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Synthesis of fluorescent and radioactive analogues of two lactosylceramides and glucosylceramide containing β-thioglycosidic bonds that are resistant to enzymatic degradation

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Abstract

Condensation of 2-*S*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-2-thiopseudourea hydrobromide with 2,3,6-tri-*O*-benzoyl-4-*O*-trifluoromethylsulfonyl-β-D-galactopyranosyl-(1 → 1)-(2*S*,3*R*,4*E*)-3-*O*-benzoyl-2-dichloroacetamido-4-octadecen-1,3-diol afforded *S*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1 → 4)-2,3,6-tri-*O*-benzoyl-4-thio-β-D-glucopyranosyl-(1 → 1)-(2*S*, 3*R*,4*E*)-3-*O*-benzoyl-2-dichloroacetamido-4-octadecen-1,3-diol in good yield. Removal of the protecting groups, followed by selective *N*-acylation of the sphingosine amino group with either a fluorescent or a radioactive fatty acid, gave labeled lactosylceramide analogues in good yield. Since these products contained a β-thioglycosidic bond between the two sugar moieties, they were totally resistant to the action of acid lysosomal glycosidases. Likewise, condensation of 2-*S*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-2-thiopseudourea hydrobromide and 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1 → 4)-2,3,6-tri-*O*-acetyl-1-*S*-acetyl-1-thio-β-D-glucopyranose with (2*R*,3*R*,4*E*)-3-*O*-benzoyl-2-dichloroacetamido-1-iodo-4-octadecen-3-ol in methanolic sodium acetate afforded the corresponding β-thioglycosides **14** and **16**, respectively, in good yield. These β-thioglycosides were converted into glucosylceramide and lactosylceramide analogues following removal of the protecting groups and by subsequent selective *N*-acylation using either a fluorescent or radioactive fatty acid *N*-succinimidyl ester. Whereas the glucosylthioceramides thus obtained proved to be completely undegradable by lysosomal glucocerebrosidase, the lactosylceramides containing the β-thioglycosidic bond between the lactose and the ceramide residues

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could be degraded by lysosomal GM1-β-galactosidase to give the corresponding glucosylthioceramides. These compounds did not yield to any further enzymatic degradation.

Keywords: Thioglycolipids; Glycolipids, labeled; Thioglycosides; Glucosylthioceramide; Lactosylthioceramide

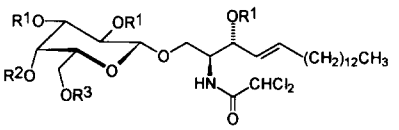
1. Introduction

Increasing evidence demonstrates that glycosphingolipids, as constituents of the outer surface of the plasma membrane, play a role in cell differentiation, morphogenesis, neoblastic transformation, and cell-surface phenomena relevant to signal transduction [for review see refs [1–4]]. Although most of the reported functions of glycosphingolipids probably occur within the plasma membrane, it is likely that most, if not all, steps in glycosphingolipid metabolism take place in intracellular compartments. Therefore, besides regulation of the individual metabolic steps, their localization and relation to intracellular lipid transport and lipid sorting has become a matter of increasing interest. A useful tool for the study of lipid transport and metabolism are labeled glycolipids that allow analysis of both their metabolism and distribution in cells.

We have observed labeled and cell-type specific glycosylation products when a radioactive or a fluorescent analogue of glucosylceramide or lactosylceramide had been fed to cultured cells. These products (analogues of globosides and gangliosides) could have been formed in the Golgi compartment from either the labeled glycosphingolipid analogues or from the labeled ceramide, the latter being derived from deglycosylation, most likely within lysosomes of the former. We have, therefore, synthesized two analogues of lactosylceramide containing a β-thioglycosidic bond either between the galactose and glucose residue or between the lactose and the ceramide moiety, and one analogue of glucosylceramide containing β-thioglucosidically linked ceramide. These analogues that contain either a fluorescent or radioactive acyl residue of medium chain length (C_8) in place of a long fatty-acyl chain cannot be degraded to the corresponding ceramides. Thus, they will be used in the study of glycosphingolipid traffic, distribution, and metabolism.

2. Results and discussion

For the preparation of a lactosylceramide analogue that is resistant to degradation by glycosidases two general synthetic approaches were considered: (*i*) the preparation of a thiolactose [5] which contains sulfur as the interglycosidic link that, after appropriate protection and activation, could be used for the glycosylation of sphingosine or ceramide derivatives. This approach would involve the formation of two glycosidic linkages. (*ii*) The coupling of the sodium salt of 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-galactopyranose in an S_N2 reaction to C-4 of an appropriate galactosylsphingosine derivative. This would take advantage of an already existing correct glycosidic bond between the sugar and the lipid part. We have chosen the latter approach as galactosylsphingosine was available in our laboratory in sufficient quantities for synthesis.



1 $R^1 = R^2 = R^3 = H$

2 $R^1 = H$, $R^2 = R^3 = p\text{-}CH_3OC_6H_4CH$

3 $R^1 = Bz$, $R^2 = R^3 = p\text{-}CH_3OC_6H_4CH$

4 $R^1 = Bz$, $R^2 = R^3 = H$

5 $R^1 = R^3 = Bz$, $R^2 = H$

6 $R^1 = R^3 = Bz$, $R^2 = CF_3SO_2$

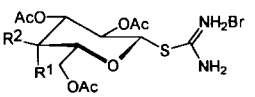
Bz = benzoyl

Scheme 1.

The necessary intermediate **6** (Scheme 1) could be obtained from **1** via simple chemistry in an overall good yield. We were unable, however, to obtain the 2,3,6-tri-*O*-benzoyl-β-D-galactopyranoside **5** in sufficient yield by selective benzoylation of **1** as described for methyl 2,3,6-tri-*O*-benzoyl-α-D-galactopyranoside [6]. Thus, an intermediate *p*-methoxybenzylidene derivative was formed that, after perbenzoylation and subsequent removal of the benzylidene group, could be selectively benzoylated at position 6 due to the known order of reactivity of OH-6 and OH-4 towards benzoylation.

After conversion of **5** into the triflate **6**, the latter could be successfully attacked by the sulfur-containing nucleophile generated in situ from 2-*S*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-2-thiopseudourea hydrobromide (Scheme 2) to afford the β-thioglycosidic linkage, with subsequent conversion into the gluco configuration of the sphingoid-bound galactose residue.

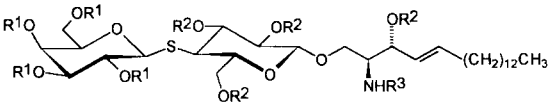
Proof for the structure of the coupling product **7** (Scheme 3) is provided by (*i*) ^1H NMR spectrometry, (*ii*) FABMS spectrometry, and (*iii*) lack of degradation of the corresponding lactosylceramides **22** and **25** (Scheme 3) by acid β-galactosidase (Fig. 1). The β-anomeric configuration of the thioglycosidic linkage is clearly shown by the spin–spin coupling of protons H-1 and H-2 of the galactose moiety ($J_{1,2}$ 9.5 Hz). The expected inversion at C-4 to the *gluco* configuration is evidenced by the coupling



17 $R^1 = H$, $R^2 = OAc$

18 $R^1 = OAc$, $R^2 = H$

Scheme 2.

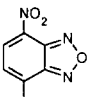


7 R^1 = Ac, R^2 = Bz, R^3 = $COCHCl_2$

19 $R^1 = R^2 = R^3 = H$

22 $R^1 = R^2 = H$, $R^3 = CO(CH_2)_6CH_2NH$-NBD

25 $R^1 = R^2 = H$, $R^3 = [1\text{-}^{14}C]\text{-}CO(CH_2)_6CH_3$

Ac = acetyl NBD = 

Scheme 3.

constant $J_{3,4}$ 11 Hz. Also due to the sulfur atom in position 4, the signal of H-4 appears 0.49 ppm to higher field than the corresponding signal for the oxygenated compound **16** (Scheme 6 below). Other NMR data are consistent with the structure assigned for this fully blocked coupling product **7**.

The synthesis of glucosyl- and lactosyl-ceramide analogues bearing a β-thioglycosidic bond between the sugar residue and the sphingosine moiety can be achieved by two different routes. In the first route the sulfur atom is introduced into the 1-position of a protected sphingosine or sphingosine precursor prior to condensation with the glycosyl donor [7]. In the second route the sugar is first converted to a derivative containing the sulfur atom in a β-anomeric linkage before condensation with a sphingosine or sphingoid derivative bearing a good leaving group [8].

Whereas Bär and Schmidt [7] were successful in coupling hepta-*O*-acetyl-α-lactosyl bromide to a thiosphingosine precursor, e.g., (2*R*,3*R*,4*E*)-1-*S*-acetyl-2-azido-3-benzoyloxy-4-octadecen-1-thiol, this approach was unsuccessful in our hands when starting directly from natural sphingosine. Though sphingosine could be readily converted into the required intermediate, e.g., (2*R*,3*R*,4*E*)-1-*S*-acetyl-3-benzoyloxy-2-dichloroacetamido-4-octadecen-1-thiol, its transformation into the corresponding 1-thiol by mild alkaline treatment could not be sufficiently achieved. Instead we obtained an intractable mixture of products, some of which seemed to be derived by elimination of the thioacetyl group of (2*R*,3*R*,4*E*)-1-*S*-acetyl-3-benzoyloxy-2-dichloroacetamido-4-octadecen-1-thiol. These were not further characterized. Since we were fortunate in having sufficient quantities of sphingosine, i.e., (2*S*,3*R*,4*E*)-2-amino-octadecen-1,3-diol, we pursued the other route in which an appropriately protected sphingosine containing iodine as a good leaving group, i.e., (2*R*,3*R*,4*E*)-3-*O*-benzoyl-2-dichloroacetamido-1-iodo-octadecen-3-ol (**13**, Scheme 4), could be successfully condensed to afford the desired β-thioglycosides in a one-flask reaction starting from the well-known 2-*S*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-2-thiopseudourea hydrobromide (**17**, Scheme 2) and 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1 → 4)-2,3,6-tri-*O*-acetyl-1-*S*-acetyl-1-thio-β-D-glucopyranose (**15**, Scheme 5), respectively. A similar approach

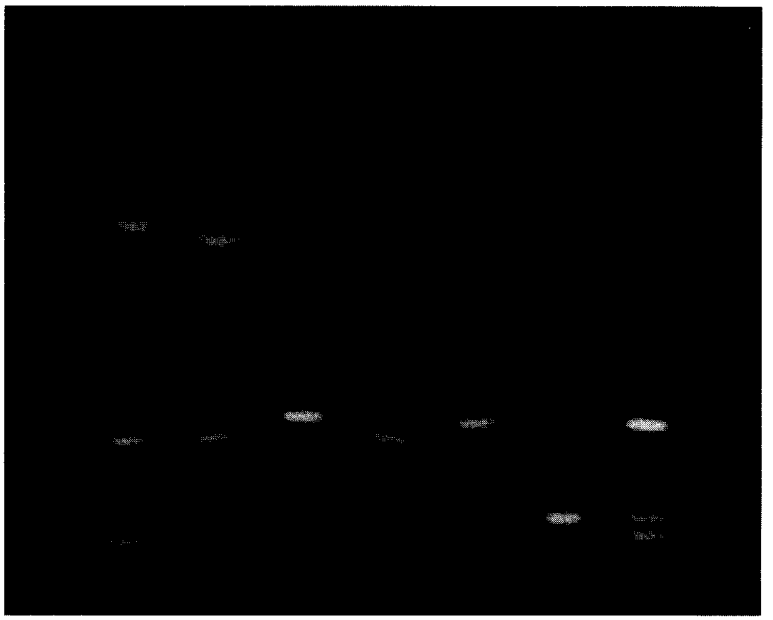


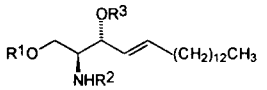
Fig. 1. TLC separation of the products obtained from fluorescent analogues of thioglycosphingolipids **22**, **23** and **24** and their *O*-glycosidic counterparts by treatment with both GM1-β-galactosidase and glucocerebrosidase.

