DOI: 10.1002/ejoc.200800670

Carbamate Linker Strategy in Solid-Phase Synthesis of Amino-Functionalized Glycoconjugates for Attachment to Solid Surfaces and Investigation of Protein-Carbohydrate Interactions

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Keywords: Solid-phase synthesis / Glycoconjugates / Carbohydrates / Proteins / Carbohydrate protein interactions / Carbamate linker / Gel-phase ^{19}F NMR spectroscopy / Microtiter plates

Amino-functionalized serine-based galactose and glucose neoglycolipids were prepared by solid-phase synthesis using a carbamate strategy for anchoring amino functionalities to a (2-fluoro-4-hydroxymethylphenoxy)acetic acid linker resin. Key synthetic steps were monitored with gel-phase ^{19}F NMR spectroscopy. Cleavage from the solid support was performed with trifluoroacetic acid. The terminal amine of the neoglycolipids was conjugated with didecyl squarate and then immobilized in amino-functionalized microtiter plates and the glycoconjugates were successfully probed with a galactose-binding lectin.

Introduction

Carbohydrates are the most abundant of the four major classes of biomolecules, which also include proteins, lipids, and nucleic acids. While proteins and nucleic acids are generally linear polymers, carbohydrates can be highly branched and have complex structures, a tendency that results in almost unlimited structural variation. In cells, carbohydrates are found as glycoconjugates where the carbohydrate is attached to other macromolecules forming e.g. glycoproteins and glycolipids. Glycoconjugates are involved in a wide variety of biological processes, for example viral entry, signal transduction, inflammation, cell–cell interactions, bacteria–host interactions, fertilization, and development.[1] For that reason it is of great interest to further investigate the role of glycoconjugates in biological systems.

Recently, carbohydrate microarray techniques have been developed to study carbohydrate–protein interactions.[2–8] In addition microarray techniques are applicable also to study small molecules in general.[9] These techniques have many advantages including high-throughput, minimal consumption of analyte and immobilized molecules, and direct characterization of interactions. For glycoconjugates the presentation on the array surface mimics the multivalent presentation on cell surfaces. The number and type of glycoconjugates, the array surface material, and the method used to attach the glycoconjugate to the surface can vary. Recent studies have examined several attachment methods on different types of surfaces.[2–6] A method where two different amines cross-react with dialkyl squarate under mildly basic conditions is very efficient and can be used for both primary and secondary amines.[10–13]

Because oligosaccharides and glycoconjugates are complicated to synthesize and the isolation of pure and well-defined material from natural sources is demanding, efficient methods for the preparation of these compounds are needed. Glycosphingolipids, like β-galactosylceramide **1**, are abundant in nature and involved in numerous biological recognition events.[14] In cell membranes the hydrophobic part of the glycolipid is buried in the lipid layer and the hydrophilic part, the carbohydrate portion, is exposed to the surroundings for recognition. Because the lipid part of **1** contains two stereocenters and one defined double-bond simplified analogs have been synthesized.[15] The serine-based neoglycolipid **2** is such an analog.[16,17] The synthesis of compound **2**, subsequent conjugation to amino-functionalized plates and evaluation with lectins have been described.[16,17] In one report the synthesis was carried out on solid phase using a linker and protective groups containing fluorine that enable gel-phase ^{19}F NMR spectroscopy to measure yield and stereochemical outcome during synthesis.[16] The carboxylic acid in compound **2** was coupled to amino-functionalized microtiter plates and the resulting neoglycolipid surface was subsequently probed with biotin-

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labeled RCA_{120} from *Ricinus communis* with affinity for α- and β-Gal, followed by horseradish peroxidase conjugated avidin (avidin-HRP) for detection.

In this paper we describe the solid-phase synthesis of the amino-functionalized biotin model compounds **8a** and **8b** and serine-based neoglycolipids **3** and **17** (Figure 1 and Schemes 2 and 3) using a carbamate-linker strategy and gel-phase ^{19}F NMR spectroscopy as monitoring technique. The amino-functionalized conjugates were immobilized in amino-functionalized microtiter plates using squaric acid chemistry to give small molecule surfaces that were probed with labeled avidin and a galactose-binding lectin.

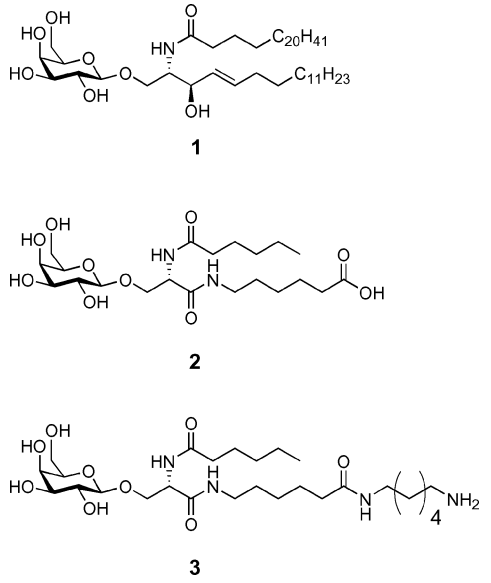


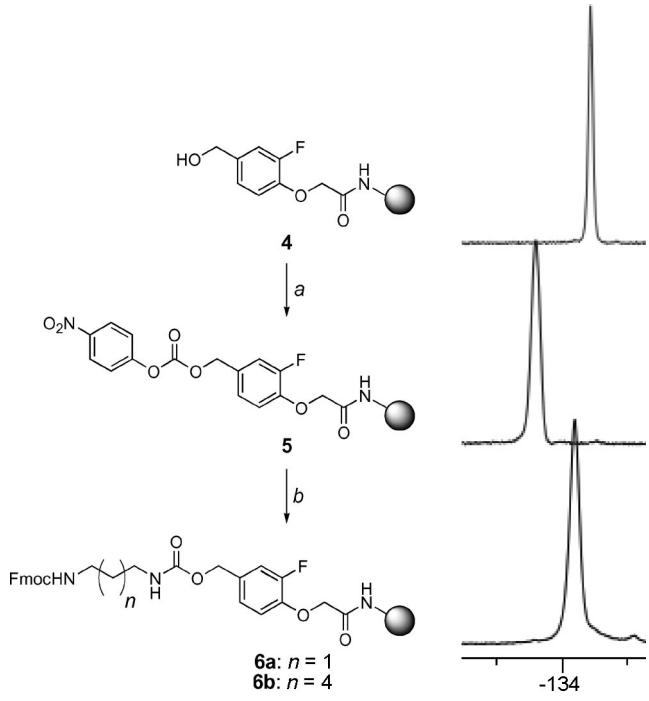
Figure 1. β-Galactosylceramide **1** and the serine-based analogs **2** and **3**.

Results and Discussion

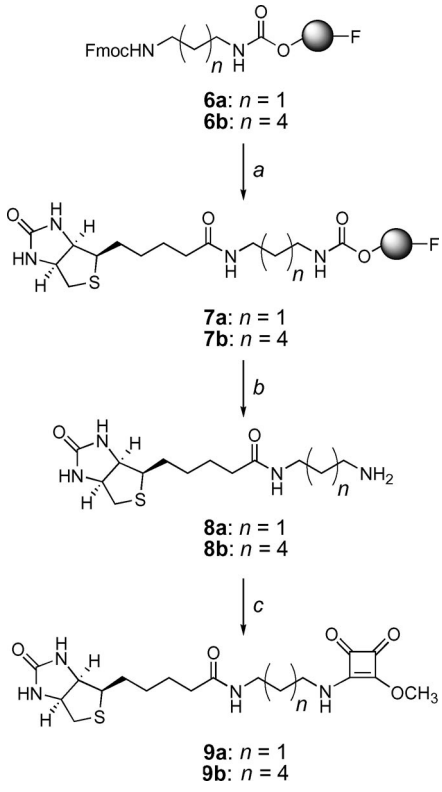
In previous studies compound **2** (Figure 1) was covalently linked to secondary amines in CovaLink™ microtiter plates through the addition of *N*-hydroxysuccinimide (NHS) and 1-[3-(diethylamino)propyl]-3-ethylcarbodiimide (EDC) in water.[16,17] This immobilization procedure required relatively large amounts of the valuable neoglycolipid, mM concentrations in the coupling buffer, and thus a more efficient strategy would be beneficial. One strategy is based on the fact that two different amines efficiently crosslink through dialkyl squarate under mildly basic conditions.[10–13] On the basis of this we designed the amino functionalized neoglycolipid **3** (Figure 1). In order to enable solid-phase synthesis a linker suitable for attachment of amines was needed and we decided to use a carbamate-linker strategy[18,19] based on the previously described linker [2-fluoro-4-(hydroxymethyl)phenoxy]acetic acid.[16] The carbamate linkage is expected to withstand conditions throughout the glycoconjugate synthesis and subsequent cleavage can be performed with acid of moderate strength, e.g. trifluoroacetic acid (TFA).[18,19] The fluorinated linker enables monitoring of key reactions with gel-phase ^{19}F NMR spectroscopy.[20]

