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New GM1 Ganglioside Derivatives for Selective Single and Double Labelling of the Natural Glycosphingolipid Skeleton

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Dedicated to Professor Ytzhak Apeloig on the occasion of his 65th birthday

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Selective single and double labelling of the natural ganglioside GM1 enables one to introduce various markers into different parts of the glycosphingolipid molecule without changing the natural skeleton. To that end, *N*-Fmoc-2-amino-, *N*-Fmoc-18-amino- and *S*-(ethoxythiocarbonyl)-18-mercaptostearic acids have been prepared and coupled with the primary amino group in the sphingosine part of lyso-GM1 and deAc-deAcyl-GM1 gangliosides. The products of these coupling reactions – building blocks **16a**, **16b**, **16c**, **26** and **27** – may be used for the synthesis of GM1 derivatives with one or two fluorescent dye moieties or other labels of various polarities. Examples of various labelling strategies, using hydrophilic and lipophilic photostable fluorescent dyes, have been made available. The GM1 derivatives **17a**, **22a** and **23c** labelled with the fluorescent dye ATTO 647N or the doubly labelled derivative **25b** can be used as probes in fluorescence correlation spectroscopy (in conventional microscopy or stimulated emission depletion nanoscopy) to study the diffusion of lipid analogues in model or live cell membranes.

Introduction

Gangliosides,[1] glycosphingolipids with sialic acid residues (Figure 1), are components of the plasma membranes of mammalian cells and are particularly abundant in neuronal membranes. A ganglioside consists of a hydrophobic ceramide moiety that contains the sphingosine residue acylated with a long fatty acid chain and a structurally very variable hydrophilic oligosaccharide chain. The last of these contains at least one *N*-acetylneuraminic (sialic) acid residue and one 2-deoxy-2-(acetylamino)galactose fragment.

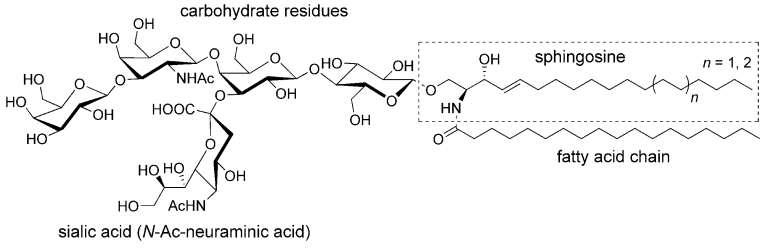

Figure 1. Structure of the ganglioside GM1.

Ganglioside derivatives containing radioactive,[2a–2c] photoreactive,[2d] paramagnetic[2e] or fluorescent labels have been used in studies of cell surfaces as well as processes occurring on them.

Such labels may influence the physical properties of a ganglioside, however, and under certain measurement conditions the behaviour of a labelled ganglioside may differ from that of the endogenous lipid. This may be especially true for a fluorescent dye with a bulkiness and polarity that

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may introduce steric or lipophilic changes into the labelled lipid. Previous fluorescence microscopy studies have, for example, demonstrated distinct partitioning of differently labelled sphingomyelin derivatives into liquid-disordered and ordered phases in model membranes[3] or have shown diverse dynamical behaviour of these derivatives in the plasma membranes of living cells.[4] Single-molecule-based fluorescence studies of lipid dynamics such as fluorescence correlation spectroscopy (FCS)[5] or its combination with stimulated emission depletion (STED) far-field fluorescence nanoscopy[4] require bright and photostable fluorescent labels. It is therefore of utmost importance to develop a ganglioside-labelling strategy that provides high flexibility and an optimized procedure with a minimal bias.

Here we present a novel synthesis approach to GM1 lipid derivatives allowing for flexible introduction of one or more fluorescent markers of various types (e.g., different polarities) into different parts of the glycosphingolipid molecule without changing the natural skeleton. STED- and two-colour FCS measurements of the fluorescent GM1 analogues prepared in this study illustrate the excellent applicability of the chosen model compounds for dynamic live-cell and multicolor experiments.

Results and Discussion

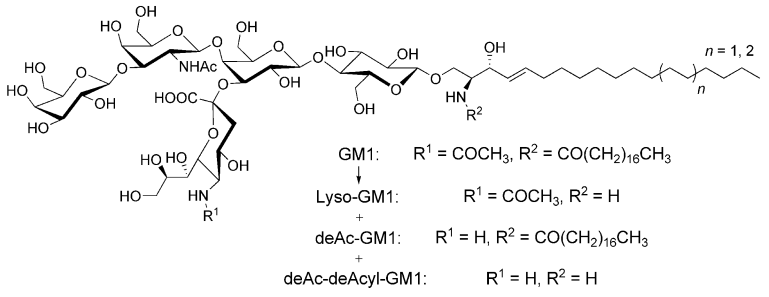
1. GM1 Labelling Approaches

Various sites in the GM1 molecule (Figure 1) have previously been decorated with different fluorescent dyes. The sialic acid residue is a convenient labelling site in gangliosides. It is possible chemically to modify the sialic acid residues directly on the surfaces of living cells for attaching the markers. Numerous reports deal with oxidative cleavage of the CH(OH)CH(OH) group in the sialic acid residue to afford an aldehyde, followed by treatment variously with hydrazides of fluoresceine,[6] rhodamine B,[6a,6d,7] lissamine rhodamine,[6a,8] Lucifer yellow CH[7–9] or eosin.[10] GM1 without its sialic acid residue was labelled with Alexa Fluor 488 dye.[11] The compound deAc-GM1 (Scheme 1), lacking one acetyl group at the sialic acid moiety (with the free amino group of the neuraminic acid), has been labelled with the BODIPY dye.[12] Lysoganglioside GM1 (Scheme 1) has been labelled (directly or through linkers containing up to 12 carbon atoms, which mimic the natural acyl chain) with the fluorescent dyes fluoresceine,[13] tetramethylrhodamine,[14] lissamine rhodamine,[15] NBD,[16] pyrene,[2a,2e,17] sulforhodamine 101,[18] "dark-red" fluorophore (DY650),[19] 9-anthrylvinyl- and 3-perylenoyl-fluorophores,[20] as well as with various BODIPY derivatives.[11,12,15,16e,21] None of the reported modifications (except for[12]) left the GM1 molecule intact, but variously changed the sialic acid residue, shortened the natural C_{18} acyl chain or enzymatically oxidized the terminal galactose unit.[22]

A great step forward towards a GM1 derivative with a structure only slightly perturbed by a fluorescent label was made in 2005, with the synthesis of a GM1 variant with a relatively small NBD fluorophore directly attached through its amino group to the α-position of the stearic acid residue.[23] Unfortunately, the brightness and photostability of the NBD fluorophore is rather low, impeding its application in FCS experiments.

We changed the labelling strategy, introducing one photochemically stable marker either to the α- or the ω-position of the stearic acid residue, or labelling both the stearic acid fragment and the neuraminic acid moiety with two different dyes.[24] The first modifications were conceived to leave the native GM1 skeleton (MW 1545/1573) formally intact, and "only" to add a considerable mass to it (the apolar and lipophilic ATTO 647N with MW 746, or the polar and hydrophilic RhS with MW 655). The α-position of the stearic acid residue is favorable for the attachment both of hydrophilic and of lipophilic dyes. Hydrophilic dyes may be embedded into the polar domain containing the hydrated "head" groups that are not far away from the start of the acyl chains. To provide a certain freedom in orientation, the polar hydrophilic labels should be attached to the α-position through linkers. A lipophilic label such as the organic dye ATTO 647N at the α-position of the stearic acid residue would be expected to be incorporated into the nonpolar domain of the lipid bilayer with minimal perturbation.[4]

Derivatization of the α-position creates an additional stereogenic center, so two diastereomeric GM1 derivatives are therefore generally obtained. They may differ in their chromatographic mobilities, and the interpretation of the analytical data may be not as easy as in the case of a single diastereomer. Shifting the labelling position to the end of the C_{18} chain not only removes all complications associated with the presence of two diastereomers, but also secures the location of a fluorophore within the non-polar lipid domain.



Scheme 1. Products of the alkaline hydrolysis of the GM1 ganglioside according to Sonnino.[27b]

We introduce ganglioside derivatives with *N*-protected α- and ω-aminostearic acid components, as well as with the *S*-protected ω-mercaptostearic acid, which after deblocking of the thiol group might provide additional labelling site for thiol-reactive fluorophores. Polar dyes are not recommended for "ω-labelling" of the ceramide moiety because they might cause "looping" and perturbation of the lipid bilayer.[25]

Finally, we obtained GM1 derivatives labelled with two different fluorescent dyes: a hydrophilic sulfonated rhodamine (RhS)[24a] at the polar head group (attached to the amino group in the neuraminic acid) and a hydrophobic one (ATTO 647N) bound through a short C_4 spacer with the amino group in the sphingosine part. In this case the hydrophobic ATTO 647N dye with the C_4 spacer mimics the natural fatty acid residue. Analogues of GM1 labelled with two different dyes may find applications in multicolor or Förster resonance energy transfer (FRET) experiments.

At last, we synthesized ganglioside derivatives retaining the natural structure of GM1 with two anchoring sites: one free amino group in the neuraminic acid and the second protected one in the fatty acid residue either in the α- or in the ω-position. These compounds may serve as precursors for GM1 derivatives labelled with two different (fluorescent) probes. An additional "degree of freedom" allows one to attach not only dyes of various polarities, but also other markers (e.g., spin- and radioactive labels), to the required site of GM1.

2. Starting Materials – Derivatives of GM1 and Fluorescent Dyes

Lysoganglioside GM1 (lyso-GM1, deAcyl-GM1) was used as a starting material for the preparation of the GM1 derivatives. Lyso-GM1 has a free amino group in the ceramide residue, without the natural fatty acid chain (Scheme 1), and has been prepared from the natural ganglioside GM1 either by enzymatic methods[14a,16c,26] or chemically.[2a,27,28] Alkaline hydrolysis of the GM1 ganglioside with KOH in *n*-propanol in the absence of oxygen results in the cleavage of the long fatty acid acyl chain with a high degree of selectivity[27b] (Scheme 1). The cleavage of one or two acetyl groups in an oligosaccharide domain of a ganglioside (in alkaline medium) is inhibited by the negatively charged carboxy group in the sialic acid residue.[27a] The by-products, deAc-GM1 and deAc-deAcyl-GM1 (Scheme 1), were collected and used either for labelling the neuraminic acid residue or for introducing two different fluorescent labels, respectively. The starting natural ganglioside GM1 that we used[29] is a mixture of two compounds differing in their lipid compositions. The long-chain base part consists of C_{18} and C_{20} *erythro*-sphingosines in approximately equal amounts.

Lyso-GM1 may be used for the synthesis of GM1 derivatives with fluorescent labels directly attached to the free amino group in the sphingosine residue. In this case a fluorophore mimics the native long acyl chain. Another option is to decorate the fatty acid chain of the ganglioside molecule with a fluorescent dye. Although the latter approach is more technically sophisticated, it is more flexible with respect to positioning of the fluorescent dye, and it also formally keeps the global structure of the natural GM1 intact. Obviously, the final goal can be achieved by two different approaches: either the selected position of the stearic acid is first labelled with a fluorophore and the whole intermediate is then coupled with the amino group in lyso-GM1 or, alternatively, an activated fatty acid with a protected binding site is coupled with lyso-GM1 followed by deprotection and treatment with an activated fluorescent dye. The high cost of lyso-GM1 makes the first approach advantageous, because this precious compound is used here only in the last crucial step. The second approach was reported in 2005: Schwarzmann et al.[23] coupled 2-azidostearic acid with lyso-GM1, reduced the azido group with H_2S and treated the corresponding amine with the reactive NBD fluoride, thus introducing the NBD fluorophore into the α-position of the stearic acid residue in the native GM1. In this procedure, the valuable lyso-GM1 is used in an earlier step, after which two more steps follow.