Each glycolipid (1 nmol) as indicated below was dissolved in 91 mM sodium phosphate–citrate buffer (100 μL, pH 4.5) containing Triton-X 100 (0.4% w/v) and taurodeoxycholate (0.8% w/v). GM1-β-galactosidase (18 pKatal) and glucocerebrosidase (0.9 pKatal) were added, and each mixture was incubated for 3 h at 37°C. After desalting on LiChroprep RP-18 the (glyco)lipids were separated by TLC with 70:15:2 chloroform–methanol–water, and the plate was photographed under UV light.

Substrates are as follows: lane 6: *S*-β-D-galactopyranosyl-(1 → 4)-4-thio-β-D-glucopyranosyl-(1 → 1)-(2*S*,3*R*,4*E*)-2-[8-*N*-(7-nitrobenz-1,3-diazol-2-oxa-4-yl)amino]octanamido-4-octadecen-1,3-diol (**22**); lane 3: *S*-β-D-glucopyranosyl-(1 → 1)-(2*R*,3*R*,4*E*)-3-hydroxy-2-[8-*N*-(7-nitrobenz-1,3-diazol-2-oxa-4-yl)amino]octanamido-4-octadecen-1-thiol (**23**); lane 5: β-D-galactopyranosyl-(1 → 4)-*S*-β-D-glucopyranosyl-(1 → 1)-(2*R*,3*R*,4*E*)-3-hydroxy-2-[8-*N*-(7-nitrobenz-1,3-diazol-2-oxa-4-yl)amino]octanamido-4-octadecen-1-thiol (**24**); lane 2: β-D-glucopyranosyl-(1 → 1)-(2*S*,3*R*,4*E*)-2-[8-*N*-(7-nitrobenz-1,3-diazol-2-oxa-4-yl)amino]octanamido-4-octadecen-1,3-diol; lane 4: β-D-galactopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 1)-(2*S*,3*R*,4*E*)-2-[8-*N*-(7-nitrobenz-1,3-diazol-2-oxa-4-yl)amino]octanamido-4-octadecen-1,3-diol.

Reference lipids from top to bottom are as follows: lane 1: (2*S*,3*R*,4*E*)-2-[8-*N*-(7-nitrobenz-1,3-diazol-2-oxa-4-yl)amino]octanamido-4-octadecen-1,3-diol, β-D-glucopyranosyl-(1 → 1)-(2*S*,3*R*,4*E*)-2-[8-*N*-(7-nitrobenz-1,3-diazol-2-oxa-4-yl)amino]octanamido-4-octadecen-1,3-diol, β-D-galactopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 1)-(2*S*,3*R*,4*E*)-2-[8-*N*-(7-nitrobenz-1,3-diazol-2-oxa-4-yl)amino]octanamido-4-octadecen-1,3-diol; lane 7: **23**, **22**, **24**.

had also been successfully applied by Hasegawa et al. [8] for the synthesis of a lactosylthioceramide via the "azido" sphingoid precursor containing the *p*-tolylsulfonyl moiety as a good leaving group. In our hands this condensation reaction proceeded under very mild conditions (sodium acetate in methanol was used as base), thus making use of a precise amount of sodium methoxide in dry methanol unnecessary.



8 $R^1 = R^3 = H$, $R^2 = COCHCl_2$

9 $R^1 = t\text{-}BuPh_2Si$, $R^2 = COCHCl_2$, $R^3 = H$

10 $R^1 = t\text{-}BuPh_2Si$, $R^2 = COCHCl_2$, $R^3 = Bz$

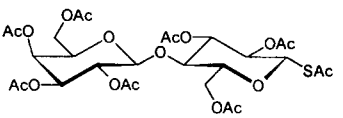
11 $R^1 = H$, $R^2 = COCHCl_2$, $R^3 = Bz$

12 $R^1 = CH_3SO_2$, $R^2 = COCHCl_2$, $R^3 = Bz$

13 $R^1 = I$, $R^2 = COCHCl_2$, $R^3 = Bz$

t-$BuPh_2Si$ = *tert*-butyldiphenylsilyl

Scheme 4.



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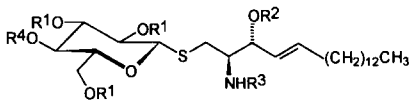
Scheme 5.

Starting from sphingosine, the necessary intermediate (2*R*,3*R*,4*E*)-3-*O*-benzoyl-2-dichloroacetamido-1-iodo-octadecen-3-ol (**13**, Scheme 4) could be obtained in 53% overall yield in six synthetic steps. These are as follows: Selective *N*-dichloroacetylation of sphingosine, followed by selective *tert*-butyldiphenylsilylation, gave (2*S*,3*R*,4*E*)-1-*O*-*tert*-butyldiphenylsilyl-2-dichloroacetamido-4-octadecen-1,3-diol (**9**) in 92% yield. This product was benzoylated to give (2*S*,3*R*,4*E*)-3-*O*-benzoyl-1-*O*-*tert*-butyldiphenylsilyl-2-dichloroacetamido-4-octadecen-1,3-diol (**10**) in 97% yield. Removal of the protecting group at position 1 with tetrabutylammonium fluoride gave (2*S*,3*R*,4*E*)-3-*O*-benzoyl-2-dichloroacetamido-4-octadecen-1,3-diol (**11**) in 70% yield, after purification on silica gel. The structure of **11** was unambiguously proved by 500 MHz ^1H NMR spectroscopy. The observed signals exhibited a sharp singlet at δ 5.92 that integrated for one proton, which demonstrated the presence of the dichloroacetamido group. The proton of the hydroxy group resonated at δ 2.65 as a multiplet. The *ortho*, *meta* and *para* proton(s) of the benzoyloxy residue resonated at δ 8.05, 7.47 and 7.60, respectively. The other signals are also consistent with the structure of **11**.

The free hydroxy group at position 1 was mesylated to give (2*S*,3*R*,4*E*)-3-*O*-benzoyl-2-dichloroacetamido-1-*O*-methylsulfonyl-4-octadecen-1,3-diol (**12**) in 90% yield. The structure of this compound could be clearly demonstrated by mass spectrometry. Peaks observed for $[M + H^+]$ at m/z 592, 594 and 596 indicated the presence of two chlorine atoms as expected for the dichloroacetamido residue. In addition, a similar



14 $R^1 = R^4 = Ac$, $R^2 = Bz$, $R^3 = COCHCl_2$

16 $R^1 = Ac$, $R^2 = Bz$, $R^3 = COCHCl_2$, $R^4 = Ac_4Gal$

20 $R^1 = R^2 = R^3 = R^4 = H$

21 $R^1 = R^2 = R^3 = H$, $R^4 =$Gal

23 $R^1 = R^2 = R^4 = H$, $R^3 = CO(CH_2)_6CH_2NH$-NBD

24 $R^1 = R^2 = H$, $R^4 =$ Gal, $R^3 = CO(CH_2)_6CH_2NH$-NBD

26 $R^1 = R^2 = R^4 = H$, $R^3 = [1\text{-}^{14}C]\text{-}CO(CH_2)_6CH_3$

27 $R^1 = R^2 = H$, $R^4 =$ Gal, $R^3 = [1\text{-}^{14}C]\text{-}CO(CH_2)_6CH_3$

Gal = ß-D-galactopyranosyl

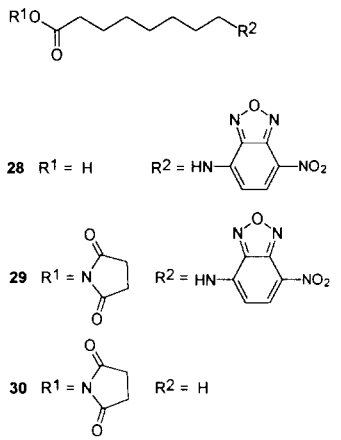
Ac_4Gal = 2,3,4,6-tetra-*O*-acetyl-ß-D-galactopyranosyl

Scheme 6.

triplet occurred at m/z 470, 472 and 474 for $[M + H^+ - PhCO_2H]$, again indicative for the presence of the benzoyloxy residue. Displacement of the mesyloxy group of **12** with sodium iodide in acetone afforded (2*R*,3*R*,4*E*)-3-*O*-benzoyl-2-dichloroacetamido-1-iodo-4-octadecen-3-ol (**13**) in 94% yield. The structure of **13** was confirmed by mass spectrometry, showing signals for $[M + H^+]$ at m/z 624, 626 and 628. Again signals were found for $[M + H^+ - PhCO_2H]$ at m/z 502, 504 and 506.

Finally, removal of all protecting groups of the condensation products **7**, **14**, and **16** with 0.2 M potassium hydroxide in methanol under argon afforded *S*-β-D-galactopyranosyl-(1 → 4)-4-thio-β-D-glucopyranosyl-(1 → 1)-(2*S*,3*R*,4*E*)-2-amino-4-octadecen-1,3-diol (**19**, Scheme 3), *S*-β-D-glucopyranosyl-(1 → 1)-(2*R*,3*R*,4*E*)-2-amino-3-hydroxy-4-octadecen-1-thiol (**20**, Scheme 6), and β-D-galactopyranosyl-(1 → 4)-*S*-β-D-glucopyranosyl-(1 → 1)-(2*R*,3*R*,4*E*)-2-amino-3-hydroxy-4-octadecen-1-thiol (**21**, Scheme 6), respectively, in good yield. These "lyso" compounds could easily be converted into the desired radioactive and fluorescent glycolipid analogues by selective *N*-acylation [9] with the *N*-succinimidyl ester of [1-^{14}C]octanoic acid and 8-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]octanoic acid (Scheme 7), respectively.

