ChemPubSoc Europe

DOI: 10.1002/cbic.201700414

CHEMBIOCHEM Full Papers

Gb_3 Glycosphingolipids with Fluorescent Oligoene Fatty Acids: Synthesis and Phase Behavior in Model Membranes

Lukas J. Patalag[+],[a] Jeremias Sibold[+],[b] Ole M. Schütte,[b] Claudia Steinem,*[b] and Daniel B. Werz*[a]

Glycosphingolipids are involved in a number of physiological and pathophysiological processes, and they serve as receptors for a variety of bacterial toxins and viruses. To investigate their function in lipid membranes, fluorescently labeled glycosphingolipids are highly desirable. Herein, a synthetic route to access Gb_3 glycosphingolipids with fluorescently labeled fatty acids, consisting of pentaene and hexaene moieties either at the terminus or in the middle of the acyl chain, has been developed. The fluorescent properties of the Gb_3 derivatives were investigated in small unilamellar vesicles composed of a raft-like mixture. Phase-separated giant unilamellar vesicles (GUVs) allowed the quantification of the apparent partitioning coefficients of the Gb_3 compounds by means of confocal fluorescence laser scanning microscopy. The determined partition coefficients demonstrate that the Gb_3 derivatives are preferentially localized in the liquid-disordered (l_d) phase. To analyze whether the compounds behave like their physiological counterparts, Cy3-labeled (Cy: cyanine) Shiga toxin B subunits (STxB) were specifically bound to Gb_3-doped GUVs. However, the protein was favorably localized in the l_d phase, in contrast to results reported for STxB bound to naturally occurring Gb_3, which is discussed in terms of the packing density of the lipids in the liquid-ordered (l_o) phase.

Introduction

Eukaryotic plasma membranes of animals comprise a large number of lipids, including glycerophospholipids, cholesterol (Chol), and sphingomyelin (SM). In addition, the outer leaflet of the plasma membrane harbors glycosphingolipids, which can serve as receptor molecules for neighboring cells and are involved in the formation of raft domains through interactions with Chol.[1,2] Glycosphingolipids are also specific attachment sites for a number of bacteria, viruses, and proteins.[3] Among them, bacterial toxins are known to bind to gangliosides or globosides. For example, cholera toxin uses the ganglioside GM_1 to enter the cell,[4] whereas Shiga toxin (STx) binds specifically to the globoside Gb_3.[5–7] Cholera toxin, a typical AB_5 protein, is produced by *Vibrio cholerae* and has been widely used as a marker for raft-like liquid-ordered (l_o) domains in phase-separated membranes.[2,8] The bacterial STx is produced by *Shigella dysenteriae* and by enterohemorrhagic strains of *Escherichia coli*. With the AB_5 structure, it harbors five B subunits (STxB) capable of binding to the trisaccharidic carbohydrate structure of Gb_3.[5,9–11] Clustering of Gb_3 upon STxB binding has been shown to induce invaginations as the first step of internalization of the toxin into the cell.[6] Although STxB can serve as a marker to localize Gb_3 in the membrane, virtually nothing is known about the distribution of Gb_3, its dynamics, and interaction with other lipid components in the absence of bound STxB. To localize glycosphingolipids in membranes by optical microscopy, a fluorescent label is required. Several attempts have been made to label the gangliosides GM_1 and GM_3.[8,12–15] The fluorescent probes were attached to either the fatty acid side chain[16] or the sugar chains.[14,17] However, if characterized in detail, the behavior of these ganglioside derivatives were quite different from those of the native molecules. In particular, derivatives with bulky fluorophores in the hydrophobic part of the membrane do not partition into the l_o phase as expected.[13] The same holds true for the commercially available fatty acid-labeled (nitrobenzoxadiazole (NBD)-labeled) Gb_3. Gangliosides tagged with hydrophilic fluorophores are capable of partitioning into the l_o phase.[14] However, their binding properties to viruses and proteins, including toxins, might be hampered due to the bulky fluorophore at the head group.

To design a fluorescently labeled Gb_3 molecule able to partition into the l_o phase and serve as a receptor for proteins, such as STxB, a fatty acid labeled structure is desirable. The envisioned fatty acid should fit into the tightly packed l_o phase. Goñi and co-workers showed that polyene ceramide analogues could serve as membrane probes.[18] They designed a polyene ceramide analogue termed pentaene I, which appeared to par-

[a] L. J. Patalag,[+] Prof. Dr. D. B. Werz
TU Braunschweig, Institut für Organische Chemie
Hagenring 30, 38106 Braunschweig (Germany)
E-mail: d.werz@tu-braunschweig.de

[b] J. Sibold,[+] Dr. O. M. Schütte, Prof. Dr. C. Steinem
Georg-August-Universität Göttingen
Institut für Organische und Biomolekulare Chemie
Tammannstrasse 2, 37077 Göttingen (Germany)
E-mail: claudia.steinem@chemie.uni-goettingen.de

[+] These authors contributed equally to this work.

Supporting information and the ORCID identification numbers for the authors of this article can be found under https://doi.org/10.1002/cbic.201700414: detailed experimental procedures, synthetic methods, chemical compound information, and supplementary results for Dye731-DOPE.

tition into the gel phase of a membrane, and the fluorescence emission of this molecule was more intense in the gel than that in the fluid phase. However, in contrast to this finding, the group concluded from differential calorimetry data that pentaene I partitioned preferentially in the fluid phase.

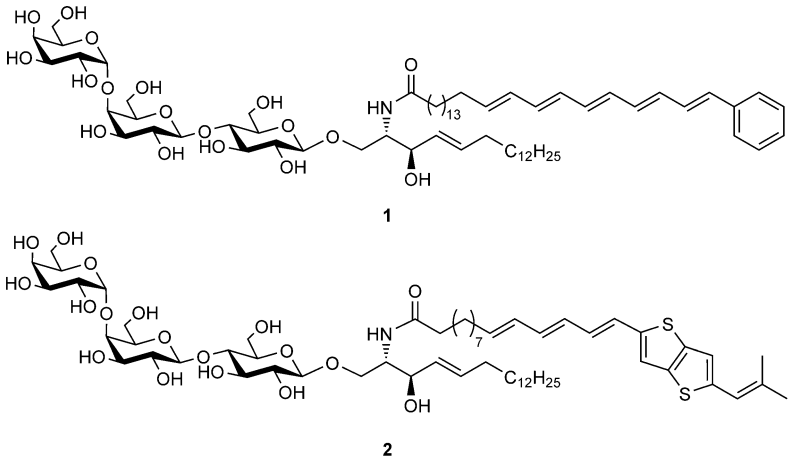
Herein, we have taken this general approach and designed fluorescently labeled Gb_3 molecules with fatty acids consisting of pentaene and hexaene moieties either at the terminus or in the middle of the acyl chain,[19] with the hypothesis that they can partition into the l_o phase of phase-separated lipid bilayers and are still able to bind the protein STxB.

