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Note

Oxidation of the primary hydroxyl group of galactose of galactaosyl ceramide analogue by chemical method—precursors for the synthesis of labeled conjugates

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ABSTRACT

Isotopic labeling of the C-6 of a model glycosphingolipid (2*S*, 3*R*, 4*E*)-2-(1-adamantanacetamido)-3-hydroxy-4-octadecenyl-β-D-galactopyranoside, GalCAda, is described. Oxidation of (2*S*, 3*R*, 4*E*)-2-(1-adamantanacetamido)-3-(benzoyloxy)-4-octadecenyl-2,3,4-tri-*O*-benzoyl-β-D-galactopyranoside with *o*-iodoxybenzoic acid gave the dialdoside derivative in good yield. Reduction of the dialdoside with sodium borodeuteride gave the deuterium labeled D-GalCAda, with a cumulative yield of 35%.

Glycosphingolipids (GSLs) present on the cell surface play a variety of important roles[1]—intercellular recognition,[2,3] growth regulation,[4] differentiation,[5] microbial adhesion,[6,7] and receptors for bacterial toxins.[7] In addition, some GSL-metabolites are important intracellular second messengers.[5] We have demonstrated that substituting the acyl chain of GSLs with an adamantanacetyl group results in glycoconjugates that are significantly more water-soluble than the corresponding natural lipids.[8] Further, these conjugates have been shown to inhibit GSL–protein interactions associated with *E. coli* and HIV infections.[9,10]

Our biochemical investigations required labeled natural and model GSLs. Oxidation of the C-6 of terminal sugars—galactose, galactosamine, and *N*-acetyl galactosamine, of GSLs with galactose-oxidase followed by reduction of the dialdoside with tritiated sodium borohydride gave the radio-labeled GSLs.[11–13] However, this enzymatic oxidation is highly selective. Our attempts to oxidize the galactose residues of globotriaosyl ceramide were unsuccessful. Platinum-catalyzed hydrogenation of the sphingosine double bond as a method to radiolabel GSLs[14] was unsuitable for our applications. Catalytic hydrogenation is likely to reduce and/or isomerize the unsaturations present in the sphingosine and acyl chains. Such modifications to the ceramide chains can significantly alter the receptor function of the GSLs.[15]

Using GalCAda as a model compound, we describe an efficient chemical procedure to label the C-6 of galactose (see Scheme 1). GalCAda was used to circumvent the difficulties in characterization of natural GSLs. For example, natural GalC has more than 20 different ceramide-subtypes due to the variations in the acyl chain. When the optimized conditions for GalCAda were directly applied to the natural GalC, identical results were obtained.

For the protection of the primary hydroxyl group of the sugar, two methods were investigated: protection as a formyl ester or as trityl ether. Primary hydroxyls of methyl glucoside can be selectively converted to formyl esters with trichloro triazine/dimethyl formamide.[16] However, our attempts to achieve this with GalCAda were not successful. Tritylation with TrBr in Py gave greater than 70% of the desired product with significant (~20%) ditrityl derivatives. Formation of a thick precipitate during the reaction possibly hampered smooth progress. Using DMAP in a mixed solvent system THF–Py significantly reduced the formation of precipitate and gave the monotrityl ether, **1,** in greater than 95% yield.

The hydrolysis of the trityl ether group of precursor **5** with TFA gave significant acetyl group migration to the primary hydroxyl group. Interestingly, the degree of migration was influenced by the nature of the ceramide structure; in natural GalC the migration was less than 10% whereas for GalCAda, it was greater than 50%. However, benzoyl migration was less than 5% when identical hydrolysis conditions were employed for compound **2**. Treatment of **2** with TFA–AcOH in a mixed solvent AcOH–*i*PrOH gave compound **3** with no benzoyl ester hydrolysis. Oxidation of **3** with pyridinium chlorochromate[17] or by Swern-oxidation[18] failed to give the desired product. The dialdoside **4** was obtained in high yield by reacting **3** with *o*-iodoxybenzoic acid in DMSO–fluorobenzene at

