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PAPER

Dual purpose *S*-trityl-linkers for glycoarray fabrication on both polystyrene and gold†

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There is a wide range of immobilisation reactions to tether substrates to a variety of surfaces for array-based analysis. Most of these immobilisation strategies are specific for a particular surface and require an additional linker to be attached to the substrate or the surface. Furthermore, the analysis of functionalised surfaces is often restricted to certain analytical techniques and therefore, different immobilisation strategies for different surfaces are desirable. Here we have tested an *S*-tritylated linker for non-covalent or covalent immobilisation of mannosides to polystyrene or gold surfaces. *S*-Tritylated mannosides with varying linkers were readily synthesised and used to add to biorepulsive maleimide-terminated preformed SAMs after *in situ* deprotection of the *S*-trityl group. In addition, *S*-tritylated mannosides themselves formed stable glycoarrays on polystyrene microtiter plates. The glycoarrays were successfully analysed by MALDI-ToF mass spectrometry, SPR spectroscopy, and interrogated with GFP-transfected *Escherichia coli* cells. This work has shown that a dual purpose linker can be used on multiple surfaces to form arrays allowing for different testing as well as analytical approaches.

Introduction

Microarrays are valuable tools in the analysis of biological interactions in fundamental research and in high-throughput screening and have promising applications as diagnostic devices in the clinic.[1,2] Among many different microarrays, glycoarrays are carbohydrate-functionalised surfaces, which have received much attention in the glycosciences for the investigation of the molecular details of carbohydrate–protein interactions,[3–7] and to study cellular adhesion such as in the context of carbohydrate-specific bacterial colonisation of surfaces.[8]

A key step in the preparation of glycoarrays, which can consist of a variety of materials such as gold, glass or polystyrene, is the immobilisation of the glycoconjugates on the respective surface.[9–14] A variety of methods have been utilised for this step, involving covalent or non-covalent attachments. Common covalent immobilisation techniques include the amide formation *via* direct amine coupling into activated esters[15,16] or *via* native chemical ligation (NCL),[17] 1,3-dipolar cycloaddition,[18] or Diels–Alder cycloaddition.[19,20] Each surface type, however, requires specific methods for functionalisation. For example, for the formation of self-assembled monolayers (SAMs) on gold, thiol-functionalised derivatives are needed, in order to form Au–S bonds on the surface. Hence, immersion of gold wafers in a solution of thiol-functionalised glycosides has been developed into a common method for the preparation of glyco-SAMs.[18,19,21] Moreover, carbohydrate thiols have been utilised in the formation of glycoarrays through Michael-type addition to maleimide-terminated surfaces.[22] For the formation of glycoarrays on polystyrene, prefunctionalised microtiter plates have been employed for covalent immobilisation.[8,23] However, direct non-covalent array formation on polystyrene is especially appealing as it requires no additional immobilisation agents. As polystyrene is inherently hydrophobic, hydrophobic interactions or π–π interactions between the glycoconjugate and the polymer surface can be used in this case to produce robust glycoarrays.[3,9,13,24–26]

In the course of our work on the preparation and biological testing of glycoamino acids, we have found that *S*-trityl-protected low molecular weight glycoconjugates are readily made and purified.[27,28] This has prompted us to test the direct application of *S*-tritylated carbohydrate derivatives for the preparation of glycoarrays on different surfaces. De-tritylation would lead to thiol-modified glycoconjugates to allow immobilisation on plain gold or on preformed maleimide-terminated biorepulsive SAMs. On the other hand, the trityl protecting

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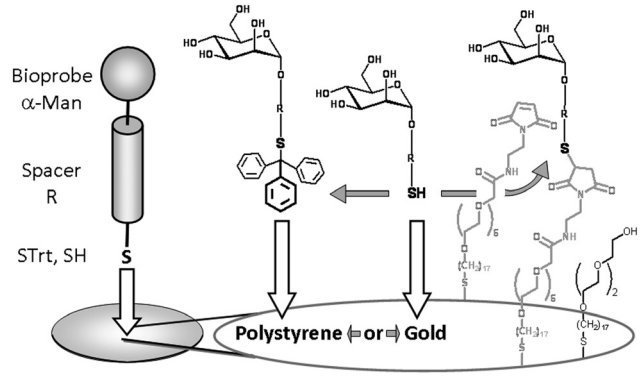


Fig. 1 Dual linkers: thio-functionalised bioprobes (*e.g.* carbohydrates as shown for α-D-mannosides) can be directly attached to polystyrene surfaces in their *S*-tritylated form (left), or added as thiols to gold or maleimide-terminated surfaces (right).

group should also allow preparation of glycoarrays on simple polystyrene microtiter plates, by hydrophobic interactions between the molecule's trityl fragment and the hydrophobic surface (Fig. 1). In this account we report the synthesis of *S*-tritylated glycosides, their utilisation in glycoarray fabrication on gold as well as on polystyrene and the interrogation of the prepared surfaces with lectins as well as live bacterial cells.

