Chemical synthesis of globotriose and galabiose: relative stabilities of their complexes with *Escherichia coli* Shiga-like toxin-1 as determined by denaturation-titration with guanidinium chloride

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Globotriose [α-D-Gal-(1→4)-β-D-Gal-(1→4)-D-Glc] is the carbohydrate moiety of the globotriosyl ceramide (Gb_3), also known as the germinal centre B-cell differentiation antigen CD77, a glycolipid present on the plasma membrane of certain mammalian cells. In Gb_3, globotriose functions as the cell-surface receptor for Shiga toxin and for the Shiga-like toxins (verocytotoxins). Here we report the chemical synthesis of globotriose and the corresponding terminal disaccharide, galabiose [α-D-Gal-(1→4)-β-D-Gal]. Globotriose and galabiose are attached *via* a linker to CNBr-activated Sepharose to generate affinity matrices that permit the one-step purification of recombinant Shiga-like toxin-1 from crude *E. coli* homogenates. Toxin is released from either of the immobilised saccharides by elution with 6 M guanidinium chloride. After dilution of the denaturant, the released toxin had full catalytic activity. Denaturation-titration experiments show that the bound toxin is released from galabiose-Sepharose at 2.3 M guanidinium chloride, while its release from globotriose-Sepharose requires a higher concentration of 4.8 M. These results indicate that the glucose component of globotriose contributes ~2.6 kcal mol^{-1} to the binding energy relative to galabiose.

Introduction

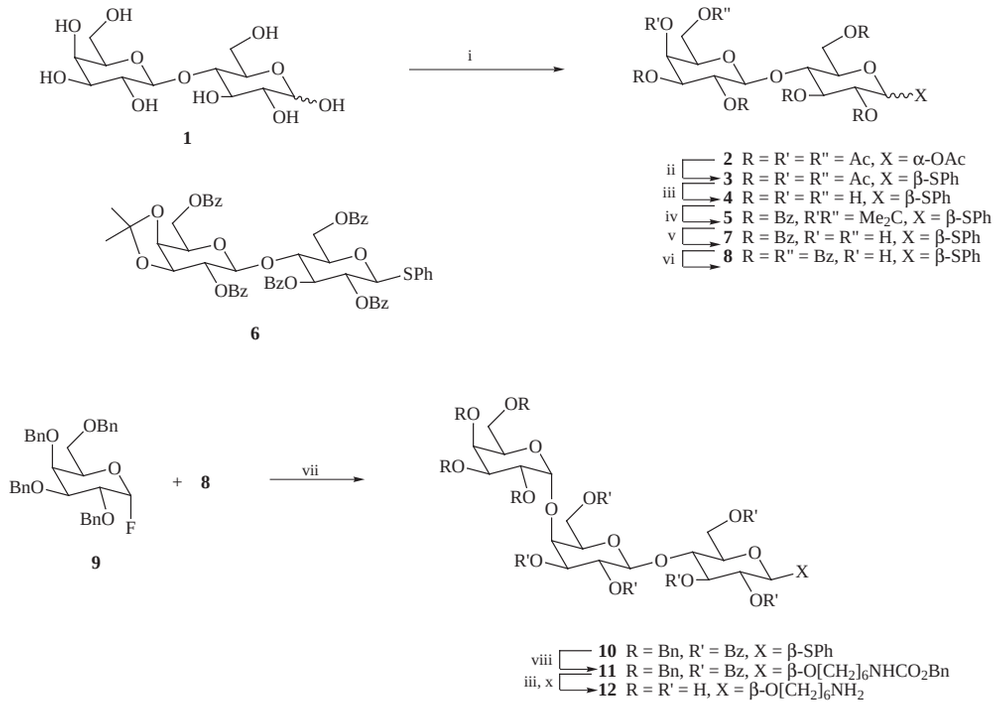
The plasma membrane glycolipid globotriosyl ceramide (Gb_3) functions as the receptor on sensitive cells responsible for the binding and subsequent internalisation of Shiga toxin (ST), produced by the bacterium *Shigella dysenteriae*, and the Shiga-like toxins (SLTs, also known as verocytotoxins) produced by enterohaemorrhagic strains of *Escherichia coli*.[1–4] ST and SLTs consist of a single catalytically active polypeptide of 32 kDa (the A subunit) non-covalently associated with a pentamer of identical Gb_3-binding polypeptides (the B subunits) each of 7.7 kDa. During endocytic uptake the A chain is cleaved at a furin-sensitive site to generate a catalytic A1 domain which remains disulfide-bonded to a B chain-interacting A2 domain. The A1 fragment can subsequently enter the cytosol from the endoplasmic reticulum lumen. Within the cytosol, A1 acts as an *N*-glycosidase that cleaves a specific adenine residue from a highly conserved loop in 28S ribosomal RNA. The adenine residue that is removed by the toxin normally plays an essential role during protein synthesis by binding elongation factors. Ribosomes containing toxin-depurinated 28S RNA are therefore unable to synthesise proteins, which in turn leads rapidly to cell death.[5–7] Recently, Gb_3 has been shown to act as a receptor for binding of *Burkholderia* (*Pseudomonas*) *cepacia* to epithelial cells *via* an adhesin in cystic fibrosis patients. Infection by *B. cepacia* can give rise to rapidly fatal deterioration with pneumonia and septicaemia, the so-called 'cepacia syndrome'.[7,8]

Recent outbreaks of food poisoning involving the SLT-producing *E. coli* strain 0157:H7 have focused attention on the diseases caused by this organism. SLTs are involved in the etiology of haemorrhagic colitis and the more serious haemolytic uraemic syndrome (HUS), where they bind to and enter the microvascular endothelial cells of the gastrointestinal tract and the kidney respectively. Intestinal and kidney cells are vulnerable to the toxins since, unlike most human cell types, they have a significant cell surface content of Gb_3. The high specificity of the carbohydrate moiety of Gb_3 for SLTs suggests that it may have potential as a therapeutic agent. For example, free receptor or receptor immobilised on an appropriate inert non-toxic matrix could potentially be used in the case of *E. coli* 0157 infections to intercept SLTs before they reach target cells in the kidney. As a preliminary to such studies, we now describe the chemical synthesis of globotriose and galabiose, and show that the immobilised carbohydrates are capable of specifically binding SLT-1 from *E. coli* 0157.

Chemical synthesis

Syntheses of globotriose or derivatives have been reported by a number of groups.[9–16] The objective of our synthesis was to prepare the trisaccharide thioglycoside building block **10** (Scheme 1). This was expected to be chemically stable but easy to activate for the preparation of a variety of derivatives. Thiophenyl β-lactoside **4** was prepared from lactose **1** in three high-yielding steps *via* the polyacetates **2** and **3**.[9] Formation of the isopropylidene derivative of glycoside **4**, followed by benzoylation without isolation, was carefully controlled (see Experimental section) to favour formation of the 4,6- (kinetic) derivative **5**, rather than the 3,4- (thermodynamic) derivative **6** that was formed with longer reaction times in the isopropylidenation reaction. The yield of the 4,6-isopropylidene derivative **5** was favoured by using an excess of 2,2-dimethoxypropane and a controlled amount of acid catalyst. Benzoylation to the fully protected intermediate **5** followed by removal of the isopropylidene group gave the intermediate **7**. This was selectively benzoylated on the 6′-hydroxy group by using benzoyl cyanide. By careful control of the reaction conditions, the required 6-benzoylated product **8** was obtained in 90% yield.

The acceptor **8** was coupled with the glycosyl fluoride donor **9** as in an analogous reaction described by Nicolaou[9] to give the protected target trisaccharide glycoside **10** in 68–95% yield. The yield was dependent on the ratio of donor to acceptor. With a ratio of 3.4:1 the product was obtained in 95% yield, based on acceptor. However, in practice, it was more economical to use a ratio of 1:1 as the excess of the more valuable acceptor **8** could be recovered quantitatively by chromatography of the product mixture.



Scheme 1 *Reagents*: i, Ac_2O, DMAP, pyridine; ii, $PhSSiMe_3$, ZnI_2; iii, MeONa, MeOH; iv, $MeC(OMe)_2Me$, TsOH; v, TFA; vi, PhCOCN, NEt_3; vii, $SnCl_2$, AgOTf; viii, $HO[CH_2]_6NHCO_2Bn$, AgOTf, Br_2; ix, cyclohexene, 20% $Pd(OH)_2$–C

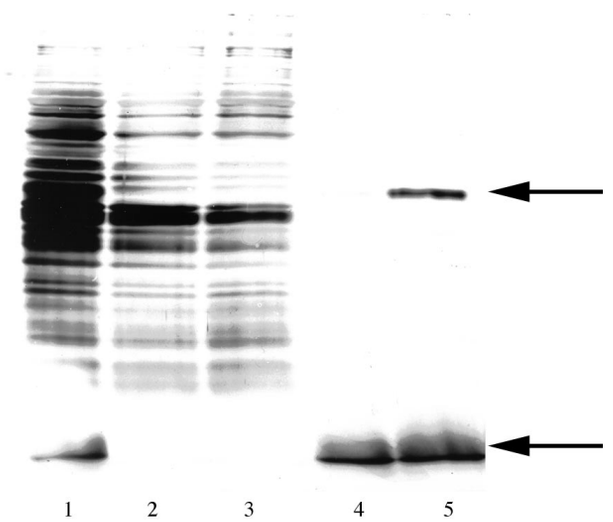


Fig. 1 Binding of *E. coli* Shiga-like toxin 1 to immobilised globotriose. Silver-stained SDS-PAGE of various protein fractions. Lanes 1: total *E. coli* proteins applied to the column; Lanes 2 and 3: *E coli* proteins that do not bind to the globotriose matrix; Lanes 4 and 5: *E. coli* Shiga-like toxin 1 eluted from the matrix in 6 M guanidinium chloride. Upper arrow: A chain; lower arrow: B chain.