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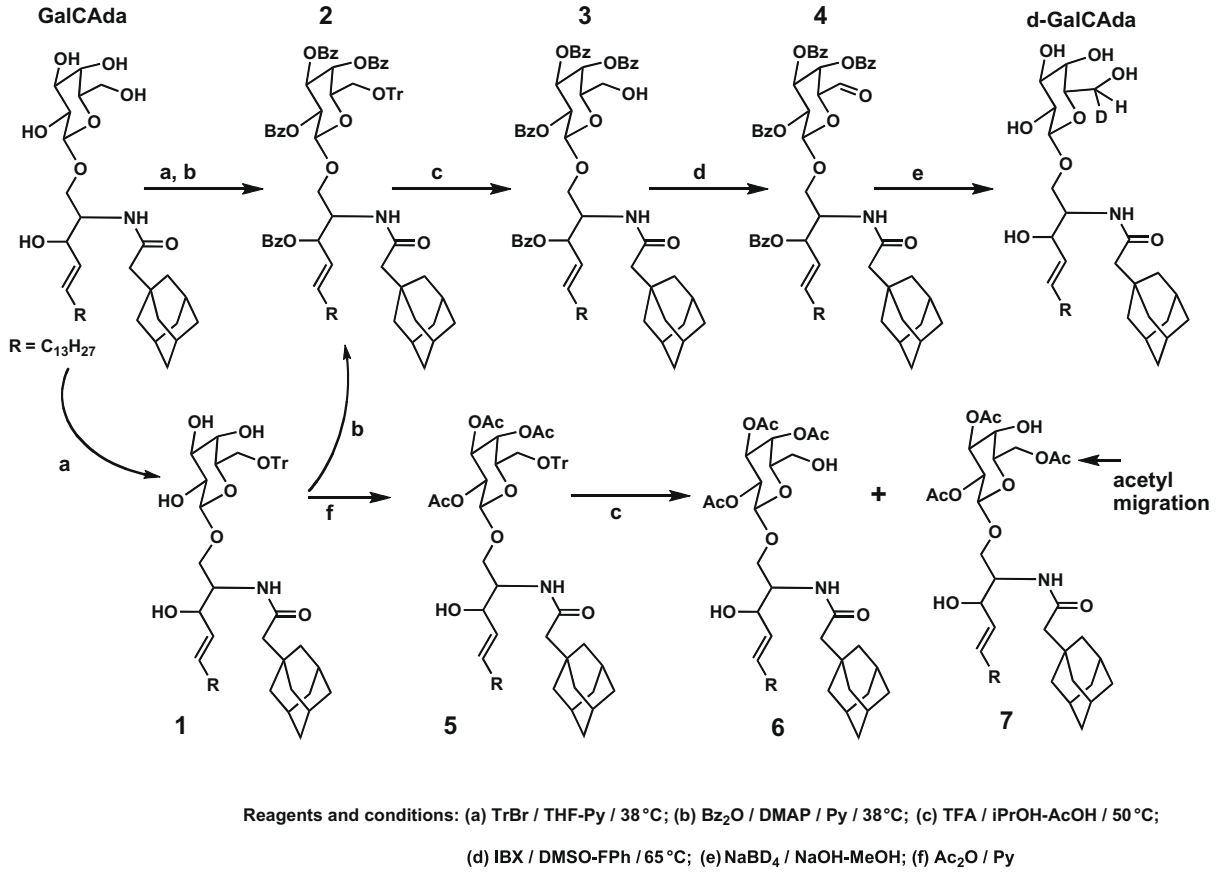


Scheme 1. Intermediates and reaction conditions involved in the synthesis of D-GalCAda from GalCAda.

65 °C.[19] Deuterium labeled compound D-GalCAda was obtained by reduction of **4** with $NaBD_4$ in MeOH–NaOH.[18]

The ESMS of GalCAda (Fig. 1A and C) gave the parent molecular ion ($M+Na^+$) and cluster-ion ($2M+Na^+$) peaks at 660.43 and 1297.87, respectively. The deuterium compound D-GalCAda (Fig. 1B and D) gave the expected shifts for the same ions, ($M+Na^+$) and cluster-ion ($2M+Na^+$) peaks at 661.44 and 1299.90, respectively. Comparison of the isotope-pattern of A to C and B to D, respectively, shows that the pattern is virtually identical, suggesting that the deuterium substitution is greater than 95%. Comparison of MS/MS spectra of (M+Na) ion of GalCAda and D-GalCAda shows that only the glycone derived daughter ions are shifted by 1 mass unit, suggesting that greater than 95% of deuterium is in the sugar (Fig. 2). Scheme 2 depicts a putative analysis of the fragmentation of GalCAda and D-GalCAda, where paths a and b are consistent with previous observations.[20] Daughter ions at 135.1 and 264.3 are reasonable since the former is a tertiary carbonium ion and the latter a resonance stabilized ion, probably formed by the loss of a ketene.[20]