Results and Discussion

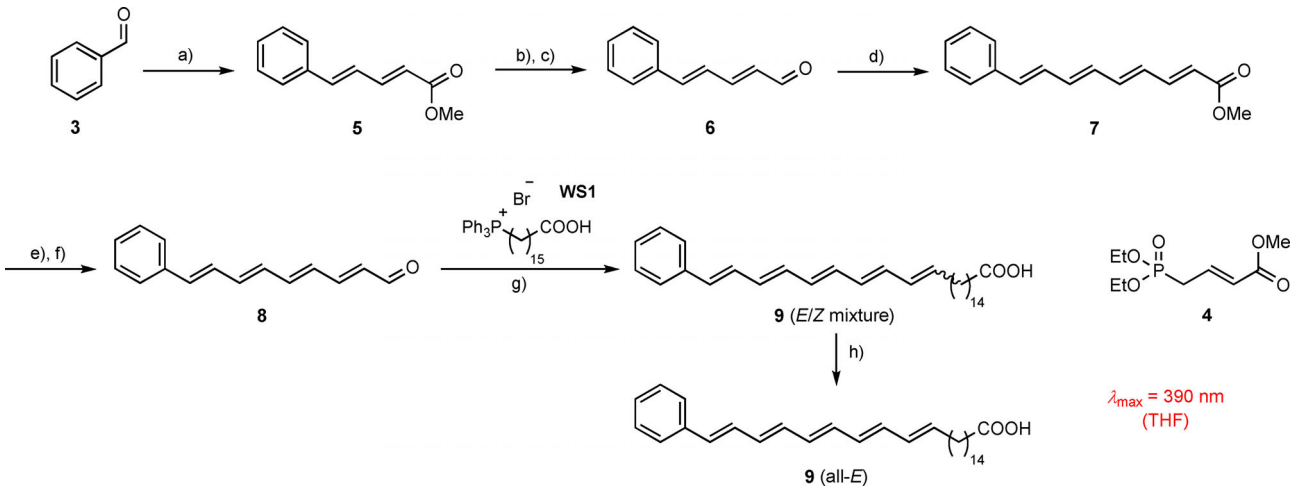
Syntheses of glycosphingolipids with fluorescent fatty acids

Chemical synthesis of Gb_3 glycosphingolipids enabled us to access two glycoconjugates, **1** and **2**, differing in their fatty acid part (Scheme 1). In order not to alter the membrane structure and dynamics to a major extent, we did not consider fatty acids with embedded polar and/or bulky fluorescent moieties, such as NBD,[21] pyrene,[22] boron-dipyrromethene (BODIPY),[23] boron complexes of iminopyrrolide ligands (BOIMPY),[24] or benzothiadiazole,[25] but prepared fatty acids consisting of pentaene and hexaene moieties either at the terminus or in the middle of the acyl chain.[19] Their slim shape and hydrophobic scaffold should only disturb the membrane integrity to a minor extent.[26] However, these molecular entities proved to be rather unstable in the presence of both air and strong laser beams. In addition, an absorption far below $\lambda = 400$ nm appears to be ill-suited to standard fluorescence microscopy. To increase the stability of the fluorescent core structure and improve the advantageous spectroscopic properties without largely increasing the structural bulkiness, we designed two new fluorescent fatty acids with either a phenyl (**1**) or a thienothienyl residue (**2**) in conjugation to an oligoene system (Scheme 1). Both aryl moieties extend the π system and are expected to red-shift the absorption and emission spectra.

We commenced our synthesis with benzaldehyde (**3**), which was subjected to a Horner–Wadsworth–Emmons reaction by using allyl phosphonate **4**, leading to unsaturated ester **5** in 79% yield (Scheme 2). The phosphonate allows the introduc-



Scheme 1. Gb_3 glycoconjugates **1** and **2** with fluorescent fatty acids based on terminal oligoene systems.



Scheme 2. Synthesis of pentaene fatty acid **9** with a phenyl substituent at the terminus. a) **4**, lithium bis(trimethylsilyl)amide (LiHMDS; 79%); b) diisobutyl aluminium hydride (DIBAL); c) MnO_2 (74%); d) **4**, *n*BuLi (24%); e) DIBAL; f) MnO_2 (55%); g) LiHMDS, *n*BuLi (20%); h) I_2 (cat.).

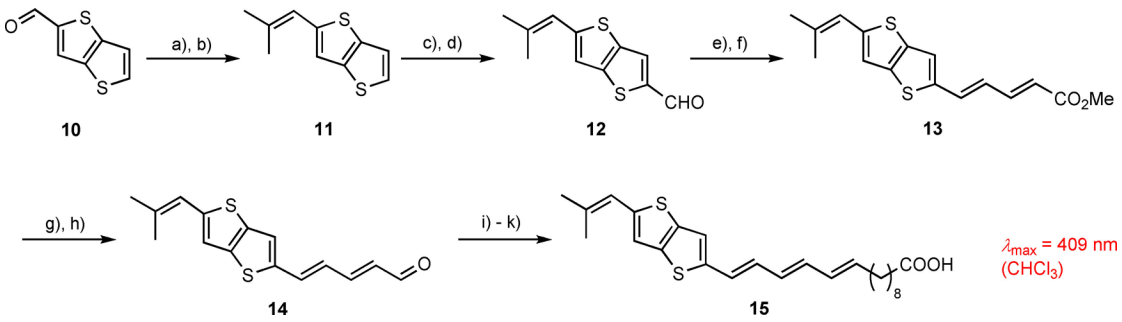
tion of two *E*-configured C=C double bonds in a one-pot process. Reduction to the alcohol and subsequent oxidation with manganese dioxide led to aldehyde **6**.

