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Stereoselective synthesis of head group of archaeal phospholipid PGP-Me to investigate bacteriorhodopsin-lipid interactions†

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Phosphatidylglycerophosphate methyl ester (PGP-Me), a major constituent of archaeal purple membrane, is essential for the proper proton-pump activtiy of bacteriorhodopsin (bR). We carried out the first synthesis of the bis-phosphate headgroup of PGP-Me using H-phosphonate chemistry that led to the production of a simplified PGP-Me analogue with straight alkyl chains. To investigate the role of this head group in structural and functional integrity of bR, the analogue was used to reconstitute bR into liposomes, in which bR retained the original trimeric structure and light-induced photocycle activity. Enhanced ordering of an alkyl chain of ^{2}H-labelled analogue was observed on ^{2}H NMR spectra upon interaction with bR. These results together sugget that the PGP head group plays a role in the proper functioning of bR through lipid-protein interaction.

It is scientifically important to investigate lipid-protein interaction (LPI), which is a key factor for the proper functioning of membrane-integral proteins. However, the interpretation of experimental results on LPI is usually difficult due to the highly complex and dynamic nature of biological membranes. Thus, as shown in the pioneering studies by Racker and coworkers,[1-3] artificial liposomes reconstituted with purified membrane proteins provide a useful model system[4-9] to investigate the interplay between membrane proteins and their surrounding lipids.[10-12]

Bacteriorhodopsin (bR) is a light-driven proton pump of *Halobacterium halobium* that is localized in the purple membrane (PM) in a trimeric form.[13-15] Because of the thermal stability and facile preparation, reconstituted bR into artificial membranes has been regarded as a simple and well-characterised model to investigate LPI at the atomistic level.[16] Membrane models composed of phospholipids (and sterols) have been used to reproduce the original properties of biomembranes to a certain extent. Among those, neutral phosphatidylcholine (PC) are often used for the reconstitution of bR.[5-9] However, large differences between the chemical structures of PCs and PM lipids, particularly in their head groups, lead to an altered membrane environment that disrupts the assembly and functionality of bR. It has been frequently reported that the two-dimensional crystalline structure of bR molecules are disassembled in dimyristoyl-PC (DMPC) vesicles above the phase transition temperature,[5,8,17] while bR in natural PM is stable even at high temperatures;[18] among the other artificial lipids, PCs with fluorine-substituted acyl chains have been found to provide a more suitable environment for bR.[9] For a better understanding of the atomistic mechanism underlying LPI, structurally stable membrane proteins such as bR should be investigated in depth by using model lipids with various head groups .

Phosphatidylglycerophosphate methyl ester (PGP-Me, **1** in Fig. 1), which is the major constituent of PM lipids,[19,20] possesses an acidic bisphosphate headgroup. In addition, **1** contains methyl-branched alkyl chains that are bound to glycerol via ether linkages as opposed to straight acyl chains in PCs. Previous studies have suggested that the double charged head group of **1** is important for the array structure and proton pump activity of bR.[21-24] Thus, a molecular probe, in which the polar head of PGP-Me is preserved, may exhibit superior performance of LPI in comparison with PCs with neutral head groups. In addition, higher affinity of the negatively charged bisphosphate moiety to the surface of the protein may somehow facilitate the observation of the transient LPI; lipid molecules usually undergo very rapid exchange between the annular shell and the bulk phase in membranes.[10,11] However, to our knowledge, there is no previous report on a stereoselective synthetic method for the head group of PGP-Me.

In this study we established the first stereoselective route to furnish the polar head of PGP-Me based on H-phosphonate chemistry, and synthesised a PGP-Me analogue **2** bearing two simple non-branched side chains that was used for the reconstitution of bR. The analogue **2** was subjected to circular dichroism (CD) and laser flash photolysis for evaluating its ability to stabilise the

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structure and functions of membrane-bound bR. Based on ^{2}H NMR experiments for ^{2}H-labled analogue **3** of PGP-Me, a moderate increase in acyl chain order was observed upon interaction with bR.

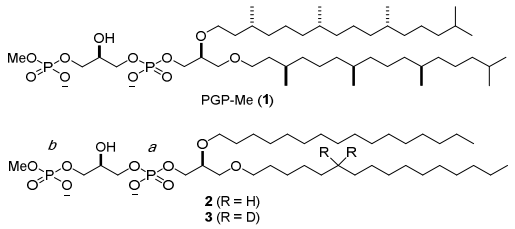


Fig. 1 Chemical structures of compounds 1-3 with two phosphate esters *a* and *b*.