1. Experimental

1.1. Material

Suppliers of reagents and solvents: Caledon—dichloromethane (CH_2Cl_2), methanol (MeOH), chloroform ($CHCl_3$), *iso*-propanol (*i*PrOH), hexanes, Silica gel 60 (35–70 μm), and aluminum-backed nanosilica plates (alugram NanoSIL GI UV254, Macherey & Nagel). Aldrich—tetrahydrofuran (THF), pyridine (Py), dimethyl sulfoxide (DMSO), fluorobenzene (FPh), diethyl ether (Et_2O), trityl bromide (TrBr), 2 M ammonia solution in methanol ($MeOH–NH_3$), LC–MS Chromasolv® methanol (MeOH–MS), acetic acid (AcOH), trifluoroacetic acid (TFA), dimethylamino pyridine (DMAP), benzoic anhydride (Bz_2O), phosphorus pentoxide, orcinol, 2,4 dinitro phenyl hydrazine, sodium borodeuteride ($NaBD_4$), and 3 Å molecular sieves. Commercial Alcohols Inc. (Brampton, Ont)—ethanol (EtOH). Sigma—galactoaosyl ceramide. Waters Associates, Mississauga, ON—silica-based C-18 reverse-phase cartridge (Sep-Pack™). GalCAda[10] and *o*-iodoxybenzoic acid (IBX)[21] were synthesized by published procedures.

Air sensitive reagents were handled under an inert atmosphere. Molecular sieves (3 Å) were activated at 250 °C for 24 h and were allowed to cool to room temperature under vacuum. Dry solvent mixtures: THF:Py, 2:1 (THF–Py); DMSO:FPh, 1:2 (DMSO–FPh), and Py were dried over activated molecular sieves (3 Å) for 16 h. MeOH and EtOH were stored over K_2CO_3. Unless specified, Teflon-lined screw-capped Kimax® glass tubes were used as reaction tubes and for the storage of products. GSL precursors were dried in a P_2O_5 desiccator. Thin layer chromatograms (TLCs) developing sprays: orcinol spray—0.5% orcinol in 6 M sulfuric acid, 2,4-DNP spray—2% 2,4-DNP in 0.7 M sulfuric acid in MeOH. Plates were sprayed with the developing reagent and heated in an oven at 140 °C until color developed (2–4 min). Estimating concentrations: Yield of compounds **1**–**4** were determined by comparison to standard GalCAda TLCs developed with orcinol.

1.2. Mass spectroscopic analysis

Electro spray mass spectroscopic (ESMS) analyses were performed on a $QSTAR_{XL}$ MS/MS spectrometer with a nano-spray source (ABI/MDS Sciex, Concord, ON). The sample was loaded in a PicoTip™ EMITTER and was attached to the nano-spray source.