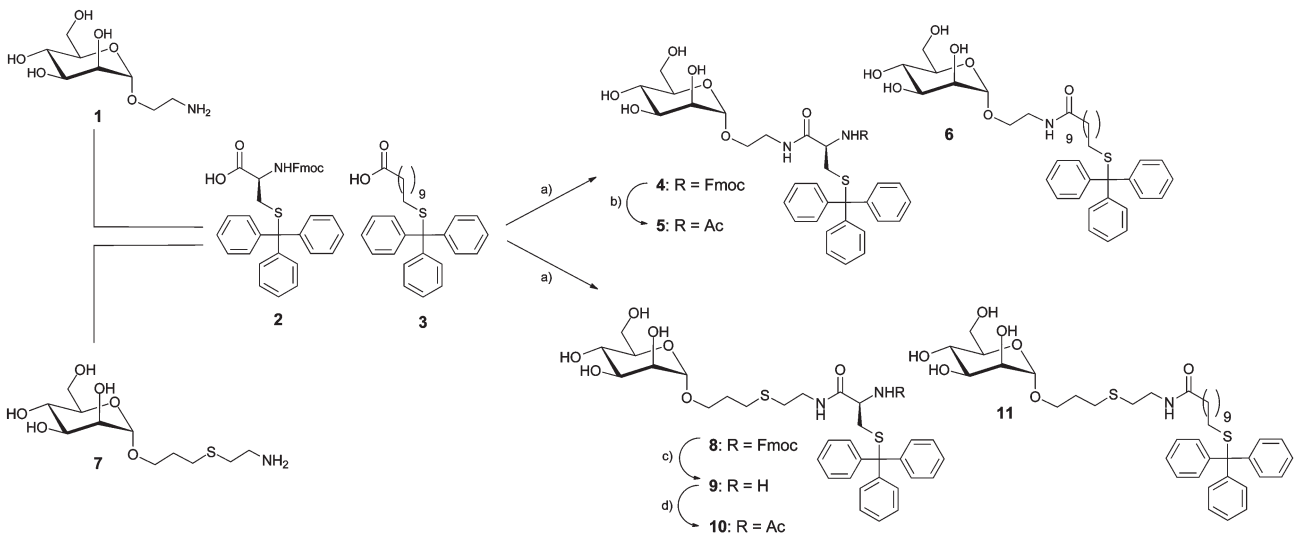
Results and discussion

Synthesis of *S*-tritylated glycoconjugates

As we have long-standing interest in the investigation of mannose-specific lectins, in particular mannose-specific bacterial adhesion, we have made a selection of four *S*-tritylated mannoside derivatives for this study, **5**, **6**, **10**, and **11** (Scheme 1). We have shown earlier that preparation of mannosides **5** and **6** is readily accomplished by coupling of the well-known 2-aminoethyl mannoside **1**[29] with the commercially available cysteine derivatives Fmoc–Cys(Trt)–OH (**2**) or 11-tritylsulphanyl-undecanoic acid (**3**),[9,30] leading to the peptide-coupled mannosides **4**[27] and **6**,[9] respectively. Removal of the Fmoc protecting group and *N*-acetylation can be effected in one pot yielding the known *S*-tritylated glycoamino acid **5**.[27] Then, it was important to add *S*-tritylated mannoside derivatives to the collection having a longer spacer, because this usually facilitates immobilisation of the respective compound on a surface. Therefore, 6-amino-4-thiahexyl mannoside **7**[8,31] was made and subjected to peptide coupling with the *S*-tritylated thiols **2** and **3**, using HATU and DIPEA. In analogy to the preparation of **4** and **6**, this reaction led to the trityl-functionalised mannosides **8** and **11**, having considerably longer spacers than their analogues **4** and **6**. Removal of the Fmoc protecting group in **8** led to **9** and then acetylation to the *N*-acetylated target molecule **10** (Scheme 1).

Fabrication and interrogation of glycoarrays on gold

Initially we tested, if the prepared *S*-tritylated glycosides can be immobilised on gold with concomitant removal of the trityl protecting group. Thus, *in situ* de-tritylation of **5**, **6**, **10**, and **11** was effected overnight by treatment with trifluoroacetic acid (TFA) and triethylsilane (TES) in dichloromethane.[28] Then, the solvent was removed and the crude free thiol dissolved in PBS buffer, centrifuged and the solution applied to the gold surface according to the standard protocol for preparation of SAMs.[17] To test if the immobilisation of *in situ* deprotected mannosides was successful, the prepared glycoarrays were analysed by MALDI-ToF mass spectrometry.[32] This mass spectrometric protocol is a reliable method for the analysis of SAMs on gold, in which typically the masses of the disulphides of the respective thiols are detected.[17] Also here, the detected peaks correspond to the disulphides of the thiols derived from **5**, **6**, **10**, and **11** (*cf.* ESI, Fig. S16–S19†). Thus, the MS analysis showed the success of glycoarray formation after *in situ* de-tritylation. In addition,



Scheme 1 Synthesis of *S*-tritylated α-D-mannosides **5**, **6**, **10**, and **11**. Reaction conditions: (a) HATU, DIPEA, DMF, 0 °C → room temp., overnight, 81% (**4**, from **1** and **2**), 74% (**6**, from **1** and **3**), 84% (**8**, from **7** and **2**), 78% (**11**, from **7** and **3**); (b) (i) morpholine, DMF, room temp., quant. (ii) Ac_2O, DIPEA, room temp., 4 h, quant.; (c) morpholine, DMF, room temp., 67%; (d) pyridine, Ac_2O, room temp., overnight, 96%.

tritylated **6** was deprotected and purified to deliver the pure thiol **6-SH**. When pure **6-SH** was employed for glycoarray fabrication, mass spectrometric analysis gave very similar results as when the *in situ* deprotection–immobilisation approach was employed (*cf.* ESI, Fig. S15†). The efficiency of immobilisation of *in situ* deprotected thiols greatly simplifies fabrication of glycoarrays on gold. *S*-Tritylated derivatives are much easier to purify than free thiols, owing to their greater hydrophobicity. In addition, free thiols are prone to oxidation, forming the respective disulphides, a problem which is circumvented in the *in situ* deprotection approach.

The same *in situ* de-tritylation protocol was employed on preformed maleimide-terminal self-assembled monolayers (SAMs) on gold (*cf.* Fig. 1). These SAMs include biorepulsive oligoethyleneglycol units,[33] which are important in biological studies to avoid nonspecific protein adsorption. The *S*-tritylated bioprobes **5**, **6**, **10**, and **11** were treated as above and the non-purified mixtures directly applied to the maleimide-functionalised surface. After 1.5 h reaction time, the surface was rinsed with ethanol and again analysed by MALDI-ToF MS. The MALDI MS spectra showed the corresponding masses of the coupled ligands (ESI, Fig. S24–S27†). Comparison with the coupling results obtained with previously deprotected and purified thiols revealed that glycoarray formation after *in situ* deprotection is similarly effective.

As an additional method to test glycoarray formation on gold, SPR spectroscopy was used. Here, the mannose-specific lectin concanavalin A (ConA) was employed for interrogation of glycoarrays prepared after *in situ* de-tritylation of a pair of comparable mannosides, **10** having the shorter spacer, and **11** having a longer spacer incorporated. The crude thiols were added to a maleimide-terminated biorepulsive SAM on gold and ConA was allowed to interact with the formed glycoarray. In both cases, the expected carbohydrate–lectin interactions were detected, suggesting glycoarray formation (Fig. 2). For the array formed from thiol derived from **11**, a much stronger interaction with ConA was measured than for the analogous case using **10**. This suggests that (tritylated) thiols having the bioprobe attached to a rather long spacer are better suited for surface immobilisation.

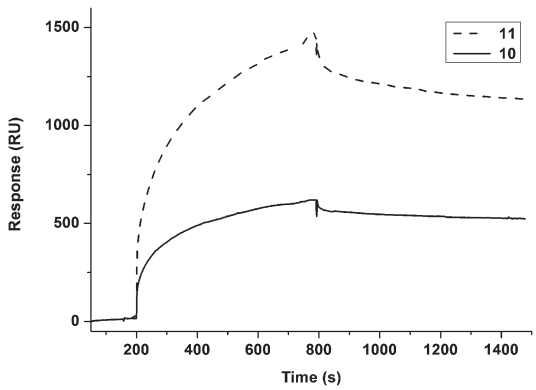


Fig. 2 SPR spectroscopy on surfaces functionalised with **10** and **11** using the lectin ConA was used to prove the formation of glycoarrays after *in situ* deprotection of tritylated mannosides **10** and **11**.

Fabrication and interrogation of glycoarrays on polystyrene

The next step was to employ the *S*-trityl group in mannosides **5**, **6**, **10**, and **11** (Scheme 1) to anchor these molecules to a hydrophobic polystyrene surface, as it has been shown earlier for another type of tritylated molecules.[13] For the non-covalent functionalisation of polystyrene microplates hydrophobic molecules have been used regularly. From these studies it is known that the π–π interactions established between the polystyrene surface and the aromatic trityl fragment are strong enough to guarantee a robust direct immobilisation on polystyrene microtiter plates.