We embarked on the synthesis of fluorescent GM1 derivatives by the first approach. As a photostable hydrophilic fluorophore we chose the water-soluble analogue of rhodamine Q (RhS),[24a] available in this laboratory in sufficient amounts (Figure 2, dye **1**, Table 1, **1e**–**h**). The α-position of the stearic acid is more easily derivatized, and very polar dyes at this position may adopt locations at the interface between a polar and a nonpolar domain or, if the linker is long enough, even in an aqueous phase. We also used other dyes for the labelling of ganglioside derivatives (Figure 2). They represent highly photostable fluorophores suitable for STED and FCS experiments and are also applicable in conventional high-resolution light microscopy. These dyes ab-

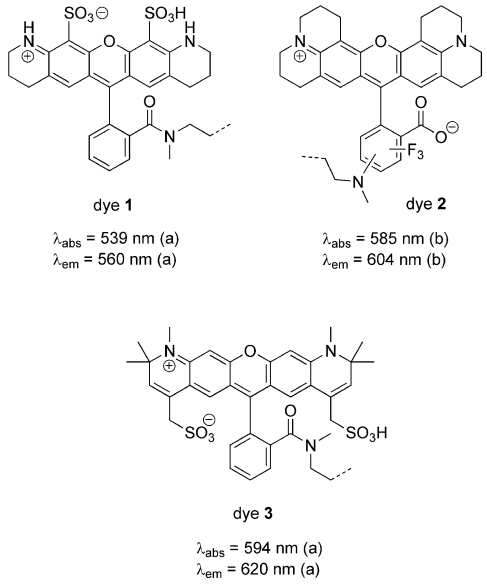
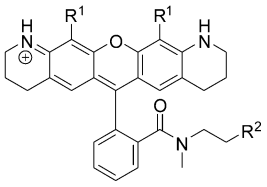


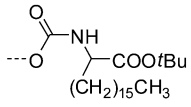
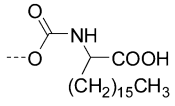
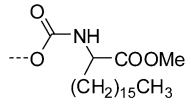
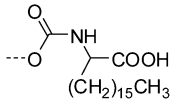
Figure 2. Fluorescent dyes used in this study and their absorption and emission maxima: a) in water, and b) in methanol.

sorb at 539–594 nm and emit at 560–620 nm, where undesirable autofluorescence of biological samples or cell membranes (visible below 550 nm) is negligible. The commercially available lipophilic ATTO 647N dye with λ_{abs} = 644 nm and λ_{em} = 669 nm is widely used in biological applications.

Table 1. Model fluorescent dye **1** and its conjugates with 2-aminostearic acid.



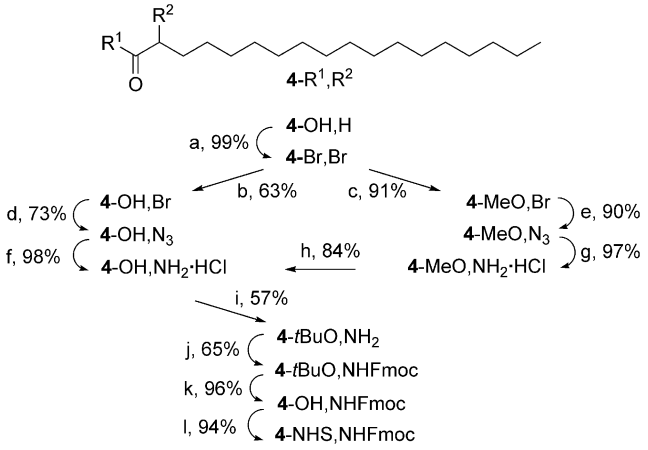
1

	R^1	R^2
1a[a]	H	OH
1b[a]	H	COOMe
1c[b]	H	COOH
1d[c]	H	OCONHS
1e[24a]	SO_3H	OC(O)O*p*NP[d]
1f[24a]	SO_3H	OH
1g[24a]	SO_3H	OCONHS
1h[24a]	SO_3H	CONHS
1i	SO_3H	
1j	H	
1k	SO_3H	
1l	SO_3H	

[a] For the preparation, see the Supporting Information. [b] The acid **1c** could not be obtained from the ester **1b**. [c] The carbonate **1d** could not be obtained from the alcohol **1a**. [d] *p*NP = *p*-nitrophenyl.

3. Synthesis and Coupling Reactions of α- and ω-Aminostearic Acids and Their Derivatives

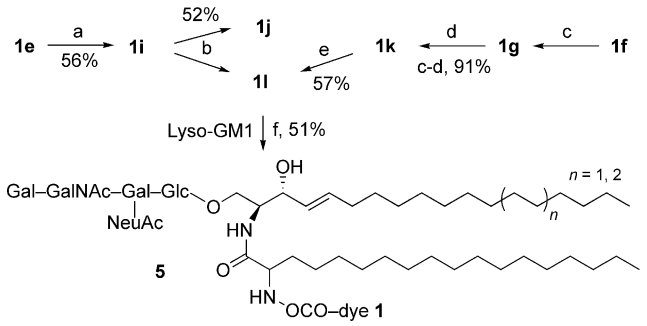
It was planned to introduce fluorescent labels at the α-position of the stearic acid residue of GM1 along two different routes. The first was based on the initial preparation of 2-aminostearic acid labelled with the fluorescent dye **1**, followed by conjugation with lyso-GM1. However, direct acylation of 2-aminostearic acid (**4**-OH,NH_2·HCl; cf. Scheme 2) with *N*-succinimidyl esters of the fluorophore **1h** was found to be impossible, due to the very low solubility of this amino acid, so soluble esters of 2-aminostearic acid were used in coupling reactions with fluorescent dyes reactive towards amino groups.



Scheme 2. α-Aminostearic acid and its esters: a) P, Br_2, 85 °C, 12 h; b) H_2O, 70 °C, 1 h; c) MeOH, 80 °C, 4 h; d) NaN_3, DMF, room temp., 48 h; e) NaN_3, MeOH, reflux, 3 d; f) H_2, Pd/C, EtOH, HCl in EtOH (5 M), room temp., 18 h; g) H_2, Pd/C, MeOH, HCl in *i*PrOH (5–6 M), room temp., 4 h; h) MeOH, aq. NaOH (1 M), room temp., 4 h; i) *t*BuOAc, aq. $HClO_4$ (70%), 0 °C to room temp., 24 h; j) FmocCl, $NaHCO_3$, 1,4-dioxane, room temp., 24 h; k) TFA, Et_3SiH, CH_2Cl_2, 4 °C, 20 h; l) bis(*N*-succinimidyl) carbonate, NEt_3, THF, acetone, room temp., 48 h.

Methyl 2-aminostearate (**4**-MeO,NH_2·HCl) was prepared from methyl 2-bromostearate (**4**-MeO,Br)[30] which was in turn synthesized by trapping the bromination product obtained from stearic acid under Vollhard–Zelinski conditions with methanol. The bromo ester **4**-MeO,Br was then converted into the azido ester **4**-MeO,N_3,[31] and this was reduced to the amino ester **4**-MeO,NH_2·HCl.[32] The analogous *tert*-butyl ester can easily be prepared by treatment of the free amino carboxylic acid with *tert*-butyl acetate in the presence of aq. $HClO_4$ (70%). *tert*-Butyl 2-aminostearate (**4**-*t*BuO,NH_2)[33] was synthesized from **4**-OH,NH_2·HCl, which could be obtained either from 2-bromostearic acid (**4**-OH,Br)[30] via the azide **4**-OH,N_3[34] (to avoid treatment of **4**-OH,Br with ammonia in an autoclave[35]) or by saponification of the methyl ester **4**-MeO,NH_2·HCl.[36] The choice and further use either of methyl or of *tert*-butyl 2-aminostearate depends on the compatibility of a given fluorophore with basic or acidic conditions required for the deprotection of the carboxy group. Sulfonated rhodamines, for example, are sensitive to strong acids, which cleave the sulfonic acid residues off the aromatic rings. This cleavage was indeed observed when deprotection of the adduct **1i** (prepared from the RhS derivative **1e** and the *tert*-butyl ester **4**-*t*BuO,NH_2) was attempted by exposure to trifluoroacetic acid in the presence of Et_3SiH. The disulfonic acid **1l**

could not therefore be prepared from the *tert*-butyl ester **1i**, because **1i** lost both sulfonic acid residues under acidic conditions (Scheme 3).



Scheme 3. Synthesis of the labelled α-aminostearic acid **1l** and the fluorescent ganglioside GM1 derivative **5**: a) **4**-*t*BuO,NH_2, NEt_3, DMF, room temp., 4 d; b) TFA, Et_3SiH, CH_2Cl_2, room temp., 24 h; c) bis(*N*-succinimidyl) carbonate, NEt_3, DMAA, room temp., 1 h; d) **4**-MeO,NH_2·HCl, room temp., 48 h; e) LiI, THF, 100 °C, 48 h; f) HATU, NEt_3, DMSO, room temp., 24 h.

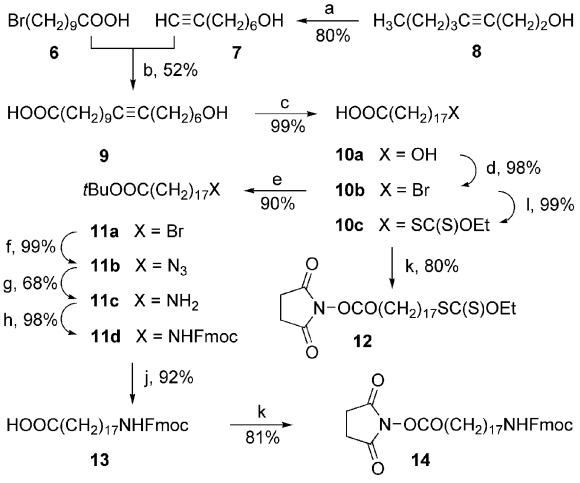
Eventually, methyl 2-aminostearate (**4**-MeO,NH_2·HCl) was used in the reaction with the NHS carbonate of the sulfonated rhodamine **1g** prepared from the alcohol **1f**[37] (Scheme 3). The methyl ester group in compound **1k** was cleaved under mild conditions,[38] leaving the fluorophore intact. In the last step, the labelled acid **1l** was coupled with lyso-GM1 (with use of the activating agent HATU), and so the ganglioside derivative **5** with the hydrophilic fluorescent label at C-2 of its long fatty acid chain was obtained.

All attempts to prepare the nonpolar and amino-reactive rhodamine Q-based dye **1d** (see Table 1) failed. Instead of the colored NHS carbonate **1d**, a colorless solution was obtained from the alcohol **1a** and bis(*N*-succinimidyl) carbonate, probably because the latter compound attacked the more nucleophilic *N*-atoms instead of the hydroxy group. In an attempt to synthesize the NHS ester corresponding to the acid **1c**, the methyl ester **1b** was first prepared from rhodamine Q and methyl 2-(methylamino)propionate (with the coupling reagent HATU), but saponification of **1b** failed to give the required acid **1c**.

Synthesis of 2-aminostearic acid labelled with the ATTO 647N dye gave unsatisfactory results. The high cost precluded the use of more than 1 mg of the NHS ester of ATTO 647N, and the small amounts of labelled materials obtained did not allow this synthetic sequence to be completed. The alternative route, along which the active fluorescent dye is coupled with the lipid in the last step, does not have this drawback.

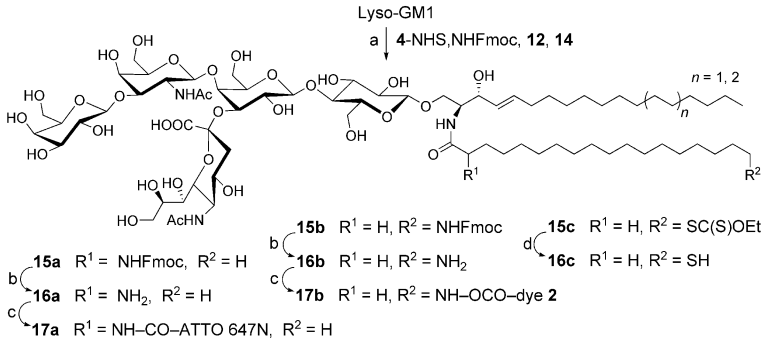
This second general approach to GM1 derivatives with fluorescent markers attached to the α-positions of their stearic acid residues requires a single precursor for all available amino-reactive fluorophores. The same is true for the ω-position in the stearic acid moiety. Therefore, an active ester of the α- or ω-aminostearic acid with suitable protection of the amino group should be prepared, coupled with lyso-GM1 and then deprotected, leaving all other GM1 functionalities intact. The *N*-(9-fluorenylmethyl)oxycarbonyl (Fmoc) group is a good protecting group for that purpose, because it may be removed by treatment with dilute solutions of piperidine or NH_3 in DMF or methanol, and these reagents do not react with any other functional groups in GM1.[2d] The synthesis of the NHS ester of the Fmoc-protected α-aminostearic acid **4**-NHS,NHFmoc is shown in Scheme 2. Compound **4**-*t*BuO,NH_2 was treated with FmocCl in dioxane, the α-carboxy group was then deprotected with TFA in CH_2Cl_2,[33] and the acid **4**-OH,NHFmoc was activated with bis(*N*-succinimidyl) carbonate to yield the desired active ester **4**-NHS,NHFmoc.

