Glycoconjugate J (1990) 7:229-234

Enzymic Glycosphingolipid Synthesis on Polymer Supports.
II. Synthesis of Lactosyl Ceramide*

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Received March 15/June 11, 1990.

Key words: glycosphingolipid, synthesis, polymer support, lactosyl ceramide, D-galactosyltransferase

In a first example of a polymer-supported enzymic synthesis of glycosphingolipids, synthetic (2*S*,3*R*,4*E*)-2-amino-1-(β-D-glucopyranosyloxy)-3-hydroxy-4-octadecene (glucosylsphingosine) was converted to the *N*-4-carboxymethyl-2-nitrobenzyloxycarbonyl derivative and was subsequently attached to a water-soluble polymer. The material served as an acceptor in the D-galactosyltransferase reaction (36% transfer yield) and further photolysis, acylation and chromatography afforded (2*S*,3*R*,4*E*)-1-[4-*O*-(β-D-galactopyranosyl)-β-D-glucopyranosyloxy]-3-hydroxy-2-octadecanoylamino-4-octadecene (lactosylceramide, 54% yield).

Earlier experiments focused on methodology necessary for the preparation of acceptors suitable for polymer-supported enzymic synthesis of glycosphingolipids [1]. Potential acceptors were derived from native glycosphingolipids or sphingosine atttached in both cases through a light-sensitive linkage to a water soluble poly(acrylamide) (**P**). It was assumed that employing a water soluble polymer as a carrier might provide for improved accessibility in chemical and enzymic condensation steps and that photochemical removal from the polymer (shown for model compounds) would be readily accomplished. Homogeneous, well-characterized synthetic glycosylsphingosine derivatives are considered as prime choice precursors for the preparation of similar acceptors. The present work examines specifically (2*S*,3*R*,4*E*)-2-amino-1-(β-D-glucopyranosyloxy)-3-hydroxy-4-octadecene (glucosylsphingosine) [2] as a starting material for the construction of an acceptor for D-galactosyltransferase.

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* A preliminary account of this work has appeared: U. Zehavi, M. Herchman, R.R. Schmidt and T. Bär, 5th European Symp. Carbohydrates, Prague, Czechoslovakia, August 1989, Abs. C-10; 10th Internat. Symp. Glycoconjugates, Jerusalem, Israel, September 1989, Abs. 7.

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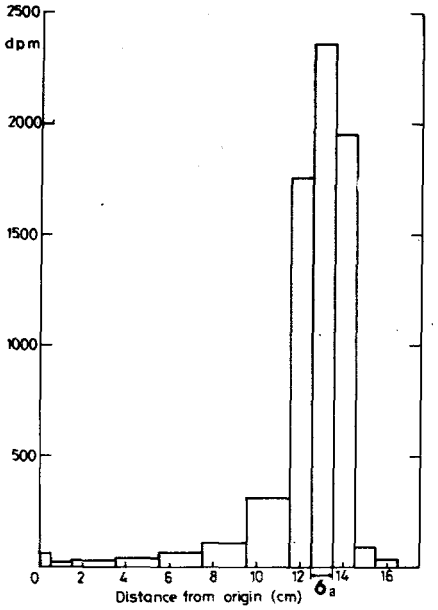
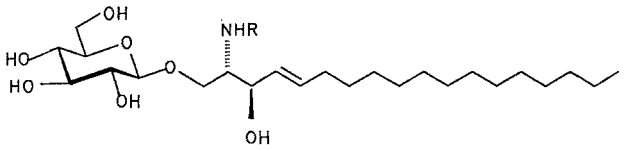


Figure 1. TLC (Merck plate, chloroform/methanol/water, 56/38/10, by vol) separation of radioactive products released by irradiation of compound **5** followed by acylation (octadecanoyl chloride). The marker (**6a**) was run alongside the products and was detected by iodine vapour.

Results and Discussion

Synthetic (2*S*,3*R*,4*E*)-2-amino-1-(β-D-glucopyranosyloxy)-3-hydroxy-4-octadecene [2] (synthetic glucosyl sphingosine, **1**) was conveniently converted to the *N*-4-carboxymethyl-2-nitrobenzyloxycarboxyl derivative (**2**). This 2-nitrobenzyl urethane is, as expected [3], light sensitive and possesses a methyl ester that can be readily converted into the hydrazide (**3**, not isolated) under conditions not affecting the urethane [1].