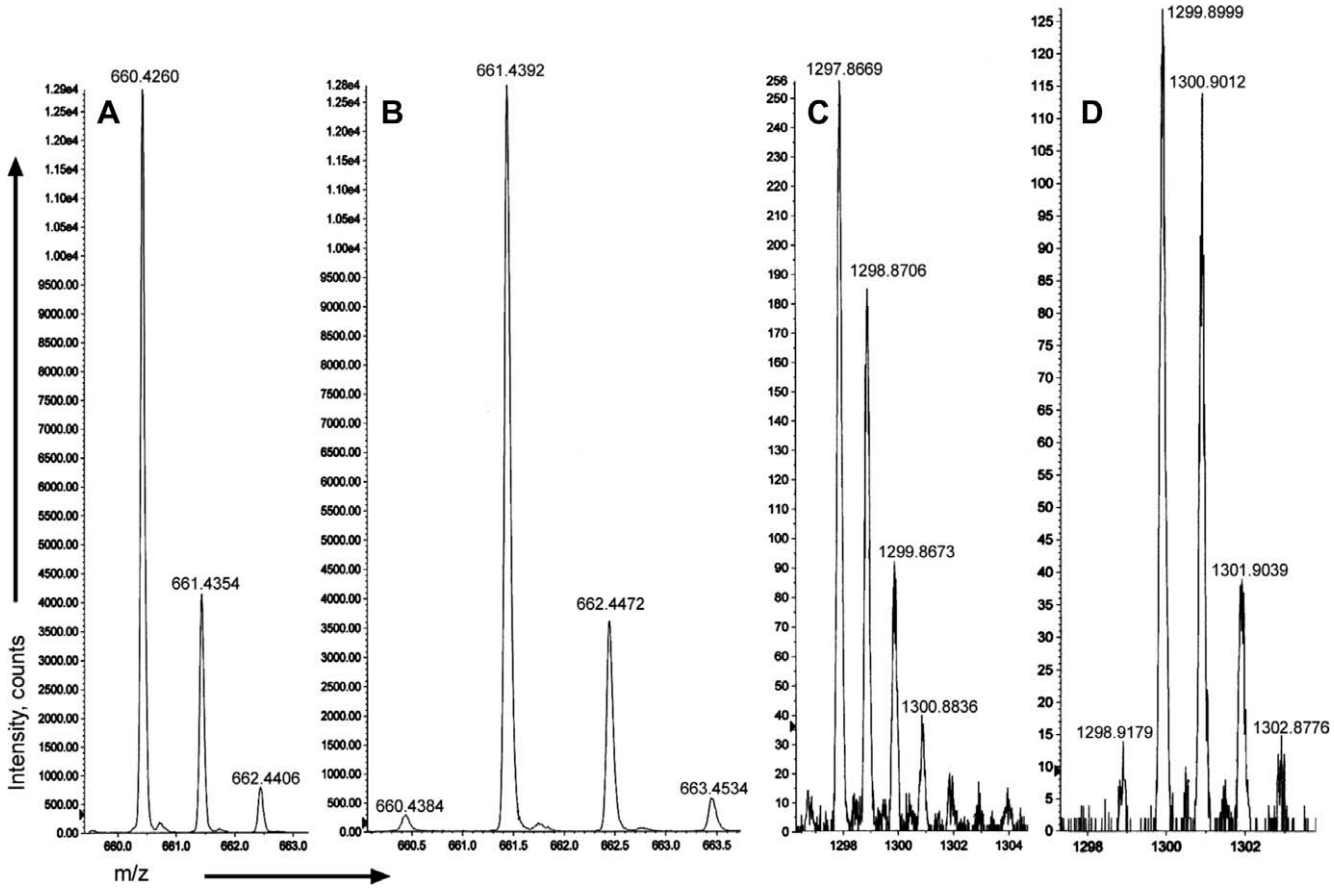


Figure 1. Electrospray mass spectra of GalCAda and D-GalCAda. GalCAda: A and C, ($M+Na^+$) and ($2M+Na^+$), respectively. D-GalCAda: B and D, ($M+Na^+$) and ($2M+Na^+$), respectively.

MS/MS spectra were recorded on a $QSTAR_{XL}$ MS/MS spectrometer with an o-MALDI source (ABI/MDS Sciex, Concord, ON). Sample (1 μL) was mixed with an equal volume of DHB and was spotted on a MALDI-plate. Compounds were dissolved in MeOH–NaCl solution to a final concentration of 0.5 μg/μL. MeOH–NaCl was prepared by adding a satd aq NaCl solution (3 μL) to MeOH–MS (10 mL). Theoretical mass was calculated with ChemDraw®.

1.3. Synthesis

1.3.1. (2*S*, 3*R*, 4*E*)-2-(1-Adamantanacetamido)-3-hydroxy-4-octadecenyl-6-*O*-trityl-β-D-galactopyranoside (1)

A dry sample of GalCAda (6.5 mg, 10.2 μmol) was dissolved in dry THF–Py (1.2 mL) and the following reagents were added in sequence—DMAP solution (42 μL of 0.5 M solution in THF–Py, 21 μmol) and TrBr (30 mg, 93 μmol). The mixture was then stirred at 38 °C and progress of the reaction was monitored every 1.5 h. Monitoring reaction progress: an aliquot (10 μL) of reaction mixture was added to EtOH (100 μL), dried, residue dissolved in $CHCl_3$:MeOH, 98:2 (20 μL), and was analyzed by TLC ($CHCl_3$:MeOH:water, 90:10:0.5). Once greater than 95% of the starting material was consumed (2.5–3.5 h), EtOH was added (6 mL) and dried. The oily material was suitable for the next step without further purification. ESMS, (ion) (found, expected): ($M+Na^+$) (902.313, 902.554).