For the synthesis of this fluorescent short-chain fatty acid, we modified the procedures described [10,11] for the synthesis of the corresponding fluorescent hexanoic as well as dodecanoic acid analogues as these methods of preparation did not work effectively in our hands. The reaction of a moderate excess of 4-chloro-7-nitrobenzofurazan (NBD-Cl) with 8-aminooctanoic acid in methanol formed a highly fluorescent product from a non-fluorescent precursor in good yield when sodium methoxide was added in small increments over time just to keep the reaction mixture slightly alkaline. Most of the unavoidable colored side products could be removed by adsorption onto



Scheme 7.

aluminum oxide, thus facilitating purification of the fluorescent short-chain fatty acid on silica gel. The structure of the desired fluorescent acid was confirmed by mass spectrometry. The melting point and the R_f value observed were in good agreement with those noted by others [10,12] for the corresponding fluorescent fatty acids with a chain length of 6 or 12 carbon atoms, respectively.

The resistance to acid glycosidases of the fluorescent thioglycosphingolipids, *S*-β-D-galactopyranosyl-(1 → 4)-4-thio-β-D-glucopyranosyl-(1 → 1)-(2*S*,3*R*,4*E*)-2-[8-*N*-(7-nitrobenz-1,3-diazol-2-oxa-4-yl)amino]octanamido-4-octadecen-1,3-diol (**22**, Scheme 3), *S*-β-D-glucopyranosyl-(1 → 1)-(2*R*,3*R*,4*E*)-3-hydroxy-2-[8-*N*-(7-nitrobenz-1,3-diazol-2-oxa-4-yl)amino]octanamido-4-octadecen-1-thiol (**23**, Scheme 6), and β-D-galactopyranosyl-(1 → 4)-*S*-β-D-glucopyranosyl-(1 → 1)-(2*R*,3*R*,4*E*)-3-hydroxy-2-[8-*N*-(7-nitrobenz-1,3-diazol-2-oxa-4-yl)amino]octanamido-4-octadecen-1-thiol (**24**, Scheme 6) in comparison to their appropriate *O*-glycosidic counterparts was tested (see Experimental). Lane 6 of Fig. 1 demonstrates that the fluorescent lactosylceramide analogue **22** with sulfur in the interglycosidic link remained totally resistant to the action of a mixture of GM1-β-galactosidase and glucocerebrosidase. This enzyme mixture also failed to split the glucosylthioceramide analogue **23** (lane 3), whereas the fluorescent lactosylceramide analogue **24** with the β-thioglycosidic bond between the lactose and the sphingosine moiety was degalactosylated, as was expected, to give **23** (lane 5). In contrast, under the same conditions the corresponding *O*-glycosidic counterparts, i.e., β-D-glucopyranosyl-(1 → 1)-(2*S*,3*R*,4*E*)-2-[8-*N*-(7-nitrobenz-1,3-diazol-2-oxa-4-yl)amino]octanamido-4-octadecen-1,3-diol (lane 2) as well as β-D-galactopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 1)-(2*S*,3*R*,4*E*)-2-[8-*N*-(7-nitrobenz-1,3-diazol-2-oxa-4-yl)amino]octanamido-4-octadecen-1,3-diol (lane 4), were readily degraded to yield the corresponding fluorescent ceramide analogue (lanes 2 and 4). In case of the lactosylceramide analogue the intermediate glucosylceramide analogue is also seen (lane 4).

It is interesting to note a small increase of relative mobility of the sulfur-containing compounds (cf. lanes 1 and 7).

It has been shown before that a fluorescent analogue of a short-chain ceramide, i.e., (2*S*,3*R*,4*E*)-2-[6-*N*-(7-nitrobenz-1,3-diazol-2-oxa-4-yl)amino]hexanamido-4-octadecen-1,3-diol, is metabolized in cultured cells to give fluorescent sphingomyelin and glucosylceramide [13]. We have observed some glycosylation products besides sphingomyelin after feeding labeled short-chain analogues of glucosyl- and lactosylceramide to cultured cells. It remains to be established if the observed metabolites were derived directly from the applied glycolipids or from their deglycosylation products, i.e., ceramides. With the analogues of lactosyl- and glucosyl-ceramide that cannot be catabolized to the corresponding ceramide, we shall now be able to study this postendocytotic glycosylation in more detail.

3. Experimental

Materials.—Galactosylsphingosine [β-D-galactopyranosyl-(1 → 1)-(2*S*,3*R*,4*E*)-2-amino-4-octadecen-1,3-diol] was prepared from galactosylceramide [14], which was isolated from human brain as previously described [14]. Sphingosine [(2*S*,3*R*,4*E*)-2-amino-4-octadecen-1,3-diol] was prepared according to ref. [15]. GM1-β-Galactosidase [16], prepared from human liver, was available in this laboratory. Glucocerebrosidase was a generous gift of Dr H. Aerts, Amsterdam. 4-Chloro-7-nitro-benzofurazan (NBD-Cl) and 8-aminooctanoic acid were obtained from Fluka Chemie AG, Buchs, Switzerland. [1-^{14}C]Octanoic acid (148 GBq/mol) was purchased from Amersham Life Science, Braunschweig, Germany. 6-*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)aminohexanoic acid was purchased from Molecular Probes Inc, Eugene, Oregon, USA. Precoated thin-layer Kieselgel 60 plates (0.25 mm layer thickness), Silica Gel Si-60 (15–40 μm) and LiChroprep RP-18 were obtained from E. Merck, Darmstadt, Germany. All other chemicals were of the highest purity available. All mixtures of solvents are given in proportions of volumes.

Thin-layer chromatography.—The progress of reactions and column chromatographic elution profiles were routinely followed by thin-layer chromatography (TLC) in tanks (Camag, Muttenz, Switzerland) with vapor saturation. R_f values were determined from plates of 20 cm height. The detection on TLC plates of compounds was carried out by spraying the plates with a mixture of 500:10:2 acetic acid–sulfuric acid–anisaldehyde, followed by heating for 10 min at 120°C [17]. Radioactive products were localized and quantified by radioscanning using an automatic TLC linear analyzer (Tracemaster 40, Berthold, Wildbad, Germany), and radioactive bands on TLC plates were visualized by exposure to X-ray-sensitive film (Kodak, Rochester, USA). Fluorescent products were localized and quantified by fluorescence scanning using a model CS 910 TLC-scanner and C-R4A integrator from Shimadzu, Düsseldorf, Germany (excitation 475 nm, emission < 550 nm). Fluorescent bands on TLCs were photographed on Polaroid 667 film in a Polaroid camera attached to a Camag Reprostar II Camag, Muttenz, Switzerland (excitation at 366 nm).

Removal of salts and other polar components from reaction products.—Amphiphilic reaction products were freed of salts and other polar and water-soluble materials by reversed-phase chromatography similarly to the procedure described [18]. Briefly,

Pasteur pipettes containing a small cotton plug were filled with a 1:1 slurry of LiChroprep RP-18 in methanol, and washed successively with methanol, 1:1 methanol–water, and water. The "minicolumns" thus prepared were ready for use. Lipophilic reaction products were adsorbed onto the minicolumns from a solution in 4:6 methanol–water and then eluted with methanol after salts and other polar material had been washed out.

HPLC.—Final purification of reaction products was carried out by high-performance liquid chromatography on columns of various sizes (0.4 × 15, 1 × 25 and 2 × 25 cm) containing reversed-phase material (ProSep C_{18}, with 5 μm mean particle diameter; Latek, Heidelberg, Germany).

Fast atom bombardment mass spectra (FABMS).—FABMS spectra were recorded either in the negative- or positive-ion mode on a ZAB HF instrument (VG Analytical, Manchester, UK) [19].

500 MHz ^1H NMR and 125 MHz ^{13}C NMR spectroscopy.—A Bruker AMX 500 NMR spectrometer (Karlsruhe, Germany) was used for ^1H NMR and ^{13}C NMR measurements. Chemical shifts (δ) are indicated in ppm relative to internal tetramethylsilane (Me_4Si).

Quantification of fluorescence.—Fluorescence was quantified with a Shimadzu RF 5000 spectrofluorophotometer (Shimadzu, Düsseldorf, Germany) in 1:1 chloroform–methanol in a range between 1 nM and 1 μM using 6-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoic acid as a standard (excitation 475 nm, emission 518 nm).

Quantification of radioactivity.—Radioactivity was determined using a Tri-Carb 1900 liquid scintillation counter (Canberra Packard, Frankfurt, Germany), using the Ultima Gold (Canberra Packard) scintillation cocktail.

Elemental analyses.—Elemental analyses were carried out with a CHN-O-Rapid analyzer (Heraeus, Osterode, Germany).

β-D-Galactopyranosyl-(1 → 1)-(2S,3R,4E)-2-dichloroacetamido-4-octadecen-1,3-diol (**1**).—To a solution of galactosylsphingosine (420 mg, 0.91 mmol) in dry methanol (20 mL) and *N,N*-diisopropylethylamine (0.5 mL), cooled to −20°C, aliquots (200 μL) of a solution of dichloroacetyl chloride (0.77 mL, 8.25 mmol) in dry dichloromethane (4.25 mL) were added over a period of 1 h with vigorous stirring. Addition of dichloroacetyl chloride was continued until no more ninhydrin-positive material could be detected. After completion of the reaction 50 mL water were added to the mixture, and the solution was extracted six times with ethyl acetate (75 mL). The organic phase was evaporated to a syrup that was chromatographed on a column of silica gel 200 g with 9:1 chloroform–methanol, to give compound **1** (460 mg, 0.80 mmol, 88%) as an amorphous mass: $R_f = 0.61$ (60:40:9 chloroform–methanol–2 M ammonia); ^1H NMR (500 MHz, $CDCl_3$): galactose unit δ 3.38 (dd, 1 H, $J_{5,6a}$ 4 Hz, $J_{6a,6b}$ 10 Hz, H-6a), 3.39 (m, 1 H, H-5), 3.44 (dd, 1 H, $J_{5,6a}$ 7.5 Hz, $J_{6a,6b}$ 10 Hz, H-6b), 3.51 (dd, 1 H, $J_{3,4}$ 3.25 Hz, $J_{2,3}$ 10 Hz, H-3), 3.77 (d, 1 H, $J_{3,4}$ 3.25 Hz, H-4), 4.11 (d, 1 H, $J_{1,2}$ 7.5 Hz, H-1), 4.13 (dd, 1 H, $J_{1,2}$ 7.5 Hz, $J_{2,3}$ 10 Hz, H-2); sphingosine unit δ 0.75 (t, 3 H, CH_3), 1.1–1.27 (m, 22 H, 11 CH_2), 1.89 (m, 2 H, H-6), 3.62 (dd, 1 H, $J_{1a,2}$ 5 Hz, $J_{1a,1b}$ 11.5 Hz, H-1a), 3.71 (dd, 1 H, $J_{1b,2}$ 6.5 Hz, $J_{1a,1b}$ 11.5 Hz, H-1b), 3.83 (s, 5 H, OH), 3.85 (m, 1 H, H-2), 4.06 (m, 1 H, H-3), 5.34 (dd, 1 H, $J_{3,4}$ 7.5 Hz, $J_{4,5}$ 15 Hz, H-4), 5.60 (td, 1 H, $J_{5,6}$ 6.5 Hz, $J_{4,5}$ 15 Hz, H-5), 7.02 (d, 1 H, NH); protecting group unit δ 5.93

(s, 1 H, $CHCl_2$); FABMS: ($C_{26}H_{47}Cl_2NO_8$, MW 572.56) [M + Na^+] at *m/z* 594, 596, 598. Anal. Calcd for $C_{26}H_{47}Cl_2NO_8 \cdot 0.8H_2O$ (586.97): C, 53.20; H, 8.35; N, 2.39. Found: C, 53.16; H, 8.56; N, 2.37.