Subsequently, the hydrazide function in compound **3** was reacted with poly(acrylamide)-poly(*N*-acryloxysuccinimide) (PAN), yielding (following ultrafiltration) polymer **4**. The latter, containing β-*gluco* substituents served in the presence of α-lactalbumin as an acceptor to D-galactosyltransferase (lactose synthase) giving rise to a lactosylsphingosine polymer (**5**). Photolysis (350 nm) of the 2-nitrobenzyl urethane groups in the polymer **5**, in the presence of an insoluble hydrazine-substituted polymer, decreased the side reactions of the newly formed amino functions with photochemical by-products [4] and provided, following acylation, (2*S*,3*R*,4*E*)-1-[4-*O*-(β-D-galactopyranosyl)-β-D-glucopyranosyloxy]-3-hydroxyl-2-octadecanoylamino-4-octadecene (**6**, lactosyl ceramide) chromatographically identical with a synthetic marker (Fig. 1).



1 R = H

2 R = 4-carboxymethyl-2-nitrobenzyloxycarbonyl

3 R = 4-hydrazinocarbonyl-2-nitrobenzyloxycarbonyl

The methodology presented in this paper could be broadened to provide additional glycosyl sphingosine-substituted polymers derived from modified native or synthetic precursors. These polymers may subsequently serve as acceptors to glycosyltransferases taking part in the biosynthesis of glycosphingolipids. Furthermore, it is suggested that these polymeric acceptors will aid in the study of several consecutive steps in the metabolism of glycosphingolipids, including those reactions catalyzed by glycosyltransferases and glycosidases, by allowing the facile isolation of radioactive reaction products [5].

