methods

N-Azidoacetylmannosamine-mediated chemical tagging of gangliosides

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Abstract Peracetylated *N*-α-azidoacetylmannosamine (Ac_4ManNAz) is metabolized by cells to CMP-azidosialic acid. It has been demonstrated previously that in this way azidosialic acid-containing glycoproteins are formed that can be labeled on the cell surface by a modified Staudinger ligation. Here, we first demonstrate that the same procedure also results in the formation of azidosialic acid-containing gangliosides. Deoxymannojirimycin, an inhibitor of *N*-glycan processing in proteins, decreases the total cell surface labeling in Jurkat cells by ~25%. Inhibition of ganglioside biosynthesis with *N*-[5-(adamantan-1-yl-methoxy)-pentyl]1-deoxynojirimycin reduces cell surface labeling by ~75%. In conclusion, exposure of cells to Ac_4ManNAz allows in vivo chemical tagging of gangliosides.—Bussink, A. P., P. F. van Swieten, K. Ghauharali, S. Scheij, M. van Eijk, T. Wennekes, G. A. van der Marel, R. G. Boot, J. M. F. G. Aerts, and H. S. Overkleeft. ***N*-Azidoacetylmannosamine-mediated chemical tagging of gangliosides.** ***J. Lipid Res.*** **2007.** 48: **1417–1421.**

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Glycoconjugate-metabolizing glycosyltransferases and glycosidases have become important drug targets in recent years. Nature provides numerous lead compounds, primarily polyhydroxylated alkaloids (denominated iminosugars), that exert important biological activities by inhibiting specific glycosidases (1, 2). Effective therapeutic strategies based on interfering with glycoprocessing enzymes have been described. Two iminosugar-based drugs are now used in the clinic. Miglitol (*N*-hydroxyethyldeoxynojirimycin), inhibiting the intestinal glycosidases sucrase and maltase, is used for the treatment of diabetes mellitus type II (3). Miglustat (*N*-butyldeoxynojirimycin), inhibiting the glycosyltransferase glucosylceramide synthase, is in use for the treatment of Gaucher disease (4–6). Glucosylceramide synthase, the transferase responsible for the assembly of glucosylceramide from UDP-glucose and ceramide, is a key enzyme in the biosynthesis of neutral glycosphingolipids and sialic acid-containing gangliosides. More recently, the reduction of ganglioside levels was identified as a therapeutic approach for diabetes mellitus type II (7–9). Partial inhibition of glucosylceramide synthase, therefore, also appears as an attractive therapeutic target for the prevention and treatment of diabetes mellitus type II.

As part of our efforts to obtain effective glucosylceramide synthase inhibitors, we searched for a means to monitor the inhibitory effect of selected iminosugars on ganglioside biosynthesis in living cells. Bertozzi and coworkers (10–13) earlier developed a strategy for in vivo labeling of cell surface glycoproteins. Their approach is based on the finding that *N*-α-azidoacetylmannosamine is accepted by the CMP-sialic acid biosynthesis machinery. The resulting CMP-azidosialic acid in turn is recognized by sialic acid transferases, leading to the biosynthesis and cell surface expression of azidosialic acid containing *N*-linked glycoproteins. The azide can next be chemoselectively tagged by a modified Staudinger ligation. To promote the formation of azidosialic acid, Saxon and Bertozzi (10) established that peracetylated *N*-α-azidoacetylmannosamine (Ac_4ManNAz) is vividly taken up by cells and efficiently metabolized to *N*-α-azidoacetylmannosamine by cytosolic esterases. The realization that gangliosides, like GM3, also contain a sialic acid residue at the nonreducing end led us to explore whether the strategy developed by Saxon and Bertozzi (10) would also have merit in cell surface labeling of gangliosides. Here, we demonstrate the validity of this with the finding that Jurkat cells cultured in the presence of Ac_4ManNAz express azidosialic acid-containing glycosphingolipids at levels at least as high as

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those of azidosialic acid-containing *N*-linked glycoproteins. We further show that cell surface labeling of azidosialic acid-containing glycoproteins and gangliosides can be suppressed independently by the proper selection of iminosugars: those that inhibit *N*-linked glycan processing mannosidases and those that inhibit glucosylceramide synthase, respectively.

MATERIALS AND METHODS

Chemicals

Peracetylated *N*-azidoacetylmannosamine was synthesized as described previously (11). Phosphine-biotin was synthesized as described (14). *N*-[5-(Adamantan-1-yl-methoxy)-pentyl]1-deoxynojirimycin was synthesized as reported (15). All other chemical solvents and reagents were of analytical grade, obtained from commercial suppliers, and used without further purification unless stated otherwise.