Synthesis of Model Compounds

To evaluate the carbamate-linker strategy we synthesized the biotinylated model compounds **8a** and **8b** (Scheme 2) that also can be used to optimize array conditions using labeled avidin. Polystyrene resins grafted with polyethyleneglycol e.g. Tentagel™ are excellent for solid-phase glycoconjugate synthesis and gel-phase ^{19}F NMR spectroscopy.[20–23] Resin **4**[16] was converted into the carbonate **5** through base-catalyzed reaction with *p*-nitrophenyl chloroformate (Scheme 1).[18,19] Under basic conditions *N*-Fmoc-1,3-diaminopropane and *N*-Fmoc-1,6-diaminohexane reacted with the carbonate yielding the carbamate resin **6a** and **6b**. The reactions were monitored with gel-phase ^{19}F NMR spectroscopy and all conversions were complete as illustrated for **6b** (Scheme 1). Fmoc-Deprotection was performed using piperidine (20% in DMF) and biotin was coupled to the amine with *N*,*N*′-diisopropylcarbodiimide (DIC) and 1-hydroxy-7-azabenzotriazole (HOAt) to give resin **7a** and **7b** (Scheme 2). Cleavage from the resin with trifluoroacetic acid (TFA 90% in water) at room temperature yielded **8a** in quantitative yield and **8b** in 70% yield based on the initial loading of TentaGel-NH_2. Functionalization of **8a** and **8b** was performed with dimethyl squarate in DMF and triethylamine giving **9a** and **9b** in quantitative yields.



Scheme 1. Synthesis of the carbamate resin **6a** and **6b** and gel-phase ^{19}F NMR spectra recorded for resin **4**, **5**, and **6b**. a) *N*-methyl morpholine, *p*-nitrophenyl chloroformate, CH_2Cl_2; b) *N*-Fmoc-1,3-diaminopropane hydrochloride or *N*-Fmoc-1,6-diaminohexane hydrochloride, *N*,*N*-diisopropylethylamine, DMF.



Scheme 2. Synthesis of model compounds **9a** and **9b**. a) i) Piperidine (20% in DMF); ii) HOAt, DIC, D-biotin, DMF; b) 3 × TFA (90% in H_2O), quantitative yield and 70% yield, respectively; c) dimethyl squarate, TEA, DMF, quantitative yield for both **9a** and **9b**.

Immobilization and Detection of Compounds 9a and 9b in Amino-Functionalized Microtiter Plates

Compounds **9a** and **9b** were dissolved in sodium hydrocarbonate buffer (pH 9) and serially diluted (1.0 mM to 0.24 nM for **9a** and 1.1 mM to 2.2 nM for **9b**) in transparent amino-functionalized microtiter plates and incubated for 18 h at room temperature. The immobilized model compounds **9a** and **9b** were detected with avidin-HRP (4 μg/mL in PBS containing 0.05% Tween 20, 100 μL) and subsequent addition of substrate solution. Optimization resulted in conditions producing good signal-to-noise ratio allowing efficient detection of the immobilized biotin (Figure 2, a and b). Neither blocking with bovine serum albumin (BSA)[16] nor blocking with acetic anhydride (20% in water)[13] improved the results for the biotin avidin-HRP combination. In all three cases unspecific binding of biotin avidin-HRP was low. A second improvement was to replace a washing step with Cova buffer by water. These results indicate that the carbamate linker and squarate array strategy are promising for more complex compounds.

To further explore the potential of the strategy the model compounds **9a** and **9b** (2.5 mM to 0.61 μM) were also immobilized in white amino-functionalized microtiter plates and subsequently probed with fluorescein isothiocyanate labeled avidin (avidin-FITC) (Figure 2, c and d). During optimization of this protocol it was found that blocking with BSA gave less unspecific binding of avidin-FITC compared to no blocking or blocking with acetic anhydride.

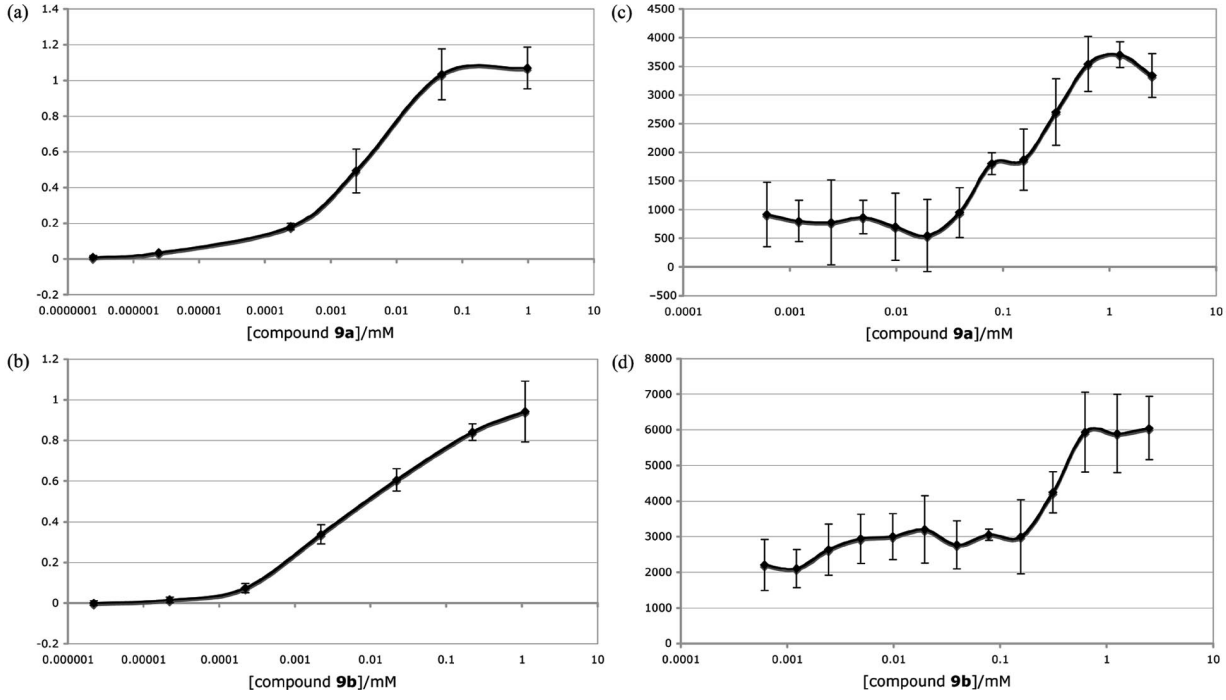
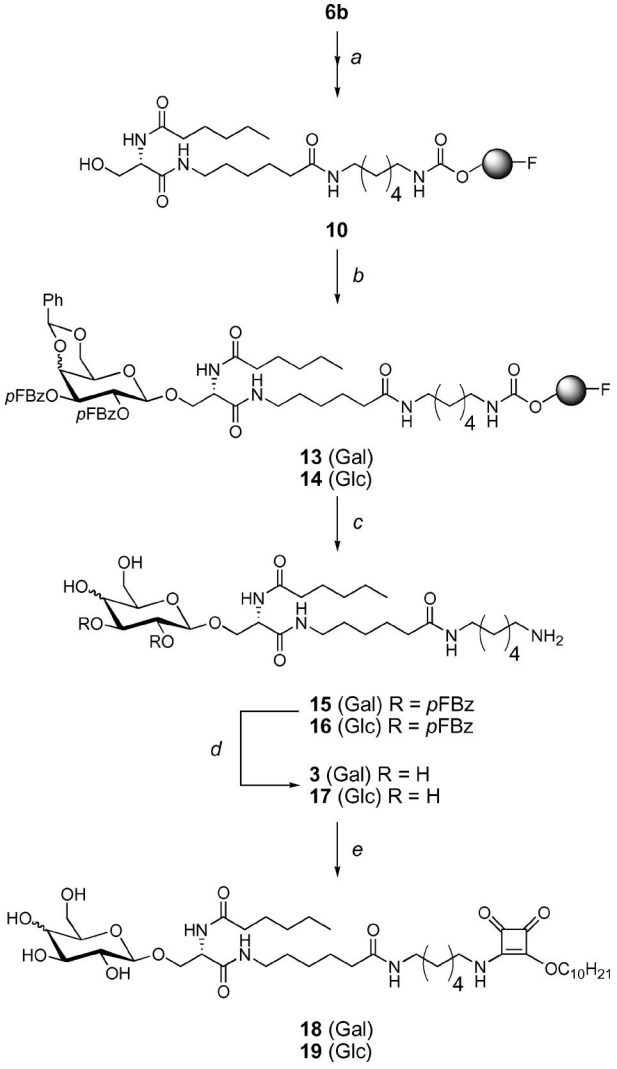


