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Purification of Subunit B of Shiga Toxin Using a Synthetic Trisaccharide-Based Affinity Matrix

Vince Pozsgay,*,† Loc Trinh,†,‡ Joseph Shiloach,‡ John B. Robbins,† Arthur Donohue-Rolfe,§ and Stephen B. Calderwood⊥

Laboratory of Developmental and Molecular Immunity, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892, Laboratory of Cellular and Molecular Biology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892, Department of Comparative Medicine, Tufts University School of Veterinary Medicine, 200 Westboro Road, North Grafton, Massachusetts 01536, and Infectious Diseases Unit, Massachusetts General Hospital, Boston, Massachusetts 02114. Received May 1, 1995⊗

The blood group P_1 antigenic trisaccharide (**3**), which is the receptor-binding ligand of Shiga-like toxins, is synthesized in a spacer-equipped form (**32**) from 2-(trimethylsilyl)ethyl glucoside **5** and the 1-thiogalactoside building blocks **10** and **22** in a stereocontrolled, stepwise fashion. Covalent attachment of **32** to hydrazine group-containing agarose gel by reductive amination provided the P_1 trisaccharide-containing affinity sorbent which was used for preparative scale isolation of subunit B of Shiga toxin.

INTRODUCTION

The Shiga and Shiga-like families of secreted toxins play an important role in the pathogenesis of infections caused by *Shigella dysenteriae* type 1 and various enterotoxic strains of *Escherichia coli* and are associated with a number of clinical manifestations of infections such as hemorrhagic colitis and hemolytic uremic syndrome (*1, 2*). These toxins are multimeric proteins consisting of a single enzymatic subunit (subunit A, MW 32 kDa) and a symmetrically arranged, pentameric subunit B (MW 7.7 kDa) (*1, 2*). The latter was shown to mediate the binding of the holotoxin to cell-surface glycolipids as the first step of its endocytosis. Galabiose (**1**) was identified as the minimum binding receptor that recognizes the toxin, placed either terminally or internally in glycolipids (*3*). The binding was stronger with P blood group (*4*) determinant trisaccharides, such as the P_k antigen (globotriose, **2**) and the P_1 antigen (**3**) (Chart 1). More recent reports confirmed these findings and showed that the α-(1→4) linkage between the two galactose residues is essential for the recognition (*5, 6*). It has been suggested (*5*) that specific adsorbents of Shiga (Shiga-like) toxins may aid the development of serologic tests for rapid diagnosis of *E. coli* infections, and receptor-analog oligosaccharides that inhibit the binding of these toxins to their cell-surface receptors can be candidates for therapeutics against such diseases (*6*). Three methods were used for the isolation and purification of the toxin components. Functional subunits A and B were isolated from the holotoxin in small quantities by molecular sieve chromatography (*7*). In an elegant method, the P_1 glycoprotein of the hydatid cyst fluid was covalently attached to Sepharose 4B and the receptor-based affinity material was used to isolate subunit B in a single step on the mg scale (*8*). Unfortunately, the low capacity of this column precludes scale-up. Recently, large scale purification was also reported (*9*). Although the latter approach provided pure subunit B in the 100 mg level, it required a two-step chromatographic protocol including ion-exhange chromatography on DEAE-Sephadex followed by chromatofocusing using Polybuffer Exchanger 94 and, finally, by ammonium-sulfate precipitation. We now report a simple and efficient method for the preparative scale isolation of subunit B of Shiga toxin, using a receptor−analog affinity sorbent. Specifically, we describe the chemical synthesis of a glycosyl donor derivative of the P_1 trisaccharide antigen and the covalent attachment of the P_1 trisaccharide (**3**) through a chemical anchor to a solid support, using a general method reported earlier from this laboratory (*10*). We also demonstrate the utility of the affinity material for the rapid isolation of pure subunit B in a semiautomated fashion.

EXPERIMENTAL PROCEDURES

General. Melting points were taken on a Meltemp capillary melting point apparatus and are uncorrected. All chemicals were of commercial grade and were used without purification. Anhydrous solvents were obtained from Aldrich. Optical rotations were measured at 22 °C with a Perkin-Elmer type 241MC or 341 polarimeter for $CHCl_3$ solutions, except where indicated otherwise. Column chromatography was performed on silica gel 60 (0.040−0.063 mm). The NMR spectra were obtained at 296 K, on a Varian XL-300 or a Gemini 300 spectrometer operating at 300 MHz for ^1H and at 75.5 MHz for ^{13}C. Internal references: TMS (0.000 ppm for ^1H for solutions in organic solvents), acetone (2.225 ppm for ^1H and 31.00 ppm for ^{13}C for solutions in D_2O) and $CDCl_3$ (77.00 ppm for ^{13}C for solutions in $CDCl_3$). The methylene carbon resonances were identified by a DEPT-135 experiment. Subscripts A-C refer to the individual sugar residues, with A standing for the reducing-end unit. Protons linked to the same carbon atom are differentiated by an asterisk. Aglyconic atoms are denoted by a prime for compounds **28**-**32**. For compound **32**, the assignments for the hydrated form are shown in italics. The fast atom bombardment mass spectra were run on a JEOL SX102 mass spectrometer using 6 keV xenon atoms to ionize

* To whom correspondence should be addressed. Phone (301) 402-0036. Fax: (301) 402-9857. E-mail: vipo@helix.nih.gov.
† NICHD.
‡ NIDDK.
§ Tufts University School of Veterinary Medicine.
⊥ Massachusetts General Hospital.
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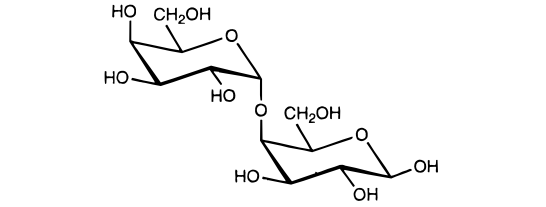
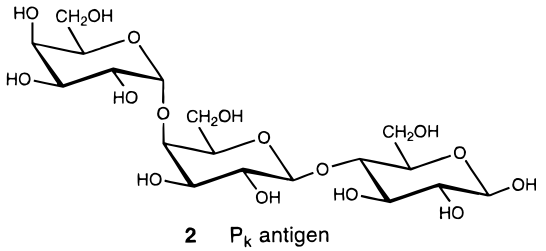
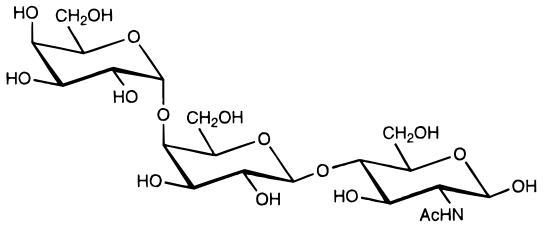
Chart 1[a]



