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Synthetic Glycosphingolipids for Live-Cell Labeling

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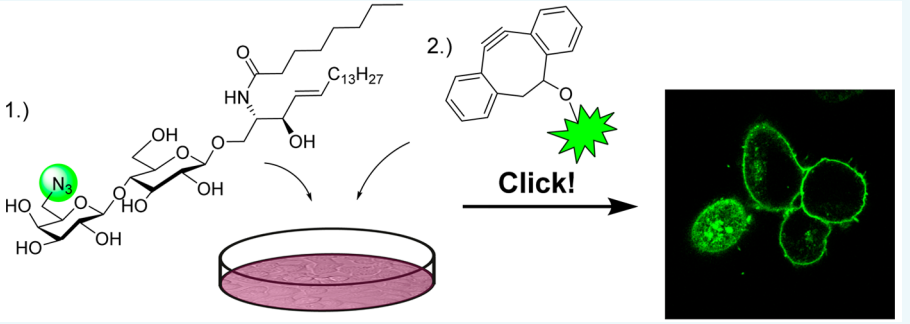
Supporting Information



ABSTRACT: Glycosphingolipids are an important component of cell membranes that are involved in many biological processes. Fluorescently labeled glycosphingolipids are frequently used to gain insight into their localization. However, the attachment of a fluorophore to the glycan part or—more commonly—to the lipid part of glycosphingolipids is known to alter the biophysical properties and can perturb the biological function of the probe. Presented here is the synthesis of novel glycosphingolipid probes with mono- and disaccharide head groups and ceramide moieties containing fatty acids of varying chain length (C_4 to C_{20}). These glycosphingolipids bear an azide or an alkyne group as chemical reporter to which a fluorophore can be attached through a bioorthogonal ligation reaction. The fluorescent tag and any linker connected to it can be chosen in a flexible manner. We demonstrate the suitability of the probes by selective visualization of the plasma membrane of living cells by confocal microscopy techniques. Whereas the derivatives with the shorter fatty acids can be directly applied to HEK 293T cells, the hydrophobic glycosphingolipids with longer fatty acids can be delivered to cells using fusogenic liposomes.

INTRODUCTION

Glycosphingolipids are an important class of lipids found in cell membranes. They play a major role in many biological processes, such as the formation of membrane microdomains and the interaction of pathogenic microbes or their secreted toxins with host cells.[1,2] A large number of glycosphingolipid derivatives have been prepared to gain insight into their biological function.[3] The toolbox of available probes includes lipids with fluorophores, photoreactive cross-linkers, and radiolabels.[4] Fluorescently labeled derivatives in particular have been widely used to study trafficking of glycosphingolipids and to monitor the formation of lipid microdomains.[5,6] In most of these labeled lipids, the fluorophore is ligated to the sphingosine backbone.[7] Synthetically, this can be conveniently achieved by de-*N*-acylation of natural glycosphingolipids and reacylation with a fluorescent dye.[8−11] Although several of these compounds are commercially available, their altered biophysical properties can perturb their biological function.[4,5,12−14] For example, Sezgin et al.[15] and Honigmann et al.[16] showed that size, polarity, charge, and position of the dye within a lipid molecule can dramatically influence the biological function in the plasma membrane as compared to its natural counterpart. The hydrophilic glycan part has also been used for ligation with a fluorescent dye.[15,17−20] In this case, the synthesis is more challenging and variation of the dye is not easily possible. Alternatively, the fluorescently labeled B subunits of bacterial toxins, such as Cholera toxin (CtxB) or Shiga toxin (StxB), can be used to visualize glycosphingolipids.[21] These microbial proteins selectively bind specific glycolipids but, due to their multimeric structure, they can also influence lipid organization by multivalent cross-linking.[21−24]

During the last years, several research groups including our own[25−29] made use of a chemical reporter strategy to study glycoconjugates in living cells.[30,31] In this approach, synthetic biomolecules with a small chemical reporter group are applied to cells, metabolically incorporated into biomolecules, and subsequently labeled in a bioorthogonal reaction, for example, with a fluorescent dye. This approach, however, leads to labeling of the whole glycome including glycolipids and glycoproteins,[32] and the isolation of individual labeled glycolipids in preparative scale is not feasible. In addition, it has been shown that different cell lines differ in their ability to discriminate among variant forms of a carbohydrate which may lead to preferential incorporation into different glycolipids.[33]

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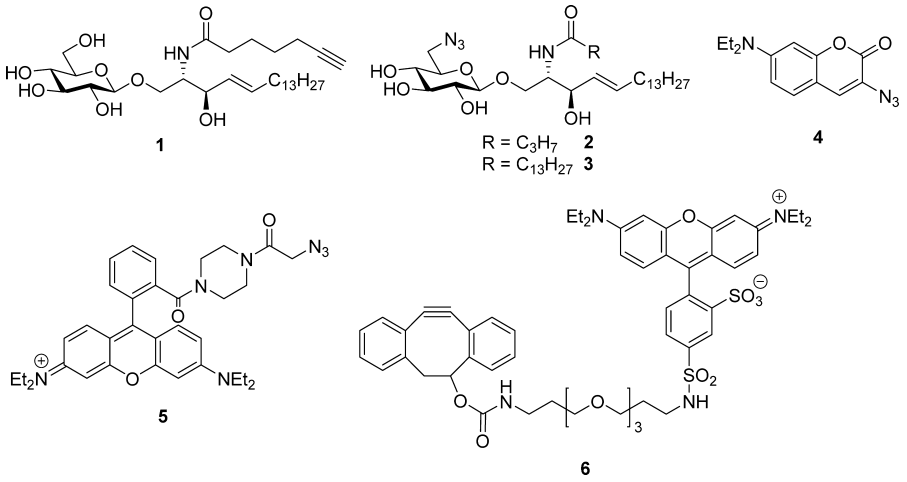


Figure 1. Alkyne- and azide-labeled glucosylceramides and fluorescent dyes.

Thus, the application of synthetic lipid derivatives modified by a reporter group that allows for flexible attachment of a probe is desirable. This strategy[34] has, for example, been adopted for the study of phospholipids,[35−38] sphingolipids,[39] and lipids with a choline headgroup.[40] However, synthetically demanding glycosphingolipids with chemical reporter groups have been reported only rarely.[41] Here, we present the synthesis of azide- and alkyne-tagged glycosphingolipids and their visualization in cell membranes by subsequent click reaction with various fluorescent dyes.

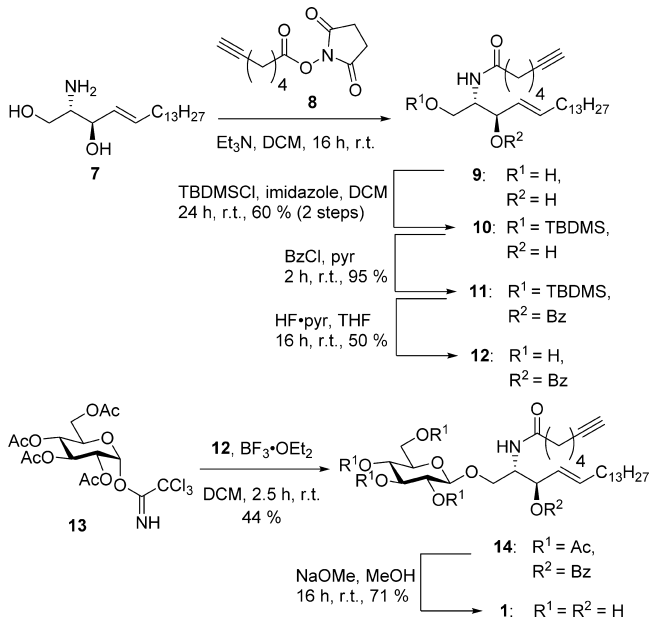
RESULTS AND DISCUSSION

We designed two types of glucosylceramide analogs (Figure 1), one bearing an alkyne group in the *N*-acyl chain (**1**), and the other with an azido group in the glucose moiety (**2**, **3**). The latter has the advantage that the lipid part of the molecule is not altered, a fact that might be crucial for studies concerning the organization of cell membranes. The length of the *N*-acyl chain varies in nature and can affect the biological function of the glycolipid.[42−44] Due to their better solubility, short-chain analogs are usually used in biological applications.[4] For sufficient solubility lipid derivatives should have a logP value similar to that of *N*-acetyl sphingosine.[45] Based on the estimation of logP values with the ALOGPS-2.1 software[46] we expected azidoglucosylceramide **2** with an *N*-butanoyl moiety to be a promising candidate for cell studies. For comparison, derivative **3** with an *N*-tetradecanoyl chain was also synthesized.

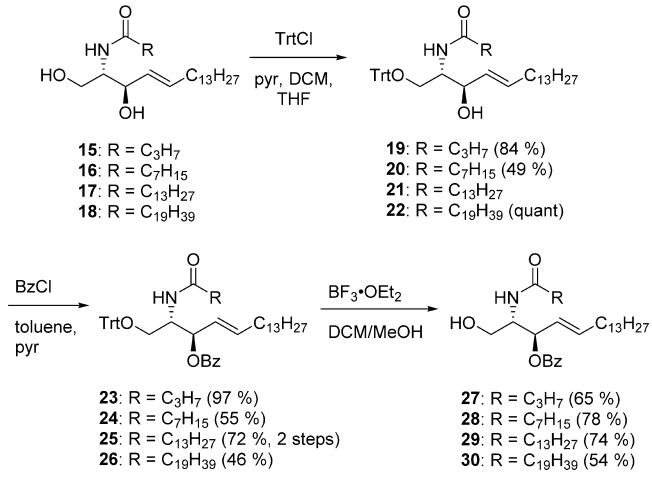
The synthesis of glucosylceramide **1** is depicted in Scheme 1. Commercially available sphingosine **7** was *N*-acylated with succinimidyl ester **8**, and the resulting amide **9** was protected at the secondary hydroxy group by a sequence of silylation, benzoylation, and desilylation. The obtained primary alcohol **12** was reacted with trichloroacetimidate **13**[47] to provide protected *β*-glucosylceramide **14**. *O*-Deacylation, finally, gave glucosylceramide **1**.

To prepare glycosphingolipids containing fatty acids of different chain length, we protected the ceramide derivatives **15**−**18** at the secondary hydroxy group by a sequence similar to that of the preparation of **12** (Scheme 2). Tritylation of the primary alcohol followed by protection of the secondary alcohol with benzoyl chloride and detritylation gave ceramide derivatives **27**−**30**. The synthesis of glucosylceramides **2** and **3** is depicted in Scheme 3. The acetylated 6-azido glucose

Scheme 1. Synthesis of Glucosylceramide 1



Scheme 2. Synthesis of Benzoyl-Protected Ceramide Derivatives 27−30



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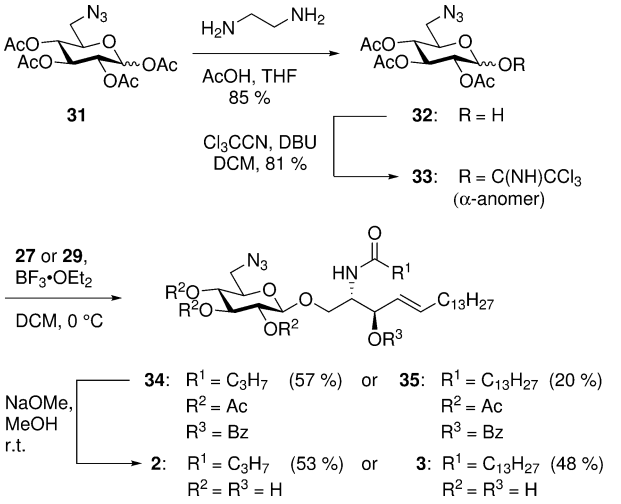
Scheme 3. Synthesis of Azide-Modified Glucosylceramides 2 and 3



derivative **31**[48] was anomerically deprotected with ethylene diamine/acetic acid according to the procedure described by Zhang and Kováč[49] and converted to the α-trichloroacetimidate **33**. Schmidt glycosylation[50] with ceramide **27** or **29** resulted in protected glucosylceramides **34** or **35** that were de-*O*-acylated with sodium methoxide in methanol to provide glucosylceramide **2** and **3**, respectively.