1.3.2. (2*S*, 3*R*, 4*E*)-2-(1-Adamantanacetamido)-3-(benzoyloxy)-4-octadecenyl-2,3,4-tri-*O*-benzoyl-6-*O*-trityl-β-D-galactopyranoside (2)

To compound **2** (10.2 μmol, product from Section 1.3.1), a solution of DMAP (1 mmol, 2 mL of 0.5 M solution in dry Py) and Bz_2O (5 mmol, 5 mL of 1 M solution in dry Py) were added and the resulting mixture was stirred at 38 °C for 3 h. Then Py was evaporated and the residue was suspended in hexanes (20 mL) and transferred into a separating funnel. The organic phase was washed with saturated Na_2CO_3 (3–5 times, 5 mL each), with water (once, 10 mL), dried over anhydrous Na_2SO_4, and filtered. Removal of solvent from the filtrate gave an oily substance. Solvent for TLC analysis was *i*PrOH:CH_2Cl_2, 1:10. Silica gel chromatography: silica was suspended in $CHCl_3$:MeOH, 98:2 and added to a column 1 cm in diameter to a final bed volume of 3 cm (height). The column was equilibrated with the following solvents: MeOH–NH_3 (5 mL), CH_2Cl_2 (2 times, 10 mL each), and with hexanes (2 times, 10 mL each). Crude sample was dissolved in a minimum volume of hexanes, loaded, and eluted with the following solvents (number of fractions, volume of each fraction): hexanes (3, 5 mL), 1:3, CH_2Cl_2:hexanes (4, 6 mL); 1:1, CH_2Cl_2:hexanes (4, 6 mL); CH_2Cl_2 (4, 5 mL), and $CHCl_3$:MeOH, 98:2 (8, 3 mL). The product was eluted in the $CHCl_3$:MeOH, 98:2 fractions. Yield was 80%. ESMS, (ion) (found, expected): ($M+H^+$) (1296.682, 1296.678); ($M+Na^+$) (1318.677, 1318.659); ($M+EtOH+Na^+$) (1364.734, 1364.701).

1.3.3. (2*S*, 3*R*, 4*E*)-2-(1-Adamantanacetamido)-3-(benzoyloxy)-4-octadecenyl-2,3,4-tri-*O*-benzoyl-β-D-galactopyranoside (3)

A dried sample of **2** (1 mg, 1.5 μmol) was dissolved in solvent-mixture *i*PrOH:AcOH, 1:4 (0.25 mL), TFA was added (15 μmol, 30 μL of 0.5 M TFA in AcOH) and stirred at 50 °C. Monitoring reaction progress: an aliquot (5 μL) of sample was added to Et_2O (0.5 mL) and the organic layer was washed with water (2 times, 0.5 mL each) and with $KHCO_3$ until no effervescence (2–4 times, each 0.5 mL 1 M solution). The organic layer was dried, the residue dissolved in CH_2Cl_2 (25 μL), and analyzed by TLC (10:1, CH_2Cl_2:*i*-