This species is converted into tetraene ester **7** with phosphonate **4**. The respective aldehyde **8** was again obtained by a reduction/oxidation protocol. To install the fifth double bond of the pentaene system, a Wittig reaction proved to be the method of choice. A mixture of the corresponding (*E*/*Z*) isomers was obtained in 20% yield. Treatment with catalytic amounts of iodine triggered a gentle isomerization to give the desired all-*E* isomer **9**. Due to the enlarged π system, the absorption maximum of **9** is red-shifted by about 35 nm (from $\lambda = 350$ to 385 nm) in comparison to a non-arylated counterpart.

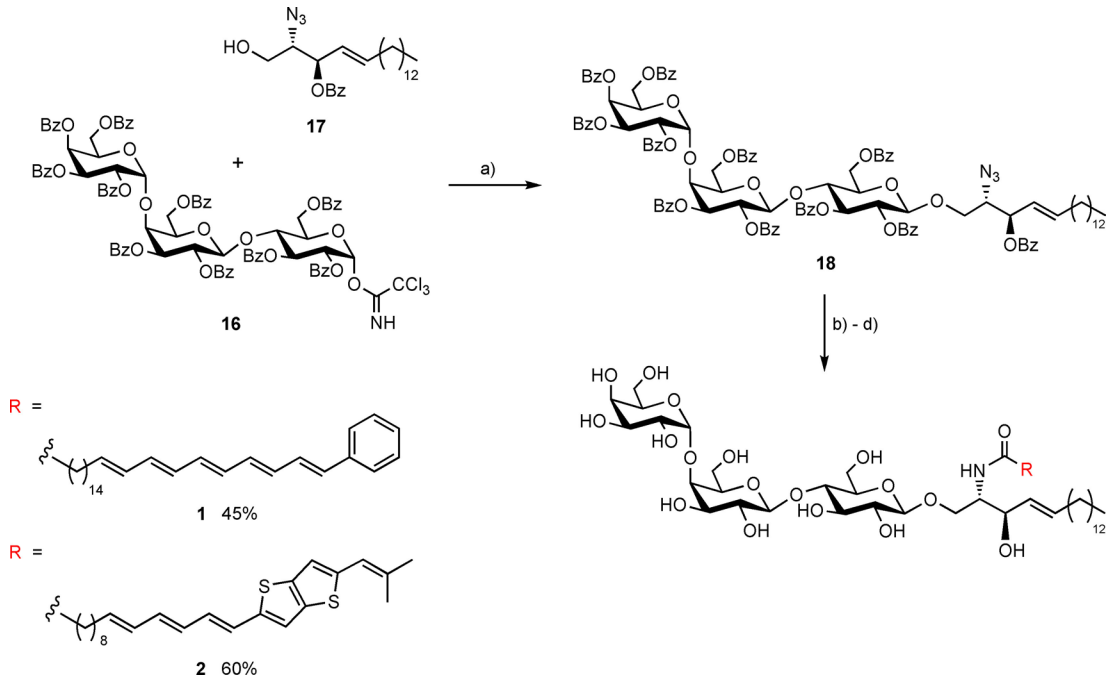
The synthetic route to thienothienyl-modified fatty acid **15** resembles that previously described for **9**. First, thienothiophene-2-carbaldehyde (**10**) was engaged in a Wittig reaction to give isopropylidene-equipped product **11**, which was subjected to a formal carbonylation by using DMF as a carbonyl source to afford **12** in 70% yield (Scheme 3).

For the formation of the unsaturated diene, Horner–Wadsworth–Emmons reagent **4** was employed.[27] The emerging ester **13** was transformed via an alcohol into the corresponding aldehyde **14**, which proved to be the crucial intermediate for elongation to the final fatty acid through a Wittig reaction.[28] Catalytic amounts of iodine allowed isomerization to all-*E*-system **15**.

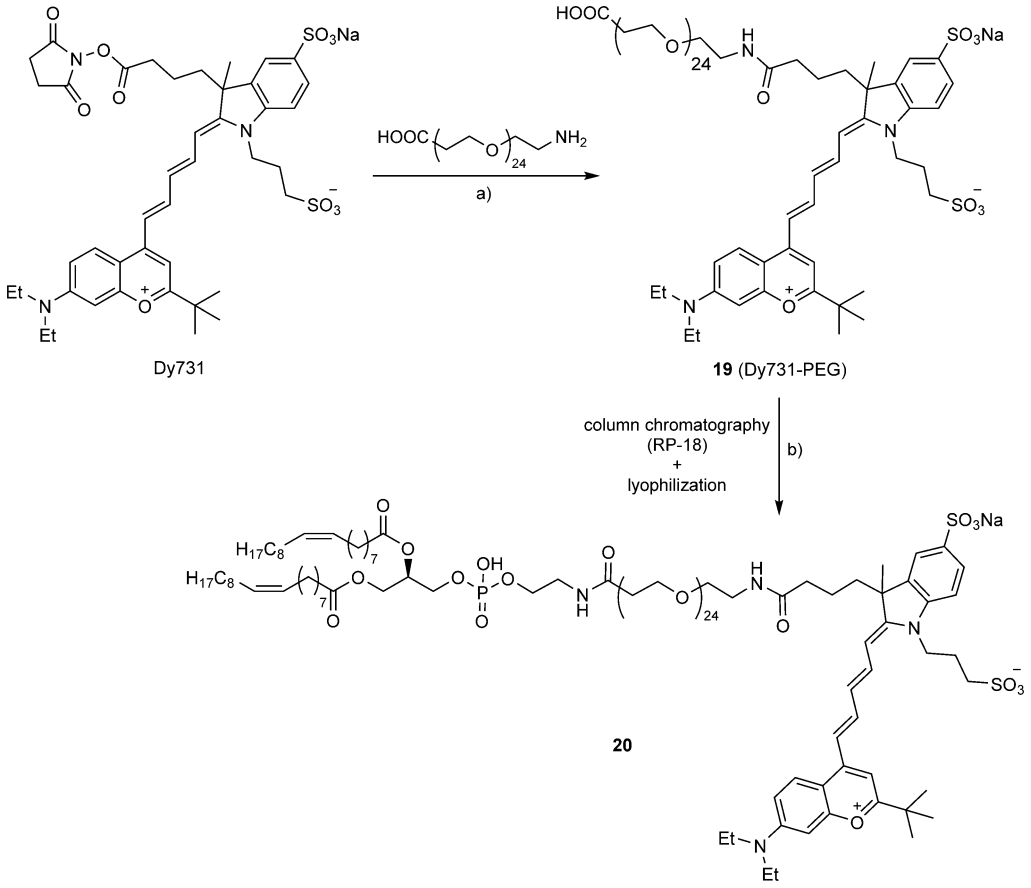
To obtain the desired glycolipids **1** and **2**, perbenzoylated globotriaosyl trichloroacetimidate **16** was submitted to a glycosylation reaction with azide-protected sphingosine alcohol **17** (Scheme 4).[10,11] Under the influence of boron trifluoride as a Lewis acid, protected trisaccharide **18** was obtained in 41% yield. Staudinger reduction, subsequent amide formation by using our novel fatty acids, and saponification under Zemplén conditions led to glycosphingolipids **1** and **2**.



