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Solid-phase synthesis of serine-based glycosphingolipid analogues for preparation of glycoconjugate arrays

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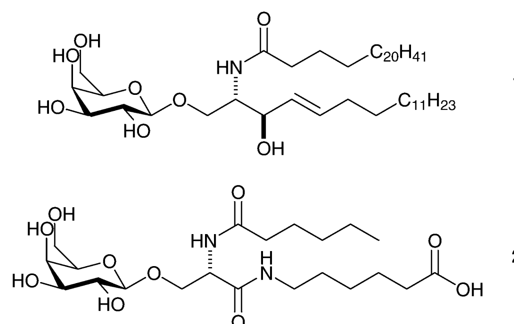
Synthetic glycolipids with defined structures are important tools in the study of glycolipid biology. In this paper we describe a solid-phase synthesis of three galactosylated serine-based glycosphingolipid analogues using the novel linker 2-fluoro-4-(hydroxymethyl)-phenoxyacetic acid. Gel-phase ^{19}F-NMR spectroscopy was used to measure the yield and stereochemical outcome of the solid-phase glycosylations. Under NIS–TfOH promotion, α- and β-selective glycosylations were performed at room temperature with thioglycoside donors carrying fluorine labelled protective groups. Finally, the glycolipids were covalently linked to microtiter plates and labelled lectins with different selectivity for α- and β-galactosides could bind to the glycolipid arrays.

Introduction

Glycolipids constitute an important and large group of biomolecules with diverse biological functions. They are, for example, building blocks in cell walls[1] and important epitopes in molecular recognition mechanisms in eukaryote cell–cell signalling,[2] microbial infections[3,4] and cancer.[2] There is a large structural variety among the glycolipids, especially in the carbohydrate part, and small variations in the structure can result in a drastic change in the biological function. Hence, to study the biology of glycolipids pure and well-characterised derivatives are needed. Isolation of pure and structurally defined glycolipids from natural sources is, however, cumbersome. This imposes a need for efficient methods for the preparation of synthetic glycolipids. Many efforts have been made in the synthesis of natural glycolipids and their analogues. However, despite the progress made in this area, examples of parallel or combinatorial synthesis of glycolipid libraries are rare. Glycolipid libraries have great potential for high-throughput strategies to study glycobiology using, for example, microarray techniques.[5] Microarrays have emerged as important analytical tools in molecular biology, with nucleotide and protein arrays leading the way. Recently, small carbohydrate arrays have been prepared with a variety of methods and both commercial[6–8] and non-commercial[9] arrays are available. Oligosaccharides, glycoproteins, and glycolipids have been non-covalently attached to nitrocellulose,[10,11] glass[12,13] or polystyrene[14] slides, and microtiter plates.[9,10,15–20] Covalent attachments have been performed on gold surfaces,[21–25] glass slides,[6,26–30] microspheres[31] and microtiter plates.[7,32–35] The glycoconjugate arrays have been used to study receptor–carbohydrate and enzyme–carbohydrate interactions, to identify and study the specificity of different carbohydrate-binding proteins, to find inhibitors to the same proteins, and to study the carbohydrate binding properties of intact cells.

Glycosphingolipids like β-galactosylceramide (**1**) are abundant in nature and participate in numerous molecular recognition events.[36] Consequently, many of the >200 naturally occurring glycosphingolipids identified so far[37] have been synthesised,[38] but microarray applications are rare.[10] Due to the relative complexity in the lipid portions of glycosphingolipids, with at least two stereo centres and defined double bonds, several families of simplified analogues have been synthesised.[38] One of these families is the serine-based glycolipids like **2**, which have been used in several different applications[39–50] since they appeared in 1994.[51] When ceramides are present in cell membranes, their hydrophobic parts are buried in the lipid layer, while the



polar head is exposed to the surroundings for recognition. In the study of carbohydrate–protein interactions it is therefore important that the ceramide analogues mimic the polar head group of the ceramide. For the serine-based glycolipids, this is accomplished by the serine moiety together with its two adjacent amide bonds. The possibility for these neoglycolipids to act as ceramide analogues has been demonstrated in the inhibition of HIV invasion of cells,[41–43] in transglycosylations by ceramide glycanase[47] and in the promotion of β-glucocerebrosidase activity.[44] Moreover, the structure of glycolipids like **2** can easily be varied using different ω-amino acids, hydroxy-amino acids, fatty acids and glycosyl donors. The synthesis can be performed on the solid phase, which simplifies library preparation and the terminal carboxylic acid enables covalent array preparation. Solid-phase synthesis is being increasingly used in carbohydrate chemistry.[52] However, its main drawback is the lack of methods to analyse products when still attached to the solid support. One strategy that circumvents this obstacle is the use of fluorinated protecting groups and linkers that enable reaction monitoring with gel-phase ^{19}F-NMR spectroscopy (ref. 53 and further references therein). Due to the requirement for stereo- and regiochemical control in carbohydrate chemistry on-resin analysis is a major challenge for routine solid-phase carbohydrate synthesis,[52] especially since glycosylations are very sensitive to changes in both the glycosyl donor and acceptor structure.[54–56] This sensitivity makes it almost impossible to optimise a few test reactions and use the results for the construction of a larger library, the way solid-phase library synthesis is normally performed.[57] Parallel synthesis of glycolipid libraries using efficient synthetic protocols and monitoring techniques will provide pure and structurally defined compounds for the preparation of glycolipid microarrays. In this paper we describe the synthesis of three serine-based glycosphingolipid analogues

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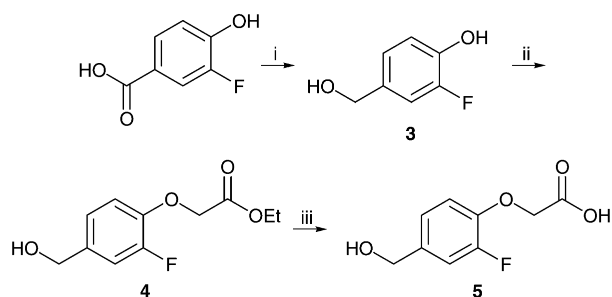


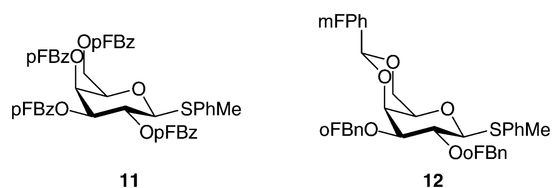
Scheme 1 Synthesis of the fluorinated linker **5**. (i) BH_3·DMS, $B(OMe)_3$, THF; (ii) α-bromoacetic acid ethyl ester, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), MeCN, 57% over two steps; (iii) LiOH, THF–MeOH–water 3 : 1 : 1, 90%.

using solid-phase glycosylations monitored with gel-phase ^{19}F-NMR spectroscopy. In addition, we demonstrate how glycolipid arrays can be prepared by covalent attachment of glycolipids to a microtiter plate and subsequent detection with carbohydrate recognising proteins.