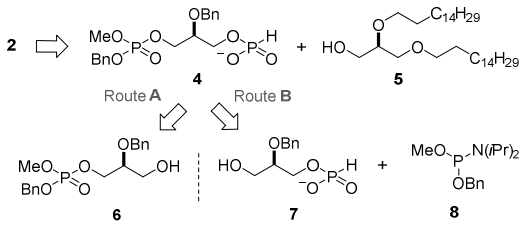
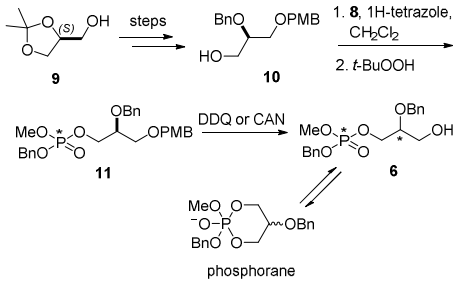


Fig. 2 Retrosynthetic analysis of compound **2**.

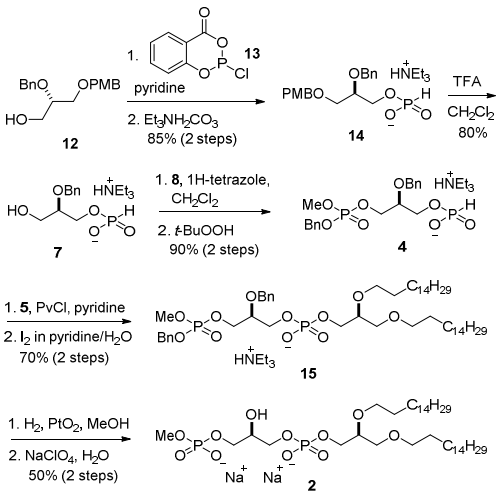
Retrosynthetically, we envisioned that **2** would be constructed by H-phosphonate coupling[25-27] between **4** and **5**. Compound **4** could be obtained via phosphorylation either from phosphate **6** (Route A), or from H-phosphonate derivative **7** and a known phosphoramidite **8** (Route B) (Fig. 2). As shown in Scheme 1, a protected glycerol **10** was synthesised from commercially available (*S*)-solketal **9** using a procedure that was partially modified from that in previous reports.[28] Compound **10** was phosphorylated with **8**/1H-tetrazole followed by oxidation with *tert*-butyl hydroperoxide (*t*-BuOOH) to produce phosphotriester **11**. ^{1}H NMR spectra revealed that **11** was a mixture of diastereoisomers with respect to the *sn*-2 carbon and phosphate centre at a diastereomeric ratio of 2:1 based on the methylene proton signals of the benzyl phosphonate ester. To introduce the second phosphorus, the *p*-methoxybenzyl (PMB) group was removed by oxidative cleavage using typical conditions (2,3-dichloro-5,6-dicyanobenzoquinone (DDQ), wet CH_2Cl_2; ammonium cerium (IV) nitrate (CAN), acetone/H_2O (9/1 v/v)). However, in each case, the benzyl methylene protons of isolated **6** showed two pairs of doublets in a ca. 1:1 ratio, which was distinctly different from that of the substrate, indicating that an isomerisation reaction occurred during deprotection and/or purification. Presumably, the hydroxyl group of **6** underwent intramolecular nucleophilic attack at the phosphotriester to give a phosphorane intermediate,[29] resulting in the racemisation of the *sn*-2 asymmetric centre in **6** (Scheme 1).

We turned our attention to an alternative approach based on Route B (Fig. 2) via an H-phosphonate intermediate, which could prevent he racemisation. As shown in Scheme 2, compound **12**, which was synthesised from (*R*)-solketal,[28] was phosphorylated using 2-chloro-4H-1,3,2-benzodioxaphosphinin-4-one **13** in pyridine followed by hydrolysis to afford H-phosphonate **14**. The PMB group in **14** was removed by trifluoroacetic acid (TFA) to give H-phosphonate **7** with a high enantiomeric excess (> 95%) as determined by ^{1}H NMR using Chirabite-AR[30] as a chemical shift reagent.[31] Phosphorylation of **7** with **8** followed by selective oxidation of the phosphite triester generated *in situ*, resulted in a high yield of bisphosphate **4**. Using pivaloyl chloride (PvCl) as a condensing agent, in the presence of **5**[32], **4** was completely converted into a phosphite, which was then oxidised with iodine followed by hydrolysis with water to give **15**. Deprotection of benzyl ethers in the presence of Adams′ catalyst followed by ion exchange using $NaClO_4$ produced **2** with a modest yield (50%) for two steps.