Scheme 3. Synthesis of thienothienyl-modified fatty acid **15**. a) *i*PrP$^+$Ph$_3$ I$^-$, WS2; b) NaHMDS (95%); c) lithium tetramethylpiperidide; d) DMF (70%); e) **4**; f) *n*BuLi (82%); g) DIBAL; h) MnO_2 (36%); i) Ph$_3$P$^+$(CH$_2$)$_9$COOH Br$^-$, WS3; j) *n*BuLi; k) I_2 (cat.) (67%).



Scheme 4. Assembly of Gb$_3$ glycosphingolipids **1** and **2** with fluorescent fatty acids. BF_3·OEt_2, 4 Å MS, CH_2Cl_2/hexane (1:1) (41%); b) PPh_3, benzene/H_2O; c) 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU), *N*,*N*-diisopropylethylamine (DIPEA), **9** or **14**; d) NaOMe, CH_3OH.



Scheme 5. Preparation of fluorescent Dy731-DOPE (**20**). The fluorophore is attached through a long PEG chain to the respective lipid. a) NEt_3, MeCN, 3 days (dark); b) DOPE (2 equiv), DIPEA (4 equiv), HATU (1.5 equiv), DMF/$CHCl_3$, 35 °C, 14 h (65% over 2 steps).

Apart from the fluorescent labeling of the glycolipids, 1,2-di-(9*Z*-octadecenoyl)-*sn*-glycero-3-phosphoethanolamine (DOPE) also had to be labeled by another dye. Therefore, we attached the fluorophore Dy731 through amide coupling to a long poly(ethylene glycol) (PEG) linker ($n=24$). Emerging product **19** was further conjugated to DOPE to afford **20** (Scheme 5). This highly amphiphilic conjugate was purified by reversed-phase column chromatography (RP-18).

Excitation and emission spectra of compounds 1 and 2

First, the optical properties of compounds **1** and **2** reconstituted into small unilamellar vesicles (SUVs) composed of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC)/SM/Chol/Gb_3 (40:35:20:5) were analyzed (Figure 1). The excitation spectrum of **1** shows two characteristic bands at $\lambda=347$ and 366 nm and a shoulder at $\lambda=330$ nm, which is typical for the vibrational resolution of polyene chromophores.[29] Similarly, two excitation bands are found for **2**; however, these are shifted to longer wavelengths ($\lambda=369$ and 391 nm) as a result of the thiophene moiety in the chain. Compared with a sphingosine-like chain containing a pentaene,[18] the excitation spectra are slightly red-shifted owing to the extended π system. The fluorescence emission spectrum of compound **1** is broad, ranging from $\lambda=$ 400 to 650 nm with a fine structure with three maxima at $\lambda=$ 410, 434, and 458 nm. Compound **2** also shows a broad fluorescence emission spectrum in the range of 400–650 nm with one maximum at $\lambda\approx440$ nm, which is very similar to previously reported pentaene systems.[18]

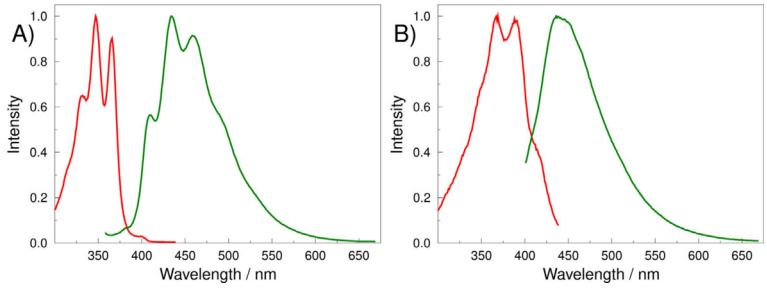


Figure 1. Excitation (red) and emission (green) spectra of the fatty acid labeled Gb_3-species **1** (A) and **2** (B) in SUVs composed of DOPC/SM/Chol/Gb_3 (40:35:20:5). The fluorescence excitation spectra were measured from $\lambda=300$ to 445 nm, with $\lambda_{em}=465$ nm for both Gb_3 species. Fluorescence emission was excited at A) $\lambda_{ex}=348$ nm, recorded from $\lambda=358$ to 669 nm, and at B) $\lambda_{ex}=390$ nm, recorded from $\lambda=400$ to 669 nm.

Fluorescence emission spectra in phase-separated lipid bilayers

In phase-separated liquid-ordered (l_o)/liquid-disordered (l_d) membranes, glycosphingolipids are discussed to preferentially partition into the l_o phase. To investigate the partition behavior of the fluorescent Gb_3 species **1** and **2** in phase-separated lipid bilayers, again a lipid mixture composed of DOPC/SM/Chol/Gb_3 (40:35:20:5) was used; this is known to phase separate.[10,11,30] For a quantitative analysis of the partition of the Gb_3 species in giant unilamellar vesicles (GUVs), as described below, the l_d phase needs to be unambiguously assigned; this is achieved by using an l_d phase marker. To first investigate whether the synthesized fluorescent Gb_3 species were compatible with an l_d phase marker, SUVs composed of the given lipid mixture were prepared, in which DOPC (0.5 mol%) was replaced by a fluorescent dye that partitioned into the l_d phase. In the first attempt, sulforhodamine-1,2-dihexanoyl-*sn*-glycero-3-phosphoethanolamine (Texas Red-DHPE) was chosen.[13,31] The resulting fluorescence spectra (Figure 2, red lines) clearly indicate that FRET between the fluorescent Gb_3 species and the Texas Red dye occurs as a result of an overlap of the emission spectra of compounds **1** and **2** and the absorption spectrum of Texas Red. From this result, we conclude that Texas Red-DHPE is not suited to quantify the partition of the Gb_3 species in phase-separated lipid bilayers. A fluorescent dye that partitions preferentially into the l_d phase with an absorption spectrum shifted to longer wavelengths that does not overlap with the emission spectra of the Gb_3 species is required. Thus, we synthesized Dy731-DOPE, which had an absorption maximum of $\lambda =$ 732 nm. SUVs composed of DOPC/SM/Chol/Gb_3/Dy731-DOPE (39.5:35:20:5:0.5) show the fluorescence emission spectra depicted in Figure 2 (green lines). Only a very small FRET peak at $\lambda \approx 760$ nm is observed. FRET does not significantly decrease the emission of the donor, that is, compounds **1** and **2**, respectively, which makes this dye suited for further investigations to quantify the partition of the two compounds in phase-separated GUVs.