1 Galabiose



2 P_k antigen (globotriose)



3 P_1 antigen

[a] Minimum carbohydrate structures recognized by subunit B of Shiga toxin. The reducing end unit is shown as the β anomer.

the samples which were desorbed from a mixture of dithiothreitol and dithioerythritol from glycerol or from 3-nitrobenzyl alcohol as the matrix. Chemical ionization mass spectra (CI-MS) were obtained by using NH_3 as the ionizing gas. Elemental analyses were performed by Atlantic Microlab, Inc., Norcross, GA. Affinity chromatography was performed in a Pharmacia FPLC system.

***Vibrio cholerae* Strain.** *V. cholerae* strain 0395-N1 (pSBC32) containing the gene of subunit B of Shiga toxin but lacking in the gene for subunit A has been described by Acheson *et al.* (*9*).

Protein Determination. Protein determination was carried out by the bicinchonic acid (BCA) method (*27*), using the BCA protein assay reagent of Pierce (Rockfort, IL). Bovine serum albumin was used as the reference.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). SDS−PAGE was performed in Novex (16% Tris−tricine) slab gels, 8 × 8 cm, and stained with Coomassie blue R 350 stain, obtained from Pharmacia Biotechnology (Uppsala, Sweden).

Antigenicity of Subunit B of Shiga Toxin. Purified subunit B of Shiga toxin was reacted with a monoclonal antisubunit B rabbit monoclonal antibody as described earlier (*9*).

Chemical Synthesis of the Affinity Sorbent. *2-(Trimethylsilyl)ethyl 3-O-benzyl-4,6-O-benzylidene-2-deoxy-2-phthalimido-β-D-glucopyranoside (**4**).* Compound **4** prepared essentially as described in ref 11 was obtained in crystalline form: mp 103−105 °C (hexane), $[\alpha]_D$ +43° (*c* 0.7) (lit. (*11*) syrup, $[\alpha]_D$ +39° (*c* 0.7, $CHCl_3$)). Anal. ($C_{33}H_{37}NO_7Si$) C,H,N.

*Methyl 2,3,4,6-Tetra-O-acetyl-1-thio-β-D-galactopyranoside (**6**).* To a solution of 1,2,3,4,6-penta-*O*-acetyl-β-D-galactopyranoside (127 g, 325 mmol) in anhydrous CH_2Cl_2 (350 mL) were added $BF_3{\cdot}Et_2O$ (100 mL, 813 mmol) (*12*) and liquid MeSH (25 g, 520 mmol) at 0 °C. The solution was allowed to reach 22 °C in 1 h. TLC (1:1 EtOAc−hexane) indicated complete conversion of the starting material to a slightly faster-moving compound. The solution was slowly poured into ice-cold, aqueous $NaHCO_3$ in a 4 L Erlenmeyer flask under stirring. The organic layer was washed with H_2O, dried (Na_2SO_4), and concentrated. The resulting solids were treated with diethyl ether (300 mL). Filtration afforded **6** (109 g, 88.5%), which was used for the next step without further purification. Recrystallization of a sample afforded analytically pure **6** having physical properties identical to those of an independent preparation (*13c*).

*Methyl 4,6-O-Benzylidene-1-thio-β-D-galactopyranoside (**8**).* To a stirred mixture of **7** (*13*) (60 g, 303 mmol) and benzaldehyde dimethyl acetal (200 mL, 800 mmol) in DMF (100 mL) was added a catalytic amount of 10-camphorsulfonic acid (*14*). After 12 h, Et_3N (excess) was added, and approximately half of the volatiles were evaporated under vacuum. The residual solution was stirred with H_2O and hexane at ambient temperature for 1 h to afford a crystalline product which was isolated by filtration followed by washing alternatively with water and hexane and drying to give colorless **8** (69 g, 76%): mp 148−151 °C; $[\alpha]_D$ −32° (*c* 0.9) (lit. (*15*) mp 149−151; $[\alpha]_D$ −34° (*c* 0.5, H_2O)); NMR ($CDCl_3$) ^1H, δ 5.516 (s, 1 H, *CHPh*), 4.322 (dd, 1 H, $J_{5,6}$ = 1.5 Hz, $J_{6,6*}$ = 12.2 Hz, H-6), 4.263 (d, 1 H, $J_{1,2}$ = 9.3 Hz, H-1), 4.205 (br d, 1 H, H-4), 4.000 (dd, 1 H, $J_{5,6*}$ = 1.7 Hz, H-6*), 3.814 (t, 1 H, $J_{1,2}$ = $J_{2,3}$ = 9.3 Hz, H-2), 3.650 (dd, 1 H, H-3), 3.482 (d, 1 H, H-5), and 2.180 (s, 3 H, CH_3S); ^{13}C, δ 129.3, 128.3, and 126.4 (aromatic), 101.4 (*C*HPh), 85.0 (C-1), 75.9, 73.8, 69.9, and 68.8 (C-2,3,4,5), 69.2 (C-6), and 10.9 (CH_3S). Anal. ($C_{14}H_{18}O_5S$) C,H,S.