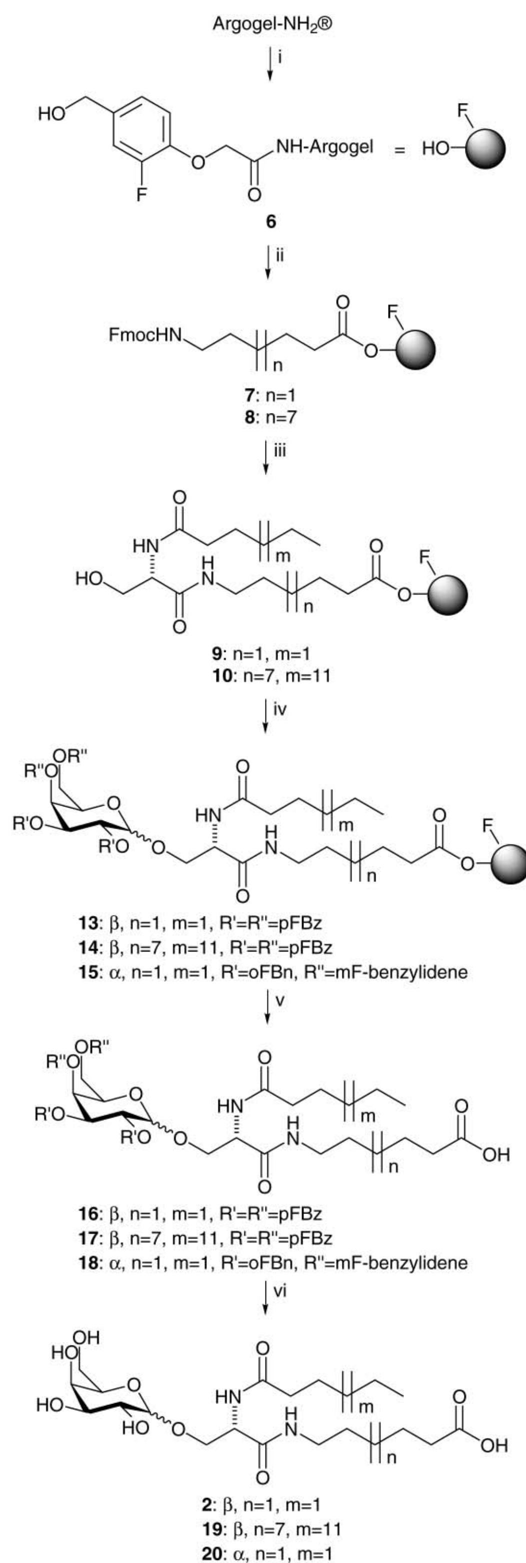
Results and discussion

Synthesis of glycolipids

For the synthesis of glycolipid libraries a method based on glycosylations of resin-bound serine-based lipids was envisioned. The novel linker **5**, 2-fluoro-4-(hydroxymethyl)-phenoxyacetic acid, was efficiently made in 51% total yield from 3-fluoro-4-hydroxy-benzoic acid in a three-step synthesis that is suitable for large-scale preparation (Scheme 1). This linker has the advantage that the final glycolipid can be cleaved under both acidic and basic conditions. This enables cleavage of fully protected glycolipids with both acid- and base-labile protecting groups. In solid-phase synthesis the purification after cleavage is often critical since the intermediate products have not been purified. Therefore, it is beneficial to have the potential to purify the glycolipid in both its apolar, protected form and in the polar, deprotected form. The linker **5** is an analogue to 3-fluoro-4-(hydroxymethyl)-phenoxyacetic acid that has previously been used as a linker in solid-phase synthesis monitored with gel-phase ^{19}F-NMR spectroscopy[58] and is sufficiently stable to withstand acidic glycosylations.[59] Attachment of **5** to Argogel®-NH_2 with *N*,*N*′-diisopropyl-carbodiimide and 1-hydroxy-7-azabenzotriazole activated amide bond formation gave the linker resin **6** in quantitative yield as indicated by monitoring with bromophenol blue (Scheme 2). Argogel®-NH_2 is a resin with good swelling and diffusion properties, leading to fast reactions and high-resolution gel-phase ^{19}F-NMR spectra.[59] With the linker in place the lipid part was assembled using standard peptide synthesis conditions. The Fmoc-ω-amino acids were coupled to the hydroxyl resin **6** using 1-(mesitylene-2-sulfonyl)-3-nitro-1,2,4-triazole (MSNT) and 1-methyl-imidazole (MeIm) to give **7** and **8** in quantitative yields according to gel-phase ^{19}F-NMR analysis. Fmoc-deprotections were carried out with 20% piperidine in DMF and amides were formed using *N*,*N*′-diisopropyl-carbodiimide (DIC), 1-hydroxy-benzotriazole (HOBt), and monitored with bromophenol blue to give lipids **9** and **10** (Scheme 2). To start to explore the scope of the method, both α- and β-galactosides were made, using the previously described galactosyl donors **11**[60] and **12**.[53] The glycosylations



were performed in the absence of light, using *N*-iodosuccinimide (NIS) and trifluoromethanesulfonic acid (TfOH) as the promoter system.[60] The donor **11** has a benzoate ester in the 2-position, allowing neighbouring-group participation, which leads to a 1,2-*trans* linked β-galactoside with good selectivity.[61] The fluorinated protecting groups made it possible to monitor both the yield and the selectivity of the reaction. Submitting the



Scheme 2 Glycolipid synthesis. (i) **5**, DIC, 1-hydroxy-7-azabenzotriazole, DMF; (ii) *N*-Fmoc-6-aminohexanoic acid or *N*-Fmoc-12-aminododecanoic acid, MSNT, MeIm, DMF; (iii) (a) piperidine, DMF; (b) Fmoc-serine, DIC, HOBt, DMF; (c) piperidine, DMF; (d) hexanoic acid or palmitic acid, DIC, HOBt, DMF; (iv) **11**, NIS, TfOH, CH_2Cl_2 or **12**, NIS, TfOH, CH_2Cl_2–THF 1 : 1; (v) TFA (95% aq), 60 °C or LiOH (20–33 mM, THF–water 1 : 2); (vi) NaOMe, MeOH or H_2 (1 atm), Pd/C, AcOH

Table 1 Investigation of the α-glycosylation of resin **9** using the galactosyl donor **12**