Figure 2. a) and b) Binding of avidin-HRP to the immobilized model compounds **9a** and **9b**. c) and d) binding of avidin-FITC to the immobilized model compounds **9a** and **9b**. The compounds were serially diluted and covalently immobilized in wells of clear (a and b) or white (c and d) aminofunctionalized microtiter plates and probed with the given avidin conjugate. The concentrations of the model compounds added to the well are given on the *x*-axis. The points represent the average of triplicate runs and error bars are set to ± one standard deviation. In the graphs, the average value of a triplicate of controls was subtracted from the observed values (triplicates).

The data clearly show the efficiency of the immobilization chemistry and that detection can be based on both absorbance and fluorescence. Detection with avidin-HRP was superior compared to avidin-FITC both in terms of background level and signal to noise. Binding of avidin to the two model compounds appeared to be unaffected by the two spacer lengths used (see Figure 2, compare part a with b and c with d).

Synthesis of Neoglycolipids

For preparation of neoglycolipids the longer spacer, i.e. resin **6b**, was chosen as starting material. The serine-based lipid part **10** was assembled from **6b** under standard peptide synthesis conditions (Scheme 3).[16] The galactose and glucose donors **11** and **12** (Figure 3) were synthesized according to previously described methods.[20] The fluorinated benzoyl groups and the fluorinated linker make it possible to monitor the outcome of the glycosylations with gel-phase ^{19}F NMR spectroscopy. *N*-Bromosuccinimide (NBS) and tetrabutylammonium trifluoromethanesulfonate (QOTf) were used as the promoter system.[24] Glycosylation of **10** with **11** (3 equiv.), QOTf (0.65 equiv.), and NBS (4 equiv.) resulted in 50% glycosylation according to gel-phase ^{19}F NMR spectroscopy. When the procedure was repeated the yield increased to 65% yield. Glycosylation of **10** with **12** under similar conditions gave 35% glycosylation and when the procedure was repeated, 57% yield according to gel-phase ^{19}F NMR spectroscopy. Although the ^{19}F NMR spectra allowed estimation of the glycosylation yields the spectra were of relatively poor quality. The reason for the strong broadening of the fluorine signals is not known. Compound **13** and **14** were cleaved from the resin by addition of TFA/H_2O, 9:1 giving the partially deprotected neoglycolipids **15** and **16** in 24% and 12% yield, respectively, based on the initial loading of TentaGel-NH_2 resin. The *p*-fluorobenzoate protecting groups were removed using NaOMe in methanol to give the deprotected neoglycolipids **3** and **17**. Both products were problematic to purify with flash-column chromatography. Preparative reversed-phase LC-MS gave partially pure **3** and it was therefore decided to carry out purification after functionalization with didecyl squarate. Compound **3** and **17** were treated with didecyl squarate (ca. 3.3 equiv.)[13] to give **18** and **19** in 20% and 27%, respectively after purification with reversed-phase HPLC. Didecyl squarate gives a more lipophilic product than dimethyl squarate, which was considered important from a purification point of view. The total yield based on the initial loading of the resin was 4.8% for **18** and 2.4% for **19** over 13 steps corresponding to an average yield of 75–80% in each step.



Scheme 3. Synthesis of squaric amide ester neoglycolipid **18** and **19**. [a]a) i) Piperidine (20% in DMF); ii) Fmoc-6-aminohexanoic acid, HOBt, DIC, DMF, BFB; iii) Piperidine (20% in DMF); iv) Fmoc-L-serine HOBt, DIC, DMF, BFB; v) piperidine (20% in DMF); vi) hexanoic acid, HOBt, DIC, DMF, BFB; b) **11** or **12**, QOTf, NBS, MS 3 Å, CH_2Cl_2, 65% and 57% respectively; c) TFA/H_2O, 9:1, 24% and 12% yield respectively; d) NaOMe (0.20 M in MeOH), MeOH; e) didecyl squarate, TEA, DMF, 20% and 27% yield respectively.

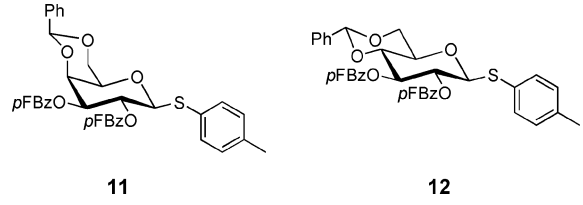
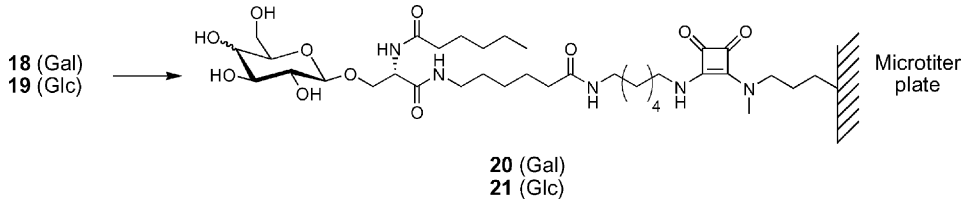


Figure 3. Glycosyl donors.

Immobilization and Detection of the Neoglycolipids in Amino-Functionalized Microtiter Plates

The neoglycolipid **18** was immobilized in transparent CovaLink™ microtiter plates as described for the biotin derivatives **9a** and **9b**. Compound **18** was dissolved in sodium hydrocarbonate buffer (pH 9) and serially diluted (0.1 mM–48 nM) on the microtiter plates and incubated for 24 h at room temperature. The immobilized neoglycolipid was detected with biotin-conjugated lectin from *Ricinus communis* (RCA_{120}, 5 μg/mL in PBS containing 0.05% Tween 20,



Scheme 4. Immobilization of squaric amide ester neoglycolipid **18** and **19** in amino-functionalized microtiter plates. a) $NaHCO_3$ buffer (pH 9), CovaLink™ (Nunc A/S, Denmark) microtiter plate.