As fluorescent dyes we used coumarin azide **4**[51] as well as rhodamine azide **5** (for synthesis see Supporting Information) to label alkyne-modified glycolipid **1**. Coumarin azide **4** is fluorogenic and has the advantage to only fluoresce after reaction of the azide with alkyne-modified **1**. For visualizing azidoglucosylceramide **2** and **3**, we synthesized lissamine-labeled dibenzocyclooctyne (DIBO-lissamine) **6** (see Supporting Information) which can be employed in a copper-free labeling procedure.[52]

To investigate incorporation into cell membranes, glycolipids were applied to HEK 293T cells as a solution in Hanks' Minimum Essential Medium (HMEM) containing 1 equiv of defatted bovine serum albumin (dfBSA)[53] for 30 min at 4 °C to prevent endocytosis. Then, cells were washed two times with phosphate-buffered saline (PBS) and labeled with the fluorescent dye at 4 °C. Subsequent confocal microscopy of cells was performed at ambient temperature. The Cu(I)-catalyzed labeling of alkyne-modified glucosylceramide **1** using azides **4** or **5** was carried out according to the protocol of Hong et al.,[54,55] which has been shown to be compatible with living cells. In our case, however, tris(benzyltriazolylmethyl)amine (TBTA)[56] gave superior results than the hydrophilic copper ligand tris(3-hydroxypropyltriazolylmethyl)amin (THPTA). Probably, the hydrophobicity of the resulting copper-TBTA complex facilitates efficient catalysis within the lipid bilayer. **1** caused staining of the plasma as well as intracellular membranes (Figure 2A–D). The staining pattern is similar to that obtained with fluorescent ceramide and glucosylceramide analogs bearing a fluorophore in the hydrophobic part[57] and shows that the lipid can reach intracellular membranes. We speculated that an analog of **1** in which the glucose moiety is replaced with a lactose moiety would not be able to cross the cell membrane spontaneously due to its bigger hydrophilic headgroup. Cell experiments with such an alkyne-labeled lactosylceramide indeed showed that only the plasma membrane was stained, but the overall fluorescence intensity was rather weak (not shown). Possibly, the molecule becomes too hydrophilic after the click reaction and is washed out to some extent, resulting in reduced membrane staining. This finding might reflect one of the general drawbacks of lipids that are labeled with a hydrophilic dye in the hydrophobic part of the molecule.

Azidoglucosylceramide **2** was labeled by copper-free click reaction with DIBO-lissamine **6** and caused selective staining of the plasma membrane (Figure 2E, F). The same result was obtained after labeling with commercially available DIBO-AlexaFluor-488. In this case the fluorescent background was even a bit lower (Figure 2G, H). Azidoglucosylceramides with significantly longer *N*-acyl chains such as C_{10} or C_{14} (**3**) apparently were not incorporated into the cell membrane under these conditions (not shown), which underscores that the solubility of glycolipids might be a limiting factor. However, different mechanisms how glycolipids are taken up and incorporated into cell membranes including the formation of micelles have to be considered.[58] We speculated that a bigger hydrophilic carbohydrate moiety can compensate for longer lipid chains. Therefore, we tested modified lactosylceramides, which are also important natural glycosphingolipids. According to estimations of logP values, azidolactosylceramide **48** with an *N*-octanoyl moiety was expected to have a suitable polarity and, thus, was synthesized (Scheme 4). For comparison we also synthesized derivative **47** with an *N*-butanoyl moiety and **49** with an *N*-eicosanoyl moiety. In these compounds, the azide tag replaces the 6′-OH group of the lactosyl moiety. Allyl lactoside **36** was selectively TBDMS-protected at this position via a stannane[59] followed by acetylation of the remaining hydroxy groups to produce intermediate **38**. After desilylation, the 6′-OH group was mesylated and then substituted with sodium azide. The resulting lactosyl building block **41** was converted into trichloroacetimidate **43** and used as glycosyl donor for reaction with ceramides **27**–**30** to give, after final deprotection, azide-modified lactosylceramides **47**–**49**.

47 and **48** were applied to HEK 293T cells and labeled with DIBO-lissamine **6** (Figure 3A–C) at 4 °C in the same manner as described for glucosylceramide **2**. In both cases, selective visualization of the plasma membrane with comparable fluorescence intensity was achieved. These results demonstrate that a lactosylceramide can be incorporated into the plasma membrane, even when it contains an *N*-octanoyl chain. Compound **49** featuring a longer *N*-eicosanoyl chain, however, could not be delivered to the cells. In further experiments we could show that the incorporation of **47** and **48** and subsequent staining with DIBO-lissamine **6** can also be carried out at ambient temperature leading to comparable staining (Figure S1). We could not observe any endocytosis under these conditions.

With azidolactosylceramide **48** we also carried out a two-step labeling with DIBO-biotin conjugate **50**[52] and streptavidin-AlexaFluor-647 resulting in a bright staining of the plasma membrane and extremely low background fluorescence (Figures 3D, E, and S2). In contrast, with glycosphingolipids **1**, **2**, or **47** two-step labeling via a biotin derivative and streptavidin did not lead to any observable membrane staining. An explanation for this finding might be the fact that these glycolipids are washed out of the membrane after reaction with DIBO-biotin **50** and binding to streptavidin due to their shorter membrane anchor.

As mentioned above, the hydrophobic glycosphingolipids **3** and **49** could not be incorporated into the plasma membrane of

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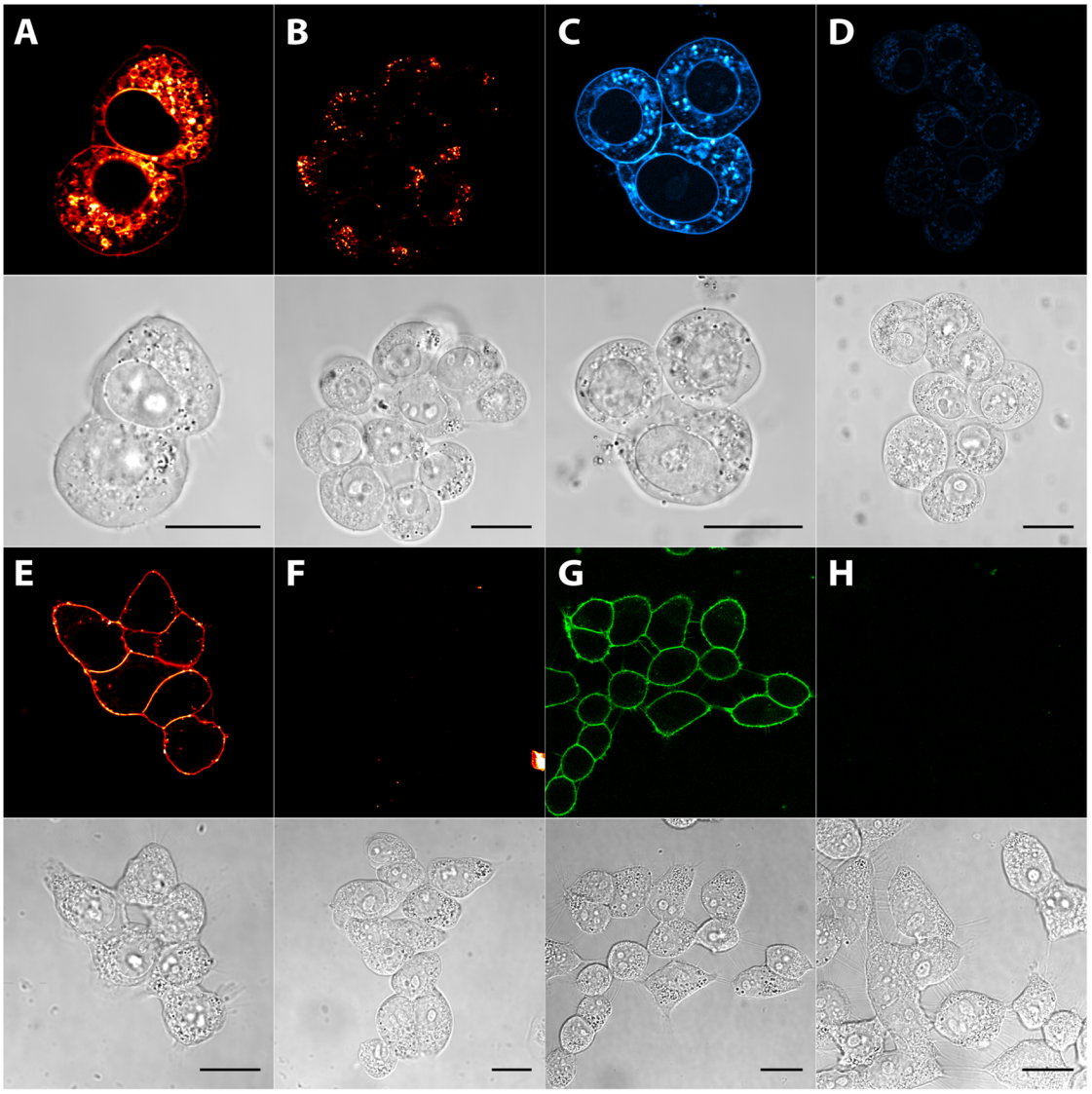

Figure 2. Cell experiments with labeled glucosylceramides **1** (A–D) and **2** (E–H). HEK 293T cells were treated with 5 μM **1** (A, C) or without **1** (B, D) for 30 min at 4 °C followed by labeling with 2 μM rhodamine azide **5** (A, B) or 50 μM coumarin azide **4** (C, D) in the presence of 50 μM $CuSO_4$, 100 μM TBTA, 1 mM aminoguanidine, and 10 mM sodium ascorbate for 30 min at 4 °C. In experiments with azidoglucosylceramide **2**, HEK 293T cells were treated with 10 μM **2** (E, G) or without **2** (F, H) for 30 min at 4 °C followed by fluorescence labeling with 2 μM DIBO-lissamine **6** (E, F) or 2 μM DIBO-AlexaFluor-488 (G, H) for 30 min at 4 °C. Confocal microscopy was performed at r.t. Scale bar: 20 μm.

HEK 293T cells. To overcome this limitation we investigated the use of fusogenic liposomes which have been shown to enhance the incorporation of biomolecules.[60,61] We used fusogenic liposomes prepared of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) as neutral and positively charged lipids, respectively, and TexasRed-labeled 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (DHPE) as lipid with an extended conjugated π electron system that enhances fusion efficacy.[60] The TexasRed dye, furthermore, allows monitoring of the fusion of the liposomes with the plasma membrane. The glycolipids **3** and **49**, respectively, were embedded in fusogenic liposomes, incubated with HEK 293T cells for 10 min at 37 °C, and subsequently labeled with DIBO-AlexaFluor-488 for 30 min at room temperature (Figure 4). Both glycolipids **3** (Figure 4A) and **49** (Figure 4B) show a bright membrane staining with only minor background (Figure 4C).

CONCLUSIONS

In summary, we have described the synthesis of new glycosphingolipid derivatives containing azide or alkyne reporter groups in either the lipid or the glycan moiety. Visualization of these glycolipids within membranes of living cells could be achieved by subsequent labeling through a bioorthogonal ligation reaction. Glycosphingolipids with longer *N*-acyl chains could be successfully delivered to cells using fusogenic liposomes. This strategy allows one to choose various dyes in a flexible manner which is a useful feature since different fluorophores can have different spectroscopic properties and can differ with respect to size, charge, and polarity. The small size of the azide and alkyne reporter groups enables future studies of lipid transport, accumulation, and metabolism. Thus, we expect our probes to offer new opportunities for studying the biological roles of glycosphingolipids.