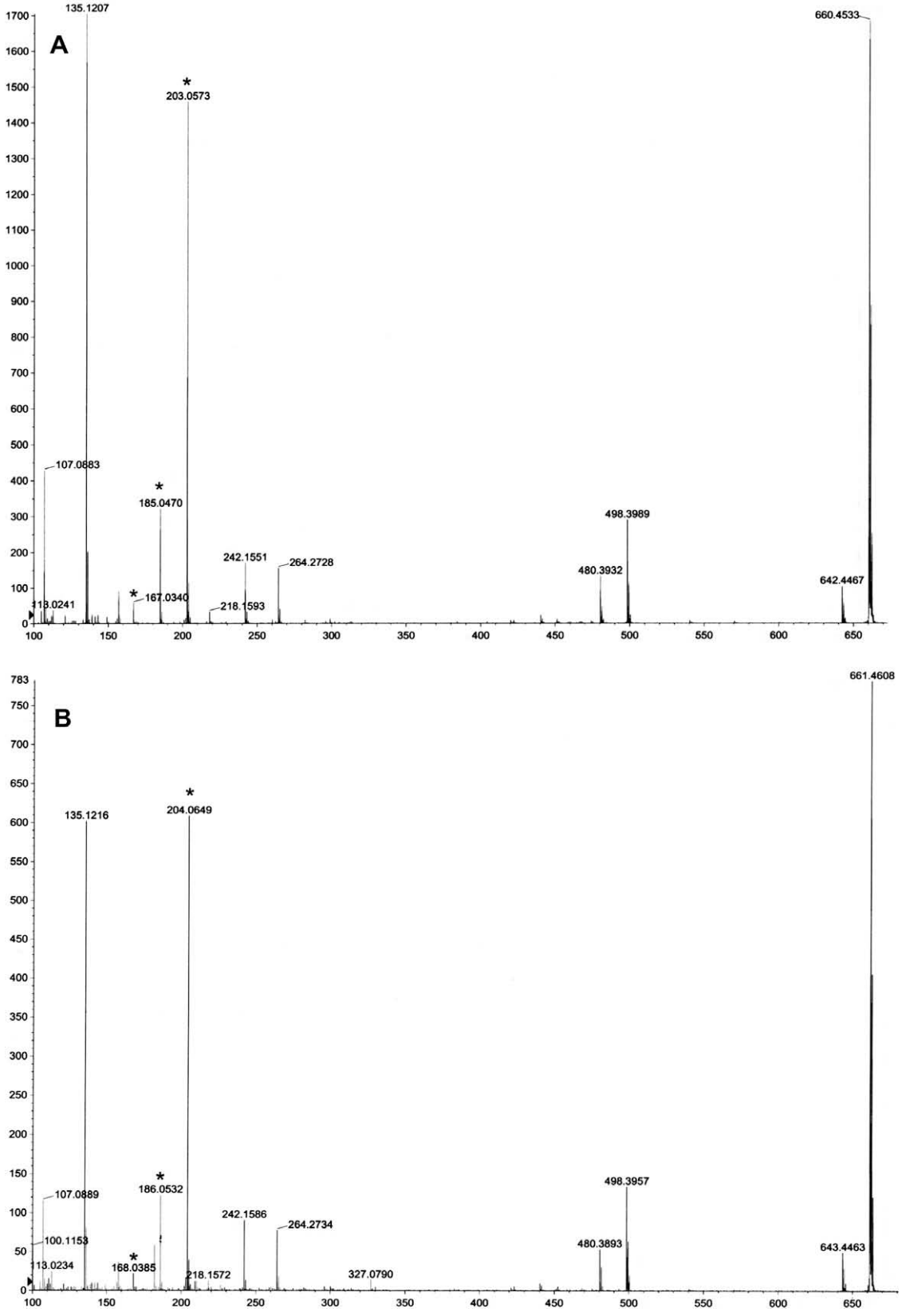
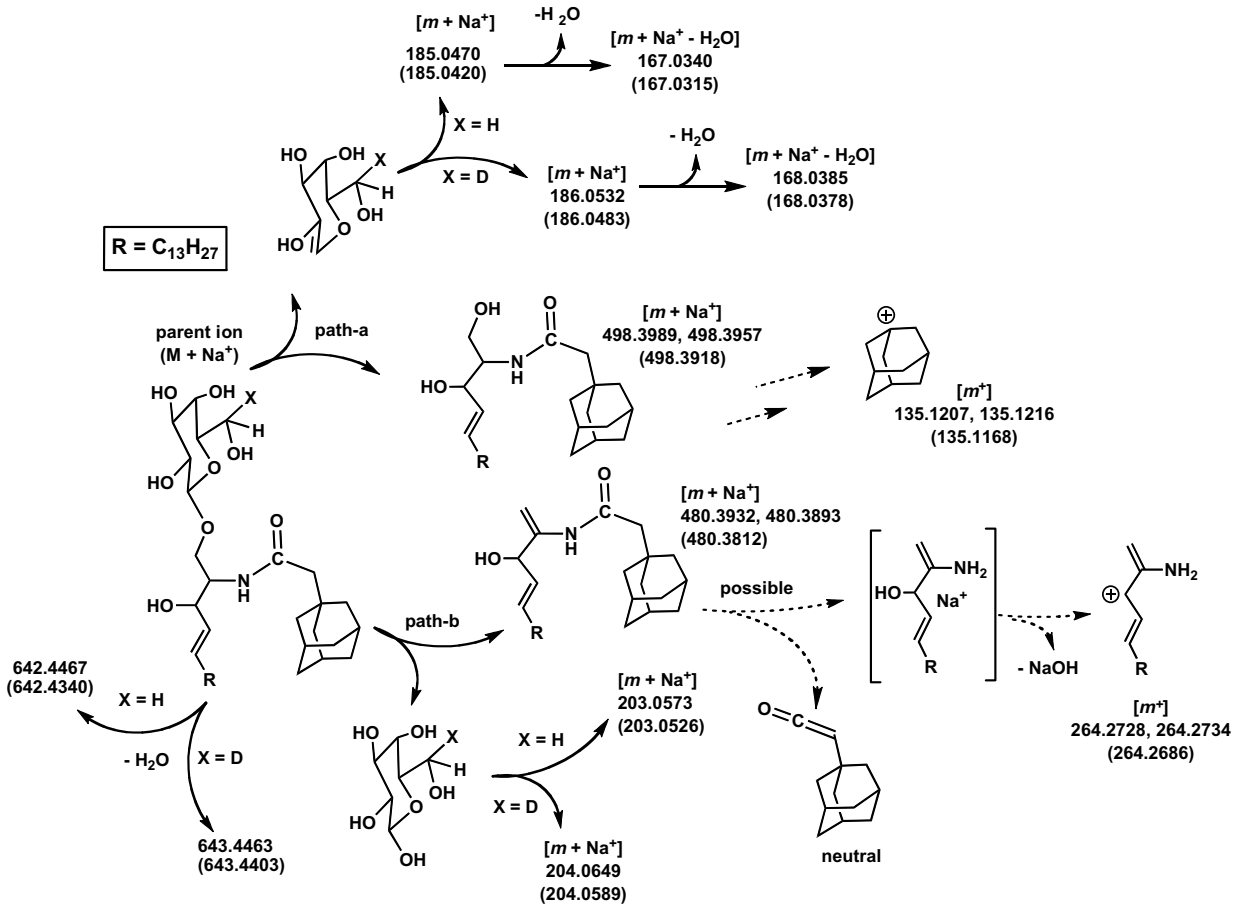