Cell culture conditions

Jurkat cells were grown and maintained in RPMI 1640 medium with 2 mM L-glutamine (Biowhittaker, Baltimore, MD) containing 10% fetal calf serum (Gibco, Carlsbad, CA) at 5% CO_2. Cells were seeded at $\sim 1.5 \times 10^5$ ml in 5 ml flasks for flow cytometry or 50 ml flasks for lipid analysis. Cell viability was assessed during maintenance and before and after the various stages of labeling using trypan blue dye exclusion.

Both peracetylated *N*-azidoacetylmannosamine and *N*-[5-(adamantan-1-yl-methoxy)-pentyl]1-deoxynojirimycin were added to cell cultures from stock solutions in DMSO. The final concentration of DMSO in the culture medium was 1% (v/v). Deoxymannojirimycin (DMM; Sigma-Aldrich, Nieuwegein, The Netherlands) was added from stock solution in ethanol. As negative controls, equal volumes of the appropriate solvent were added to the cell culture.

Labeling of cell surface azides

After 3 days of incubation in the presence of 50 μM peracetylated *N*-azidoacetylmannosamine, cells were collected by centrifugation at 1,500 rpm for 10 min, washed three times in cold PBS, and resuspended in PBS containing 2% (v/v) fetal calf serum. The cells were distributed on a six-well plate in 1 ml, after which an equal amount of 0.5 mM biotin-phosphine in PBS was added. After incubation at room temperature for 3 h under mild shaking, the cells were collected by centrifugation and washed three times in cold PBS. Either the cells were labeled with streptavidin-FITC for the purpose of flow cytometry or total lipids were isolated as described below. FITC labeling was accomplished by incubation of cells with 1 ml of 1:1,000 streptavidin-FITC (Gibco) in PBS for 1 h in the dark at 4°C, after which the cells were washed three times in cold PBS. Flow cytometry was performed using a FACSscan (Becton Dickinson, Palo Alto, CA) with settings optimized for FITC fluorescence.

Isolation of gangliosides and ganglioside ligation product

Lipids were extracted with chloroform-methanol (1:1, v/v), and phase separation was performed according to Bligh and Dyer (16). The aqueous phase was evaporated to dryness under N_2. The samples were desalted on a SPE C18 column (Bakerbond, Deventer, The Netherlands). In short, the dried fractions were dissolved in 1 ml of water containing 0.1 M NaCl (pH 4.5). The solution was applied to the column, which had been preequilibrated with 2 ml of the same watery solution. Subsequently, the column was desalted with 30 ml of water, after which the lipids were eluted with 20 ml of a mixture containing equal volumes of chloroform and methanol. The eluent was evaporated to dryness under N_2.

In vitro Staudinger ligation of metabolically labeled gangliosides

Desalted gangliosides were dissolved in 500 μl of a mixture of equal volumes of chloroform and methanol containing phosphine-biotin in a final concentration of 1 mM. Next, 100 μl of water was added, and the reaction was allowed to proceed overnight at room temperature under mild stirring, after which the organic solvents and the water were evaporated to dryness under N_2.

Ganglioside detection

Gangliosides were detected by analysis of the acidic glycolipid fraction obtained after Folch extraction using chloroform-methanol-water (65:25:4) as a solvent (17). Gangliosides were quantified after the release of oligosaccharides from glycosphingolipids by ceramide glycanase detection (18). The oligosaccharides were labeled at their reducing end with the fluorescent compound anthranilic acid (2-aminobenzoic acid) before analysis using normal-phase high-performance liquid chromatography.

Glucosylceramide synthase activity in living cells was determined using as substrate fluorescently labeled 6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoyl-sphingosine(C6-NBD-ceramide) (19). Briefly, cells were incubated with 150 μM lipid and harvested at different time points. Lipids were extracted and separated by thin-layer chromatography, and NBD-ceramide and NBD-glucosylceramide were quantified (19). Endogenous cell surface GM3 was visualized by flow cytometry using monoclonal anti-GM3 antibody (Seikagu, Tokyo, Japan) and FITC-conjugated secondary antibodies according to the procedure described previously (20).