For coupling to a solid support a 6-aminohexanol spacer arm was chosen. This was converted into the benzoyloxycarbonyl derivative and coupled to the trisaccharide thioglycoside **10** by *in situ* glycosyl bromide formation and activation with silver trifluoromethanesulfonate (triflate).[17] By this means the spacer arm was attached in 84% yield to give exclusively the β-glycosidic product **11**. A two-step deprotection sequence gave the unprotected globotriose with an attached spacer arm, compound **12**. This was coupled to CNBr-activated Sepharose 4B.

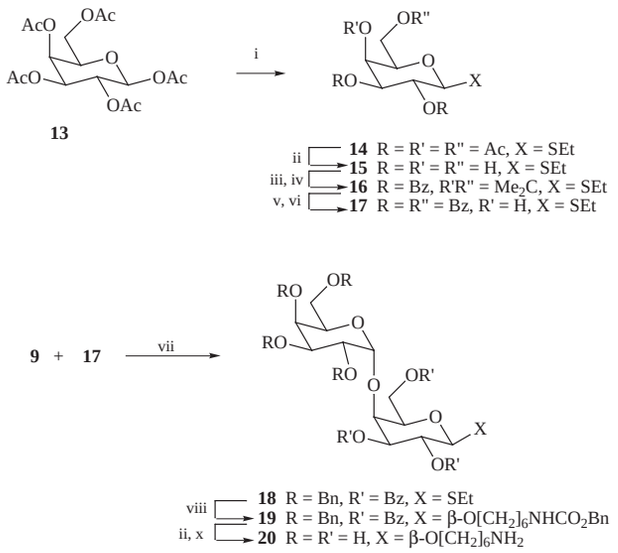
The immobilised trisaccharide was highly effective in selectively binding Shiga toxin (Fig. 1). Lanes 1–3 show the total periplasmic protein mixture and the mixture eluted from the column in 0.5 M NaCl in phosphate-buffered saline (PBS), respectively. Lanes 4 and 5 show the protein mixture subsequently eluted with 6 M guanidinium chloride (pH 6.7).

We were interested to study the interaction between the B_5 pentamer and the corresponding disaccharide derivative α-D-Gal-(1→4)-β-D-GalO$[CH_2]_6$NH-Sepharose (galabioseO$[CH_2]_6$-NH-Sepharose). Such a study would help to illuminate the question of docking the B monomer with the carbohydrate acceptor. It would also have a practical outcome in showing whether or not the disaccharide would provide adequate binding for purification purposes. This was a likely outcome since globotriose immobilised on amino-Fractogel[18] had been shown to be effective for the purification of the toxin. In this material, the integrity of the reducing glucose unit must have been destroyed. The disaccharide analogue was synthesised as shown in Scheme 2 by following a procedure closely analogous to that used for the synthesis of the globotriose preparation. In practice, it proved to be as effective as the immobilised trisaccharide for the toxin's purification.

Biological results

Release of Shiga toxin from affinity columns is routinely carried out by elution with 6 M guanidinium chloride. Following a different procedure, experiments were carried out on binding and release of SLT-1 B chain produced in *E. coli* strain JM105 transformed with pSLTwt (a plasmid coding the SLT-1 operon under control of the lac promoter). In these experiments, the concentration of guanidinium chloride in the eluent was increased stepwise from 0 M to 6 M and the concentration at which toxin was released was determined by estimation of protein in the eluate. The results are shown in Fig. 2a,b. Also shown, in Fig. 2c, is the elution profile for a commercial sample of trisaccharide immobilised on Fractogel. The elution profiles clearly show that toxin is released from the disaccharide column at a concentration significantly lower than that required to release it from the trisaccharide column. The results obtained with the commercial material (Fig. 2c) were very similar to those obtained with the disaccharide column (Fig. 2b), consistent with the suggestion, above, that the recognition unit in this preparation is the terminal disaccharide component of the original globotriose.

The interaction between guanidinium chloride and proteins has been extensively studied, particularly in relation to protein folding and unfolding.[19] Tanford has described an approach to



Scheme 2 *Reagents*: i, EtSH, $SnCl_4$; ii, MeONa, MeOH; iii, $MeC(OMe)_2Me$, TsOH; iv, BzCl, pyridine; v, TFA; vi, BzCN, Et_3N; vii, AgOTf, $SnCl_2$; viii, $HO[CH_2]_6NHCO_2Bn$, AgOTf, Br_2; ix, cyclohexene, 10% Pd–C

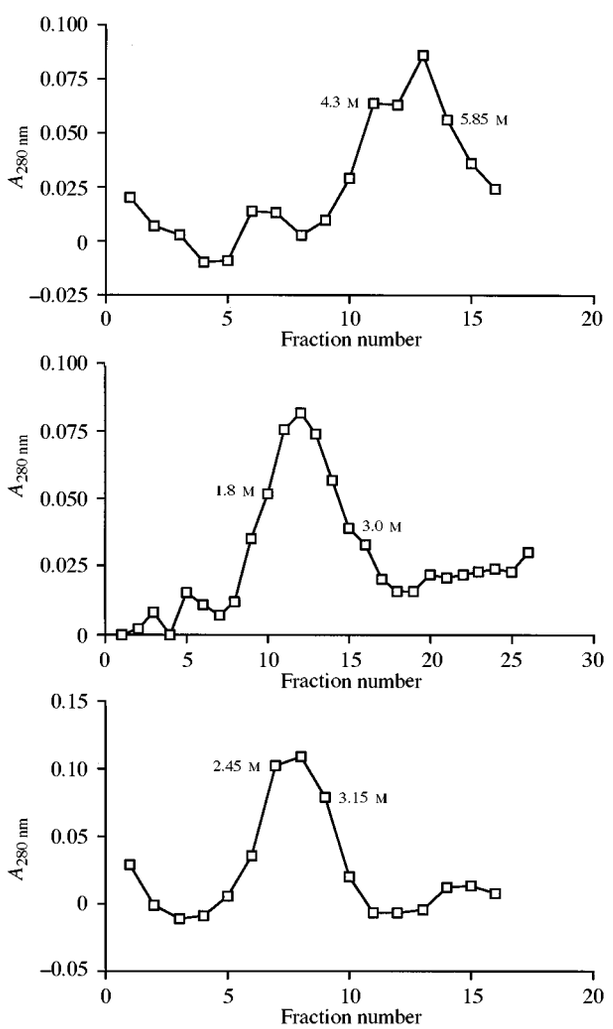


Fig. 2 Denaturation-titration of Shiga toxin B chain bound to (a) globotriose-Sepharose, (b) galabiose-Sepharose, (c) globotriose-amino-Fractogel

the estimation of the free energy of unfolding in terms of data obtained from solubility studies on amino acids and peptides [equation (1)],[20,21] where ΔG_D is the free energy of unfolding in a

$$\Delta G_D = \Delta G_D^{H_2O} + \sum_i a_i n_i \delta g_{tr,i} \quad (1)$$

solution of the denaturant, $\Delta G_D^{H_2O}$ is the corresponding free energy of unfolding in water, $\delta g_{tr,i}$ is the free energy of transfer of a group of type i from water to denaturant, n_i is the total number of groups of type i in the protein and a_i is the average fractional change in the degree of exposure of groups of type i on unfolding. Since individual a_i values are not readily accessible, an average value $\bar{a}$, is usually used, leading to the unfolding equation (2). Values of δg_{tr} have been determined

$$\Delta G_D = \Delta G_D^{H_2O} + \bar{a}\sum_i n_i \delta g_{tr,i} \quad (2)$$

for transfer of the peptide group and amino acid side chains from water to guanidinium chloride solutions of different concentrations. If the populations, n_i, of specific amino acids in a given protein, are known, $\Delta G_D^{H_2O}$, the free energy of unfolding in water, can be calculated if $\bar{a}$ and ΔG_D for a given denaturant at a specified concentration can be determined. Using the free energies of transfer from amino acids given by Tanford and from the known amino acid sequence of verotoxin B subunits, the terms $\sum_i n_i \delta g_{tr,i}$ in equation (2) can be calculated for the concentrations of guanidinium chloride corresponding to the peaks of the elution profiles in Fig. 2a,b. These are 19.08 kcal mol^{-1} (for 2.25 M guanidinium chloride) and 32.11 kcal mol^{-1} for 5.28 M guanidinium chloride† (Fig. 2a). If it is assumed that the peak values of the guanidinium chloride concentrations for the trisaccharide (Fig. 2a) and disaccharide (Fig. 2b) correspond to the mid-points of the denaturation curves, then $\Delta G_D = 0$ [equation (2)] for both cases. Accordingly the difference in free energies of unfolding in water, $\Delta\Delta G_D^{H_2O}$, with simultaneous release of carbohydrate, of B subunits bound to disaccharide and trisaccharide respectively, is given by equation (3).

