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Supporting Information

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Solid-Phase Oligosaccharide Tagging (SPOT): Validation on glycolipid-derived structures

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General experimental

PEGA 1900 resin was purchased from Versamatrix, Denmark. CPG was purchased from Millipore Corp, USA. Solid-phase chemistry was performed in plastic syringes equipped with sintered Teflon filters (50 µm pore). Oligosaccharides (LNT and LNFP) were purchased from IsoSep, Sweden. 5-Tetramethylrhodamine isothiocyanate (5-TRITC, TMR-NCS) was purchased from Molecular Probes, USA, while the hexadeutero labeled analogue was prepared using a procedure similar to the one described by Corrie *et al*.[1] but starting from 3- (N-methyl-N-trideuteromethylamino)-phenol. The remaining chemicals were purchased from Aldrich Chemical Co. and used as received. All solvent were HPLC grade and used as purchased without further purification. Silica gel, Merck grade 9385, 60 Å, 230-400 mesh was used for flash chromatography. Solution-phase NMR spectra were acquired on a Bruker Avance DRX 250 spectrometer at room temperature. ES-MS were recorded on a Bruker Daltonics, Esquire 3000 *plus* mass spectrometer (mobile phase: 50% acetonitrile in H_2O containing 0.1 % formic acid). HR-ES-MS were recorded on a MicroMass QTOF mass spectrometer (mobile phase: 50% acetonitrile in H_2O, 0.1 µL min^{-1}, sample conc. ~10 pM). Capillary electrophoresis (CE) was performed using an automated PrinCE 2-lift, model 560 CE system (Prince Technologies, The Netherlands). Separations were carried out in an uncoated fused-silica capillary of 75 µm ID with an effective length between 40-75 cm (plus 30 cm extra from the detection window to outlet), thermostatically controlled at 25 °C. The CE background electrolyte (BGE) was 50 mM borate buffer pH 9.3 containing 150 mM SDS. The capillary was initially conditioned at room temperature by rinsing at 2000 mbar for 20 min with 1 M NaOH, 10 min with water, and 10 min with BGE. Between runs the capillary was washed at 2000 mbar for 3 min with 1 M NaOH, 3 min with water, and 3 min with BGE. Detection was carried out using a fluorescence detector (Argos 250B, Flux Instruments, Switzerland) equipped with an excitation filter of 546.1/10 nm and an emission filter of 570 nm. Samples were injected hydrodynamically for 6 sec at 50 mbar and electrophoresed across a potential difference of 25 kV. All experiments were carried out at a normal polarity, i.e. inlet anodic.

Synthesis of SPOT-PEGA and SPOT-CPG

a) N-Fmoc-4-aminooxymethyl-benzoic acid (2)

To a solution of 4-aminooxymethyl-benzoic acid, hydrochloride **1**[2] (2.0 g, 0.010 mol) in dioxane (20 mL) and half saturated Na_2CO_3 solution (20 mL) was added Fmoc-Cl (2.8 g, 0.011 mol) and the mixture was stirred for 2 h. Ethyl acetate (100 mL) was added and the pH of the aqueous phase was adjusted to 1-3 by careful addition of $HCl_{(conc.)}$. The mixture was poured in to a separating funnel and the organic phase was isolated, washed once with water (100 mL), dried over Na_2SO_4 and the solvent was removed on a rotavap to give an oil that solidified upon standing. The crude product was purified by flash column chromatography (petroleum ether, ethyl acetate, AcOH 60:40:1). Yield: 3.5 g (92%) ^{1}H-NMR (250 MHz, DMSO-d6) δ = 4.05 (1H, t, *J* = 6.3 Hz), 4.27 (2H, d, *J* = 6.3 Hz), 4.51 (2H, s), 7.08-7.22 (6H, m), 7.46 (2H, d, *J* = 7.4 Hz), 7.66 (2H, d, *J* = 7.1 Hz), 7.72 (2H, d, *J* = 8.4 Hz), 10.29 (1H, br. s), 12.78 (1H, br. s).