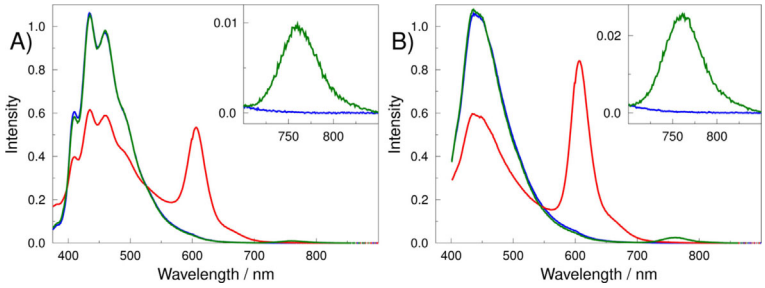


Figure 2. Fluorescence spectra of A) **1** ($\lambda_{ex} = 348$ nm) and B) **2** ($\lambda_{ex} = 390$ nm). Blue lines: fluorescence spectra obtained from vesicles composed of DOPC/SM/Chol/Gb_3 species (40:35:20:5); red lines: fluorescence spectra obtained from vesicles composed of DOPC/SM/Chol/Gb_3 species/Texas Red-DHPE (39.5:35:20:5:0.5); green lines: fluorescence spectra obtained from vesicles composed of DOPC/SM/Chol/Gb_3 species/Dy731-DOPE (39.5:35:20:5:0.5).

Partition of fluorescent Gb_3 in phase-separated GUVs

The most straightforward approach to study lipid domains is to use GUVs doped with lipid-like dyes that partition specifically in either the l_o or l_d phase.[13,31] We took fluorescence micrographs of GUVs composed of DOPC/SM/Chol/Gb_3/Dy731-DOPE (39.5:35:20:5:0.5) to quantify the partition of the fluorescent Gb_3 species in these phase-separated membranes (Figure 3). The dye Dy731-DOPE is almost exclusively localized in the l_d phase, as expected (Figure 3B and D, Figure S23 in the Supporting Information). However, compound **1** appears to be more homogeneously distributed among the l_o and l_d phase (Figure 3A). As a first estimate, we determined the fraction of Gb_3 species partitioning into the l_o phase from intensity line profiles of confocal images (Figure 3). The fluorescence intensities of the l_o and l_d phase, I_{l_o} and I_{l_d}, respectively, were determined from the peaks of the line scans, for which the two different phases were identified by the l_d phase marker Dy731-DOPE.[13] The apparent partitioning coefficient (%l_o) is calculated according to Equation (1):

$$\%\, l_o = \frac{I_{l_o} \times 100}{I_{l_o} + I_{l_d}} \qquad (1)$$

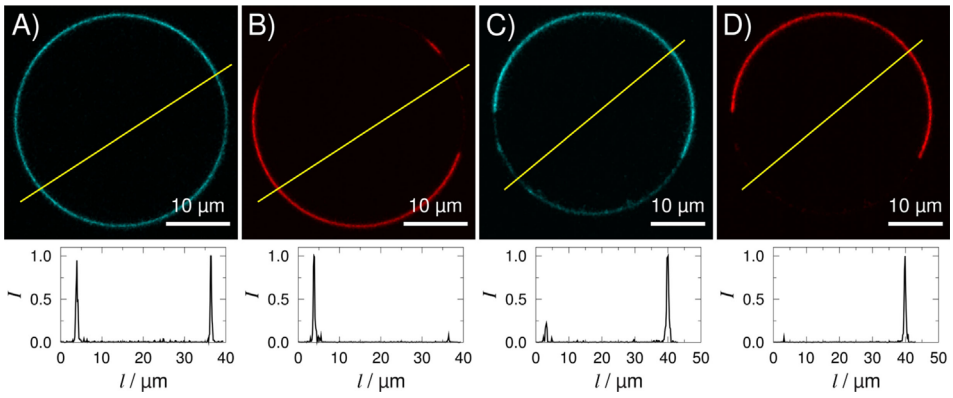


Figure 3. Confocal fluorescence images of GUVs composed of DOPC/SM/Chol/Gb_3/Dy731-DOPE (39.5:35:20:5:0.5). A) Fluorescence of **1** and B) the corresponding fluorescence of Dy731-DOPE. C) Fluorescence of **2** and D) the corresponding fluorescence of Dy731-DOPE. Line scans, which were used for the analysis, are highlighted in yellow and the intensity profiles from these line scans are shown in the bottom row.

Equation (1) neglects that the fluorescent yield could be slightly different in the two phases.[13] For compound **1**, the apparent partitioning coefficient was determined to be $\%I_o = (45 \pm 22)\%$ ($n = 2175$), which indicated that the fluorescent Gb_3 species was almost equally distributed between the I_o and I_d phase. For compound **2**, the apparent partitioning coefficient was $\%I_o = (24 \pm 10)\%$ ($n = 2003$); this demonstrated that this Gb_3 species was preferentially localized in the I_d phase.

Yet, not much is known about how Gb_3 distributes between the I_o and I_d phase prior to binding of a protein, such as STx. However, it is well established that, upon binding of STxB subunits to Gb_3-doped phase-separated GUVs, Gb_3 preferentially partitions into the I_o phase.[6,32] Thus, we analyzed whether the fluorescent Gb_3 species were recruited into the I_o phase upon binding of STxB.