Several potential labelling sites in the natural GM1 molecule provide additional possibilities in the search for labelled glycosphingolipids that will behave just like their unlabelled counterparts in a particular assay. To this end, the stearic acid was decorated with ω-amino and ω-mercapto groups. The thiol group can also serve as an anchoring site for fluorescent labels that are difficult or even impossible to use as NHS esters. In this case they may be prepared as maleimide derivatives, which readily react with thiols. The synthesis of active esters of the *N*-protected ω-amino- and *S*-protected ω-mercaptostearic acids is shown in Scheme 4.



Scheme 4. Active esters **12** and **14** of *N*-protected ω-amino- and *S*-protected ω-mercaptostearic acids: a) KH, $NH_2(CH_2)_2NH_2$, 80 °C, 5 h; b) $LiNH_2$/liq. NH_3, –33 °C, 18 h; c) H_2, PtO_2, Et_2O, room temp., 3 h; d) aq. HBr (48%), AcOH, reflux, 18 h; e) Ac_2O, *t*BuOH, THF, room temp., 18 h; f) NaN_3, DMF, 85 °C, 18 h; g) H_2, Pd/C, HCl (5–6 M) in *i*PrOH, EtOAc, room temp., 18 h; h) FmocCl, $NaHCO_3$, 1,4-dioxane, room temp., 24 h; j) TFA, Et_3SiH, CH_2Cl_2, 4 °C, 20 h; k) bis(*N*-succinimidyl) carbonate, NEt_3, THF, acetone, room temp., 48 h; l) KSC(S)OEt, acetone, room temp., 18 h.

An ω-aminostearic acid[39] with an *N*-protective group that could easily be removed without destroying other functionalities of GM1 was required. As in the case of ω-aminododecanoic acid,[2d] Fmoc protection, which is compatible with the *tert*-butyl ester, was chosen.[40] The target compound – *tert*-butyl ω-aminostearate (**11c**) – can be obtained from the corresponding azido ester **11b**, which is in turn easily prepared from the bromo or the hydroxy ester. As a precursor, ω-hydroxy-[41] or ω-bromostearic acid was re-



Scheme 5. Analogues of GM1 ganglioside with α-amino-, ω-amino- and ω-mercaptostearic acid residues (**16a**–**c**) and their conjugation with fluorescent dyes: a) NEt_3, DMF, Triton X-100, room temp., 24–48 h; b) piperidine in MeOH (20%), room temp., 2 h; c) ATTO 647N NHS ester or **2**-OCONHS, NEt_3, DMSO, room temp., 24–48 h; d) aq. NH_3, room temp., 24 h; DTT, MeOH, room temp., 4 h.

quired.[42] The synthesis of the ω-hydroxystearic acid **10a** (Scheme 4) started with the "acetylene zipper" reaction[43] of oct-3-yn-1-ol (**8**) in which the triple bond was moved to the end of the carbon–carbon chain. The resulting oct-7-yn-1-ol (**7**) was coupled with 10-bromodecanoic acid (**6**) in liquid ammonia in the presence of lithium amide to give 18-hydroxyoctadec-11-ynoic acid (**9**) in 50% yield.[41c] It turned out to be necessary to use freshly distilled ammonia; otherwise the impurities dramatically inhibited the coupling reaction. The triple bond in **9** was hydrogenated over PtO_2 to yield ω-hydroxyoctadecanoic acid (**10a**).[41c] Direct conversion of the ω-hydroxy group to afford the corresponding ω-bromo acid **10b**[44] furnished the common precursor for both the ω-amino- and the ω-mercaptostearic acids. Toward those target compounds, **10b** was converted into the *tert*-butyl ester **11a**,[45] in which the *tert*-butyl group not only protects the carboxy function, but also improves the solubility. The bromo ester **11a** was transformed into the azido ester **11b**, which was hydrogenated over Pd/C to afford the amino ester **11c** as the hydrochloride. To avoid undesired transesterification, alcohols should not be used as solvents for this step. The amino group in **11c** readily reacted with FmocCl to yield the urethane **11d**, and the *tert*-butyl ester was then cleaved with TFA in the presence of Et_3SiH as a scavenger of *tert*-butyl cations. The Fmoc-protected amino acid **13** was treated with bis(*N*-succinimidyl) carbonate, and the active ester **14** was isolated and kept at –20 °C. No synthesis of the ω-mercaptostearic acid has been reported so far. To introduce a protected thiol group, ω-bromostearic acid (**10b**) was treated with potassium xanthogenate in anhydrous acetone at room temperature,[46] and the resulting acid **10c** was converted into the NHS ester **12** (Scheme 4).

With three protected NHS esters at our disposal, the synthesis of the corresponding GM1 derivatives with additional "anchoring" sites was initiated. To that end, the lysoganglioside was treated with an excess of the corresponding active ester (**4**-NHS,NHFmoc, **12**, **14**) in anhydrous DMF/THF solution in the presence of NEt_3 as a base and the non-ionic detergent Triton X-100 at room temperature (Scheme 5).[2d] The reactions were each complete within one to two days as evidenced by TLC. The products, the *N*-Fmoc-protected 2- and 18-amino derivatives **15a** and **15b** with R_f values intermediate between those of lyso-GM1 and the corresponding active esters of stearic acids, were very readily visible on the TLC plates because of their strong UV absorptions at 254 nm. They were isolated by column chromatography on silica gel with a mixture of $CHCl_3$, MeOH and H_2O (60:35:5) as eluent. Treatment with piperidine in methanol (20%) at room temperature was sufficient for complete deprotection of the amino group. The compound GM1-2-NH_2 (**16a**) with a ^{14}C-labelled acyl group in the sialic acid residue was first synthesized by Schwarzmann et al. from lyso-GM1 and the NHS ester of 2-azidostearic acid.[23] In this current investigation, the new amino group was introduced into the GM1 molecule not by reduction of the azido ester, but by removal of an Fmoc protective group. This facilitated the detection and chromatographic separation of the intermediate **15a**, and it did not require the use of poisonous and noxious hydrogen sulfide. The *S*-protecting group in the mercapto-modified ganglioside **15c** was cleaved off by stirring the compound with aqueous ammonia at room temperature for one day (Scheme 5), and the obtained disulfide dimer of the thiol **16c** was reduced by stirring with dithiotreitol in methanol followed by dialysis against water and lyophilization to furnish pure **16c**.

4. ^{1}H NMR Spectra of the GM1 Derivatives

The diagnostically important signals in the ^{1}H NMR spectra of the ganglioside derivatives **16a**–**c** and the data for the natural compound GM1 are presented in Table 2. The ^{1}H NMR spectra were recorded both in [D_4]methanol and in [D_6]DMSO solutions. Before the measurements, the samples were dissolved in D_2O and lyophilized twice to substitute the exchangeable protons with deuterium. 2D ^{1}H–^{1}H COSY spectra were used to assign the signals. The ^{1}H NMR spectra of ganglioside derivatives differ only as a result of the modifications in their ceramide residues, whereas the chemical shifts of the protons in the oligosaccharide moieties of GM1-2-NH_2 (**16a**), GM1-18-NH_2 (**16b**) and (GM1-18-S)$_2$ (**16c**) dimer were found to be very similar to those of the natural GM1. Thus, in the ^{1}H NMR spectrum

Table 2. Characteristic ^{1}H NMR chemical shifts of the natural GM1 and ganglioside derivatives GM1-2-NH_2 (**16a**), GM1-18-NH_2 (**16b**), and (GM1-18-S)$_2$ (**16c** dimer).

	GM1-2-NH_2, ppm[a] (ppm)[b]	GM1-18-NH_2, ppm[a] (ppm)[b]	(GM1-18-S)$_2$, (ppm)[b]	GM1, ppm[a] (ppm)[c]
Fatty acid chain				
2	3.53 (3.05)	2.17 (2.02)	(2.01)	2.17 (2.02)
3	1.51/1.64 (1.50)	1.59 (1.44)	(1.43)	1.58 (1.24)
$(CH_2)_n$	1.29 (1.24)	1.29 (1.24)	(1.24)	1.29 (1.24)
17	1.29 (1.24)	1.63 (1.40)	(1.60)	1.29 (1.24)
18	0.90 (0.85)	2.87 (2.60)	(2.67)	0.90 (0.85)
Sphingosine residue				
1a/1b	3.41/4.16 (–)[d]	3.54/4.21 (3.40/3.96)	(3.42/3.98)	3.38/4.15 (3.43/3.99)
2	4.00 (3.77)	3.96 (3.76)	(3.77)	3.96 (3.77)
3	4.10 (3.96)	4.07 (3.89)	(3.87)	4.07 (3.87)
4	5.46 (5.38)	5.44 (5.35)	(5.35)	5.42 (5.35)
5	5.72 (5.58)	5.68 (5.55)	(5.53)	5.66 (5.53)
6	2.03 (1.95)	2.02 (1.90)	(1.93)	2.02 (1.93)
7	1.39 (1.24)	1.37 (1.24)	(1.24)	1.38 (1.24)
$(CH_2)_n$	1.29 (1.24)	1.29 (1.24)	(1.24)	1.30 (1.24)
Methyl groups				
GalNAc	1.99 (1.75)	1.99 (1.75)	(1.74)	1.99 (1.75)
Neu5Ac	2.01 (1.86)	2.01 (1.88)	(1.86)	2.01 (1.88)
CH_3	0.90 (0.85)	0.90 (0.85)	(0.85)	0.90 (0.85)
Sialic acid residue (CH_2)				
3e	2.73 (2.56)	2.73 (2.53)	(2.52)	2.73 (2.56)
3a	1.91 (1.62)	1.90 (1.62)	(1.62)	1.91 (1.64)
Anomeric protons				
Glc(I)	4.30 (–)[d]	4.30 (4.14)	(4.15)	4.30 (4.15)
Gal(II)	4.41 (–)[d]	4.43 (4.26)	(4.26)	4.41 (4.28)
Gal(IV)	4.45 (–)[d]	4.46 (4.20)	(4.20)	4.44 (4.21)
GalNAc	4.92 (–)[d]	4.90 (4.90)	(4.89)	4.92 (4.86)

[a] Obtained at 600 MHz and 24 °C in CD_3OD. [b] Obtained at 600 MHz and 35 °C in [D_6]DMSO. [c] Reported values[47] at 500 MHz and 30 °C in [D_6]DMSO/D_2O (98:2, v/v). [d] Not resolved, due to the presence of two diastereomers.

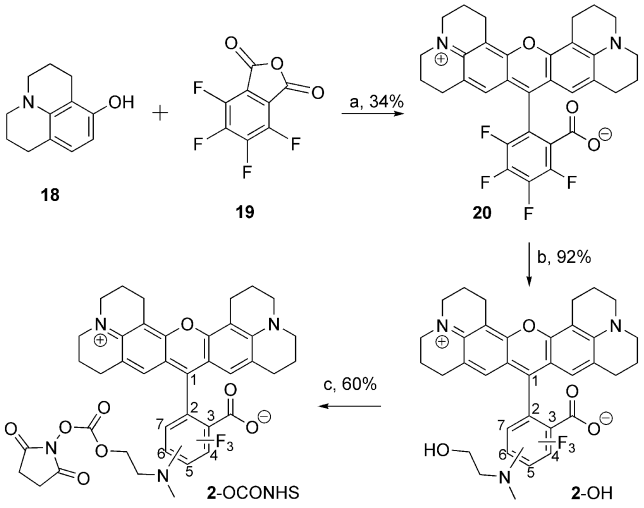
of compound **16a** with an amino group at the α-position of the stearic acid side chain, the characteristic signal of the CH_2CO group at δ = 2.17 ppm (in CD_3OD) has disappeared, whereas a new multiplet at δ = 3.53 ppm has appeared. The signal of 3-CH_2 is slightly shifted to lower field (δ = 1.51/1.64 ppm), and two broad multiplets are found as a result of the presence of an additional stereogenic center.[48] In the ^{1}H NMR spectrum of compound GM1-2-NH_2 (**16a**) in [D_4]methanol, the four signals of the anomeric protons in the carbohydrate residues appear as well-resolved doublets (at 4.30, 4.41, 4.45 and 4.92 ppm), whereas in [D_6]DMSO this part of the spectrum is not well resolved (Table 2). This effect may be due to the higher degree of solvation in [D_4]methanol and a more rigid structure of the whole oligosaccharide domain, stabilized by intramolecular hydrogen bonds, in compound GM1-2-NH_2 (**16a**) in [D_6]DMSO solution.[49] In the case of the ω-substituted fatty acid residue in compound GM1-18-NH_2 (**16b**), the signal of 17-CH_2 is only slightly shifted from 1.29 ppm [corresponding to the $(CH_2)_n$ group] to 1.63 ppm, whereas the triplet of 18-CH_2 is found at δ = 2.87 ppm (in CD_3OD, Table 2). The signals of the same groups were shifted to lower field with the change of solvent from [D_6]DMSO to [D_4]methanol, and the differences were in the range of 0.04–0.25 ppm. The use of deuterated methanol for the ^{1}H NMR measurements of ganglioside derivatives is advantageous because the residual solvent signals do not overlap with the characteristic multiplets of the GM1 derivatives.

