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Enzymic glycosphingolipid synthesis on polymer supports. III. Synthesis of G_{M3}, its analog [NeuNAcα(2-3)Galβ(1-4)Glcβ(1-3)Cer] and their *lyso*-derivatives

Uri Zehavi* and Alexandra Tuchinsky

Institute of Biochemistry, Food Sciences and Nutrition, Faculty of Agricultural, Food and Environmental Quality Sciences, The Hebrew University of Jerusalem, P.O. Box 12, Rehovot 76100, Israel

Two water-soluble polymers, carrying 0.24 meq g^{-1} of lactosyl-β(1-1)-sphingosine (7) and 0.13 meq g^{-1} of lactosyl-β(1-3)-sphingosine (8) were prepared. The polymers served as acceptors in the α-(2-3)-sialyltransferase reaction (up to 55.3 and 38.5% transfer yields, respectively). Subsequent photolysis, released compounds 11 (*lyso*-G_{M3}) and 12 (*lyso*-G_{M3} analog), respectively; acylation and chromatography afforded (5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2-3)-β-D-galactopyranosyl-(1-4)-β-D-glucopyranosyl-(1-1)-(2*S*, 3*R*, 4*E*)-2-octadecanoylamino-4-octadecene-1,3-diol (13, G_{M3}) and (5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2-3)-β-D-galactopyranosyl-(1-4)-β-D-glucopyranosyl-(1-3)-(2*S*, 3*R*, 4*E*)-2-octadecanoylamino-4-octadecene-1,3-diol (14, G_{M3} analogue), respectively, thus presenting a route to glycosphingolipids possessing the unusual glycosyl-β(1-3)-spingosine linkage.

***Keywords*: enzymic sialylation, glycosphingolipid, G_{M3}, G_{M3} analog, *lyso*-G_{M3}, *lyso*-G_{M3} analog, modified polyacrylamide, photolysis, polymer support, sialyltransferase, synthesis**

Introduction

In addition to the well established importance of glycosphingolipids (GSLs) in cellular recognition, they were shown to modulate transmembrane signaling by, for instance, directly affecting receptors or their membranal environment. Directly or indirectly, they may also influence the activity of enzymes and of signaling pathways [1–4].

Synthetic glycoconjugates are valuable tools for the elucidation of their various biological roles and eventually may become important in therapy. In the past, we have described a chemoenzymic glycosphingolipid synthesis on a light-sensitive polymer support while utilizing a bifunctional protecting group. Amino groups on carrier polymers are attached as amides *via* 4-carboxyamido-2-nitrobenzyloxycarbonyl and as urethans through the 2-amino function of sphingosine (Sph) derivatives [5, 6]. Irradiation (> 350 nm) releases the corresponding *lyso*-GSLs, namely, GSLs possessing free Sph 2-amino function.

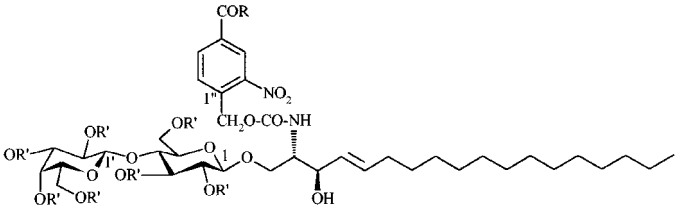
Reported here is a synthesis of *lyso*-G_{M3}-ganglioside, G_{M3}, and its analog, on polymer supports using a chemoenzymic approach (for previous G_{M3} syntheses, see [7–16]).