First, the reaction conditions for glycoarray fabrication on polystyrene were optimised and methanol was identified as the most suitable solvent for immobilisation of the prepared tritylated glycosides. Then, in order to determine the stability of produced glycoarrays against different washing conditions, a colorimetric phenol–sulphuric acid assay was performed (Fig. 3).[34–36] This assay allows quantification of glycoconjugates immobilised on surfaces. Washing with ethanol removed the immobilised glycosides completely, as expected. In contrast, washing with twice distilled water and/or PBST buffer led to negligible reduction of the carbohydrate content on the surface in the case of the glycoarrays formed from the tritylated glycosides **6**, **10**, and **11**. However, the mannoside with the shortest spacer, compound **5**, formed the least stable glycoarray on polystyrene which was washed out by water or buffer to over 50% according to the phenol–sulphuric acid assay.

In the next step, the prepared glycoarrays were tested with live bacterial cells in a GFP-assisted adhesion assay, which was established earlier.[8] Here, the genetically engineered *E. coli* strain PKL1162 was used.[8,37] Protocols for cellular adhesion assays on polystyrene microplates usually involve a blocking step with BSA or skimmed milk for example, to prevent unspecific binding of the cells to the microtiter plate surface. However, we could show that when the tritylated mannosides

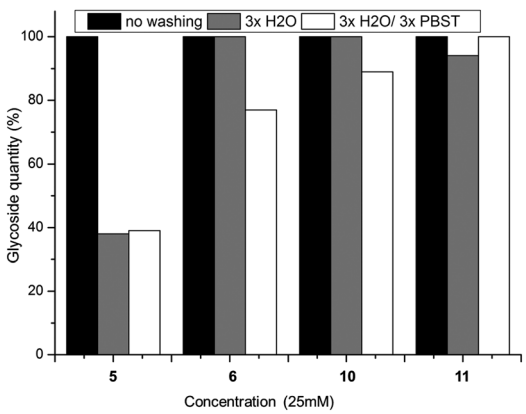


Fig. 3 Removal of compounds **5**, **6**, **10** and **11** from polystyrene surfaces using different washing steps. Microtiter plate wells were functionalised with 25 mM methanolic solutions of tritylated mannosides **5**, **6**, **10**, and **11**, then it was washed with water and/or buffer and the remaining glycoside content on the surface determined by the phenol–sulphuric colorimetric acid assay. The glycoside content without washing was defined as 100%. Six-fold washing with ethanol removed the glycoarray completely (not shown).

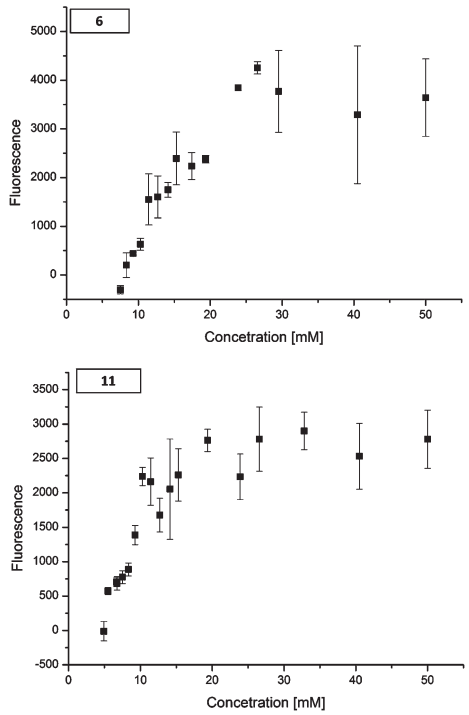


Fig. 4 Bacterial adhesion curves (GFP-tagged *E. coli* PKL1162) obtained by application of glycoarrays consisting of compounds **6** (top) and **11** (bottom) after 1 h incubation and fluorescence readout.

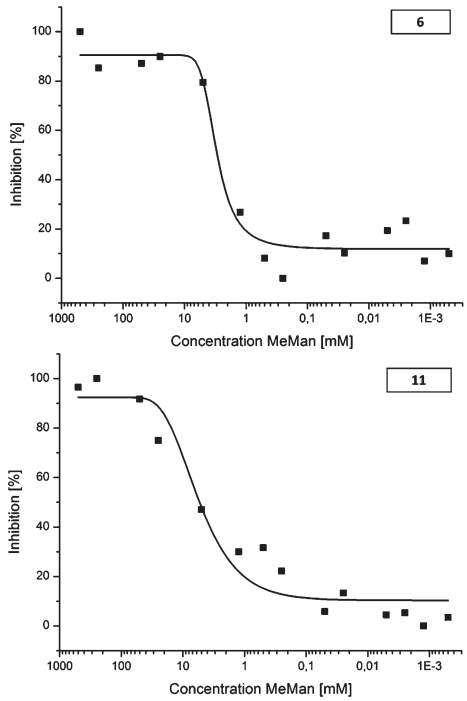


Fig. 5 Inhibition curves of competitive bacterial adhesion inhibition assays using polystyrene glycoarrays prepared from **6** (top) and **11** (bottom). Methyl α-D-mannoside was used as an inhibitor and type 1 fimbriated *E. coli* cells (PKL1162) were used to adhere to the surface.

were used for modification of polystyrene plates, no blocking step was necessary (ESI, Fig. S28†).

Glycoarrays on polystyrene were prepared using tritylated mannosides **6** and **11** at different concentrations. Concentration dependency of bacterial adhesion to these two glycoarrays was tested and was found as expected in both cases, with the intensity of the GFP fluorescence increasing with higher concentrations of the applied mannoside solutions (Fig. 4). A plateau was reached at concentrations between 20 mM and 25 mM. Mannosides **5** and **10** were less suited in this assay. Only little adhesion could be detected and no consistent concentration dependency of bacterial adhesion could be observed in the case of these mannosides linked *via* short spacers (ESI, Fig. S31†).

After having shown that tritylated mannosides such as **6** and **11** form stable glycoarrays on polystyrene microtiter plates upon direct treatment, testing of inhibition of bacterial adhesion to these surfaces could be done next. From the results obtained in the adhesion experiments, 25 mM concentrations appeared optimal to form microarrays for competitive bacterial adhesion inhibition assays. As described earlier,[38] serial dilutions of methyl α-D-mannoside (MeMan), a standard inhibitor of mannose-specific bacterial adhesion, were applied to inhibit bacterial adhesion to the two different glycoarrays formed with **6** and **11**, respectively. The obtained inhibition curves are depicted in Fig. 5. After sigmoidal fitting of the testing results, IC_{50} values could be deduced, with IC_{50} (MeMan) ~2.9 mM for the inhibition of bacterial adhesion to the surface, modified with mannoside **6** and IC_{50} (MeMan) ~5.3 mM for **11**. Thus, the surface prepared from mannosides **11** appears to be slightly more adhesive in this testing system than when **6** was used for glycoarray formation. This finding is in line with earlier results, which have indicated that mannosides having a thiahexyl aglycon moiety show a higher affinity to the mannose-specific lectin of *E. coli* than mannosides having an ethyl aglycon.[38]

Conclusions

S-Tritylated mannosides were synthesised and shown to be suitable for the fabrication of glycoarrays on different surfaces such as gold and polystyrene. An *in situ* deprotection protocol has allowed us to apply tritylated carbohydrate derivatives on plain gold as well as on maleimide-terminal preformed biorepulsive SAMs. As SAMs on gold on the one hand and polystyrene microtiter plates on the other can be used in quite different applications, *S*-tritylated glycoconjugates can be regarded as facile derivatives for orthogonal immobilisation on surfaces of opposite character.