Entry	Solvent	Temperature/ °C	Yield/%[a]	α-/β-selectivity[a]
1	CH_2Cl_2	−40	53	2 : 1
2	CH_2Cl_2	RT	Quant.	2 : 1
3	CH_2Cl_2–THF 1 : 1	−40	45	2 : 1
4	CH_2Cl_2–THF 1 : 1	RT	Quant.	4 : 1

[a] Quantification was made by integration of peaks in the ^{19}F-NMR spectra.

resin-bound lipids to four equivalents of **11** gave resin bound glycolipids **13** and **14** in a yield of *ca.* 50% and repeating the reaction increased the yield to *ca.* 75%. The β-/α-selectivity was estimated to be greater than 5 : 1. 1,2-*cis*-Linked glycosides are, on the other hand, generally more difficult to form than the *trans*-glycosides. In the absence of a participating group at the 2-position the α-glycoside is the thermodynamically favoured isomer, but the selectivity is often moderate or low. To increase the selectivity, α-glycosylations are predominantly performed at low temperatures. Our first attempt was made with five equivalents of the donor **12** and dichloromethane as solvent at −40 °C giving resin-bound glycolipid **15** in *ca.* 50% yield and an α-/β-selectivity of *ca.* 2 : 1 (Table 1, entry 1). In addition, the ^{19}F-NMR spectrum indicated that a third, unknown glycosylated product was formed at a unimolecular ratio to the β-anomer. A second glycosylation raised the yield to *ca.* 85%. However, low temperature reactions are less practical to perform, which makes them unsuitable for library synthesis, where uniform reaction conditions and simple handling are preferred. We therefore attempted to explore the possibilities to carry out selective α-glycosylations at room temperature. Increasing the temperature resulted in a quantitative yield, but the selectivity remained low (Table 1, entry 2). Another commonly used method to increase the yield of α-glycosides is to employ an ether solvent.[62–64] By performing the reaction in dichloromethane–THF (1 : 1) at room temperature, the selectivity could be raised to 4 : 1 (Table 1, entry 4). Unfortunately, the unknown product was still formed, again in equimolar ratio to the β-anomer. At low temperature the ether effect could not be observed (Table 1, entry 3). Thanks to the fluorinated protecting groups and gel-phase ^{19}F-NMR spectroscopy, this improvement in both yield and selectivity was straightforward, without the need for cleavage and purification of the products. The higher yield compared to the β-glycosylation is probably due to the higher reactivity for benzylated, relative to benzoylated, glycosyl donors.[65] The galactosyl donor **12** has previously been used to glycosylate the 3′-hydroxyl on a resin-bound lactose derivative with complete α-selectivity.[53] Cleavage of the β-glycolipid resins **13** and **14** in trifluoroacetic acid (TFA, 95% aq) at 60 °C for 3.5 h gave the protected lipids **16** and **17** in 14 and 32% yield, respectively, based on the initial loading of Argogel®-NH_2. These yields are comparable to the yields obtained in earlier studies of solid-phase glycosylations of serine.[59] The discrepancy between on-bead and cleaved yields could be explained with unreactive amino-groups on the Argogel®-NH_2 resin, lowering the initial loading. Furthermore, chromatographic purifications on a small scale inevitably lead to loss of material. The fluorobenzoates were then removed using NaOMe yielding the unprotected lipids **2** and **19** in *ca.* 80% yield. In the synthesis of the longer lipid, the filtration of the resin became very slow after attachment of the dodecanoic acid. Presumably this was due to organogel formation. Double-chained glycolipids have previously been shown to be good organogelators,[66] and this result indicates that the glycolipid **19** could be one. However, even though the filtrations were slow, the gel-phase ^{19}F-NMR spectra were of good quality and all reactions proceeded well. When the α-glycolipid **15** was cleaved in TFA (95% aq) at 60 °C for 6 h, the acid labile fluorobenzylidene group was simultaneously removed. This deprotection was accompanied by what appeared to be migration of the fluorobenzyl groups, resulting in a complex reaction mixture. To avoid this, a basic cleavage strategy was used. Reaction of the resin with LiOH (20 mM in THF–water 1 : 2) for 3 h followed by 3 h with 33 mM LiOH gave *ca.* 90% cleavage from the resin according to gel-phase ^{19}F-NMR analysis. The ^{19}F-NMR spectrum of the remaining 10% contained the same peaks as before the reaction, indicating cleavage of all three products. The crude product was purified using flash column chromatography and HPLC to give the protected α-glycolipid **18** in 23% yield. Three peaks were collected from the preparative HPLC purification. In addition to the α-glycolipid, the β-glycolipid could be isolated in 4% yield and its identity was confirmed by ^{1}H-NMR spectroscopy. The final peak was believed to be the third, enigmatic, unknown product but unfortunately ^{1}H- and ^{19}F-NMR spectroscopy indicated a complex mixture of several glycosylated compounds. Hydrogenation of the glycolipid **18** over palladium on charcoal finally yielded the deprotected glycolipid **20** in 94% yield. Notably the ^{19}F-NMR spectra of **16**, **17**, and **18** were all in agreement with the ^{19}F-NMR spectra of the resin-bound glycolipids **13**, **14**, and **15**.

To summarise, the glycolipids **2**, **19**, and **20** were synthesised in eight steps and with total yields of 11, 26, and 22%, respectively, all based on the theoretical loading of Argogel®-NH_2.

Formation of glycolipid microtiter plates

To start to explore the scope of microtiter bound glycolipids, the water-soluble glycolipids **2** and **20** were covalently linked to the amino functions of CovaLink™ microtiter plates. The CovaLink™ plates have secondary amines bound to the plate walls *via* alkyl spacers. The glycolipids were dissolved in water and serially diluted in the plates. A water solution of *N*-hydroxysuccinimide (NHS) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) was added to the wells and the plates were shaken at room temperature for 6 h. The plates were then rinsed and blocked with bovine serum albumin overnight. The glycolipids were detected with labelled lectins, biotin-labelled RCA_{120} from *Ricinus communis* with affinity for α- and β-Gal[67] (Fig. 1), followed by horseradish peroxidase-conjugated avidin, and horseradish peroxidase-conjugated BS-1 from *Bandeiraea simplicifolia* with affinity for α-Gal and α-GalNAc[67] (Fig. 2). Both glycolipids gave similar curves when detected with RCA_{120}, even though the lectins affinity for the β-anomer is twice that for the α-anomer, indicating that the method is qualitative. The α-selective BS-1, on the other hand, only bound to the α-galactosylated lipid **20**. Importantly, the plates could be regenerated with a 10% SDS solution and blocking, giving essentially the same results as before regeneration (Fig. 3). Since synthetic material is very precious this is a valuable property.