5. Synthesis of GM1 Derivatives Labelled with Fluorescent Dyes

The applicabilities of compounds **16a** and **16b** for labelling reactions with amino-reactive fluorophores were tested under standard conditions. An activated dye (1–2 equiv.) was added to a solution of a modified ganglioside in anhydrous DMSO, along with a base, and the reaction mixture was stirred at room temperature in the dark under inert gas for 1–2 d. The colored product (which was also developed with anisaldehyde reagent) was isolated on HPTLC plates. The constitutions of the adducts were confirmed by their mass spectra (with electrospray ionization), and their purities and homogenities were tested by HPTLC.

As an example of a nonpolar lipophilic dye, an analogue of rhodamine 101 bearing a tetrafluoro-substituted benzoic acid residue (Figure 2, dye **2**), synthesized from julolidine and tetrafluorophthalic anhydride,[50] was chosen (Scheme 6). Its emission maximum was found to be shifted into the red spectral region relative to that of the non-fluorinated rhodamine 101 (611 nm vs. 589 nm in MeOH). The fluorinated rhodamine **20**, upon treatment with an excess of 2-(methylamino)ethanol in DMF at room temperature, gave the compound **2**-OH. Only one fluorine atom was found to have been substituted by a secondary amino group.[51] This substitution results in small blue shifts both of the absorption and of the emission bands of compound **2**-OH (585 and 604 nm, respectively). The hydroxy group in

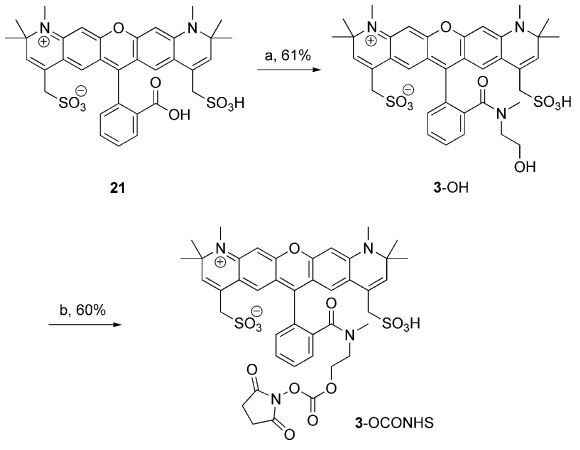
alcohol **2**-OH was transformed into the NHS carbonate by treatment with bis(*N*-succinimidyl) carbonate and a base in DMF, and the activated dye **2**-OCONHS was isolated by preparative HPLC. Its coupling with GM1-18-NH_2 (**16b**) occurred more rapidly than that of the α-amino derivative of GM1, most probably because the terminal amino group is less sterically encumbered than the α-amino group.



Scheme 6. Tetrafluororhodamine **20** and the amino-reactive NHS carbonate **2**-OCONHS: a) C_2H_5COOH, TsOH, 160 °C, 16 h; b) $CH_3NH(CH_2)_2OH$, DMF, room temp., 12 h; c) bis(*N*-succinimidyl) carbonate, NEt_3, DMF, room temp., 3 h.

Each of the compounds deAc-GM1 or lyso-GM1 (Scheme 1) has one free amino group, either in the neuraminic acid residue or in the sphingosine moiety, respectively. Amino groups are known to react readily with *N*-hydroxysuccinimidyl esters or NHS carbonates. Several amino-reactive compounds based on the highly photostable fluorescent dyes have been prepared.[24a,50] The relatively polar water-soluble sulfonated rhodamines (dye **1**, dye **3**, Figure 1) may be attached to the polar "head" groups of ganglioside derivatives, whereas a nonpolar (e.g., dye **2**, Figure 1) is suitable for labelling of lyso-GM1. Short linkers based on 2-(methylamino)ethanol and 3-(methylamino)propionic acid were used between the ganglioside and fluorescent fragments.[24a]

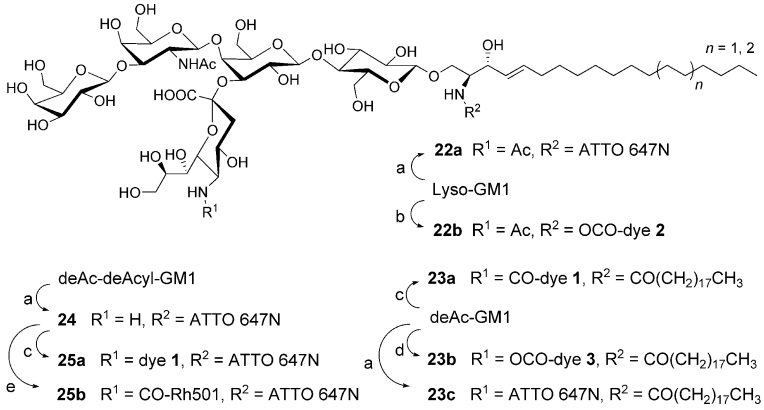
For the synthesis of the amino-reactive water-soluble dye **3**-OCONHS (Scheme 7), the carboxy group of the sulfonated rhodamine **21** was activated with HATU in the presence of triethylamine, and condensed with 2-(methylamino) ethanol. The resulting hydroxyethylamide **3**-OH showed an absorption maximum at 592 nm and emitted at 614 nm. Compound **3**-OH, on treatment with bis(*N*-succinimidyl) carbonate, gave the activated carbonate **3**-OCONHS, which was isolated by preparative HPLC.



Scheme 7. Synthesis of activated fluorescent dye **3**-OCONHS: a) $CH_3NH(CH_2)_2OH$, HATU, NEt_3, DMF, 50 °C, 24 h; b) bis(*N*-succinimidyl) carbonate, NEt_3, DMF, room temp., 1 h.

The labelling reactions (Scheme 8) were carried out as described above. The yields of the labelled compounds, after purification by HPTLC, were found to be good (ca. 80%), although the applied excesses of the activated fluorescent probes were not very high (up to 2 equiv.).

It is possible to introduce two different dyes selectively into the compound deAc-deAcyl-GM1 (Scheme 1), with its two amino groups. However, suitable ratios of reactants



Scheme 8. GM1 derivatives with fluorescent labels: a) ATTO 647N NHS ester, DMSO, NEt_3, room temp., 24 h; b) **2**-OCONHS, DMSO, NEt_3, room temp., 24 h; c) **1h**, DMSO, NEt_3, room temp., 24 h; d) **3**-OCONHS, DMSO, NEt_3, room temp., 24 h; e) Rh501-COOH,[52] HATU, NEt_3, room temp., 24 h.

should be used. In the first labelling step, an excess of deAc-deAcyl-GM1 should be applied, and in the second labelling step an excess of the active fluorophore is required. The more active amino group is located in the sphingosine residue, so the compound deAc-deAcyl-GM1 was first labelled with a lipophilic fluorescent dye (ATTO 647N) to give the derivative **24** (Scheme 8). Treatment with the second hydrophilic activated fluorophore (NHS ester **1h** in Table 1 or NHS ester of a new carboxy rhodamine Rh501[52]) gave the required products **25a** and **25b** with violet or greenish colors, which looked like superpositions of the two colors used.

6. New Intermediates for Selective Double Labelling of the Natural GM1 Skeleton

If it is necessary to label the amino group in the neuraminic acid first (e.g., with a hydrophilic dye) and then to introduce the second marker into the sphingosine part, a more complicated approach should be used.[27a] This is based on the temporary protection of the more reactive amino group in the sphingosine residue of the compound deAc-deAcyl-GM1, labelling of the free amino group in the neuraminic acid and, finally, deprotection of the amino group in the sphingosine fragment.

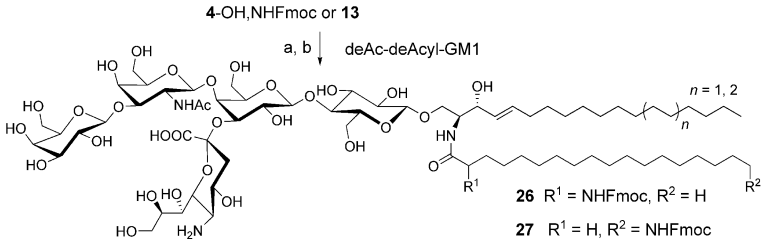
As a more convenient alternative (Scheme 9), one can first selectively reacylate deAc-deAcyl-GM1 with activated *N*-protected α- or ω-aminostearic acids (**4**-OH,NHFmoc or **13**) in order to allow labelling of the amino group at the "head" of the GM1 derivative with a polar fluorescent dye, followed by unmasking of the stearic acid amino group and its subsequent labelling with a second dye.

The compositions of compounds **26** and **27** were confirmed by mass spectrometry, and their structure assignments are based on their ^{1}H NMR spectra. The signals of the aromatic protons of the fluorenyl residue were observed for both compounds. The characteristic signal of the 5-H at the carbon atom connected with the free amino group in the neuraminic acid fragment of compound **26** was found to occur at δ = 2.80 ppm in [D$_4$]methanol.

Compound **27** was not completely soluble in MeOH, and its ^{1}H NMR spectrum was recorded in [D$_6$]DMSO solution, in which all signals were shifted to higher field (as shown for similar compounds in Table 2). The signal of 5-H in the neuraminic acid fragment of compound **27** was therefore observed at δ = 2.55 ppm, where it overlapped with the signal of the solvent. Acylation of the free amino group in the sphingosine residue with functionalized stearic acid residues resulted in shifts of the signal at δ = 2.95 ppm for 2-H in the sphingosyl chain of deAc-deAcyl-GM1 to lower fields for compounds **26** and **27**. The presence of only one signal for an acetyl group (GalNAc) in the ^{1}H NMR spectrum of compound **26**, and also in that of compound **27**, was also in accordance with previous assignments.

7. STED and Multicolor FCS Measurements with Fluorescent GM1 Lipid Analogues

STED and multicolor FCS experiments were performed with GM1 derivatives labelled with different fluorescent dyes. The left-hand panel in Figure 3 shows FCS data for ATTO 647N-labelled GM1 analogues incorporated in the plasma membranes of living PtK2 cells for conventional confocal microscopy and STED nanoscopy recordings. Diffusion of the fluorescent lipids through focal spots with diameters of ca. 240 nm (confocal) and ca. 40 nm (STED), which were placed on the plasma membrane, resulted in dropping-off FCS correlation data with decay times characteristic of the lipid transit times. Because of the smaller focal spot diameters, the correlation data for the STED recordings are shifted to shorter times. However, the shift is less than would be expected from free-diffusing species, suggesting heterogeneous diffusion of these GM1 lipids in the plasma membrane of living cells: GM1 is trapped on the nanoscale, undergoing transient (ca. 10 ms) complexation with other membrane constituents such as proteins. During trapping, the lipid dwells in areas with diameter < 20–30 nm (for details see ref.[4]). Most strikingly, the FCS data recorded for the GM1 analogues labelled with the ATTO 647N dye show no significant differences for the various labelling positions ("acyl-chain replacement" in compound **22a**, "acyl-chain addition" in **17a** or head-group labelling in **23c**). The negligible influence of the dye on the trapping characteristics demonstrates that the observed complexes are not caused by the dye label. Consequently, our observations most probably reflect the behavior of the endogeneous GM1, and the GM1 interactions seem to be assisted by molecular groups of the lipid such as hydrogen bonds of the sphingosine residue or of the hydrophilic oligosaccharide head group.[4]



Scheme 9. Analogues of GM1 ganglioside **26** and **27**, each with a free amino group in the sialic acid residue and a protected amino group in either the α- or the ω-position in the stearic acid chain: a) $SOCl_2$, 50 °C, 4 h; b) aq. $NaHCO_3$ (0.1%), Et_2O, 4 °C, 24 h.