The prepared glycoarrays were analysed by MALDI-ToF MS and were shown to be robust and suited for interrogation with lectins and live bacterial cells. Next, we will further employ this methodology in a 384 well polystyrene microtiter plate format to facilitate inhibitor screening in bacterial adhesion assays.

Experimental

Commercially available starting materials and reagents were used without further purification. Reactions requiring dry conditions were performed under an atmosphere of nitrogen using

oven-dried glassware. Anhydrous DMF was purchased from Acros. All other used solvents were purified by distillation. ConA was purchased from Vector labs. Bovine serum albumin (BSA), methyl α-D-mannopyranoside (MeMan) and polyethylene glycol sorbitan monolaurate (Tween® 20) were obtained from Sigma-Aldrich. Microtiter plates with a hydrophobic surface (Corning, no. 3540, low volume 384 wells, flat clear bottom, black polystyrene, nontreated and Corning, no. 3631, 96 wells, flat clear bottom, black polystyrene, nontreated) were obtained from Corning. 2-Aminoethyl α-D-mannopyranoside (**1**),[29] 6-amino-4-thiahexyl α-D-mannopyranoside (**7**),[8,31] *N*-(fluoren-9-yl-methoxycarbonyl)-*S*-(triphenylmethyl)-L-cysteine-[2-(α-D-mannopyranosyloxy)ethyl]amide (**4**),[27] 11-tritylsulphanyl-undecanoic acid, and 2-(11-tritylsulphanyl-undecanoyl)-aminoethyl α-D-mannopyranoside (**6**)[9] were prepared according to the literature.

Reactions were monitored by thin-layer chromatography using either silica gel 60 GF254 on aluminium foil (Merck) or RP-18 F254s on aluminium foil (Merck) with detection by UV light and charring with sulphuric acid in EtOH (10%). Merck silica gel 60 (0.040–0.063 mm) was used for flash chromatography. Analytical HPLC was performed on a Merck Hitachi LaChrom L-7000 series apparatus with a LiChrospher 100 RP-8 (5 μm, Merck) column (for HPLC chromatograms see the ESI†). Preparative MPLC was performed on a Büchi apparatus using a LiChroprep RP-18 column (40–60 μm, Merck) for reversed-phase and a LiChroprep Si 60 column (40–60 μm, Merck) for normal-phase silica gel chromatography. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker DRX-500 or a Bruker AV-600 instrument. NMR spectra were calibrated with respect to the solvent peak (in the case of $CDCl_3$ the reference was tetramethylsilane (TMS)). 2D NMR techniques (COSY, HSQC, HMBC) were used for full assignment of the spectra. ESI MS measurements were performed on a Mariner ESI-ToF 5280 instrument (Applied Biosystems). MALDI-ToF mass spectra were recorded on a Bruker Biflex-III 19 kV instrument with Cl-CCA (4-chloro-α-cyanocinnamic acid) or DHB (2,5-dihydroxybenzoic acid) as matrix. Optical rotation was measured on a Perkin-Elmer polarimeter 341 (Na-D-line: 589 nm, length of cell 1 dm). IR spectra were recorded on a Perkin-Elmer Paragon 1000 FT-IR instrument. For sample preparation a Golden Gate diamond ATR unit with a sapphire stamp was used. The SPR experiments were performed on a Biacore 3000 system (GE Healthcare, Sweden) using a gold sensor chip (GE Healthcare). For bacterial adhesion studies and phenol–sulphuric acid assays, a TECAN infinite 200 multifunction microplate reader was employed. The wavelengths of the band pass filters for excitation and emission were 485 and 535 nm, respectively. For the phenol–sulphuric acid assay absorbance at 492 nm was measured.

E. coli bacteria (PKL1162)[8,37] were used and grown in LB-media + AMP + CAM (100 mg ampicillin, 50 mg chloramphenicol L^{-1}) at 37 °C under slight agitation. Buffers were used as follows: PBS buffer solution (pH 7.2): sodium chloride (8.00 g), potassium chloride (200 mg), sodium hydrogen phosphate-dihydrate (1.44 g), and potassium dihydrogen phosphate (200 mg) were dissolved in bidist. water (1.00 L). PBST buffer solution (pH 7.2): PBS buffer + 0.05% v/v Tween® 20.

General procedure for *in situ* de-tritylation of *S*-protected mannosides

The *S*-tritylated mannosides (**5**, **6**, **10**, or **11**, 3 μmol) were dissolved in dichloromethane (1 mL). Then, triethylsilane (5 equiv.) and trifluoroacetic acid (5 equiv.) were added and the reaction mixture was left for 1.5 h at room temperature and was subsequently treated with further trifluoroacetic acid (5 equiv.) and left overnight. Thereafter, the solvent was removed *in vacuo* and the residue dissolved in 10 mM PBS to obtain a final concentration of the corresponding free thiol of 10 mM. This solution was centrifuged and the supernatant directly applied to the differently modified gold surfaces.

Fabrication of glycoarrays on gold and maleimide-terminated SAMs and their MALDI-ToF MS analysis

A disposable 64-well gold plate (Applied Biosystems) was cleaned with a Piranha solution (12 mL, 3 : 1 conc. H_2SO_4/30% H_2O_2) for 30 min, rinsed with distilled water and ethanol and dried under a stream of nitrogen. A solution of carboxylic acid-terminated linkers [HS–$(CH_2)_{17}$–$(OC_2H_4)_6$–OCH_2–COOH] and tri(ethylene glycol)-terminated alkanethiol spacers [HS–$(CH_2)_{17}$–$(OC_2H_4)_3$–OH] in dry DMSO (final concentration 0.4 mg mL^{-1}, molar ratio 1 : 4) was applied on the plate (~1 μL per well) and left overnight at room temperature to form a mixed SAM. The plate was washed with ethanol and dried under nitrogen. The carboxylic acid groups were activated by spotwise treatment with a solution of EDC, NHS and *N*-(2-aminoethyl)-maleimide (all Sigma-Aldrich, 0.180 M, 0.174 M and 0.174 M, respectively) in dry DMF for 1–2 h, followed by washing with water and ethanol and drying as above. The product formation was analysed by MALDI-ToF MS. Unless otherwise stated all MALDI-ToF MS experiments on gold surfaces were carried out on an Ultraflex II instrument (Bruker Daltonics) in positive reflection mode. A solution of matrix (2,4,6-trihydroxyacetophenone, 10 mg mL^{-1} in acetone) was applied on the gold and allowed to dry before analysis.