RESULTS

Cell surface labeling of azidosialosides

Jurkat cells were cultured for 3 days in medium containing 50 μM Ac_4ManNAz. Labeling of cell surface azidosialosides was performed as described in Materials and Methods. Cells were harvested and washed in labeling buffer and labeled for 3 h with phosphine-biotin. Next, cells were incubated with streptavidin-FITC, washed, and resuspended for flow cytometry analysis. Intense labeling of the cell surface was obtained by this procedure. The presence of Ac_4ManNAz did not influence the rate of cell proliferation. Cell viability, as assessed by trypan blue exclusion, was not affected by the procedure. Very similar results were obtained with murine B16 melanoma cells cultured in DMEM containing 10% fetal calf serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin at 10% CO_2 (data not shown).

Distinction of azide-containing *N*-linked glycoproteins and gangliosides

To distinguish between the presence of azide moieties in *N*-linked glycoproteins and gangliosides, cells were

cultured in the presence or absence of 1 mM DMM and 10 μM *N*-[5-(adamantan-1-yl-methoxy)-pentyl]1-deoxynojirimycin (AMP-DNM). DMM specifically inhibits the processing of high-mannose-type *N*-linked glycan to sialic acid-containing complex-type structures. We demonstrated previously that 1 mM DMM prevents the formation of complex-type glycan in glycoproteins in various cell types (21). Using radioactive motioning labeling, we observed that the presence of 1 mM DMM in the culture medium of Jurkat cells also completely blocked the conversion of EndoH-sensitive glycan to resistant structures in newly formed glucocerebrosidase molecules, indicating effective inhibition of glycan processing (data not shown). The presence of 1 mM DMM in the culture medium did not reduce the cell surface concentration of the ganglioside GM3, as detected by flow cytometry using antibody directed to the ganglioside.

AMP-DNM specifically inhibits the first step in glycosphingolipid biosynthesis catalyzed by glucosylceramide synthase ($IC_{50} \sim 150$ nM). Incubation of Jurkat cells with 10 μM AMP-DNM also completely inhibits in these cells the conversion of C6-NBD-ceramide to C6-NBD-glucosylceramide and subsequent glycosphingolipids (**Fig. 1**). We also observed by flow cytometry analysis that after 3 days of culture of Jurkat cells in the presence of 10 μM AMP-DNM, cell surface ganglioside GM3 is reduced by 80% (data not shown). AMP-DNM is known not to interfere with glycoprotein biosynthesis and processing (22).

Culturing of Jurkat cells for 3 days in the presence of 1 mM DMM and 0.05 mM Ac_4ManNAz resulted in a 24.0% reduction of fluorescent cell surface labeling (**Fig. 2**). In a second independent experiment, the reduction of fluorescence by DMM was 27%. The presence of

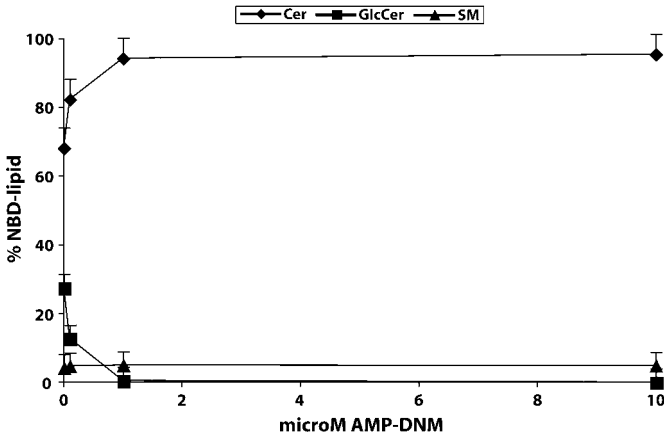
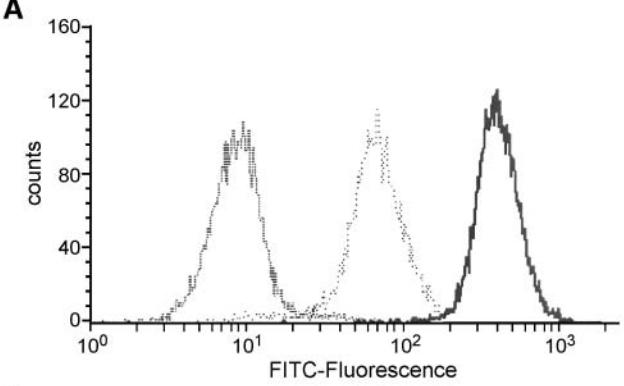