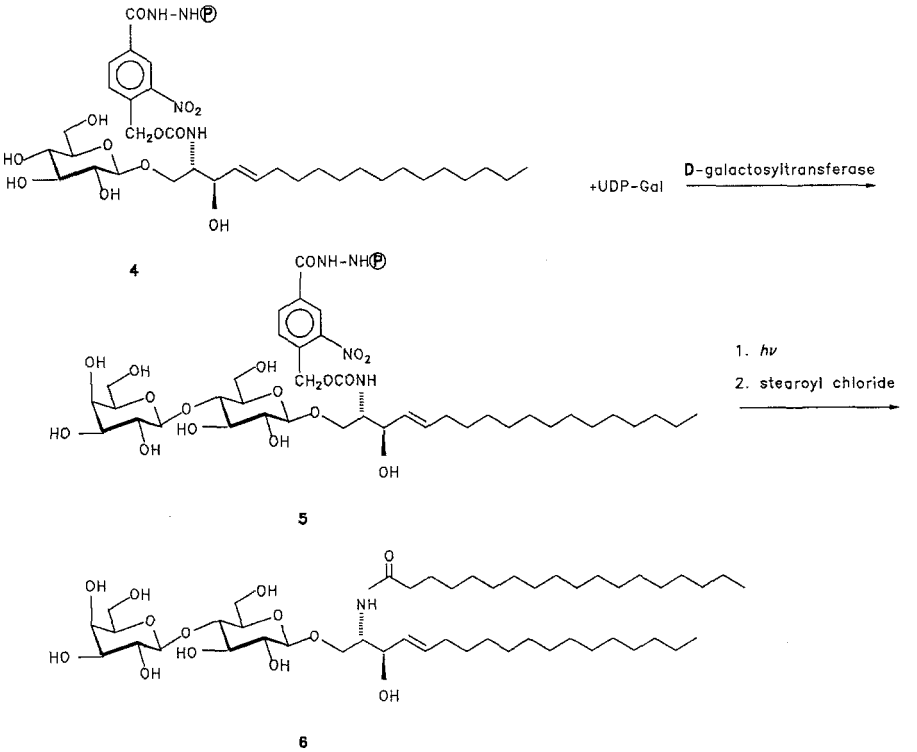
Experimental Procedures

General

Optical rotations were determined with a Bendix polarimeter. ^{1}H-NMR Spectra were recorded on a Bruker WH 270 (270 MHz) instrument; i.r. spectra, for potassium bromide discs, with a Nicolet MX-S FTIR spectrophotometer; and colorimetric measurements with a Zeiss PMQ II instrument. Photolysis was carried out in a Rayonet RPR-100 apparatus with RPR 3500 Å lamps in Pyrex glassware. D-Galactosyltransferase (EC 2.4.1.22) from bovine milk and bovine α-lactalbumin were from Sigma Chemical Co. (St. Louis, MO, USA). UDP-D-[U-^{14}C] Galactose was purchased from Radiochemical Centre (Amersham, UK.). Scintillation counting was performed on a mixture (5 ml) containing toluene (676 ml), Triton X-100 (363 ml), 1,4-bis(5-phenyl-2-oxazolyl)benzene (200 mg), and 2,5-diphenyloxazole (5 mg). Nitro derivatives described in this work are light sensitive and were routinely kept in the dark. TLC was performed on precoated Silica gel 60F_{254} sheets (E. Merck, D-6600 Darmstadt, FRG.), and compounds were detected by viewing under u.v. light or by treating with iodine vapour. Column chromatography was performed on Silica gel 60 (0.063-0.2 mm, Merck). Phenol-sulfuric acid test [6] was carried out with D-glucose standards.

*(2S,3R,4E)-2-(4-Carboxymethyl-2-nitrobenzyloxycarbonylamino)-1-(β-D-glucopyranosyloxy)-3-hydroxy-4-octadecene (**2**)*

(2*S*,3*R*,4*E*)-2-Amino-1-(β-D-glucopyranosyloxy)-3-hydroxy-4-octadecene (**1**, 63 mg) was vigorously stirred at room temperature in a mixture of 50% sodium acetate (2.1 ml in water) and tetrahydrafuran (2.1 ml). Two portions of 1-methyl-3-(4-carboxymethyl-2-nitrobenzy-



loxycarbonyl)-imidazolium chloride [1] prepared (without isolation) from methyl 4-hydroxymethyl-3-nitrobenzoate [7] (45 mg) were added at 2 h intervals and the stirring was continued overnight. The mixture was diluted with tetrahydrofuran (15 ml), the organic phase was separated and washed twice with saturated sodium chloride (2 ml each), evaporated and applied to a silica gel column (5 g, 5 mm in diameter), eluted with chloroform/methanol, 9/1 by vol (3 ml/fractions), and monitored by TLC (same solvent, Rf 0.15).

The product (**2**, 21 mg, 23%) emerged in fractions 16-20. $[\alpha]_D^{20}$ -10.9±4° (c 0.2, chloroform/methanol,1/1, by vol); i.r. data: 3420 (wide, OH, NH), 2922, 2854, 1735 (CO), 1712 (CO), 1626 and 1537 cm^{-1} (NO_2); ^{1}H-NMR data (^{2}H-dimethylsulfoxide) included: δ 8.56 (d, 1H, $J_{3,5}$ 1.6 Hz, H-3 aromatic, 8.32 (dd, 1H, $J_{5,6}$ 7.7 Hz, H-5 aromatic), 7.81 (d, 1H, H-6 aromatic), 7.34 (d, 1H, *J* 8.5 Hz), 5.44 (s, 2H, CH_2 benzylic), 5.43 (m, 1H, H-5 olefinic) 5.13 (m, 1H, H-4 olefinic), 3.92 (s, 3H, OCH_3), 1.24 (m, CH_2), 0.85 (t, 3H, *J*17,18 6.7 Hz, CH_3-18). Glucose content was determined by the phenol-sulfuric acid test. Calc.: 23.49%; Found: 22.2%.

*(2S,3R,4E)-1-(β-**D**-Glucopyranosyloxy)-3-hydroxy-2-[2-nitro-4(N-**P**-carboxyhydrazido)-benzyloxycarbonylamino]-4-octadecene (**4**)*

Compound **2** (9 mg) was treated with hydrazine/hydrazine sulfate and freeze dried [1]. The residue was dissolved in water/dimethylsulfoxide, 1/1 by vol (1 ml), triethylamine (20 µl) and PAN [8] (200 mg, Mn 6900, 0.365 mmol active ester/g) were added and the solution was stirred overnight at room temperature. The reaction mixture was then dialyzed extensively (Spectra/Por 3, Spectra Medical Industries, Los Angeles, CA, USA) against water, lyophilized and further purified on a Sephadex G-25 column (20 ml, 1 cm in diameter). Fractions of high u.v. absorbance (256 nm) were pooled and lyophilized yielding the product polymer (**4**, 137 mg, 26 µmol glucose/g) [6].