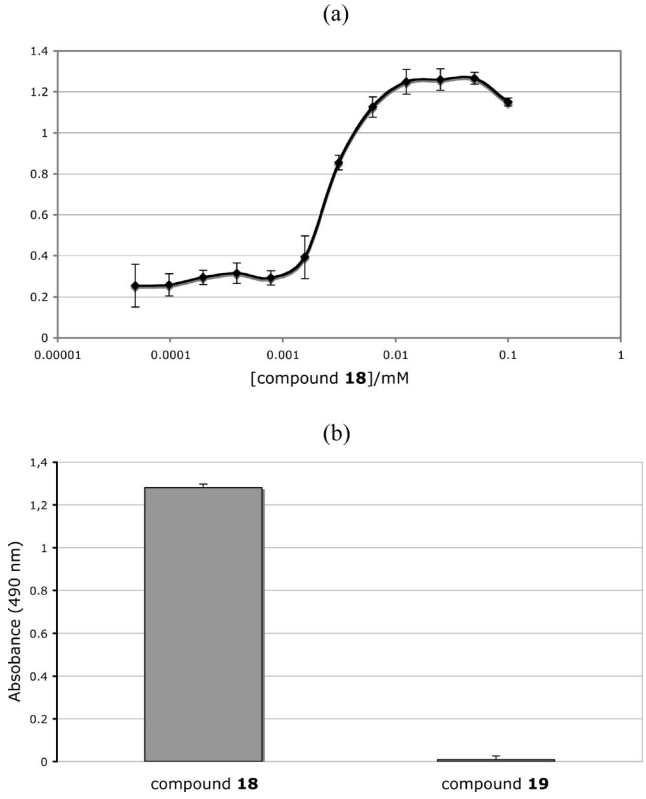


Figure 4. a) Binding of galactose selective lectin from *Ricinus communis* (RCA_{120}) to the immobilized neoglycolipid **18**. The compound was serially diluted in the wells and covalently immobilized to the amino-functionalized wells. The immobilized neoglycolipid was detected with biotin-conjugated RCA_{120} and avidin-HRP. The concentration of the neoglycolipid added to the well is given on the *x* axis. The points represent the average of triplicate runs and error bars are set to ± one standard deviation. In the graphs, the average value of a triplicate of controls was subtracted from the observed absorbencies (triplicates). b) Binding of the galactose selective lectin RCA_{120} to the immobilized neoglycolipids **18** and **19** (0.025 mM). The average value of a triplicate of controls was subtracted from the observed absorbencies (triplicates). No binding of the lectin was observed in the wells reacted with the glucose neoglycolipid **19**.

100 μL) and avidin-HRP (4 μg/mL in PBS containing 0.05% Tween 20, 100 μL). A clear dose-response is observed and indicating that the immobilization method of the neoglycolipid was effective (Figure 4, a). As low as 0.01 mM concentration of glycoconjugate **18** in the immobilization buffer resulted in maximum absorbance in contrast to the ten-fold higher concentration required when **2** was immobilized as an amide using the same lectin and type of plate (Scheme 4).[16]

The lectin RCA_{120} is specific for α- and β-galactose and will therefore not recognize glucose. To investigate whether the lectin can distinguish galactose from glucose in this setup, **18** and **19** were dissolved and diluted to 0.025 mM in $NaHCO_3$ buffer (pH 9). This concentration gives a strong signal as shown in part a of Figure 4. The compounds were immobilized and detected as described for **18**. No binding of the lectin was observed in the wells reacted with the glucose neoglycolipid **19** (Figure 4, b).

Conclusions

The carbamate-linker strategy proved to be sufficiently effective to allow solid-phase synthesis of amino-functionalized biotin model compounds and galactose and glucose neoglycolipids. With didecyl squarate the molecules were cross-linked to microtiter plate wells functionalized with secondary amines. The biotin plates were successfully probed with avidin labeled with both HRP and FITC. The glycoconjugate plates were successfully probed with a galactose specific lectin labeled with HRP. Our results indicate that this strategy can be applied for solid-phase synthesis of amino-functionalized small molecules for attachment to solid surfaces and subsequent detection with proteins carrying different labels.

Experimental Section

General: Solid-phase synthesis was performed on TentaGel HL-NH_2 resin (0.42 mmol/g) from Rapp Polymere. CH_2Cl_2 was distilled from calcium hydride and DMF was distilled under vacuum. Solvent mixtures are reported as volume (v/v) ratios. TLC was run on Silica Gel 60 F_{254} (Merck) and the spots were detected in UV-light and stained with H_2SO_4 in ethanol and heat. Silica gel (Matrex, 60 Å, 35–70 mm, Grace Amicon) and solvents of analytical grade were used for flash column chromatography. ^1H and ^{13}C NMR spectra were recorded at 298 K with a Bruker DRX-400 at 400 and 100 MHz, respectively, with $CDCl_3$ or [D_4]MeOH as solvent and residual $CHCl_3$ (δ_H = 7.27 ppm) or [D_4]MeOH (δ_H = 3.30 ppm) as internal standard for ^1H and $CDCl_3$ (δ_C = 77.23 ppm) or [D_4]MeOH (δ_C = 49.15 ppm) as internal standard for ^{13}C. Peaks that could not be assigned are not reported. *J* values are given in Hz. Gel-phase proton decoupled ^{19}F NMR spectra were recorded at 298 K with a Bruker DRX-400 at 376 MHz on resin suspensions

in $CDCl_3$ with $CFCl_3$ (δ_F = 0.00 ppm) as internal standard. Two peaks appear in the spectra around 0 ppm. One is originating from $CFCl_3$ inside the polymer and one from $CFCl_3$ outside the polymer. The peak with higher shift was used as internal standard. Preparative reversed-phase LCMS were performed with a Waters LC system equipped with an XTerra C-18 column (50 × 19 mm, 5 μm, 125 Å), eluted with a linear gradient of MeCN in water, both of which contained formic acid (0.2%) with a flow rate of 25 mL min^{-1} and detection at 214 and 254 nm. Positive and negative electrospray mass analyses were carried out with a Waters Micromass ZG 2000. Preparative HPLC separations were performed with a Beckman System Gold HPLC, using a Supelco Discovery Biowide Pore C18 column (250 × 212 mm, 5 μm) eluted with a linear gradient of MeCN in water, both of which contained trifluoroacetic acid (0.1%). The flow rate was 11 mL min^{-1} and detection at 214 nm. Analytical HPLC were performed with a Beckman System Gold HPLC, using a Supelco Discovery Biowide Pore C18 column (250 × 46 mm, 5 μm) with a flow rate of 1.5 mL min^{-1} and detection at 214 nm. In arrays; the absorbance was measured with a Tecan Infinite® 200 plate reader and fluorescence with a Wallac Victor^{2}™ 1420 Multilabel Counter. High-resolution mass spectra were recorded with a Jeol SX102 mass spectrometer. Ions were produced by a beam of Xenon atoms (6 keV) from a matrix of 3-nitrobenzyl alcohol.

Resin 5: The linker resin **4**[16] (0.424 mmol) was allowed to swell in 4 mL of dry CH_2Cl_2, *N*-methylmorpholine (0.100 mL, 0.920 mmol) was added and the mixture was cooled to 0 °C. *p*-Nitrophenyl chloroformate (0.178 g, 0.883 mmol) was dissolved in 1 mL of dry CH_2Cl_2 and added slowly under cooling. The mixture was agitated overnight at room temperature and finally the resin was washed with CH_2Cl_2, DMF and CH_2Cl_2 (5 × 5 mL) yielding **5**. According to gel-phase ^{19}F NMR the conversion was quantitative. ^{19}F NMR ($CDCl_3$): δ = –133.5 ppm.