Conclusions

We have synthesised three serine-based glycolipids using gel-phase ^{19}F-NMR spectroscopy to monitor solid-phase organic chemistry, demonstrating its utility in the synthesis of glycolipids. This synthesis is suitable for the preparation of libraries of glycolipids of varying complexity. The linearity of the synthesis gives the possibility to easily create truncated, as well as other, analogues of the target carbohydrate. Moreover, the structure

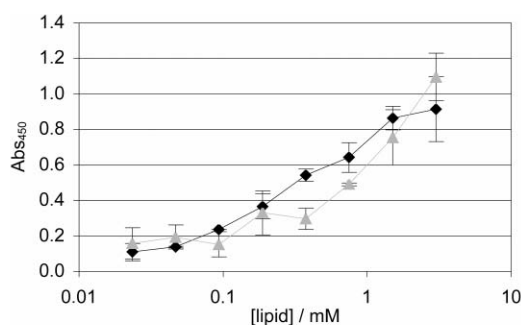


Fig. 1 Binding of the lectin from *Ricinus communis* (RCA_{120}) to the plate bound glycolipids. The lipids **2** (diamonds) and **20** (triangles) were serially diluted in the wells, followed by the coupling reagents EDC and NHS in water. The covalent attachments were performed with 50 µL of the coupling solution. The bound lipids were detected with biotin-conjugated RCA_{120} and horseradish peroxidase-labelled avidin (see the Experimental section). The average value of eight blank cells was subtracted from the absorbances in the graph. The glycolipid concentration of the final coupling solution is given on the *x*-axis. The points represents the average from duplicate runs and the error bars are set to ± one standard deviation.

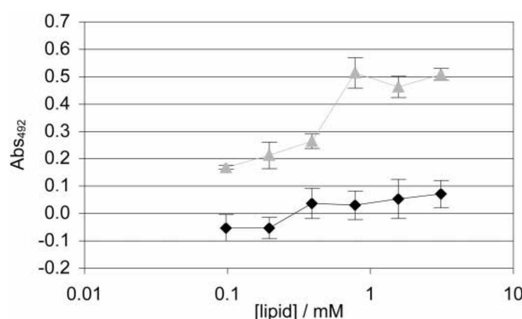


Fig. 2 Binding of the lectin from *Bandeiraea simplicifolia* (BS-1) to the plate bound glycolipids. The lipids **2** (diamonds) and **20** (triangles) were serially diluted in the wells, followed by the coupling reagents EDC and NHS in water. The covalent attachments were performed with 100 µL of the coupling solution. The bound lipids were detected with horseradish peroxidase-labelled BS-1 (see the Experimental section). The average value of eight blank cells was subtracted from the absorbances in the graph. The glycolipid concentration of the final coupling solution is given on the *x*-axis. The points represent the average from duplicate runs and the error bars are set to ± one standard deviation.

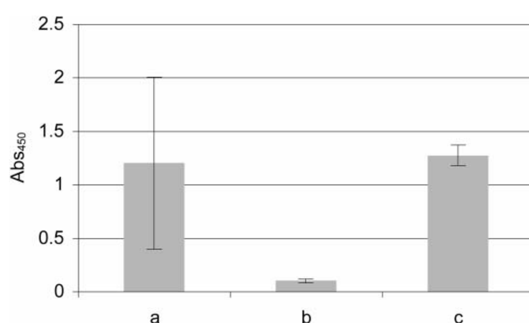


Fig. 3 Plate regeneration. (a) The lipid **2** (1.5 mM) was attached to Covalink™ wells and detected as before (*cf.* Fig. 1). (b) The proteins were stripped from the wells with sodium dodecyl sulfate (10% aq; see the Experimental section) followed by addition of horseradish peroxidase-labelled avidin and substrate solution to confirm removal of the biotin-conjugated RCA_{120}. (c) After another stripping of the wells normal detection showed the intact, well-bound glycolipid. The bars show the average absorbances from duplicate runs subtracted with the average value of two blank cells. The error bars are set to ± one standard deviation.

of the lipid part can readily be varied using different building blocks. However, the yields and selectivities in the glycosylations may hamper the purifications when making glycolipids with larger oligosaccharides. In addition, we have created glycolipid microtiter plates and showed their potential for the study of carbohydrate–protein interactions. Plate bound carbohydrates have previously been shown to be accepted as substrates for enzymatic synthesis, which could further enhance the scope of the glycolipid libraries. Currently we explore the potential for preparation and application of complex glycosphingolipid analogue arrays.

Experimental

Solid-phase syntheses were performed on Argogel®-NH_2 resin (Argonaut Technologies; 0.38 or 0.43 mmol g^{-1}). CH_2Cl_2 was distilled from calcium hydride, THF from potassium and DMF was distilled under vacuum. Before concentration, all organic solvents were dried over Na_2SO_4 and filtered. Solvent mixtures are reported as *v*/*v* ratios. TLC was run on Silica Gel 60 F_{254} (Merck) and the spots were detected in UV-light and stained with 10% aq H_2SO_4 and heat. Silica gel (Matrex, 60 Å, 35–70 µm, Grace Amicon) and solvents of analytical grade were used for flash column chromatography. The NMR-spectra were recorded on a Bruker DRX-400 spectrometer. Peaks that could not be assigned are not reported. *J* values are given in Hz. ^{13}C-NMR resonances from the fluorine-labelled protective groups are split by J_{C-F} couplings, leading to complex spectra. Therefore, signals where this problem may arise, primarily aromatic signals downfield of 110 ppm, are not reported for the compounds in question. Gel-phase proton decoupled ^{19}F-NMR spectra were recorded on resin suspensions in $CDCl_3$ with $CFCl_3$ (δ_F 0 ppm) as internal standard. Two peaks appear in the spectrum around 0 ppm. One originates from $CFCl_3$ inside the polymer and one from $CFCl_3$ outside the polymer. The peak with highest shift was used as internal standard. Preparative reversed phase LC-MS was performed on an XTerra C-18 column (50 × 19 mm, 5 µm, 125 Å), eluted with a linear gradient of MeCN (5–30% over 5 min) in water, both of which contained formic acid (0.2%). A flow rate of 25 mL min^{-1} was used and detection was at 214 and 254 nm and with positive and negative electrospray mass analysis. Low pressure reversed phase purification was performed on Supelclean solid-phase extraction tubes with LC-8 or LC-18 packings. Positive fast atom bombardment mass spectra were recorded on a Jeol SX 102 mass spectrometer. Ions were produced by a beam of Xenon atoms (6 keV).