^{13}C-NMR (63 MHz, DMSO-d6) δ = 47.04, 66.03, 76.91, 120.51, 125.41, 127.45, 128.03, 128.89, 129.33, 129.63, 130.82, 141.19, 144.01, 157.12, 167.48. MS (ES) m/z = 389 (MH^+).

b) Preparation of 3

N-Fmoc-4-aminooxymehtyl-benzoic acid (**2**) (1.0 g, 2.57 mmol) was dissolved in DMF (15 mL) and Cs_2CO_3 (0.42 g, 1.28 mmol) was added followed by stirring for 5 min at rt. tert-Butyl bromoacetate (0.55 g, 2.28 mmol) was added and the mixture was heated to 50°C for 30 min and then cooled to rt again. CH_2Cl_2 (70 mL) was added and the mixture was poured in to a separating funnel and washed with half saturated $NaHCO_3$ solution (3 × 50 mL) and then water (2 × 50 mL), dried over $MgSO_4$. The solvent was removed under reduced pressure to give the crude product as an oil. After flash column chromatography (20-40% ethyl acetate in petroleum ether) a white solid (**3**) was obtained in a yield of 1.15 g (89%). ^{1}H-NMR (250 MHz, $CDCl_3$) δ = 1.37 (9H, s), 4.06 (1H, t, *J* = 6.7 Hz), 4.37 (2H, d, *J* = 6.7 Hz), 4.61 (2H, s), 4.67 (2H, S), 7.11-7.18 (2H, m), 7.22-7.27 (4H, m), 7.43 (2H, d, *J* = 7.5 Hz), 7.60 (2H, d, *J* = 7.3 Hz), 7.75 (1H, s), 7.93 (2H, dd, *J* = 1.7 Hz, 6.6 Hz). ^{13}C-NMR (63 MHz, $CDCl_3$) δ = 26.31, 45.28, 59.95, 65.52, 75.99, 80.84, 118.30, 123.27, 125.41, 126.10, 126.93, 127.23, 128.30, 139.29, 139.58, 141.73, 155.70, 163.94, 165.15. MS (ES) m/z = 542 (MK^+).

c) Preparation of cleavable hydroxylamine linker (4)

The white solid (**3)** (1.15 g, 2.28 mmol) obtained in the previous experiment was stirred in a 50% solution of CF_3CO_2H in CH_2Cl_2 (30 mL) for 3 h and evaporated to dryness. The oily residue was taken up in a small amount of ethyl acetate and the product was precipitated as a fine white powder by slow addition of hexanes to the solution. The product was filtered and washed a couple of times with hexanes and dried under vacuum to give the desired product (**4**) in an almost quantitative yield (1.0 g, 98%). ^{1}H-NMR (250 MHz, DMSO-d6) δ = 4.07 (1H, t, *J* = 6,3 Hz), 4.28 (2H, d, *J* = 6,3 Hz), 4.53 (2H, s), 4.62 (2H, S), 7.07-7.14 (2H, m), 7.17-7.26 (4H, m), 7.46 (2H, d, *J* = 7.4 Hz), 7.66 (2H, d, *J* = 7.2 Hz), 7.77 (2H, d, *J* = 8.3 Hz), 10.30 (1H, br. s). ^{13}C-NMR (63 MHz, DMSO-d6) δ = 47.04, 61.62, 66.04, 76.79, 120.50, 125.41, 127.46, 128.03, 129.05, 129.69, 141.19, 142.20, 144.00, 157.12, 165.50, 169.47. MS (ES) m/z = 446 ($M-H^+$). HRMS (ES) calculated ($C_{25}H_{21}NO_7Na^+$): 470.1210 found: 470.1224.

d) SPOT-PEGA resin

1 g of commercial PEGA 1900 resin (loading of amino groups: 0.23 mmol/g) swelled in methanol was washed repeatedly with DMF to ensure complete removal of the methanol. The linker (**4**) (308 mg, 0.69 mmol), TBTU (207 mg, 0.64 mmol) and DIPEA (119 mg, 0.92 mmol) were mixed in DMF (10 mL) and left to pre-activate for 5 min before adding the mixture to the resin. After 3 h the reagents were removed by suction and the resin was washed with CH_2Cl_2 (5 × 20 mL).