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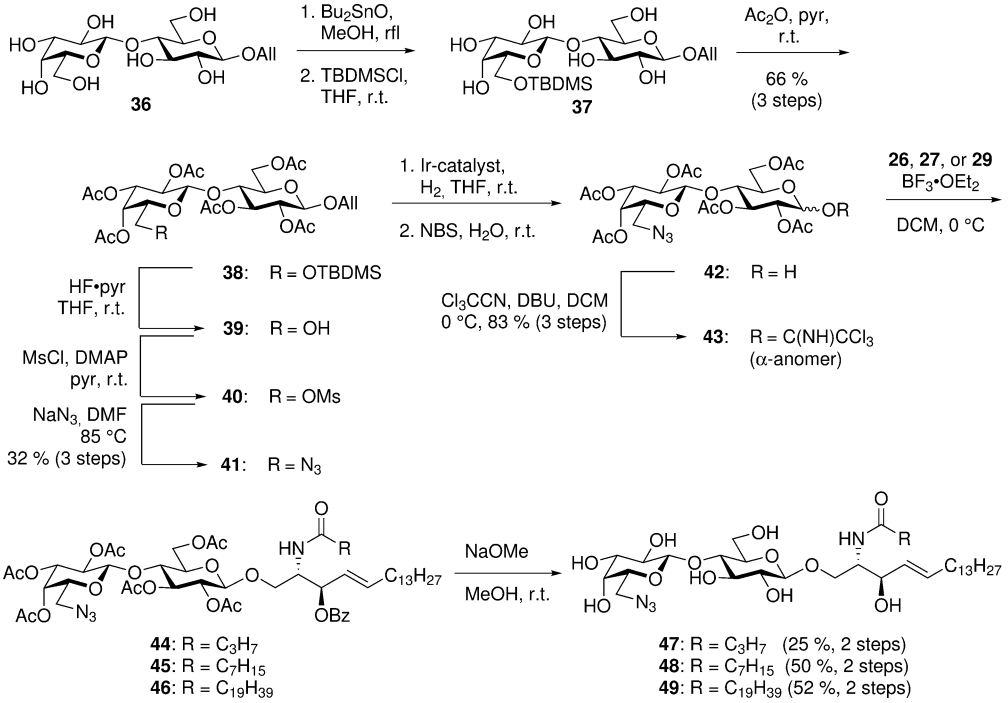
Scheme 4. Synthesis of Azidolactosylceramides 47−49



EXPERIMENTAL PROCEDURES

General. All chemicals were purchased from ABCR, Acros, Merck, or Sigma-Aldrich. AlexaFluor reagents were purchased from Invitrogen. Sphingosine was obtained from Biolab Chemicals. Technical solvents were distilled prior to use. Dry solvents were purchased from Sigma-Aldrich and Acros. Reactions were monitored by TLC on silica gel 60 F254 (Merck) with detection by UV light (λ = 254 nm). Additionally, treatment with cerium reagent (5 g molybdatophosphoric acid, 2.5 g ceric sulfate tetrahydrate, 25 mL sulfuric acid, 225 mL water), or 15% (v/v) sulfuric acid in ethanol, followed by gentle heating, were used for visualization. Preparative flash column chromatography was performed on silica gel Geduran 60 (40−60 μm, Merck). Nuclear magnetic resonance (NMR) spectra were recorded at room temperature on Avance III 400 and Avance III 600 instruments from Bruker. Chemical shifts are reported relative to solvent signals ($CDCl_3$: δ_H = 7.26 ppm, δ_C = 77.16 ppm; DMSO-d_6: δ_H = 2.50 ppm, δ_C = 39.5 ppm; CD_3OD: δ_H = 3.31 ppm, δ_C = 49.0 ppm; D_2O: δ_H = 4.79 ppm). Signals were assigned by first-order analysis and, when feasible, assignments were supported by two-dimensional ^{1}H, ^{1}H and ^{1}H, ^{13}C correlation spectroscopy (COSY, HMBC, NOESY, and HSQC). ESI mass spectra were recorded on an Esquire 3000 plus instrument (Bruker Daltonics) or on an LCMS-2020 (Shimadzu). High-resolution ESI-TOF mass spectra were recorded on a micrOTOF II instrument (Bruker Daltonics). Elemental analyses were performed on a vario EL instrument from Elementar.

Synthesis of Alkyne-Labeled Glucosylceramide 1. *Succinimidyl hept-6-ynoate (**8**).* Hept-6-ynoic acid (500 mg, 3.96 mmol) was dissolved in 20 mL dry DCM. *N*-Hydroxysuccinimide (456 mg, 3.96 mmol) and DCC (817 mg, 3.96 mmol) were added and the mixture was stirred for 16 h at room temperature. The formed precipitate was filtered off. The filtrate was evaporated to yield **8** (822 mg, 93%) as a white solid, which was used without further purification. R_f = 0.52 (petroleum ether/EtOAc 1:1); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.78 (s, 4 H, 2 × NC(O)CH_2), 2.60 (t, J = 7.4 Hz, OC(O)CH_2), 2.20 (td, J = 6.9, 2.6 Hz, 2 H, C$\underline{H}_2$C≡CH), 1.93 (t, J = 2.6 Hz, 1 H, C≡CH), 1.87−1.79 (m, 2 H, C(O)CH_2C$\underline{H}_2$), 1.64−1.56 (m, 2 H, C$\underline{H}_2$$CH_2$C≡CH); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 169.3 (2 × NC(O)), 168.4 (C(O)), 83.6 (C≡CH), 69.1 (C≡$\underline{C}$H), 30.5 (C(O)$\underline{C}H_2$), 27.4 ($\underline{C}H_2CH_2$C≡CH), 25.7 (2 × succinimidyl-CH_2), 23.6 (C(O)$CH_2\underline{C}H_2$), 18.0 ($\underline{C}H_2$C≡CH); ESI-MS calculated $[M + H]^+$ = 224.1, $[M + Na]^+$ = 246.1, found $[M + H]^+$ = 224.2, $[M + Na]^+$ = 246.2; CHN analysis calculated C 59.19, H 5.87, N 6.27, O 28.67, found C 59.91, H 6.35, N 6.53.

*(2S,3S,E)-2-Hept-6-ynamido-3-hydroxy-octadec-4-en-1-yl β-D-glucopyranoside (**1**).* D-*Erythro*-sphingosine (**7**, 250 mg, 0.74 mmol) was dissolved in 15 mL DCM/THF 2:1. Succinimidyl hept-6-ynoate (**8**) (200 mg, 0.89 mmol) and Et_3N (311 μL, 226 mg, 2.23 mmol) were added and the reaction was stirred for 16 h at room temperature. The mixture was diluted with 50 mL DCM and washed with 1× 1 M HCl solution, 2× saturated $NaHCO_3$ solution, and 1× brine. The organic phase was dried with $MgSO_4$ and evaporated under reduced pressure. The crude ceramide intermediate was dissolved in 10 mL dry DCM and stirred with TBDMSCl (840 μL of a 1 M solution in THF) and imidazole (95 mg, 1.4 mmol) for 24 h at room temperature. The mixture was diluted with 20 mL DCM and washed with 1× 1 M HCl solution, 1× saturated $NaHCO_3$ solution, and 1× brine. The organic phase was dried with $MgSO_4$ and evaporated under reduced pressure. The crude product was purified by silica column chromatography (eluent petroleum ether/ethyl acetate 3:1). **10** was obtained as a colorless solid (240 mg, 0.42 mmol, 60% over two steps). Analytical data for intermediate **10**: R_f = 0.29 (petroleum ether/EtOAc 3:1); ^{1}H NMR (250 MHz, $CDCl_3$): δ = 6.24 (d, J = 7.8 Hz, 1 H, NH), 5.81−5.72 (m, 1 H, CH═C$\underline{H}$$CH_2$), 5.55−5.46 (m, 1 H, C$\underline{H}$═$CHCH_2$), 4.16 ('t', J = 4.4 Hz, 1 H, C$\underline{H}$OH), 3.96−3.89 (m, 2 H, C$\underline{H}$NH, CH_2OSi),

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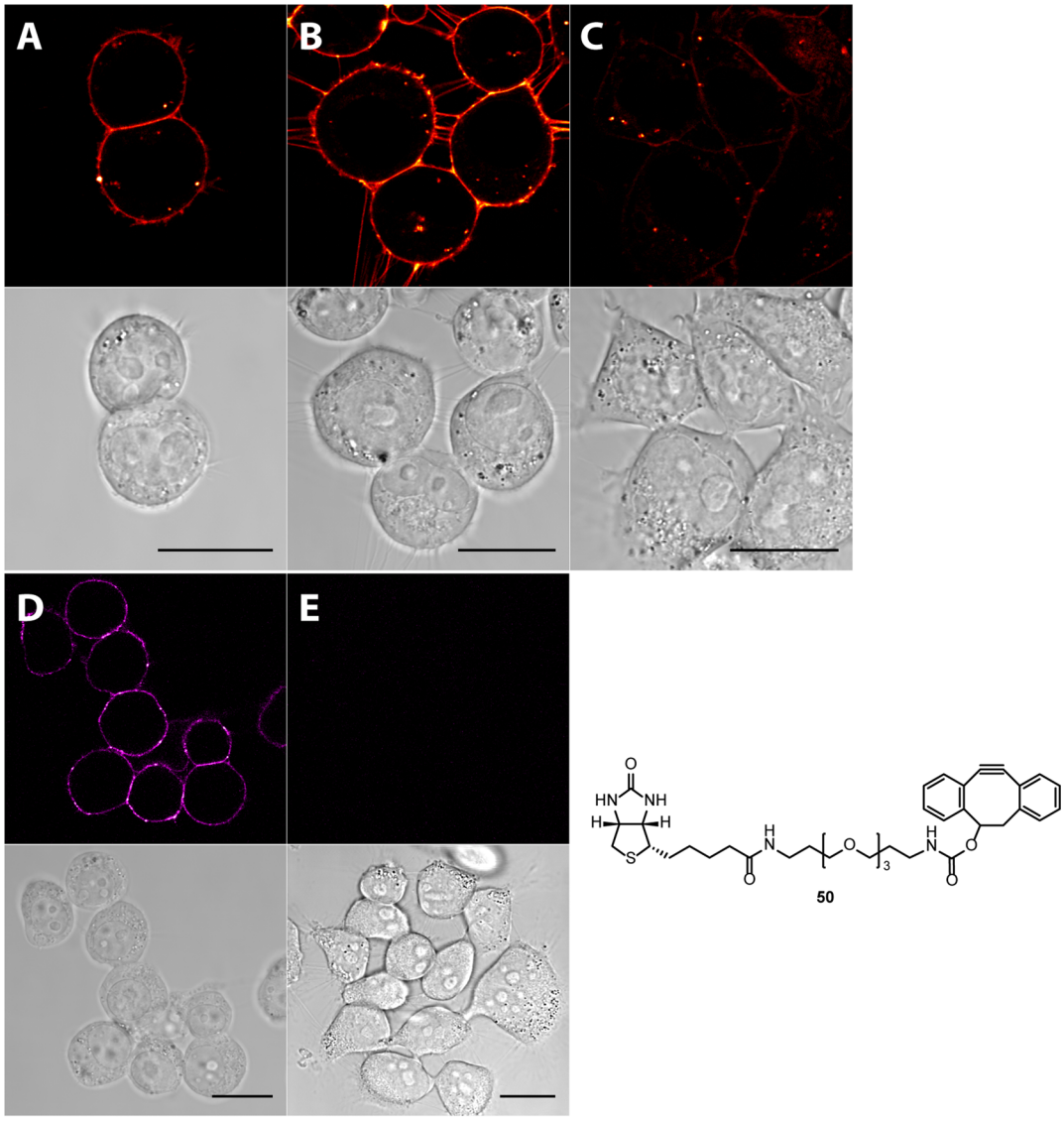

Figure 3. Cell experiments with azide-labeled lactosylceramides **47** and **48**. HEK 293T cells were treated with 10 μM **47** (A), 10 μM **48** (B), or without glycolipid (C) for 30 min at 4 °C followed by labeling with 2 μM DIBO-lissamine **5**. For a two-step labeling procedure, HEK 293T cells were treated with 10 μM (D) or without **48** (E) for 30 min at 4 °C. Then 20 μM DIBO-biotin **50** was added for 30 min at 4 °C followed by labeling with streptavidin-AlexaFluor-647 (15 min, 4 °C). Confocal microscopy was performed at r.t. Scale bar: 20 μm.

3.76−3.73 (m, 1 H, CH_2OSi), 2.28−2.19 (m, 4 H, $C(O)CH_2$, $CH_2C{\equiv}CH$), 2.07−2.02 (m, 2 H, $CH{=}CHCH_2$), 1.94 (t, J = 2.6 Hz, 1 H, $C{\equiv}CH$), 1.80−1.72 (m, 2 H, $CH_2CH_2C{\equiv}CH$), 1.61−1.53 (m, 2 H, $C(O)CH_2CH_2$), 1.38−1.21 (m, 22 H, 11× CH_2), 0.91−0.85 (m, 12 H, CH_2CH_3, $SiC(CH_3)_3$), 0.06 (2 s, 6 H, $Si(CH_3)_2$).