2-Fluoro-4-(hydroxymethyl)-phenoxyacetic acid (5)

3-Fluoro-4-hydroxybenzoic acid (3.00 g, 21.1 mmol) was dissolved in THF (50 mL) and added slowly to trimethyl borate (14.0 mL, 123 mmol) and borane dimethyl sulfide complex (8.0 mL, 84 mmol) in THF (150 mL). The solution was stirred at room temperature for 23 h, evaporated and coevaporated with MeOH yielding 3.1 g crude benzyl alcohol **3** as a grey solid. Compound **3** and ethyl bromoacetate (4.8 mL, 43 mmol) were stirred at room temperature in MeCN (180 mL) and 1,8-diazabicyclo[5.4.0]undec-7-ene (4.9 mL, 33 mmol) was added dropwise. The solution was refluxed for 20.5 h, cooled to room temperature, poured into EtOAc (300 mL) and washed with HCl (0.05 M aq, 2 × 150 mL) and brine (150 mL). The organic phase was evaporated and concentrated *in vacuo* overnight and flash column chromatography (CH_2Cl_2–MeOH 30 : 1–20 : 1) yielded ethyl ester **4** (2.82 g, 57% over two steps) as a pale yellow oil. The ester **4** was dissolved in THF–MeOH–water (170 mL, 3 : 1 : 1) and the solution was cooled to 0 °C. LiOH (1 M aq, 17 mL, 17 mmol) was added dropwise and the resulting solution was stirred at 0 °C for 35 min. The solution was allowed to reach room temperature and stirred for additional 2.5 h. After cooling to 0 °C the solution was acidified with HCl (1 M aq) and extracted with EtOAc (1 × 250 mL, 2 × 50 mL). The organic phases were washed with brine (1 × 200 mL) and concentrated *in vacuo* yielding the title compound **5** (2.23 g, 90%) sufficiently pure for further use. A small portion was further purified by flash column chromatography for characterisation. δ_H (400 MHz;

CD_3OD; CHD_2OD) 7.11 (1 H, dd, *J* 12.3, 1.6, Ar), 7.06–7.02 (1 H, m, Ar), 6.98 (1 H, t, *J* 8.34, Ar), 4.67 (2 H, s, OCH_2CO), 4.55 (2 H, s, OCH_2O); δ_C (100 MHz; CD_3OD; CD_3OD) 172.7, 67.4, 64.3; *m*/*z* (FAB) 200.0485 (M^+ $C_9H_9FO_4$ requires 200.0485).

Resin 6

Argogel®-NH_2 (0.60 g, 0.23 mmol) was allowed to swell in DMF and washed with piperidine (20% in DMF, 2 × 5 mL) and distilled DMF (3 × 5 mL). The linker **5** (68 mg, 0.34 mmol) was dissolved in distilled DMF (5 mL) followed by addition of *N*,*N′*-diisopropyl-carbodiimide (DIC, 53 µL, 0.34 mmol) and 1-hydroxy-7-azabenzotriazole (62 mg, 0.46 mmol). The solution was stirred at room temperature for 20 min and transferred to the resin followed by bromophenol blue (114 µL, 2 mM in DMF, 228 nmol). The mixture was agitated for 18 h followed by thorough washing of the resin to give **6**. δ_F (376 MHz; $CDCl_3$; $CFCl_3$): −134.5.

Resins 7 and 8

N-Fmoc-6-aminohexanoic acid (0.32 g, 0.91 mmol) (for **7**) or *N*-Fmoc-12-aminododecanoic acid (0.40 g, 0.91 mmol) (for **8**) and 1-(2-mesitylenesulfonyl)-3-nitro-1*H*-1,2,4-triazole (0.27 g, 0.91 mmol) were dissolved in CH_2Cl_2 (5 mL) and the solution was added to resin **6** (0.23 mmol). 1-Methyl imidazole (55 µL, 1.0 mmol) was added and the mixture was agitated for 18 h followed by thorough washing of the resin to give **7** or **8**, respectively. δ_F(376 MHz; $CDCl_3$; $CFCl_3$): −133.8 (resin **7**), −133.9 (resin **8**).

Resins 9 and 10

The resins **7** and **8** (0.23 mmol each) were treated with piperidine (20% in DMF, 2 × 5 mL, 10 min) and washed with DMF (2 × 5 mL) and distilled DMF (3 × 5 mL). Fmoc-serine-OH (0.30 g, 0.91 mmol) was dissolved in distilled DMF (5 mL) followed by addition of DIC (0.14 mL, 0.91 mmol) and 1-hydroxybenzotriazole (HOBt, 185 mg, 1.37 mmol). The solution was transferred to the resin **7** or **8** followed by addition of bromophenol blue (114 µL, 2 mM in DMF, 228 nmol). The reaction was agitated for 18 h and the resin was washed thoroughly, treated with piperidine (20% in DMF, 2 × 5 mL, 10 min) and washed with DMF (2 × 5 mL) and distilled DMF (3 × 5 mL). Hexanoic acid (0.11 mL, 0.91 mmol; to **7**) or palmitic acid (0.23 g, 0.91 mmol; to **8**) was dissolved in distilled DMF (5 mL) followed by addition of DIC (0.14 mL, 0.91 mmol) and HOBt (185 mg, 1.37 mmol). The solution was transferred to the resin followed by bromophenol blue (114 µL, 2 mM in DMF, 228 nmol). The reaction was agitated for 18 h and the resin was washed thoroughly to give resin **9** or **10**.

Resins 13 and 14

The resin **9** or **10** (0.11 mmol), 4-methylphenyl 2,3,4,6-tetra-*O*-(4-fluorobenzoyl)-1-thio-β-D-galactopyranoside (**11**) (0.35 g, 0.46 mmol), and N-iodosuccinimide (NIS, 0.10 g, 0.46 mmol) were dried under vacuum in the absence of light. The galactose donor **11** and NIS were dissolved in distilled CH_2Cl_2 (5 mL) and added to the resin. The mixture was agitated for 5 min and trifluoromethanesulfonic acid (TfOH, 1.0 M in CH_2Cl_2, 44 µL, 45 µmol) was added. The reaction was agitated for 3.5 h at room temperature in the absence of light and the resin was washed thoroughly to give resin **13** or **14**. ^{19}F-NMR analysis indicated 50 and 62% yield for **13** and **14**, respectively. The reaction was repeated after which the ^{19}F-NMR spectra showed 75 and 76% yield, respectively. δ_F(376 MHz; $CDCl_3$; $CFCl_3$; major isomer): −104.2, −104.6, −104.8, −105.2, −133.8 (resin **13**), −104.1, −104.5, −104.7, −105.1, −133.8 (resin **14**).