Fig. 1. Inhibition of glucosylceramide synthesis in Jurkat cells by *N*-[5-(adamantan-1-yl-methoxy)-pentyl]1-deoxynojirimycin (AMP-DNM). Jurkat cells were incubated for 1 h with 6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoyl-sphingosine (C6-NBD-ceramide) (Cer), and the formation of C6-NBD-glucosylceramide (GlcCer) and C6-NBD-sphingomyelin (SM) after 4 h was monitored as described in Materials and Methods. Lysosomal degradation of C6-NBD-glucosylceramide was prevented by the presence of 1 mM conduritol B-epoxide. Cells were exposed during incubation and chase with the indicated amounts of AMP-DNM. Cellular NBD-sphingolipid is put at 100%. Error bars represent ± SD.

A



B

Jurkat cells	Fluorescence intensity (%)	SD
No addition	100	5.1
AMP-DNM	13.5	2.1
DMM	76.0	5.6
AMP-DNM + DMM	5.1	2.2

Fig. 2. Cell surface labeling of cells cultured with peracetylated *N*-α-azidoacetylmannosamine (Ac_4ManNAz) in the absence or presence of deoxymannojirimycin (DMM) and AMP-DNM. Jurkat cells were labeled with Ac_4ManNAz and incubated with phosphine-biotin and then streptavidin-FITC, as described in Materials and Methods. Cell surface labeling was determined by FACSscan. A: Example of the effect of AMP-DNM. Overlay histogram showing labeled cells cultured in the absence of AMP-DNM, on the right, and the downward shift in cells cultured with 10 μM AMP-DNM (dotted), in the middle. Cells cultured in absence of Ac_4ManNAz but exposed to the Staudinger reagent are shown on the left. Note the log scale on the x axis. B: Overview of the impact of AMP-DNM (10 μM) and DMM (1 mM) on the labeling of Jurkat cells cultured in the presence of Ac_4ManNAz.

10 μM AMP-DNM led to an 86.5% reduction of fluorescence (Fig. 2). In the second independent experiment, the reduction of fluorescence by AMP-DNM was 68%. The combined presence of DMM and AMP-DNM led to an almost complete (95%) loss of fluorescence in all experiments. Very similar observations were made with murine melanoma cells (data not shown). Our findings suggest that a very large proportion of the cell surface azidosialosides in Jurkat cells are found in sialic acid-containing glycosphingolipids.

Demonstration of the formation of azidoGM3

To further substantiate that azidogangliosides are indeed formed in Jurkat cells exposed to Ac_4ManNAz, we isolated gangliosides and analyzed their oligosaccharides released by ceramide glycanase treatment and fluorescent labeling with anthranilic acid (AA). In Jurkat cells, the gangliosides almost exclusively consist of GM3 molecules. **Figure 3** shows that two trisaccharides derived from GM3 were detected. The retention time of the first peak coincides with the normal sialic acid-galactose-glucose-AA generated from GM3. Incubation with phosphine reagent did not influence its chromatographic behavior. The second peak shifted in its retention time after incubation with the phosphine reagent, indicating that it indeed contains an azide moiety.

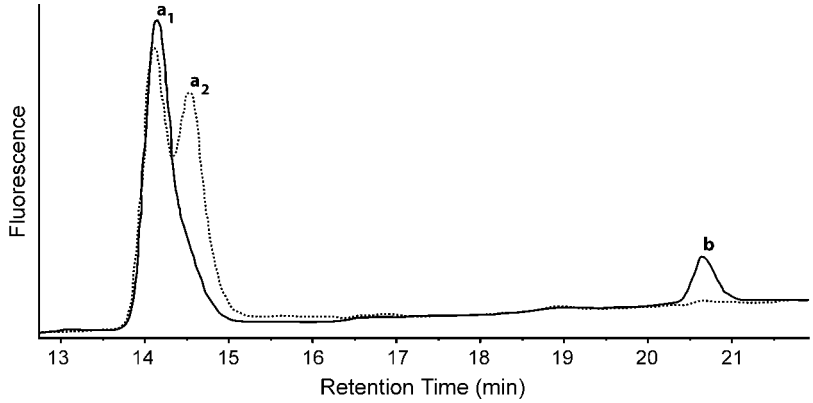


Fig. 3. Analysis of oligosaccharides derived from GM3. Demonstration of azidoGM3 formation in cells cultured with Ac_4ManNAz. Jurkat cells were labeled with Ac_4ManNAz, and glycosphingolipids were isolated as described in Materials and Methods. Oligosaccharides were removed from glycosphingolipids by ceramide glycanase digestion, labeled with anthranilic acid, and separated by HPLC as described in Materials and Methods. Oligosaccharides were reacted with phosphine-biotin or incubated identically without the agent. The dotted line, a double peak of two closely related molecular species (a_1 and a_2), displays the oligosaccharides not exposed to the Staudinger reagent. The solid line represents the chromatogram of oligosaccharides exposed to the Staudinger reagent, revealing a selective shift of a_2 to b.