$$\Delta\Delta G_D^{H_2O} = \bar{a}(32.1 - 19.1) = 13\bar{a} \quad (3)$$

The overall equilibria involved in the binding and release of the B chain are shown in Fig. 3, where B_N represents the B chain

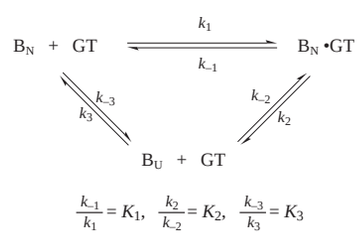


Fig. 3 Equilibria between immobilised globotriose, native Shiga toxin B chain (B_N) and unfolded Shiga toxin B chain (B_U)

in its native state, B_N·GT its complex with globotriose, GT, and B_U the unfolded B chain. Because this is a thermodynamic cycle, we can write equation (4) where the subscripts correspond to those of the equilibrium constants of Fig. 3. For the corresponding equilibria for disaccharide (galabiose) binding, indicated by primes, we can write equation (5). However,

$$-\Delta G_3 = \Delta G_1 - \Delta G_2 \quad (4)$$

$$-\Delta G_3' = \Delta G_1' - \Delta G_2' \quad (5)$$

$-\Delta G_3 = -\Delta G_3'$, since both correspond to the unfolding of the B chain in the absence of bound carbohydrate. Accordingly equations (4) and (5) lead to equation (6). This leads to the

† 1 cal = 4.184 J.

$$\Delta G_1 - \Delta G_1' = \Delta G_2 - \Delta G_2' \quad (6)$$

conclusion that the free-energy difference for unfolding-release of carbohydrate between globotriose and galabiose complexes is equal to the difference between the binding energies of the two carbohydrates. The unfolding-carbohydrate release term is $\Delta G_2 - \Delta G_2'$ which corresponds to $\Delta\Delta G_D^{H_2O}$ in equation (3). This was calculated as $\bar{a} \times 13.0$ kcal mol^{-1}. The value of $\bar{a}$ for this system has not been determined. However, values reported in the literature for globular proteins vary about a median value of 0.3, typically rising as high as 0.5 and going down to 0.17.[22] The value for verotoxin B chain is likely to be at the lower end of this range for the following reason. The parameter $\bar{a}$ is a measure of the fractional increase in exposure of a group in going from the native to the unfolded state. For individual peptide groups and side chains, $\bar{a}_i$, in general, will be larger for groups and side chains buried inside a protein than for those on the surface. For a sphere, the ratio of surface area to volume varies as $3/r$ where r is the radius. As the radius decreases, the ratio of surface area to volume increases. It follows that for roughly spherical globular proteins, the ratio surface groups to interior groups will increase as the size of the protein decreases. Accordingly, in general, it can be predicted that the smaller the protein, the smaller will be the value of $\bar{a}$ unless the protein contains a high proportion of amino acids with side chains for which δ_{gtr} is small or negligible. If a value of 0.2 is assumed for verotoxin B chain unfolding. $\Delta\Delta G_D^{H_2O} = 13.0 \times 0.2 = 2.6$ kcal mol^{-1}. Using microcalorimetry, it has been determined that the free energy of binding of soluble globotriose to verotoxin B chain is −3.6 kcal mol^{-1}.[23] The binding energy for galabiose can thus be estimated as 1 kcal mol^{-1}. This value is clearly too low and suggests that unfolding of the B chain should have an $\bar{a}$-value less than 0.2. However, it should be noted that the conditions under which the microcalorimetric measurements were made (β-methyl glycoside of globotriose and B pentamer in solution) and those of the present study (soluble B pentamer and immobilised globotriose/galabiose) were different. The microcalorimetric measurement was of binding of globotriose to B pentamer. However, in the experiments with immobilised globotriose/galabiose it appears more probable that the binding observed was between single B monomers and carbohydrate for the following reasons. First, it would be expected that dissociation of B pentamer into subunits would be promoted at concentrations of guanidinium chloride different from those required to unfold the protein with simultaneous release of carbohydrate. In this case, release of B chains bound to immobilised globotriose/galabiose would be expected to occur in two bursts, one corresponding to pentamer dissociation with release of four monomer units, and the other to unfolding with simultaneous release from globotriose of the remaining monomer. This was not seen. It is possible that pentamer dissociation and unfolding required similar concentrations of denaturant so that protein would be released on a single burst. However, this conclusion is ruled out by the fact that markedly different concentrations of denaturant were required to release B chains from bound galabiose and guanidinium. The experiment with galabiose showed that B chain could be unfolded in the presence of 2.25 M guanidinium chloride. Clearly, B_5 pentamer must be dissociated at or below this concentration. However, in the experiment with bound globotriose there was negligible release of B chain with 2.25 M guanidinium chloride. It follows that, in both experiments, B monomer only was bound. The possibility of multivalent attachment is ruled out by the average spacing between activated sites in the CNBr-activated Sepharose used to immobilise the carbohydrate. This was too large to permit binding of two or more carbohydrate molecules at a distance apart small enough to permit simultaneous binding to a B_5 pentamer. The only other possible conclusion would be that when B pentamer was bound to globotriose, binding of subunits to each other was significantly greater than when it was bound to galabiose.

In the absence of X-ray crystallographic data for globotriose bound to B pentamer, Nyholm *et al.*[24] have studied the interaction by molecular modelling. According to these studies, the glucose component of globotriose interacts only weakly with B monomer *via* a putative hydrogen bond from the 6-OH group to the carbonyl group of Gly60. Although the addition of glucose in going from galabiose to globotriose might alter the conformational stability of the galabiose component of globotriose, the addition of one stabilising hydrogen bond (worth ~1 kcal mol^{-1} net in binding energy) would be consistent with the change in binding energy estimated above from the denaturation-elution studies.

Given the weak binding of globotriose to B pentamer ($K_s = 1–2 \times 10^{-3}$) and the weaker binding of galabiose, it is surprising that both are so effective in immobilised form for the purification of the protein. It seems likely that the microenvironment of the bound galabiose and globotriose may be important in this respect. This possibility deserves further study, particularly in relation to the influence of microenvironment on the binding of proteins to membrane-associated carbohydrate receptors *in vivo*.

Experimental

NMR spectra were recorded on a Bruker WH250 (250 MHz, ^{1}H; 62.5 MHz, ^{13}C) or a WH400 (400 MHz, ^{1}H; 100 MHz, ^{13}C) spectrometer. Chemical shifts are reported in parts per million (δ) relative to tetramethylsilane; J-values are given in Hz. Optical rotations were determined with an Optical Activity LTD AA-1000 polarimeter at 589 and 546 nm; $[\alpha]_R$-values are given in units of 10^{-1} deg cm^2 g^{-1}. Mps were measured with a Gallenkamp Melting Point Apparatus in an open tube and are uncorrected. All reactions were carried out under nitrogen and anhydrous conditions with dry, freshly distilled solvents where necessary. Yields are usually taken after purification and refer to chromatographically pure materials unless otherwise noted. All reactions were monitored by TLC on 0.25 mm silica gel 60 plates (60F-254) (Merck) with UV light and/or basic aq. $KMnO_4$ (2 g $KMnO_4$ and 5 g K_2CO_3 solved in 100 cm^3 distilled water). Moisture- and air-sensitive operations were generally carried out in a glove bag under dry nitrogen. Solvents were evaporated off under reduced pressure at 45 °C bath temperature. Column chromatography was performed following the 'flash'-method using silica gel 60 (Merck). Light petroleum had boiling range 40–60 °C.

Phenyl 2,3-di-*O*-benzoyl-4,6-isopropylidene-β-D-galactopyranosyl-(1→4)-2,3,6,tri-*O*-benzoyl-1-thio-β-D-glucopyranoside 5

To a stirred solution of lactose glycoside **4** (3.0 g, 6.9 mmol)[9] and toluene-*p*-sulfonic acid (TsOH) (98 mg, 0.35 mmol) in dry DMF (20 cm^3) was added 2,2-dimethoxypropane (3.6 cm^3, 29.3 mmol). After 90 min the mixture was quenched by the addition of triethylamine (1 cm^3), concentrated, and dried under reduced pressure for 2 h. The residue was re-dissolved in dry pyridine (20 cm^3). Benzoyl chloride (6 cm^3) was added at 0 °C. The cooling bath was removed after 30 min and the mixture was stirred at rt for a further 2.5 h. The mixture was diluted with ethyl acetate (100 cm^3) and CH_2Cl_2 (100 cm^3), washed (successively) five times with 0.2 M aq. copper(II) sulfate and once with distilled water. The organic layer was dried ($MgSO_4$) and the solvent was evaporated under reduced pressure. The resulting oil was dried under reduced pressure and purified by flash chromatography (8 × 20 cm; toluene–ethyl acetate [19 : 1]) to give derivative **5** (4.70 g, 68%), R_f 0.23 (toluene–ethyl acetate [9 : 1]); mp 122 °C; δ_H(400 MHz; $CDCl_3$) 1.15 (3 H, s, CH_3), 1.23 (3 H, s, CH_3), 2.86 (1 H, br d, H-5′), 3.37 (1 H, dd, J 1.8 and 12.7, H^b-6′), 3.49 (1 H, dd, J 2.7 and 12.7, H^a-6′), 3.90 (1 H, ddd, J 2.1, 5.1 and 9.4, H-5), 4.11 (1 H, dd, J 9.4 and 9.1, H-4),