Resin 15

The resin **9** (73 µmol), galactose donor **12** (0.24 g, 0.39 mmol), and NIS (82 mg, 0.37 mmol) were protected from light and dried under vacuum overnight. CH_2Cl_2 (1.5 mL) and THF (1.5 mL) were added followed by TfOH (0.28 M in CH_2Cl_2, 51 µL, 15 µmol) and the mixture was shaken at room temperature for 370 min. The resin was filtered and washed with DMF (5 × 5 mL), piperidine (20% in DMF; 2 × 4 mL), CH_2Cl_2, THF, MeOH, CH_2Cl_2 and DMF (5 × 5 mL each). Gel-phase ^{19}F-NMR spectroscopy showed 103% yield and an α-/β-selectivity of 4 : 1 together with an unknown glycosylated product in unimolecular ratio to the β-anomer. δ_F (376 MHz; $CDCl_3$; $CFCl_3$; major isomer) −113.7, −118.5, −119.5, −133.8.

N^{α}-Hexanoyl-3-*O*-(2,3,4,6-tetra-*O*-(4-fluorobenzoyl)-β-D-galactopyranosyl)-L-seryl-6-aminohexanoic acid (16)

A solution of trifluoroacetic acid (TFA) and water (9 : 1, 5 mL) was added to the resin **13** (0.11 mmol) and the mixture was stirred for 3.5 h at 60 °C. The resin was filtered and washed with TFA and the combined solutions were concentrated *in vacuo*. Flash column chromatography (CH_2Cl_2–MeOH 40 : 1–30 : 1–20 : 1) gave **16** (14 mg, 14% based on the loading of Argogel®-NH_2). δ_H(400 MHz; $CDCl_3$; $CHCl_3$) 8.16–7.94 (6 H, m, Ar), 7.81–7.74 (2 H, m, Ar), 7.22–7.04 (6 H, m, Ar), 6.97–6.90 (2 H, m, Ar), 6.55 (1 H, t, *J* 5.7, CH_2NH), 6.46 (1 H, d, *J* 6.8, CHN*H*), 5.95 (1 H, d, *J* 3.3, 4-H), 5.76 (1 H, dd, *J* 10.3 and 8.0, 2-H), 5.60 (1 H, dd, *J* 10.4 and 3.4, 3-H), 5.02 (1 H, d, *J* 8.1, 1-H), 4.71–4.56 (2 H, m, 5-H and CH_2CH(NH)CO), 4.48–4.40 (2 H, m, 6-H), 4.08 (1 H, dd, *J* 10.7 and 4.3, OCH_2CH(NH)CO), 3.72 (1 H, dd, *J* 10.5 and 9.1, OCH_2CH(NH)CO), 3.28–3.10 (2 H, m, $NHCH_2$), 2.36 (2 H, t, *J* 6.77, CH_2CO), 2.15–2.08 (2 H, m, CH_2CO), 0.88 (3 H, t, *J* 6.86, CH_3); δ_C(100 MHz; $CDCl_3$; $CDCl_3$) 102.63, 72.02, 71.73, 70.61, 70.23, 68.39, 62.35, 52.08, 39.49, 36.45, 31.56, 28.94, 26.10, 25.30, 24.34, 22.54, 14.10; δ_F(376 MHz; $CDCl_3$; $CFCl_3$) −104.2, −104.3, −104.7, −105.0; *m*/*z* (FAB) 989.3093 (M + Na^+. $C_{49}H_{50}F_4N_2NaO_{14}$ requires 989.3096).

N^{α}-Palmitoyl-3-*O*-(2,3,4,6-tetra-*O*-(4-fluorobenzoyl)-β-D-galactopyranosyl)-L-seryl-12-aminododecanoic acid (17)

A solution of TFA and water (9 : 1, 5 mL) was added to resin **14** (0.11 mmol) and the mixture was stirred for 3.5 h at 60 °C. The resin was filtered and washed with TFA and the combined solutions were concentrated *in vacuo*. Flash column chromatography (CH_2Cl_2–MeOH 20 : 1–10 : 1 + 1% HOAc, heptane–EtOAc 1 : 5–0 : 1) gave **17** (38 mg, 28% based on the loading of Argogel®-NH_2). δ_H(400 MHz; $CDCl_3$; $CHCl_3$) 8.12–7.93 (6 H, m, Ar), 7.81–7.74 (2 H, m, Ar), 7.22–7.03 (6 H, m, Ar), 6.97–6.89 (2 H, m, Ar), 6.61–6.54 (2 H, m, 2 × NH), 5.95 (1 H, d, *J* 3.4, 4-H), 5.72 (1 H, dd, *J* 10.4 and 8.1, 2-H), 5.57 (1 H, dd, *J* 10.4 and 3.4, 3-H), 4.99 (1 H, d, *J* 8.1, 1-H), 4.71–4.55 (2 H, m, 5-H and CH_2CH(NH)CO), 4.48–4.38 (2 H, m, 6-H), 4.10 (1 H, dd, *J* 10.4 and 4.8, OCH*H*CH(NH)CO), 3.75 (1 H, t, *J* 10.4, OCH*H*CH(NH)CO), 3.22–3.13 (1 H, m, NHCH*H*), 3.09–2.99 (1 H, m, NHCH*H*), 2.34 (2 H, t, *J* 7.2, CH_2CO), 2.13 (2 H, t, *J* 7.7, CH_2CO), 0.89 (3 H, t, *J* 6.4, CH_3); δ_C(100 MHz; $CDCl_3$; $CDCl_3$) 102.45, 71.91, 71.80, 70.29, 70.01, 68.41, 67.28, 62.30, 52.12, 39.92, 36.49, 34.08, 32.12, 31.12, 26.82, 25.68, 24.97, 24.87, 22.89, 14.31; δ_F(376 MHz; $CDCl_3$; $CFCl_3$) −104.2, −104.6, −104.8, −105.1; *m*/*z* (FAB) 1235.5402 (M − H^+ + 2 Na^+. $C_{65}H_{81}F_4N_2Na_2O_{14}$ requires 1235.5414).