4,6-O-p-Methoxybenzylidene-β-D-galactopyranosyl-(1 → 1)-(2S,3R,4E)-2-dichloroacetamido-4-octadecen-1,3-diol (**2**).—A solution of β-D-galactopyranosyl-(1 → 1)-(2*S*,3*R*,4*E*)-2-dichloroacetamido-4-octadecen-1,3-diol (**1**, 375 mg, 0.656 mmol), 4-methoxybenzaldehyde dimethyl acetal (170 μL, 0.98 mmol), and *p*-toluenesulfonic acid monohydrate (2 mg) in *N*,*N*-dimethylformamide (1 mL) was stirred under argon at 50°C for 2 days. The mixture was poured into a stirred solution of cold 2.5 mM sodium hydroxide (4 mL). After 1 h the mixture was extracted with diethyl ether (5 × 2 mL), and the combined organic phases were dried under a nitrogen jet. The residue was purified by column chromatography on silica gel (200 g) using 2:1 *n*-hexane–ethyl acetate to yield compound **2** (215.5 mg, 0.312 mmol, 47.5%): $R_f = 0.59$ (9:1 chloroform–methanol); ^{1}H NMR (500 MHz, $CDCl_3$): galactose unit δ 3.75 (t, 1 H, $J_{5,6}$ 8.5 Hz, H-5), 4.03 (m, 2 H, H-6), 4.12 (d, 1 H, $J_{3,4}$ 3 Hz, H-4), 4.23 (dd, 1 H, $J_{3,4}$ 3 Hz, $J_{2,3}$ 10 Hz, H-3), 4.27 (dd, 1 H, $J_{1,2}$ 7.5 Hz, $J_{2,3}$ 10 Hz, H-2), 4.33 (d, 1 H, $J_{1,2}$ 7.5 Hz, H-1); sphingosine unit δ 0.88 (t, 3 H, CH_3), 1.2–1.4 (m, 22 H, 11 CH_2), 2.03 (m, 2 H, H-6), 3.43 (s, 1 H, OH), 3.63 (dd, 1 H, $J_{1a,2}$ 5 Hz, $J_{1a,1b}$ 10 Hz, H-1a), 3.82 (dd, 1 H, $J_{1b,2}$ 6.5 Hz, $J_{1a,1b}$ 10 Hz, H-1b), 4.26 (m, 1 H, H-3), 4.27 (m, 1 H, H-2), 5.51 (dd, 1 H, $J_{3,4}$ 6 Hz, $J_{4,5}$ 15 Hz, H-4), 5.77 (td, 1 H, $J_{5,6}$ 6 Hz, $J_{4,5}$ 15 Hz, H-5), 7.47 (d, 1 H, NH); protecting group unit δ 3.80 (s, 3 H, OCH_3), 5.49 (s, 1 H, $CHCl_2$), 6.00 (s, 1 H, *p*-$CH_3OC_6H_4CH$), 6.89 (m, 2 H, H-$Ph_{3,5}$), 7.41 (m, 2 H, H-$Ph_{2,6}$); FABMS: ($C_{34}H_{53}Cl_2NO_9$, MW 690.70) [M + Na^+] at *m/z* 712, 714, 716. Anal. Calcd for $C_{34}H_{53}Cl_2NO_9 \cdot 0.5H_2O$ (699.71): C, 58.36; H, 7.78; N, 2.00. Found: C, 58.17; H, 7.80; N, 1.77.

2,3-Di-O-benzoyl-4,6-O-p-methoxybenzylidene-β-D-galactopyranosyl-(1 → 1)-(2S,3R,4E)-3-O-benzoyl-2-dichloroacetamido-4-octadecen-1,3-diol (**3**).—To a solution of 4,6-*O*-*p*-methoxybenzylidene-β-D-galactopyranosyl-(1 → 1)-(2*S*,3*R*,4*E*)-2-dichloroacetamido-4-octadecen-1,3-diol (**2**, 205 mg, 0.296 mmol) in anhydrous pyridine (2 mL), cooled to −20°C, was added benzoyl chloride (104 μL, 0.89 mmol) in small portions over a period of 30 min with stirring. The bath temperature was kept below −10°C for 4 h and then was allowed to increase slowly to room temperature. After the addition of ethyl acetate (20 mL), the solution was washed twice successively with 5 mL each of cold M hydrogen chloride, satd sodium hydrogencarbonate, and cold water. The organic phase was evaporated to dryness, and the residue was freeze-dried from benzene. The crude product (271 mg, 0.270 mmol, 91%) was used without purification but for a small portion for analysis that was purified by HPLC on ProSep C_{18} in a stainless steel column (0.4 × 15 cm) using 85:15 methanol–water; $R_f = 0.53$ (1:1 *n*-hexane–ethyl acetate); ^{1}H NMR (500 MHz, $CDCl_3$): galactose unit δ 3.66 (m, 1 H, H-5), 4.07 (dd, 1 H, $J_{5,6a}$ 2 Hz, $J_{6a,6b}$ 12.5 Hz, H-6a), 4.25 (dd, 1 H, $J_{5,6b}$ 1.5 Hz, $J_{6a,6b}$ 12.5 Hz, H-6b), 4.55 (d, 1 H, $J_{3,4}$ 3.5 Hz, H-4), 4.78 (d, 1 H, $J_{1,2}$ 8 Hz, H-1), 5.35 (dd, 1 H, $J_{3,4}$ 3.5 Hz, $J_{2,3}$ 10.5 Hz, H-3), 5.85 (dd, 1 H, $J_{1,2}$ 8 Hz, $J_{2,3}$ 10.5 Hz, H-2); sphingosine unit δ 0.88 (t, 3 H, CH_3), 1.2–1.35 (m, 22 H, 11 CH_2), 1.98 (m, 2 H, H-6), 3.88 (dd, 1 H, $J_{1a,2}$ 4 Hz, $J_{1a,1b}$ 10.5 Hz, H-1a), 4.20 (dd, 1 H, $J_{1b,2}$ 4 Hz, $J_{1a,1b}$ 10.5 Hz, H-1b), 4.41 (m, 1 H, H-2), 5.52 (dd, 1 H, $J_{3,4}$ 7 Hz, $J_{4,5}$ 15 Hz, H-4), 5.65 (m, 1 H,

H-3), 5.84 (td, 1 H, $J_{5,6}$ 6.5 Hz, $J_{4,5}$ 15 Hz, H-5), 6.82 (d, 1 H, NH); protecting group unit δ 3.81 (s, 3 H, OCH_3), 5.48 (s, 1 H, *p*-$CH_3OC_6H_4CH$), 5.69 (s, 1 H, $CHCl_2$), 6.88 (m, 2 H, H-$Ph_{3,5}$), 7.35–7.93 (m, 15 H, H-Bz), 8.02 (m, 2 H, H-$Ph_{2,6}$); FABMS: ($C_{55}H_{65}Cl_2NO_{12}$, MW 1003.02) [M + Na^+] at *m/z* 1024, 1026, 1028.

*2,3-Di-*O*-benzoyl-β-*D*-galactopyranosyl-(1 → 1)-(2*S*,3*R*,4*E*)-3-*O*-benzoyl-2-dichloroacetamido-4-octadecen-1,3-diol* (**4**).—To a solution of 2,3-di-*O*-benzoyl-4,6-*O*-*p*-methoxybenzylidene-β-D-galactopyranosyl-(1 → 1)-(2*S*,3*R*,4*E*)-3-*O*-benzoyl-2-dichloroacetamido-4-octadecen-1,3-diol (**3**, 260 mg, 0.259 mmol) in 1:1 chloroform–methanol (5 mL) was added 2 mg of *p*-toluenesulfonic acid monohydrate, and the solution was stirred under argon for 4 h at room temperature. After addition of *N*,*N*-diisopropylethylamine, the mixture was dried under a stream of nitrogen. The residue was chromatographed on silica gel (200 g) using 1:1 *n*-hexane–ethyl acetate to afford **4** as an amorphous product (158.5 mg, 0.179 mmol, 69%); $R_f = 0.26$ (1:1 *n*-hexane–ethyl acetate); ^{1}H NMR (500 MHz, $CDCl_3$): galactose unit δ 3.53 (t, 1 H, $J_{5,6}$ 3 Hz, H-5), 3.75 (dd, 1 H, $J_{5,6a}$ 3 Hz, $J_{6a,6b}$ 10 Hz, H-6a), 4.06 (dd, 1 H, $J_{5,6b}$ 3 Hz, $J_{6a,6b}$ 10 Hz, H-6b), 4.43 (d, 1 H, $J_{3,4}$ 3 Hz, H-4), 4.67 (d, 1 H, $J_{1,2}$ 8 Hz, H-1), 5.27 (dd, 1 H, $J_{3,4}$ 3 Hz, $J_{2,3}$ 10 Hz, H-3), 5.85 (dd, 1 H, $J_{1,2}$ 8 Hz, $J_{2,3}$ 10 Hz, H-2); sphingosine unit δ 0.88 (t, 3 H, CH_3), 1.2–1.4 (m, 22 H, 11 CH_2), 2.02 (m, 2 H, H-6), 3.69 (dd, 1 H, $J_{1a,2}$ 3 Hz, $J_{1a,1b}$ 12.5 Hz, H-1a), 3.86 (dd, 1 H, $J_{1b,2}$ 4 Hz, $J_{1a,1b}$ 12.5 Hz, H-1b), 4.43 (m, 1 H, H-2), 5.50 (dd, 1 H, $J_{3,4}$ 7.5 Hz, $J_{4,5}$ 15 Hz, H-4), 5.83 (m, 1 H, H-3), 6.02 (td, 1 H, $J_{5,6}$ 6 Hz, $J_{4,5}$ 15 Hz, H-5), 6.88 (d, 1 H, NH); protecting group unit δ 5.78 (s, 1 H, $CHCl_2$), 7.37–8.02 (m, 15 H, H-Bz); FABMS: ($C_{47}H_{59}Cl_2NO_{11}$, MW 884.89) [M + Na^+] at *m/z* 906, 908, 910. Anal. Calcd for $C_{47}H_{59}Cl_2NO_{11} \cdot 0.5H_2O$ (893.90): C, 63.15; H, 6.77; N, 1.56. Found: C, 63.01; H, 6.94; N, 1.45.