Surface plasmon resonance (SPR) analysis using 10 and 11

The gold-coated sensor was cleaned with a Piranha solution, rinsed with water and ethanol and dried in a stream of nitrogen. The formation of self-assembling monolayers was performed in the same way as described above. The chip was washed with ethanol, dried under nitrogen and mounted onto a chip holder following the instructions in the supplier's manual. After docking in the instrument the sensor was equilibrated with PBS buffer (10 mM, degassed and filtered) at a flow rate of 10 μL min^{-1}. For surface activation, 70 μL of a 1 : 1 mixture of freshly prepared solutions of NHS (0.4 M) and EDC (0.1 M) in water were injected. The reference spot (channel 1) was blocked by injecting 70 μL of aminoethanol-hydrochloride (1 M). Additional channels were modified with *N*-(2-aminoethyl)-maleimide (10 mM, flow rate 10 μL min^{-1}) for 10 min and then treated with *in situ* deprotected mannosides **10** and **11** for about an hour at a flow rate of 3 μL min^{-1}.

Binding studies were carried out using the lectin ConA (10 μg mL^{-1}, 250 μL) in buffer (0.15 M NaCl, 1 mM $CaCl_2$, 1 mM

$MnCl_2$, pH 7.0) at a flow rate of 25 μL min^{-1}. After the injection a 600 s dissociation sequence was followed.

Fabrication of glycoarrays on polystyrene

A series of 5 to 50 mM stock solutions of tritylated mannosides **5**, **6**, **10**, and **11** (in MeOH) were prepared. A 12 μL sample of each solution was pipetted into a 384-well polystyrene microtiter plate, which was dried by standing overnight at ambient temperature. Each well was then washed three times with deionised water and three times with PBST buffer (20 μL per well each).

Phenol–sulphuric acid assay

To test the stability of glycoarrays formed by immobilisation of **5**, **6**, **10** or **11** against different washing conditions, these compounds were immobilised as described above followed by 6 washing cycles with ethanol (20 μL per well each) followed by phenol–sulphuric acid assay. 12 μL of solutions of trityl-protected carbohydrate (50 mM, 25 mM, 12.5 mM solutions in MeOH) were pipetted into a 384-well microtiter plate and the plate was allowed to dry by standing overnight at ambient temperature. The wells were then washed three times with deionised water and three times with PBST (20 μL per well each). The phenol–sulphuric acid assay was performed according to a literature-known method.[35] A 5% phenol solution (4.2 μL per well) was pipetted to the wells, followed by the addition of concentrated H_2SO_4 (21 μL per well). The mixture was incubated for 30 min at room temperature, and the absorbance measured at 492 nm (A492) to determine the amount of carbohydrate immobilised on the microtiter plate. The amount of immobilised **5**, **6**, **10** and **11** was estimated from the ratio of the absorption at 492 nm of immobilised compounds (subjected to 3 washing cycles with deionised water and/or PBST) to the A492 of the corresponding control (unwashed). Washings with ethanol (6 washing cycles using 20 μL ethanol per well) removed the glycoarrays completely according to the phenol–sulphuric acid assay.

GFP-based bacterial adhesion assay

Determination of bacterial adhesion. Trityl-protected carbohydrates (**5**, **6**, **10**, and **11**) were immobilised on 384-well microtiter plates as described above. The wells were incubated with *E. coli* PKL1162 (2 mg mL^{-1} PBS buffer) for 1 h (37 °C, 120 rpm), and subsequently washed with the same buffer (3 × 20 μL per well). *E. coli* binding to the mannoside-functionalised surface was monitored by fluorescence measurements at 485/535 nm using a microplate reader.

Inhibition of bacterial adhesion with methyl α-D-mannoside (MeMan). Compounds **6** and **11** (12 μL per well, 25 mM) were immobilised on 384-well microtiter plates as described above. Then, 5 μL of a serial dilution of the standard inhibitor MeMan (1 μM–1000 mM) were pipetted to the plate followed by addition of 5 μL of *E. coli* (PKL1162) solution (4 mg mL^{-1} PBS buffer). The plate was incubated for 1 h (37 °C, 120 rpm) and the wells were then washed with PBS buffer (3 × 20 μL per well). Fluorescence was read out at 485/535 nm.

2-(11-Sulphhydryl-undecanoyl)aminoethyl α-D-mannopyranoside (6-SH)

The tritylated mannoside **6** (50 mg, 75.1 μmol) was dissolved in dichloromethane (1 mL), triethylsilane (60 μL, 376 μmol) and trifluoroacetic acid (58 μL, 751 μmol) were added and the reaction mixture was stirred for 2 h at ambient temperature. The solvent was removed under reduced pressure and the crude product was purified by RP-MPLC (120 g RP-18, A: methanol, B: water, A: 60% → 95%, 90 min) yielding the deprotected title compound **6-SH** (31.3 mg, 73.6 μmol, 98%) after lyophilisation. R_f 0.33 (methanol–water, 3 : 1); $[\alpha]_D^{26}$ = +40.4 (*c* = 0.5, methanol); ^{1}H NMR (500 MHz, CD_3OD, 300 K): δ = 4.76 (d, ^3J = 1.7 Hz, 1H, H-1_{Man}), 3.83 (dd, ^2J = 11.7 Hz, ^3J = 2.3 Hz, 1H, H-$6a_{Man}$), 3.81 (dd, ^3J = 1.7 Hz, ^3J = 3.4 Hz, 1H, H-2_{Man}), 3.75 (m_c, 1H, OC*H*HCH$_2$NH), 3.72–3.67 (m, 2H, H-3_{Man}, H-$6b_{Man}$), 3.60 (dd ~ t, ^3J = 9.5 Hz, 1H, H-4_{Man}), 3.56–3.51 (m, 2H, H-5_{Man}, OCH*H*CH$_2$NH), 3.45–3.32 (m, 2H, OCH$_2$C*H*$_2$NH), 2.49 (t, ^3J = 7.1 Hz, 2H, CH$_2$*CH*$_2$SH), 2.19 (t, ^3J = 7.5 Hz, 2H, HN(O)CC*H*$_2$CH$_2$), 1.59 (m_c, 4H, HN(O)CCH$_2$C*H*$_2$, OCH$_2$C*H*$_2$CH$_2$S), 1.40 (m_c, 2H, CH$_2$C*H*$_2$CH$_2$), 1.35–1.25 (m, 12H, CH$_2$C*H*$_2$CH$_2$) ppm; ^{13}C NMR (125 MHz, CD_3OD, 300 K): δ = 176.5 (C(O)NH), 101.7 (C-1_{Man}), 74.8 (C-5_{Man}), 72.6 (C-3_{Man}), 72.1 (C-2_{Man}), 68.6 (C-4_{Man}), 67.3 (OCH$_2$CH$_2$NH), 62.9 (C-6_{Man}), 40.2 (OCH$_2$*C*H$_2$NH), 37.1 (HN(O)C*C*H$_2$CH$_2$), 35.2 (OCH$_2$*C*H$_2$CH$_2$S), 30.6, 30.5, 30.4, 30.3, 30.2, 29.4 (6 CH$_2$*C*H$_2$CH$_2$), 27.0 (HN(O)CCH$_2$*C*H$_2$), 25.0 (CH$_2$*C*H$_2$SH) ppm; HR-ESI MS: calcd for $C_{38}H_{72}N_2NaO_{14}S_2$ (disulphide): *m*/*z* 867.4317 [M + Na]$^+$; found: *m*/*z* 867.4309 [M + Na]$^+$; IR (ATR): $\tilde{\nu}$ = 3308, 2918, 2850, 1637, 1554, 1463, 1132, 1057, 1031, 975 cm^{-1}.