*Methyl 2,3-Di-O-acetyl-4,6-O-benzylidene-1-thio-β-D-galactopyranoside (**9**).* To a stirred solution of compound **8** (68 g, 227 mmol) in pyridine (100 mL) was added Ac_2O (100 mL) at 0 °C. After 3 h the volatiles were evaporated under vacuum. Extractive workup of the residue afforded **9** as a syrup (74 g, 85%), which was sufficiently pure for the next step. Column chromatographic purification (1:1 hexane−EtOAc) afforded analytically pure **9** as a syrup: $[\alpha]_D$ +44° (*c* 1.2); NMR ($CDCl_3$) ^1H, δ 5.486 (dd, 1 H, H-2), 5.486 (s, 1 H, C*H*Ph), 4.990 (dd, 1 H, $J_{2,3}$ = 9.9 Hz, $J_{3,4}$ = 3.4 Hz, H-3), 4.407 (br d, 1 H, H-4), 4.376 (d, 1 H, $J_{1,2}$ = 9.8 Hz, H-1), 4.330 (dd, 1 H, $J_{5,6}$ = 1.5 Hz, $J_{6,6*}$ = 12.4 Hz, H-6), 3.998 (dd, 1 H, $J_{5,6*}$ = 1.6 Hz, H-6*), 3.570 (m, 1 H, H-5), 2.239, 2.075, and 2.180 (3 s, 9 H, CH_3CO and CH_3S); ^{13}C, δ 170.5 and 169.4 (*C*=O), 137.5−126.3 (aromatic), 101.1 (*C*HPh), 81.9 (C-1), 73.5, 72.8, 69.5, and 69.0 (C-2,3,4,5), 65.7 (C-6), 20.80 and 20.75 (CH_3CO), and 10.1 (CH_3S). Anal. ($C_{18}H_{22}O_7S$) C,H,S.

*Methyl 2,3,4-Tri-O-acetyl-6-O-benzyl-1-thio-β-D-galactopyranoside (**10**).* To a stirred mixture of **9** (38 g, 99.4 mmol), $NaCNBH_3$ (30 g, 477 mmol), and 4A molecular sieves in tetrahydrofuran (500 mL) was added dropwise a saturated solution of HCl in diethyl ether (~130 mL) at 0 °C until TLC (2:1 hexane−EtOAc) indicated complete conversion (*16*). EtOAc (150 mL) was added, and the mixture was filtered through a layer of Celite 577. The solution was carefully neutralized with aqueous $NaHCO_3$ at 0 °C. The mixture was equilibrated between $CHCl_3$ and H_2O. The $CHCl_3$ layer was dried (Na_2SO_4) and then concentrated. A solution of the syrupy residue in pyridine (100 mL) was treated with Ac_2O (100 mL) at 25 °C. After 12 h the volatiles were removed under vacuum. Extractive workup of the residue afforded crystalline **10** (31.5 g, 74%): mp 102−104 °C; $[\alpha]_D$ +44° (c 1.2); NMR ($CDCl_3$) ^1H, δ 7.32−7.24 (aromatic), 5.516 (dd, 1 H, $J_{4,5}$ = 1.1 Hz, H-4), 5.239 (t, 1 H, $J_{1,2}$ = $J_{2,3}$ = 9.8 Hz, H-2), 5.061 (dd, 1 H, $J_{3,4}$ = 3.2 Hz, H-3), 4.550 and 4.414 (2 d,

2 H, $J \sim 12$ Hz, CH_2 of Bn), 4.395 (d, 1 H, H-1), 3.898 (ddd, 1 H, H-5), 3.547 (dd, 1 H, $J_{5,6} = 6.0$ Hz, $J_{6,6*} = 9.4$ Hz, H-6), 3.454 (dd, 1 H, $J_{5,6*} = 7.0$ Hz, H-6*), 2.184, 2.067, 2.053, and 1.982 (4 s, 12 H, CH_3CO and CH_3S); ^{13}C, δ 170.1, 170.0, and 169.7 (*C*=O), 137.4−127.9 (aromatic), 83.5 (C-1), 75.8, 72.0, 67.7, and 66.7 (C-2,3,4,5), 75.8 (*C*H_2 of Bn), 67.3 (C-6), 20.7 and 20.6 (*C*H_3-CO), and 11.5 (*C*H_3S). Anal. ($C_{19}H_{26}O_8S$) C,H,S.

*2,3,4-Tri-O-acetyl-6-O-benzyl-β-D-galactopyranosyl chloride (**11**).* To a solution of compound **10** (13.4 g, 31.4 mmol) in anhydrous CH_2Cl_2 (100 mL) was added a solution of Cl_2 in CCl_4 [containing ∼2.6 g (37 mmol) of Cl_2] at 0 °C (*17*). After 20 min, 1-hexene (excess) was added and the solution was concentrated to give **11** as a solid (11.5 g, 88%): $[\alpha]_D$ −2° (c 1.4); NMR ($CDCl_3$) ^{1}H, δ 7.37−7.23 (aromatic), 5.499 (dd, 1 H, H-4), 5.361 (dd, 1 H, $J_{1,2} = 8.7$ Hz, $J_{2,3} = 10.1$ Hz, H-2), 5.232 (d, 1 H, H-1), 5.003 (dd, 1 H, $J_{3,4} = 3.4$ Hz, H-3), 4.561 and 4.413 (2 d, 2 H, $J \sim 12$ Hz, CH_2 of Bn), 3.941 (ddd, 1 H, H-5), 3.594 (dd, 1 H, $J_{5,6} = 6.0$ Hz, $J_{6,6*} = 9.6$ Hz, H-6), 3.487 (dd, 1 H, $J_{5,6*} = 6.8$ Hz, H-6*), 2.080, 2.074, and 1.982 (3 s, 9 H, CH_3CO); ^{13}C, δ 170.0, 169.8, and 169.1 (*C*=O), 137.2, 128.4, and 128.0 (aromatic), 88.3 (C-1), 75.9 (*C*H_2 of Bn), 73.6, 71.1, 70.9, 67.1, and 67.0 (C-2,3,4,5,6), 20.6 and 20.5 (*C*H_3CO). Anal. ($C_{19}H_{23}ClO_{8)}$ C,H,Cl.