4.25 (1 H, apparent d, *J* 3.6, H-4′), 4.34 (1 H, dd, *J* 12.0 and 5.1, H^b-6), 4.68 (1 H, dd, *J* 12.0 and 2.1, H^a-6), 4.75 (1 H, d, *J* 7.9, H-1′), 4.90 (1 H, d, *J* 10.2, H-1), 5.04 (1 H, dd, *J* 10.4 and 3.6, H-3′), 5.70 (1 H, dd, *J* 10.2 and 7.9, H-2), 5.84 (1 H, dd, *J* 7.9 and 9.1, H-3), 7.00–7.60 (18 H, m, ArH) and 7.85–8.0 (12 H, m, ArH); δ_C(100 MHz; $CDCl_3$) 18.9 (CH_3), 28.3 (CH_3), 61.5 (C-6′), 62.4 (C-6), 65.8 (C-4′), 66.7 (C-5′), 69.5 (C-2′), 70.6 (C-2), 72.6 (C-3′), 74.9 (C-3), 76.3 (C-4), 76.7 (C-5), 85.5 (C-1), 98.7 (CMe_2), 101.1 (C-1′), 127.9, 128.0, 128.1, 128.2, 128.2, 128.3, 128.6, 128.7, 128.8, 129.0, 129.1, 129.4, 129.5, 129.6, 129.7 and 129.8 (Ar CH), 131.4, 132.8, 132.9, 133.0, 133.1 and 133.1 (ArC) and 164.7, 164.9, 165.2, 165.4 and 165.9 (CO); *m*/*z* (FAB) 1041 (M + 2Na, 0.1%), 980 (0.2) 885 (0.5), 411 (6.8), 353 (0.6), 323 (0.3), 289 (0.8), 231 (1.9), 165 (1.1), 154 (5.6), 149 (1.3), 136 (6.8), 123 (1.3), 105 (100), 77 (22) and 52 (8.6).

Phenyl 2,3-di-*O*-benzoyl-β-D-galactopyranosyl-(1→4)-2,3,6,tri-*O*-benzoyl-1-thio-β-D-glucopyranoside 7

To a cold (0 °C) solution of compound **5** (4.7 g, 4.72 mmol) in THF (20 cm^3) and distilled water (7.3 cm^3) was added TFA (17 cm^3). After the mixture had been stirred for 15 min a solid precipitated. The cooling bath was removed and additional THF (15 cm^3) was added. The reaction was monitored by TLC. After 45 min the mixture was poured into saturated aq. $NaHCO_3$ and the product was extracted with ethyl acetate (4 × 20 cm^3). The combined organic layers were dried ($MgSO_4$) and the solvents were evaporated off under reduced pressure. The crude solid were purified by flash chromatography with toluene–ethyl acetate 2:1 as eluent to give glycoside **7** (3.90 g, 87%), mp 211 °C; R_f 0.23 (toluene–ethyl acetate [2:1]); 0.42 (toluene–ethyl acetate [1:1]); $[\alpha]_D^{23}$ + 88.4 (*c* 1.00, $CHCl_3$); δ_H(250 MHz; $CDCl_3$) 3.20–3.43 (3 H, m), 3.87–3.96 (1 H, m, H-5), 4.10 (1 H, dd, *J* ~9.4 and 9.4), 4.19 (1 H, d, *J* 3.2), 4.42 (1 H, dd, *J* 5.5 and 11.9, H^b-6), 4.65 (1 H, dd, *J* 2.0 and 11.9, H^a-6), 4.77 (1 H, d, *J* 8.1, H-1′), 4.90 (1 H, d, *J* 9.9, H-1), 5.09 (1 H, dd, *J* 3.2 and 10.5), 5.39 (1 H, dd, *J* ~9.5 and 9.5), 5.78–5.80 (2 H, m), 7.03–7.68 (20 H, m, ArH) and 7.85–8.02 (10 H, m, ArH); *m*/*z* (FAB-CI^+) 978 (M + Na^+, 0.2%), 845 (1.3), 475 (1.6), 371 (8.0), 353 (1.7), 249 (4.0), 231 (1.0), 201 (1.0), 189 (1.2), 165 (1.4), 154 (5.2), 136 (5.6), 105 (100) and 77 (20).

Phenyl 2,3,6-tri-*O*-benzoyl-β-D-galactopyranosyl-(1→4)-2,3,6,tri-*O*-benzoyl-1-thio-β-D-glucopyranoside 8

To a solution of glycoside **7** (2.01 g, 2.09 mmol) in dry DMF (30 cm^3) was added dry trimethylamine (0.35 cm^3, 2.5 mmol). After cooling of this mixture to −20 °C (solid CO_2 in CCl_4), benzoyl cyanide (0.25 cm^3, 2.09 mmol) was added. The cooling bath was replaced by an ice-bath after 15 min. The reaction was quenched with methanol (1 cm^3) after a total reaction time of 30 min. The reaction mixture was diluted with ethyl acetate–dichloromethane (1:1) (400 cm^3) and washed with distilled water (4 × 75 cm^3). The organic layer was dried ($MgSO_4$) and the solvents were evaporated off. The crude solid was purified by flash chromatography with ethyl acetate–toluene (1:9) as eluent to give *glycoside* **8** (1.97 g, 90%), mp 131 °C (Found: C, 68.1; H, 4.9. $C_{60}H_{50}O_{16}S$ requires C, 68.04; H, 4.76%); $[\alpha]_D^{23}$ +51.9 (*c*, 1.026, $CHCl_3$); R_f 0.19 (toluene–ethyl acetate 9:1); δ_H(400 MHz; $CDCl_3$) 3.61 (1 H, dd, *J* 6.5 and 11.2, H^b-6′), 3.71 (1 H, t, *J* 6.5, H^a-6′), 3.92 (1 H, ddd, *J* 1.8, 5.4 and 9.7, H-5), 4.07 (1 H, dd, *J* 6.2 and 11.3, H-5′), 4.10 (1 H, d, *J* 9.7, H-4), 4.15 (1 H, br d, *J* 3.7, H-4′), 4.47 (1 H, dd, *J* 5.4 and 12.1, H^b-6), 4.61 (1 H, dd, *J* 1.8 and 12.1, H^a-6), 4.77 (1 H, d, *J* 7.9, H-1′), 4.94 (1 H, d, *J* 10.0, H-1), 5.16 (1 H, dd, *J* 3.3 and 10.3, H-3′), 5.41 (1 H, dd, *J* ~9.0 and 9.0, H-2), 5.75 (1 H, dd, *J* 7.9 and 10.3, H-2′), 5.80 (1 H, dd, *J* ~9.0 and 10.0, H-3), 7.06–7.61 (23 H, m, ArH) and 7.88–8.00 (12 H, m, ArH); δ_C(100 MHz; $CDCl_3$) 61.7 and 62.6 (C-6 and -6′), 66.6, 70.0, 70.3 and 72.5 (C-2, -3, -2′ and -3′), 74.0 and 74.1 (C-5 and -5′), 76.1 and 76.8 (C-4 and -4′), 85.6 (C-1), 101.1 (C-1′), 127.9, 128.1, 128.2, 128.3, 128.5, 128.7, 128.8, 128.9, 129.0, 129.3, 129.4, 129.5 and 129.7 (Ar CH), 131.8, 132.7, 133.0, 133.1, 133.2 and 133.3 (Ar C), 164.9, 165.1, 165.5, 165.2 (br) and 165.9 (CO); *m*/*z* (FAB-CI^+) 1081 (M + Na, 0.2%), 949 (1.5), 705 (0.3), 579 (0.8), 475 (10.6), 353 (4.1), 231 (1.0), 154 (9.3), 137 (5.5), 105 (100) and 77 (18.8).

Phenyl 2,3,4,6-tetra-*O*-benzyl-α-D-galactopyranosyl-(1→4)-2,3,6,tri-*O*-benzoyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-benzoyl-1-thio-β-D-glucopyranoside 10