Compound **10** (240 mg, 0.42 mmol) was dissolved in 2 mL dry pyridine and treated with benzoyl chloride (72 μL, 87 mg, 0.62 mmol). The reaction was stirred at room temperature for 2 h, diluted with 20 mL water, and extracted three times with diethyl ether. The combined organic layers were dried with Na_2SO_4 and evaporated under reduced pressure. After purification by silica column chromatography (eluent petroleum ether/ethyl acetate 8:1 to 6:1) **11** was obtained as a slightly yellow, viscous oil (250 mg, 0.39 mmol, 95%). Analytical data for intermediate **11**: R_f = 0.42 (petroleum ether/EtOAc 6:1); ^{1}H NMR (250 MHz, $CDCl_3$): δ = 8.05−8.00 (m, 2 H, aromat.), 7.57−7.52 (m, 1 H, aromat.), 7.47−7.40 (m, 2 H, aromat.), 5.84−5.79 (m, 1 H, $CH{=}CHCH_2$), 5.58−5.50 (m, 2 H, $CH{=}CHCH_2$, CHOBz), 4.43−4.33 (m, 1 H, CHNH), 3.83 (dd, J = 3.0, 10.2 Hz, 1 H, CH_2OSi), 3.65 (dd, J = 4.1, 10.2 Hz, 1 H, CH_2OSi), 2.25−2.17 (m, 4 H, $C(O)CH_2$, $CH_2C{\equiv}CH$), 2.06−1.98 (m, 2 H, $CH{=}CHCH_2$), 1.93 (t, J = 2.6 Hz, 1 H, $C{\equiv}CH$), 1.78−1.72 (m, 2 H, CH_2), 1.62−1.53 (m, 2 H, CH_2), 1.23 (br, 22 H, 11× CH_2), 0.91−0.85 (m, 12 H, CH_2CH_3, $SiC(CH_3)_3$), 0.00 (s, 3 H, $SiCH_3$), −0.01 (s, 3 H, $SiCH_3$).

Compound **11** (250 mg, 0.39 mmol) was dissolved in 2 mL THF and treated with 40 μL HF·pyridine (approximately 70% HF). The reaction was stirred for 16 h at room temperature, diluted with 10 mL DCM, and washed three times with saturated $NaHCO_3$ solution. The organic phase was dried with $MgSO_4$ and evaporated under reduced pressure. After silica column chromatography (eluent petroleum ether/ethyl acetate 3:2 to 2:3), compound **12** was obtained as a colorless solid (102 mg, 0.19 mmol, 50%). Analytical data for intermediate **12**: R_f = 0.22 (petroleum ether/EtOAc 1:1); ^{1}H NMR (250 MHz, $CDCl_3$): δ = 8.04−8.01 (m, 2 H, aromat.), 7.62−7.56 (m, 1 H,

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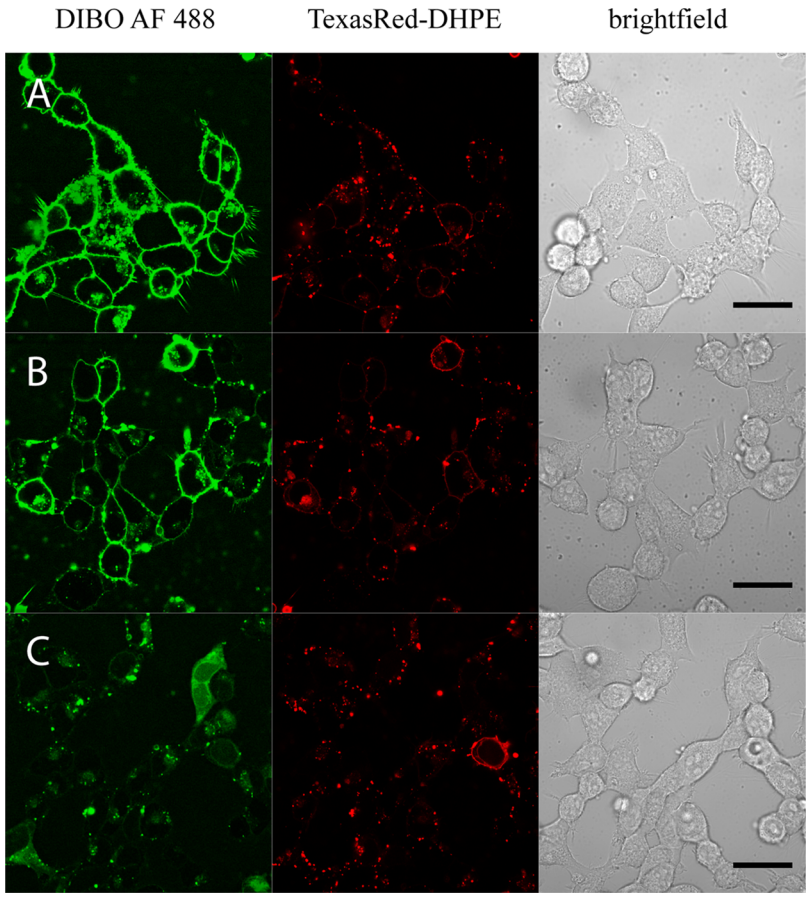

Figure 4. Cell experiments with azide-labeled glucosylceramide **3** and **49** using fusogenic liposomes. HEK 293T cells were treated with **3** (A), **49** (B), or without glycolipid (C) in fusogenic liposomes (DOTAP/DOPE/TexasRed-DHPE 1:1:0.05) for 10 min at 37 °C followed by labeling with DIBO-AlexaFluor-488 (2 μM, 30 min, rt). Scale bar: 30 μm.

aromat.), 7.48−7.42 (m, 2 H, aromat.), 6.19 (d, J = 8.9 Hz, NH), 5.91−5.80 (m, 1 H, CH═C<u>H</u>CH$_2$), 5.64−5.49 (m, 2 H, C<u>H</u>═CHCH$_2$, CHOBz), 4.33−4.22 (m, 1 H, C<u>H</u>NH), 3.78−3.65 (m, 2 H, C<u>H</u>$_2$OH), 2.26−2.16 (m, 4 H, C(O)CH$_2$, C<u>H</u>$_2$C≡CH), 2.07−1.99 (m, 3 H, OH, CH═CHC<u>H</u>$_2$), 1.94 (t, J = 2.6 Hz, C≡CH), 1.80−1.68 (m, 2 H, CH$_2$), 1.63−1.49 (m, 2 H, CH$_2$), 1.40−1.18 (m, 22 H, 11× CH$_2$), 0.94−0.84 (m, 3 H, CH$_3$).

Ceramide derivative **12** (102 mg, 0.194 mmol) and trichloroacetimidate **13**[47] (111 mg, 0.233 mmol) were dissolved in 1 mL dry DCM. After addition of $BF_3{\cdot}OEt_2$ (48 μL, 55 mg, 0.388 mmol) the mixture was stirred for 2.5 h at room temperature. The mixture was diluted with DCM and washed once each with 1 M HCl solution and saturated $NaHCO_3$ solution. The organic phase was dried with $MgSO_4$ and the solvent was removed under reduced pressure. After purification by silica column chromatography (eluent toluene/acetone 9:1), **14** was obtained as a colorless solid (72 mg, 84 μmol, 44%). Analytical data for intermediate **14**: R_f = 0.54 (toluene/acetone 4:1); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.02 (d, J = 7.9 Hz, 2 H, aromat.), 7.56 (t, J = 7.5 Hz, 1 H, aromat.), 7.44 (t, J = 7.5 Hz, 2 H, aromat.), 5.92−5.84 (m, 2 H, CH═C<u>H</u>CH$_2$, NH), 5.55 ('t', J = 6.9 Hz, 1 H, CHOBz), 5.47 (dd, J = 7.2, 15.1 Hz, 1 H, C<u>H</u>═CHCH$_2$), 5.19 ('t', J = 9.5 Hz, 1 H, H-3), 5.04 ('t', J = 9.8 Hz, H-4), 4.96 ('t', J = 8.8 Hz, 1 H, H-2), 4.50−4.45 (m, 2 H, C<u>H</u>NH, H-1), 4.13 (dd, J = 4.3, 12.2 Hz, 1 H, H-6a), 4.05−3.97 (m, 2 H, H-6b, CH$_2$OGlc), 3.71−3.62 (m, 2 H, CH$_2$OGlc, H-5), 2.21−2.18 (m, 4 H, C(O)CH$_2$, C<u>H</u>$_2$C≡CH), 2.06−2.00 (m, 11 H, 3× C(O)CH$_3$, CH═CHC<u>H</u>$_2$), 1.97 (s, 3 H, C(O)CH$_3$), 1.96−1.94 (m, 1 H, C≡CH), 1.82−1.70 (m, 2 H, CH$_2$), 1.59−1.51 (m, 2 H, CH$_2$), 1.36−1.18 (m, 22 H, 11× CH$_2$), 0.87 (t, J = 6.5 Hz, 3 H, CH$_3$); ESI-MS: calculated $[M + H]^+$ = 842.5, $[M + Na]^+$ = 864.5, found $[M + H]^+$ = 842.3, $[M + Na]^+$ = 864.4. Protected glycolipid **14** (72 mg, 84 μmol) was dissolved in 3 mL dry MeOH and treated with 42 μL of a 1 M solution of NaOMe in MeOH. The reaction was stirred for 16 h at room temperature. After neutralization with strongly acidic ion-exchange resin, the solvent was removed under reduced pressure. The crude product was purified by silica column chromatography (eluent DCM/MeOH 9:1). Glucosylceramide **1** was obtained as a colorless solid (35 mg, 60 μmol, 71%). R_f = 0.22 (DCM/MeOH 9:1); ^{1}H NMR (400 MHz, CD_3OD): δ = 7.86 (d, J = 9.1 Hz, 1 H, NH), 5.70 (dt, J = 15.3, 6.7 Hz, 1 H, CH═C<u>H</u>CH$_2$), 5.49−5.43 (m, 1 H, C<u>H</u>═CHCH$_2$), 4.26 (d, J = 7.7 Hz, 1 H, H-1), 4.13 (dd, J = 10.1, 5.2 Hz, 1 H, CH$_2$OGlc), 4.08 (t, J = 7.8 Hz, 1 H), 4.03−3.97 (m, 1 H, C<u>H</u>NH), 3.87 (dd, J = 11.8, 1.8 Hz, 1 H, H-6a), 3.68−3.61 (m, 2 H, CH$_2$OGlc, H-6b), 3.40−3.34 (m, 1 H), 3.29−3.26 (m, 2 H), 3.21 (dd, J = 9.2, 7.7 Hz, 1 H), 2.23−2.17 (m, 5 H, C≡CH, C(O)CH$_2$, CH═CHC<u>H</u>$_2$), 2.06−2.01 (m, 2 H, CH$_2$), 1.75−1.66 (m, 2 H, CH$_2$), 1.57−1.49 (m, 2 H, CH$_2$), 1.41−1.25 (s, 22 H, 11× CH$_2$), 0.90 (t, J = 6.7 Hz, 3 H, CH$_3$); ^{13}C NMR (101 MHz, CD_3OD): δ = 175.6 (C(O)), 135.0 (CH═<u>C</u>HCH$_2$), 131.2 (<u>C</u>H═CHCH$_2$), 104.7 (C1), 84.7 (<u>C</u>≡CH), 78.0, 77.9, 75.2, 73.1, 71.6, 69.9 (C≡<u>C</u>H), 69.8 (CH$_2$OGlc), 62.7, 54.9 (<u>C</u>HNH), 36.7 (C(O)CH$_2$), 33.4 (CH═CH<u>C</u>H$_2$),

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33.1, 30.8−30.7 (m), 30.5−30.4 (m), 29.2, 26.2, 23.8, 18.9 (CH_2), 14.4 (CH_3); ESI-MS: calculated $[M + H]^+$ = 570.4, $[M + Na]^+$ = 592.4, found $[M + H]^+$ = 570.2, $[M + Na]^+$ = 592.2; HR-ESI-MS: calculated $[M + H]^+$ = 570.40004, found $[M + H]^+$ = 570.39922.