To examine LPI by using ^{2}H NMR, a ^{2}H-labelled analogue **3** was prepared from **4** with an *sn*-3-6,6-dideuterated alkyl chain by employing the same method as used for **2** to detect the mobility of the analogue molecule in membrane.[28, 33-35]



Scheme 1 Synthesis of compound **6**.



Scheme 2 Synthesis of compound **2**.

With the synthetic analogues **2** and **3** in hand, we first evaluated the functions of the PGP-Me head group in restoring the protein structure and biological activity of bR. In PM, bR is aggregated into protein trimers to form a two-dimensional hexagonal lattice, and the assembly of bR is known to demonstrate the split Cotton effect with a negative peak at ca. 600 nm in visible CD spectroscopy.[18] As shown in Fig. 3**a** and **b**, the CD spectra of partially delipidated bR (dbR), where ca. 76% of the original PM lipids were removed, showed a small and an invisible negative peak at 600 nm at 25 °C and 50 °C, respectively, indicating that the trimer structure was largely disassembled. On the other hand, reconstitution of dbR into dipalmitoylphosphatidylcholine (DPPC) and **2** liposomes restored an asymmetric split-Cotton effect that was similar to that with PM at 25 °C, indicating the presence of the trimer structure. When the temperature was raised to 50 °C, the biphasic pattern was retained in PM, which supported the notion that bR has high thermodynamic stability at high temperature.[18] However, the CD spectrum of DPPC/dbR was markedly changed from that at 50 °C, exhibiting a monophasic curve with a maximum peak at ca. 560 nm. This result indicate that bR trimers were largely dispersed as monomers in the DPPC membrane. A similar phenomenon has been reported for DMPC liposomes, in which trimeric bR disaggregated into monomers.[5,8,17] More interestingly, **2**/dbR vesicles showed a similar

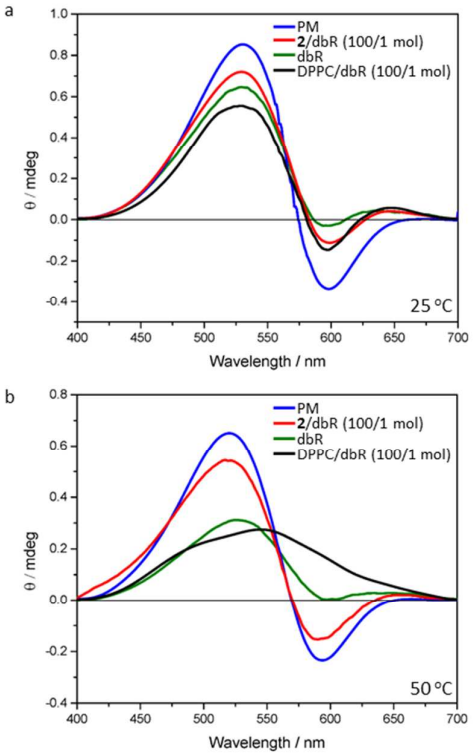


Fig. 3 CD spectra of PM, **2**/dbR (100/1 mol), dbR, and DPPC/dbR (100/1 mol) at temperatures of 25 °C (a) and 50 °C (b).

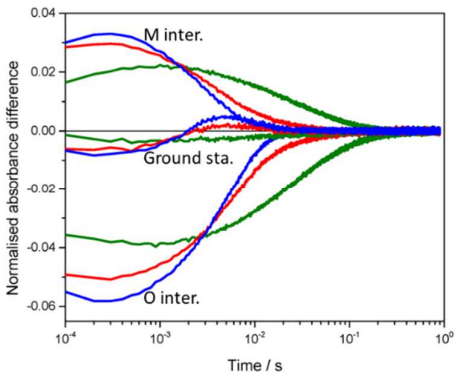


Fig. 4 Flash-induced absorbance changes at 410 nm (M intermediate), 560 nm (Ground state), and 640 nm (O intermediate) for dbR (green), **2**/dbR (100/1 mol) (red) and PM (blue) at 25 °C.

CD spectrum as that of PM at 50 °C, implying that bR could retain its lattice structure in **2**/dbR complexes in the fluid phase at higher temperatures. Since the phase transition temperature of **2** was determined by differential scanning calorimetry to be 41 °C,[28] which is the same as that of DPPC, the difference between **2** and DPPC is possibly not due to differences in their phase status, but rather due to the stabilising effect of the head group in **2**, which could partly reproduce that of the original PGP-Me.