*(2S,3R,4E)-1-[4-O-(β-D-galactopyranosyl)-β-D-glucopyranosyloxy]-3-hydroxy-2-[2-nitro-4-(N'-**P**-carboxyhydrazido)-benzyloxycarbonylamino]-4-octadecene (**5**)*

Polymer **4** (120 mg) UDP-Gal (8.27 µmol) labeled by UDP-D-[U-^{14}C]galactose (327100 dpm/µmol), α-lactalbumin (4 mg) and D-galactosyltransferase (0.4 unit, EC 2.4.1.22) from bovine milk in 25 mM sodium cacodylate buffer (4 ml, pH 7.0 containing 3 mM manganous chloride and 0.1% mercaptoethanol) were incubated for 24 h at 37°C. The product (**5**) was purified by extensive dialysis (Diaflo YM2) until only negligible radioactivity emerged in the eluate. The polymer (**5**) (9.48 µmol galactose/g) representing 36% transfer yield, was collected after lyophilization.

*(2S,3R,4E)-1-[4-O-(β-D-galactopyranosyl)-β-D-glucopyranosyloxy]-3-hydroxy-2-octadecanoylamino-4-octadecene (**6**)*

a). Lactosylsphingosine [2] (310 mg, 500 µmol) was suspended in 35 ml of tetrahydrofuran and 19 ml of a saturated sodium acetate solution. Under vigorous stirring octadecaynoyl chloride (stearoyl chloride, 151 mg, 500 µmol) was added dropwise. The reaction was complete within 10-30 min (TLC analysis). The mixture was diluted with 200 ml of tetrahydrofuran, the water layer was separated and the organic solution was washed with saline (50 ml). After evaporation, the crude product was chromatographed over silica gel. Elution with chloroform/methanol, 85/15 by vol, afforded 355 mg (80% yield) of the product (**6**) as a viscous oil, $[\alpha]_D^{20}$ -9.3° (c 1.0, pyridine), ^{1}H-NMR data (250 MHz, ^{2}H-dimethylsulfoxide): δ 7.50 (d, 1H, NH, *J* 8.6 Hz), 5.52 (m, 1H, C=C**H**-CH_2), 5.32 (dd, 1H, C**H**=CH-CH_2, *J* 6.5 Hz, *J* 15.2 Hz), 5.19 (m, 2H, 2 OH), 4.91 (d, 1H, OH, *J* 4.8 Hz), 4.81 (d, 1H, OH, *J* 3.3 Hz), 4.72-4.50 (m, 4H, 4 OH), 4.18 (m, 2H, 2H-1), 4.10-3.00 (m, 15H, 2H-2, 2H-3, 2H-4, 2H-5,2H-6, 2H-6', CH_2-O, CH-N), 2.10-1.90 (m, 4H, NCO-CH_2, C=CH-C**H**$_2$), 1.45 (m, 2H, CH_2), 1.20 (m, 50H, 25 CH_2), 0.85 (m, 6H, 2 CH_3).

b). Hydrazine substituted (0.2 mmol/g), 2% cross-linked styrene-divinylbenzene copolymer (100 mg) suspended by stirring in a solution of polymer **5** (82 mg) in water/tetrahydrofuran, 1/1 by vol (50 ml), was irradiated at ambient temperature for 9.5 h. The insoluble polymer was filtered off, washed with chloroform/methanol, 1/1 by vol, and the filtrates were evaporated. The residue dissolved in tetrahydrofuran (1 ml) - 50% sodium acetate (1 ml) was stirred vigorously, octadecanoyl chloride (3 mg) was added and the stirring was continued at room temperature for 2 h. The mixture was diluted with tetrahydrofuran (7 ml), the organic

phase was separated, washed twice with saturated sodium chloride (1 ml each) and evaporated at high vacuum. TLC (chloroform/methanol/water, 56/38/10 by vol) demonstrated a major component at R_F 0.68 corresponding to an authentic sample (**6a**). TLC carried out subsequently with up to 1/10 of the product on Merck or Baker (USA.) plates (Si-HPF, same solvent) yielded (following extraction with chloroform/methanol, 1/1 by vol, evaporation and counting) the product (**6**) in 54% yield (Fig. 1).

Acknowledgement

Support of this research by G.I.F., the German-Israeli Foundation for Scientific Research and Development, is gratefully acknowledged.

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