Synthesis of Azidoglucosylceramide 2. *2,3,4-Tri-O-acetyl-6-azido-6-deoxy-D-glucopyranose (**32**).* Glucose derivative **31**[48] was subjected to anomeric deprotection following the procedure described by Zhang and Kováč.[49] Ethylenediamine (352 μL, 315 mg, 5.25 mmol) was dissolved in 60 mL THF. Acetic acid (355 μL, 372 mg, 6.2 mmol) was added dropwise. **31** (1.78 g, 4.77 mmol) was added and the mixture was stirred for 3 days at room temperature. The solvent was removed under reduced pressure. The remaining material was dissolved in 100 mL DCM and extracted twice with 50 mL each of 1 M HCl solution, saturated $NaHCO_3$ solution, and brine. The organic phase was dried with $MgSO_4$ and the solvent was removed under reduced pressure. The crude product was purified by silica column chromatography (eluent petroleum ether/ethyl acetate 2:1 to 1:1). **32** was obtained as a colorless solid (1.35 g, 4.07 mmol, 85%; mixture of isomers α/β = 3:1). R_f = 0.35 (petroleum ether/EtOAc 1:1); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.48 ('t', J = 9.8 Hz, 1 H, H-3$^\alpha$), 5.43 (d, J = 3.6 Hz, 1 H, H-1$^\alpha$), 5.19 ('t', J = 9.5 Hz, 1 H, H-3$^\beta$), 5.01−4.93 (m, 2 H, H-4$^\beta$, H-4$^\alpha$), 4.88−4.83 (m, 1 H, H-2$^\beta$), 4.83 (dd, J = 3.6, 10.2 Hz, 1 H, H-2$^\alpha$, 4.73 (d, J = 8.5 Hz, 1 H, H-1$^\beta$), 4.19 (ddd, J = 3.1, 5.8, 9.9 Hz, 1 H, H-5$^\alpha$), 3.67 (ddd, J = 3.0, 5.9, 9.8 Hz, 1 H, H-5$^\beta$), 3.35−3.32 (m, 2 H, H-6a/b$^\beta$), 3.30−3.24 (m, 2 H, H-6a/b$^\alpha$), 2.04 (m, 6 H, 2× C(O)CH_3), 1.99 (m, 6 H, 2× C(O)CH_3), 1.97 (m, 6 H, 2× C(O)CH_3); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 171.5, 170.7 (2× C(O)$^\beta$), 170.4, 170.3, 169.9 (3× C(O)$^\alpha$), 169.7 (C(O)$^\beta$), 95.5 (C1$^\beta$), 90.1 (C1$^\alpha$), 73.20 (C5$^\beta$), 73.17 (C2$^\beta$), 72.4 (C3$^\beta$), 71.3 (C2$^\alpha$), 69.87, 69.82 (C4$^\alpha$, C3$^\alpha$), 69.7 (C4$^\beta$), 68.4 (C5$^\alpha$), 51.2 (C6$^\alpha$), 51.1 (C6$^\beta$), 21.1 (CH_3^β), 20.75, 20.73, 20.69 (3× CH_3^α), 20.65, 20.64 (2× CH_3^β); ESI-MS: calculated $[M + H]^+$ = 332.1, found $[M + H]^+$ = 332.2.

*2,3,4-Tri-O-acetyl-6-azido-6-deoxy-α-D-glucopyranosyl trichloroacetimidate (**33**).* **32** (1.2 g, 3.6 mmol) and trichloroacetonitrile (4.33 mL, 6.24 g, 43.2 mmol) were dissolved in 10 mL dry DCM at room temperature and treated with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, 135 μL, 138 mg, 0.91 mmol). TLC showed complete reaction after 5 min. The mixture was filtered over a small portion of silica gel. The filtrate was evaporated under reduced pressure. The remaining material was purified by silica column chromatography (eluent: petroleum ether/ethyl acetate 3:2). **33** was obtained as a slightly yellowish solid (1.29 g, 2.91 mmol, 81%). R_f = 0.47 (petroleum ether/EtOAc 1:1); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.72 (s, 1 H, NH), 6.58 (d, J = 3.7 Hz, 1 H, H-1), 5.55 ('t', J = 9.8 Hz, 1 H, H-3), 5.16−5.09 (m, 2 H, H-2, H-4), 4.18 (ddd, J = 2.7, 5.5, 10.1 Hz, 1 H, H-5), 3.40 (dd, J = 2.7, 13.6 Hz, 1 H, H-6a), 3.32 (dd, J = 5.5, 13.6 Hz, 1 H, H-6b), 2.05 (s, 3 H, C(O)CH_3), 2.03 (s, 3 H, C(O)CH_3), 2.01 (s, 3 H, C(O)CH_3); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 170.1, 170.0, 169.6 (3× C(O)), 160.8 (C═NH), 92.8 (C1), 90.8 (CCl_3), 71.3 (C5), 69.83 (C3), 69.82, 69.1 (C2, C4), 50.8 (C6), 20.8, 20.7, 20.6 (3× C(O)$\underline{C}H_3$); ESI-MS: calculated $[M + Na]^+$ = 497.0, found $[M + Na]^+$ = 496.9; CHN analysis: calculated C 35.35, H 3.60, N 11.78, O 26.91, Cl 22.36, found, C 34.55, H 4.02, N 11.51.

*N-((2S,3R,E)-3-Hydroxy-1-(trityloxy)octadec-4-en-2-yl)-butyramide (**19**).* 200 mg (0.52 mmol) ceramide **15** and 174 mg (0.63 mmol) trityl chloride were dissolved in 3 mL of a mixture of pyridine, THF and DCM (1:1:1). The mixture was stirred for 36 h at room temperature. The solvent was removed under reduced pressure and the crude product was purified by silica chromatography (eluent petroleum ether/ethyl acetate 2:1). **19** was obtained as a colorless solid (275 mg, 0.44 mmol, 84%). R_f = 0.41 (petroleum ether/EtOAc 1:1); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.35−7.32 (m, 6 H, aromat.), 7.26−7.16 (m, 9 H, aromat.), 6.03 (d, J = 8.0 Hz, 1 H, NH), 5.61−5.53 (m, 1 H, CH═C$\underline{H}$CH$_2$), 5.22−5.17 (m, 1 H, C$\underline{H}$═CHCH$_2$), 4.14−4.09 (m, 1 H, C$\underline{H}$OH), 4.03−3.98 (m, 1 H, C$\underline{H}$NH), 3.32 (dd, J = 9.7, 3.7 Hz, 1 H, CH$_2$OTrt), 3.24 (dd, J = 9.7, 4.0 Hz, 1 H, CH$_2$OTrt), 2.13 (t, J = 7.4 Hz, 2 H, C(O)CH_2), 1.88−1.81 (m, 2 H, CH═CHC$\underline{H}_2$), 1.66−1.57 (m, 2 H, C(O)$CH_2\underline{C}H_2$), 1.25−1.14 (m, 22 H, 11x CH_2), 0.91 (t, J = 7.4 Hz, 3 H, butyl-CH_3), 0.81 (t, J = 6.8 Hz, 3 H, sphingosine-CH_3); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 173.3 (C(O)), 143.4 (C aromat.), 133.6 (CH═$\underline{C}$HCH$_2$), 128.8 ($\underline{C}$H═CHCH$_2$), 128.6, 128.1, 127.4 (3x C aromat.), 87.5 (CPh_3), 74.5 (COH), 63.2 (COTrt), 53.4 (CNH), 38.9 (C(O)$\underline{C}H_2$), 32.3 (CH═CH$\underline{C}$H$_2$), 32.1, 29.83 (br), 29.79, 29.76, 29.65, 29.5, 29.4, 29.2, 22.8 (9× CH_2), 19.3 (C(O)$CH_2\underline{C}H_2$), 14.3, 14.0 (2× CH_3); ESI-MS: calculated $[M + H]^+$ = 612.4, $[M + Na]^+$ = 634.4, found $[M + H]^+$ = 612.6, $[M + Na]^+$ = 634.5.

*(2S,3R,E)-2-Butyramido-1-(trityloxy)octadec-4-en-3-yl benzoate (**23**).* 275 mg (0.44 mmol) ceramide **19** and 124 mg (0.88 mmol) benzoyl chloride were dissolved in 5 mL toluene/pyridine 4:1. The reaction was stirred for 16 h at room temperature. The solvent was removed under reduced pressure. The remaining material was dissolved in a small amount of DCM and was washed with 2× 1 M HCl solution and 2× saturated $NaHCO_3$ solution. The organic phase was dried with $MgSO_4$ and evaporated under reduced pressure. The crude product was purified by silica chromatography (eluent: petroleum ether/ethyl acetate 4:1). **23** was obtained as a colorless solid (307 mg, 0.43 mmol, 97%). R_f = 0.59 (petroleum ether/EtOAc 1:1); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.18−8.16 (m, 3 H, aromat.), 7.95−7.93 (m, 2 H, aromat.), 7.40−7.37 (m, 6 H, aromat.), 7.23−7.17 (m, 9 H, aromat.), 5.92−5.85 (m, 1 H, CH═C$\underline{H}$CH$_2$), 5.73−5.67 (m, 2 H, CHOBz, NH), 5.48−5.42 (m, 1 H, C$\underline{H}$═CHCH$_2$), 4.53−4.47 (m, 1 H, C$\underline{H}$NH), 3.45 (dd, J = 9.5, 3.5 Hz, 1 H, C$\underline{H}_2$OTrt), 3.20 (dd, J = 9.5, 4.1 Hz, 1 H, C$\underline{H}_2$OTrt), 2.09 (t, J = 7.2 Hz, 2 H, C(O)CH_2), 2.02−1.97 (m, 2 H, CH═CHC$\underline{H}_2$), 1.64−1.57 (m, 2 H, C(O)$CH_2\underline{C}H_2$), 1.33−1.18 (m, 22 H, 11× CH_2), 0.92 (t, J = 7.4 Hz, 3 H, butyl-CH_3), 0.88 (t, J = 6.9 Hz, 3 H, sphingosine-CH_3); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 172.4, 162.5 (2× C(O)), 143.6 ($\underline{C}$ aromat.), 137.4 (CH═$\underline{C}$HCH$_2$), 134.7, 133.1, 130.7, 129.8 (m), 129.0, 128.7, 128.5, 128.1, 128.0, 127.2 ($\underline{C}$ aromat.), 125.2 ($\underline{C}$H═CHCH$_2$), 87.0 (CPh_3), 74.5 (COBz), 60.5 (COTrt), 51.2 (CNH), 39.0 (C(O)$\underline{C}H_2$), 32.1, 29.81 (m), 29.80, 29.7, 29.6, 29.5, 29.4, 29.0, 22.8, 21.2 (CH_2), 19.3 (C(O)$CH_2\underline{C}H_2$), 14.3, 13.9 (2× CH_3).

*(2S,3R,E)-2-Butyramido-1-hydroxyoctadec-4-en-3-yl benzoate (**27**).* 307 mg (0.43 mmol) ceramide **23** were dissolved in a mixture of 1 mL dry toluene and 80 μL dry MeOH, cooled to 0 °C and treated with 158 μL (182 mg, 1.28 mmol) BF_3·OEt_2. After stirring the mixture for 20 min at 0 °C, 20 mL DCM were added and the mixture was extracted once with saturated $NaHCO_3$ solution. The organic phase was dried with $MgSO_4$ and the solvent was removed under reduced pressure. The remaining material was purified by silica chromatography (eluent petroleum ether/ethyl acetate 1:1, then 2:3). **27** was obtained as a colorless solid (132 mg, 0.28 mmol, 65%). R_f =

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0.15 (petroleum ether/EtOAc 1:1); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.04−8.02 (m, 2 H, aromat.), 7.61−7.56 (m, 1 H, aromat.), 7.47−7.43 (m, 2 H, aromat.), 6.13 (d, J = 8.7 Hz, 1 H, NH), 5.85 (dt, J = 14.6, 6.7 Hz, 1 H, CH═C$\underline{H}$CH$_2$), 5.64−5.56 (m, 1 H, C$\underline{H}$═CHCH$_2$), 5.57−5.51 (m, 1 H, CHOBz), 4.32−4.26 (m, 1 H, C$\underline{H}$NH), 3.74 (dd, J = 12.0, 3.8 Hz, 1 H, C$\underline{H}_2$OH), 3.69 (dd, J = 12.0, 3.2 Hz, 1 H, C$\underline{H}_2$OH), 2.74 (br s, 1 H, OH), 2.21−2.13 (m, 2 H, C(O)CH$_2$), 2.06−2.01 (m, 2 H, CH═CHC$\underline{H}_2$), 1.69−1.60 (m, 2 H, C(O)CH$_2$C$\underline{H}_2$), 1.38−1.19 (m, 22 H, 11× CH$_2$), 0.93 (t, J = 7.4 Hz, 3 H, butyl-CH$_3$), 0.87 (t, J = 6.8 Hz, 3 H, sphingosine-CH$_3$); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 173.4, 166.7 (2× C(O)), 137.7 (CH═$\underline{C}$HCH$_2$), 133.6, 129.9, 128.7 (3× C aromat.), 125.0 ($\underline{C}$H═CHCH$_2$), 74.8 (CHOBz), 62.0 (CH$_2$OH), 53.5 (CHNH), 38.9 (C(O)$\underline{C}$H$_2$), 32.4 (CH═CH$\underline{C}$H$_2$), 32.1, 29.81, 29.80, 29.7, 29.6, 29.5, 29.3, 29.0 (CH$_2$), 22.8 (sphingosine-CH$_3$), 19.2 (butyl-CH$_3$); ESI-MS: calculated [M + H]$^+$ = 474.4, [M + Na]$^+$ = 496.4, found [M + H]$^+$ = 474.7, [M + Na]$^+$ = 496.6.