*2-(Trimethylsilyl)ethyl O-(2,3,4-Tri-O-acetyl-6-O-benzyl-β-D-galactopyranosyl)-(1→4)-3,6-di-O-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranoside (**12**).* To a stirred mixture of **5** (*11*) (10.5 g, 17.8 mmol), **11** (20.0 g, 48.2 mmol), 2,6-di-*tert*-butyl-4-methylpyridine (8 g, 38.9 mmol), and 4A molecular sieves (15 g) in CH_2Cl_2 (150 mL) was added AgOTf (25 g, 97.3 mmol), at −40 °C (*18*). The reaction mixture was allowed to reach 25 °C in 1 h and then was treated with aqueous $NaHCO_3$. The mixture was filtered, and the solids were washed with $CHCl_3$. Concentration of the organic phase afforded a syrup, which was chromatographed (3:1 hexane−EtOAc) to give amorphous **12** (13.3 g, 77%): $[\alpha]_D$ +10° (c 0.8); NMR ($CDCl_3$) ^{1}H, δ 7.55−6.98 (aromatic), 5.542 (br d, 1 H, H-4$_B$), 5.278 (dd, 1 H, $J_{1,2} = 8.0$ Hz, $J_{2,3} = 10.4$ Hz, H-2$_B$), 5.268 (d, 1 H, $J_{1,2} = 8.1$ Hz, H-1$_A$), 5.018 (dd, 1 H, $J_{3,4} = 3.5$ Hz, H-3$_B$), 4.730 (d, 1 H, H-1$_B$), 2.154 and 2.104 (2 s, 9 H, CH_3CO), 1.04−0.81 (m, 2 H, H_2CSi), and 0.129 (s, 9 H, $SiMe_3$); ^{13}C, δ 170.0, 169.9, and 169.2 (C=O), 138.6−123.1 (aromatic), 100.2 (C-1$_B$), 97.7 (C-1$_A$), 74.3, 73.5, 73.3, 67.5, 66.8, and 66.7 (C-6$_A$,6$_B$, *C*H_2CH$_2$Si, and *C*H_2 of Bn), 55.7 (C-2$_A$), 20.7 (2C) and 20.5 (*C*H_3CO), 17.6 (*C*H_2Si), and −1.6 ($SiMe_3$); FABMS *m/z* 966 [(M + H − H_2)$^+$], 940 {[M + H − HO(CH$_2$)$_2$SiMe$_3$ + HOSiMe$_3$]$^+$}, 850 {[M + H − HO(CH$_2$)$_2$-SiMe$_3$]$^+$}. Anal. ($C_{52}H_{61}NO_{15}Si$) C,H,N.

*2-(Trimethylsilyl)ethyl O-(6-O-Benzyl-β-D-galactopyranosyl)-(1→4)-3,6-di-O-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranoside (**13**).* To a solution of **12** (13 g, 13.4 mmol) in MeOH (100 mL) was added a catalytic amount of NaOMe at 25 °C. The usual workup afforded **13** as a syrup (10.9 g, 96%): $[\alpha]_D$ +47° (*c* 1.4); NMR ($CDCl_3$) ^{13}C, δ 138.5−123.1 (aromatic), 103.4 ($^1J_{C\text{-}1,H\text{-}1} = 160$ Hz, C-1$_B$), 97.7 ($^1J_{C\text{-}1,H\text{-}1} = 163$ Hz, C-1$_A$), 74.3, 73.3 (2C), 69.2, 68.2, and 66.8 (C-6$_A$,6$_B$, *C*H_2CH$_2$Si, and *C*H_2 of Bn), 55.9 (C-2$_A$), 17.8 (*C*H_2Si), and −1.6 ($SiMe_3$). Anal. ($C_{46}H_{55}NO_{12}$-Si) C,H,N.

*2-(Trimethylsilyl)ethyl O-(6-O-benzyl-3,4-O-isopropylidene-β-D-galactopyranosyl)-(1→4)-3,6-di-O-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranoside (**14**).* To a solution of **13** (10 g, 11.9 mmol) in 2,2-dimethoxypropane (40 mL, 325 mmol) was added a catalytic amount of 10-camphorsulfonic acid at 25 °C (*19*). After 2 h, the solution was treated with an excess of Et_3N. Extractive workup ($CHCl_3$/H_2O) afforded **14** as a syrup (10.8 g, 94%): $[\alpha]_D$ +49° (*c* 1.0); NMR ($CDCl_3$) ^{13}C, δ 138.5−123.1 (aromatic), 109.9 (*C*Me_2), 102.6 ($^1J_{C\text{-}1,H\text{-}1} = 160$ Hz, C-1$_B$), 97.9 ($^1J_{C\text{-}1,H\text{-}1} = 163$ Hz, C-1$_A$), 74.6, 73.6, 73.4, 69.2, 68.2, and 66.7 (C-6$_A$,6$_B$, *C*H_2CH$_2$Si, and *C*H_2 of Bn), 55.8 (C-2$_A$), 28.2 and 26.3 [(*C*H_3)$_2$C], 17.7 (*C*H_2Si), and −1.6 ($SiMe_3$). Anal. ($C_{49}H_{59}NO_{14}Si$·EtOAc) C,H,N.

*2-(Trimethylsilyl)ethyl O-(2,6-di-O-benzyl-3,4-O-isopropylidene-β-D-galactopyranosyl)-(1→4)-3,6-di-O-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranoside (**15**).* To a stirred solution of **14** (23 g, 26.3 mmol) in DMF (140 mL) was added NaH (2.8 g of a 60% suspension in oil, ∼70 mmol) at 0 °C. After 30 min, the mixture was treated with benzyl bromide (10 mL, 84 mmol). The reaction mixture was stirred for 4 h at 0 °C. The usual workup followed by column chromatographic purification (4:1 hexane−EtOAc) afforded **15** as a syrup (21 g, 82%): $[\alpha]_D$ +41° (*c* 1.4); NMR ($CDCl_3$) ^{13}C, δ 102.2 (C-1$_B$), 97.5 (C-1$_A$), 74.3, 73.4 (2C), 73.1, 69.1, 67.9, and 66.6 (C-6$_A$,6$_B$, *C*H_2CH$_2$Si, and *C*H_2 of Bn), 55.7 (C-2$_A$), 27.9 and 26.3 [(*C*H_3)$_2$*C*], 17.7 (*C*H_2Si), and −1.6 ($SiMe_3$). Anal. ($C_{56}H_{65}NO_{12}Si$) C,H,N.