DISCUSSION

Our study reveals that exposure of Jurkat cells to Ac_4ManNAz results in the formation of chemically tagged ganglioside, in particular the most abundant ganglioside, GM3. The proportion of azidoGM3 is ~60% of total GM3, suggesting a very efficient incorporation of azidosialic acid in gangliosides. This is not entirely surprising, because different forms of sialic acid occur in nature, species that are either acylated or glycolated at the N atom. Apparently, the azide group modification in sialic acid is equally well tolerated by the ganglioside biosynthetic machinery. It is of interest that the proportion of cell surface-tagged glycoproteins is actually lower than that of gangliosides. Again, this is not entirely surprising if one considers the estimated ratio of ganglioside to glycoprotein molecules at the cell surface. Gangliosides are largely located at the cell surface. Because nearly all ganglioside is GM3 in Jurkat cells, these cells contain per gram wet weight ~200 nmol of sialic acid associated with glycolipid. Assuming that ~1% of all cellular protein is cell membrane glycoprotein with an average mass of 50 kDa, Jurkat cells would contain ~20 nmol of glycoprotein per gram wet weight. To explain the observed ratio (3:1) of FITC-labeled ganglioside to glycoprotein, it would require on average at least three sialic acids per membrane glycoprotein.

The pioneering work of Bertozzi and colleagues (10–13) has led to a convenient procedure to chemically tag sialic acid-containing glycoconjugates that can be subsequently labeled at the cell surface. Our study reveals that this also includes gangliosides besides glycoproteins. This approach may have interesting applications. First, it offers a novel tool to screen synthetic and natural compounds that interfere in ganglioside biosynthesis and/or trafficking to the cell surface. Second, given the postulated role for GM3 in modulating insulin receptor-mediated signaling, it may be of interest to analyze the impact of various synthetic tags on this process, which is impaired in type 2 diabetes mellitus.

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REFERENCES

1. Fleet, G. W., L. E. Fellows, and B. Winchester. 1990. Plagiarizing plants: amino sugars as a class of glycosidase inhibitors. *Ciba Found. Symp.* **154:** 112–122.
2. Gruters, R. A., J. J. Neefjes, M. Tersmette, R. E. de Goede, A. Tulp, H. G. Huisman, F. Miedema, and H. L. Ploegh. 1987. Interference with HIV-induced syncytium formation and viral infectivity by inhibitors of trimming glucosidase. *Nature.* **330:** 74–77.
3. Scott, L. J., and C. M. Spencer. 2000. Miglitol: a review of its therapeutic potential in type 2 diabetes mellitus. *Drugs.* **59:** 521–549.
4. Platt, F. M., G. R. Neises, R. A. Dwek, and T. D. Butters. 1994. N-Butyldeoxynojirimycin is a novel inhibitor of glycolipid biosynthesis. *J. Biol. Chem.* **269:** 8362–8365.
5. Cox, T., R. Lachmann, C. Hollak, J. Aerts, S. van Weely, M. Hrebicek, F. Platt, T. Butters, R. Dwek, C. Moyses, et al. 2000. Novel oral treatment of Gaucher's disease with N-butyldeoxynojirimycin (OGT918) to decrease substrate biosynthesis. *Lancet.* **355:** 1481–1485.
6. Aerts, J. M., C. E. Hollak, R. G. Boot, J. E. Groener, and M. Maas. 2006. Substrate reduction therapy of glycosphingolipid storage disorders. *J. Inherit. Metab. Dis.* **29:** 449–456.
7. Yamashita, T., A. Hashiramoto, M. Haluzik, H. Mizukami, S. Beck, A. Norton, M. Kono, S. Tsuji, J. L. Daniotti, N. Werth, et al. 2003. Enhanced insulin sensitivity in mice lacking ganglioside GM3. *Proc. Natl. Acad. Sci. USA.* **100:** 3445–3449.
8. Kabayama, K., T. Sato, F. Kitamura, S. Uemura, B. W. Kang, Y. Igarashi, and J. Inokuchi. 2004. TNF alpha-induced insulin resistance in adipocytes as a membrane microdomain disorder: involvement of ganglioside GM3. *Glycobiology.* **15:** 21–29.
9. Aerts, J. M., R. Ottenhoff, A. S. Powlson, A. Grefhorst, M. van Eijk, P. F. Dubbelhuis, F. Kuipers, M. J. Serlie, T. Wennekes, H. S. Overkleeft, et al. Pharmacological inhibition of glucosylceramide synthase enhances insulin sensitivity. *Diabetes.* Epub ahead of print. February 7, 2007; doi:10.2337/db06-1619.