Similar to mammalian rhodopsin, the biological functions of bR in PM are based on the protein structural change triggered by light-induced isomerization of retinal.[36-39] The photocycle kinetics of the reconstituted bR were examined by laser flash photolysis to evaluate the influence of membrane lipids on the protein function. Fig. 4 shows the absorbance changes at 410 nm, 640 nm, and 560 nm at 25 °C due to the increase and decay of the M-intermediate, O-intermediate, and ground state, respectively.[9] The proton pump activity of bR was highly perturbed by partial delipidation of PM, implying a significant change in the conformation of bR, as inferred from the slow-down of photocycle kinetics (Fig. 4, green line). Coupled with the CD results, this finding indicates the importance of surrounding lipids in maintaining the structural and functional integrity of bR. As seen in Fig. 4, **2**/dbR liposomes showed similar kinetic profiles as those of PM in both magnitude and lifetime although the photocycle was slightly increased, again implying that the original activity of bR was restored in the artificial membranes containing **2**. Similar transient absorption changes at 410 nm, 640 nm, and 560 nm of **2**/dbR and PM were obtained in the liquid crystalline phase at 50 °C (Fig. S2†).

Based on visible CD and laser flash photolysis, it is likely that the dynamic formation of structural units by interaction between bR and **2** is important to retain the original structure and functions of bR in the membranes. In order to obtain detailed information on the molecular dynamics of this process, bR was incorporated into the liposomes composed of analogue **2** and **3** for solid-state ^{31}P and ^{2}H NMR measurements, respectively.

First, we examined formation of the lamellar bilayer structure of **2** using solid state ^{31}P NMR spectroscopy. There are two phosphate

ester groups *a* and *b* in **3**, which give rise to two different ^{31}P signals; one in the terminal position (*b*) is known to show a narrow peak near 0 ppm while the other between two glycerol moieties (*a*) shows a typical pattern for the axially symmetric system.[40] As shown in Fig. 5, the ^{31}P NMR spectrum of **2** gave rise to three sets of signals, two of which fitted well with those of PGP-Me in bilayer structure while the sharp isotropic peak at 0 ppm indicated the presence of rapidly moving lipid aggregates. Thus, analogue **2** tends to form micelles, probably due to its smaller side chains than those of natural PGP-Me. For further NMR measurements, we used plain-water dispersions of **2** due to the propensity that the lipid forms micelles more easily in buffer than in pure water, particularly without bR, which prevented us from using buffer for suspending **2** in aqueous media. The effects of buffer on ^{31}P NMR spectra were examined as shown in Fig. S4; no noticeable difference in lipid mobility and packing was observed between the presence and absence of buffer.

In Fig. 6**a**, the ^{2}H NMR spectra of **3** without bR provided two patterns, comprising of a centre peak and an anisotropic Pake doublet with a splitting of 23.1 kHz. The strong central peak was assignable to isotropic components such as micelles as observed in the ^{31}P NMR spectrum. The addition of dbR significantly altered the spectra (Fig. 6**b**). The reconstituted **3**/dbR (100/1 mol) showed an obviously enhanced magnitude with a quadrupolar splitting of 25.6 kHz. The difference in splitting magnitude implies that the interaction between **3** and bR reduced its mobility (or wobbling) and resulted in the formation of higher order alkyl chains. In addition, the formation of micelles were greatly reduced as indicated by the decreased isotropic peak intensity. Since the rate of exchange of annular lipids with bulk lipids is much faster than the NMR time scale,[41] only one pair of splitting was observed in the **3**/bR membrane. After delipidation to obtain dbR, a few PM lipids, mainly composed of PGP-Me and other lipids, remained on the protein surface as determined by mass chromatography. Thus, the membrane preparation of **3**/PM lipids

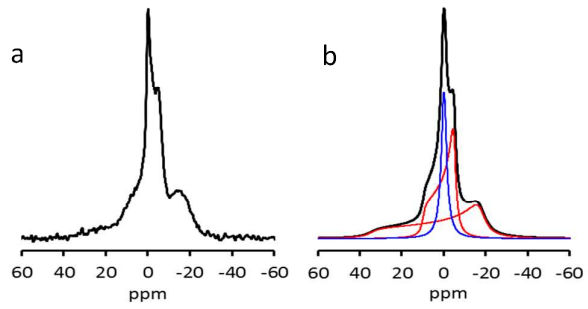


Fig. 5 ^{31}P NMR spectrum of aqueous multilamellar dispersion of **2** (a), and simulated spectra by Simpson (b) showing isotropic signal such as that due to micelles and anisotropic signal. The spectrum was measured with 60% hydrated membrane dispersions at 50°C. Simulated ^{31}P static spectrum (b) composed of two uniaxially symmetric powder patterns due to two phosphate esters (red, 79 mol%) and one isotropic pattern (blue, 21 mol%) corresponding to lamellar structure and micelle, respectively.