N-(Fluoren-9-yl-methoxycarbonyl)-*S*-(triphenylmethyl)-L-cysteine-[6-(α-D-mannopyranosyloxy)-3-thiahexyl]amide (8)

A mixture of Fmoc-L-Cys(Trt)-OH (**2**, 2.46 g, 4.18 mmol), mannoside **1** (1.37 g, 4.60 mmol), and HATU (1.91 g, 5.02 mmol) was dried for 1 h under vacuum and then dissolved in dry DMF (40 mL). It was cooled to 0 °C, DIPEA (853 μL, 5.02 mmol) was added and the reaction mixture stirred overnight at ambient temperature under a nitrogen atmosphere. The solvent was removed under reduced pressure and the crude product was purified by column chromatography (methanol–ethyl acetate, 1 : 12 → 1 : 9) yielding the title compound **8** (3.01 g, 3.48 mmol, 84%) as a colourless foam. R_f 0.38 (methanol–ethyl acetate, 1 : 9); $[\alpha]_D^{22}$ = +21.8 (*c* = 0.5, methanol); ^{1}H NMR (500 MHz, CD_3OD, 300 K): δ = 7.78 (m_c, 2H, H-aryl$_{Fmoc}$), 7.66 (d, ^3J = 6.8 Hz, 2H, H-aryl$_{Fmoc}$), 7.40–7.34 (m, 8H, H-aryl$_{Trt}$, H-aryl$_{Fmoc}$), 7.30–7.19 (m, 14H, H-aryl$_{Trt}$, H-aryl$_{Fmoc}$), 4.72 (d, ^3J = 1.6 Hz, 1H, H-1_{Man}), 4.41 (dd, ^2J = 10.6 Hz, ^3J = 7.1 Hz, 1H, C*H*H$_{Fmoc}$), 4.30 (dd, ^2J = 10.6 Hz, ^3J = 6.8 Hz, 1H, CH*H*$_{Fmoc}$), 4.23 (dd ~ t, ^3J = 6.8 Hz, 1H, CH$_{Fmoc}$), 3.93 (dd, ^3J = 8.4 Hz, ^3J = 5.5 Hz, 1H, H-α_{Cys}), 3.82 (dd, ^2J = 11.7 Hz, ^3J = 2.4 Hz, 1H, H-$6a_{Man}$), 3.78 (dd, ^3J = 3.3 Hz, ^3J = 1.6 Hz, 1H, H-2_{Man}), 3.77–3.74 (m, 1 H, OC*H*HCH$_2$CH$_2$S), 3.71 (dd,

^2J = 11.7 Hz, ^3J = 5.8 Hz, 1H, H-6b$_{Man}$), 3.67 (dd, ^3J = 9.4 Hz, ^3J = 3.3 Hz, 1H, H-3$_{Man}$), 3.61 (dd ~ t, ^3J = 9.6 Hz, 1H, H-4$_{Man}$), 3.54–3.50 (m, 1H, H-5$_{Man}$), 3.49–3.43 (m, 1H, OCH*H*CH$_2$CH$_2$S), 3.34–3.23 (m, 2H, SCH$_2$C*H*$_2$NH), 2.62–2.49 (m, 6H, OCH$_2$CH$_2$C*H*$_2$S, SC*H*$_2$CH$_2$NH, H-β$_{Cys}$), 1.79 (m$_c$, 2H, OCH$_2$C*H*$_2$CH$_2$S) ppm; ^{13}C NMR (125 MHz, CD$_3$OD, 300 K): δ = 172.7 (C(O)NH), 158.0 (OC(O)NH), 146.0 (C-aryl$_{Trt}$), 145.1, 142.6 (C-aryl$_{Fmoc}$), 130.8, 129.0 (CH-aryl$_{Trt}$), 128.8, 128.2 (CH-aryl$_{Fmoc}$), 127.9 (CH-aryl$_{Trt}$), 126.3, 120.9 (CH-aryl$_{Fmoc}$), 101.6 (C-1$_{Man}$), 74.7 (C-5$_{Man}$), 72.7 (C-3$_{Man}$), 72.2 (C-2$_{Man}$), 72.0 (C$_{q,Trt}$), 68.6 (C-4$_{Man}$), 68.1 (CH$_{2,Fmoc}$), 66.9 (OCH$_2$CH$_2$CH$_2$S), 62.9 (C-6$_{Man}$), 55.7 (C-α$_{ys}$), 48.4 (CH$_{Fmoc}$), 40.1 (SCH$_2$*C*H$_2$NH), 35.2 (C-β$_{Cys}$), 31.9 (S*C*H$_2$CH$_2$NH), 30.6 (OCH$_2$*C*H$_2$CH$_2$S), 29.3 (OCH$_2$CH$_2$*C*H$_2$S) ppm; MALDI-ToF MS (DHB): calcd for C$_{48}$H$_{52}$N$_2$NaO$_9$S$_2$: *m/z* 887.30 [M + Na]$^+$; found: *m/z* 887.50 [M + Na]$^+$; calcd for C$_{48}$H$_{52}$KN$_2$O$_9$S$_2$: *m/z* 903.27 [M + K]$^+$; found: *m/z* 903.48 [M + K]$^+$; IR (ATR): $\tilde{\nu}$ = 3316, 3055, 2924, 1705, 1660, 1521, 1490, 1445, 1318, 1230, 1130, 1084, 1029, 974, 739 cm^{-1}.