* To whom correspondence should be addressed. Tel: 972-8-9481914; Fax: 972-8-9476189; E-mail: ZEHAVI@AGRI.HUJI.AC.IL

Results and discussion

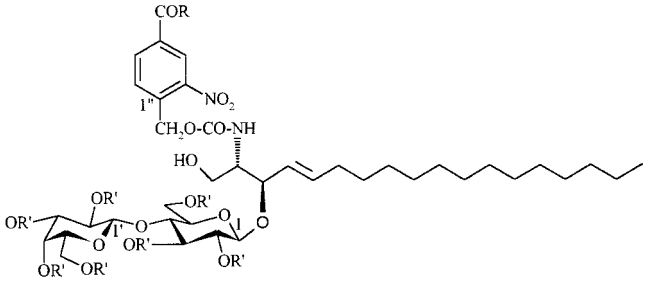
Two light-sensitive 2-nitrobenzyl derivatives of lactosylspingosine were obtained from (2*S*, 3*R*, 4*E*)-2-(4-carboxymethyl-2-nitrobenzyloxycarbonylamino)-3-hydroxy-1-[4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-2,3,6-tri-*O*-acetyl-β-D-glucopyranosyloxy]-4-octadecene (**1**) and (2*S*, 3*R*, 4*E*)-2-(4-carboxymethyl-2-nitrobenzyloxycarbonylamino)-1-hydroxy-3-[4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-2,3,6-tri-*O*-acetyl-β-D-glucopyranosyloxy]-4-octadecene (**2**) in high yield [17]. While compound **1** possesses a normal Glcβ(1-1) linkage, compound **2** has an unusual Glcβ(1-3) linkage to the derivatized sphingosine moiety. Compounds **3** and **4** were obtained by saponification of compound **1** and **2**, respectively (Schemes 1 and 2).

The water-soluble polymer polyacrylamide-poly(*N*-acryloxysuccinimide) (PAN, [18]) reacted with *N*-benzyloxycarbonyl-1,2-ethylene-diamine [19] yielding polymer **5** [20]. Subsequent hydrogenolysis gave an amino-functionalized polyacrylamide, possessing a four atom spacer **6** (0.51 meq g^{-1} NH_2), respectively (Scheme 3).



1 R=OCH_3, R'=OAc
3 R=OH, R'=OH
7 R=NH-CH_2-CH_2-NH-CO-(P), R'=OH , (0.24 meq/g)

Scheme 1



2 R=OCH_3, R'=OAc
4 R=OH, R'=OH
8 R=NH-CH_2-CH_2-NH-CO-(P), R'=OH , (0.13 meq/g)

Scheme 2

Coupling of polymer **6** with the light sensitive compounds **3** and **4** employing 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDCD) in water lead to a lactosyl-β-(1-1)-Sph polymer **7** (0.24 meq g^{-1} LacSph) and a lactosyl-β(1-3)-Sph polymer **8** (0.13 meq g^{-1} LacSph). These were then sialylated using Galβ-1,4-GlcNAcα-2,3-(*N*)-sialyltransferase including ^{14}C labeled CMP-NeuNAc as a *N*-acetyl neuraminyl donor and calf intestinal alkaline phosphatase was included in the reaction mixture to destroy the nucleotide phosphate inhibitor (CMP), that is released during the glycosyltransferase reaction, to give immobilized *lyso*-G_{M3} (**9**) and its analog (**10**).

In another work, we have shown only 33.1% yield in the sialytransferase reaction using a water-soluble support carrying lactose, which is not an optimal substrate for the enzyme (Tuchinsky and Zehavi, to be published). It was also demonstrated that a flexible spacer-arm, extending from the polymer, is helpful in rendering the acceptor saccharide more available to the glycosyltransferase on both insoluble and water-soluble polymers [21, 23]. In the present cases, the transfer yields, that may have been further improved due to the presence of an hydrophobic aglycon [14], were 55.3% for polymer **7** and only 38.5% for the more sterically hindered saccharide on the acceptor polymer **8**.