*2-(Trimethylsilyl)ethyl O-(2,6-di-O-benzyl-β-D-galactopyranosyl)-(1→4)-3,6-di-O-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranoside (**16**).* A solution of **15** (20 g, 20.6 mmol) in 4:1 AcOH−H_2O (100 mL) was kept at 80 °C for 2 h. Evaporation of the volatiles under vacuum followed by column chromatographic purification (2:1 hexane−EtOAc) afforded **16** as a syrup (15 g, 78%): $[\alpha]_D$ +33° (*c* 1.5); NMR ($CDCl_3$) ^{13}C, δ 138.7−123.1 (aromatic), 103.0 (C-1$_B$), 97.7 (C-1$_A$), 75.0, 74.3, 73.5, 73.1, 69.1, 68.0, and 66.6 (C-6$_A$,6$_B$, *C*H_2CH$_2$Si, and *C*H_2 of Bn), 55.7 (C-2$_A$), 17.7 (*C*H_2Si), and −1.6 ($SiMe_3$); CIMS *m/z* 954 [(M + Na)$^+$], 930 [(M + H − H_2)$^+$], 904 {[M + H − HO(CH$_2$)$_2$SiMe$_3$ + HOSiMe$_3$]$^+$}, 814 {[M + H − HO(CH$_2$)$_2$SiMe$_3$]$^+$}. Anal. ($C_{53}H_{61}NO_{12}Si$) C,H,N.

*2-(Trimethylsilyl)ethyl O-(2,3,6-Tri-O-benzyl-β-D-galactopyranosyl)-(1→4)-3,6-di-O-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranoside (**17**).* A stirred mixture of **16** (7.0 g, 7.5 mmol), Bu_2SnO (2.0 g, 8 mmol), and toluene (150 mL) was refluxed under a Dean-Stark adapter for 2 h, and then approximately half of the volatiles were evaporated under vacuum. Bu_4NBr (2.5 g, 7.8 mmol) and benzyl bromide (1.2 mL, 10.1 mmol) were added, and the mixture was stirred at 40 °C for 3 h. The mixture was concentrated under vacuum. Column chromatography (2:1 hexane−EtOAc) of the residue afforded **17** as a syrup (7.5 g, 98%): $[\alpha]_D$ +25° (*c* 1.2); NMR ($CDCl_3$) ^{13}C, δ 133.7−123.2 (aromatic), 102.9 (C-1$_B$), 97.8 (C-1$_A$), 75.4, 74.4, 73.5, 73.0, 72.0, 68.8, 67.9, and 66.6 (C-6$_A$,6$_B$, *C*H_2CH$_2$Si, and *C*H_2 of Bn), 55.7 (C-2$_A$), 17.5 (*C*H_2Si), and −1.7 ($SiMe_3$); CIMS *m/z* 1094 [(M + $SiMe_3$)$^+$], 994 {[M + H − HO(CH$_2$)$_2$SiMe$_3$ + HOSiMe$_3$]$^+$}, 904 [(M + H − HO(CH$_2$)$_2$-SiMe$_3$)$^+$]. Anal. ($C_{60}H_{67}NO_{12}Si$) C,H,N.

*Methyl 2-O-Benzyl-6-O-(tert-butyldiphenylsilyl)-3,4-O-isopropylidene-1-thio-β-D-galactopyranoside (**19**).* To a solution of methyl 6-*O*-(*tert*-butyldiphenylsilyl)-3,4-*O*-isopropylidene-1-thio-β-d-galactopyranoside [**18**, (*20*)] (7.7 g, 15.8 mmol) in DMF (60 mL) was added NaH (0.7 g of a 60% suspension in oil, ∼17.5 mmol) at 0 °C. After 30 min, the mixture was treated with benzyl bromide (3.5 mL, 29.4 mmol). The reaction mixture was stirred for 2 h at 0 °C. The usual workup followed by column chromatographic purification (8:1 hexane−EtOAc) afforded **19** as a crystalline solid (7.6 g, 83%): mp 108−110 °C; $[\alpha]_D$ −4° (*c* 1.1); NMR ($CDCl_3$) ^{1}H, δ 7.72−7.23 (aromatic), 4.860 and 4.747 (2 d, 2 H, $J \sim 12$ Hz, CH_2 of Bn), 4.316 (dd, 1 H, $J_{3,4} = 5.5$ Hz, $J_{4,5}$ 2.0 Hz, H-4), 4.284 (d, 1 H, H-1), 4.224 (dd, 1 H, H-3), 3.925 (m, 1 H, H-6,6*), 3.825 (m, 1 H, H-5), 4.442 (dd, 1 H, $J_{1,2} = 9.6$ Hz, H-2), 2.156 (s, 3 H, CH_3S), 1.436 and 1.358 [2 s, 6 H, (CH_3)$_2$C)], and 1.050 (s, 9 H, CMe_3); ^{13}C, δ 135.6−127.7 (aromatic), 109.8 [*C*(CH$_3$)$_2$], 84.5 (C-1), 79.7, 78.9, 76.8, and 73.5 (C-

2,3,4,5), 73.5 (CH_2 of Bn), 62.7 (C-6), 27.9 and 26.3 [$(CH_3)_2C$], 26.7 [$C(CH_3)_3$)], and 12.6 (CH_3S). Anal. ($C_{56}H_{65}NO_{12}Si$) C,H,N.