Resin 6a: The carbonate linker resin **5** (0.350 mmol) was washed with dry DMF (2 × 5 mL) and then allowed to swell in dry DMF (5 mL). *N*-Fmoc-1,3-Diaminopropane hydrochloride (0.300 g, 1.01 mmol) and *N*,*N*-diisopropylethylamine (0.180 mL, 1.01 mmol) were added and agitated at room temperature for 16 h. The resin was washed with DMF (10 × 5 mL), agitated with DMF for 10 min, washed with DMF, THF and CH_2Cl_2 (3 × 5 mL) to give **6**. According to gel-phase ^{19}F NMR the conversion was quantitative. ^{19}F NMR ($CDCl_3$): δ = –134.2 ppm.

Resin 6b: The carbonate linker resin **5** (0.424 mmol) was washed with dry DMF (2 × 5 mL) and then allowed to swell in dry DMF (5 mL). *N*-Fmoc-1,6-Diaminohexane hydrochloride (0.394 g, 1.05 mmol) and *N*,*N*-diisopropylethylamine (0.190 mL, 1.09 mmol) were added and agitated at room temperature for 22 h. The resin was washed with DMF (10 × 5 mL), agitated with DMF for 10 min, washed with DMF, THF and CH_2Cl_2 (3 × 5 mL) to give **6**. According to gel-phase ^{19}F NMR the conversion was quantitative. ^{19}F NMR ($CDCl_3$): δ = –134.2 ppm.

Resin 7a: Resin **6a** (0.173 mmol) was treated with piperidine (20% in DMF) for 5 min and washed with DMF (3 × 5 mL) and distilled DMF (3 × 5 mL). 1-Hydroxy-7-azabenzotriazole (HOAt) (0.141 g, 1.04 mmol) and D-biotin (0.170 g, 0.696 mmol) were dissolved in distilled DMF (5 mL), *N*,*N*′-diisopropylcarbodiimide (DIC) (0.107 mL, 0.691 mmol) was added and the mixture was stirred for 15 min at room temperature. The solution was added to the resin followed by bromophenolblue (BFB) (20 μL, 2 mM in DMF). The mixture was agitated at room temperature for 16 h and washed with CH_2Cl_2, DMF, THF, MeOH, DMF and CH_2Cl_2 (10 mL of each). According to gel-phase ^{19}F NMR the conversion was quantitative. ^{19}F NMR ($CDCl_3$): δ = –134.0 ppm.

Resin 7b: Resin **6b** (0.075 mmol) was treated with piperidine (20% in DMF) for 5 min and washed with DMF (3 × 5 mL) and distilled DMF (3 × 5 mL). 1-Hydroxy-7-azabenzotriazole (HOAt) (0.065 g, 0.474 mmol) and D-biotin (0.077 g, 0.315 mmol) were dissolved in distilled DMF (5 mL), *N*,*N*′-diisopropylcarbodiimide (DIC) (0.049 mL, 0.316 mmol) was added and the mixture was stirred for 15 min at room temperature. The solution was added to the resin followed by bromophenolblue (BFB) (20 μL, 2 mM in DMF). The mixture was agitated at room temperature for 16 h and washed with CH_2Cl_2, DMF, THF, MeOH, DMF and CH_2Cl_2 (10 mL of each). According to gel-phase ^{19}F NMR the conversion was quantitative. ^{19}F NMR ($CDCl_3$): δ = –134.0 ppm.

1-Amino-3-(biotinoylamino)propane (8a): TFA (90% in H_2O, 6 mL) was added to resin **7a** (0.075 mmol) and shaken at room temperature for 2 h. This procedure was repeated twice, the resin was filtered and washed thoroughly with MeOH, CH_2Cl_2 and MeOH. The solvents were combined and concentrated in vacuo. The product was purified by preparative LC-MS (100% H_2O to 100% MeCN, 10 min) yielding **8a** (60 mg, quantitative yield based on resin loading). ^{1}H NMR ([D$_4$]MeOH): δ = 4.52–4.49 [m, 1 H, C*H*(NH)CH$_2$], 4.33–4.30 [m, 1 H, C*H*(NH)CH], 3.23–3.19 (m, 1 H, C*H*S), 3.15–3.12 (m, 2 H, C*H*$_2$NHCO), 2.95–2.91 [m, 3 H, NH$_2$CH$_2$, C*H*H(S)CH], 2.72–2.69 [m, 1 H, C*H*H(S)CH], 2.26–2.22 (m, 2 H, NHCOC*H*$_2$), 1.88–1.83 (m, 2 H, COCH$_2$CH$_2$CH$_2$C*H*$_2$CH), 1.81–1.75 (m, 2 H, NH$_2$CH$_2$C*H*$_2$CH$_2$NH), 1.73–1.54 (m, 2 H, COCH$_2$C*H*$_2$CH$_2$CH$_2$CH), 1.49–1.43 (m, 2 H, COCH$_2$CH$_2$C*H*$_2$-CH$_2$CH) ppm. MS (ES$^+$) calcd. for $C_{13}H_{25}N_4O_2S$ 301.17 *m*/*z* [M + H]$^+$, observed 301.08.

1-Amino-6-(biotinoylamino)hexane (8b): TFA (90% in H_2O, 6 mL) was added to resin **7b** (0.075 mmol) and shaken at room temperature for 2 h. This procedure was repeated twice, the resin was filtered and washed thoroughly with MeOH, CH_2Cl_2 and MeOH. The solvents were combined and concentrated in vacuo. The product was purified by flash column chromatography (four gradient steps: $CHCl_3$, $CHCl_3$/MeOH, 1:1, $CHCl_3$/MeOH/H_2O, 65:35:7, MeOH/H_2O, 1:1) yielding **8b** (18 mg, 70% yield based on resin loading). Data for **8b** in agreement with those previously published.[25]

3-[3-(Biotinoylamino)propylamino]-4-methoxy-3-cyclobuten-1,2-dione (9a): Compound **8a** (0.030 g, 0.100 mmol) was dissolved in distilled DMF (7 mL), dimethyl squarate (0.057 g, 0.400 mmol) and triethylamine (56 μL, 0.402 mmol) were added and the mixture was stirred at room temperature for 19 h and followed by TLC. The reaction mixture was lyophilized and purified by flash column chromatography (five gradient steps: heptane, heptane/$CHCl_3$, 1:1, $CHCl_3$, $CHCl_3$/MeOH, 1:1, MeOH) giving **9a** (59 mg, quantitative yield). ^{1}H NMR ([D$_4$]MeOH, mixture of two rotamers): δ = 4.57–4.50 [m, 1 H, C*H*(NH)CH$_2$], 4.38–4.36 (m, 3 H, C*H*$_3$OC), 4.33–4.28 [m, 1 H, C*H*(NH)CH], 3.71–3.66 (m, 1 H, C*H*$_2$NHC), 3.51–3.45 (m, 1 H, C*H*$_2$NHC), 3.25–3.19 [m, 3 H, C*H*$_2$(NH)CO, C*H*S], 2.97 [dd, *J* = 12.7 and 4.9 Hz, 1 H, C*H*$_2$(S)CH], 2.74 [d, *J* = 12.6 Hz, 1 H, C*H*$_2$(S)CH], 2.26 (t, *J* = 7.4 Hz, 2 H, C*H*$_2$CONH) 1.83–1.75 (m, 2 H, NHCH$_2$C*H*$_2$CH$_2$NH), 1.75–1.54 (m, 2 H, COCH$_2$C*H*$_2$CH$_2$CH$_2$CH, COCH$_2$CH$_2$CH$_2$C*H*$_2$CH), 1.49–1.42 (m, 2 H, COCH$_2$CH$_2$C*H*$_2$CH$_2$CH) ppm. ^{13}C NMR ([D$_4$]MeOH): δ = 207.2, 193.4, 191.6, 176.2, 174.5, 166.0, 63.3, 61.6, 61.2, 57.0, 43.2, 42.7, 41.0, 37.3, 37.1, 36.8, 31.5, 31.2, 29.8, 29.5, 26.8 ppm. $[a]_D^{25}$ = +30 (MeOH). HRMS (FAB) calcd. for $C_{18}H_{26}N_4NaO_5S$ 433.1522 [M + Na]$^+$, found 433.1531.