Partition of Gb_3 in the presence of bound STxB

GUVs composed of DOPC/SM/Chol/Gb_3/Dy731-DOPE (39.5:35:20:5:0.5) were incubated with 60 nM unlabeled STxB (Figure 4). Again, for compound **1**, an apparent partitioning coefficient was determined to be $\%I_o = (41 \pm 21)\%$ ($n = 2103$); this clearly demonstrates that the receptor lipid Gb_3 does not change its partition behavior upon STxB binding. The same result was obtained for compound **2**, with an apparent partitioning coefficient of $\%I_o = (24 \pm 11)\%$ ($n = 2329$).

To further prove that, indeed STxB has been bound to the Gb_3 species, Cy3-labeled (Cy: cyanine) STxB was used. For both fluorescent Gb_3 species, STxB binds to the I_d phase (Figure 5), in contrast to results obtained for natural (Figure S24)[6,32] or synthetic[10,11] unlabeled Gb_3 species. This result clearly indicates that the fluorescent Gb_3 species do not behave like natural Gb_3 molecules, owing to the altered fatty acid structure.

It is known that sphingolipids containing BODIPY-FL or NBD as fluorophores in the fatty acid chain prevent their incorporation into the I_o phase, owing to bulkiness inserted into the hydrophobic part of the membrane.[12,13,15,31] Moreover, it has been shown that GM_1, which is the natural receptor lipid of cholera toxin, partitions and binds cholera toxin exclusively in the I_d phase, if the acyl chain is labeled with BODIPY-FL, in contrast to what has been reported for unlabeled GM_1.[12,13,33] However, the pentane moiety used in **1** was expected to fit into the I_o-phase structure because the length of the fatty acid matches that of the naturally occurring Gb_3 molecules, with fatty acid chain lengths of 24 carbon atoms, while the aromatic ring is attached at the very end. Our results, however, clearly indicate that the pentaene structure, with the terminal aromatic ring, is apparently already sufficiently bulky to prevent close packing of the Gb_3 molecules in the I_o phase upon STxB binding. The same holds true for **2**, which is even largely excluded from the I_o phase. In the absence and in the presence of bound STxB, the thiophene moiety, which is positioned further

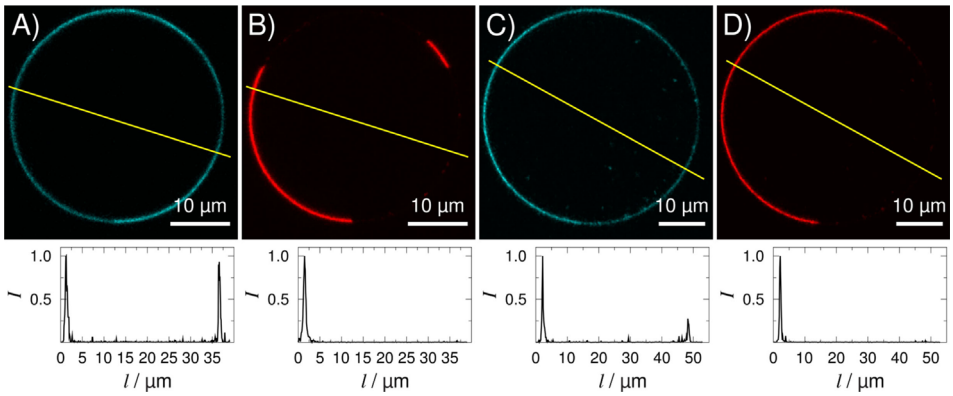


Figure 4. Confocal fluorescence images of GUVs composed of DOPC/SM/Chol/Gb_3/Dy731-DOPE (39.5:35:20:5:0.5) with 60 nM STxB in solution. A) Fluorescence of **1** and B) the corresponding fluorescence of Dy731-DOPE. C) Fluorescence of **2** and D) the corresponding fluorescence of Dy731-DOPE. Line scans, which were used for the analysis, are highlighted in yellow and the intensity profiles from these line scans are shown in the bottom row.

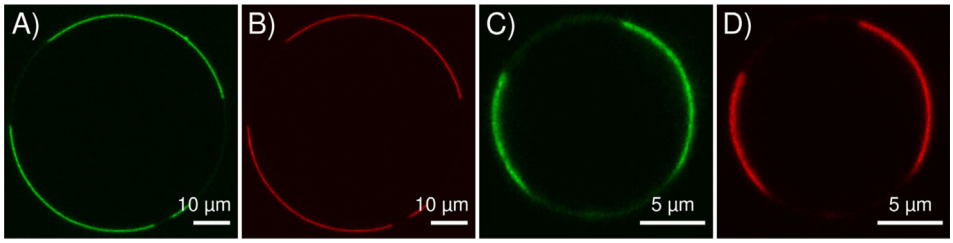


Figure 5. Confocal fluorescence images of GUVs composed of DOPC/SM/Chol/Gb_3/Dy731-DOPE (39.5:35:20:5:0.5) with 60 nM Cy3-labeled STxB in solution. A) Fluorescence of Cy3-STxB bound to **1** and B) the corresponding fluorescence of Dy731-DOPE. C) Fluorescence of Cy3-STxB bound to **2** and D) the corresponding fluorescence of Dy731-DOPE.

to the center of the lipid monolayer, is already too bulky to be placed in the l_o phase.

Conclusion

Two new fluorescently labeled Gb_3 globosides were synthesized, containing pentaene or hexaene moieties that allowed localization of Gb_3 molecules in phase-separated lipid bilayers. Because the molecules are labeled with fatty acids, binding of STx to the head group of the receptor lipid is preserved. However, the phase behavior of the fatty acid labeled Gb_3 species is greatly influenced, as shown by STxB binding, which binds to the l_d phase and not to the l_o phase, as known from naturally occurring Gb_3-containing membranes. This finding strongly suggests that even small changes in the packing density in the l_o phase can largely alter the phase behavior of the glycosphingolipids. To combine a fluorescence label with a glycosphingolipid that retains its natural phase behavior, a more promising approach would be to use head group labeled lipids with a long spacer and attached to a site at the head group of the glycosphingolipid not involved in protein binding.