S-(Triphenylmethyl)-L-cysteine-[6-(α-D-mannopyranosyloxy)-3-thiahexyl]amide (9)

The Fmoc-protected cysteinyl mannoside **8** (406 mg, 467 μmol) was dissolved in dry DMF (5 mL) under a nitrogen atmosphere and morpholine (250 μL, 2.87 mmol) was added. The reaction mixture was stirred for 1 h at ambient temperature and then another portion of morpholine (250 μL, 2.87 mmol) was added. This was repeated after 3 h and 4 h and stirred overnight. After 18 h the volatile compounds were removed under reduced pressure and the crude product was purified by column chromatography (methanol–ethyl acetate–TEA, 100 : 20 : 1) yielding the title compound **9** (200 mg, 311 μmol, 67%) as a colourless syrup. R_f 0.28 (RP-18, methanol–water, 4 : 1); $[\alpha]_D^{22}$ = +22.8 (*c* = 0.26, methanol); ^{1}H NMR (500 MHz, CD$_3$OD, 300 K): δ = 7.41 (m$_c$, 6H, H-aryl$_{Trt}$), 7.29 (m$_c$, 6H, H-aryl$_{Trt}$), 7.22 (m$_c$, 3H, H-aryl$_{Trt}$), 4.74 (d, ^3J = 1.6 Hz, 1H, H-1$_{Man}$), 3.85–3.77 (m, 3 H, OC*H*HCH$_2$CH$_2$S, H-6a$_{Man}$, H-2$_{Man}$), 3.71 (dd, ^2J = 11.7 Hz, ^3J = 5.7 Hz, 1H, H-6b$_{Man}$), 3.69 (dd, ^3J = 9.6 Hz, ^3J = 3.4 Hz, 1H, H-3$_{Man}$), 3.61 (dd ~ t, ^3J = 9.6 Hz, 1H, H-4$_{Man}$), 3.55–3.46 (m, 2H, H-5$_{Man}$, OCH*H*CH$_2$CH$_2$S), 3.41–3.30 (m, 2H, SCH$_2$C*H*$_2$NH), 3.11 (dd ~ t, ^3J = 6.5 Hz, 1H, H-α$_{Cys}$), 2.65–2.59 (m, 4H, OCH$_2$CH$_2$C*H*$_2$S, SC*H*$_2$CH$_2$NH), 2.55 (dd, ^2J = 12.1 Hz, ^3J = 6.1 Hz, 1H, H-βa$_{Cys}$), 2.40 (dd, ^2J = 12.1 Hz, ^3J = 7.0 Hz, 1H, H-βb$_{Cys}$), 1.90 (s, 3H, NHCOC*H*$_3$), 1.83 (m$_c$, 2H, OCH$_2$C*H*$_2$CH$_2$S) ppm; ^{13}C NMR (125 MHz, CD$_3$OD, 300 K): δ = 175.5 (C(O)NH), 146.1 (C-aryl$_{Trt}$), 130.8, 129.0, 127.9 (CH-aryl$_{Trt}$), 101.6 (C-1$_{Man}$), 74.7 (C-5$_{Man}$), 72.7 (C-3$_{Man}$), 72.2 (C-2$_{Man}$), 68.6 (C-4$_{Man}$), 67.8 (C$_{q,Trt}$), 66.9 (OCH$_2$CH$_2$CH$_2$S), 62.9 (C-6$_{Man}$), 55.3 (C-α$_{Cys}$), 40.0 (SCH$_2$*C*H$_2$NH), 38.3 (C-β$_{Cys}$), 32.0 (S*C*H$_2$CH$_2$NH), 30.7 (OCH$_2$*C*H$_2$CH$_2$S), 29.3 (OCH$_2$CH$_2$*C*H$_2$S) ppm; HR-ESI MS: *m/z* = [Trt]$^+$ 243.1242; calcd for C$_{33}$H$_{43}$N$_2$O$_7$S$_2$: *m/z* 643.2506 [M + H]$^+$; found: *m/z* 643.2534 [M + H]$^+$; calcd for C$_{33}$H$_{42}$NaN$_2$O$_7$S$_2$: *m/z* 665.2326 [M + Na]$^+$; found: *m/z* 665.2323 [M + Na]$^+$; MALDI-ToF MS (DHB): calcd for C$_{33}$H$_{42}$NaN$_2$O$_7$S$_2$: *m/z* 665.23 [M + Na]$^+$; found: *m/z* 665.47 [M + Na]$^+$; IR (ATR): $\tilde{\nu}$ = 3303, 3062, 2921, 1658, 1519, 1488, 1443, 1318, 1261, 1129, 1084, 1054, 1024, 975, 803, 743 cm^{-1}.

N-(Acetyl)-*S*-(triphenylmethyl)-L-cysteine-[6-(α-D-mannopyranosyloxy)-3-thiahexyl]amide (10)

The glycoamino acid **9** was dissolved in pyridine (2 mL) and acetic anhydride (110 μL, 1.17 mmol) was added. The reaction mixture was stirred overnight at room temperature. Then solvents were removed under reduced pressure, it was codistilled with toluene three times (10 mL each) and the crude product was subjected to RP-MPLC (60 g RP-18, A: methanol, B: water, A: 40% → 95%, 120 min) yielding the title compound **10** (154 mg, 225 μmol, 96%) after lyophilisation. R_f 0.31 (ethyl acetate); $[\alpha]_D^{22}$ = +29.4 (*c* = 0.5, methanol); HPLCt_R = 2.64 min (A = water, B = methanol, A: 20%, 10 min, 1.2 mL min^{-1}); ^{1}H NMR (500 MHz, CD$_3$OD, 300 K): δ = 7.38 (m$_c$, 6H, H-aryl$_{Trt}$), 7.30 (m$_c$, 6H, H-aryl$_{Trt}$), 7.23 (m$_c$, 3H, H-aryl$_{Trt}$), 4.73 (d, ^3J = 1.6 Hz, 1H, H-1$_{Man}$), 4.19 (m$_c$, 1H, H-α$_{Cys}$), 3.83 (dd, ^2J = 11.7 Hz, ^3J = 2.4 Hz, 1H, H-6a$_{Man}$), 3.81–3.77 (m, 2H, OC*H*HCH$_2$CH$_2$S, H-2$_{Man}$), 3.71 (dd, ^2J = 11.7 Hz, ^3J = 5.6 Hz, 1H, H-6b$_{Man}$), 3.70–3.67 (m, 1H, H-3$_{Man}$), 3.61 (dd ~ t, ^3J = 9.6 Hz, 1H, H-4$_{Man}$), 3.55–3.50 (m, 1H, H-5$_{Man}$), 3.50–3.45 (m, 1H, OCH*H*CH$_2$CH$_2$S), 3.36–3.24 (m, 2H, SCH$_2$C*H*$_2$NH), 3.62–2.56 (m, 4H, OCH$_2$CH$_2$C*H*$_2$S, SC*H*$_2$CH$_2$NH), 2.56 (dd, ^2J = 12.4 Hz, ^3J = 6.3 Hz, 1H, H-βa$_{Cys}$), 2.49 (dd, ^2J = 12.4 Hz, ^3J = 7.7 Hz, 1H, H-βb$_{Cys}$), 1.91 (s, 3H, NH(O)CC*H*$_3$), 1.82 (m$_c$, 2H, OCH$_2$C*H*$_2$CH$_2$S) ppm; ^{13}C NMR (125 MHz, CD$_3$OD, 300 K): δ = 173.0 (CH$_3$*C*(O)NH), 172.4 (C(O)NH), 145.9 (C-aryl$_{Trt}$), 130.7, 129.0, 128.0 (CH-aryl$_{Trt}$), 101.6 (C-1$_{Man}$), 74.7 (C-5$_{Man}$), 72.7 (C-3$_{Man}$), 72.2 (C-2$_{Man}$), 68.6 (C-4$_{Man}$), 68.0 (C$_{q,Trt}$), 66.9 (OCH$_2$CH$_2$CH$_2$S), 62.9 (C-6$_{Man}$), 54.0 (C-α$_{Cys}$), 40.1 (SCH$_2$*C*H$_2$NH), 34.9 (C-β$_{Cys}$), 31.9 (S*C*H$_2$CH$_2$NH), 30.7 (OCH$_2$*C*H$_2$CH$_2$S), 29.3 (OCH$_2$CH$_2$*C*H$_2$S), 22.5 (NHCO*C*H$_3$) ppm; HR-ESI MS: calcd for C$_{35}$H$_{44}$N$_2$NaO$_8$S$_2$: *m/z* 707.2431 [M + Na]$^+$; found: *m/z* 707.2426 [M + Na]$^+$; MALDI-ToF MS (Cl-CCA): calcd for C$_{35}$H$_{44}$N$_2$NaO$_8$S$_2$: *m/z* 707.24 [M + Na]$^+$; found: *m/z* 707.33 [M + Na]$^+$, calcd for C$_{35}$H$_{44}$KN$_2$O$_8$S$_2$: *m/z* 723.22 [M + K]$^+$; found: *m/z* 723.31 [M + K]$^+$; IR (ATR): $\tilde{\nu}$ = 3284, 2926, 1645, 1535, 1489, 1443, 1372, 1202, 1131, 1085, 1055, 1031, 975, 742, 698, 675 cm^{-1}.