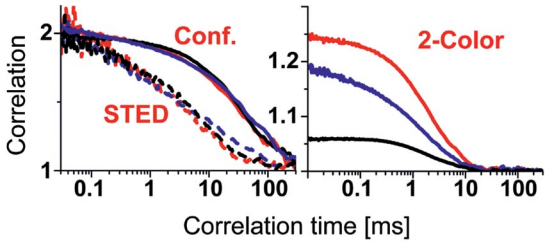


Figure 3. STED-FCS measurements with fluorescent GM1 lipid analogues in living cells (left-hand panel, all data normalized to 2) and multicolor FCS measurements of doubly labelled GM1 in model membranes (right-hand panel). Left-hand panel: ATTO 647N-labelled GM1 (black: compound **22a** (Scheme 8) with "acyl-chain replacement", blue: compound **17a** (Scheme 5) with "acyl-chain addition", red: compound **23c** (Scheme 8) with headgroup labelling) with confocal (solid lines "Conf.") and STED recording (dotted lines "STED"), focal spots of ca. 240 nm and ca. 40 nm in diameter. Right-hand panel: FCS data for the doubly labelled compound **25b** (Scheme 8) with lipophilic ATTO 647N dye replacing the acyl chain and a new hydrophilic "blue" dye at the carbohydrate "head" of GM1 with confocal recording following 488 nm laser excitation only (blue: autocorrelation of "blue" dye fluorescence, red: autocorrelation of ATTO 647N fluorescence, black: cross-correlation of the simultaneous excited blue dye and ATTO 647N fluorescence).

The right-hand panel in Figure 3 shows FCS data for compound **25b** (Scheme 8) with a confocal recording for this GM1 derivative doubly labelled with a hydrophilic "blue" dye (at the polar carbohydrate "head") and the apolar ATTO 647N dye replacing the long fatty acid chain: autocorrelation of the red ATTO 647N (red line) and of the blue fluorescence (blue line) and cross-correlation of the red and blue fluorescence (black line) following 488 nm excitation only. The significant cross-correlation demonstrates that the lipids are indeed doubly labelled. The occurrence of red fluorescence reveals Förster resonance energy transfer (FRET) from the blue to the red dye (ATTO 647N fluorescence is hardly directly excited only by 488 nm laser light). FRET is not 100%, however, because the blue fluorescence is not completely quenched. Consequently, this lipid analogue might serve as a suitable control sample in multicolor FCS experiments on membranes: as a standard for studying the interaction of two differently colored molecules, for example.[53] Comparison of the auto- and cross-correlation amplitudes reveals that the red and blue fluorescence coincide only for about 30% of all lipid molecules.[53] If the homogeneity of the doubly labelled substance **25b** (verified by thin-layer chromatography) and mass spectroscopy data are taken into consideration, we can exclude incomplete double labelling (at least to an extent of 70%). Either parts of the dyes are photobleached or inactive or take on a conformation leading to complete or inactive FRET,[54] or overlap of detection of blue and red fluorescence is incomplete.

All correlation data point to the fluorescent GM1 lipid analogues as appropriate probes for studying lipid dynamics in living cells and shed new light on longstanding biological questions such as details of lipid rafts.[4]

Conclusions

Building blocks **16a**, **16b**, **16c**, **26** and **27** may be used for the synthesis of GM1 derivatives bearing one or two fluorescent dyes or other labels of various polarities. Introduction of new labelling sites into the natural GM1 framework enables one to attach markers to different parts of the GM1 molecule. The use of suitable protected 2-amino-, 18-amino- and 18-mercaptostearic acid residues offers the potential for insertion of the (fluorescent) labels either into the interface between the polar "head" group and the nonpolar domain, or into the lipophilic part of the natural GM1 ganglioside. Another marker may replace the acetyl group of the sialic acid residue in the polar carbohydrate "head" group. Flexible labelling strategies help to minimize the perturbations of the natural GM1 skeleton caused by the introduction of large markers. The presented approach may be used for the labelling of other glycosphingolipids as well. The outlined ganglioside synthesis allows for very flexible labelling and represents a promising approach in clarification of important details relating to ganglioside dynamics and activity in living cells.

Experimental Section

General: Ganglioside GM1 was purchased from ALEXIS Biochemicals (AXXORA, Lörrach, Germany), and ATTO 647N NHS ester from ATTO-Tec GmbH (Siegen, Germany, www.atto-tec.com). Triton X-100 was obtained from Acros Organics, and for dialysis the Spectra/Por Dialysis Membrane (6–8 KDa MWCO) from Spectrum (www.spectrapor.com) was used. Melting points (uncorrected) were determined in capillaries with a SMP 10 apparatus (Bibby Sterling Ltd, UK). Routine NMR spectra were recorded with a Varian Mercury 300 spectrometer at 300.5 (^{1}H), 75.5 (^{13}C and APT), and 282.4 MHz (^{19}F). ^{1}H and ^{13}C NMR spectra were also recorded with Varian Inova 600 (600 MHz) and Varian Inova 500 (125.7 MHz) instruments, respectively. Chemical shifts (δ) are given in ppm. All spectra are referenced to tetramethylsilane as an internal standard (δ = 0 ppm) based on the signals of residual protons of the deuterated solvents: 7.26 ppm for $CHCl_3$, 3.31 ppm for CHD_2OD and 2.50 ppm for [D_5]DMSO. Multiplicities of signals are described as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad. EI-MS were recorded with Finnigan MAT 95 (70 eV) and ESI-MS with Finnigan LCQ spectrometers. HPLC system (Knauer): Smartline pump 1000 (2×), UV detector 2500, column thermostat 4000, mixing chamber, injection valve with 20 and 100 µL loops for analytical and preparative columns, respectively; six-port-three-channel switching valve; analytical column: Eurosphere-100 C18, 5 µm, 250×4 mm; preparative column: Eurosphere-100 C18, 5 µm, 250×8 mm; solvent A: MeCN + TFA (0.1% v/v), solvent B: H_2O + TFA (0.1% v/v); temperature 25 °C. Analytical TLC was performed with MERCK ready-to-use plates (silica gel 60 F_{254}), which were developed with molybdatophosphoric acid solution (5% in EtOH), ninhydrin and anisaldehyde reagents. High-performance silica gel thin-layer plates (HPTLC, 0.2 mm Kieselgel 60, 10×10 cm) were purchased from VWR International (Darmstadt, Germany). Flash chromatography: MERCK silica gel, grade 60, 0.04–0.063 mm, Polygoprep 60–50 C18 (Macherey–Nagel, Düren, Germany). Elemental analyses were carried out at the Mikroanalytisches Laboratorium des Instituts für Organische und Biomolekulare Chemie der Georg-

August-Universität Göttingen. THF and diethyl ether were dried with sodium benzophenone ketyl. Organic solutions were dried with $MgSO_4$ or Na_2SO_4. All reactions were carried out with magnetic stirring under positive argon or nitrogen pressure by standard techniques with vacuum/inert gas manifold, unless stated otherwise. Thin-layer chromatography of GM1 derivatives was performed with HPTLC plates at room temperature and the following solvent systems (all solvent ratios are given by volume). 1) Analytical: A) $CHCl_3$/MeOH/aqueous $CaCl_2$ (30 mM)/aqueous KCl (100 mM) 50:50:4:8, B) $CHCl_3$/MeOH/aqueous $CaCl_2$ (15 mM) 60:35:8. 2) Preparative: C) $CHCl_3$/MeOH/H_2O 60:35:8, D) $CHCl_3$/MeOH/H_2O 60:35:5, E) $CHCl_3$/MeOH/H_2O 70:25:5, F) MeOH/1-butanol/H_2O 2:2:1.

STED and Multicolour FCS Experiments: Inclusion of the fluorescent lipid analogues in the plasma membranes of living PtK2 cells and STED FCS measurements were performed as reported.[4] Multicolour experiments were performed on the same setup with the STED modus switched off. Here, pulsed 488 nm laser light (LDH 485, Picoquant, Berlin, Germany, repetition rate 80 MHz, pulse width ca. 80 ps) was applied for excitation of the fluorescence of the "blue" Rh501 dye and ATTO 647N dye. FRET between Rh501 and ATTO 647N dyes allowed for cross-correlation of the fluorescence signals of both dyes, and a finite cross-correlation amplitude demonstrated coinciding occurrence of both labels.[53] The amplitudes $G_{red}(0)$, $G_{blue}(0)$ and $G_{cross}(0)$ of the red and blue autocorrelation and cross-correlation data, respectively, allow for the calculation of the relative fraction of lipid molecules with coinciding blue and red fluorescence: $[G_{cross}(0) - 1]/\{0.5\ [(G_{red}(0) - 1) + (G_{blue}(0) - 1)]\}$.[53] The doubly labelled GM1 lipid was incorporated into a supported lipid bilayer. These planar bilayers were prepared by the procedure described by Chiantia et al.[55] Briefly, the lipid DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine, Avanti Polar Lipids Inc., Alabaster, AL, USA) and the fluorescent lipid analogues were mixed in organic solvents ($CHCl_3$ and MeOH) at a lipid concentration of 10 mg mL^{-1}. The concentrations of the fluorescent lipid analogues were ca. 0.01 mol-%. After solvent evaporation, the lipid film was slowly rehydrated with a buffer solution [NaCl (150 mM), HEPES (10 mM)] and resuspended by vigorous vortexing. After sonication of the suspension at room temperature, a small aliquot was diluted in a buffer solution [NaCl (150 mM), HEPES (10 mM), $CaCl_2$ (3 mM)] and deposited on a glass coverslip.

Lyso-GM1: This compound was prepared as described previously.[27] $C_{55}H_{97}N_3O_{30}$ (sphingosine C_{18}, M_1 1279), $C_{57}H_{101}N_3O_{30}$ (sphingosine C_{20}, M_2 1307). ESI-MS (negative mode): m/z = 1278.7 $[M_1 - H]^-$, 1306.8 $[M_2 - H]^-$.

***N*-Hydroxysuccinimidyl (2*R*/*S*)-[(9-Fluorenylmethoxycarbonyl)amino]octadecanoate (4-NHS,NHFmoc):** A solution of **4**-OH,NHFmoc (45 mg, 0.086 mmol) and bis(*N*-succinimidyl) carbonate (33 mg, 0.129 mmol) in anhydrous acetone (4 mL) was added to a solution of NEt_3 (25 μL) in anhydrous THF (6 mL), and the mixture was stirred at room temperature for 48 h. Concentration under reduced pressure and filtration through a pad of SiO_2 (30 mL) with elution with EtOAc gave the title compound (50 mg, 94%) as an oil. ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 0.88 (t, 3 H, CH_3), 1.25 (m, 28 H, CH_2), 1.75–2.13 (m, 2 H, 3-H), 2.85 (s, 4 H, CH_2 in NHS), 4.24 (t, J = 7.4 Hz, 1 H, 9′-H), 4.45 (m, 2 H, CH_2O), 4.75 (m, 1 H, 2-H), 5.21 (m, 1 H, NH), 7.32 (m, 2 H, 2′/7′-H), 7.40 (m, 2 H, 3′/6′-H), 7.60 (d, J = 7.4 Hz, 2 H, 1′/8′-H), 7.77 (d, J = 7.4 Hz, 2 H, 4′/5′-H) ppm. ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ = 14.1 (CH_3), 22.7 (CH_2, C-17), 24.9 (CH_2, C-4), 25.5 (2 × CH_2 in NHS), 29.0–29.7 (11 × CH_2, C-5 to C-15), 31.9 (CH_2, C-3), 32.7 (CH_2, C-16), 47.0 (CH, C-9′), 52.3 (CH, C-2), 67.2 (CH_2), 119.9 (2 × CH, C-1′/8′), 125.0 (2 × CH, C-2′/7′), 127.1 (2 × CH, C-4′/5′), 127.7 (2 × CH, C-3′/6′), 141.3 (2 × C), 143.6 (C), 143.8 (C), 155.5 (CO), 168.4 (CO), 168.5 (2 × CO in NHS) ppm. ESI-MS (positive mode): m/z = 641.3 $[M + Na]^+$, 1259.1 $[2M + Na]^+$. HR-MS (ESI, positive mode): found: 641.3569; calcd. for $C_{37}H_{50}N_2O_6$: 641.3561 $[M + Na]^+$.