Figure 2. MSMS spectra of the parent-ion ($M+Na^+$). (A) GalCAda and (B) D-GalCAda. Asterisks indicate daughter ions containing the sugar moiety.

PrOH). Usually, the reaction was completed in 2 h. If substantial (>20%) starting material remained after 2 h, an appropriate amount of TFA solution was added (based on % starting material remaining) and heated until completion. Upon completion, workup was similar to the procedure described in monitoring reaction progress. Silica gel chromatography: silica was suspended in $CHCl_3$:MeOH, 98:2 and added to a column 1 cm in diameter to a final bed volume of 3 cm (height). The column was equilibrated with CH_2Cl_2 (2



Scheme 2. Putative fragmentation analysis of the (M+Na$^+$) ion of GalCAda and D-GalCAda. Mass data presentation: for daughter ions containing the hexose—[ion], observed, (calculated); and other daughter ions—[ion], observed for X = H, observed for X = D, (calculated). M and *m* denote parent and fragments, respectively.

times, 10 mL each). Crude sample was dissolved in a minimum volume of CH_2Cl_2, loaded, and eluted with the following solvents (number of fractions, volume of each fraction): CH_2Cl_2:$CHCl_3$, 2:1 (4, 3 mL); CH_2Cl_2:$CHCl_3$, 1:1 (4, 3 mL); $CHCl_3$ (4, 3 mL). The product was eluted in the $CHCl_3$ fractions. Yield was 80%. ESMS, (ion) (found, expected): (M+H$^+$) (1054.577, 1054.568); (M+Na$^+$) (1076.527, 1076.549).

1.3.4. (2*S*, 3*R*, 4*E*)-2-(1-Adamantanacetamido)-3-(benzoyloxy)-4-octadecenyl-2,3,4-tri-*O*-benzoyl-β -D-*galacto*-hexodialdoside (4)

A dried sample of **3** (0.2 mg, 0.2 μmol) was dissolved in DMSO–FPh (0.4 mL) and a solution of IBX (0.3 μmol, 15 μL of 0.02 M solution in DMSO) was added. The reaction mixture was stirred at 65 °C for 2 h. Upon completion, Et_2O was added (5 mL) and the organic layer was washed with saturated Na_2CO_3 solution (twice, 2 mL each), dried over Na_2SO_4, and filtered. Removal of solvent from the filtrate gave an oily material that can be used without further purification. Solvent for TLC analysis, *i*PrOH:CH_2Cl_2, 1:10.

1.3.5. (2*S*, 3*R*, 4*E*)-2-(1-Adamantanacetamido)-3-hydroxy-4-octadecenyl-[6-^{2}H]-β-D-galactopyranoside (D-GalCAda)