*(2S,3S,E)-2-Butyramido-3-(benzoyloxy)-octadec-4-en-1-yl 2,3,4-tetra-O-acetyl-6-azido-6-deoxy-β-D-glucopyranoside (**34**).* Ceramide **27** (132 mg, 0.28 mmol) and trichloroacetimidate **33** (159 mg, 0.33 mmol) were dissolved in 1 mL dry DCM and cooled to 0 °C. After addition of BF_3·OEt_2 (68 μL, 79 mg, 0.56 mmol), the reaction was stirred for 2 h at 0 °C, diluted with 20 mL DCM and washed with 10 mL saturated $NaHCO_3$ solution. The organic phase was dried with $MgSO_4$ and evaporated under reduced pressure. The crude product was purified by silica column chromatography (eluent toluene/acetone 9:1 to 4:1). **34** was obtained as a colorless solid (125 mg, 0.16 mmol, 57%). R_f = 0.45 (toluene/acetone 4:1); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.10 (d, J = 7.3 Hz, 2 H, aromat.), 7.63 ('t', J = 7.4 Hz, 1 H, aromat.), 7.51 ('t', J = 7.7 Hz, 2 H, aromat.), 5.97−5.87 (m, 2 H, CH═C$\underline{H}$CH$_2$, NH), 5.61 ('t', J = 6.8 Hz, 1 H, CHOBz), 5.54 (dd, J = 15.2, 7.4 Hz, 1 H, C$\underline{H}$═CHCH$_2$), 5.25 ('t', J = 9.5 Hz, 1 H, H-3), 5.03−4.98 (m, 2 H, H-2, H-4), 4.60−4.53 (m, 1 H, C$\underline{H}$NH), 4.59 (d, J = 7.9 Hz, 1 H, H-1), 4.12 (dd, J = 10.0, 4.5 Hz, 1 H, CH$_2$OGlc), 3.78 (dd, J = 10.1, 4.1 Hz, 1 H, CH$_2$OGlc), 3.72 (ddd, J = 9.7, 6.7, 2.8 Hz, 1 H, H-5), 3.31 (dd, J = 13.4, 6.7 Hz, 1 H, H-6a), 3.24 (dd, J = 13.4, 2.8 Hz, 1 H, H-6b), 2.24−2.18 (m, 2 H, C(O)CH$_2$), 2.11−2.06 (m, 11 H, CH═CHC$\underline{H}_2$, 3× C(O)-CH$_3$), 1.74−1.65 (m, 2 H, C(O)CH$_2$C$\underline{H}_2$), 1.42−1.27 (m, 22 H, 11× CH$_2$), 1.00 (t, J = 7.4 Hz, 3 H, butyl-CH$_3$), 0.94 (t, J = 6.8 Hz, 3 H, sphingosine-CH$_3$); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 170.3, 169.7, 169.6, 165.6, 163.8 (5× C(O)), 137.6 (CH═$\underline{C}$HCH$_2$), 133.2, 130.3, 129.8, 129.1 (4× C aromat.), 124.6 ($\underline{C}$H═CHCH$_2$), 100.4 (C1), 74.6 (COBz), 73.7 (C5), 72.5 (C3), 71.4, 69.6 (C2, C4), 67.5 (CH$_2$OGlc), 51.0 (C6), 50.9 (CNH), 38.8 (C(O)CH$_2$), 32.4 (CH═CH$\underline{C}$H$_2$), 32.0, 29.8−29.7 (m), 29.6, 29.5, 29.3, 29.0, 22.8, 20.7, 19.2 (CH$_2$), 14.2 (sphingosine-CH$_3$), 13.8 (butyl-CH$_3$); ESI-MS: calculated [M + Na]$^+$ = 808.4, found [M + Na]$^+$ = 808.6.

*(2S,3S,E)-2-Butyramido-3-hydroxy-octadec-4-en-1-yl 6-azido-6-deoxy-β-D-glucopyranoside (**2**).* Glycolipid **34** (125 mg, 0.16 mmol) was dissolved in 1 mL dry MeOH and treated with 48 μL of a 0.5 M solution of NaOMe in MeOH. The reaction was stirred for 90 min at room temperature and then neutralized with strongly acidic ion-exchange resin. The solvent was removed under reduced pressure and the remaining material was purified by silica column chromatography (eluent DCM/MeOH 9:1). **2** was obtained as a colorless solid (47 mg, 84 μmol, 53%). R_f = 0.20 (DCM/MeOH 9:1); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 6.54 (d, J = 7.8 Hz, 1 H, NH), 5.75 (dt, J = 14.8, 6.8 Hz, 1 H, CH═C$\underline{H}$CH$_2$), 5.45 (dd, J = 15.5, 6.1 Hz, 1 H, C$\underline{H}$═CHCH$_2$), 4.35 (d, J = 7.5 Hz, 1 H, H-1), 4.21−4.15 (m, 2 H, C$\underline{H}$NH, C$\underline{H}$OH), 4.02−3.98 (m, 1 H, CH$_2$OGlc), 3.76−3.71 (m, 1 H, CH$_2$OGlc), 3.63−3.33 (m, 9 H, H-2, H-3, H-4, H-5, H-6a/b, OH), 2.22 (t, J = 7.3 Hz, C(O)CH$_2$), 2.07−1.98 (m, 2 H, CH═CHC$\underline{H}_2$), 1.69−1.60 (m, 2 H, C(O)-CH$_2$C$\underline{H}_2$), 1.39−1.19 (m, 22 H, 11× CH$_2$), 0.95 (t, J = 7.4 Hz, 3 H, butyl-CH$_3$), 0.88 (t, J = 6.8 Hz, 3 H, sphingosine-CH$_3$); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 174.8 (C(O)), 135.0 (CH═$\underline{C}$HCH$_2$), 128.2 ($\underline{C}$H═CHCH$_2$), 103.0 (C1), 76.4, 75.7, 73.5 (C2), 73.3 (CHNH$\underline{C}$HOH), 71.2, 69.3 (CH$_2$OGlc), 53.4 (CHNH), 51.7 (C6), 38.8 (C(O)$\underline{C}$H$_2$), 32.5 (CH═CH$\underline{C}$H$_2$), 32.1, 29.87, 29.82, 29.7, 29.5, 29.3, 22.8, 19.4 (CH$_2$), 14.3, 13.9 (2× CH$_3$); ESI-MS: calculated [M + H]$^+$ = 557.4, [M + Na]$^+$ = 579.4, [M-H]$^-$ = 555.4 found [M + H]$^+$ = 557.2, [M + Na]$^+$ = 579.2, [M-H]$^-$ = 555.2; HR-ESI-MS: calculated [M + H]$^+$ = 557.39088; found [M + H]$^+$ = 557.38981.

For synthesis and analytical data of azidoglucosylceramide **3**, see Supporting Information.

Synthesis of Azidolactosylceramide 47. *Allyl 2,3,4-tri-O-acetyl-6-O-tert-butyldimethylsilyl-β-D-galactopyranosyl-(1 → 4)-2,3,6-tri-O-acetyl-β-D-glucopyranoside (**38**).* To obtain a lactose derivative with an orthogonal protecting group at the 6′-position, a procedure reported by Glen et al.[59] was followed with modifications. Allyl lactoside **36**[62] (12 g, 31.4 mmol) and dibutyltin(IV) oxide (7.97 g, 32 mmol) were dissolved in 700 mL dry MeOH and refluxed for 4 h. The mixture was evaporated under reduced pressure and dried thoroughly at vacuum. 500 mL dry THF and *tert*-butyldimethylsilyl chloride (5.06 g, 33.4 mmol) were added and the mixture was stirred for 3 days at room temperature. Approximately 20 mL of the solution was taken out and evaporated. The obtained material was subjected to silica column chromatography (eluent DCM/MeOH 9:1), which caused slow decomposition of the product. However, enough of **37** was obtained to be characterized by NMR. The major part of the silylation reaction was treated with 200 mL each of acetic anhydride and pyridine and stirred for 16 h at room temperature. The solvent was removed under reduced pressure and the remaining material was purified by silica chromatography (eluent petroleum ether/ethyl acetate 3:2 to 1:1). **38** was obtained as a colorless solid (14.6 g, 20.7 mmol, 66%). Analytical data of allyl 6-*O*-*tert*-butyldimethylsilyl-β-D-galactopyranosyl-(1 → 4)-β-D-glucopyranoside (**37**): R_f = 0.14 (DCM/MeOH 9:1); ^{1}H NMR (400 MHz, CD_3OD): δ = 6.02−5.90 (m, 1 H, CH$_2$C$\underline{H}$═CH$_2$), 5.37−5.29 (m, 1 H, CH$_2$CH═C$\underline{H}_2$), 5.18−5.14 (m, 1 H, CH$_2$CH═C$\underline{H}_2$), 4.37 (d, J = 7.5 Hz, 1 H, anomeric H), 4.40−4.31 (m, 1 H, C$\underline{H}_2$CH═CH$_2$), 4.33 (d, J = 7.8 Hz, 1 H, anomeric H), 4.18−4.10 (m, 1 H, C$\underline{H}_2$CH═CH$_2$), 3.89−3.76 (m, 5 H), 3.63−3.47 (m, 6 H), 3.42−3.37 (m, 1 H), 0.92 (s, 9 H, SiC(CH$_3$)$_3$), 0.11 (s, 6 H, 2× SiCH$_3$); ^{13}C NMR (101 MHz, CD_3OD): δ = 135.8 ($\underline{C}$H═CH$_2$), 117.6 (CH═$\underline{C}$H$_2$), 105.3, 103.4 (C1, C1′), 80.9, 76.9, 76.51, 76.45, 75.0, 74.9, 72.6, 71.2, 69.8, 63.1, 62.0 (C2−C6, C2′-C6′), 26.6 (SiC($\underline{C}$H$_3$)$_3$), 22.0 (Si$\underline{C}$(CH$_3$)$_3$), −5.1, −5.2 (2× SiCH$_3$). Analytical data of allyl 2,3,4-tri-*O*-acetyl-6-*O*-*tert*-butyldimethylsilyl-β-D-galactopyranosyl-(1 → 4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside (**38**): R_f = 0.39 (petroleum ether/EtOAc 1:1); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.86−5.76 (m, 1 H, CH$_2$C$\underline{H}$═CH$_2$), 5.42 ('d', J = 3.2 Hz, 1 H, H-4′), 5.26−5.20 (m, 1 H, CH$_2$CH═C$\underline{H}_2$), 5.17 ('t', J = 9.3 Hz, 1 H, H-3), 5.19−5.15 (m, 1 H, CH$_2$CH═C$\underline{H}_2$), 5.06 (dd, J = 10.4, 7.8 Hz, 1 H, H-2′), 4.96 (dd, J = 10.4, 3.4 Hz, 1 H, H-3′), 4.89 (dd, J = 9.5, 8.0 Hz, 1 H, H-2), 4.49 (d, J = 8.0 Hz, 1 H, H-1), 4.48−

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4.43 (m, 1 H, H-6a), 4.44 (d, J = 7.7 Hz, 1 H, H-1′), 4.28 (ddt, J = 13.2, 4.9, 1.5 Hz, 1 H, OC$\underline{H}_2$CH=CH_2), 4.13−4.01 (m, 2 H, H-6b, OC$\underline{H}_2$CH=CH_2), 3.79 ('t', J = 9.5 Hz, 1 H, H-4), 3.69−3.62 (m, 2 H, H-5′, H-6a′), 3.59−3.51 (m, 2 H, H-5, H-6b′), 2.10 (s, 3 H, C(O)CH_3), 2.10 (s, 3 H, C(O)CH_3), 2.02 (s, 3 H, C(O)CH_3), 2.02 (s, 3 H, C(O)CH_3), 2.01 (s, 3 H, C(O)CH_3), 1.94 (s, 3 H, C(O)CH_3), 0.83 (s, 9 H, SiC$(CH_3)_3$), 0.00 (s, 3 H, $SiCH_3$), −0.02 (s, 3 H, $SiCH_3$); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 170.6, 170.3, 170.1, 169.9, 169.8, 169.4 (6× $\underline{C}$(O)CH_3), 133.4 ($CH_2\underline{C}H$=CH_2), 117.7 (CH_2CH=$\underline{C}H_2$), 101.1 (C1′), 99.4 (C1), 76.1 (C4), 73.6 (C5′), 73.1 (C3), 72.7 (C5), 71.8 (C2), 71.4 (C3′), 70.1 ($\underline{C}H_2CH$=CH_2), 69.6 (C2′), 66.7 (C4′), 62.1 (C6), 60.1 (C6′), 25.8 (SiC$(\underline{C}H_3)_3$), 21.0, 20.9, 20.81, 20.77, 20.75, 20.66, (6× C(O)$\underline{C}H_3$), 18.2 (Si$\underline{C}(CH_3)_3$), −5.56, −5.64 (2× $SiCH_3$); ESI-MS: calculated $[M + H]^+$ = 749.3, $[M + Na]^+$ = 771.3, found $[M + H]^+$ = 749.1, $[M + Na]^+$ = 771.1; CHN analysis: calculated C 52.93, H 7.00, O 36.32, Si 3.75, found C 52.69, H 7.01.