Adduct 5: HATU (3.3 mg, 8.60 μmol) was added to a solution of compound **1l** (5.5 mg, 5.75 μmol) and NEt_3 (10 μL) in DMSO (0.1 mL), followed after 20 min by lyso-GM1 (3 mg, 2.30 μmol). The mixture was stirred at room temperature for 24 h and concentrated under reduced pressure. The title product was isolated by preparative TLC (silica gel) with elution with $CHCl_3$/MeOH/H_2O (60:35:5) to yield a red powder (2.6 mg, 51%). ESI-MS (positive mode): m/z = 2214.03 $[M_1 + H]^+$, 2242.07 $[M_2 + H]^+$. HR-MS (ESI, negative mode): found 1105.9927, 1120.0092; calcd. for $C_{103}H_{159}N_7O_{41}S_2$ and $C_{105}H_{163}N_7O_{41}S_2$: 1105.9934 $[M_1 - 2H]^{2-}$, 1120.0090 $[M_2 - 2H]^{2-}$.

18-Hydroxyoctadec-11-ynoic Acid (9): A solution of oct-7-yn-1-ol (2.08 g, 16.5 mmol) in anhydrous THF (13 mL) was added over 15 min to a solution of $LiNH_2$ [prepared from Li (0.3 g) and $Fe(NO_3)_3 \cdot 9H_2O$ (27 mg) in liquid ammonia (200 mL) under Ar], and the mixture was stirred for 1 h. A solution of 10-bromodecanoic acid (1.60 g, 6.4 mmol) in anhydrous THF (26 mL) was added, and the reaction mixture was stirred for 18 h. After evaporation of NH_3, the reaction mixture was quenched with ice, acidified to pH 3 (concd. aq. HCl), extracted with Et_2O and dried with Na_2SO_4. The title product was isolated by chromatography on SiO_2 (300 mL) with elution with hexane/EtOAc (2:1 + 1% AcOH) to yield a colorless solid (1.0 g, 52%), m.p. 69 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.18–1.50 (m, 18 H, CH_2), 1.50–1.66 (m, 4 H, CH_2), 2.05–2.17 (m, 4 H, $CH_2C{\equiv}$), 2.30 (t, J = 7.5 Hz, 2 H, CH_2CO), 3.62 (t, J = 6.6 Hz, 2 H, CH_2O), 6.49 (br. s, 1 H, OH) ppm. ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 18.7 (2 × CH_2, C-10/13), 24.7 (CH_2, C-16), 25.2 (CH_2, C-3), 28.6 (CH_2), 29.0 (5 × CH_2), 29.1 (CH_2, C-5), 29.2 (CH_2, C-6), 32.5 (CH_2, C-17), 34.0 (CH_2, C-2), 62.9 (CH_2O), 80.1 (C), 80.3 (C), 179.3 (CO) ppm. CI-MS (NH_3): m/z (%) = 314.5 $[M + NH_4]^+$ (100). $C_{18}H_{32}O_3$ (296.44): calcd. C 72.93, H 10.88; found C 72.67, H 10.87.

18-(Ethoxycarbonothioylthio)octadecanoic Acid (10c): A solution of potassium xanthogenate (0.12 g, 0.77 mmol) in acetone (10 mL) was added to a solution of **10b** (0.20 g, 0.55 mmol) in anhydrous acetone (15 mL), and the mixture was stirred at room temperature for 18 h under an inert atmosphere. The solvent was evaporated under reduced pressure, the residue was dissolved in Et_2O (50 mL), and aq. HCl (10 mL, 1 M) was added. The organic layer was washed with water (2 × 20 mL) and brine (20 mL), dried and concentrated in vacuo to give the title compound (0.22 g, 99%) as a colorless solid, m.p. 79 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.18–1.37 (m, 26 H, CH_2), 1.42 (t, J = 7.1 Hz, 3 H, CH_3), 1.55–1.75 (m, 4 H, CH_2, 3/17-H), 2.34 (t, J = 7.5 Hz, 2 H, CH_2-2), 3.10 (t, J = 7.5 Hz, 2 H, CH_2-18), 4.64 (q, J = 7.1 Hz, 2 H, CH_2O) ppm. ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 13.8 (CH_3), 24.7 (CH_2, C-3), 28.3 (CH_2), 28.9 (CH_2), 29.0 (CH_2), 29.1 (CH_2), 29.2 (CH_2), 29.4 (2 × CH_2), 29.5 (CH_2), 29.6 (6 × CH_2), 34.0 (CH_2, C-17), 35.9 (CH_2 C-2), 69.7 (CH_2O), 180.1 (CO), 215.3 (C=S) ppm. ESI-MS (negative mode): m/z (%) = 403.0 $[M - H]^-$ (100), 807.9 $[2M - H]^-$ (76); ESI-MS (positive mode): m/z (%) = 405.2 $[M + H]^+$ (27), 427.2 $[M + Na]^+$ (100), 831.5 $[2M + Na]^+$ (24). HR-MS (ESI, positive mode): found: 427.2312; calcd. for $C_{21}H_{40}O_3S_2$: 427.2311 $[M + Na]^+$.

***tert*-Butyl 18-Bromooctadecanoate (11a):** Trifluoroacetic anhydride (0.67 mL, 4.8 mmol) was slowly added at 0 °C to a solution of **10b** (0.9 g, 2.5 mmol) in THF (7.5 mL). After 1 h, *t*BuOH (2.8 mL) was added, and the solution was stirred at room temperature overnight.

The reaction mixture was poured into sat. aq. $NaHCO_3$ (30 mL) and extracted with CH_2Cl_2 (3 × 30 mL), and the combined organic solutions were washed with brine (30 mL) and dried. After evaporation of the solvents, the residue was purified by flash chromatography on SiO_2 (100 mL). Elution with hexane/EtOAc (16:1) afforded the title compound (0.94 g, 90%) as a colorless solid with m.p. 46 °C. ^1H NMR (300 MHz, $CDCl_3$): δ = 1.17–1.33 (m, 26 H, CH_2), 1.41 (s, 9 H, CH_3), 1.48–1.61 (m, 2 H, CH_2), 1.76–1.89 (m, 2 H, CH_2), 2.17 (t, J = 7.5 Hz, 2 H, CH_2CO), 3.38 (t, J = 6.9 Hz, 2 H, CH_2Br) ppm. ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 25.1 (CH_2, C-3), 28.1 (3 × CH_3), 28.2 (CH_2, C-16), 28.8 (CH_2, C-4), 29.1 (CH_2), 29.3 (CH_2), 29.4 (CH_2), 29.5 (2 × CH_2), 29.6 (6 × CH_2), 32.8 (CH_2, C-17), 34.0 (CH_2Br), 35.6 (CH_2, C-2), 79.8 (C), 173.3 (CO) ppm. CI-MS (NH_3): m/z (%) = 436.4 and 438.4 $[M + NH_4]^+$ (100). $C_{22}H_{43}BrO_2$ (419.48): calcd. C 62.99, H 10.33; found C 62.83, H 10.69.

***tert*-Butyl 18-Azidooctadecanoate (11b):** Compound **11a** (0.84 g, 2.0 mmol) was stirred with NaN_3 (0.16 g, 2.4 mmol) in anhydrous DMF (13 mL) at 85 °C for 18 h. The solvent was evaporated in vacuo, the mixture was diluted with CH_2Cl_2 (50 mL), and the solution was washed with water (3 × 20 mL), dried with Na_2SO_4 and concentrated to give the title compound (0.76 g, 99%) as a colorless solid, m.p. 42 °C. ^1H NMR (300 MHz, $CDCl_3$): δ = 1.23 (m, 26 H, CH_2), 1.42 (s, 9 H, CH_3), 1.45–1.65 (m, 4 H, CH_2, 3/17-H), 2.17 (t, J = 7.5 Hz, 2 H, CH_2CO), 3.23 (t, J = 7.0 Hz, 2 H, CH_2N) ppm. ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 25.1 (CH_2, C-3), 26.7 (CH_2), 28.1 (3 × CH_3), 28.8 (CH_2), 29.1 (2 × CH_2), 29.3 (CH_2), 29.5 (3 × CH_2), 29.6 (6 × CH_2), 35.6 (CH_2, C-2), 51.5 (CH_2N), 79.8 (C), 173.3 (CO) ppm. CI-MS (NH_3): m/z (%) = 399 $[M + NH_4]^+$ (100), 356 (28), 357 (25), 343 (45). $C_{22}H_{43}N_3O_2$ (381.60): calcd. C 69.24, H 11.36, N 11.01; found C 69.08, H 11.94, N 10.75.

***tert*-Butyl 18-Aminooctadecanoate Hydrochloride (11c):** A solution of **11b** (0.57 g, 1.5 mmol) in EtOAc (60 mL), together with HCl (5–6 M) in *i*PrOH (0.75 mL), was added to a suspension of Pd/C (10%, 0.11 g, Merck, oxidized form) in EtOAc (10 mL). The mixture was stirred under H_2 (1 atm) at room temperature overnight, diluted with EtOAc (100 mL), filtered through Celite® and concentrated in vacuo to give the title compound (0.4 g, 68%) as a colorless solid. ^1H NMR (300 MHz, $CDCl_3$): δ = 1.22 (m, 26 H, CH_2), 1.42 (s, 9 H, CH_3), 1.47–1.63 (m, 2 H, CH_2), 1.65–1.82 (m, 2 H, CH_2), 2.17 (t, J = 7.5 Hz, 2 H, CH_2CO), 2.94 (m, J = 7.5 Hz, 2 H, CH_2N) ppm. ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 25.1 (CH_2, C-3), 26.7 (CH_2, C-16), 28.1 (3 × CH_3), 28.9 (CH_2), 29.1 (CH_2), 29.2 (CH_2), 29.3 (CH_2), 29.5 (2 × CH_2), 29.6 (5 × CH_2), 29.7 (2 × CH_2), 35.6 (CH_2, C-2), 39.7 (CH_2NH_2), 79.9 (C), 173.4 (CO) ppm. CI-MS (NH_3): m/z (%) = 356.4 $[M + H]^+$ (100), 711 $[2M + H]^+$ (8). HR-MS (ESI, positive mode): found: 356.3524; calcd. for $C_{22}H_{45}NO_2$: 356.3523 $[M + H]^+$.

***tert*-Butyl 18-[(9-Fluorenylmethoxycarbonyl)amino]octadecanoate (11d):** FmocCl (0.295 g, 1.14 mmol) was added in small portions to a cold solution of **11c** (0.37 g, 0.95 mmol) in 1,4-dioxane (60 mL), followed by $NaHCO_3$ (0.44 g). The reaction mixture was stirred at room temperature for 24 h, diluted with $CHCl_3$ (100 mL), washed with water (2 × 50 mL) and dried. After evaporation of the solvents under reduced pressure, the residue was filtered through silica gel (75 g) with elution with CH_2Cl_2 to afford the title compound (0.53 g, 97%) as a colorless solid, m.p. 91–93 °C. ^1H NMR (300 MHz, $CDCl_3$): δ = 1.24 (m, 26 H, CH_2), 1.42 (s, 9 H, CH_3), 1.44–1.63 (m, 4 H, CH_2, 3/17-H), 2.18 (t, J = 7.5 Hz, 2 H, CH_2CO), 3.16 (dt, J = 6.8, 7.4 Hz, 2 H, CH_2N), 4.20 (t, J = 6.9 Hz, 1 H, CH), 4.38 (d, J = 6.9 Hz, 2 H, CH_2), 4.76 (m, 1 H, NH), 7.29 (t, J = 7.2 Hz, 2 H, 2′/7′-H), 7.38 (t, J = 7.2 Hz, 2 H, 3′/6′-H), 7.58 (d, J = 7.4 Hz, 2 H, 1′/8′-H), 7.75 (d, J = 7.4 Hz, 2 H, 4′/5′-H) ppm. ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 25.1 (CH_2, C-3), 25.8 (CH_2), 28.1 (3 × CH_3), 29.1 (CH_2), 29.3 (2 × CH_2), 29.5 (3 × CH_2), 29.6 (4 × CH_2), 29.7 (2 × CH_2), 30.0 (CH_2), 35.7 (CH_2, C-2), 41.2 (CH_2NH), 47.4 (CH), 66.5 (CH_2), 79.8 (C), 119.8 (2 × CH, C-1′/8′), 125.0 (2 × CH, C-2′/7′), 127.0 (2 × CH, C-4′/5′), 127.6 (2 × CH, C-3′/6′), 141.4 (2 × C), 144.1 (2 × C), 156.4 (CO), 173.3 (CO) ppm. ESI-MS (positive mode): m/z (%) = 1177.2 $[2M + Na]^+$ (100), 600 $[M + Na]^+$ (45). $C_{37}H_{55}NO_4$ (577.84): calcd. C 76.91, H 9.59, N 2.42; found C 76.80, H 9.96, N 2.26.