6-(10-Tritylsulphanyl-undecanoyl)amino-4-thiahexyl-α-D-mannopyranoside (11)

11-Tritylsulphanyl-undecanoic acid (**3**, 194 mg, 420 μmol) and HATU (319 mg, 840 μmol) were dried for 1 h under vacuum, dry DMF (2.50 mL) and DIPEA (288 μL, 1.68 mmol) were added and the mixture was stirred for 20 min under a nitrogen atmosphere at ambient temperature. Simultaneously in a different reaction vessel **7** (150 mg, 504 μmol) was dried for 1 h under vacuum, dissolved in dry DMF (2.50 mL) and stirred for 20 min under a nitrogen atmosphere at ambient temperature. The reaction mixture with the preactivated 11-tritylsulphanyl-undecanoic acid (**3**) and HATU was cooled to 0 °C, the solution of mannoside **7** was added and it was stirred under a nitrogen atmosphere at ambient temperature overnight. Then, all volatile compounds were removed under reduced pressure and the crude product was subjected to MPLC (150 g silica column, A: ethyl acetate, B: methanol, A: 99% → 85%, 120 min) and RP-MPLC (60 g RP-18, A: methanol, B: water, A: 50% → 95%, 120 min) yielding the title compound **11** (242 mg, 327 μmol, 78%) as a

colourless foam. R_f 0.29 (ethyl acetate), $[\alpha]_D^{22}$ = +25.3 (c = 0.5, methanol); HPLCt_R = 6.11 min (A = water, B = methanol, A: 20%, 10 min, 1.2 mL min^{-1}); ^{1}H NMR (500 MHz, CD_3OD, 300 K): δ = 7.38 (m_c, 6H, H-aryl$_{Trt}$), 7.26 (m_c, 6H, H-aryl$_{Trt}$), 7.19 (m_c, 3H, H-aryl$_{Trt}$), 4.75 (d, ^3J = 1.7 Hz, 1H, H-1$_{Man}$), 3.85–3.80 (m, 2H, H-6a$_{Man}$, OC*H*HCH$_2$CH$_2$S), 3.79 (dd, ^3J = 3.3 Hz, ^3J = 1.7 Hz, 1H, H-2$_{Man}$), 3.72 (dd, ^2J = 11.7 Hz, ^3J = 5.6 Hz, 1H, H-6b$_{Man}$), 3.70 (dd, ^3J = 9.3 Hz, ^3J = 3.3 Hz, 1H, H-3$_{Man}$), 3.62 (dd ~ t, ^3J = 9.5 Hz, 1H, H-4$_{Man}$), 3.56–3.48 (m, 2H, H-5$_{Man}$, OCH*H*CH$_2$CH$_2$S), 3.37–3.32 (m, 2H, SCH$_2$C*H*$_2$NH), 2.63 (m_c, 4H, OCH$_2$CH$_2$C*H*$_2$S, SC*H*$_2$CH$_2$NH), 2.17 (t, ^3J = 7.5 Hz, 2H, HN(O)CC*H*$_2$CH$_2$), 2.11 (t, ^3J = 7.4 Hz, 2H, C*H*$_2$STrt), 1.89–1.81 (m, 2H, OCH$_2$C*H*$_2$CH$_2$S), 1.59 (quint., ^3J = 7.6 Hz, 2H, HN(O)CCH$_2$C*H*$_2$), 1.38–1.09 (m, 14H, CH$_2$C*H*$_2$CH$_2$) ppm; ^{13}C NMR (125 MHz, CD_3OD, 300 K): δ = 176.4 (C(O)NH), 146.5 (C-aryl$_{Trt}$), 130.8, 128.8, 127.7 (CH-aryl$_{Trt}$), 101.6 (C-1$_{Man}$), 74.7 (C-5$_{Man}$), 72.7 (C-3$_{Man}$), 72.2 (C-2$_{Man}$), 68.6 (C-4$_{Man}$), 67.6 (C$_{q,Trt}$), 66.9 (O*C*H$_2$CH$_2$CH$_2$S), 62.9 (C-6$_{Man}$), 40.1 (SCH$_2$*C*H$_2$NH), 37.1 (HN(O)C*C*H$_2$CH$_2$), 32.9 (CH$_2$STrt), 32.1 (S*C*H$_2$CH$_2$NH), 30.7 (OCH$_2$*C*H$_2$CH$_2$S), 30.5, 30.4, 30.4, 30.3, 30.1, 30.0, 29.7 (7 CH$_2$*C*H$_2$CH$_2$), 29.3 (OCH$_2$CH$_2$*C*H$_2$S), 27.0 (HN(O)CCH$_2$*C*H$_2$) ppm; HR-ESI MS: calcd for $C_{41}H_{57}NNaO_7S_2$: m/z 762.3469 [M + Na]$^+$; found: m/z 762.3453 [M + Na]$^+$; MALDI-ToF MS (DHB): calcd for $C_{41}H_{57}NNaO_7S_2$: m/z 762.35 [M + Na]$^+$; found: m/z 762.52 [M + Na]$^+$; calcd for $C_{41}H_{57}KNO_7S_2$: m/z 778.32 [M + K]$^+$; found: m/z 778.52 [M + K]$^+$; IR (ATR): $\tilde{\nu}$ = 3296, 2922, 2852, 1643, 1544, 1488, 1443, 1130, 1084, 1054, 1030, 975, 811, 741, 697, 676, 616 cm^{-1}.

Abbreviations

DIPEA	*N*,*N*-diisopropylethylamine,
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide,
HATU	*O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyl-uronium hexafluorophosphate,
NHS	*N*-hydroxysuccinimide,
PBS	phosphate buffered saline,
PBST	phosphate buffered saline + 0.05% v/v Tween® 20,
TEA	triethylamine.

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