*Allyl 2,3,4-tri-O-acetyl-β-D-galactopyranosyl-(1 → 4)-2,3,6-tri-O-acetyl-β-D-glucopyranoside (**39**).* Lactoside **38** (14.8 g, 20.9 mmol) was dissolved in 200 mL dry THF and treated with 11 mL HF·pyridine (approximately 70% HF). The reaction was stirred for 3 h at room temperature and then neutralized with solid $NaHCO_3$ until gas formation ceased. Solids were filtered off and the filtrate was evaporated under reduced pressure. The remaining material was purified by silica column chromatography (eluent petroleum ether/ethyl acetate 1:2 to 1:3). **39** was obtained as a colorless solid (7.58 g, 11.9 mmol, 57%). R_f = 0.17 (petroleum ether/EtOAc 1:2); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.86−5.77 (m, 1 H, $CH_2C\underline{H}$=CH_2), 5.33 ('d', J = 3.4 Hz, 1 H, H-4′), 5.26−5.21 (m, 1 H, CH_2CH=$C\underline{H}_2$), 5.20−5.15 (m, 2 H, CH_2CH=$C\underline{H}_2$, H-3), 5.11 (dd, J = 10.4, 7.8 Hz, 1 H, H-2′), 4.97 (dd, J = 10.4, 3.4 Hz, 1 H, H-3′), 4.90 (dd, J = 9.4, 7.9 Hz, 1 H, H-2), 4.52 (d, J = 6.0 Hz, 1 H, H-1), 4.50 (d, J = 6.0 Hz, 1 H, H-1′), 4.50−4.46 (m, 1 H, H-6a), 4.30−4.25 (m, 1 H, C$\underline{H}_2$CH=CH_2), 4.08−4.03 (m, 2 H, H-6b, C$\underline{H}_2$CH=CH_2), 3.83 ('t', J = 9.3 Hz, 1 H, H-4), 3.72−3.65 (m, 2 H, H-5′, H-6′a), 3.59 (ddd, J = 9.7, 5.3, 2.2 Hz, 1 H, H-5), 3.51−3.44 (m, 1 H, H-6′b), 2.58 (br, 1 H, OH), 2.14 (s, 3 H, C(O)CH_3), 2.10 (s, 3 H, C(O)CH_3), 2.03 (s, 3 H, C(O)CH_3), 2.03 (s, 3 H, C(O)CH_3), 2.02 (s, 3 H, C(O)CH_3), 1.96 (s, 3 H, C(O)CH_3); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 171.1, 170.6, 170.3, 170.2, 169.8, 169.3 (6× $\underline{C}$(O)CH_3), 133.4 ($CH_2\underline{C}H$=CH_2), 117.7 (CH_2CH=$\underline{C}H_2$), 101.2 (C1), 99.3 (C1′), 76.2 (C4), 74.1 (C5′), 73.5 (C3), 72.6 (C5), 71.8 (C2), 71.1 (C3′), 70.1 ($\underline{C}H_2CH$=CH_2), 69.6 (C2′), 67.7 (C4′), 62.2 (C6), 60.8 (C6′), 21.0, 20.98, 20.82, 20.78, 20.74, 20.66 (6× C(O)$\underline{C}H_3$); ESI-MS: calculated $[M + H]^+$ = 635.2, $[M + Na]^+$ = 657.2, $[M + K]^+$ = 673.2, found $[M + H]^+$ = 635.2, $[M + Na]^+$ = 657.0, $[M + K]^+$ = 673.0; CHN analysis: calculated C 51.10, H 6.04, O 42.86, found C 50.82, H 6.35.

*Allyl 2,3,4-tri-O-acetyl-6-O-methylsulfonyl-β-D-galactopyranosyl-(1 → 4)-2,3,6-tri-O-acetyl-β-D-glucopyranoside (**40**).* Lactoside **39** (3.5 g, 5.52 mmol) was dissolved in 50 mL dry pyridine and cooled to 0 °C. Mesyl chloride (643 μL, 8.27 mmol) and 4-(dimethylamino)pyridine (122 mg, 1 mmol) were added and the mixture was stirred for 16 h, while warming to room temperature. The solvent was removed under reduced pressure. The remaining material was dissolved in 200 mL DCM and washed 3× with 50 mL each of 1 M HCl solution, saturated $NaHCO_3$ solution, and once with 50 mL brine. The organic phase was dried with $MgSO_4$ and evaporated under reduced pressure. **40** was obtained as a colorless solid, which was used without further purification (3.46 g, 4.85 mmol, 88%). R_f = 0.40 (petroleum ether/EtOAc 1:2); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.86−5.77 (m, 1 H, $CH_2C\underline{H}$=CH_2), 5.38 (dd, J = 3.6, 0.7 Hz, 1 H, H-4′), 5.26−5.21 (m, 1 H, CH_2CH=$C\underline{H}_2$), 5.21−5.15 (m, 2 H, CH_2CH=$C\underline{H}_2$, H-3), 5.09 (dd, J = 10.4, 7.8 Hz, 1 H, H-2′), 4.96 (dd, J = 10.4, 3.4 Hz, 1 H, H-3′), 4.88 (dd, J = 9.4, 7.9 Hz, 1 H, H-2), 4.53 (d, J = 8.1 Hz, 1 H, H-1′), 4.51 (d, J = 8.1 Hz, 1 H, H-1), 4.49−4.45 (m, 1 H, H-6a), 4.29−4.25 (m, 1 H, C$\underline{H}_2$CH=CH_2), 4.23−4.14 (m, 2 H, H-6′a/b), 4.09−4.02 (m, 2 H, H-6b, C$\underline{H}_2$CH=CH_2), 3.97−3.94 (m, 1 H, H-5′), 3.82 ('t', J = 9.5 Hz, 1 H, H-4), 3.59 (ddd, J = 9.8, 5.0, 2.0 Hz, 1 H, H-5), 3.05 (s, 3 H, SO_2CH_3), 2.14 (s, 3 H, C(O)CH_3), 2.10 (s, 3 H, C(O)CH_3), 2.04 (s, 3 H, C(O)CH_3), 2.03 (s, 3 H, C(O)CH_3), 2.02 (s, 3 H, C(O)CH_3), 1.94 (s, 3 H, C(O)CH_3); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 170.5, 170.2, 170.1, 169.9, 169.7, 169.2 (6× $\underline{C}$(O)CH_3), 133.4 ($CH_2\underline{C}H$=CH_2), 117.7 (CH_2CH=$\underline{C}H_2$), 100.9 (C1′), 99.3 (C1), 76.2 (C4), 73.0 (C3), 72.6 (C5), 71.8 (C2), 70.9 (C5′), 70.8 (C3′), 70.1 ($\underline{C}H_2CH$=CH_2), 69.1 (C2′), 66.6 (C4′), 64.8 (C6′), 62.1 (C6), 37.8 (SO_2CH_3), 21.0 (2×), 20.8, 20.712, 20.706, 20.6 (6× C(O)$\underline{C}H_3$); ESI-MS: calculated $[M + H]^+$ = 713.2, $[M + Na]^+$ = 735.2, found $[M + H]^+$ = 713.1, $[M + Na]^+$ = 735.0; CHN analysis: calculated: C 47.19, H 5.66, O 42.65, S 4.50, found: C 45.53, H 5.66, S 4.76.

*Allyl 2,3,4,6-tri-O-acetyl-6-azido-6-deoxy-β-D-galactopyranosyl-(1 → 4)-2,3,6-tri-O-acetyl-β-D-glucopyranoside (**41**).* Lactoside **40** (2.8 g, 3.93 mmol) was dissolved in 30 mL dry DMF and treated with NaN_3 (2.54 g, 39 mmol). The resulting suspension was stirred for 16 h at 85 °C. Solids were filtered off and the filtrate was evaporated under reduced pressure. The remaining material was dissolved in 100 mL DCM and washed twice with 100 mL each of saturated $NaHCO_3$ solution and brine. The organic phase was dried with $MgSO_4$ and evaporated under reduced pressure. After silica column chromatography (eluent petroleum ether/ethyl acetate 1:1 to 2:3), **41** was obtained as a colorless solid (1.93 g, 2.93 mmol, 74%). R_f = 0.36 (petroleum ether/EtOAc 1:2); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.86−5.76 (m, 1 H, $CH_2C\underline{H}$=CH_2), 5.32 (dd, J = 3.4, 0.9 Hz, 1 H, H-4′), 5.26−5.20 (m, 1 H, CH_2CH=$C\underline{H}_2$), 5.19−5.16 (m, 2 H, CH_2CH=$C\underline{H}_2$, H-3), 5.07 (dd, J = 10.4, 7.8 Hz, 1 H, H-2′), 4.92 (dd, J = 10.3, 3.5 Hz, H-3′), 4.89 (dd, J = 9.5, 7.9 Hz, 1 H, H-2), 4.51−4.47 (m, 3 H, H-1, H-1′, H-6a), 4.30−4.25 (m, 1 H, C$\underline{H}_2$CH=CH_2), 4.10−4.02 (m, 2 H, H-6b, C$\underline{H}_2$CH=CH_2), 3.84 ('t', J = 9.5 Hz, 1 H, H-4), 3.73−3.70 (m, 1 H, H-5′), 3.58 (ddd, J = 9.9, 5.0, 2.1 Hz, 1 H, H-5), 3.43 (dd, J = 12.7, 7.2 Hz, 1 H, H-6a′), 3.23 (dd, J = 12.7, 5.9 Hz, 1 H, H-6b′), 2.14 (s, 3 H, C(O)CH_3), 2.09 (s, 3 H, C(O)CH_3), 2.03 (s, 3 H, C(O)CH_3), 2.01 (s, 3 H, C(O)CH_3), 2.00 (s, 3 H, C(O)CH_3), 1.94 (s, 3 H, C(O)CH_3); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 170.5, 170.2, 170.1, 169.9, 169.7, 169.1 (6× C(O)), 133.4 ($CH_2\underline{C}H$=CH_2), 117.7 (CH_2CH=$\underline{C}H_2$), 100.7 (C1), 99.4 (C1′), 75.7 (C4), 72.8 (C3), 72.7 (C5), 72.2 (C5′), 71.7 (C2), 71.0 (C3′), 70.1 ($\underline{C}H_2CH$=CH_2), 69.2 (C2′), 67.5 (C4′), 62.0 (C6), 50.2 (C6′), 21.0, 20.9, 20.8, 20.7 (2×), 20.6 (6× C(O)$\underline{C}H_3$); ESI-MS: calculated $[M + H]^+$ = 660.2, $[M + Na]^+$ = 682.2, $[M + K]^+$ = 698.2, found $[M + H]^+$ = 660.1, $[M + Na]^+$ = 682.0, $[M + K]^+$ = 698.0; CHN analysis: calculated C 49.16, H 5.65, N 6.37, O 38.81; found C 49.21, H 5.81, N 5.94.