3-[6-(Biotinoylamino)hexylamino]-4-methoxy-3-cyclobuten-1,2-dione (9b): Compound **8b** (0.012 g, 0.034 mmol) was dissolved in distilled DMF (5 mL), dimethyl squarate (0.029 g, 0.205 mmol) and triethylamine (20 μL, 0.143 mmol) were added and the mixture was stirred at room temperature for 91 h and followed by TLC. The reaction mixture was lyophilized and purified by flash column chromatography ($CHCl_3$ to $CHCl_3$/MeOH/H_2O, 65:35:7) giving **9b** (22 mg, quantitative yield). ^{1}H NMR ([D$_4$]MeOH): δ = 4.51 [dd, J = 7.8 and 4.3 Hz, 1 H, C*H*(NH)CH_2], 4.38 (s, 3 H, CH_3OC), 4.35–4.31 [m, 1 H, C*H*(NH)CH], 3.62 (t, J = 6.9 Hz, 1 H, CH_2NHC), 3.42 (t, J = 6.9 Hz, 1 H, CH_2NHC), 3.26–3.16 [m, 3 H, CH_2(NH)CO, C*H*S], 2.95 [dd, J = 12.7 and 5.0 Hz, 1 H, CH_2(S)CH], 2.71 [d, J = 12.7 Hz, 1 H, CH_2(S)CH], 2.22 (t, J = 7.3 Hz, 2 H, CH_2CONH) 1.85–1.29 (m, 14 H, NHCH_2CH_2CH_2CH_2CH_2CH_2, COCH_2CH_2CH_2CH_2CH) ppm. ^{13}C NMR ([D$_4$]MeOH): δ = 206.5, 196.9, 193.5, 191.6, 175.9, 166.0, 63.3, 61.6, 61.1, 59.8, 57.0, 45.5, 45.2, 41.0, 40.1, 36.8, 29.7, 29.5, 26.9, 24.2 ppm. $[\alpha]_D^{25}$ = +27 (MeOH). HRMS (FAB) calcd. for $C_{21}H_{32}N_4NaO_5S$ 475.1991 [M + Na]$^+$, found 475.1996.

Resin 10: Resin **6** (0.424 mmol) was treated with piperidine (20% in DMF, 2 × 5 mL, 10 min) and washed with DMF (3 × 5 mL) and distilled DMF (2 × 5 mL). Fmoc-6-aminohexanoic acid (0.597 g, 1.69 mmol), HOBt (0.346 g, 2.56 mmol) and DIC (0.264 mL, 1.69 mmol) were dissolved in distilled DMF (5 mL) and stirred at room temperature for 10 min. The solution was transferred to the resin followed by addition of BFB (20 μL, 2 mM in DMF). The suspension was agitated at room temperature for 4 h until the resin turned yellow. The resin was washed with DMF and CH_2Cl_2 (5 × 5 mL). The resin (0.424 mmol) was treated with 20% piperidine in DMF (5 mL) for 2 × 10 min. Washed with DMF (3 × 5 mL) and distilled DMF (2 × 5 mL). Fmoc-L-serine (0.566 g, 1.729 mmol), HOBt (0.342 g, 2.53 mmol) and DIC (0.264 mL, 1.69 mmol) were dissolved in distilled DMF and stirred at room temperature for 10 min. The solution was transferred to the resin followed by addition of BFB (20 μL, 2 mM in DMF). The suspension was agitated at room temperature overnight until the resin turned yellow. The resin was washed with DMF and CH_2Cl_2 (5 × 5 mL). The resin (0.424 mmol) was treated with 20% piperidine in DMF (5 mL) for 2 × 10 min. Washed with DMF (3 × 5 mL) and distilled DMF (2 × 5 mL). Hexanoic acid (0.212 mL, 1.692 mmol), HOBt (0.349 g, 2.583 mmol) and DIC (0.264 mL, 1.696 mmol) were dissolved in distilled DMF and stirred at room temperature for 10 min. The solution was transferred to the resin followed by addition of BFB (20 μL, 2 mM in DMF). The suspension was agitated at room temperature overnight until the resin turned yellow. The resin was washed with DMF and CH_2Cl_2 (5 × 5 mL) to give resin **10**. ^{19}F NMR ($CDCl_3$): δ = –134.1 ppm.

4-Methylphenyl 2,3-Di-*O*-(4-fluorobenzoyl)-4,6-*O*-benzylidene-1-thio-β-D-galactopyranoside (11) and 4-Methylphenyl 2,3-Di-*O*-(4-fluorobenzoyl)-4,6-*O*-benzylidene-1-thio-β-D-glucopyranoside (12): 4-Fluorobenzoyl chloride (2.5 equiv.) was added dropwise to a solution of 4,6-*O*-benzylidene-1-thio-β-D-galactopyranoside (3.53 g, 9.42 mmol) or 4,6-*O*-benzylidene-1-thio-β-D-glucopyranoside[26] (0.700 g, 1.87 mmol) in pyridine (40 mL or 12 mL). The solution was stirred for 20 or 3 h, respectively, at room temperature and then diluted with CH_2Cl_2, washed with saturated aqueous $NaHCO_3$ (3×) and H_2O. The organic phase was dried with Na_2SO_4, and concentrated. Residual pyridine was removed by co-evaporation with toluene. The residue was purified by flash column chromatography with gradient elution of heptane/ethyl acetate (5:1→4:1 for **11** and 8:1→4:1 for **12**) giving **11** and **12** as a colorless solid (6.00 g and 1.13 g, respectively) in quantitative yields. **11**: ^{1}H NMR ($CDCl_3$): δ = 8.02–7.90 (m, 4 H, ArH), 7.50 (d, J = 8.1 Hz, 2 H, ArH), 7.41–7.32 (m, 5 H, ArH), 7.11–7.04 (m, 4 H, ArH), 6.99 (t, J = 8.7 Hz, 2 H, ArH), 5.70 (t, J = 9.8 Hz, 1 H, 2-H), 5.49 (s, 1 H, PhC*H*), 5.31 (dd, J = 3.3 and 10.0 Hz, 1 H, 3-H), 4.88 (d, J = 9.8 Hz, 1 H, 1-H), 4.55 (d, J = 3.0 Hz, 1 H, 4-H), 4.44 (dd, J = 1.3 and 12.4 Hz, 1 H, 6-H), 4.08 (dd, J = 1.5 and 12.4 Hz, 1 H, 6-H), 3.74 (s, 1 H, 5-H), 2.35 (s, 3 H, CH_3) ppm. ^{19}F NMR ($CDCl_3$): δ = –105.2 and –105.6 ppm. ^{13}C NMR ($CDCl_3$): δ = 167.4, 167.3, 165.2, 164.8, 164.7, 164.1, 138.7, 137.6, 134.6, 132.7, 132.6, 132.5, 132.4, 129.7, 129.2, 128.2, 126.9, 126.6, 125.9, 125.8, 125.4, 125.3, 115.9, 115.8, 115.6, 115.5, 101.1, 85.3, 74.3, 73.7, 69.9, 69.2, 67.5, 21.4 ppm. HRMS (FAB) calcd. for $C_{34}H_{28}F_2NaO_7S$ 641.1421 [M + Na]$^+$ found 641.1423. $[\alpha]_D^{25}$ = +31 (MeOH); m.p. 83 °C; **12**: ^{1}H NMR ($CDCl_3$): δ = 8.00–7.91 (m, 4 H, ArH), 7.40–7.29 (m, 7 H, Ar), 7.14–6.99 (m, 6 H, ArH), 5.73 (t, J = 9.4 Hz, 1 H, 3-H), 5.53 (s, 1 H, PhC*H*-), 5.39 (dd, J = 9.27 and 0.65 Hz, 1 H, 2-H), 4.94 (d, J = 9.98 Hz, 1 H, 1-H), 4.46 (dd, J = 5.0 and 5.76 Hz, 1 H, 5-H), 3.89–3.84 (m, 2 H, 4-H, 6-H$_a$), 3.75–3.70 (m, 1 H, 6-H$_b$), 2.35 (s, 3 H, CH_3) ppm. ^{19}F NMR ($CDCl_3$): δ = –105.2 and –105.6 ppm. ^{13}C NMR ($CDCl_3$): δ = 101.5, 87.1, 78.5, 73.6, 71.3, 70.9, 68.5, 21.2 ppm. HRMS (FAB) calcd. for $C_{34}H_{28}F_2NaO_7S$ (641.6): 641.1421 [M + Na]$^+$ found 641.1410. $[\alpha]_D^{25}$ = +289 ($CHCl_3$); m.p. 149–151 °C.