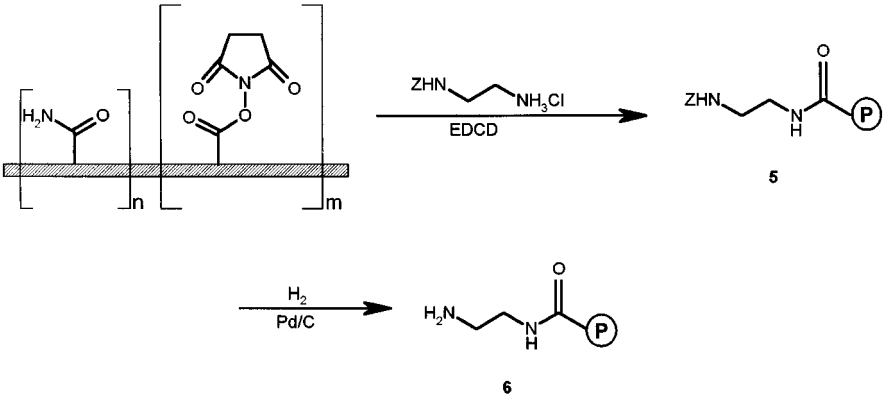
Photolysis (> 350 nm) of the 2-nitrobenzyl urethan groups in the sialylated polymers **9** and **10** provided compounds **11** (*lyso*-G_{M3}) and **12** (*lyso*-G_{M3} analog), respectively. Acylation with stearoyl chloride afforded compounds **13**, chromatographically identical with a synthetic marker of G_{M3}, and compound **14**, a G_{M3} analog, slower in chromatography than G_{M3}, respectively (Schemes 4 and 5; Figures 1 and 2).

The present paper describes not only a useful chemoenzymic synthesis of compounds **11–14**, the sialyltransferase reaction circumvents the need for elaborate protecting groups chemistry with usually low overall yield. The mild, high yielding, photochemical release of compound **11** from polymer **9** (and the same analogy applies to compound **12**) could be particularly advantageous in more complex cases where less stable *lyso*-GSLs are to be released. The synthesis of compounds **12** and **14** opens a route to a new family of GSLs containing glycosyl-β(1-3)-Sph linkages.

Experimental procedures

General

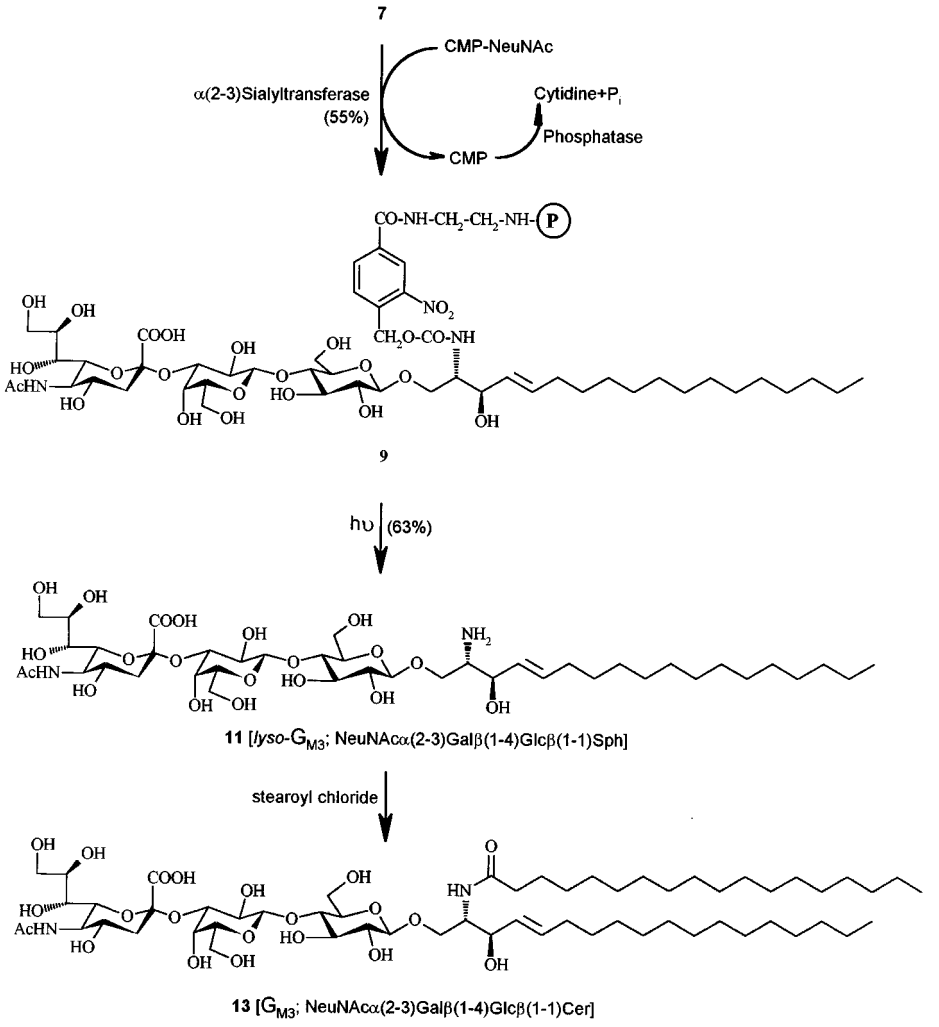
Described in [23]; additionally, CMP-[^{14}C]-NeuNAc (11.3 Gbq $mmol^{-1}$) was purchased from New England Nuclear Research Products (Boston, USA). CMP-NeuNAc,