A small portion of the resin was removed for a Kaiser test, which confirmed a successful coupling of the linker to the resin. Likewise, the loading of linker on the resin was determined and found to be

approximately 0.20 mmol/g by comparison with a standard curve after release of the Fmoc-group. The hydroxylamine protecting group (Fmoc) was now removed from the remaining resin by treatment with 20% piperidine in DMF (15 mL for 2 min and 15 mL for18 min) followed by extensive washings with DMF (5×20 mL) and CH_2Cl_2 (7×20 mL). The resin was dried under high vacuum for 24 h to give the final SPOT-PEGA resin, which was used for all subsequent experiments.

e) Coupling of spacer (5) to CPG

AMP CPG (765 mg, loading of amino groups: 60 µmol/g, 38 µmol.) was washed with DMF (3 × 2 mL), 50% DIPEA in DMF (3 × 2 mL), and DMF (3 × 2 mL). The beads were treated with a mixture of spacer **5**[3] (57 mg, 115 µmol), TBTU (37 mg, 115 µmol), and DIPEA (20 µL, 115 µmol) in DMF for 3 h at rt. The resin was washed with DMF (3 × 2 mL), CH_2Cl_2 (3 × 2 mL), and treated with 50% Ac_2O in pyridine for 30 min at rt. The beads were washed with CH_2Cl_2 (3 × 2 mL), DMF (3 × 2 mL), CH_2Cl_2 (3 × 2 mL), and treated with 50% TFA in CH_2Cl_2 for 1 h at rt. Washed with CH_2Cl_2 (3 × 2 mL) and DMF (3 × 2 mL). The coupling of spacer **5**, Ac_2O-pyridine, and TFA treatment were repeated giving compound **6**.

f) SPOT-CPG resin

Resin **6** (38 µmol) was washed with DMF (3 × 2 mL). The beads were treated with a mixture of **4** (51 mg, 115 µmol), TBTU (37 mg, 115 µmol), and DIPEA (27 µL, 153 mmol) in DMF at rt over night. The beads were washed with DMF (3 × 2 mL), CH_2Cl_2 (3 × 2 mL), and treated with 50% of Ac_2O in pyridine for 30 min at rt, washed with CH_2Cl_2 (3 × 2 mL), DMF (3 × 2 mL), CH_2Cl_2 (3 × 2 mL), and dried. The loading was determent to 50 µmol/g as described for SPOT-PEGA. The Fmoc group was removed using 20% piperidine in DMF for 2 × 10 min at rt and washed with, DMF (3 × 2 mL), CH_2Cl_2 (3 × 2 mL), and methanol (3 × 2 mL), and CH_2Cl_2 (3 × 2 mL). The resin was dried in vacuum.

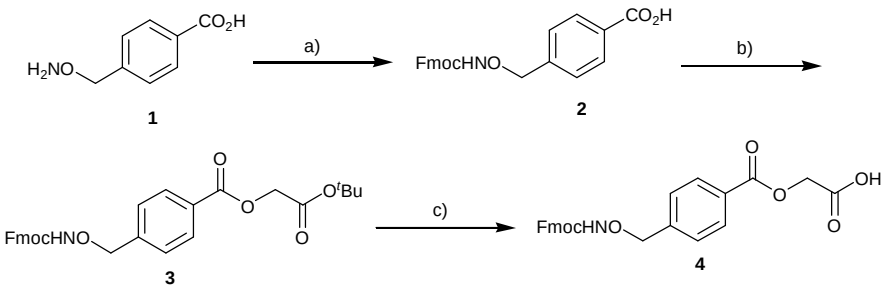
Enzymatic release of the oligosaccharide from glycolipid GM_1.

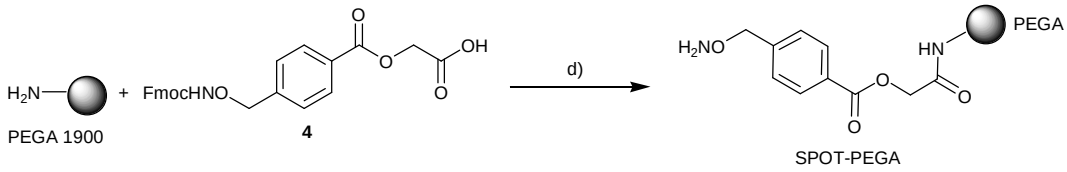
GM_1[4] (100 µg) was dissolved in buffer (100µl, 20 mM acetate, pH 5,5 + 0.2% T-100) and a solution of recombinant endoglycoceramidase II (5 µl, 10 mU, Takara-bio, Cat. No. 4460) was added. The mixture was incubated at 37° C for 20 h followed by removal of solvent by freeze-drying.