A 100 cm^3 two necked flask was charged with glycoside **8** (1.0 g, 0.94 mmol), silver triflate (481 mg, 1.87 mmol), tin(II) chloride (365 mg, 1.92 mmol) and freshly activated 4 Å molecular sieves (3.0 g). The mixture was dried for 2 h under high vacuum (<0.1 mbar)‡ and the flask was filled again with dry nitrogen. Dry diethyl ether (12 cm^3) and dry dichloromethane (6 cm^3) were added and the mixture was cooled to 0 °C (ice-bath). The galactopyranosyl fluoride **9**[9] (554 mg, 1.13 mmol) as a solution in dry dichloromethane (4 cm^3) was added and the reaction mixture was stirred for 3 h at 0 °C. When the reaction had finished (TLC), ethyl acetate (25 cm^3) was added. The suspension was filtered through Celite and the filter pad was washed thoroughly with dichloromethane. The organic layer was washed successively with saturated aq. $NaHCO_3$ (3 × 50 cm^3) and distilled water (50 cm^3). The organic phase was dried ($MgSO_4$) and the solvent was evaporated off under reduced pressure. Purification by flash chromatography (toluene–ethyl acetate [19:1]) gave protected trisaccharide **10** (1.02 g, 69%). The unchanged acceptor **8** (313 mg, 31%) was recovered during the purification. For *protected trisaccharide* **10** (Found: C, 72.2; H, 5.6. $C_{94}H_{84}O_{21}S$ requires C, 71.38; H, 5.35%); R_f 0.44 (toluene–ethyl acetate [9:1]); mp 76 °C; $[\alpha]_D^{23}$ +53.3 (*c* 1.00, $CHCl_3$); $[\alpha]_{546}^{23}$ +61.9 (*c* 1.00, $CHCl_3$); δ_H(250 MHz; $CDCl_3$) 2.98 (1 H, dd, *J* 8.5 and 4.9), 3.35 (1 H, t, *J* 8.9), 3.45–3.55 (1 H, m), 3.70 (1 H, t, *J* 6.48), 3.82–4.95 (20 H, m), 5.05 (1 H, dd, *J* 10.74 and 2.87), 5.29 (1 H, d, *J* 3.47), 5.34 (1 H, dd, *J* ~9.7 and 9.7), 5.74 (1 H, dd, *J* 10.71 and 7.81), 5.81 (1 J, dd, *J* ~9.2 and 9.2), 7.02–7.62 (45 H, m), 7.75–7.85 (2 H, m, ArH) and 7.90–8.05 (8 H, m, ArH); δ_C(62.5 MHz; $CDCl_3$) 61.9, 62.6, 67.2, 69.7, 70.7, 72.5, 72.8, 73.2, 73.9, 74.3, 74.6, 74.8, 74.9, 75.3, 75.7, 76.2, 76.9, 78.9, 85.6 and 93.4 (internal C), 101.0 and 101.1 (C-1 and -1′), 127.1, 127.2, 127.2, 127.3, 127.5, 127.6, 127.7, 127.8, 127.9, 127.9, 128.0, 128.0, 128.1, 128.1, 128.2, 128.2, 128.3, 128.3, 128.4, 128.6, 128.9, 129.4, 129.5, 129.6, 129.6, 129.7, 129.8, 132.9, 133.0 and 133.0 (Ar CH), 131.6, 137.9, 138.1, 138.3, 138.5, 138.6, 138.7 and 138.8 (Ar C), 164.9, 165.0, 165.1, 165.5, 165.6 and 166.3 (CO); *m*/*z* (FAB-CI^+) 1605 (M + Na^+, 25.2%), 1473 (M^+ − SPh, 11.2), 949 (51.5), 829 (5.0), 705 (27.1), 565 (55.2), 503 (14.5), 475 (100), 431 (8.6), 353 (37.2), 335 (16.6) and 271 (31.0) {Found: *m*/*z* [FAB (M + Na)], 1603.5154. $C_{94}H_{84}NaO_{21}S$ requires *m*/*z* 1603.5099}.

Benzyl *N*-(6-hydroxyhexyl)carbamate

A solution of 6-aminohexanol (3.67 g, 31.3 mmol) in aq. sodium hydroxide [2 M; 15.8 cm^3 (31.3 mmol NaOH)] was cooled to 0 °C (ice-bath). To this mixture was added benzyl chloroformate (4.7 cm^3, 31.3 mmol) over a period of 10 min. The cooling ice-bath was removed and the reaction mixture was stirred for a further 1 h. Distilled water (100 cm^3) was added and the product was extracted with dichloromethane (3 × 150 cm^3). The combined organic fractions were washed successively with HCl (1 M; 100 cm^3), saturated aq. $NaHCO_3$ (100 cm^3) and distilled water (100 cm^3). The organic phase was dried ($MgSO_4$) and the solvent was evaporated off under reduced pressure. Crystallisation (ethyl acetate–light petroleum) gave the *title carbonate* (6.82 g, 87%) as a solid, mp 81 °C (Found: C, 67.0; H, 8.4; N, 5.6. $C_{14}H_{21}NO_3$ requires C, 66.91; H, 8.42; N, 5.57%); R_f 0.23 (ethyl acetate–light petroleum [1:1]); δ_H(250 MHz; $CDCl_3$) 1.31–1.63 (8 H, m, 4 × CH_2), 3.21 (2 H, t, *J* 6.7, CH_2N), 3.64

‡ 1 bar = 10^5 Pa.

(2 H, t, *J* 6.4, C*H*$_2$OH), 4.79 (1 H, br s, NH), 5.11 (2 H, s, C*H*$_2$Ph) and 7.28–7.41 (5 H, m, ArH); δ_C(62.5 MHz; $CDCl_3$) 25.1, 26.2, 29.8 and 32.4 (4 × CH_2), 40.7 (NCH_2), 62.4 (COH), 66.4 (O*C*Ph), 127.9 and 128.3 (Ar CH), 136.4 (Ar C) and 156.3 (CO).

6-(Benzyloxycarbonylamino)hexyl 2,3,4,6-tetra-*O*-benzyl-α-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-benzoyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-benzoyl-β-D-glucopyranoside 11

A 50 cm^3 two-necked flask was charged with protected trisaccharide **10** (1.0 g, 0.63 mmol), benzyl *N*-(6-hydroxyhexyl)-carbamate (694 mg, 2.76 mmol), silver triflate (2.2 g, 8.7 mmol) and 4 Å molecular sieves (4.0 g). After drying of the solid mixture under high vacuum for 60 min, dry dichloromethane (15 cm^3) was added. The mixture was cooled to 0 °C (ice-bath) and 9.2 cm^3 of previously prepared and cooled bromine solution (100 mm^3 of Br_2 in 10 cm^3 of dichloromethane) was added in portions over a period of 5 min. After being stirred for 2 h at 0 °C the mixture was filtered through Celite and the filter pad was thoroughly rinsed with CH_2Cl_2 (250 cm^3). The organic layer was washed successively with saturated aq. $NaHCO_3$ (3 × 100 cm^3) and distilled water (100 cm^3). The solution was dried ($MgSO_4$) and evaporated under reduced pressure. Purification by flash chromatography with toluene–ethyl acetate (12:1) as eluent followed by a second flash chromatographic purification with toluene–ethyl acetate (19:1) as eluent gave *trisaccharide glycoside* **11** (914 mg, 84%) as a solid, mp 59 °C (Found: C, 70.61; H, 5.9; N, 0.9. $C_{102}H_{99}NO_{24}$ requires C, 71.11; H, 5.79; N, 0.81%); R_f 0.22 (toluene–ethyl acetate [9:1]; 0.72 toluene–ethyl acetate [2:1]); $[\alpha]_D^{23}$ +47.7 (*c* 0.64, $CHCl_3$); $[\alpha]_{546}^{23}$ +56.9 (*c* 0.64, $CHCl_3$); δ_H(250 MHz; $CDCl_3$) 1.05–1.60 (8 H, m, CH_2), 2.87–3.07 (2 H, m), 3.30–3.47 (2 H, m), 3.67 (1 H, m), 3.72–4.90 (24 H, m), 4.98–5.18 (2 H, m, CO_2CH_2Ph), 5.36 (2 H, m), 5.71–5.88 (2 H, m), 7.05–7.62 (45 H, m, ArH), 7.79–7.85 (2 H, m, ArH) and 7.90–8.07 (8 H, m, ArH); δ_C(62.5 MHz; $CDCl_3$) 25.2, 26.0, 29.0, 29.4, 40.7, 61.9, 62.3, 66.3, 67.1, 69.6, 69.7, 69.7, 72.2, 72.5, 72.8, 72.9, 73.1, 73.2, 74.3, 74.7, 75.3, 75.5, 75.7, 76.2 and 78.9 (aliphatic and internal carbon atoms), 101.1, 101.0, 100.8 (C-1, -1′ and -1″), 127.1, 127.1, 127.2, 127.3, 127.4, 127.8, 127.9, 127.9, 127.9, 128.0, 128.1, 128.1, 128.1, 128.2, 128.3, 128.4, 128.8, 128.9, 129.3, 129.4, 129.4, 129.5, 129.6, 129.7, 132.9, 132.9, 132.9 and 133.0 (Ar CH), 136.5, 138.0, 138.3, 138.7 and 138.8 (Ar C) and 164.8, 165.0, 165.1, 165.4, 165.6 and 166.3 (CO); *m*/*z* (FAB-CI$^+$) 1762 (M + K, 37%), 1723 (M + Na, 100), 1723 (M, 14), 1589 (12), 1472 (10), 1291 (27.5), 1247 (8.2), 1201 (8.0), 1183 (6.7) and 1039 (13.7).