N^{α}-Hexanoyl-3-*O*-(2,3-di-*O*-(2-fluorobenzyl)-4,6-*O*-(3-fluorobenzylidene)-α-D-galactopyranosyl)-L-seryl-6-aminohexanoic acid (18)

Resin **15** (0.25 mmol) was swelled in THF, filtered and THF (6 mL) was added followed by LiOH (30 mM aq, 12 mL, 0.36 mmol). The mixture was shaken at room temperature for 190 min. More LiOH (2 M aq, 0.12 mL, 0.24 mmol) was added and the mixture was shaken at room temperature for another 190 min. The resin was filtered and washed with MeOH (3 × 15 mL) and CH_2Cl_2–MeOH (2 : 1, 3 × 15 mL). The combined filtrates were acidified (pH *ca.* 4) with Amberlite® IR 120 (plus),

filtrated, evaporated and concentrated *in vacuo*. The residue was extracted between CH_2Cl_2–toluene–EtOAc (1 : 1 : 1) and water. The organic phase was evaporated and concentrated *in vacuo* yielding *ca.* 0.15 g crude product. Flash column chromatography (CH_2Cl_2–MeOH 30 : 1) and preparative normal phase HPLC yielded the protected glycolipid **18** (46 mg, 23% based on the loading of Argogel®-NH_2). Gel-phase ^{19}F-NMR analysis of the resin showed 80–90% cleavage. δ_H(400 MHz; $CDCl_3$; $CHCl_3$) 7.54 (1 H, td, *J* 7.6 and 1.6, Ar), 7.38–7.23 (6 H, m, Ar), 7.17–6.98 (6 H, m, Ar, CH_2NH), 6.60 (1 H, d, *J* 6.4, CHN*H*), 5.49 (1 H, s, mFPhC*H*), 5.26 (1 H, d, *J* 3.6, 1-H), 4.96 (1 H, d, *J* 10.7, oFPhCH*H*), 4.84–4.74 (3 H, m, oFPhCH*H* and oFPhCH_2), 4.42–4.35 (1 H, m, CH_2CH(NH)CO), 4.32 (1 H, d, *J* 3.0, 4-H), 4.25 (1 H, dd, *J* 12.6 and 1.0, 6-H), 4.12 (1 H, dd, *J* 10.0 and 3.6, 2-H), 4.03 (1 H, dd, *J* 12.6 and 1.4, 6-H), 3.96–3.89 (2 H, m, 3-H, OCH*H*CH(NH)CO), 3.71 (1 H, br s, 5-H), 3.45 (1 H, t, *J* 10.6, OCH*H*CH(NH)CO), 3.16–3.05 (1 H, m, CH*H*NH), 2.70–2.60 (1 H, m, CH*H*NH), 2.30 (2 H, t, *J* 7.3, CH_2CO), 2.18 (2 H, t, *J* 7.6, CH_2CO), 0.89 (3 H, t, *J* 7.0, CH_3); δ_C(100 MHz; $CDCl_3$; $CDCl_3$) 98.5, 77.0, 75.8, 74.0, 69.5, 67.5, 63.2, 51.2, 39.3, 36.6, 34.0, 31.6, 29.0, 26.3, 25.4, 24.4, 22.6, 14.1; δ_F(376 MHz; $CDCl_3$; $CFCl_3$) −119.6, −118.4, −113.8; *m/z* (FAB) 823.3389 (M + Na^+. $C_{42}H_{51}F_3N_2NaO_{10}$ requires 823.3393); β-anomer (8.8 mg, 4% based on the loading of Argogel®-NH_2) δ_H(400 MHz; $CDCl_3$; $CHCl_3$) 7.52–7.21 (7 H, m, Ar), 7.14–6.93 (6 H, m, Ar, CH_2NH), 6.66 (1 H, d, *J* 6.8, CHN*H*), 5.53 (1 H, s, mFPhCH), 4.92–4.74 (5 H, m, oFPhCH_2, CH_2CH(NH)CO), 4.61 (1 H, d, *J* 7.6, 1-H), 4.33 (1 H, d, *J* 12.0, 6-H), 4.25 (1 H, d, *J* 3.2, 4-H), 4.08 (1 H, d, *J* 12.0, 6-H), 3.97 (1 H, dd, *J* 11.4 and 5.5, OCH*H*CH(NH)CO), 3.82–3.67 (2 H, m, 2-H, OCH*H*CH(NH)CO), 3.61 (1 H, dd, *J* 9.7 and 3.4, 3-H), 3.55 (1 H, br s, 5-H), 3.23–3.12 (1 H, m, CH*H*NH), 3.03–2.92 (1 H, m, CH*H*NH), 2.26–2.12 (4 H, m, CH_2CO), 0.88 (3 H, t, *J* 7.1, CH_3); δ_F(376 MHz; $CDCl_3$; $CFCl_3$) −119.5, −119.2, −113.5.

N^{α}-Hexanoyl-3-*O*-(β-D-galactopyranosyl)-L-seryl-6-amino-hexanoic acid (2)

The protected glycolipid **16** (15 mg, 15 µmol) was dissolved in MeOH (14.3 mL) and NaOMe (0.20 M, 0.75 mL, 0.15 mmol) was added dropwise under constant stirring. After 55 min acetic acid was added and the solution was concentrated. The residue was purified on a C-8 column and filtered through a pasteur pipette with silica-gel to give **2** (6 mg, 80%). δ_H(400 MHz; CD_3OD; CD_2HOD) 4.56 (1 H, t, *J* 5.2, CH_2CH(NH)CO), 4.25 (1 H, d, *J* 7.6, 1-H), 4.12 (1 H, dd, *J* 10.3 and 5.2, OCH*H*CH(NH)CO), 3.84 (1 H, d, *J* 2.9, 4-H), 3.82–3.68 (3 H, m, 6-H and OCH*H*CH(NH)CO), 3.59–3.44 (3 H, m, 2-H, 3-H and 5-H), 3.20 (2 H, t, *J* 6.5, CH_2CO), 2.26 (2 H, t, *J* 7.4, CH_2CO), 0.91 (3 H, t, *J* 6.8, CH_3); δ_C(100 MHz; CD_3OD; CD_3OD) 181.35, 176.50, 172.20, 105.43, 76.89, 75.08, 72.53, 70.76, 70.59, 62.76, 55.07, 40.64, 37.01, 32.76, 30.20, 28.00, 27.18, 26.62, 24.24, 23.62, 14.47; *m/z* (FAB) 523.2247 (M − H^+ + 2 Na^+. $C_{21}H_{37}N_2Na_2O_{10}$ requires 523.2238).

N^{α}-Palmitoyl-3-*O*-(β-D-galactopyranosyl)-L-seryl-12-amino-dodecanoic acid (19)

The protected glycolipid **17** (14 mg, 12 µmol) was dissolved in MeOH (14 mL) and NaOMe (0.20 M, 0.75 mL, 0.15 mmol) was added dropwise under constant stirring. After 1 h acetic acid and ice were added and the solution was concentrated. Flash column chromatography (CH_2Cl_2–MeOH 10 : 1) and purification through a C-8 column (MeOH–H_2O 1 : 9–3 : 7–1 : 1) gave **19** (7.5 mg, 82%). δ_H(400 MHz; [d6]DMSO; [d5]DMSO) 4.39–4.32 (1 H, m, CH_2CH(NH)CO), 4.02 (1 H, d, *J* 7.1, 1-H), 3.95 (1 H, dd, *J* 9.6 and 4.4, OCH*H*CH(NH)CO), 3.64–3.18 (6 H, m, OCH*H*CH(NH)CO, 2-H, 3-H, 5-H, and 6-H), 0.83 (3 H, t, *J* 6.7, CH_3); δ_C(100 MHz; [d6]DMSO; [d6]DMSO) 176.57, 172.33, 169.66, 104.43, 79.17, 75.36, 73.16, 70.44, 69.72, 67.93, 60.36, 52.92, 35.09, 31.28, 26.10, 25.72, 25.19, 22.08, 13.93; *m/z* (FAB) 747.4752 (M − H^+ + 2 Na^+. $C_{37}H_{69}N_2Na_2O_{10}$ requires 747.4742).

