **4**.

Standard Conditions for Ligation of GFP to 2, 3, and 4. For a typical ligation, **2**, **3**, or **4** (4 mg) and β-octylglucoside (4 mg) were dissolved in 1:1 CH_2Cl_2:MeOH and dried in a 2.0-mL Eppendorf tube to a film under a stream of N_2 and then in vacuo. The mixture was resuspended in the GFP-MESNa ligation buffer (400 μL) and mixed for 24 h at room temperature with MESNa (12 mg) under argon. The protein solutions were then dialyzed against phosphate-buffered saline (PBS), containing 0.05% β-octylglucoside, 1 mM methionine, and 1 mM tris(2-carboxylethyl)phosphine hydrochloride (TCEP).

Triton X-114 Detergent Partitioning.[18] A solution of GFP-**2**, GFP-**3**, or GFP-**4** (10 μL in PBS) was dissolved in ice-cold PBS (190 μL). Precondensed Triton X-114 (40 μL, ∼12% in PBS) was added to this solution, which was mixed thoroughly and then heated at 37 °C for 15 min (until the solution turned cloudy). The cloudy suspension was then centrifuged (14 000 rpm) for 5 min at room temperature, and the upper and lower layers were diluted to 200 μL in PBS and quantitated by fluorescence spectroscopy.

Gel Electrophoresis of Modified GFPs. Protein samples were separated by polyacrylamide gel electrophoresis (PAGE) under reducing conditions using a Criterion 12% Bis-Tris gel with MES buffer. Gels were stained with Coomassie blue.

Chloroform/Methanol/Water Protein Precipitation Procedure.[19] To 100 μL of an aqueous solution of GFP-**2**, GFP-**3**, or GFP-**4** was added 400 μL of methanol. This solution was mixed and centrifuged (9000*g*) for 10 s, and 200 μL of chloroform was added. The resulting solution was mixed and centrifuged (9000*g*) for 10 s. Next, 300 μL of ddH_2O was added, and the solution was mixed and centrifuged (9000*g*) for 1 min. The upper aqueous phase was removed without disturbing the precipitated protein found between the upper and lower (organic) phases. Finally, 300 μL of methanol was added to the lower phase to precipitate the protein. This suspension was centrifuged (9000*g*) for 2 min, and the supernatant was discarded. The precipitated protein was dissolved in neat formic acid (20 μL), subjected to reverse-phase HPLC, and analyzed by ESI-MS or MALDI-TOF MS.

Incorporation of Modified GFPs into Supported Lipid Bilayers. Supported lipid bilayers were formed on glass coverslips by standard vesicle fusion techniques.[22] Briefly, 97% (mol % in $CHCl_3$) DOPC and 3% (mol % in $CHCl_3$) Marina Blue DHPE were mixed in $CHCl_3$, and then the $CHCl_3$ was removed by evaporation. The dried lipid film was hydrated to 2 mg/mL with ddH_2O and extruded with high pressure through a 100-nm filter to form SUVs.

The supported lipid bilayers were formed on clean #1 borosilicate coverslips cleaned previously with Piranha solution (1:4 H_2O_2:H_2SO_4). *Caution: Piranha is highly reactive with organics and is a self-heating solution.* A supported lipid bilayer was formed on one of these coverslips by addition of PBS to the above vesicle solution to give a final concentration of 137 mM NaCl, 10 mM phosphate salts, and 1 mg/mL of vesicles. The coverslip was then dropped face-down onto a droplet of this mixture. The slide was submerged in PBS to rinse unbound vesicles and kept under this solution for the duration of the experiment.

The supported lipid bilayer was blocked for 40 min with a 0.01% (w/v) solution of BSA in PBS to prevent nonspecific binding of protein. After rinsing with PBS, 1 μg of the modified GFP (GFP-Cys, GFP-**2**, GFP-**3**, or GFP-**4**) was added to the mixture to give a final concentration of 1 μg/mL of the modified GFP. The supported lipid bilayers were left in the dark for 12 h at room temperature to allow insertion of the modified GFPs into the bilayers. Before FCS analysis, the samples were rinsed extensively with PBS to remove excess GFP from the solution. The Marina Blue labeled lipids were used to confirm fluidity of the lipid bilayer via fluorescence recovery after photobleaching (FRAP) experiments (data not shown).

Acknowledgment. We gratefully acknowledge J.-P. Pellois for GFP protein expression advice, H. van Halbeek for NMR advice, D. King and the Howard Hughes Medical Institute Mass Spectrometry Facility for MS help and analyses, and J. Czlapinski for many helpful discussions. M.G.P. was supported by an HHMI predoctoral fellowship. This research was supported by National Institutes of Health Grant GM59907 (to C.R.B.) and by the Director, Office of Science, Office of Basic Energy Sciences, of the U.S. Department of Energy under Contract No. DE-AC03-76SF00098 (to J.T.G.).

Supporting Information Available: Synthetic procedures, analytical data, ^{1}H NMR spectra for compounds **2**−**7**, **9**, **10**, **12**−**15**, **17**−**26**, **28**−**39**, and **41**, HMQC spectra for compounds **17** and **31**, mass spectra for GFP-**2**, GFP-**3**, and GFP-**4**, and detailed experimental procedures for FCS measurements. This material is available free of charge via the Internet at http://pubs.acs.org.

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