10. Saxon, E., and C. R. Bertozzi. 2000. Cell surface engineering by a modified Staudinger reaction. *Science.* **287:** 2007–2010.
11. Saxon, E., S. J. Luchansky, H. C. Hang, C. Yu, S. C. Lee, and C. R. Bertozzi. 2002. Investigating cellular metabolism of synthetic azidosugars with the Staudinger ligation. *J. Am. Chem. Soc.* **124:** 14893–14902.
12. Laughlin, S. T., N. J. Agard, J. M. Baskin, I. S. Carrico, P. V. Chang, A. S. Ganguli, M. J. Hangauer, A. Lo, J. A. Prescher, and C. R. Bertozzi. 2006. Metabolic labeling of glycans with azido sugars for visualization and glycoproteomics. *Methods Enzymol.* **415:** 230–250.
13. Prescher, J. A., D. H. Dube, and C. R. Bertozzi. 2004. Chemical remodelling of cell surfaces in living animals. *Nature.* **430:** 873–877.
14. Ovaa, H., P. F. van Swieten, B. M. Kessler, M. A. Leeuwenburgh, E. Fiebiger, A. M. C. H. van den Nieuwendijk, P. J. Galardy, G. A. van der Marel, H. L. Ploegh, and H. S. Overkleeft. 2003. Chemistry in living cells: detection of active proteasomes by a two-step labeling strategy. *Angew. Chem. Int. Ed. Engl.* **42:** 3626–3629.
15. Wennekes, T., R. J. B. H. N. van den Berg, W. Donker, G. A. van der Marel, A. Strijland, J. M. F. G. Aerts, and H. S. Overkleeft. 2007. Development of adamantan-1-yl-methoxy-functionalized 1-deoxynojirimycin derivatives as selective inhibitors of glucosylceramide metabolism in man. *J. Org. Chem.* **72:** 1088–1097.
16. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Med. Sci.* **37:** 911–917.
17. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226:** 497–509.
18. Neville, D. C., V. Coquard, D. A. Priestman, D. J. te Vruchte, D. J. Sillence, R. A. Dwek, F. M. Platt, and T. D. Butters. 2004. Analysis of fluorescently labeled glycosphingolipid-derived oligosaccharides following ceramide glycanase digestion and anthranilic acid labelling. *Anal. Biochem.* **331:** 275–282.
19. van Weely, S., M. B. van Leeuwen, I. D. Jansen, M. A. de Bruijn, E. M. Brouwer-Kelder, A. W. Schram, M. C. Sa Miranda, J. A. Barranger, E. M. Petersen, J. Goldblatt, et al. 1991. Clinical phenotype of Gaucher disease in relation to properties of mutant glucocerebrosidase in cultured fibroblasts. *Biochim. Biophys. Acta.* **1096:** 301–311.
20. Tagami, S., J. Inokuchi Ji, K. Kabayama, H. Yoshimura, F. Kitamura, S. Uemura, C. Ogawa, A. Ishii, M. Saito, Y. Ohtsuka, S. Sakaue, and Y. Igarashi. 2002. Ganglioside GM3 participates in the pathological conditions of insulin resistance. *J. Biol. Chem.* **277:** 3085–3092.
21. Aerts, J. M., S. Brul, W. E. Donker-Koopman, S. van Weely, G. J. Murray, J. A. Barranger, J. M. Tager, and A. W. Schram. 1986. Efficient routing of glucocerebrosidase to lysosomes requires complex oligosaccharide chain formation. *Biochem. Biophys. Res. Commun.* **141:** 452–458.
22. Aerts, J. M., C. Hollak, R. Boot, and A. Groener. 2003. Biochemistry of glycosphingolipid storage disorders: implications for therapeutic intervention. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **358:** 905–914.