*Methyl 2-O-benzyl-3,4-O-isopropylidene-1-thio-β-D-galactopyranoside (**20**).* To a solution of compound **19** (7.0 g, 12.1 mmol) in tetrahydrofuran (50 mL) was added Bu_4NF (6.0 mL of a 1.4 M solution in tetrahydrofuran, ~8.4 mmol) at 25 °C. After 12 h, the solution was concentrated. Column chromatography (3:1 → 1:1 hexane−EtOAc) afforded **20** as a syrup (4.0 g, 97%): $[\alpha]_D$ +4° (*c* 1.0); NMR ($CDCl_3$) ^{1}H, δ 7.42−7.24 (aromatic), 4.850 and 4.746 (2 d, 2H, *J* ~ 11 Hz, CH_2 of Bn), 4.322 (d, 1 H, $J_{1,2}$ = 9.6 Hz, H-1), 4.250 (t, 1 H, $J_{2,3} = J_{3,4}$ = 6 Hz, H-3), 4.182 (dd, 1 H, $J_{4,5}$ = 1.9 Hz, H-4), 3.98−3.75 (m, 3 H, H-5,6,6*), 3.451 (dd, 1 H, H-2), 2.190 (s, 3 H, CH_3S), 1.442 and 1.351 [2 s, 6 H, $(CH_3)_2C$)]; ^{13}C, δ 137.7, 128.3, and 127.8 (aromatic), 110.1 [$C(CH_3)_2$], 84.3 (C-1), 79.6, 78.5, 76.7, and 73.9 (C-2,3,4,5), 73.3 (CH_2 of Bn), 62.4 (C-6), 27.7 and 26.2 [$(CH_3)_2C$], and 12.7 (CH_3S). Anal. ($C_{17}H_{24}O_5S$) C,H,S.

*Methyl 2-O-Benzyl-1-thio-β-D-galactopyranoside (**21**).* A solution of **20** (3.8 g, 11.2 mmol) in 3:1 AcOH−H_2O (40 mL) was kept at 80 °C for 3 h and then concentrated under vacuum. Toluene (3 × 50 mL) was added to and evaporated from the residue. TLC (1:1 hexane−EtOAc) indicated the disappearance of **20** and the formation of a major and a minor product. A solution of the residue in MeOH was treated with a catalytic amount of NaOMe. After 2 h, the solution was neutralized (Dowex 50 × 2, H^+) and concentrated. Trituration of the residue in hexane afforded **21** as a crystalline solid (3.2 g, 95%): mp 157−159 °C; $[\alpha]_D$ +0.4° (*c* 0.7, MeOH); NMR ($CDCl_3$) ^{1}H, δ 7.48−7.24 (aromatic), 4.864 and 4.815 (2 d, 2H, *J* = 10.5 Hz, CH_2 of Bn), 4.316 (d, 1 H, $J_{1,2}$ = 9.3 Hz, H-1), 3.920 (dd, 1 H, H-4), 3.797 (dd, 1 H, $J_{5,6}$ = 6.6 Hz, $J_{6,6*}$ = 11.4 Hz, H-6), 3.721 (dd, 1 H, $J_{5,6*}$ = 5.3 Hz, H-6*), 3.638 (dd, 1 H, $J_{2,3}$ = 9.1 Hz, $J_{3,4}$ = 3.2 Hz, H-3), 3.557 (t, 1 H, H-2), 5.51 (m, 1 H, H-5), and 2.237 (s, 3 H, CH_3S); ^{13}C, δ 137.6−127.0 (aromatic), 84.9 (C-1), 78.3, 77.8, 74.3, and 68.8 (C-2,3,4,5), 74.6 (CH_2 of Bn), 60.6 (C-6), and 11.4 (CH_3S). Anal. ($C_{17}H_{24}O_5S$) C,H,S.

*Methyl 3,4,6-Tri-O-acetyl-2-O-benzyl-1-thio-β-D-galactopyranoside (**22**).* To a solution of **21** (2.9 g, 9.6 mmol) in pyridine (20 mL) was added Ac_2O (20 mL) at 0 °C. The reaction mixture was allowed to reach 20 °C in 1 h, and then the volatiles were removed under vacuum. Column chromatography (5:2 hexane−EtOAc) afforded **22** as a syrup (4.0 g, 97%): $[\alpha]_D$ +15° (*c* 1.0); NMR ($CDCl_3$) ^{1}H, δ 7.36−7.25 (aromatic), 5.403 (dd, 1 H, $J_{3,4}$ = 3.4 Hz, H-4), 5.017 (dd, 1 H, $J_{2,3}$ = 9.5 Hz, H-3), 4.85 and 4.62 (2 d, 2 H, *J* ~ 11 Hz, CH_2 of Bn), 4.463 (d, 1 H, $J_{1,2}$ = 9.7 Hz, H-1), 4.160 (dd, 1 H, $J_{5,6}$ = 6.7 Hz, $J_{6,6*}$ = 11.2 Hz, H-6), 4.085 (dd, 1 H, $J_{5,6*}$ = 6.6 Hz, H-6*), 3.871 (br t, 1 H, H-5), 3.675 (t, 1 H, H-2), 2.266, 2.131, 2.036, and 1.946 (4 s, 12 H, 3 CH_3CO and CH_3S); ^{13}C, δ 170.5, 170.2, and 170.0 (C=O), 137.7−127.9 (aromatic), 85.8 (C-1), 75.6, 74.0, 73.9, 67.6 (C-2,3,4,5), 75.4 (CH_2 of Bn), 61.4 (C-6), 20.5 (CH_3CO), and 12.9 (CH_3S); CIMS *m/z* 444 [(M + NH_3)$^+$], 379 [(M + H-CH_3SH)$^+$]. Anal. ($C_{20}H_{26}O_8S$) C,H,S.