18-[(9-Fluorenylmethoxycarbonyl)amino]octadecanoic Acid (12): Et_3SiH (50 μL) and TFA (0.5 mL) were added at 0 °C to a solution of **11d** (0.144 g, 0.25 mmol) in CH_2Cl_2 (0.8 mL), and the mixture was kept at 4 °C for 20 h. Evaporation in vacuo gave the title compound (0.12 g, 92%) as a colorless solid; m.p. 122 °C. ^1H NMR (300 MHz, [D$_6$]DMSO): δ = 1.21 (m, 26 H, CH_2), 1.32–1.54 (m, 4 H, CH_2, 3/17-H), 2.17 (t, J = 7.3 Hz, 2 H, CH_2CO), 2.97 (dt, J = 5.6, 6.0 Hz, 2 H, CH_2N), 4.21 (t, J = 6.6 Hz, 1 H, 9′-H), 4.29 (d, J = 6.6 Hz, 2 H, CH_2O), 7.20 (m, 1 H, NH), 7.32 (t, J = 7.4 Hz, 2 H, 2′/7′-H), 7.41 (t, J = 7.4 Hz, 2 H, 3′/6′-H), 7.68 (d, J = 7.3 Hz, 2 H, 1′/8′-H), 7.88 (d, J = 7.4 Hz, 2 H, 4′/5′-H) ppm. ^{13}C NMR (75.5 MHz, [D$_8$]THF): δ = 24.3 (CH_2, C-3), 24.8 (CH_2, C-17), 27.6 (CH_2, C-16), 29.8 (CH_2), 30.0 (CH_2), 30.3 (2 × CH_2), 30.4 (CH_2), 30.5 (5 × CH_2), 30.9 (CH_2), 34.0 (CH_2), 34.2 (CH_2, C-2), 41.5 (CH_2NH), 48.3 (CH), 67.5 (CH_2), 120.5 (2 × CH, C-1′/8′), 125.8 (2 × CH, C-2′/7′), 127.5 (2 × CH, C-4′/5′), 128.1 (2 × CH, C-3′/6′), 142.2 (2 × C), 145.4 (2 × C), 156.9 (CO), 174.2 (CO) ppm. ESI-MS (positive mode): m/z (%) = 1087.4 $[2M + 2Na – H]^+$ (10), 1065.3 $[2M + Na]^+$ (100), 544.5 $[M + Na]^+$ (10), 522.2 $[M + H]^+$ (2); ESI-MS (negative mode): m/z (%) = 1063.6 $[2M + Na – 2H]^-$ (30), 1041.3 $[2M – H]^-$ (100), 520.0 $[M – H]^-$ (2). HR-MS (ESI, positive mode): found: 522.3580; calcd. for $C_{33}H_{47}NO_4$: 522.3578 $[M + H]^+$.

***N*-Hydroxysuccinimidyl 18-[(9-Fluorenylmethoxycarbonyl)amino]octadecanoate (13):** Compound **13** was prepared as described above for compound **4**-NHS,NHFmoc, yield 0.10 g (81%) of a colorless solid. ^1H NMR (300 MHz, $CDCl_3$): δ = 1.17–1.33 (m, 26 H, CH_2), 1.34–1.54 (m, 2 H, CH_2), 1.65–1.80 (m, 2 H, CH_2), 2.58 (t, J = 7.5 Hz, 2 H, CH_2CO), 2.81 (s, 4 H, CH_2 in NHS), 3.17 (m, 2 H, CH_2N), 4.20 (t, J = 6.9 Hz, 1 H, CH), 4.38 (d, J = 6.9 Hz, 2 H, CH_2), 4.73 (m, 1 H, NH), 7.29 (t, J = 7.4 Hz, 2 H, 2′/7′-H), 7.38 (t, J = 7.4 Hz, 2 H, 3′/6′-H), 7.58 (d, J = 7.4 Hz, 2 H, 1′/8′-H), 7.75 (d, J = 7.4 Hz, 2 H, 4′/5′-H) ppm. ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 24.5 (CH_2, C-3), 25.6 (2 × CH_2), 26.7 (CH_2, C-17), 28.8 (CH_2), 29.1 (CH_2), 29.3 (2 × CH_2), 29.5 (2 × CH_2), 29.6 (5 × CH_2), 30.0 (CH_2), 30.3 (CH_2), 30.9 (CH_2), 41.1 (CH_2NH), 47.3 (CH), 66.4 (CH_2), 119.9 (2 × CH, C-1′/8′), 125.0 (2 × CH, C-2′/7′), 127.0 (2 × CH, C-4′/5′), 127.6 (2 × CH, C-3′/6′), 141.3 (2 × C), 144.0 (2 × C), 156.4 (CO), 168.7 (CO), 169.2 (2 × CO in NHS) ppm. ESI-MS (positive mode): m/z (%) = 1259.3 $[2M + Na]^+$ (100), 641.5 $[M + Na]^+$ (67). HR-MS (ESI, positive mode): found: 641.3569; calcd. for $C_{37}H_{50}N_2O_6$: 641.3561 $[M + Na]^+$.

***N*-Hydroxysuccinimidyl 18-(Ethoxycarbonothioylthio)octadecanoate (14):** Compound **14** was prepared as described above for compound **4**-NHS,NHFmoc, yield 0.10 g (80%) of a colorless solid, m.p. 79 °C. ^1H NMR (300 MHz, $CDCl_3$): δ = 1.20–1.32 (m, 26 H, CH_2), 1.40 (t, J = 7.1 Hz, 3 H, CH_3), 1.60–1.78 (m, 4 H, CH_2, 3/17-H), 2.58 (t, J = 7.5 Hz, 2 H, CH_2-2), 2.82 (s, 4 H, CH_2), 3.09 (t, J = 7.5 Hz, 2 H, CH_2-18), 4.62 (q, J = 7.1 Hz, 2 H, CH_2O) ppm. ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 13.8 (CH_3), 24.6 (CH_2, C-3), 25.6 (2 × CH_2), 28.4 (CH_2), 28.8 (CH_2), 28.9 (CH_2), 29.1 (CH_2), 29.2 (CH_2), 29.4 (CH_2), 29.5 (CH_2), 29.6 (2 × CH_2), 29.7 (5 × CH_2), 31.0

(CH_2, C-17), 35.9 (CH_2, C-2), 69.7 (CH_2O), 168.5 (CO), 170.0 (2 × CO), 215.0 (C=S) ppm. ESI-MS (positive mode): *m/z* (%) = 502.3 $[M + H]^+$ (27), 524.2 $[M + Na]^+$ (100), 1025.5 $[2M + Na]^+$ (35). HR-MS (ESI, positive mode): found: 524.2471; calcd. for $C_{25}H_{43}NS_2O_5$: 524.2475 $[M + Na]^+$.

GM1-2-NHFmoc (15a): Compound **4**-NHS,NHFmoc (48 mg, 78.0 μmol) in anhydrous THF (0.3 mL) was added to a solution of lyso-GM1 (10 mg, 7.8 μmol), Triton X-100 (0.13 mL) and NEt_3 (2 μL) in anhydrous DMF (0.2 mL). The mixture was stirred at room temperature for 24 h and was then concentrated under reduced pressure. The product was isolated by chromatography on SiO_2 (20 mL) with elution with $CHCl_3$/MeOH/H_2O (60:35:5), to yield a colorless powder (7 mg, 50%) after lyophilization. ESI-MS (negative mode): *m/z* = 1781.9 $[M_1 - H]^-$, 1809.9 $[M_2 - H]^-$. HR-MS (ESI, negative mode): found: 1781.9476, 1809.9780; calcd. for $C_{88}H_{142}N_4O_{33}$ and $C_{90}H_{146}N_4O_{33}$: 1781.9484 $[M_1 - H]^-$, 1809.9797 $[M_2 - H]^-$.

GM1-18-NHFmoc (15b): Compound **15b** was obtained by the procedure described above for compound **15a**, starting from lyso-GM1 (10 mg, 7.7 μmol), to yield a colorless solid (8.4 mg, 60%). ESI-MS (negative mode): *m/z* = 1781.8 $[M_1 - H]^-$, 1810.0 $[M_2 - H]^-$. HR-MS (ESI, positive mode): found: 892.4850, 906.5009; calcd. for $C_{88}H_{142}N_4O_{33}$ and $C_{90}H_{146}N_4O_{33}$: 892.4851 $[M_1 + 2H]^{2+}$ and 906.5007 $[M_2 + 2H]^{2+}$.

GM1-18-SC(S)OEt (15c): Compound **15c** was obtained by the procedure described above for compound **15a**, starting from lyso-GM1 (10 mg, 7.7 μmol), to yield a colorless solid (6.9 mg, 54%). ESI-MS (negative mode): *m/z* (%) = 831.9 $[M_1 - 2H]^{2-}$ (40), 845.9 (57) $[M_2 - 2H]^{2-}$, 1664.8 $[M_1 - H]^-$ (67), 1692.9 $[M_2 - H]^-$ (100). HR-MS (ESI, positive mode): found: 831.9160, 845.9322; calcd. for $C_{76}H_{135}N_3O_{32}S_2$ and $C_{78}H_{139}N_3O_{32}S_2$: 831.9162 $[M_1 - 2H]^{2-}$, 845.9319 $[M_2 - 2H]^{2-}$.

GM1-2-NH_2 (16a): Compound **15a** (6 mg, 3.3 μmol) was stirred with a solution of piperidine in MeOH (20%, 0.4 mL) at room temperature for 2 h, and the mixture was concentrated under reduced pressure. Two diastereomers of the title product were separated and isolated by chromatography on SiO_2 (10 g). Elution with $CHCl_3$/MeOH/H_2O (60:35:5), followed by dissolution in water and freeze-drying, gave colourless powders of the two compounds with different R_f values [0.31 and 0.19, 2.7 mg (52%) and 2.3 mg (44%), respectively] and the same mass spectra. ESI-MS (negative mode): *m/z* = 1560.1 $[M_1 - H]^-$, 1588.0 $[M_2 - H]^-$. HR-MS (ESI, positive mode): found: 781.4510, 795.4667; calcd. for $C_{73}H_{132}N_4O_{31}$ and $C_{75}H_{136}N_4O_{31}$: 781.4511 $[M_1 + 2H]^{2+}$, 795.4667 $[M_2 + 2H]^{2+}$.

GM1-18-NH_2 (16b): Compound **16b** was obtained by the procedure described above for compound **16a**, to yield a colorless powder (5.4 mg, 77%). ESI-MS (negative mode): *m/z* = 1560.1 $[M_1 - H]^-$, 1588.1 $[M_2 - H]^-$. HR-MS (ESI, positive mode): found: 792.4416, 806.4573; calcd. for $C_{73}H_{132}N_4O_{31}$ and $C_{75}H_{136}N_4O_{31}$: 792.4420 $[M_1 + H + Na]^{2+}$, 806.4577 $[M_2 + H + Na]^{2+}$.