5

6

Scheme 3



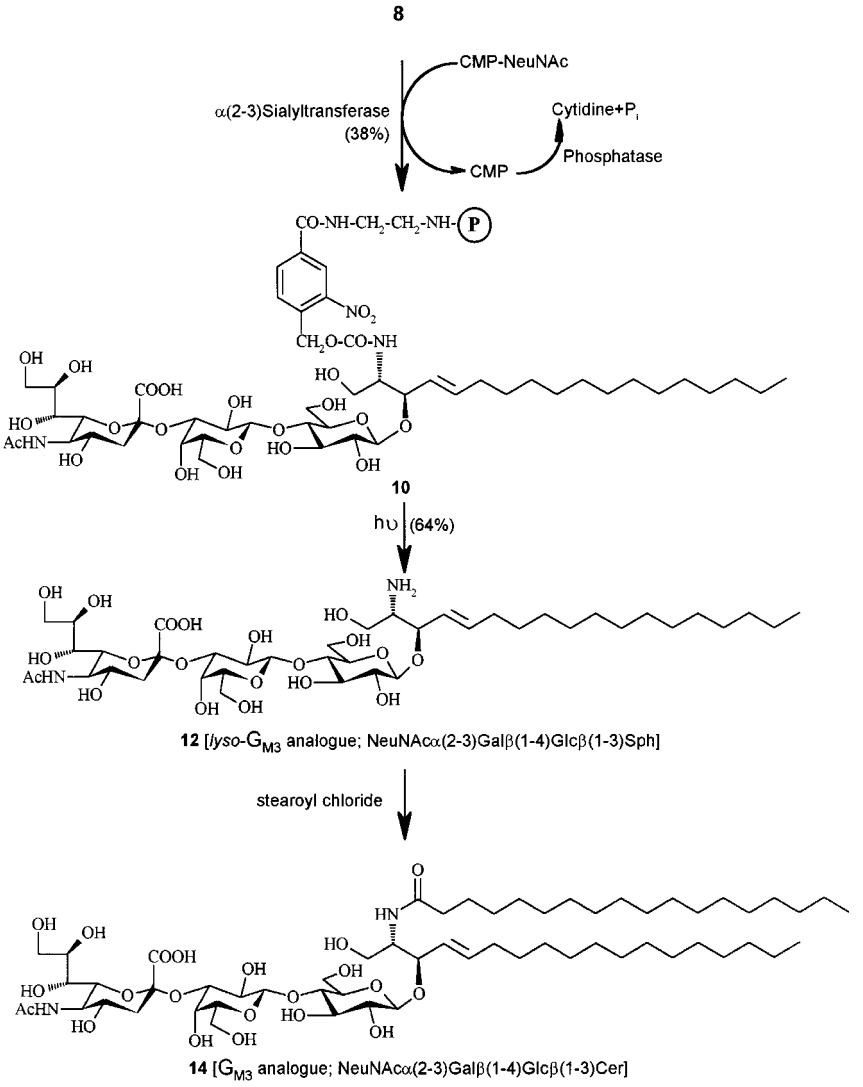
Scheme 4

Triton X-100 and bovine α-lactalbumin were from Sigma Chemical Co. (St Louis, MO 63178, USA). Calf intestinal alkaline phosphatase (EC 3.13.1) was obtained from Boehringer Menaheim. Recombinant rat liver Gal*β*-1,4-GlcNAcα-2,3-(*N*)-sialyltransferase (EC 2.4.99.6) was a gift from Dr. James C. Paulson (Cytel Corp., La Jolla, CA, USA). Sep-Pak C-18 reversed phase cartridges were purchased from Waters Associates (Mississauda, Ont., Canada), and were conditioned before use by washing with 10 ml of methanol and 20 ml of water [10]. G_{M3}, NeuNAcα(2-3)Gal*β*(1-4)Glc*β*(1-1)Cer, was purchased from Accurate Chemical Company (NY, USA). All the reactions were performed at least in duplicate.

(2*S*, 3*R*, 4*E*)-2-(4-carboxymethyl-2-nitrobenzyloxycarbonylamino)-3-hydroxy-1-[4-*O*-(2,3,4,6-tetra-*O*-acetyl-*β*-D-galactopyranosyl)-2,3,6-tri-*O*-acetyl-*β*-D-glucopyranosyloxy]-4-octadecene (**1**) and (2*S*, 3*R*, 4*E*)-2-(4-carboxymethyl-2-nitrobenzyloxycarbonylamino)-1-hydroxy-3-[4-*O*-(2,3,4,6-tetra-*O*-acetyl-*β*-D-galactopyranosyl)-2,3,6-tri-*O*-acetyl-*β*-D-glucopyranosyloxy]-4-octadecene (**2**) were prepared according to [17].