*2,3,6-Tri-*O*-benzoyl-β-*D*-galactopyranosyl-(1 → 1)-(2*S*,3*R*,4*E*)-3-*O*-benzoyl-2-dichloroacetamido-4-octadecen-1,3-diol* (**5**).—2,3-Di-*O*-benzoyl-β-D-galactopyranosyl-(1 → 1)-(2*S*,3*R*,4*E*)-3-*O*-benzoyl-2-dichloroacetamido-4-octadecen-1,3-diol (**4**, 155 mg, 0.175 mmol) was dissolved in dry pyridine (0.5 mL) and cooled to −30°C. Benzoyl chloride (22.4 μL, 0.193 mmol) was added, and the mixture was stirred for 2 h at −30°C and then raised to room temperature. Workup was carried out as for **3**. The crude product was purified on silica gel (200 g) using 8:2 *n*-hexane–ethyl acetate to yield **5** (111 mg, 0.112 mmol, 64%): $R_f = 0.35$ (7:3 *n*-hexane–ethyl acetate); ^{1}H NMR (500 MHz, $CDCl_3$): galactose unit δ 2.1, 2.3 (2s, 2 OH), 4.00 (t, 1 H, $J_{5,6}$ 6.5 Hz, H-5), 4.27 (dd, 1 H, $J_{5,6a}$ 6.5 Hz, $J_{6a,6b}$ 11.5 Hz, H-6a), 4.29 (d, 1 H, $J_{3,4}$ 3 Hz, H-4), 4.50 (dd, 1 H, $J_{5,6b}$ 6.5 Hz, $J_{6a,6b}$ 11.5 Hz, H-6b), 4.78 (d, 1 H, $J_{1,2}$ 8 Hz, H-1), 5.35 (dd, 1 H, $J_{3,4}$ 3 Hz, $J_{2,3}$ 10 Hz, H-3), 5.77 (dd, 1 H, $J_{1,2}$ 8 Hz, $J_{2,3}$ 10 Hz, H-2); sphingosine unit δ 0.88 (t, 3 H, CH_3), 1.18–1.38 (m, 22 H, 11 CH_2), 2.00 (m, 2 H, H-6), 3.73 (dd, 1 H, $J_{1a,2}$ 3 Hz, $J_{1a,1b}$ 10 Hz, H-1a), 4.23 (dd, 1 H, $J_{1b,2}$ 4 Hz, $J_{1a,1b}$10 Hz, H-1b), 4.41 (m, 1 H, H-2), 5.49 (dd, 1 H, $J_{3,4}$ 7.5 Hz, $J_{4,5}$ 15 Hz, H-4), 5.67 (dd, 1 H, $J_{2,3}$ 7.5 Hz, $J_{3,4}$ 7.5 Hz, H-3), 5.89 (td, 1 H, $J_{5,6}$ 6.5 Hz, $J_{4,5}$ 15 Hz, H-5), 6.80 (d, 1 H, NH); protecting group unit δ 5.70 (s, 1 H, $CHCl_2$), 7.34–8.02 (m, 20 H, H-Bz); FABMS: ($C_{54}H_{63}Cl_2NO_{12}$, MW 988.99) [M + Na^+] at *m/z* 1010, 1012, 1014.

*2,3,6-Tri-*O*-benzoyl-4-*O*-trifluoromethylsulfonyl-β-*D*-galactopyranosyl-(1 → 1)-(2*S*,3*R*,4*E*)-3-*O*-benzoyl-2-dichloroacetamido-4-octadecen-1,3-diol* (**6**).—To a cooled (−20°C) solution of **5** (75 mg, 76 μmol) in dry pyridine (100 μL) and dry

dichloromethane (400 μL), under argon atmosphere, was slowly added trifluoromethanesulfonic anhydride (44 μL, 189 μmol). After 2 h TLC showed the reaction to be completed. The mixture was diluted with dichloromethane (2 mL) and washed with cold M hydrogen chloride (4 mL). Following phase separation the aqueous layer was extracted twice with dichloromethane (3 mL). The combined organic phases were concentrated under a nitrogen stream, and the residue was purified on silica gel (50 g) using a linear gradient from 9:1 *n*-hexane–ethyl acetate (300 mL) to 7:3 *n*-hexane–ethyl acetate (300 mL). The product **6** (63.5 mg, 57 μmol, 75%) was a white powder: $R_f = 0.39$ (7:3 *n*-hexane–ethyl acetate); ^{1}H NMR (500 MHz, $CDCl_3$): galactose unit δ 4.16 (dd, 1 H, $J_{5,6a}$ 7 Hz, $J_{6a,6b}$ 11.5 Hz, H-6a), 4.26 (t, 1 H, $J_{5,6}$ 7 Hz, H-5), 4.52 (dd, 1 H, $J_{5,6b}$ 7 Hz, $J_{6a,6b}$ 11.5 Hz, H-6b), 4.81 (d, 1 H, $J_{1,2}$ 8 Hz, H-1), 5.49 (d, 1 H, $J_{3,4}$ 3 Hz, H-4), 5.56 (dd, 1 H, $J_{3,4}$ 3 Hz, $J_{2,3}$ 10.5 Hz, H-3), 5.72 (dd, 1 H, $J_{1,2}$ 8 Hz, $J_{2,3}$ 10.5 Hz, H-2); sphingosine unit δ 0.88 (t, 3 H, CH_3), 1.20–1.39 (m, 22 H, 11 CH_2), 2.00 (m, 2 H, H-6), 3.77 (dd, 1 H, $J_{1a,2}$ 4 Hz, $J_{1a,1b}$ 9.5 Hz, H-1a), 4.22 (dd, 1 H, $J_{1b,2}$ 3.5 Hz, $J_{1a,1b}$ 9.5 Hz, H-1b), 4.42 (m, 1 H, H-2), 5.48 (dd, 1 H, $J_{3,4}$ 7.5 Hz, $J_{4,5}$ 15 Hz, H-4), 5.62 (dd, 1 H, $J_{2,3}$ 7.5 Hz, $J_{3,4}$ 7.5 Hz, H-3), 5.90 (td, 1 H, $J_{5,6}$ 6.5 Hz, $J_{4,5}$ 15 Hz, H-5), 6.75 (d, 1 H, NH); protecting group unit δ 5.67 (s, 1 H, $CHCl_2$), 7.34–8.05 (m, 20 H, H-Bz).

S-*(2,3,4,6-Tetra*-O-*acetyl-β*-D-*galactopyranosyl)-(1 → 4)-2,3,6-tri*-O-*benzoyl-4-thio-β*-D-*glucopyranosyl-(1 → 1)-(2*S,*3*R,*4*E*)-*3*-O-*benzoyl-2-dichloroacetamido-4-octadecen-1,3-diol* (**7**).—To a solution of 2-*S*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-2-thiopseudourea hydrobromide (**18**, 130 mg, 0.267 mmol) in 1:1 acetone–methanol (0.5 mL) was added potassium carbonate (100 mg) and **6** (60 mg, 53 μmol). After stirring under argon for 2 h at room temperature, dichloromethane (3 mL) was added, and the salts were removed by washing the mixture three times with water (3 mL). Following purification on silica gel (50 g) using 7:3 *n*-hexane–ethyl acetate as eluent, **7** (48 mg, 36 μmol, 68% with respect to **6**) was obtained: $R_f = 0.59$ (1:1 *n*-hexane–ethyl acetate); ^{1}H NMR (500 MHz, $CDCl_3$): galactose unit δ 3.89 (t, 1 H, $J_{5,6}$ 6 Hz, H-5), 3.97 (dd, 1 H, $J_{5,6a}$ 6 Hz, $J_{6a,6b}$ 11 Hz, H-6a), 4.03 (dd, 1 H, $J_{5,6b}$ 6 Hz, $J_{6a,6b}$ 11 Hz, H-6b), 4.85 (d, 1 H, $J_{1,2}$ 9.5 Hz, H-1), 4.92 (dd, 1 H, $J_{3,4}$ 3.5 Hz, $J_{2,3}$ 9.5 Hz, H-3), 5.04 (dd, 1 H, $J_{1,2}$ 9.5 Hz, $J_{2,3}$ 9.5 Hz, H-2), 5.37 (d, 1 H, $J_{3,4}$ 3.5 Hz, H-4); glucose unit δ 3.29 (dd, 1 H, $J_{3,4}$ 11 Hz, $J_{4,5}$ 11 Hz, H-4), 4.07 (m, 1 H, H-5), 4.55 (dd, 1 H, $J_{5,6a}$ 2 Hz, $J_{6a,6b}$ 12 Hz, H-6a), 4.70 (d, 1 H, $J_{1,2}$ 8 Hz, H-1), 4.75 (dd, $J_{5,6b}$ 4 Hz, $J_{6a,6b}$ 12 Hz, H-6b), 5.42 (dd, 1 H, $J_{1,2}$ 8 Hz, $J_{2,3}$ 8.5 Hz, H-2), 5.66 (dd, 1 H, $J_{2,3}$ 8.5 Hz, $J_{3,4}$ 11 Hz, H-3); sphingosine unit δ 0.88 (t, 3 H, CH_3), 1.17–1.32 (m, 22 H, 11 CH_2), 1.95 (m, 2 H, H-6), 3.69 (dd, 1 H, $J_{1a,2}$ 4 Hz, $J_{1a,1b}$ 10 Hz, H-1a), 4.16 (dd, 1 H, $J_{1b,2}$ 3 Hz, $J_{1a,1b}$ 10 Hz, H-1b), 4.38 (m, 1 H, H-2), 5.43 (dd, 1 H, $J_{3,4}$ 7.5 Hz, $J_{4,5}$ 15 Hz, H-4), 5.58 (m, 1 H, H-3), 5.84 (td, 1 H, $J_{5,6}$ 7 Hz, $J_{4,5}$ 15 Hz, H-5), 6.70 (d, 1 H, NH); protecting group unit δ 1.53, 1.91, 2.02, 2.10 (4s, 12 H, 4 CH_3CO), 5.67 (s, 1 H, $CHCl_2$), 7.31–8.02 (m, 20 H, H-Bz); FABMS: ($C_{68}H_{81}Cl_2NO_{20}S$, MW 1335.35) [M + Na^+] at *m/z* 1356, 1358, 1360.

Preparation of 2-S-*(2,3,4,6-tetra*-O-*acetyl-β*-D-*glucopyranosyl)-2-thiopseudourea hydrobromide* (**17**) *and* 2-S-*(2,3,4,6-tetra*-O-*acetyl-β*-D-*galactopyranosyl)-2-thiopseudourea hydrobromide* (**18**).—These compounds were prepared from the corresponding 2,3,4,6-tetra-*O*-acetyl-α-D-hexopyranosyl bromides essentially as described [20].

(2S,3R,4E)-2-Dichloroacetamido-4-octadecen-1,3-diol (**8**).—A solution of sphingosine (750 mg, 2.5 mmol) in dry methanol (40 mL) and *N,N*-diisopropylethylamine (0.6 mL), cooled to −20°C, was treated with dichloroacetyl chloride as described for **1**. Following warmup and addition of water (40 mL), the reaction product was isolated by extraction into ethyl acetate (75 mL). After evaporation of the solvent the crude product [single spot on TLC $R_f = 0.4$ (ethyl acetate)] was freeze-dried from benzene to give **8** (1.00 g, 2.43 mmol) that was sufficiently pure for the next reaction step. FABMS: ($C_{20}H_{37}Cl_2NO_3$, MW 410.38) [M + Na^+] at *m/z* 432, 434, 436, and [M + H^+ − H_2O] at *m/z* 392, 394, 396.