Resins 13 and 14: Resin **10**, NBS, QOTf and glycosyl donors **11** or **12** were dried separately under vacuum overnight. Dried molecular sieves 3 Å (0.080 g), dry CH_2Cl_2 (3 mL), **11** (3 equiv.) or **12** (3.4 equiv.) and tetrabutylammonium trifluoromethanesulfonate (QOTf) (0.65 equiv. or 0.82 equiv.) were added to the resin **10** (0.106 mmol) or (0.120 mmol). The resins were agitated for 10 min. *N*-bromosuccinimide (NBS) (4 eq or 5 equiv.) was added and the mixtures were agitated at room temperature in absence of light for 5 h or 4.5 h. The resins were washed with CH_2Cl_2, 20% piperidine in DMF, DMF and CH_2Cl_2 (5 × 5 mL). According to gel-phase ^{19}F NMR the yield were approximately 50% and 35% respectively. The procedure was repeated to give the resins **13** and **14** in approximately 65% and 57% total yield according to gel-phase ^{19}F NMR spectroscopy. **13**: ^{19}F NMR ($CDCl_3$): δ = –104.9 and –105.3 (2 × 4-*F*PhCO$_2$) and –134.1 (linker) ppm; **14**: ^{19}F NMR ($CDCl_3$): δ = –104.7 and –105.4 (2 × 4-*F*PhCO$_2$) and –134.0 (linker) ppm.

{*N*-Hexanoyl-3-*O*-[2,3-di-*O*-(4-fluorobenzoyl)-β-D-galactopyranosyl]-L-seryl}-6-aminohexanoyl-6-aminohexylamine (15) and {*N*-Hexanoyl-3-*O*-[2,3-di-*O*-(4-fluorobenzoyl)-β-D-glucopyranosyl]-L-seryl}-6-aminohexanoyl-6-aminohexylamine (16): The partially protected neoglycolipid **15** and **16** were obtained from the resins **13** and **14** by addition of TFA/H_2O, 9:1 (35 mL or 47 mL). The resins were agitated at room temperature for 4 h and then thoroughly washed with TFA/H_2O, 9:1, CH_2Cl_2, THF and CH_2Cl_2. The filtrates were collected, combined and concentrated to give 88 mg or 120 mg of crude product in total. Purification by preparative LC-MS gave **15** (21 mg, 24% yield) and **16** (8 mg, 12% yield). **15**: ^{19}F NMR ($CDCl_3$): δ = –104.7, –105.1. MS (ES$^-$) calcd. for $C_{41}H_{57}F_2N_4O_{11}$ 819.40 *m*/*z* (M – H)$^-$, observed 819.70; **16**: MS (ES$^+$) calcd. for $C_{41}H_{59}F_2N_4O_{11}$ 821.42 *m*/*z* [M + H]$^+$, observed 821.47.

[*N*-Hexanoyl-3-*O*-(β-D-galactopyranosyl)-L-seryl]-6-aminohexanoyl-6-aminohexylamine (3) and [*N*-Hexanoyl-3-*O*-(β-D-glucopyranosyl)-L-seryl]-6-aminohexanoyl-6-aminohexylamine (17): The partially protected neoglycolipid **15** (0.021 g, 0.026 mmol) or **16** (0.008 mg, 0.010 mmol) was dissolved in dry MeOH (15 mL) and NaOMe in MeOH (0.20 M, 10 equiv.) was added dropwise. The solutions were stirred at room temperature for 1 h or 2 h. The reactions were quenched by addition of AcOH and ice to pH 4 and the mixtures were concentrated giving 46 mg of **3** and 16 mg of **17**. Crude **3** was dissolved in MeOH and purified by preparative LC-MS

chromatography and lyophilized to give 0.024 g. From the ^{1}H NMR a peak at 9 ppm could be detected, which was identified as formic acid salt formed during purification. Therefore **17** was not purified and an exact yield could not be calculated. **3**: ^{1}H NMR ([D$_4$]MeOH): δ = 4.55–4.50 [m, 1 H, OCH$_2$C*H*(NH)CO], 4.26 (d, *J* = 6.9 Hz, 1 H, 1-H), 4.18–4.11 [m, 1 H, OCH*H*CH(NH)CO], 3.86 (s, 1 H, 4-H) 3.81–3.68 [m, 3 H, 6-H, OCH*H*CH(NH)CO], 3.56–3.45 (m, 3 H, 2-H, 3-H, 5-H), 3.21–3.12 [m, 4 H, 2×CO(NH)-C*H*$_2$], 0.94 (t, *J* = 6.7 Hz, 3 H, C*H*$_3$) ppm. MS (ES$^+$) calcd. for $C_{27}H_{53}N_4O_9$ 577.38 *m*/*z* [M + H]$^+$, observed 577.43; **17**: ^{1}H NMR ([D$_4$]MeOH): δ = 4.54 [t, *J* = 4.85, 1 H, OCH$_2$C*H*(NH)CO], 4.30 (d, *J* = 7.6 Hz, 1 H, 1-H), 4.15 [dd, *J* = 5.5 Hz and 10.3 Hz, 1 H, OCH*H*CH(NH)CO], 3.88 (d, *J* = 11.5 Hz, 1 H, 4-H), 3.73 [dd, *J* = 5.6 and 10.3 Hz, 1 H, OC*H*HCH(NH)CO], 3.22–3.12 [m, 5 H, 2-H, 3-H, 5-H, CO(NH)-CH$_2$C*H*$_2$], 2.91 [t, *J* = 7.4 Hz, 2 H, CO(NH)-C*H*$_2$], 0.92 (t, *J* = 6.4 Hz, 3 H, C*H*$_3$) ppm. MS (ES$^+$) calcd. for $C_{27}H_{53}N_4O_9$ 577.38 *m*/*z* [M + H]$^+$, observed 577.72.