6-(Benzyloxycarbonylamino)hexyl 2,3,4,6-tetra-*O*-benzyl-α-D-galactopyranosyl-(1→4)-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside

A solution of trisaccharide glycoside **11** (800 mg, 0.46 mmol) in methanol (7 cm^3) was treated with sodium methoxide (102 mg, 1.88 mmol). The mixture was stirred for 5 h at rt, neutralised with Amberlyst 15 ion-exchange resin, and filtered through a pad of Celite. The solvent was evaporated under reduced pressure. The product was purified by flash chromatography with dichloromethane–methanol (15:1) as eluent to give 6-benzyloxycarbonylamino)hexyl 2,3,4,6-tetra-*O*-benzyl-α-D-galactopyranosyl-(1→4)-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside (423 mg, 84%), mp 40 °C; R_f 0.16 (CH_2Cl_2–CH_3OH [15:1]); $[\alpha]_D^{23}$ −7.5 (*c* 0.54, $CHCl_3$); $[\alpha]_{546}^{23}$ −9.6 (*c* 0.54, $CHCl_3$); δ_H(250 MHz; $CDCl_3$) 1.20–1.68 (8 H, m, 4 × CH_2), 2.99 (2 H, t, *J* 6.5, CH_2NCO), 3.17–4.28 (22 H, m), 4.32 (1 H, d, *J* 10.76, C*H*$_2$Ph), 4.40 (1 H, d, *J* 10.75, C*H*$_2$Ph), 4.54 (1 H, d, *J* 11.34, H-1), 4.67 (1 H, d, *J* 11.92, H-1′), 4.75 (1 H, d, *J* 3.75, H-1″), 4.79 (2 H, s, COOC*H*$_2$Ph), 4.90 (1 H, d, *J* 11.91), 4.95 (1 H, d, *J* 11.3), 5.09 (2 H, s) and 7.20–7.48 (25 H, m, ArH); δ_C(62.5 MHz; $CDCl_3$) 25.3, 26.1, 29.2, 29.6, 29.6, 40.7, 60.0, 62.4, 62.5, 65.7, 66.4, 69.8, 69.8, 71.4, 72.6, 73.4, 73.7, 73.8, 74.0, 74.3, 74.4, 74.8, 75.4, 100.8, 102.5 and 103.9 (last three C-1, -1′ and -1″) and 126.7, 127.4, 127.8, 127.9, 128.0, 128.1, 128.1, 128.2, 128.3, 128.4, 128.4, 128.6, 128.7, 129.7, 129.8, 130.7, 136.4, 136.8, 137.1 and 137.7 (Ar C); *m*/*z* (FAB-CI$^+$) 1121 (M + Na$^+$, 3.2%), 1099 (M + H$^+$, 3.2), 1098 (M, 4.1), 391 (27.2), 377 (14.5), 197 (6.3), 181 (20.4), 149 (100) and 121 (6.7) ($C_{60}H_{75}NO_{18}$ = 1098.26).

6-Aminohexyl α-D-galactopyranosyl-(1→4)-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside 12

6-(Benzyloxycarbonylamino)hexyl 2,3,4,6-tetra-*O*-benzyl-α-D-galactopyranosyl-(1→4)-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside (200 mg, 0.182 mmol) was dissolved in methanol (4 cm^3). $Pd(OH)_2$/C (20%; 600 mg) and cyclohexene (4 cm^3) were added. The mixture was boiled under reflux for 4.5 h and then was filtered (Celite). The filter pad was washed thoroughly with both methanol and distilled water. The filtrate was evaporated under reduced pressure and the residue was purified by flash chromatography with CH_3OH–aq. ammonia (4:1) as eluent to give *glycoside* **12** (70.0 mg, 60%), mp 185 °C (Found: *m*/*z*, 604.2801. $C_{24}H_{46}NO_{16}$ requires *m*/*z*, 604.2803); R_f 0.20 (CH_3OH–conc. NH_3 [4:1]); δ_H(250 MHz; $CDCl_3$) 1.15–1.65 (8 H, m, CH_2), 2.40 (2 H, m), 2.83 (1 H, m), 3.14–3.28 (2 H, m), 3.44–3.98 (18 H, m), 4.28 (1 H, t, *J* 6.5), 4.40 (2 H, t, *J* 7.3), 4.71 (1 H, d, *J* 7.3, H-1″) and 4.82 (1 H, d, *J* 11.62, H-1′); δ_H(400 MHz; D_2O) 1.36 (4 H, m, CH_2CH_2), 1.59 (4 H, m, CH_2CH_2), 4.86 (1 H, d, *J* 3.8, H-1), 2.87 (2 H, t, *J* 7.2, *C*H_2NH$_2$), 3.25 (1 H, m, H-2), 3.51–4.06 (18 H, m), 4.31 (1 H, t, *J* 6.3, H-5′), 4.44 (1 H, d, *J* 8.0, H-1), 4.47 (1 H, d, *J* 7.8, H-1′) and 4.91 (1 H, d, *J* 3.8, H-1″); δ_C(62.5 MHz, D_2O) 25.2, 26.0, 28.3, 29.1 and 40.3 (CH_2), 60.7 (C-6), 61.0 (C-6′), 61.1 (C-6″), 69.2 (C-2″), 69.6 (C-4″), 69.8 (C-3″), 71.1 (OCH_2), 71.4 (C-5″), 71.5 (C-5 or -2′), 72.8 (C-3′), 73.6 (C-2), 75.2 (C-3), 75.4 (C-2′ or -5), 76.1 (C-5′), 78.0 (C-4′), 79.3 (C-4), 100.9 (C-1″), 102.6 (C-1) and 103.9 (C-1′); *m*/*z* (FAB-CI$^+$) 604 (M + H$^+$, 47.5%), 370 (8.1), 307 (5.4), 299 (6), 278 (21), 259 (15), 245 (16), 225 (6), 215 (16), 207 (35.5), 199 (8), 186 (100), 167 (27.5), 153 (17), 137 (9), 131 (9.5), 118 (15), 115 (46) and 107 (10.5).

Coupling of 6-aminohexyl α-D-galactopyranosyl-(1→4)-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside 12 to CNBr-activated Sepharose 4B

Dry Sepharose (1 g) was swollen in aq. HCl (1 mM) for 15 min. The acidic solution was removed by suction without allowing the gel to dry. The gel was transferred into a previously prepared solution of glycoside **12** (9.5 mg) in aq. $NaHCO_3$ (0.1 M $NaHCO_3$ and 0.5 M NaCl, pH 10.3; 15 cm^3). The mixture was shaken for 4 h at 25 °C (110 rpm). The coupling solution was filtered by suction and the gel was again placed in aq. $NaHCO_3$ (0.1 M $NaHCO_3$ and 0.5 M NaCl, pH 10.3; 15 cm^3). Ethanolamine was added to deactivate the gel. The mixture was shaken for 2 h, the solvent was removed by suction, and the gel was washed successively with aq. HCl (1 mM) and aq. NaCl (0.5 M). It was stored in aq. NaCl (0.5 M) containing NaN_3 to prevent bacterial contamination.

Ethyl 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-galactopyranoside 14

The peracetylated galactopyranoside **13** (Sigma) (6.14 g, 15.74 mmol) was dissolved in $ClCH_2CH_2Cl$ (200 cm^3) containing 4 Å molecular sieves (5 g) and ethanethiol (2.31 cm^3, 31.25 mmol). The mixture was stirred at −30 °C for 1 h, $SnCl_4$ (2.00 cm^3, 17.02 mmol) was added, and the reaction medium was allowed to warm to −17 °C over a period of 1 h. The reaction was quenched by addition of saturated aq. $NaHCO_3$ (150 cm^3). The mixture was stirred for 1 h and filtered through a pad of Celite. The layers were separated and the aqueous layer was extracted with CH_2Cl_2 (150 cm^3). The organic layers were combined, washed with water (200 cm^3), dried ($MgSO_4$), and evaporated. Crystallisation (Et_2O–light petroleum) afforded *thioglycoside* **14** as a crystalline solid (4.1 g, 80%); mp 77–78 °C (Found: C, 48.94; H, 6.2. $C_{16}H_{24}O_9S$ requires C, 49.00; H,

6.12%); R_f 0.41 (light petroleum–Et_2O [2:8]); $[a]_D^{28}$ −8.6 (*c* 0.98, $CHCl_3$); δ_H(250 MHz; $CDCl_3$) 1.22 (3 H, t, *J* 7.4, CH_3CH_2), 1.92 (3 H, s, $COCH_3$), 1.98 (3 H, s, $COCH_3$), 2.00 (3 H, s, $COCH_3$), 2.09 (3 H, s, $COCH_3$), 2.56–2.74 (2 H, m, SCH_2CH_3), 3.84–3.90 (1 H, m), 4.00–4.14 (2 H, m), 4.43 (1 H, d, *J* 9.8, H-1), 4.98 (1 H, dd, *J* 10.0 and 3.3, H^a-6), 5.17 (1 H, dd, *J* ~9.9 and 9.9) and 5.36 (1 H, dd, *J* 3.3 and 1.0, H-4); δ_C(62.5 MHz; $CDCl_3$) 14.7 (SCH_2CH_3), 20.5, 20.6, 20.7 and 24.3 (SCH_2CH_3), 61.3 (C-6), 67.0, 67.1, 71.7, 74.2, 83.9 (C-1) and 169.5, 170.0, 170.1 and 170.3 (CO).

Ethyl 1-thio-β-D-galactopyranoside 15

To a solution of thioglycoside **14** (4.03 g, 10.28 mmol) in MeOH (100 cm^3) was added a catalytic amount of NaOMe (55.5 mg, 1.02 mmol). The mixture was kept for 17 h at rt, neutralised with Amberlyst 15, filtered and concentrated under reduced pressure to yield deprotected thioglycoside **15** quantitatively as a syrup, $[a]_D^{24}$ −23.2 (*c* 0.59, MeOH); δ_H(250 MHz; D_2O) 1.03 (3 H, t, *J* 7.4, SCH_2CH_3), 2.44–2.60 (2 H, m, SCH_2CH_3), 3.27–3.57 (5 H, m), 3.73 (1 H, d, *J* 3.1, H-4) and 4.24 (1 H, d, *J* 9.5, H-1); δ_C(62.5 MHz; D_2O) 15.3 (SCH_2CH_3), 25.0 (SCH_2CH_3), 61.9 (C-6), 69.6, 70.4, 74.7, 79.7 and 86.4 (C-1); *m/z* (FAB) 247 (M + Na^+).