$(GM1\text{-}18\text{-}S)_2$ (16c Dimer): Compound **15c** (4.8 mg, 2.9 μmol) was stirred in aqueous ammonia solution (25%, 0.5 mL) at room temperature for 24 h, and the mixture was then lyophilized and purified by chromatography on SiO_2 (10 mL) with elution with $CHCl_3$/MeOH/H_2O (60:35:5) to give compound **16c** as a dimer (4.3 mg, 96%). ESI-MS (negative mode): *m/z* (%) = 1604.8 (81) $[M_1 - 2H]^{2-}$, 1590.4 (100) $[M_2 - 2H]^{2-}$, 1576.8 (31) $[M_3 - 2H]^{2-}$, 283.3 (27). HR-MS (ESI, negative mode): found: 3209.7485, 3181.7223, 3153.6886; calcd. for $C_{150}H_{268}N_6O_{62}S_2$, $C_{148}H_{264}N_6O_{62}S_2$, $C_{146}H_{260}N_6O_{62}S_2$: 3209.7440, 3181.7131, 3153.6818 $[M - H]^-$.

GM1-18-SH (16c): Compound **16c** dimer (2.0 mg, 0.63 μmol) was stirred with DTT (4.8 mg, 0.03 mmol) in MeOH (0.1 mL) at room temperature for 4 h, concentrated in vacuo, dialysed against water with reduced oxygen content and addition of a small amount of EDTA, and lyophilized to give the title compound in quantitative yield. ESI-MS (negative mode): *m/z* = 1576.8 $[M_1 - H]^-$, 1604.9 $[M_2 - H]^-$. HR-MS (ESI, negative mode): found: 1576.8439, 1604.8734; calcd. for $C_{73}H_{131}N_3O_{31}S$ and $C_{75}H_{135}N_3O_{31}S$: 1576.8414 $[M_1 - H]^-$, 1604.8727 $[M_2 - H]^-$.

Adduct 17a: One of the diastereomers of compound **16a** (1 mg, 0.63 μmol) and the NHS ester of the fluorescent dye ATTO 647N (1 mg, 1.26 μmol) were dissolved in anhydrous DMSO (0.1 mL), NEt_3 (5 μL) was added, and the solution was stirred at room temperature for 24 h. After evaporation of the solvent under reduced pressure, the title compound was isolated by HPTLC eluting with $CHCl_3$/MeOH/H_2O (60:35:5). Yield: 1.0 mg (72%) of a blue powder. ESI-MS (positive mode): *m/z* = 2188.27 $[M_1 + H]^+$, 2216.31 $[M_2 + H]^+$, 1095.15 $[M_1 + 2H]^{2+}$, 1109.16 $[M_2 + 2H]^{2+}$. HR-MS (ESI, positive mode): found: 1106.1312, 1120.1484; calcd. for $C_{115}H_{181}N_7O_{33}$ and $C_{117}H_{185}N_7O_{33}$: 1106.1333 $[M_1 + H + Na]^{2+}$, 1120.1489 $[M_2 + H + Na]^{2+}$.

Compound 17b: The title compound was obtained by the procedure described above for compound **17a**, starting from **16b** (1.0 mg, 0.63 μmol) and **2**-OCONHS (1.0 mg, 1.26 μmol), to yield a violet powder (1.1 mg, 80%). ESI-MS (positive mode): *m/z* = 2205.1 $[M_1 + H]^+$, 2227.1 $[M_1 + Na]^+$, 2233.2 $[M_2 + H]^+$, 2255.1 $[M_2 + Na]^+$. HR-MS (ESI, positive mode): found: 1114.0566, 1128.0715; calcd. for $C_{109}H_{164}F_3N_7O_{36}$ and $C_{111}H_{168}F_3N_7O_{36}$: 1114.0657 $[M_1 + H + Na]^{2+}$, 1128.0724 $[M_2 + H + Na]^{2+}$.

Compound 22b: Lyso-GM1 (1.0 mg, 0.77 μmol) and **2**-OCONHS (0.9 mg, 1.15 μmol) were stirred with NEt_3 (5 μL) in DMSO (0.2 mL) at room temperature for 24 h, and the solvent was evaporated under reduced pressure. The title product was isolated by preparative TLC (silica gel) with elution with $CHCl_3$/MeOH/H_2O (60:35:5), to yield a violet powder (1.2 mg, 81%). ESI-MS (negative mode): *m/z* = 1922.9 $[M_1 - H]^-$, 1950.9 $[M_2 - H]^-$. HR-MS (ESI, positive mode): found: 970.9431, 987.4361; calcd. for $C_{91}H_{129}N_6O_{35}$ and $C_{93}H_{133}N_6O_{35}$: 970.9431 $[M_1 + H + Na]^{2+}$, 987.4365 $[M_2 + H + Na]^{2+}$.

Compound 23a: The title compound was obtained by the procedure described above for compound **22b**, starting from deAc-GM1 (1.00 mg, 0.7 μmol) and **1h** (0.75 mg, 1.0 μmol), to yield a red powder (1.1 mg, 77%). ESI-MS (negative mode): *m/z* = 1070 $[M_1 - 2H]^{2-}$, 1084 $[M_2 - 2H]^{2-}$. HR-MS (ESI, negative mode): found: 1083.5034 $[M - 2H]^{2-}$; calcd. for $C_{103}H_{160}N_6O_{39}S_2$: 1083.5009.

Compound 23b: This compound was obtained by the procedure described above for compound **22b**, starting from deAc-GM1 (1.5 mg, 1.0 μmol) and **3**-OCONHS (1.75 mg, 2.0 μmol), to yield a violet powder (1.7 mg, 79%). ESI-MS (negative mode): *m/z* (%) = 1132 (64) $[M_1 - 2H]^{2-}$, 1146 (100) $[M_2 - 2H]^{2-}$. HR-MS (ESI, negative mode): found: 1131.5294, 1145.5453; calcd. for $C_{109}H_{168}N_6O_{40}S_2$ and $C_{111}H_{172}N_6O_{40}S_2$: 1131.5296 $[M_1 - 2H]^{2-}$, 1145.5453 $[M_2 - 2H]^{2-}$.

Compound 23c: This compound was obtained by the procedure described above for compound **22b**, starting from deAc-GM1 (1.0 mg, 0.67 μmol) and ATTO 647N NHS ester (1.1 mg, 1.33 μmol), to yield a blue powder (1.0 mg, 71%). ESI-MS (positive mode): *m/z* (%) = 1066.63 (80) $[M_1 + 2H]^{2+}$, 1080.65 (100) $[M_2 + 2H]^{2+}$. HR-MS (ESI, positive mode): found: 1066.6311, 1080.6469; calcd. for $C_{113}H_{178}N_6O_{32}$ and $C_{115}H_{182}N_6O_{32}$: 1066.6316 $[M_1 + 2H]^{2+}$, 1080.6472 $[M_2 + 2H]^{2+}$.

Compound 24: The title product was obtained by the procedure described above for compound **22b**, starting from ATTO 647N-

NHS ester (1.0 mg, 1.19 μmol) and deAc-deAcyl-GM1 (3.0 mg, 2.37 μmol), to yield a blue powder (1.0 mg, 45%). ESI-MS (positive mode): *m/z* = 945.0 $[M_1 + H + Na]^{2+}$, 959.1 $[M_2 + H + Na]^{2+}$. HR-MS (ESI, positive mode): found: 933.5005, 944.4913, 958.5072; calcd. for $C_{95}H_{144}N_6O_{31}$ and $C_{97}H_{148}N_6O_{31}$: 933.5011 $[M_1 + 2H]^{2+}$, 944.4921 $[M_1 + H + Na]^{2+}$, 958.5077 $[M_2 + H + Na]^{2+}$.

Compound 25a: The title product was obtained by the procedure described above for compound **22b**, starting from **24** (1.0 mg) and **1h** (1.0 mg), to yield a violet powder (0.5 mg, 37%). ESI-MS (positive mode): *m/z* = 2503.13 $[M_1 + H]^+$, 2531.16 $[M_2 + H]^+$. HR-MS (ESI, positive mode): found: 1252.0609, 1263.0525, 1247.0433; calcd. for $C_{125}H_{171}N_9O_{40}S_2$, $C_{127}H_{175}N_9O_{40}S_2$: 1252.0605 $[M_1 + 2H]^{2+}$, 1263.0515 $[M_1 + 2Na]^{2+}$, 1247.0425 $[M_2 + 2Na]^{2+}$.

Compound 25b: The title product was obtained by the procedure described above for compound **22b**, starting from **24** (0.5 mg) and Rh501 (1.0 mg),[52] to yield a greenish powder (0.3 mg, 44%). ESI-MS (negative mode): *m/z* = 1272 $[M_1 - 2H]^{2-}$, 1286 $[M_2 - 2H]^{2-}$.

Compound 26: Compound **4**-OH,NHFmoc (2.1 mg, 3.95 μmol) was stirred with $SOCl_2$ (0.2 mL) at 50 °C for 4 h, and the excess of $SOCl_2$ was evaporated in vacuo to give the corresponding chloroanhydride. A solution of chloroanhydride in Et_2O (0.2 mL) was added at 0 °C to a solution of deAc-deAcyl-GM1 (5.0 mg, 3.95 μmol) in aq. $NaHCO_3$ (0.1%, 0.4 mL) and Et_2O (0.4 mL), and the mixture was stirred at 4 °C for 24 h. After evaporation of the solvents in vacuo, the title compound was isolated by chromatography on SiO_2 (10 mL) with elution with $CHCl_3$/MeOH/H_2O (60:35:8). Yield: 1.1 mg (16%) of a colorless powder after lyophilization. ESI-MS (negative mode): *m/z* = 1740 $[M_1 - H]^-$, 1769 $[M_2 - H]^-$. HR-MS (ESI, positive mode): found: 893.4617, 907.4771; calcd. for $C_{86}H_{140}N_4O_{32}$, $C_{88}H_{144}N_4O_{32}$: 893.4618 $[M_1 + 2Na]^{2+}$, 907.4774 $[M_2 + 2Na]^{2+}$.

Compound 27: The title compound was prepared as described for compound **26**, starting from compound **13** (3.4 mg, 6.48 μmol) and deAc-deAcyl-GM1 (8.2 mg, 6.48 μmol), to yield a colorless powder (2.1 mg, 18%). ESI-MS (negative mode): *m/z* = 1740 $[M_1 - H]^-$, 1769 $[M_2 - H]^-$. HR-MS (ESI, positive mode): found: 882.4701, 896.4865; calcd. for $C_{86}H_{140}N_4O_{32}$, $C_{88}H_{144}N_4O_{32}$: 882.4708 $[M_1 + H + Na]^{2+}$, 896.4864 $[M_2 + H + Na]^{2+}$.

Abbreviations:[1c,1d] GM1, II^3Neu5AcGgOse$_4$Cer, β-Gal-(1–3)-β-GalNAc-(1–4)-[α-Neu5Ac-(2–3)]-β-Gal-(1–4)-β-Glc-(1–1)-Cer; deAc-GM1, II^3NeuGgOse$_4$Cer, β-Gal-(1–3)-β-GalNAc-(1–4)-[α-Neu-(2–3)]-β-Gal-(1–4)-β-Glc-(1–1)-Cer; deAc-deAcyl-GM1, II^3NeuGgOse$_4$LCB, β-Gal-(1–3)-β-GalNAc-(1–4)-[α-Neu-(2–3)]-β-Gal-(1–4)-β-Glc-(1–1)-LCB; Lyso-GM1, deAcyl-GM1, II^3Neu5AcGgOse$_4$LCB, β-Gal-(1–3)-β-GalNAc-(1–4)-[α-Neu5Ac-(2–3)]-β-Gal-(1–4)-β-Glc-(1–1)-LCB; Neu, neuraminic acid; Neu5Ac, *N*-acetylneuraminic acid; Cer, ceramide; LCB, long-chain bases; NBD, 4-amino-7-nitrobenz-2-oxa-1,3-diazole; BODIPY, 4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene; HPLC, high pressure liquid chromatography; HPTLC, high performance thin layer chromatography; THF, tetrahydrofuran; DMF, *N*,*N*-dimethylformamide; DMAA, *N*,*N*-dimethylacetamide; DMSO, dimethyl sulfoxide; TFA, trifluoroacetic acid; HATU, *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; FmocCl, 9-fluorenylmethyl chloroformate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid.

Supporting Information (see also the footnote on the first page of this article): Synthesis and properties of compounds **1a**, **1b**, **1i**, **1j**, **1k**, **1l**, **2**-OH, **2**-OCONHS, **3**-OH, **3**-OCONHS, **4**-*t*BuO,NHFmoc, **4**-OH,NHFmoc, **10a** and **10b**; chromatographic behaviour of the selected GM1 derivatives bearing the fluorescent labels.

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