(2*S*, 3*R*, 4*E*)-2-(4-Carboxy-2-nitrobenzyloxycarbonylamino)-1-[4-*O*-(*β*-D-galactopyranosyl)-*β*-D-glucopyranosyloxy]-3-hydroxy-4-octadecene (**3**)

Compound **1** (0.2 g, 0.23 mmol) was suspended in water (10 ml) and treated with sodium hydroxide (1 M, 7 ml) for 20 min. The solution was neutralized with a cation exchange resin (Amberlite IR-120, H^+-form), the suspension was filtered and the solution was freeze dried giving compound **3** (0.18 g, 94%), m.p. 168–171°, α_D^{31} 3.7 ± 1.9° (c. 3.1, water), R_f 0.15 (1:1, methanol-chloroform), ^{1}H-NMR (CD_3OD): δ 8.66 (d, 1H, $J_{3'',5''}$ 1.4 Hz, H-3″), 8.29 (dd, 1H, $J_{5'',6''}$ 7.9 Hz, H-5″), 7.78 (d, 1H, H-6″), 5.71 (m, 1H, H-5, vinyl, Sph), 5.50 (s, 2H, benzylic CH_2), 5.47 (m, 1H, H-4, vinyl, Sph), 4.35 (d, 1H, $J_{1',2'}$ 7.4 Hz, H-1′), 4.31 (d, 1H, $J_{1,2}$ 7.8 Hz, H-1), 4.17 (dd, 1H, $J_{1b,2}$ 1.6 Hz, Sph H-1b), 4.08 (t, 1H, $J_{1a,2}$ 7.5 Hz, Sph H-1a), 3.89–3.42 (m, 14H,



Scheme 5

saccharide protons), 2.03 (ms, 2H, Sph), 1.24–1.17 (ms, 22H, Sph), 0.88 (t, 3H, $J_{17,18}$ 6.6 Hz, CH_3-18, Sph), lit. $J_{1,2}$ 7.8 Hz [23].

Anal. calc. for total sugar: 40.4%. Found: 41.0%. MS: calc. 846.0 (M). Found: 844.9 $(M\text{-}H)^-$.