Ethyl 2,3-di-*O*-benzoyl-4,6-*O*-isopropylidene-1-thio-β-D-galactopyranoside 16

To a solution of thioglycoside **15** (1.00 g, 4.46 mmol) in DMF (9 cm^3) were added a catalytic amount of TsOH (138.9 mg, 0.73 mmol) and 2,2-dimethoxypropane (2.18 cm^3, 17.83 mmol). After 90 min the reaction mixture was quenched by the addition of triethylamine (1.3 cm^3), evaporated under reduced pressure and dried overnight under reduced pressure. The residue was dissolved in pyridine (15 cm^3), and benzoyl chloride (1.55 cm^3, 13.39 mmol) was added dropwise at 0 °C. After 30 min the reaction mixture was allowed to warm to rt and stirred for a further 3.5 h. The reaction mixture was diluted with ethyl acetate (50 cm^3) and washed with water (3 × 50 cm^3). The organic layer was dried ($MgSO_4$), and evaporated under reduced pressure. Flash chromatography (5% EtOAc in toluene) afforded the *protected glycoside* **16** (1.00 g, 50%) as a foam (Found: C, 63.6; H, 5.9. $C_{25}H_{28}O_7S$ requires C, 63.57; H, 5.92%); R_f 0.57 (toluene–EtOAc [7:3]); $[a]_D^{29}$ +87.5 (*c* 1.15, $CHCl_3$); δ_H(250 MHz; $CDCl_3$) 1.30 (3 H, t, *J* 7.4, SCH_2CH_3), 1.37 (3 H, s, CH_3), 1.47 (3 H, s, CH_3), 2.34–2.96 (2 H, m, SCH_2CH_3), 3.58 (1 H, br s, H-5), 4.04–4.06 (2 H, m, H_2-6), 4.58 (1 H, dd, *J* 0.8, 3.4, H-4), 4.66 (1 H, d, *J* 9.8, H-1), 5.28 (1 H, dd, *J* 3.4 and 9.8, H-3), 5.90 (1 H, t, *J* 9.8, H-2), 7.22–7.50 (7 H, m, ArH) and 7.95–8.00 (3 H, m, ArH); δ_C(100 MHz; $CDCl_3$) 14.6 (SCH_2CH_3), 18.4 (CH_3), 22.8 (SCH_2CH_3), 28.9 (CH_3), 62.6 (C-6), 66.7 (C-4), 67.1 (C-5), 69.6 (C-2), 73.8 (C-3), 82.5 (C-1), 98.8 (CMe_2), 128.0, 128.1, 128.8, 129.1, 129.4, 129.6 and 129.7 (ArCH), 132.9 and 131.3 (ArC) and 165.2 and 169.9 (CO).

Ethyl 2,3,6-tri-*O*-benzoyl-1-thio-β-D-galactopyranoside 17

To a solution of glycoside **16** (0.92 g) in THF (11 cm^3) at 0 °C were added water (2.5 cm^3) and TFA (7 cm^3). After 50 min the reaction was quenched by addition of saturated aq. $NaHCO_3$ (4 cm^3) and the product was extracted with EtOAc (2 × 60 cm^3). The combined organic layers were washed with brine (20 cm^3), dried ($MgSO_4$), and evaporated under reduced pressure. The residue was co-evaporated twice with toluene and dried overnight under reduced pressure. The residue was dissolved in DMF (15 cm^3) and the solution was cooled to −20 °C. Triethylamine (0.33 cm^3) and benzoyl cyanide (0.24 cm^3) were added and the mixture was stirred for 15 min at −20 °C and for a further 20 min at 0 °C. The reaction was quenched with MeOH (1 cm^3). The resulting mixture was diluted with EtOAc (50 cm^3), washed successively with water (20 cm^3) and brine (20 cm^3), dried ($MgSO_4$), and evaporated under reduced pressure. Flash chromatography (10% EtOAc in toluene) afforded the *selectively deprotected glycoside* **17** (1.05 g, 96%) as a solid, mp 121–122 °C (Found: C, 64.9; H, 5.25. $C_{29}H_{28}O_8S$ requires C, 64.94; H, 5.25%); R_f 0.57 (toluene–EtOAc [7:3]); $[a]_D^{32}$ +59.4 (*c* 1.02, $CHCl_3$); δ_H(250 MHz; $CDCl_3$) 1.32 (3 H, t, *J* 7.4, SCH_2CH_3), 2.70–2,86 (2 H, m, SCH_2CH_3), 4.10 (1 H, dd, *J* ~6.4 and 6.4, H-4), 4.36–4.40 (1 H, m, H-5), 4.54–4.72 (2 H, m, H_2-6), 4.75 (1 H, d, *J* 9.8, H-1), 5.40 (1 H, dd, *J* 2.9 and 9.8, H-3), 5.83 (1 H, dd, *J* ~9.8 and 9.8, H-2), 7.32–7.61 (10 H, m, ArH) and 7.94–8.06 (5 H, m, ArH); δ_C(100 MHz; $CDCl_3$) 14.8 (SCH_2CH_3), 24.1 (SCH_2CH_3), 62.7 (C-6), 67.3, 67.7, 74.9 and 75.9 (C-2, -3, -4 and -5), 83.8 (C-1), 128.2, 128.3, 128.8, 129.2, 129.3, 129.6 and 129.7 (ArCH), 133.1, 133.2 and 133.3 (ArC) and 165.3, 165.6 and 166.3 (CO).

Ethyl 2,3,4,6-tetra-*O*-benzyl-α-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-benzoyl-1-thio-β-D-galactopyranoside 18

To glycoside **17** (0.33 g, 0.61 mmol) were added silver triflate (0.43 g, 1.70 mmol), tin(II) chloride (0.24 g, 1.31 mmol) and activated molecular sieves (4 Å). The mixture was dried for 2 h under reduced pressure, cooled to 0 °C, and dissolved in Et_2O (12 cm^3)–CH_2Cl_2 (6 cm^3). A solution of glycosyl fluoride **9** (0.46 g, 0.86 mmol) in Et_2O (3 cm^3) was added. The mixture was stirred for 3.5 h at 0 °C, EtOAc (50 cm^3) was added, and the suspension was filtered through a pad of Celite. The organic layer was washed three times with saturated aq. $NaHCO_3$ (30 cm^3) and once with brine (30 cm^3), dried ($MgSO_4$), and evaporated under reduced pressure. Flash chromatography (40% Et_2O in light petroleum) gave *disaccharide glycoside* **18** (0.33 g, 51%) as an amorphous solid, mp 44–45 °C (Found: C, 71.2; H, 5.8. $C_{63}H_{62}O_{13}S$ requires C, 71.47; H, 5.85%); R_f 0.46 (silica, Et_2O–light petroleum [1:1]); $[a]_D^{24}$ +61.5 (*c* 0.72, $CHCl_3$); δ_H(100 MHz; $CDCl_3$) 1.25 (3 H, t, *J* 7.4, SCH_2CH_3), 2.69–2.88 (2 H, m, SCH_2CH_3), 2.89–2.92 (1 H, m, H^a-6′), 3.38 (1 H, t, *J* 8.8, H^b-6′), 4.00–4.10 (5 H, m, H-2′, -4′, -5′ and CH_2Ph), 4.15 (1 H, dd, *J* 2.5 and 10.2, H-3′), 4.27–4.34 (1 H, m, H-5), 4.44 (1 H, d, *J* 2.8, H-4), 4.47 (1 H, d, *J* 11.0, CH_2Ph), 4.68–4.87 (8 H, m, H-1, H_2-6, 3 × CH_2Ph), 4.94 (1 H, d, *J* 3.4, H-1′), 5.27 (1 H, dd, *J* 2.8 and 10.7, H-3), 5.84 (1 H, dd, *J* ~10.0 and 10.0, H-2), 7.12–7.59 (30 H, m, ArH) and 7.88–8.01 (5 H, m, ArH); δ_C(100 MHz; $CDCl_3$) 14.9 (SCH_2CH_3), 23.6 (SCH_2CH_3), 62.3 (C-6), 67.3 (C-6′), 67.8 (C-2), 69.6 (C-5), 72.5 (CH_2Ph), 72.7 (CH_2Ph), 73.8 (CH_2Ph), 74.7 (C-5′), 74.8 (CH_2Ph), 75.2 (C-3), 75.4 (C-4), 75.8 (C-4′), 76.3 (C-3′), 78.8 (C-2′), 83.5 (C-1), 100.6 (C-1′), 127.2, 127.2, 127.3, 127.8, 127.9, 128.0, 128.1, 128.1, 128.2, 128.3, 128.3, 128.9, 129.3, 129.5, 129.7, 133.0, 133.0 and 133.1 (ArCH), 138.1, 138.2, 138.5 and 138.7 (ArC) and 165.2, 165.9 and 166.2 (CO).