3-{[*N*-Hexanoyl-3-*O*-(β-D-galactopyranosyl)-L-seryl]-6-aminohexanoyl-6-aminohexylamino}-4-decyloxy-3-cyclobutene-1,2-dione (18) and 3-{[*N*-Hexanoyl-3-*O*-(β-D-glucopyranosyl)-L-seryl]-6-aminohexanoyl-6-aminohexylamino}-4-decyloxy-3-cyclobutene-1,2-dione (19): Compound **3** (0.015 g, 0.026 mmol) or **17** (0.008 g crude product) and 3,4-didecyloxy-cyclobut-3-ene-1,2-dione[13] (3.3 equiv.) were dissolved in distilled DMF (5 mL and 2 mL, respectively). TEA (1.65 equiv.) was added and the solutions were stirred at room temperature for 20 h. The DMF was removed under vacuum giving 0.069 g of **18** and 0.020 g of **19**. Purification by preparative HPLC gave **18** (4.2 mg, 20% yield) and **19** (2.2 mg, 27% yield). **18**: ^{1}H NMR ([D$_4$]MeOH): δ = 4.77–4.68 (m, 2 H, COC*H*$_2$C$_9$H$_{19}$), 4.56 [t, *J* = 5.3 Hz, 1 H, CH$_2$C*H*(NH)CO], 4.27 (d, *J* = 7.4 Hz, 1 H, 1-H), 4.14 [dd, *J* = 4.7 and 10.4 Hz, 1 H, OCH*H*CH(NH)CO], 3.95–3.69 [m, 4 H, 4-H, OCH*H*CH(NH)CO, 6-H], 3.67–3.48 (m, 3 H, 2-H, 3-H, 5-H), 3.44 (t, *J* = 6.9 Hz, 2 H, COC*H*$_2$), 3.27–3.14 (m, 4 H, 2×CONHC*H*$_2$), 1.89–1.78 (m, 4 H, OCH$_2$C*H*$_2$, COCH$_2$C*H*$_2$), 1.44–1.33 (m, 4 H, 2×C*H*$_2$-CH$_3$), 0.98–0.91 (m, 6 H, 2×C*H*$_3$). ^{13}C NMR ([D$_4$]MeOH): δ = 176.3, 174.8, 172.1, 105.3, 77.1, 74.9, 74.7, 72.5, 70.6, 70.4, 62.7, 54.9, 45.5, 45.2, 40.3, 40.2, 37.1, 36.8, 33.1, 32.6, 31.9, 31.6, 31.2, 30.6, 30.4, 30.3, 30.2, 30.0, 27.6, 27.5, 27.4, 27.2, 27.0, 26.7, 26.6, 26.4, 23.7, 23.7, 23.5. $[\alpha]_D^{25}$ = +19 (MeOH). HRMS (FAB) calcd. for $C_{41}H_{72}N_4NaO_{12}$ 835.5044 [M + Na]$^+$, observed 835.5045; **19**: ^{1}H NMR ([D$_4$]-MeOH): δ = 4.71–4.65 [m, 2 H, CO(C)OC*H*$_2$C$_9$H$_{19}$], 4.54 [t, *J* = 4.9 Hz, 1 H, OCH$_2$C*H*(NH)CO], 4.29 (d, *J* = 7.7 Hz, 1 H, 1-H), 4.12 [dd, *J* = 5.5 and 10.3 Hz, 1 H, OCH*H*CH(NH)CO], 3.82 (d, *J* = 11.5 Hz, 1 H, 4-H), 3.76 [dd, *J* = 5.6 and 10.3 Hz, 1 H, OC*H*HCH(NH)CO], 3.70–3.65 (m, 1 H, 5-H), 3.42 (t, *J* = 7.8 Hz, 2 H, COC*H*$_2$) 3.38–3.12 [m, 10 H, 2-H, 2×6-H, 3-H, 2×CO(NH)-C*H*$_2$CH$_2$, COC*H*$_2$], 1.87–1.75 [m, 2 H, CO(C)OCH$_2$C*H*$_2$C$_8$H$_{17}$] 0.93–0.88 (m, 6 H, 2 Me). ^{13}C NMR ([D$_4$]MeOH): δ = 185.5, 184.8, 176.4, 176.1, 172.6, 172.1, 104.2, 77.9, 77.7, 74.9, 74.4, 71.4, 71.0, 70.3, 62.7, 62.4, 47.7, 45.4, 40.0, 36.8, 36.5, 32.8, 32.6, 30.2, 29.9, 27.0, 26.3, 23.3, 14.1. $[\alpha]_D^{25}$ = –13.4 (MeOH). HRMS (FAB) calcd. for $C_{41}H_{72}N_4NaO_{12}$ 835.5044 [M + Na]$^+$, observed 835.5024.

Formation of Model Compound Microtiter Plates

Detection with Avidin-HRP: Compound **9a** and **9b** in Na_2HCO_3 buffer (aq., pH 9) were serially diluted (1.0 mM to 0.24 nM for **9a** and 1.1 mM to 2.2 nM for **9b**) in transparent microtiter plate wells (CovaLink™, Nunc A/S, Denmark) and incubated with shaking for 18 h at room temperature. Control wells were incubated with buffer alone. All wells were washed with Milli-Q water (1×150 μL, 1 min and 2×150 μL, 10 min) and incubated with avidin-HRP (Vector Laboratories, 4 μg/mL in PBS containing 0.05% Tween 20, 100 μL) for 45 min. The wells were washed with Milli-Q water (2×150 μL, 1 min and 1×150 μL, 10 min) and citric acid buffer (150 μL, 10 min), incubated with substrate solution [6 mg of *O*-phenylenediamine and 5 μL H_2O_2 (30% aq.) in phosphate-citric acid buffer (0.1 M, pH 5, 10 mL), 100 μL] for 20 min in the dark and the substrate reaction was stopped with H_2SO_2 (1 M aq.) (100 μL). The optical density was measured at 490 nm against a reference at 650 nm. All concentrations and controls were performed in triplicate samples.

Detection with Avidin-FITC: Compound **9a** and **9b** in Na_2HCO_3 buffer (aq., pH 9) were serially diluted (2.5 mM to 0.61 μM) in white microtiter plate wells (CovaLink™, Nunc A/S, Denmark) and incubated with shaking for 18 h at room temperature. Control wells were incubated with buffer alone. All wells were washed with Milli-Q water (1×150 μL, 1 min and 2×150 μL, 10 min) and blocked by incubation overnight with BSA (1% w/v in PBS, 200 μL) at 4 °C. The wells were washed with PBS containing BSA (1% w/v) and Triton X-100 (0.05%) (2×200 μL, 1 min) and PBS (1×200 μL, 1 min). Avidin-FITC (from egg white, Sigma, 50 μg/mL in PBS, 100 μL) were added to the wells and incubated with shaking for 3 h. The wells were washed with Milli-Q water (2×150 μL, 1 min and 1×150 μL, 10 min) and phosphate-citric acid buffer (0.1 M, pH 5, 100 μL) was added to each well. The fluorescence was measured at 485/535 (ex/em).

Formation of Neoglycolipid Microtiter Plates: A stock solution (0.5 mM) of neoglycolipid **18** in $NaHCO_3$ buffer (pH 9) was prepared and serially diluted in transparent CovaLink™ (Nunc A/S, Denmark) microtiter plate wells, resulting in 100 μL neoglycolipid solution in each well in the concentration range of 48 nM to 0.1 mM. Control wells were incubated with buffer alone. The plate was shaken at room temperature for 24 h, emptied, washed with Cova buffer (2×150 μL, 1 min and 1×150 μL, 10 min) and water (1×150 μL, 1 min). Any remaining amino-groups were blocked with acetic anhydride (20% in water) (100 μL, 2 h), emptied and washed with water (2×150 μL, 1 min and 1×150 μL, 10 min). Biotin-conjugated lectin from *Ricinus communis* (RCA_{120}, Vector Laboratories, 5 μg/mL in PBS containing 0.05% Tween 20, 100 μL) was added to the wells. The plate was shaken for 70 min at room temperature and the wells were emptied and washed with PBS with 0.05% Tween 20 (2×150 μL, 1 min). Avidin-HRP (Vector Laboratories, 4 μg/mL in PBS containing 0.05% Tween 20, 100 μL) was added to the wells. The plate was shaken at room temperature for 45 min, emptied and washed with Cova buffer (2×150 μL, 1 min and 1×150 μL, 10 min) and phosphate-citric acid buffer (0.1 M, pH 5) (1×150 μL, 1 min and 1×150 μL, 10 min). Substrate solution [6 mg of *O*-phenylenediamine and 5 μL H_2O_2 (30% aq.) in phosphate-citric acid buffer (0.1 M, pH 5, 10 mL), 100 μL] was added to the wells. The plate was incubated with shaking in the dark at room temperature for 15 min and absorbance was measured at 490 nm against a reference at 650 nm. All concentrations and controls were performed in triplicate samples.

Supporting Information (see also the footnote on the first page of this article): ^{1}H NMR spectra for compounds **9a**, **9b**, **18**, and **19**.

Acknowledgments

This work was supported by the Swedish Research Council and the J. C. Kempe Foundation.

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Received: July 8, 2008
Published Online: December 4, 2008