(100/4 w/w) was subjected to NMR measurements to confirm that the ordering effect of the proteins observed in Fig. 6**b** was due to bR. As shown in Fig. 6**c**, the addition of PM lipids slightly decreased the splitting magnitude of **3**, probably due to the disordering nature of the methyl-branched alkyl chains in PM lipids. These observations imply that the enhancement of quadrupolar splitting in **3**/dbR was largely due to LPI; quadrupolar splitting is known to be markedly influenced by subtle changes in membrane conditions, and thus, further NMR experiments under the different lipid composition, buffer and pH may be necessary to confirm the present results. These results again reveal that the head group of PGP-Me is crucial for providing an appropriate environment for bR to form its intrinsic structure and proper function. The role of the phytanyl chains of PM lipids including PGP-Me remains unknown in the present study. Chemical synthesis of this tertamethyl-hexadecanol with three stereogenic centres poses another synthetic challenge, and we are currently attempting to prepare a ^{2}H-labelled probe to examine the effects of the hydrophobic portions of PM lipids on bR in a further study on LPI.

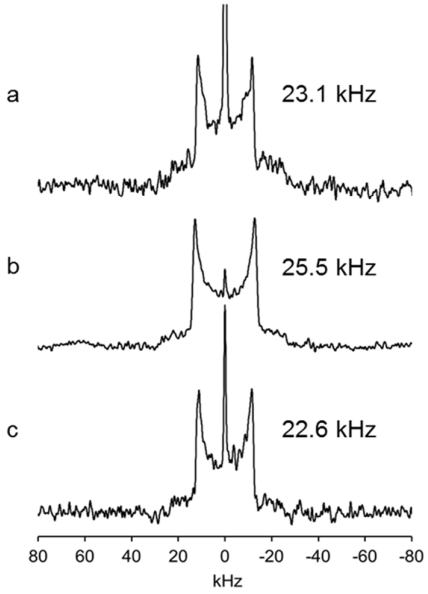


Fig. 6 ^{2}H NMR spectra of aqueous multilamellar dispersions of (a) **3**, (b) **3**/dbR (100/1 mol), and (c) **3**/PM lipids (100/4 w/w). All the spectra were measured with 60% hydrated membrane dispersions at 50 °C.

Previous ^{2}H NMR studies on LPI have revealed that lipids are important to reproduce the original structure and function of embedded proteins.[42,43] Standard phospholipids, such as DPPC in this study, sometimes cause dissociation of protein-protein interactions, resulting in unnatural contacts of model lipids with the intrinsic protein-protein interfaces. Annular lipids usually stay on the protein interface for less than a microsecond,[11] which hampers the selective observation of their associated form. The order parameter of **3** upon interacting with bR is increased by 10% (Fig. 6**a** and 6**b**), which is relatively high as compared with the ordering effect of rhodopsin on a saturated chain of PC.[42] Thus, the firm association of

3 with bR may be one of reasons for stabilisation of the trimeric assemblage; hydrophobic matching is also known to play an important role in oligomerisation and functions of rhodopsin.[42,43] The analogues **2**/**3** provide a native-like environment for bR, which grossly reproduce its original light-activated photocycle even at a high temperature. The unique double-charged head group of the analogue possibly play an essential role in forming the lipid/protein functional complex. Further comparison between **2** and usual PC such as DPPC will unveil a key role of this unique head group, eventually leading to a better understanding of general functions of membrane lipids to stabilize the structure of integral membrane proteins.

Conclusions

The polar head of PGP-Me and its analogue with straight side chains were stereoselectively synthesised using H-phosphonate chemistry. The PGP-Me analogue **2** was adopted to provide a model lipid environment for the photoreceptor membrane protein bR. In the artificial membranes, bR grossly reproduced the original trimeric structure and light-activated photocycling. The unique double-charged head group of the analogue should play an important role in forming the lipid/protein functional complex. Using a ^{2}H-labelled PGP-Me analogue **3**, the interactions between lipids and proteins were detected by solid-state ^{2}H NMR as indicated by the higher order of the probe in the bR-containing membranes. These results indicate the usefulness of the synthetic analogue as a tool for investigating the LPIs occurring in PM.

Acknowledgements

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