*2-(Trimethylsilyl)ethyl O-(3,4,6-Tri-O-acetyl-2-O-benzyl-α-D-galactopyranosyl)-(1→4)-O-(2,3,6-tri-O-benzyl-β-D-galactopyranosyl)-(1→4)-3,6-di-O-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranoside (**23**).* To a stirred mixture of disaccharide **17** (7.0 g, 6.85 mmol), thioglycoside **22** (6.1 g, 14.3 mmol), 2,6-di-*tert*-butyl-4-methylpyridine (3 g, 14.6 mmol), and 4A molecular sieves (3 g) in diethyl ether (100 mL) at 25 °C was added MeOTf (4 mL, 35 mmol) over a period of 96 h. The usual workup, followed by column chromatographic purification (3:1 hexane−EtOAc), afforded **23** as an amorphous solid (6.9 g, 73%): $[\alpha]_D$ +48° (*c* 0.4); NMR ($CDCl_3$) ^{1}H, 7.8−6.9 (aromatic), 5.381 (dd, 1 H, $J_{3,4}$ = 3.3 Hz, $J_{4,5}$ = 1.3 Hz, H-4$_C$), 5.258 (dd, 1 H, $J_{1,2}$ = 3.2 Hz, $J_{2,3}$ = 10.1 Hz, H-3$_C$), 5.053 (d, 1 H, H-1$_C$), 3.770 (dd, 1 H, H-2$_C$), 1.988, 1.848, and 1.851 (3 s, 9 H, 3 CH_3CO), and −0.177 [s, 9 H, ($SiMe_3$)]; ^{13}C, δ 179.9 and 169.6 (C=O), 139.2−127.9 (aromatic), 102.6, 99.3, and 97.8 (C-1$_A$,1$_B$,1$_C$), 80.3, 79.4, 78.1, 77.1, 75.1, 74.1, 73.8, 72.3, 70.0, 68.4, and 66.0 (C-2$_B$,2$_C$,3$_A$,3$_B$,3$_C$,4$_A$,4$_B$,4$_C$,5$_A$,5$_B$,5$_C$), 75.2, 74.7, 73.5, 73.0, 72.9, 72.6, 68.0, 66.8, and 66.5 (C-6$_B$,6$_C$, 6 CH_2 of Bn, CH_2CH_2Si), 60.9 (C-6$_A$), 55.8 (C-2$_A$), 20.6 and 20.5 (CH_3CO), 17.8 (CH_2Si), and −1.6 ($SiMe_3$); FABMS *m/z* 1398 [(M + H − H_2)$^+$]. Anal. ($C_{79}H_{89}NO_{20}Si$) C,H,N.

*2-(Trimethylsilyl)ethyl O-(3,4,6-Tri-O-acetyl-α-D-galactopyranosyl)(1→4)-O-(β-D-galactopyranosyl)-(1→4)-2-deoxy-2-phthalimido-β-D-glucopyranoside (**24**).* A mixture of **23** (6.8 g, 5 mmol), 10% Pd−C (1.5 g), EtOH (50 mL), and AcOH (5 mL) was stirred under hydrogen at 25 °C at 345 kPa for 4 days. Removal of the catalyst by filtration and the volatiles under vacuum afforded amorphous **24** (3.94 g, 92%): $[\alpha]_D$ +47° (*c* 1); NMR ($CDCl_3$) ^{1}H, 7.78−7.70 (aromatic), 5.413 (br d, 1H, H-4$_C$), 5.175 (dd, 1H, H-3$_C$), 5.235 (d, 1H, $J_{1,2}$ = 8, 7 Hz, H-1$_A$), 5.068 (d, 1H, $J_{1,2}$ = 3.7 Hz, H-1$_C$), 4.567 (d, 1H, $J_{1,2}$ = 8.9 Hz, H-1$_B$), 2.119, 2.058, and 1.993 (3 s, 9 H, 3 CH_3CO), and −0.150 (s, 9 H, $SiMe_3$); ^{13}C, δ 171.4, 171.2, and 170.4 (C=O of Ac), 168.6 (C=O of Phth), 134.3 and 131.7 (aromatic), 103.9 and 100.8 (C-1$_B$,1$_C$), 97.8 (C-1$_A$), 80.6, 78.4, 74.9, 74.6, 73.0, 71.6, 70.2 (2C), 70.1, 68.3, 67.3, 67.2, and 66.9 (C-2$_B$,2$_C$,3$_A$,3$_B$,3$_C$,4$_A$,4$_B$,4$_C$,5$_A$,5$_B$,5$_C$, and CH_2CH_2Si), 62.0, 61.2, and 60.7 (C-6$_A$,6$_B$,6$_C$), 56.4 (C-2$_A$), 20.7, 20.6, and 20.5 (CH_3CO), 17.7 (CH_2Si), and −1.7 ($SiMe_3$); FABMS *m/z* 882 [(M + Na)$^+$], 840 {[M + H − HO(CH_2)$_2$$SiMe_3$ + $HOSiMe_3$]$^+$}, 742 {[M + H − HO(CH_2)$_2$$SiMe_3$]$^+$}. Anal. ($C_{37}H_{53}NO_{20}Si$) C,H,N.

*2-(Trimethylsilyl)ethyl O-(2,3,4,6-Tetra-O-acetyl-α-D-galactopyranosyl)-(1→4)-O-(2,3,6-tri-O-acetyl-β-D-galactopyranosyl)-(1→4)-3,6-di-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranoside (**25**).* A solution of **24** (3.7 g, 4.3 mmol), 4-(dimethylamino)pyridine (100 mg, 0.8 mmol), pyridine (10 mL), and Ac_2O (10 mL) was kept at 25 °C for 12 h. Removal of the volatiles at 30 °C under vacuum followed by column chromatographic purification (2:1 hexane−EtOAc) afforded amorphous **25** (4.5 g, 94%): $[\alpha]_D$ +52° (*c* 0.6); NMR ($CDCl_3$) ^{1}H 7.89−7.73 (aromatic), 5.727 (dd, 1 H, $J_{2,3}$ = 10 Hz, $J_{3,4}$ = 7.8 Hz, H-3$_A$), 5.564 (br d, 1 H, H-4$_C$), 5.398 (d, 1 H, $J_{1,2}$ = 8.4 Hz, H-1$_A$), 5.377 (dd, 1 H, $J_{2,3}$ = 11 Hz, $J_{3,4}$ = 3.2 Hz, H-3$_C$), 5.173 (dd, 1 H, $J_{1,2}$ = 3.5 Hz, H-2$_C$), 5.130 (dd, 1 H, $J_{1,2}$ = 7.7 Hz, $J_{2,3}$ = 10.7 Hz, H-2$_B$), 4.997 (d, 1 H, $J_{1,2}$ = 3.5 Hz, H-1$_C$), 4.800 (dd, 1 H, $J_{3,4}$ = 2.4 Hz, H-3$_B$), 4.614 (d, 1 H, H-1$_B$), 4.185 (H-2$_A$), 2.155, 2.138, 2.083, 2.070, 2.056, 1.964, and 1.949 (7 s, 27 H, 9 CH_3CO), and −0.127 (s, 9 H, $SiMe_3$); ^{13}C, δ 171−169.2 (C=O of Ac), 134.3−123.3 (aromatic), 100.8, 99.3, and 97.2 (C-1$_A$,1$_B$,1$_C$), 76.9, 76.7, 72.6, 72.3, 71.5, 71.4, 68.9, 68.6, 67.8, 67.1, 66.9, and 66.8 (C-2$_B$,2$_C$, 3$_A$,3$_B$,3$_C$,4$_A$,4$_B$,4$_C$,5$_A$,5$_B$,5$_C$, and CH_2CH_2Si), 62.3, 61.1, and 60.1 (C-6$_A$,6$_B$,6$_C$), 54.7 (C-2$_A$), 20.4−20.0 (CH_3CO), 17.3 (CH_2Si), and −2.1 ($SiMe_3$); FABMS *m/z* 1134 [(M + Na)$^+$], 1084 {[M + H − HO(CH_2)$_2$$SiMe_3$ + $HOSiMe_3$]$^+$}, 1052 [(M + H − AcOH)$^+$], 994 {[M + H − HO(CH_2)$_2$-$SiMe_3$]$^+$}, 934 [(994 − AcOH)$^+$]. Anal. ($C_{49}H_{65}NO_{26}Si$) C,H,N.