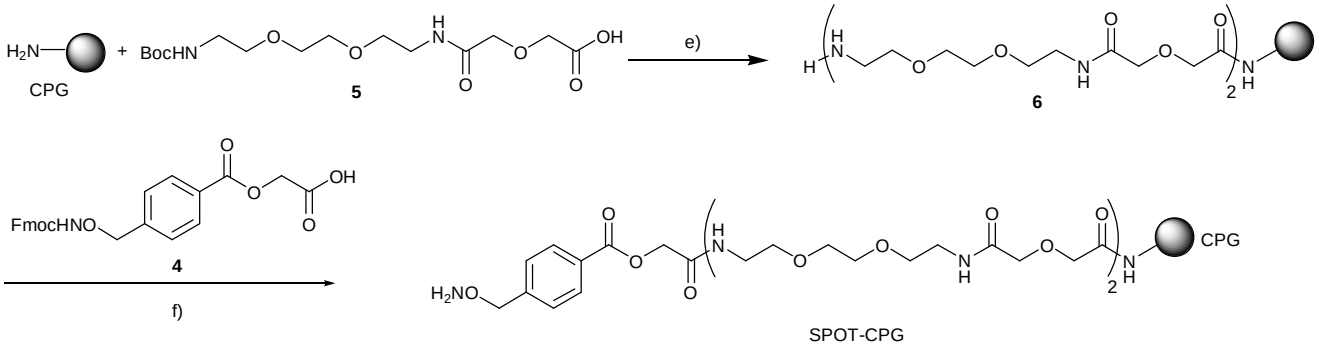
SPOT-labeling of the released GM_1 oligosaccharide.

The crude sample obtained from the digestion was re-dissolved in a mixture of DMSO and acetic acid (7:3, 100 µl) and added to 5 mg of PEGA resin (1 µmol of capture groups, >10 fold excess compared to released oligosaccharide) and left to incubate for 4 h at 75° C in a sealed tube. The beads were transferred to a 1 mL plastic syringe equipped with a scintered Teflon filter and mounted on a vaccum manifold. The resin was washed with MeOH (3 × 0.5 mL), DMF (2 × 0.5 mL), DMF containing 5% diisopropylethylamine (1 x 0.5 mL), MeOH (3 × 0.5 mL). The remaining hydroxylamines were capped

using 50% Ac_2O in MeOH (200 µL) for 1 hour, and washed with MeOH (5 × 0.5 mL). The oximes were reduced using BH_3-pyridine complex (8M solution in pyridine, 1000 equiv. compared to the oligosaccharide, ~10µL) in a mixture of 50% CCl_3CO_2H in water (20 µL) and MeOH (70 µL) for 15 min. The beads were washed with MeOH (3 × 0.5 mL), DMF containing 5% diisopropylethylamine (1 x 0.5 mL) and DMF (3 × 0.5 mL). The reduced product was labeled with TRITC (10 equiv. compared to the sugar) in DMF (100 µL) for 4 h at rt. The labeling was followed by extensive washing with DMF (5 × 0.5 mL), MeOH (5 × 0.5 mL), CH_2Cl_2 (3 × 0.5 mL), MeOH (3 × 0.5 mL), and water (3 × 0.5 mL). Finally, the product was cleaved from the support using 1M LiOH (100 µL) for 1h at rt. The supernatant, now containing the labeled oligosaccharide, was removed from the beads followed by washing of the beads with water (3 × 200 µL). The combined fractions were neutralized using 10% AcOH, diluted further to volume ~1-3 mL. For mass spectrometric and CE analysis a small portion of the sample was de-salted by adsorption on a Sep-Pak column (C-18), washed with water, and eluted with 20% acetonitrile in water.







[1] Corrie, J. E. T. & Craik, J. S., *J. Chem. Soc., Perkins Tran. 1*, 2967-2973 (1994).

[2]The material was prepared in a similar manner to that previously described: Deles, J., Kolaczkowska, E. & Superson, A. *Pol. J. Chem*. **53**, 1025-1032 (1979).

[3] The material was prepared in a similar manner to that previously described: Pieters, R., Fennema, M., Kellogg, R. M. & Janssen, D. B. *Bioorg. Med. Chem. Lett.* **9**, 161-166 (1999).

[4] Gift from Prof. Guido Tettamanti, University of Milano.