*(2S,3R,4E)-1-*O*-tert-Butyldiphenylsilyl-2-dichloroacetamido-4-octadecen-1,3-diol* (**9**).—To a solution of 1.00 g (2.43 mmol) (2*S*,3*R*,4*E*)-2-dichloroacetamido-4-octadecen-1,3-diol (**8**) in dry dichloromethane (20 mL) and tetrahydrofuran (2 mL), triethylamine (600 μL, 4.33 mmol), and *N,N*-dimethylaminopyridine (20 mg, 163 μmol) were added. Then *tert*-butylchlorodiphenylsilane (370 μL, 2.7 mmol) was added, and the solution was stirred for 7 h at room temperature. The reaction was stopped by adding methanol (0.5 mL), and the solvent was evaporated under nitrogen. The residue was chromatographed on silica gel (200 g) using 8:1 *n*-hexane–ethyl acetate as eluent to afford 1.51 g (2.31 mmol, 95%) of **9**: $R_f = 0.67$ (8:1 *n*-hexane–ethyl acetate); FABMS: ($C_{36}H_{55}Cl_2NO_3Si$, MW 648.83) [M + Na^+] at *m/z* 670, 672, 674, and [M + H^+ − H_2O] at *m/z* 630, 632, 634.

*(2S,3R,4E)-3-*O*-Benzoyl-1-*O*-tert-butyldiphenylsilyl-2-dichloroacetamido-4-octadecen-1,3-diol* (**10**).—To a cold (0°C) solution of 1.50 g (2.31 mmol) of (2*S*,3*R*,4*E*)-1-*O*-*tert*-butyldiphenylsilyl-2-dichloroacetamido-4-octadecen-1,3-diol (**9**) in pyridine (5 mL) was added benzoyl chloride (0.53 mL, 4.6 mmol), and the suspension was stirred for 1 h at 0°C, and then overnight at room temperature. Then 0.1 M hydrogen chloride (120 mL) was added, and the product extracted into ethyl acetate (60 mL). The ethyl acetate layer was washed with water, 0.1 M sodium hydrogencarbonate, and then it was evaporated. Chromatography on silica gel (200 g) of the crude product with 8:1 *n*-hexane–ethyl acetate gave 1.69 g of **10** (2.23 mmol, 96%): $R_f = 0.58$ (4:1 *n*-hexane–ethyl acetate); FABMS: ($C_{43}H_{59}Cl_2NO_4Si$, MW 752.94) [M + Na^+] at *m/z* 774, 776, 778, and [M + H^+] at *m/z* 752, 754, 756, and [M + H^+ − $PhCO_2H$] at *m/z* 630, 632, 634.

*(2S,3R,4E)-3-*O*-Benzoyl-2-dichloroacetamido-4-octadecen-1,3-diol* (**11**).—A solution of 1.65 g (2.18 mmol) of (2*S*,3*R*,4*E*)-3-*O*-*tert*-butyldiphenylsilyl-2-dichloroacetamido-4-octadecen-1,3-diol (**10**) in 0.17 M tetrabutylammonium fluoride in dry tetrahydrofuran (18 mL) was stirred overnight at 35°C. After the addition of M hydrogen chloride (10 mL), the product was extracted into ethyl acetate (60 mL). Evaporation of the organic solvent afforded a crude product that was purified by silica gel chromatography (200 g) with 7:3 *n*-hexane–ethyl acetate to give **11** (785 mg, 1.53 mmol, 70%): $R_f = 0.29$ (7:3 *n*-hexane–ethyl acetate); ^{1}H NMR (500 MHz, $CDCl_3$): sphingosine unit δ 0.88 (t, 3 H, CH_3), 1.22–1.29 (m, 20 H, 10 CH_2), 1.36 (m, 2 H, H-7), 2.05 (m, 2 H, H-6), 2.65 (m, 1 H, OH), 3.71 (m, 1 H, H-1a), 3.80 (m, 1 H, H-1b), 4.21 (m, 1 H, H-2), 5.59 (dd, 1 H, $J_{2,3}$ 8 Hz, $J_{3,4}$ 8 Hz, H-3), 5.61 (dd, 1 H, $J_{3,4}$ 8 Hz, $J_{4,5}$ 15 Hz, H-4), 5.91 (dd, 1 H, $J_{5,6}$ 7 Hz, $J_{4,5}$ 15 Hz, H-5), 7.05 (d, 1 H, $J_{NH,2}$ 9 Hz, NH); protecting group unit δ 5.92 (s, 1 H, $CHCl_2$), 7.47 (m, 2 H, H-$Bz_{3,5}$), 7.60 (m, 1 H, H-Bz_4), 8.05

(m, 2 H, H-$Bz_{2,6}$); FABMS: ($C_{27}H_{41}Cl_2NO_4$, MW 514.53) $[M + Na^+]$ at *m/z* 536, 538, 540, and $[M + H^+ - PhCO_2H]$ at *m/z* 392, 394, 396. Anal. Calcd for $C_{27}H_{41}Cl_2NO_4$ (514.53): C, 63.03; H, 8.03; N, 2.72. Found: C, 63.19; H, 8.36; N, 2.75.

(2S,3R,4E)-3-O-Benzoyl-2-dichloroacetamido-1-O-methylsulfonyl-4-octadecen-1,3-diol (**12**).—To a cooled (−10°C) solution of (2*S*,3*R*,4*E*)-3-*O*-benzoyl-2-dichloroacetamido-4-octadecen-1,3-diol (**11**, 410 mg, 0.8 mmol) in pyridine (1.3 mL) was added methanesulfonyl chloride (93 μL, 1.2 mmol), and the suspension was kept overnight at 0°C. The suspension was then diluted with cold M hydrogen chloride (40 mL), and the product was extracted into dichloromethane (40 mL). The organic layer was washed with cold water (20 mL) and M sodium hydrogencarbonate (20 mL). After evaporation of the solvent and further purification by silica gel chromatography (200 g) with 7:3 *n*-hexane–ethyl acetate, 427 mg (0.72 mmol, 90%) of **12** was isolated: $R_f = 0.30$ (7:3 *n*-hexane–ethyl acetate); FABMS: ($C_{28}H_{43}Cl_2NO_6S$, MW 592.61) $[M + H^+]$ at *m/z* 592, 594, 596, and $[M + H^+ - PhCO_2H]$ at *m/z* 470, 472, 474. Anal. Calcd for $C_{28}H_{43}Cl_2NO_6S$ (592.61): C, 56.75; H, 7.31; N, 2.36. Found: C, 56.95; H, 7.45; N, 2.30.

(2R,3R,4E)-3-O-Benzoyl-2-dichloroacetamido-1-iodo-4-octadecen-3-ol (**13**).—A solution of 1.5 M sodium iodide (2 mL, 3 mmol) in acetone was added to a solution of 427 mg (0.72 mmol) (2*S*,3*R*,4*E*)-3-*O*-benzoyl-2-dichloroacetamido-1-*O*-methylsulfonyl-4-octadecen-1,3-diol (**12**) in acetone (3 mL) and stirred for 7 h at 60°C. After the addition of cold water (20 mL), the product was extracted into dichloromethane (20 mL). Iodine that formed during the reaction was reduced by the addition of a drop of 5 M sodium hydrogensulfite, and the organic phase was washed once with cold water. After evaporation of dichloromethane the crude product was freeze-dried from benzene to yield **13** (425 mg, 0.68 mmol, 94%). As this compound gave a single spot on TLC ($R_f = 0.52$, 4:1 *n*-hexane–ethyl acetate), it was used without further purification. FABMS: ($C_{27}H_{40}Cl_2INO_3$, MW 624.43) $[M + H^+]$ at *m/z* 624, 626, 628, and $[M + H^+ - PhCO_2H]$ at *m/z* 502, 504, 506. Anal. Calcd for $C_{27}H_{40}Cl_2INO_3$ (624.43): C, 51.94; H, 6.46; N, 2.24. Found: C, 51.35; H, 6.57; N, 2.15.

S-*(2,3,4,6-Tetra-O-acetyl-β-D-glucopyranosyl)-(1 → 1)-(2R,3R,4E)-3-benzoyloxy-2-dichloroacetamido-4-octadecen-1-thiol* (**14**).—Under an argon atmosphere 2-*S*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-2-thiopseudourea hydrobromide (**17**, 245 mg, 500 μmol) was dissolved in M methanolic sodium acetate (0.6 mL). This solution was added to a solution of (2*R*,3*R*,4*E*)-3-*O*-benzoyl-2-dichloroacetamido-1-iodo-4-octadecen-3-ol (**13**, 125 mg, 200 μmol) in 1:1 acetone–methanol (1.4 mL). After the addition of solid potassium carbonate (42 mg, 300 μmol), the mixture was stirred for 6 h at 20°C. Potassium carbonate was removed by centrifugation, washed once with acetone (0.6 mL) and again centrifuged off. To buffer the remaining salts, acetic acid (10 μL) was added before drying the clear supernatant in a nitrogen jet. The resulting syrup was chromatographed on a column of silica gel (50 g) using a linear gradient from *n*-hexane (300 mL) to ethyl acetate (300 mL) to afford **14** (121 mg, 140 μmol, 70% with respect to the iodo compound), which was freeze-dried from benzene to give an amorphous mass: $R_f = 0.23$ (7:3 *n*-hexane–ethyl acetate); ^1H NMR (500 MHz, $CDCl_3$): glucose unit δ 3.75 (ddd, 1 H, $J_{5,6a}$ 2 Hz, $J_{5,6b}$ 5 Hz, $J_{4,5}$ 9.5 Hz, H-5), 4.14 (dd, 1 H, $J_{5,6a}$ 2 Hz, $J_{6a,6b}$ 13 Hz, H-6a), 4.23 (dd, 1 H, $J_{5,6b}$ 5 Hz, $J_{6a,6b}$ 13 Hz, H-6b), 4.51 (d, 1 H, $J_{1,2}$