*2,3,4-Tri-O-acetyl-6-azido-6-deoxy-β-D-galactopyranosyl-(1 → 4)-2,3,6-tri-O-acetyl-α/β-D-glucopyranose (**42**).* Bis(methyldiphenylphosphine)cyclooctadiene-iridium(I) hexafluorophosphate (30 mg, 0.035 mmol) was suspended in 10

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mL of dry THF and bubbled with hydrogen gas for 15 min at room temperature while stirring. The metal complex dissolved and the solution was added to a solution of lactoside **41** (1.5 g, 1.9 mmol) in 50 mL dry THF. The reaction was stirred for 60 min at room temperature. Water (5 mL) and *N*-bromosuccinimide (405 mg, 2.28 mmol) were added. After 1 min TLC showed complete reaction. The mixture was diluted with 200 mL DCM and washed with 3× 50 mL saturated $NaHCO_3$ solution and 1× 50 mL brine. The organic phase was dried with $MgSO_4$ and evaporated under reduced pressure. The crude product was purified by silica column chromatography (eluent petroleum ether/ethyl acetate 1:2 to 1:3). **42** was obtained as a colorless solid (1.17 g, 1.89 mmol, quantitative; mixture of isomers α/β = 2:1). R_f = 0.50 (petroleum ether/EtOAc 1:3); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.51 ('t', J = 9.7 Hz, 1 H, H-3$^{\alpha}$), 5.37 ('t', J = 3.3 Hz, 1 H, H-1$^{\alpha}$), 5.33 (d, J = 2.8 Hz, 1.5 H, H-4$'^{\alpha/\beta}$), 5.23 ('t', J = 9.4 Hz, 0.5 H, H-3$^{\beta}$), 5.09 (dd, J = 10.4, 7.8 Hz, 1 H, H-2$'^{\alpha}$), 5.08 (dd, J = 10.4, 7.7 Hz, 0.5 H, H-2$'^{\beta}$), 4.96 (dd, J = 10.4, 3.4 Hz, 1.5 H, H-3$'^{\alpha/\beta}$), 4.81−4.76 (m, 1.5 H, H-2$^{\alpha/\beta}$), 4.71 ('t', J = 7.8 Hz, 0.5 H, H-1$^{\beta}$), 4.54−4.47 (m, 3 H, H-6a$^{\alpha/\beta}$, H-1$'^{\alpha/\beta}$), 4.17 (ddd, J = 10.0, 4.0, 1.8 Hz, 1 H, H-5$^{\alpha}$), 4.13−4.08 (m, 1.5 H, H-6b$^{\alpha/\beta}$), 3.87−3.79 (m, 2 H, H-4$^{\alpha/\beta}$, OH$^{\beta}$), 3.76−3.73 (m, 1.5 H, H-5$'^{\alpha/\beta}$), 3.65 (ddd, J = 10.2, 5.1, 2.0 Hz, 0.5 H, H-5$^{\beta}$), 3.49−3.42 (m, 2.5 H, H-6a$'^{\alpha/\beta}$, OH$^{\alpha}$), 3.24 (dd, J = 12.8, 5.4 Hz, 1.5 H, H-6b$'^{\alpha/\beta}$), 2.15 (s, 4.5 H, C(O)$CH_3^{\alpha/\beta}$), 2.12 (s, 3 H, C(O)CH_3^{α}), 2.10 (s, 1.5 H, C(O)CH_3^{β}), 2.06 (br s, 9 H, 2× C(O)CH_3^{α}, 2× C(O)CH_3^{β}), 2.03 (s, 4.5 H, C(O)$CH_3^{\alpha/\beta}$), 1.95 (s, 4.5 H, C(O)$CH_3^{\alpha/\beta}$); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 171.0 (C(O)$^{\beta}$), 170.67 (C(O)$^{\alpha}$), 170.65 (C(O)$^{\beta}$), 170.4, 170.27 (2× C(O)$^{\alpha}$), 170.22 (C(O)$^{\beta}$), 170.19 (C(O)$^{\alpha}$), 170.14 (C(O)$^{\beta}$), 169.7 (br s, C(O)$^{\alpha/\beta}$), 169.19 (C(O)$^{\beta}$), 169.16 (C(O)$^{\alpha}$), 100.7 (C1$'^{\beta}$), 100.6 (C1$'^{\alpha}$), 95.5 (C1$^{\beta}$), 90.2 (C1$^{\alpha}$), 75.6 (2x, C4$^{\alpha/\beta}$), 73.6 (C2$^{\beta}$), 73.1 (C5$^{\beta}$), 72.3 (2×, C5$'^{\alpha/\beta}$), 72.1 (C3$^{\beta}$), 71.5 (C2$^{\alpha}$), 71.1 (C3$'^{\alpha}$), 71.0 (C3$'^{\beta}$), 69.6 (C3$^{\alpha}$), 69.3 (C2$'^{\alpha}$), 69.2 (C2$'^{\beta}$), 68.4 (C5$^{\alpha}$), 67.70 (C4$'^{\alpha}$), 67.66 (C4$'^{\beta}$), 62.1 (C6$^{\beta}$), 62.0 (C6$^{\alpha}$), 50.36 (C6$'^{\alpha/\beta}$), 21.1−20.6 (m, 6× C(O)C$H_3^{\alpha/\beta}$); ESI-MS: calculated $[M + H]^+$ = 620.2, $[M + Na]^+$ = 642.2, $[M + K]^+$ = 658.2, found $[M + H]^+$ = 620.1, $[M + Na]^+$ = 642.0, $[M + K]^+$ = 658.0; CHN analysis: calculated C 46.53, H 5.37, N 6.78, O 41.32; found C 46.18, H 5.59, N 6.22.

*2,3,4-Tri-O-acetyl-6-azido-6-deoxy-β-D-galactopyranosyl-(1 → 4)-2,3,6-tri-O-acetyl-α-D-glucopyranosyl trichloroacetimidate (**43**).* Lactose derivative **42** (1.15 g, 1.86 mmol) was dissolved in 15 mL dry DCM and cooled to 0 °C. Trichloroacetonitrile (933 μL, 9.3 mmol) and DBU (28 μL, 0.2 mmol) were added and the resulting mixture was stirred for 30 min at 0 °C. The solution was filtered through a short plug of silica gel and evaporated under reduced pressure. The crude product was purified by silica column chromatography (eluent petroleum ether/ethyl acetate 1:1 to 2:3). **43** was obtained as a colorless solid (1.19 g, 1.55 mmol, 83%). R_f = 0.55 (petroleum ether/EtOAc 1:1); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.65 (s, 1 H, NH), 6.50 (d, J = 3.8 Hz, 1 H, H-1), 5.56 ('t', J = 9.7 Hz, 1 H, H-3), 5.35−5.33 (m, 1 H, H-4'), 5.11 (dd, J = 10.3, 7.9 Hz, 1 H, H-2'), 5.06 (dd, J = 10.1, 3.8 Hz, 1 H, H-2), 4.97 (dd, J = 10.3, 3.4 Hz, 1 H, H-3'), 4.55 (d, J = 7.9 Hz, 1 H, H-1'), 4.52−4.47 (m, 1 H, H-6a), 4.15−4.08 (m, 2 H, H-5, H-6b), 3.93 ('t', J = 9.6 Hz, 1 H, H-4), 3.77−3.73 (m, 1 H, H-5'), 3.47 (dd, J = 12.9, 7.5 Hz, 1 H, H-6a'), 3.25 (dd, J = 12.9, 5.3 Hz, 1 H, H-6b'), 2.16 (s, 3 H, C(O)CH_3), 2.11 (s, 3 H, C(O)CH_3), 2.09 (s, 3 H, C(O)CH_3), 2.04 (s, 3 H, C(O)CH_3), 2.00 (s, 3 H, C(O)CH_3), 1.96 (s, 3 H, C(O)CH_3); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 170.4, 170.2, 170.1 (2×), 169.5, 169.2 (6× C(O)), 161.1 (C═NH), 100.8 (C-1'), 93.1 (C-1), 90.9 (CCl_3), 75.2 (C-4), 72.5 (C-5'), 71.2 (C-5, C-3'), 70.1 (C-2), 69.6 (C-3), 69.3 (C-2'), 67.7 (C-4'), 61.6 (C-6), 50.4 (C-6'), 21.1, 20.9, 20.80, 20.75, 20.62, 20.60 (6× C(O)CH_3); ESI-MS: calculated $[M + H]^+$ = 763.1, $[M + Na]^+$ = 785.1, found $[M + H]^+$ = 762.9, $[M + Na]^+$ = 784.6; CHN analysis: calculated C 40.88, H 4.35, N 7.33, O 13.92, Cl 13.92, found C 41.23, H 4.80, N 7.00.

For glycosylation and deacylation to **47** as well as synthesis and analytical data of **48** and **49**, see Supporting Information.

Cell Growth Conditions. HEK 293T cells were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% calf serum (DMEM + 10% CS). Mouse embryonic fibroblasts (MEFs) were cultured in DMEM with 10% fetal calf serum supplemented with nonessential amino acids and pyruvate on gelatin-coated culture dishes (0.1% gelatin in PBS). All cells were incubated at 37 °C under a water saturated atmosphere with 5% carbon dioxide and subcultured every 2−3 days.

Application of Glycolipids. Glycolipids were dissolved and stored in a mixture of chloroform/MeOH 19:1, yielding concentrations of typically 2−10 mM. 100 μL of these solutions were transferred into a 2 mL glass vial and evaporated in a nitrogen stream, followed by drying under vacuum. The remaining solid was dissolved in a defined amount of EtOH, yielding concentrations of typically 1−5 mM. A small amount of this solution was diluted with 0.34 mg mL^{-1} dfBSA (68 kDa) in HMEM to obtain a concentration of 1−10 μM glycolipid.[63] The mixture was vortexed vigorously and added to cell cultures.

For observation by fluorescence microscopy, 2 × 10^5 HEK 293T cells were seeded in 3.5 cm μ-dishes (ibidi GmbH) with a glass surface that had been coated with 1 μg mL^{-1} fibronectin and 10 μg mL^{-1} poly(L-lysine) in PBS for 1 h at 37 °C. Cells were grown for 16 h, washed with PBS, and incubated with 2 mL glycolipid solution for 30 min at 4 °C. Then the cells were washed twice with PBS and labeled by click reactions.

Application of Glycolipids Using Fusogenic Liposomes. The preparation of fusogenic liposomes was performed as described.[61] The lipids DOPE and DOTAP (1:1) were dissolved in 500 μL DCM/ethanol (20:1) in a total concentration of 1 mg mL^{-1}. 5 mol % of the azide-labeled glucosylceramide **3** or lactosylceramide **49** together with 5 mol % TexasRed-DHPE were added to the solution. The solvent was removed under reduced pressure for 2 h. The residue was diluted in 125 μL HEPES buffer (pH 7.4) to the final lipid concentration of 2.1 mg/mL and was vortexed for 2 min. The suspension was further sonicated for 10 min and stored at 4 °C before use. For incubation HEK 293T cells were treated with 75 μL of the suspension containing glycolipids **35** or **49** or without modified glycolipid in 10 mL DMEM + 10% FCS for 10 min at 37 °C.

Fluorescence Labeling. For copper-catalyzed click reactions,[54] a mixture of the following components was prepared immediately before the labeling experiments (volume for one cell culture dish): 2 mL PBS, 50 μM $CuSO_4$ (stock solution 50 mM in H_2O), 100 μM TBTA[56] (stock solution 200 mM in DMSO), 1 mM aminoguanidine (stock solution 100 mM in PBS), desired amount of fluorescent dye (stock solutions in DMSO), 10 mM sodium ascorbate (stock solution 2.5 M in H_2O). The solution was applied to HEK 293T cells in culture dishes and incubated for 30 min at 4 °C. Cells were washed twice with PBS, supplied with DMEM + 10% CS, and analyzed by fluorescence microscopy. For copper-free labeling with

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cyclooctyne derivatives, cells were incubated with a solution of 2 μM DIBO-lissamine (**6**) (stock solution 5 mM in DMSO), 2 μM DIBO-AlexaFluor-488 (stock solution 2 mM in DMSO), or 20 μM DIBO-biotin **50** (stock solution 50 mM in DMSO) in PBS for 30−60 min at 4 °C or room temperature. Cells were washed twice with PBS, supplied with DMEM + 10% CS and analyzed by fluorescence microscopy. Cells which had been labeled with DIBO-biotin **50** were incubated with streptavidin-AlexaFluor-647 (stock solution 1 mg/mL in PBS; dilution 1:500 in PBS) for 15 min at 4 °C. Cells were washed twice with PBS, supplied with DMEM + 10% CS, and analyzed by fluorescence microscopy. Samples were observed with a TCS SP5 confocal laser scanning microscope (Leica) using a 63×, 1.4 NA PLAPO oil-immersion objective (Figures 2 and 3) or LSM 780 (Zeiss) equipped with a 63×, 1.4 NA oil immersion objective and a GAsP detector array for spectral imaging (Figures 4 and S2). Fluorescence signals of labeled specimens were serially recorded with appropriate excitation wavelengths and emission bands for coumarin, AlexaFluor-488, lissamine, rhodamine, TexasRed, or AlexaFluor-647, respectively, to avoid bleed-through and cross-excitation. Images were processed with ImageJ (NIH, Bethesda, MD, USA).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.6b00177.

Experimental procedures and analytical data of remaining compounds; additional structures, additional confocal microscopy images of modified glycosphingolipids; analysis by flow cytometry (PDF)

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Notes

The authors declare no competing financial interest.

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