(2*S*, 3*R*, 4*E*)-2-(4-Carboxy-2-nitrobenzyloxycarbonyl-amino)-3-[4-*O*-(*β*-D-galactopyranosyl)-*β*-D-glucopyrano-syloxy]-1-hydroxy-4-octadecene (**4**)

This compound was prepared from compound **2** as described for compound **3** with following amounts: compound **2** (0.14 g, 0.16 mmol), sodium hydroxide (1M, 3 ml), water (20 ml). Compound **4**: (0.13 g, 92%), m.p. 135–141°, α_D^{31} 2.5 ± 1.9° (c. 3.3, water), R_f 0.15 (1 : 1, methanol-chloroform), ^{1}H-NMR (D_2O): δ 8.53 (s, 1H, H-3″, aryl), 8.17 (d, 1H, $J_{5'',6''}$ 7.9 Hz, H-5″, aryl), 7.80 (d, 1H, H-6″, aryl), 5.48 (m, 1H, H-5, vinyl, Sph), 5.18 (m, 1H, H-4, vinyl Sph), 5.02 (s, 2H, benzylic CH_2), 4.66 (d, 1H, $J_{1',2'}$ 8.2 Hz, H-1′), 4.44 (d, 1H, $J_{1,2}$ 7.8 Hz, H-1), 3.99 (m-dd, 1H, Sph H-1b), 3.92–3.54 (m, 14H, saccharide protons), 3.28 (t, 1H, $J_{2,3}$ 8.1 Hz, H-2), 2.10–2.03 (m, 2H, Sph), 1.24–1.19 (ms, 22H, Sph), 0.85 (s, 3H, CH_3-18, Sph), lit. $J_{1,2}$ 7.8 Hz [23].

Anal. calc. for total sugar: 40.4%. Found: 39.6%. MS: calc. 846.0 (M). Found: 844.9 $(M\text{-}H)^-$.

N-Ⓟ-*N*′-Benzyloxycarbonyl-diaminoethane (**5**)

A solution of *N*-benzyloxycarbonyl-1,2-diaminoethane [19] (1.5 g, 6.5 mmol) in water (50 ml) was brought to pH 11 with triethylamine, treated with 3.5 g of PAN [18]. (1.08 meq g^{-1} active ester) and gentle stirring was continued overnight. Ammonia solution (25%, 0.4 ml) was added and stirring was continued for additional 24 h. After dialysis the solution was lyophilized to afford 2.9 g of polymer **5**.

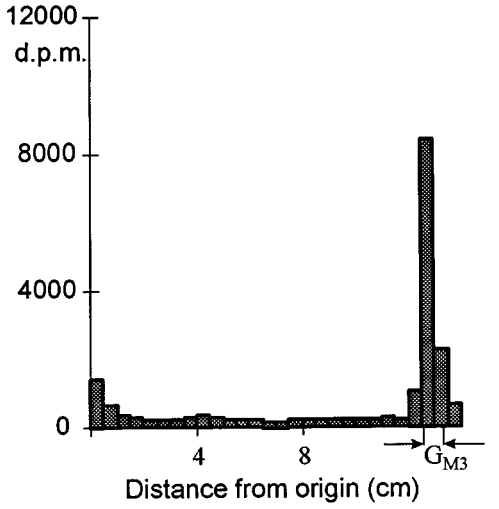


Figure 1. TLC (Merck plate, chloroform/methanol/15 mM $CaCl_2$, by vol) separation of radioactive products released by irradiation of compound **11** followed by acylation (stearoyl chloride) to yield compound **13**. The marker G_{M3}, NeuNAc*α*(2-3)Gal*β*(1-4)Glc*β*(1-1)Cer, was run alongside the product and was detected by iodine vapor.