The dried sample of **4** (0.2 μmol, product from Section 1.3.4) was dissolved in a solution of NaOH in MeOH (0.5 mL of 0.25 M solution), solid $NaBD_4$ was added (2–3 mg, excess) and the mixture was stirred at room temperature for 4 h. Addition of water (3 mL) to the reaction-mixture yielded a basic, cloudy suspension, which was then sonicated and neutralized with HCl (6 M). A Sep-Pack cartridge was wetted with MeOH (5 mL) and then exchanged with water (10 mL). The aqueous reaction mixture was then passed through the pack, and was washed with water (5 mL, twice). The product was then eluted with MeOH (10 mL) and solvent was evaporated. Silica gel chromatography: silica was suspended in $CHCl_3$:MeOH, 98:2 and added to a column 0.5 cm in diameter to a final bed volume of 1 cm (height). Crude sample was dissolved in a minimum volume of $CHCl_3$:MeOH, 98:2, loaded, and eluted with the following solvents (number of fractions, volume of each fraction): $CHCl_3$:MeOH, 98:2 (4, 0.5 mL); $CHCl_3$:MeOH:water, 90:10:0.5 (4, 0.5 mL); $CHCl_3$:MeOH:water, 90:15:1 (4, 0.5 mL). The product eluted in fractions 7–10. Solvent for TLC analysis, $CHCl_3$:MeOH:water, 90:15:1. Yield was 60%. ESMS, (ion) (found, expected): For GalCAda (Fig. 1B and D): (M+Na$^+$) (660.426, 660.445), (2M+Na$^+$) (1297.867, 1297.900). For D-GalCAda (Fig. 2B and D): (M+Na$^+$) (661.439, 661.451); (M+Na$^+$–H_2O) (643.433, 643.440), (2M+Na$^+$) (1299.900, 1299.913).

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References

1. Hakomori, S. *Sci. Am.* **1986**, *254*, 40–53.
2. Hakomori, S. *J. Biol. Chem.* **1990**, *265*, 18713–18716.
3. Iwabuchi, K.; Handa, K.; Hakomori, S. *J. Biol. Chem.* **1998**, *273*, 33766–33773.
4. Davis, R. J.. In *Advances in Lipid Research*; Bell, R., Hannun, Y. A., Merrill, A., Jr., Eds.; Academic Press: San Diego, 1993; Vol. 25, pp 131–142.

5. Hakomori, S. *Cancer Res.* **1996**, *56*, 5309–5318.
6. Karlsson, K. A. *Curr. Opin. Struct. Biol.* **1995**, *5*, 622–635.
7. Lingwood, C. A. *Trends Microbiol.* **1996**, *4*, 147–153.
8. Mylvaganam, M.; Lingwood, C. A.. In *Carbohydrate-based Drug Discovery*; Wong, C.-H., Ed.; Wiley-VCH Press: Weinheim, Germany, 2003; Vol. 2, pp 761–780.
9. Lund, N.; Branch, D.; Mylvaganam, M.; Chark, D.; Ma, X.; Sakac, D.; Binnington, B.; Fantini, J.; Puri, A.; Blumenthal, R.; Lingwood, C. *AIDS* **2006**, *20*, 1–11.
10. Mylvaganam, M.; Lingwood, C. *Biochem. Biophys. Res. Commun.* **1999**, *257*, 391–394.
11. Lampio, A.; Siissalo, I.; Gahmberg, G. *Eur. J. Biochem.* **1988**, *178*, 87–91.
12. Suzuki, Y.; Suzuki, K. *J. Lipid Res.* **1972**, *13*, 687–690.
13. Yalpani, M.; Hall, L. H. *J. Polym. Sci. Pol. Chem.* **1982**, *20*, 3399–3420.
14. Schwarzmann, G. *Biochim. Biophys. Acta* **1978**, *529*, 106–114.
15. Lingwood, C.; Mylvaganam, M. In *Methods in Enzymology*; Lee, Y. C., Lee, R. T., Eds.; Academic Press: San Diego, CA, 2003; Vol. 363, pp 264–283.
16. DeLuca, L.; Giacomelli, G.; Porcheddu, A. *J. Org. Chem.* **2002**, *67*, 5152–5155.
17. Corey, E. J.; Suggs, J. W. *Tetrahedron Lett.* **1975**, *31*, 2647–2650.
18. Singh, S.; Nambiar, S.; Porter, R. A.; Sander, T. L.; Taylor, K. G. *J. Org. Chem.* **1989**, *54*, 2300–2307.
19. Nicolaou, K. C.; Zhong, Y. L.; Baran, P. S. *J. Am. Chem. Soc.* **2000**, *122*, 7596–7597.
20. Mylvaganam, M.; Meng, L.-J.; Lingwood, C. A. *Biochemistry* **1999**, *38*, 10885–10897.
21. Dess, D. B.; Martin, J. C. *J. Am. Chem. Soc.* **1991**, *113*, 7277–7287.