N^{α}-Hexanoyl-3-*O*-(α-D-galactopyranosyl)-L-seryl-6-amino-hexanoic acid (20)

The protected glycolipid **18** (19 mg, 23 µmol) and Pd/C (10%, 21 mg) was dissolved/suspended in AcOH (7.5 mL) and the mixture was stirred under hydrogen (1 atm) at room temperature for 22 h. The mixture was filtered through Celite, which was rinsed with MeOH. The solvents were evaporated and the crude residue was concentrated *in vacuo*, loaded onto a C-18 column in water, eluted with water, and coevaporated with MeOH and hexane. Preparative reversed phase LC-MS of the residue gave the deprotected glycolipid **20** (10.5 mg, 94%). δ_H(400 MHz; [d5]pyridine; [d4]pyridine) 8.90–8.82 (1 H, m, CH_2NH), 8.70 (1 H, d, *J* 7.8, CHN*H*), 5.55 (1 H, d, *J* 3.5, 1-H), 5.38–5.29 (1 H, m, CH_2CH(NH)CO), 4.69 (1 H, dd, *J* 9.8 and 3.5, 2-H), 4.65–4.60 (1 H, m, 4-H), 4.57–4.31 (5 H, m, 3-H, 5-H, 6-H, OCH*H*CH(NH)CO), 4.29–4.20 (1 H, m, OCH*H*CH(NH)CO), 3.53–3.37 (2 H, m, CH_2NH), 2.43 (4 H, q, *J* 7.6, CH_2CO), 0.77 (3 H, t, *J* 7.1, CH_3); δ_C(100 MHz; [d5]pyridine; [d5]pyridine) 173.2, 170.8, 101.3, 73.1, 71.4, 70.9, 70.1, 69.5, 62.6, 53.2, 39.7, 36.3, 31.6, 29.6, 26.9, 25.7, 25.3, 22.6, 14.0; *m/z* (FAB) 501.2421 (M^+ + Na^+. $C_{21}H_{38}N_2NaO_{10}$ requires 501.2424).

Formation of glycolipid microtiter plates

Stock solutions (64 or 12 mM for BS-1 and RCA_{120}, respectively) of the glycolipids **2** and **20** in water were prepared and aliquots (50 or 25 µL for BS-1 and RCA_{120}, respectively) were serially diluted in CovaLink™ (Nunc A/S, Denmark) microtiter plate wells, resulting in 50 or 25 µL aqueous glycolipid solutions in each well. An equal volume of coupling reagents (*N*-hydroxysuccinimide (13 mM) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (37 mM) in water) was added to each well. Blank control wells, where neither glycolipids nor coupling reagents were added, were run in parallel. The plates were shaken at room temperature for 6 h, emptied, washed with water (100 µL; BS-1) or with water (3 × 150 µL), sat. $NaHCO_3$ (2 × 150 µL), SDS (1% in water; 3 × 150 µL) and water again (4 × 150 µL; RCA_{120}), and blocked by incubation overnight with bovine serum albumin (BSA, 1% w/v in phosphate-buffered saline (PBS), 200 µL) at 4 °C. The plates were washed with PBS containing BSA (1% w/v) and Triton X-100 (0.05%) (2 × 100 µL). Horseradish peroxidase conjugated lectin from *Bandeiraea simplicifolia* (BS-1, Sigma-Aldrich, 15 µg mL^{-1} in PBS containing 0.05% Tween 20, 100 µL) and biotin-conjugated lectin from *Ricinus communis* (RCA_{120}, Vector Laboratories, 5 µg mL^{-1} in PBS containing 0.05% Tween 20, 100 µL) were added to the wells. The plates were shaken for 70 min at room temperature and the wells were emptied. The plate with biotin-conjugated lectin was washed with Tween 20 (0.05% in PBS; 2 × 100 µL) and horseradish peroxidase-conjugated avidin (HRP-AV, Vector Laboratories, 4 µg mL^{-1}; 0.05% Tween 20 in PBS; 100 µL) was added. The plate was shaken at room temperature for 35 min and both plates were washed with Covabuffer (PBS containing NaCl (117 g L^{-1}), $MgSO_4$ (4.9 g L^{-1}) and Tween 20 (0.05%); 4 × 100 µL) and phosphate–citrate buffer (0.1 M, pH 5; 2 × 100 µL). Substrate solution (6 mg *O*-phenylenediamine and 5 µL H_2O_2 (30% aq) in phosphate–citrate buffer (0.1 M, pH 5, 10 mL); 100 µL) was added. The plate with BS-1 was incubated at room temperature for 15 min, the reaction was quenched with H_2SO_4 (1 M, aq) and the absorbance at 492 nm was measured. The plate with RCA_{120} was incubated at room temperature for 30 min and the absorbance at 450 nm was measured.

Regeneration of glycolipid microtiter plate

After the absorbance measurements, the wells were washed with water (3 × 150 µL), $NaHCO_3$ (sat. aq; 2 × 150 µL), SDS (1% in water; 3 × 150 µL) and water again (4 × 150 µL). SDS (10% in

water; 150 μL) was added to the wells and the plate was shaken at room temperature for 15 min, the wells were washed with water (3 × 150 μL), SDS (10% in water; 150 μL) was added and the plate was again shaken at room temperature for 15 min. The wells were washed with water (3 × 150 μL) and HRP-AV (4 μg mL^{-1}; 0.05% Tween 20 in PBS; 100 μL) was added. The plate was shaken at room temperature for 35 min and substrate solution was added and the absorbance measured as before. The wells were washed with water (3 × 150 μL), $NaHCO_3$ (sat. aq; 2 × 150 μL), SDS (1% in water; 3 × 150 μL) and water again (4 × 150 μL). SDS (10% in water; 150 μL) was added to the wells and the plate was shaken at room temperature for 15 min, the wells were washed with water (3 × 150 μL), more SDS (10% in water; 150 μL) was added and the plate was again shaken at room temperature for 15 min. The wells were washed with water (3 × 150 μL), blocked with BSA and RCA_{120}, HRP-AV was added and the absorbance was measured as before.

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