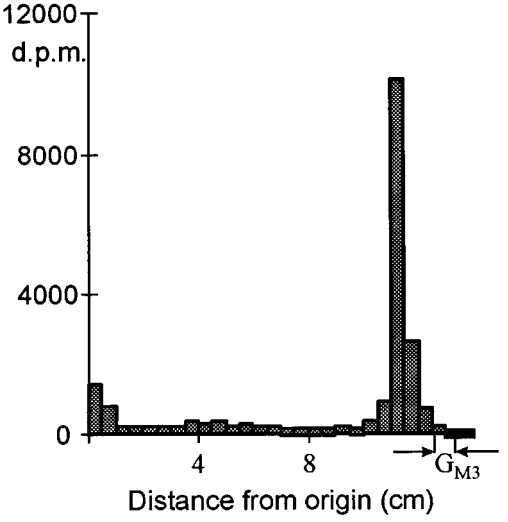


Figure 2. TLC (Merck plate, chloroform/methanol/15 mM $CaCl_2$, by vol) separation of radioactive products released by irradiation of compound **12** followed by acylation (stearoyl chloride) to yield compound **14**. The marker G_{M3}, NeuNAc*α*(2-3)Gal*β*(1-4)Glc*β*(1-1)Cer, was run alongside the product and was detected by iodine vapor.

N-Ⓟ-Diaminoethane (**6**)

Polymer **5** (2.5 g) was dissolved in water (50 ml) and hydrogenolyzed over palladium on charcoal (10%, 350 mg). After 24 h the catalyst was removed by filtration through celite. The solution was ultrafiltered through a Diaflo UM2 membrane against large excess of water and residue lyophilized to give 2.3 g **6** (0.51 meq g^{-1} NH_2). IR: 3421 (wide, OH, NH), 3218, 2926, 2854, 2818, 1664 (CO), 1452 cm^{-1}.

(2*S*, 3*R*, 4*E*)-1-(4-*O*-(*β*-D-Galactopyranosyl)-*β*-D-glucopyranosyloxy]-3-hydroxy-2-[2-nitro-4-(*N*-Ⓟ-carboxydiaminoethyl)-benzyloxycarbonylamino]-4-octadecene (**7**)

Compounds **6** (0.36 g) and **3** (0.25 g, 0.3 mmol) were dissolved in water (23 ml). The pH was adjusted to 4.7 and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDCD; 0.2 mg, 0.6 mmol) was added. Addition of the same amount of EDCD was repeated five times, accompanied by re-adjustment of the pH to 4.7 when necessary. After 4 d low molecular weight compounds were removed by ultrafiltration, and polymer **7** (0.2 g, 0.24 meq g^{-1} Lac) was obtained after lyophilization.

(2*S*, 3*R*, 4*E*)-3-(4-*O*-(*β*-D-Galactopyranosyl)-*β*-D-glucopyranosyloxy]-1-hydroxy-2-[2-nitro-4-(*N*-Ⓟ-carboxydiaminoethyl)-benzyloxycarbonylamino]-4-octadecene (**8**)

This compound was prepared from compound **4** as described for compound **7** with the following amounts: compound **4** (0.11 g, 0.1 mmol), polymer **6** (0.1 g), EDCD (0.6 mg, 1.8 mmol), water (11 ml). Polymer **8** (0.06 g, 0.13 meq g^{-1} Lac) was obtained after lyophilization.

(5-Acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-(2-3)-*β*-D-galactopyranosyl-(1-4)-*β*-D-glucopyranosyloxy-(1-1)-(2*S*, 3*R*, 4*E*)-3-hydroxy-2-[2-nitro-4-(*N*-Ⓟ-carboxydiaminoethyl)-benzyloxycarbonylamino]-4-octadecene (**9**)

The incubation mixture (100 µl) contained polymer **7** (600 µg), CMP-NeuNAc (100 µg, 156 nmol) labeled with CMP-[^{14}C]NeuNAc (212, 205 d.p.m. µmol^{-1}, 345, 611 d.p.m. mg^{-1}), α-lactalbumin (60 µg), 50 mM sodium cacodilate (pH 6.5), Triton X-100 (0.5%), calf intestinal alkaline phosphatase (3U) and Gal*β*-1,4-GlcNAcα-2,3-(*N*)-sialyltransferase (100 mU). The same amounts of labeled CMP-NeuNAc and calf intestinal alkaline phosphatase were added at regular intervals of 24 h. After 3 d of incubation at 37 °C the polymer was isolated by ultrafiltration (Dialflo YM2), washed extensively with water (until only very little radioactivity emerged in the eluants) and lyophilized. Polymer **9** (27, 671, 810 d.p.m. g^{1}, 0.13 meq g^{-1} *lyso*-G_{M3}, represented a 55.3% incorporation yield of NeuNAc.