10 Hz, H-1), 5.03 (dd, 1 H, $J_{2,3}$ 9.5 Hz, $J_{1,2}$ 10 Hz, H-2), 5.08 (dd, 1 H, $J_{4,5}$ 9.5 Hz, $J_{3,4}$ 10 Hz, H-4), 5.22 (dd, 1 H, $J_{2,3}$ 9.5 Hz, $J_{3,4}$ 10 Hz, H-3); sphingosine unit δ 0.87 (t, 3 H, CH_3), 1.2–1.3 (m, 20 H, 10 CH_2), 1.36 (m, 1 H, H-7), 2.06 (m, 2 H, H-6), 2.93 (dd, 1 H, $J_{1a,2}$ 7.5 Hz, $J_{1a,1b}$ 14 Hz, H-1a), 3.06 (dd, 1 H, $J_{1b,2}$ 5 Hz, $J_{1a,1b}$ 14 Hz, H-1b), 4.47 (m, 1 H, H-2), 5.50 (dd, 1 H, $J_{3,4}$ 6.5 Hz, $J_{4,5}$ 15 Hz, H-4), 5.67 (dd, 1 H, $J_{2,3}$ 6.5 Hz, $J_{3,4}$ 6.5 Hz, H-3), 5.95 (dd, 1 H, $J_{5,6}$ 7 Hz, $J_{4,5}$ 15 Hz, H-5), 6.86 (d, 1 H, $J_{NH,2}$ 7 Hz, NH); protecting group unit δ 2.00, 2.02, 2.02, 2.03 (4s, 12 H, 4 $COCH_3$), 5.94 (s, 1 H, $CHCl_2$), 7.46 (m, 2 H, H-$Bz_{3,5}$), 7.58 (m, 1 H, H-Bz_4), 8.04 (m, 2 H, H-$Bz_{2,6}$); ^{13}C NMR (125 MHz, $CDCl_3$): glucose unit δ 62 (1 C, C-6), 68 (1 C, C-4), 70 (1 C, C-2), 74 (1 C, C-3), 76 (1 C, C-5), 84 (1 C, C-1); sphingosine unit δ 14 (1 C, CH_3), 23 (1 C, C-17), 29–33 (11 C, 11 CH_2), 52 (1 C, C-2), 62 (1 C, C-1), 75 (1 C, C-3), 123 (1 C, C-4), 138 (1 C, C-5); protecting group unit δ 21 (4 C, 4 $COCH_3$), 66 (1 C, $CHCl_2$), 129 (2 C, C-$Bz_{2,6}$), 130 (1 C, C-Bz_1), 133 (1 C, C-Bz_4), 164 (1 C, NHCO), 165 (1 C, PhCO), 169–170 (4 C, 4 $COCH_3$); FABMS: ($C_{41}H_{59}Cl_2NO_{12}S$, MW 860.88) [M + H$^+$] at *m/z* 860, 862, 864, and [M + H$^+$ − $PhCO_2H$] at *m/z* 738, 740, 742, and [M + Na$^+$] at *m/z* 882, 884, 886. Anal. Calcd for $C_{41}H_{59}Cl_2NO_{12}S$ (860.88): C, 57.20; H, 6.91; N, 1.63. Found: C, 56.67; H, 6.61; N, 1.51.

2,3,4,6-Tetra-O-*acetyl*-β-D-*galactopyranosyl*-(1 → 4)-*2,3,6-tri*-O-*acetyl*-1-S-*acetyl*-1-*thio*-β-D-*glucopyranose* (**15**).—Potassium thioacetate (430 mg, 3.77 mmol) and 2.22 g (3.2 mmol) of 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1 → 4)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl bromide (synthesized as described [20]) were dissolved in acetone (15 mL) and stirred overnight at 25°C. (Potassium bromide precipitated and the solution turned red.) The suspension was diluted with 40 mL of water, and the crude products were extracted into ethyl acetate (3 × 30 mL). Chromatography on silica gel (200 g) using a linear gradient from 2:1 *n*-hexane–ethyl acetate (900 mL) to ethyl acetate (900 mL) afforded 1.8 g (2.6 mmol, 81%) of **15**: R_f = 0.17 (1:1 *n*-hexane–ethyl acetate); FABMS: ($C_{28}H_{38}O_{18}S$, MW 694.65) [M + H$^+$] at *m/z* 695, and [M + Na$^+$] at *m/z* 717.

2,3,4,6-Tetra-O-*acetyl*-β-D-*galactopyranosyl*-(1 → 4)-S-(*2,3,6-tri*-O-*acetyl*-β-D-*glucopyranosyl*)-(1 → 1)-(2R,3R,4E)-*3-benzoyloxy-2-dichloroacetamido-4-octadecen-1-thiol* (**16**).—Solid sodium acetate (750 mg, 9.1 mmol) was added to a solution of 162.5 mg (260 μmol) (2*R*,3*R*,4*E*)-3-*O*-benzoyl-2-dichloroacetamido-1-iodo-4-octadecen-3-ol (**13**) and 451 mg (0.65 mmol) 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1 → 4)-2,3,6-tri-*O*-acetyl-1-*S*-acetyl-1-thio-β-D-glucopyranose (**15**) in dry acetone (4 mL), and the mixture was stirred for 20 h at 25°C. The suspension was then diluted with water (40 mL), and the product was extracted into ethyl acetate (40 mL). After evaporation of the organic layer the residue was purified on silica gel (200 g) using a linear gradient from 7:3 *n*-hexane–ethyl acetate (800 mL) to ethyl acetate (800 mL) to afford 195 mg of **16** (169 μmol, 65% with respect to the iodo compound): R_f = 0.37 (1:1 *n*-hexane–ethyl acetate); ^{1}H NMR (500 MHz, $CDCl_3$): galactose unit δ 3.86 (t, 1 H, $J_{5,6}$ 7 Hz, H-5), 4.10 (dd, 1 H, $J_{5,6a}$ 7 Hz, $J_{6a,6b}$ 12 Hz, H-6a), 4.13 (dd, 1 H, $J_{5,6b}$ 7 Hz, $J_{6a,6b}$ 12 Hz, H-6b), 4.47 (d, 1 H, $J_{1,2}$ 8 Hz, H-1), 4.95 (dd, 1 H, $J_{3,4}$, 4 Hz, $J_{2,3}$ 10 Hz, H-3), 5.10 (dd, 1 H, $J_{1,2}$ 8 Hz, $J_{2,3}$ 10 Hz, H-2), 5.35 (d, 1 H, $J_{3,4}$ 4 Hz, H-4); glucose unit δ 3.64 (td, 1 H, $J_{5,6}$ 5 Hz, $J_{4,5}$ 9.5 Hz, H-5), 3.78 (dd, 1 H, $J_{4,5}$ 9.5 Hz, $J_{3,4}$ 10 Hz, H-4), 4.06

(dd, 1 H, $J_{5,6a}$ 5 Hz, $J_{6a,6b}$ 12 Hz, H-6a), 4.48 (d, 1 H, $J_{1,2}$ 10 Hz, H-1), 4.51 (dd, 1 H, $J_{5,6b}$ 5 Hz, $J_{6a,6b}$ 12 Hz, H-6b), 4.93 (dd, 1 H, $J_{1,2}$ 10 Hz, $J_{2,3}$ 10 Hz, H-2), 5.20 (dd, 1 H, $J_{2,3}$ 10 Hz, $J_{3,4}$ 10 Hz, H-3); sphingosine unit δ 0.85 (t, 3 H, CH_3), 1.2–1.4 (m, 22 H, 11 CH_2), 2.05 (m, 2 H, H-6), 2.92 (dd, 1 H, $J_{1,2}$ 5.5 Hz, $J_{1a,1b}$ 12 Hz, H-1a), 3.05 (dd, 1 H, $J_{1b,2}$ 5.5 Hz, $J_{1a,1b}$ 12 Hz, H-1b), 4.46 (tdd, 1 H, $J_{1,2}$ 5.5 Hz, $J_{2,3}$ 6 Hz, $J_{2,NH}$ 7 Hz, H-2), 5.49 (dd, 1 H, $J_{3,4}$ 6 Hz, $J_{4,5}$ 15 Hz, H-4), 5.67 (dd, 1 H, $J_{2,3}$ 6 Hz, $J_{3,4}$ 6 Hz, H-3), 5.94 (dd, 1 H, $J_{5,6}$ 7 Hz, $J_{4,5}$ 15 Hz, H-5), 6.90 (d, 1 H, $J_{NH,2}$ 7 Hz, NH); protecting group unit δ 1.96, 2.02, 2.03, 2.04, 2.04, 2.07, 2.14 (7s, 21 H, 7 $COCH_3$), 5.95 (s, 1 H, $CHCl_2$), 7.46 (m, 2 H, H-$Bz_{3,5}$), 7.58 (m, 1 H, H-Bz_4), 8.04 (m, 2 H, H-$Bz_{2,6}$); ^{13}C NMR (125 MHz, $CDCl_3$): galactose unit δ 61 (1 C, C-6), 67 (1 C, C-4), 70 (1 C, C-2), 71 (1 C, C-3), 71 (1 C, C-5), 101 (1 C, C-1); glucose unit δ 61 (1 C, C-6), 71 (1 C, C-2), 74 (1 C, C-3), 76 (1 C, C-5), 77 (1 C, C-4), 84 (1 C, C-1); sphingosine unit δ 14 (1 C, CH_3), 23 (1 C, C-17), 30–33 (11 C, 11 CH_2), 52 (1 C, C-2), 62 (1 C, C-1), 75 (1 C, C-3), 123 (1 C, C-4), 138 (1 C, C-5); protecting group unit δ 21 (7 C, 7 $COCH_3$), 66 (1 C, $CHCl_2$), 129 (2 C, C-$Bz_{3,5}$), 130 (1 C, C-Bz_1), 130 (2 C, C-$Bz_{2,6}$), 133 (1 C, C-Bz_4), 164 (1 C, NHCO), 165 (1 C, PhCO); 169–170 (7 C, 7 $COCH_3$); FABMS: ($C_{53}H_{75}Cl_2NO_{20}S$, MW 1149.13) $[M + H^+]$ at *m/z* 1148, 1150, 1152, and $[M + H^+ - PhCO_2H]$ at *m/z* 1026, 1028, 1030, and $[M + Na^+]$ at *m/z* 1170, 1172, 1174. Anal. Calcd for $C_{53}H_{75}Cl_2NO_{20}S$ (1149.13): C, 55.40; H, 6.58; N, 1.22. Found: C, 54.89; H, 6.33; N, 1.29.

S-*β*-D-*Galactopyranosyl-(1 → 4)-4-thio-β*-D-*glucopyranosyl-(1 → 1)-(2S,3R,4E)-2-amino-4-octadecen-1,3-diol* (**19**), S-*β*-D-*glucopyranosyl-(1 → 1)-(2R,3R,4E)-2-amino-3-hydroxy-4-octadecen-1-thiol* (**20**), and *β*-D-*galactopyranosyl-(1 → 4)*-S-*β*-D-*glucopyranosyl-(1 → 1)-(2R,3R,4E)-2-amino-3-hydroxy-4-octadecen-1-thiol* (**21**).—*S*-(2,3,4,6-Tetra-*O*-acetyl-*β*-D-galactopyranosyl)-(1 → 4)-2,3,6-tri-*O*-benzoyl-4-thio-*β*-D-glucopyranosyl-(1 → 1)-(2*S*,3*R*,4*E*)-3-*O*-benzoyl-2-dichloroacetamido-4-octadecen-1,3-diol (**7**, 45 mg, 33.7 *μ*mol), *S*-(2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosyl)-(1 → 1)-(2*R*,3*R*,4*E*)-3-benzoyloxy-2-dichloroacetamido-4-octadecen-1-thiol (**14**, 50 mg, 58 *μ*mol) and 2,3,4,6-tetra-*O*-acetyl-*β*-D-galactopyranosyl-(1 → 4)-*S*-2,3,6-tri-*O*-acetyl-*β*-D-glucopyranosyl-(1 → 1)-(2*R*,3*R*,4*E*)-3-benzoyloxy-2-dichloroacetamido-4-octadecen-1-thiol (**16**, 184 mg, 160 *μ*mol), respectively, were treated, under argon, for 3 h at 65°C with 0.2 M potassium hydroxide in methanol (1 mL per 4 mg of educt). After cooling to room temperature the solution was buffered with acetic acid and diluted with water (3 mL per 4 mg of educt). Each mixture thus obtained was passed over LiChroprep RP-18 to adsorb the respective saponified product. Salts and other water-soluble materials were washed out with water, and the retained lipids were subsequently eluted with methanol. The lipids **19, 20** and **21**, respectively, were purified on silica gel (50 g), using 65:25:4 chloroform–methanol–2.5 M ammonia as eluent to afford 17.5 mg (27.2 *μ*mol, 81%) of **19**, 24.5 mg (51 *μ*mol, 88%) of **20**, and 89 mg (142 *μ*mol, 89%) of **21**, respectively: R_f values for **19, 20**, and **21** = 0.12, 0.11, and 0.26, respectively, (65:25:4 chloroform–methanol–2.5 M ammonia); FABMS for **19** and **21**: ($C_{30}H_{57}NO_{11}S$, MW 639.84) $[M + H^+]$ at *m/z* 640, and $[M + Na^+]$ at *m/z* 662; FABMS for **20**: ($C_{24}H_{47}NO_6S$, MW 477.70) $[M + H^+]$ at *m/z* 478.