6-(Benzyloxycarbonylamino)hexyl 2,3,4,6-tetra-*O*-benzyl-α-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-benzoyl-1-thio-β-D-galactopyranoside 19

To glycoside **18** (0.3 g, 0.28 mmol) were added benzyl 6-hydroxyhexyl carbamate (0.31 g, 1.24 mmol), silver triflate (1.07 g, 4.19 mmol) and activated molecular sieves (4 Å). The mixture was dried under reduced pressure for 3 h and cooled to 0 °C. Dichloromethane (10 cm^3) was added and a solution of bromine (83 mm^3 in CH_2Cl_2 [10 cm^3]) was added dropwise over a period of 10 min. The mixture was stirred for 2 h, the suspension was filtered through a pad of Celite, and the filter pad was washed with CH_2Cl_2 (3 × 30 cm^3). The organic layer was washed successively with saturated aq. $NaHCO_3$ (3 × 30 cm^3) and water (30 cm^3), dried ($MgSO_4$), and evaporated under reduced pressure. Flash chromatography (10% EtOAc in toluene) afforded *glycoside* **19** (0.28 g, 83%) as a foam, R_f 0.35 (PhMe–EtOAc [9:1]) (Found: C, 72.1; H, 5.95; N, 1.1. $C_{75}H_{77}NO_{16}$ requires C, 72.25; H, 6.09; N, 1.12%); $[a]_D^{27}$ +54.7 (*c* 0.61, $CHCl_3$); δ_H(400 MHz; $CDCl_3$) 1.12–1.22 (6 H, m, $O[CH_2]_2CH_2CH_2CH_2CH_2NH$), 1.40–1.53 (2 H, m, $OCH_2CH_2[CH_2]_4NH$), 2.88–2.91 (1 H, m, H^a-6′), 2.94–3.00 (1 H, m, H^b-6′), 3.29–3.38 (1 H, m, H^a-6), 3.47–3.54 (1 H, m, OCH_2-

$[CH_2]_5NH$), 3.90–4.08 (5 H, m, H-5, -2′, CH_2Ph, OCH_2-$[CH_2]_5NH$), 4.10 (1 H, br s, H-4′), 4.18 (1 H, dd, *J* 2.5 and 10.2, H-3′), 4.32–4.35 (1 H, m, H-5′), 4.40 (1 H, d, *J* 2.5, H-4), 4.45 (2 H, d, *J* 11.0, CH_2Ph), 4.61 (1 H, m, NH), 4.66 (1 H, d, *J* 7.6, H-1), 4.71–4.88 (7 H, m, H^b-6, 2 × CH_2Ph, O$[CH_2]_5$CH_2NH), 4.92 (1 H, d, *J* 3.4, H-1′), 5.07 (2 H, s, NHCOOCH_2Ph), 5.22 (1 H, dd, *J* 2.8 and 10.5, H-3), 5.74 (1 H, dd, *J* 7.6 and 10.6, H-2), 7.10–7.59 (35 H, m, ArH) and 7.89–8.05 (5 H, m, ArH); δ_C(100 MHz; $CDCl_3$) 15.9 (CH_2), 18.9 (CH_2), 29.1 (CH_2), 29.5 (CH_2), 40.7 (CH_2NH), 62.3 (*C*H_2Ph), 66.4 (C-6), 67.3 (C-6′), 69.5 (C-5′), 69.6 (OCH_2), 69.8 (C-2), 72.3 (*C*H_2Ph), 72.5 (C-5), 72.7 ($NHCO_2$*C*H_2Ph), 73.8 (*C*H_2Ph), 74.0 (C-3), 74.6 (C-4′), 74.8 (*C*H_2Ph), 75.5 (C-4), 75.7 (C-2′), 78.8 (C-3′), 101.0 (C-1′), 101.4 (C-1), 127.2, 127.2, 127.3, 127.4, 127.8, 127.9, 128.1, 128.1, 128.2, 128.3, 128.3, 129.4, 129.5, 129.7, 132.9 and 133.0 (ArCH), 136.5, 138.1, 138.6 and 138.7 (ArC), 156.1 (CO_2NH) and 165.1, 165.9 and 166.3 (CO).

6-Aminohexyl α-D-galactopyranosyl-(1→4)-β-D-galactopyranoside 20

To a solution of glycoside **19** (0.2 g, 0.16 mmol) in MeOH (8 cm^3) at rt was added MeONa (9 mg). The mixture was stirred for 20 h, neutralised with Amberlyst 15 ion exchange resin, filtered, and evaporated under reduced pressure, and the residue was dried for 3 h under reduced pressure. A single spot was visualised by TLC, R_f 0.67 (CH_2Cl_2–MeOH [15:1]). The residue was dissolved in EtOH (5 cm^3), 10% $Pd(OH)_2$ on carbon (0.18 g) and cyclohexene (76 mm^3, 0.74 mmol) were added, and the mixture was boiled under reflux for 1.75 h before being allowed to cool to rt and filtered through a pad of Celite. Purification by flash chromatography (20% aq. NH_3 in MeOH) afforded *disaccharide glycoside* **20** (49.1 mg, 70%) (Found: C, 48.7; H, 7.7; N, 3.0. $C_{18}H_{35}NO_{11}$ requires C, 49.01; H, 7.93; N, 3.17%); R_f 0.21 (aq. NH_3–MeOH [2:8]); δ_H(400 MHz; D_2O) 1.36–1.39 (4 H, m, 2 × CH_2), 1.60–1.61 (4 H, m, 2 × CH_2), 2.92 (1 H, t, *J* 7.4, CH_2NH_2), 3.49 (1 H, dd, *J* 7.7 and 7.7, H-2), 3.61–3.92 (10 H, m, H-3, -5, -2′, -3′, H_2-6, H_2-6′, OCH_2), 3.99 (2 H, d, *J* 3.0, H-4 and -4′), 4.33 (1 H, dd, *J* ~5.6 and 5.6, H-5′), 4.41 (1 H, d, *J* 7.7, H-1) and 4.92 (1 H, d, *J* 3.8, H-1′); δ_C(100 MHz; D_2O) 25.3 (CH_2), 25.9 (CH_2) 27.6 (CH_2), 29.2 (CH_2), 40.1 (CH_2NH), 60.7 (C-6 or -6′), 61.1 (C-6′ or -6), 69.3 (C-2′), 69.6 (C-4′), 69.8 (C-3′), 71.0 (OCH_2), 71.4 (C-5′), 71.6 (C-2), 73.1 (C-3), 75.7 (C-5), 77.7 (C-4), 100.8 (C-1′) and 103.5 (C-1).

Coupling of 6-aminohexyl α-D-galactopyranosyl-(1→4)-β-D-galactopyranoside 20 to CNBr-activated Sepharose 4B

Freeze-dried CNBr-activated Sepharose 4B (1 g) was swollen for 15 min in HCl (1 M; 200 cm^3) and then was washed successively with HCl (1 mM; 200 cm^3) and aq. $NaHCO_3$ (0.1 M, pH 8.3; 5 cm^3). The gel was immediately transferred into a previously prepared solution of glycoside **20** (7 mg) in aq. $NaHCO_3$ (0.1 M, pH 8.3; 10 cm^3) and shaken (110 rpm) for 4 h at rt. The gel was transferred to a solution of ethanolamine (0.1 M) in aq. $NaHCO_3$ (0.1 M, pH 8.3; 10 cm^3) and the mixture was shaken for 2 h. The blocking solution was removed by filtration and the gel was washed successively with the following solutions: aq. $NaHCO_3$ (0.1 M pH 8.3; 100 cm^3), aq. NaOAc (0.1 M, pH 4.0; 200 cm^3) and aq. $NaHCO_3$ (0.1 M, pH 8.3; 100 cm^3) and stored in the last solution.

Comparison of the binding of Shiga toxin B pentamer to Gb_3-Sepharose with that of B pentamer to Gb_2 and Gb_3-Fractogel

pSLTwt (a plasmid coding the SLT-1 operon under control of the lac promoter) in *E. coli* strain JM 105 was grown in 1 dm^3 of Luria broth to an absorbance *A* of 0.6 at 600 nm. Cells were induced for 3 h with a final concentration of isopropyl β-D-thiogalactopyranoside of 1 mM. Cells were harvested by centrifugation and washed once in Tris-sucrose, followed by centrifugation at 5000 *g*. Cells were resuspended in Tris (pH 7.0, 1 mM), incubated on ice for 10 min, and pelleted at 10 000 *g* for 20 min. Columns of Gb_3-Sepharose, Gb_2-Sepharose and Gb_3-Fractogel (1 cm^3) were equilibrated with NaCl solution (0.5 M) in phosphate-buffered saline (PBS; NaCl, 137 mM; KCl, 2.7 mM; Na_2HPO_4, 10.1 mM; KH_2PO_4, 1.8 mM; pH 7.4). Periplasm (3 cm^3) was loaded on to each column. The columns were washed with 40 column volumes of NaCl (0.5 M) in PBS. Fractions were eluted from the columns with a stepwise gradient from 0 to 6 M of guanidinium chloride. Thus guanidinium chloride (pH 6.7, 0.5 cm^3) was placed on the column and pumped through at 0.4 cm^3 min^{-1}. Fractions (1 cm^3) were collected at 2.5 min intervals. As the guanidinium chloride was taken into the column, it was replaced by a second aliquot of 1 M denaturant and so on in steps of 0.5 M denaturant up to 6 M. Protein in the eluted fractions was measured from the absorbance at 280 nm. Fractions corresponding to peaks in the elution profile were removed for refractometry analysis to determine the concentration of guanidinium chloride by comparison with standard curves. The remainder was dialysed against PBS. Aliquots (10 mm^3) from each sample were examined by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) with silver staining to confirm the identity of the eluted protein.

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