Experimental Section

Materials: DOPC and SM from porcine brain were purchased from Avanti Polar Lipids. Chol and Texas Red-DHPE were obtained from Sigma–Aldrich. STxB and Cy3-labeled Shiga toxin B subunits (Cy3-STxB) were purified as described previously.[20]

SUVs: SUVs either composed of DOPC/SM/Chol/Gb_3 (40:35:20:5) or DOPC/SM/Chol/Gb_3/l_d marker (39.5:35:20:5:0.5) were prepared by extrusion. A solution (200 μL) of lipid in chloroform (0.5 mg mL^{-1}) was dried at the bottom of glass test tubes at 55 °C in a stream of nitrogen. The resulting lipid films were rehydrated in phosphate-buffered saline (1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 136.9 mM NaCl, pH 7.4) for 30 min followed by vortexing (30 s, three cycles in 5 min intervals) at 55 °C. The vesicle suspension was extruded 31 times by using a Liposofast mini extruder (Avestin, Ottawa, ON, Canada) through a 50 nm polycarbonate membrane.

GUVs: GUVs composed of DOPC/SM/Chol/Gb_3/l_d marker (39.5:35:20:5:0.5) were prepared by electroformation at 55 °C. A solution (100 μL) of lipid in chloroform (1 mg mL^{-1}) was deposited on indium tin oxide (ITO) slides. After removal of the solvent under reduced pressure and at a temperature of 55 °C for 3 h, the ITO slides were assembled in a chamber sealed with a Teflon ring and filled with a solution of sucrose (298 mOsmol L^{-1}). GUVs were obtained by applying a potential of $U = 1.6$ V with a frequency of $f = 12$ Hz for $t = 3$ h.

Fluorescence spectra: Fluorescence spectra were recorded on a FluoroMax-4 fluorimeter (Horiba Scientific, Edison, NJ, USA) by using poly(methyl methacrylate) (PMMA) cuvettes (VWR International, Leuven, Belgium). For both Gb_3 species, excitation spectra in a wavelength range of $\lambda = 300$–455 nm, with a resolution of 1 nm, were recorded at an emission wavelength of $\lambda = 465$ nm with a slit width of 2 nm. Fluorescence spectra were recorded with a resolution of 1 nm in a wavelength range of $\lambda = 358$–669 nm. The fluorescence of compound **1** was excited at $\lambda = 348$ nm and that of compound **2** at $\lambda = 390$ nm by using a slit width of 5 nm for excitation and 2 nm for emission.

Fluorescence microscopy: Fluorescence images were recorded with a confocal laser scanning microscope (FV 1200; Olympus, Hamburg, Germany) equipped with a water immersion objective (UPlanSApo N 60×/1.20 NA; Olympus, Hamburg, Germany). The fluorescence of both Gb_3 species was excited at $\lambda = 405$ nm and detected at $\lambda = 440$–540 nm. The excitation wavelength for Cy3-STxB was $\lambda = 561$ nm and fluorescence was detected from $\lambda = 575$ to 620 nm. Dy731-DOPE was excited with the laser line at $\lambda = 635$ nm and emission was recorded from $\lambda = 655$ to 755 nm.

Acknowledgements

We thank the DFG (SFB 803, project A05) for financial support.

Conflict of Interest

The authors declare no conflict of interest.

Keywords: fluorescence spectroscopy · glycolipids · membranes · proteins · Shiga toxin · vesicles

[1] D. Lingwood, B. Binnington, T. Róg, I. Vattulainen, M. Grzybek, U. Coskun, C. A. Lingwood, K. Simons, *Nat. Chem. Biol.* **2011**, *7*, 260–262.
[2] K. Simons, M. J. Gerl, *Nat. Rev. Mol. Cell Biol.* **2010**, *11*, 688–699.
[3] a) T. Eierhoff, B. Stechmann, W. Römer in *Molecular Regulation of Endocytosis* (Ed.: B. Ceresa), InTech, Rijeka, **2012**; b) J. A. Cho, D. J.-F. Chinnapen, E. Aamar, Y. M. te Welscher, W. I. Lencer, R. Massol, *Front. Cell. Infect. Microbiol.* **2012**, *2*, 51.
[4] a) D. E. Saslowsky, Y. M. te Welscher, D. J.-F. Chinnapen, J. S. Wagner, J. Wan, E. Kern, W. I. Lencer, *J. Biol. Chem.* **2013**, *288*, 25804–25809; b) M. L. Torgersen, G. Skretting, B. van Deurs, K. Sandvig, *J. Cell Sci.* **2001**, *114*, 3737–3747.
[5] W. Römer, L. Berland, V. Chambon, K. Gaus, B. Windschiegl, D. Tenza, M. R. E. Aly, V. Fraisier, J.-C. Florent, D. Perrais, C. Lamaze, G. Raposo, C. Steinem, P. Sens, P. Bassereau, L. Johannes, *Nature* **2007**, *450*, 670–675.
[6] B. Windschiegl, A. Orth, W. Römer, L. Berland, B. Stechmann, P. Bassereau, L. Johannes, C. Steinem, *PLoS ONE* **2009**, *4*, e6238.
[7] D. G. Pina, L. Johannes, M. A. R. B. Castanho, *Biochim. Biophys. Acta Biomembr.* **2007**, *1768*, 628–636.
[8] A. S. Klymchenko, R. Kreder, *Chem. Biol.* **2014**, *21*, 97–113.
[9] a) W. Pezeshkian, A. G. Hansen, L. Johannes, H. Khandelia, J. C. Shillcock, P. B. S. Kumar, J. H. Ipsen, *Soft Matter* **2016**, *12*, 5164–5171; b) H. Ling, A. Boodhoo, B. Hazes, M. D. Cummings, G. D. Armstrong, J. L. Brunton, R. J. Read, *Biochemistry* **1998**, *37*, 1777–1788; c) F. Eghiaian, *Biophys. J.* **2015**, *108*, 2757–2758; d) D. G. Pina, L. Johannes, *Toxicon* **2005**, *45*, 389–393.
[10] O. M. Schütte, A. Ries, A. Orth, L. J. Patalag, W. Römer, C. Steinem, D. B. Werz, *Chem. Sci.* **2014**, *5*, 3104.
[11] O. M. Schütte, L. J. Patalag, L. M. C. Weber, A. Ries, W. Römer, D. B. Werz, C. Steinem, *Biophys. J.* **2015**, *108*, 2775–2778.
[12] N. Komura, K. G. N. Suzuki, H. Ando, M. Konishi, M. Koikeda, A. Imamura, R. Chadda, T. K. Fujiwara, H. Tsuboi, R. Sheng, W. Cho, K. Furukawa, K. Furukawa, Y. Yamauchi, H. Ishida, A. Kusumi, M. Kiso, *Nat. Chem. Biol.* **2016**, *12*, 402–410.
[13] E. Sezgin, I. Levental, M. Grzybek, G. Schwarzmann, V. Mueller, A. Honigmann, V. N. Belov, C. Eggeling, U. Coskun, K. Simons, P. Schwille, *Biochim. Biophys. Acta* **2012**, *1818*, 1777–1784.
[14] A. V. Samsonov, I. Mihalyov, F. S. Cohen, *Biophys. J.* **2001**, *81*, 1486–1500.
[15] P. Sengupta, A. Hammond, D. Holowka, B. Baird, *Biochim. Biophys. Acta Biomembr.* **2008**, *1778*, 20–32.
[16] a) G. Schwarzmann, M. Wendeler, K. Sandhoff, *Glycobiology* **2005**, *15*, 1302–1311; b) C. Eggeling, C. Ringemann, R. Medda, G. Schwarzmann, K. Sandhoff, S. Polyakova, V. N. Belov, B. Hein, C. von Middendorff, A. Schönle, S. W. Hell, *Nature* **2009**, *457*, 1159–1162; c) I. Mikhalyov, A.