S-*β*-D-*Galactopyranosyl-(1 → 4)-4-thio-β*-D-*glucopyranosyl-(1 → 1)-(2S,3R,4E)-2-[8-N-(7-nitrobenz-1,3-diazol-2-oxa-4-yl)amino]octanamido-4-octadecen-1,3-diol* (**22**),

S-β-D-*glucopyranosyl-(1 → 1)-(2*R,*3*R,*4*E*)*-3-hydroxy-2-[8-*N*-(*7-nitrobenz-1,3-diazol-2-oxa-4-yl)amino]octanamido-4-octadecen-1-thiol* (**23**), and β-D-*galactopyranosyl-(1 → 4)*-S-β-D-*glucopyranosyl-(1 → 1)-(2*R,*3*R,*4*E*)*-3-hydroxy-2-[8-*N*-(*7-nitrobenz-1,3-diazol-2-oxa-4-yl)amino]octanamido-4-octadecen-1-thiol* (**24**).—*S*-β-D-Galactopyranosyl-(1 → 4)-4-thio-β-D-glucopyranosyl-(1 → 1)-(2*S*,3*R*,4*E*)-2-amino-4-octadecen-1,3-diol (**19**), *S*-β-D-glucopyranosyl-(1 → 1)-(2*R*,3*R*,4*E*)-2-amino-3-hydroxy-4-octadecen-1-thiol (**20**) and β-D-galactopyranosyl-(1 → 4)-*S*-β-D-glucopyranosyl-(1 → 1)-(2*R*,3*R*,4*E*)-2-amino-3-hydroxy-4-octadecen-1-thiol (**21**) were *N*-acylated with *N*-succinimidyl 8-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]octanoate (**29**) as described for the *N*-acylation of lysogangliosides [9]. Briefly, the glycosylated sphingosines **19, 20,** and **21** (10 μmol each) were dissolved in *N*,*N*-dimethylformamide (150 μL) and *N*,*N*-diisopropylethylamine (10 μL). The solvents were removed in a nitrogen jet to ensure elimination of volatile amino and imino compounds that might otherwise interfere with the *N*-acylation reaction. Each residue, redissolved in *N*,*N*-dimethylformamide (150 μL) and *N*,*N*-diisopropylethylamine (10 μL), was mixed with **29** (15 μmol in 162 μL of *N*,*N*-dimethylformamide) and stirred under argon for 48 h at 40°C. After evaporation of the solvents under reduced pressure, the crude products were purified by HPLC on ProSep C_{18} (1 × 25 cm) with 85:15 methanol–water to yield **22** (8.5 μmol, 85%), **23** (9 μmol, 90%), and **24** (8.6 μmol, 86%): R_f values (60:35:8 chloroform–methanol–15 mM calcium chloride) were found to be 0.58, 0.75, and 0.55 for **22**, **23**, and **24**, respectively; FABMS **22** and **24**: ($C_{44}H_{73}N_5O_{15}S$, MW 944.14) $[M + H^+]$ at *m/z* 944, $[M + H^+ - H_2O]$ at *m/z* 926, and $[M + Na^+]$ at *m/z* 966; FABMS for **23**: ($C_{38}H_{63}N_5O_{10}S$, 782.00) $[M + H^+]$ at *m/z* 782, $[M + H^+ - H_2O]$ at *m/z* 764, and $[M + Na^+]$ at *m/z* 804.

S-β-D-*Galactopyranosyl-(1 → 4)-4-thio-β-D-glucopyranosyl-(1 → 1)-(2*S,*3*R,*4*E*)*-2-[1-*^{14}*C]octanamido-4-octadecen-1,3-diol* (**25**), S-β-D-*glucopyranosyl-(1 → 1)-(2*R,*3*R,*4*E*)*-2-[1-*^{14}*C]octanamido-3-hydroxy-4-octadecen-1-thiol* (**26**) *and* β-D-*galactopyranosyl-(1 → 4)*-S-β-D-*glucopyranosyl-(1 → 1)-(2*R,*3*R,*4*E*)*-2-[1-*^{14}*C]octanamido-3-hydroxy-4-octadecen-1-thiol* (**27**).—S-β-D-Galactopyranosyl-(1 → 4)-4-thio-β-D-glucopyranosyl-(1 → 1)-(2*S*,3*R*,4*E*)-2-amino-4-octadecen-1,3-diol (**19**), *S*-β-D-glucopyranosyl-(1 → 1)-(2*R*,3*R*,4*E*)-2-amino-3-hydroxy-4-octadecen-1-thiol (**20**) and β-D-galactopyranosyl-(1 → 4)-*S*-β-D-glucopyranosyl-(1 → 1)-(2*R*,3*R*,4*E*)-2-amino-3-hydroxy-4-octadecen-1-thiol (**21**) were acylated with *N*-succinimidyl [1-^{14}C]octanoate (**30**) as described for the fluorescent derivatives **22, 23**, and **24**. Here, only 5 μmol of each lysolipid was used. The products were purified by HPLC on ProSep C_{18} (1 × 25 cm) with 85:15 methanol–water to afford **25** (4.4 μmol, 88%), **26** (4.5 μmol, 90%), and **27** (4.6 μmol, 92%): R_f values (65:35:8 chloroform–methanol–15 mM calcium chloride) were found to be 0.52, 0.70, and 0.55 for **25, 26**, and **27**, respectively; FABMS for **25** and **27**: ($C_{38}H_{71}NO_{12}S$, MW 766.04) $[M + H^+]$ at *m/z* 766, $[M + H^+ - H_2O]$ at *m/z* 748, and $[M + Na^+]$ at *m/z* 788; FABMS for **26**: ($C_{32}H_{61}NO_7S$, MW 603.89) $[M + H^+]$ at *m/z* 604, $[M + H^+ - H_2O]$ at *m/z* 586, and $[M + Na^+]$ at *m/z* 626.

*8-[*N*-(7-Nitrobenz-1,3-diazol-2-oxa-4-yl)amino]octanoic acid* (**28**).—To a solution of 8-aminooctanoic acid (160 mg, 1 mmol) in dry methanol (15 mL) were added M sodium methoxide in dry methanol (1 mL) and solid 4-chloro-7-nitro-benzofurazan (200 mg, 1 mmol). This mixture was stirred under argon for 6 h at 25°C. After further

addition of the same amounts of sodium methoxide and 4-chloro-7-nitro-benzofurazan, the mixture was stirred overnight. The reaction was then stopped by acidification with 0.1 M hydrochloric acid (30 mL). The crude products were extracted into ethyl acetate (3 × 50 mL). The fluorescent organic phase was applied to a column of aluminum oxide (120 g), and the fluorescent products were eluted with ethyl acetate. After evaporation of the solvent the crude product was chromatographed on silica gel (200 g) using 80:20:1 chloroform–methanol–water to give **28** (84 mg, 0.26 mmol, 26%) as a crystalline solid: $R_f = 0.24$ (ethyl acetate); mp 141–143°C.

N-*Succinimidyl 8[*N-*(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]octanoate* (**29**) *and* N-*succinimidyl [1-*^{14}*C]octanoate* (**30**).—These *N*-succinimidyl esters were prepared as described for *N*-succinimidyl pyrene decanoate [9]. Briefly, 8-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]octanoic acid (**28**, 34 mg, 105 μmol) and [1-^{14}C]octanoic acid (15 mg, 104 μmol), respectively, were dissolved in dry *N*,*N*-dimethylformamide (1 mL). Following the addition of *N*,*N*′-dicyclohexylcarbodiimide (22 mg, 106 μmol) and *N*-hydroxysuccinimide (13 mg, 113 μmol), the reaction mixtures were stirred under argon for 2 days at 25°C. *N*,*N*′-Dicyclohexylurea that formed during the reactions was sedimented by centrifugation for 5 min at 2000 *g*, and the clear supernatants were stored at −20°C under argon until use. The yield of **29** and **30** was 88% and 85%, respectively, as determined by fluorescence and radioscanning of the appropriate bands from TLC plates: $R_f = 0.60$ and 0.58 (ethyl acetate) for **29** and **30**, respectively.

Assay with GM1-β-galactosidase and glucocerebrosidase.—Briefly, 1 nmol of the appropriate glycolipid analogue **22**, **23**, **24** and β-D-glucopyranosyl-(1 → 1)-(2*S*,3*R*,4*E*)-2-[8-*N*-(7-nitrobenz-1,3-diazol-2-oxa-4-yl)amino]octanamido-4-octadecen-1,3-diol and β-D-galactopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 1)-(2*S*,3*R*,4*E*)-2-[8-*N*-(7-nitrobenz-1,3-diazol-2-oxa-4-yl)amino]octanamido-4-octadecen-1,3-diol, respectively, were dried from their methanolic stock solutions. These latter two glycolipid analogues were synthesized from glucosyl- and lactosyl-ceramide [21], respectively, by alkaline degradation [9], followed by *N*-acylation with **29** as described for **22**, **23** and **24**. Each glycolipid analogue was dissolved in a Eppendorf vial in 91 mM sodium phosphate–citrate buffer pH 4.5 (100 μL) containing Triton-X 100 (0.4% w/v) and taurodeoxycholate (0.8% w/v). To each solution were added both GM1-β-galactosidase (18 pKatal) and glucocerebrosidase (0.9 pKatal). The activity of the respective enzymes was determined with 4-methylumbelliferyl β-D-galactopyranoside and β-D-glucopyranoside, respectively. The resulting mixtures were incubated for 3 h at 37°C. The reactions were stopped by adding methanol (0.2 mL), and the reaction products were desalted on LiChroprep RP-18. The glycolipid analogues and their degradation products, if any, were separated by TLC with 70:15:2 chloroform–methanol–water (Fig. 1).

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