(5-Acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-(2-3)-*β*-D-galactopyranosyl-(1-4)-*β*-D-glucopyranosyloxy-(1-3)-(2*S*, 3*R*, 4*E*)-1-hydroxy-2-[2-nitro-4-(*N*-Ⓟ-carboxydiaminoethyl)-benzyloxycarbonylamino]-4-octadecene (**10**)

This compound was prepared from polymer **8** as described for polymer **9** with the following amounts in a volume

of 100 µl: polymer **8** (1.3 mg), CMP-NeuNAc (400 µg, 624 nmol) labelled with CMP-[^{14}C]-NeuNAc (212, 205 d.p.m. µmol^{-1}, 345, 611 d.p.m. mg^{-1}), α-lactalbumin (60 µg), 50 mM sodium cacodilate (pH 6.5), Triton X-100 (0.5%), calf intestinal alkaline phosphatase (9U) and Galβ-1,4-GlcNAcα-2,3-(*N*)-sialyltransferase (100 mU). Polymer **10** (13,856,740 d.p.m. g^{-1}, 0.065 meq g^{-1} *lyso*-G_{M3} analog, represented a 38.5% incorporation yield of NeuNAc.

Photochemical release of (5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-(2-3)-β-D-galactopyranosyl-(1-4)-β-D-glucopyranosyloxy-(1-1)-(2*S*, 3*R*, 4*E*)-3-hydroxy-4-octadecene (*lyso*-G_{M3}, 11) and (5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-(2-3)-β-D-galactopyranosyl-β-D-glucopyranosyloxy-(1-3)-(2*S*, 3*R*, 4*E*)-1-hydroxy-4-octadecene (*lyso*-G_{M3} analog, **12**) from polymers **9** and **10**

The photolysis of polymer **9** and **10** [24], followed by ultrafiltration and counting samples of the filtrate afforded *lyso*-G_{M3} (11) 62.8% yield (17 324 416 d.p.m. g^{-1}, 0.084 mmol g^{-1} *lyso*-G_{M3}) and *lyso*-G_{M3} analog (**12**) 64.2% yield (6 818 592 d.p.m. g^{-1}, 0.032 mmol g^{-1} *lyso*-G_{M3} analog), respectively.

(5-Acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-(2-3)-β-D-galactopyranosyl-(1-4)-β-D-glucopyranosyl-(1-1)-(2*S*, 3*R*, 4*E*)-2-octadecanoylamino-4-octadecene-1-,3-diol (**13**, G_{M3}) and (5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-(2-3)-β-D-galactopyranosyl-(1-4)-β-D-glucopyranosyl-(1-3)-(2*S*, 3*R*, 4*E*)-2-octadecanoylamino-4-octadecene-1,3-diol (**14**, G_{M3} analog)

Compounds **11** and **12** were dissolved in 200 µl of ether and 100 µl of saturated sodium acetate solution, the mixture was stirred vigorously and stearoyl chloride (20 µl) was added and the stirring was continued for 30 min at a room temperature. The mixture was diluted with 1 ml of ether and washed with saline (1 ml). The organic phase was separated, evaporated, applied to a preconditioned Sep-Pak C-18 cartridge attached to 5 ml syringe and washed with 15 ml water. The radiolabeled reaction product was eluted in 20 ml methanol-chloroform (1 : 2) affording following evaporation compounds, **13** and **14**, respectively. TLC (chloroform methanol 15 mM $CaCl_2$, 60 : 35 : 10 by vol) demonstrated a major component at R_f 0.67 corresponding to an authentic sample of G_{M3} for compound **13** and R_f 0.61 for compound **14**, respectively (Figures 1 and 2).

Acknowledgments

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