Olofsson, G. Gröbner, L. B.-A. Johansson, *Biophys. J.* **2010**, *99*, 1510 – 1519; d) R. Sachl, I. Mikhalyov, M. Hof, L. B. A. Johansson, *Phys. Chem. Chem. Phys.* **2009**, *11*, 4335 – 4343; e) E. A. Larsson, U. Olsson, C. D. Whitmore, R. Martins, G. Tettamanti, R. L. Schnaar, N. J. Dovichi, M. M. Palcic, O. Hindsgaul, *Carbohydr. Res.* **2007**, *342*, 482 – 489.

[17] a) D. J.-F. Chinnapen, W.-T. Hsieh, Y. M. te Welscher, D. E. Saslowsky, L. Kaoutzani, E. Brandsma, L. D'Auria, H. Park, J. S. Wagner, K. R. Drake, M. Kang, T. Benjamin, M. D. Ullman, C. E. Costello, A. K. Kenworthy, T. Baumgart, R. H. Massol, W. I. Lencer, *Dev. Cell* **2012**, *23*, 573 – 586; b) S. M. Polyakova, V. N. Belov, S. F. Yan, C. Eggeling, C. Ringemann, G. Schwarzmann, A. de Meijere, S. W. Hell, *Eur. J. Org. Chem.* **2009**, 5162 – 5177; c) D. Marushchak, N. Gretskaya, I. Mikhalyov, L. B.-A. Johansson, *Mol. Membr. Biol.* **2007**, *24*, 102 – 112.

[18] I. Nieves, I. Artetxe, J. L. Abad, A. Alonso, J. V. Busto, L. Fajarí, L. R. Montes, J. Sot, A. Delgado, F. M. Goñi, *Langmuir* **2015**, *31*, 2484 – 2492.

[19] L. J. Patalag, D. B. Werz, *J. Org. Chem.* **2012**, *77*, 5297 – 5304.

[20] L. Johannes, D. Tenza, C. Antony, B. Goud, *J. Biol. Chem.* **1997**, *272*, 19554 – 19561.

[21] a) N. G. Lipsky, R. E. Pagano, *Science* **1985**, *228*, 745 – 748; b) N. G. Lipsky, R. E. Pagano, *Proc. Natl. Acad. Sci. USA* **1983**, *80*, 2608 – 2612.

[22] P. Somerharju, *Chem. Phys. Lipids* **2002**, *116*, 57 – 74.

[23] a) R. E. Pagano, *J. Cell Biol.* **1991**, *113*, 1267 – 1279; b) R. D. Kaiser, E. London, *Biochim. Biophys. Acta Biomembr.* **1998**, *1375*, 13 – 22.

[24] L. J. Patalag, P. G. Jones, D. B. Werz, *Angew. Chem. Int. Ed.* **2016**, *55*, 13340 – 13344; *Angew. Chem.* **2016**, *128*, 13534 – 13539.

[25] L. J. Patalag, D. B. Werz, *Beilstein J. Org. Chem.* **2016**, *12*, 2739 – 2747.

[26] L. Kuerschner, C. S. Ejsing, K. Ekroos, A. Shevchenko, K. I. Anderson, C. Thiele, *Nat. Methods* **2005**, *2*, 39 – 45.

[27] W. S. Wadsworth, W. D. Emmons, *J. Am. Chem. Soc.* **1961**, *83*, 1733 – 1738.

[28] G. Wittig, W. Haag, *Chem. Ber.* **1955**, *88*, 1654 – 1666.

[29] M. A. Castanho, A. Coutinho, M. J. Prieto, *J. Biol. Chem.* **1992**, *267*, 204 – 209.

[30] S. L. Veatch, S. L. Keller, *Phys. Rev. Lett.* **2005**, *94*, 148101.

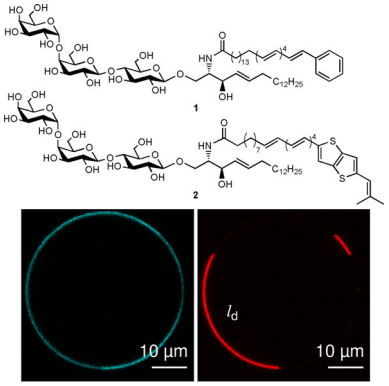
[31] T. Baumgart, G. Hunt, E. R. Farkas, W. W. Webb, G. W. Feigenson, *Biochim. Biophys. Acta Biomembr.* **2007**, *1768*, 2182 – 2194.

[32] M. Safouane, L. Berland, A. Callan-Jones, B. Sorre, W. Römer, L. Johannes, G. E. S. Toombes, P. Bassereau, *Traffic* **2010**, *11*, 1519 – 1529.

[33] K. Bacia, P. Schwille, T. Kurzchalia, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 3272 – 3277.

Manuscript received: August 1, 2017
Version of record online: ■■ ■■, 0000

Get in place! Fluorescent labels can alter the phase behavior of lipids considerably. The synthetic route to fatty acid-labeled Gb_3 derivatives that partition in the liquid-disordered phase, even after binding of Shiga toxin B subunits, is described.



L. J. Patalag, J. Sibold, O. M. Schütte, C. Steinem, D. B. Werz**

■■ – ■■

Gb_3 Glycosphingolipids with Fluorescent Oligoene Fatty Acids: Synthesis and Phase Behavior in Model Membranes