*O-(2,3,4,6-Tetra-O-acetyl-α-D-galactopyranosyl)-(1→4)-O-(2,3,6-tri-O-acetyl-β-D-galactopyranosyl)-(1→4)-3,6-di-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranose (**26**).* A mixture of **25** (5.0 g, 4.5 mmol), $CHCl_3$ (15 mL), and CF_3COOH (15 mL) was kept at 25 °C for 4 h, and then the volatiles were removed under vacuum at 25 °C. Tritu-

ration of the residue in hexane afforded **26** as an amorphous solid (3.5 g 77%): $[\alpha]_D$ +85° (*c* 0.63); NMR ($CDCl_3$) ^{1}H, 7.85−7.70 (aromatic), 5.757 (m, 1 H, $J_{2,3}$ = 10.5 Hz, H-3$_A$), 5.664 (d, 1 H, $J_{1,2}$ = 8.4 Hz, H-1$_A$), 5.565 (dd, 1 H, H-4$_C$), 5.367 (dd, 1 H, $J_{2,3}$ = 11 Hz, $J_{3,4}$ = 3.3 Hz, H-3$_C$), 5.157 (dd, 1 H, $J_{1,2}$ = 3.5 Hz, H-2$_C$), 5.116 (dd, 1 H, $J_{1,2}$ = 7.6 Hz, $J_{2,3}$ = 10.8 Hz, H-2$_B$), 4.975 (d, 1 H, $J_{1,2}$ = 3.5 Hz, H-1$_C$), 4.753 (dd, 1 H, $J_{3,4}$ = 2.5 Hz, H-3$_B$), 4.588 (d, 1 H, H-1$_B$), 4.16 (H-2$_A$), 2.129, 2.116, 2.061, 2.060, 2.055, 2.053, 2.031, 1.948, and 1.941 (9 s, 27 H, 9 C*H*$_3$CO); ^{13}C, δ 170.65, 170.61, 170.5, 170.4, 170.0, 169.7, 169.5, 168.9, 167.8 (*C*=O of Ac), 167.8 (*C*=O of Phth), 134.3−123.5 (aromatic), 100.9 and 99.5 (C-1$_B$,1$_C$), 92.3 (C-1$_A$), 76.9, 76.7, 72.7 (2C), 71.6, 71.3, 69.0, 68.8, 67.9, and 67.0 (2C) (C-2$_B$,2$_C$,3$_A$,3$_B$,3$_C$,4$_A$,4$_B$,4$_C$,5$_A$,5$_B$,5$_C$), 62.4, 61.2, and 60.2 (C-6$_A$,6$_B$,6$_C$), 56.2 (C-2$_A$), and 20.9−20.4 (*C*H$_3$CO); FABMS *m*/*z* 1050 [(M + K)$^+$], 1034 [(M + Na)$^+$], 1012 [(M + H)$^+$], 1010 [(M + H − H$_2$)$^+$]. Anal. ($C_{44}H_{53}NO_{26}$) C,H,N.

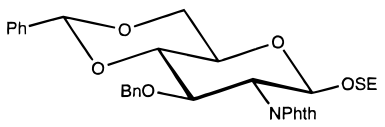
*O-(2,3,4,6-Tetra-O-acetyl-α-D-galactopyranosyl)-(1→4)-O-(2,3,6-tri-O-acetyl-β-D-galactopyranosyl)-(1→4)-3,6-di-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl Trichloroacetimidate (**27**).* To a stirred solution of **26** (2.38 g, 2.4 mmol) in CH_2Cl_2 (20 mL) were added at −30 °C CCl_3CN (4.0 mL, 40 mmol) and 1,8-diazabicyclo[5.4.0]undec-7-ene (600 μL, 4 mmol) (*21*, *22*). The solution was stirred at −20 °C for 1 h and then was allowed to reach ∼20 °C in 2 h. The reaction mixture was applied to a column of silica gel suspended in hexane. Elution with hexane → hexane−EtOAc (1:2) followed by crystallization from diisopropyl ether−hexane gave **27** (2.22 g, 82%): mp 154 °C dec; $[\alpha]_D$ +59° (*c* 0.5); NMR ($CDCl_3$) ^{1}H, 6.643 (d, 1 H, $J_{1,2}$ = 8.9 Hz, H-1$_A$), 5.868 (dd, 1 H, $J_{2,3}$ = 10.5 Hz, H-3$_A$), 5.577 (dd, 1 H, H-4$_C$), 5.380 (dd, 1 H, $J_{2,3}$ = 11 Hz, $J_{3,4}$ = 3.3 Hz, H-3$_C$), 5.170 (dd, 1 H, $J_{1,2}$ = 3.5 Hz, H-2$_C$), 5.134 (dd, 1 H, $J_{1,2}$ = 7.7 Hz, $J_{2,3}$ = 10.7 Hz, H-2$_B$), 4.980 (d, 1 H, $J_{1,2}$ = 3.6 Hz, H-1$_C$), 4.744 (dd, 1 H, $J_{3,4}$ = 2.5 Hz, H-3$_B$), 4.578 (d, 1 H, H-1$_B$), 2.153−1.953 (8 s, 27 H, 9 C*H*$_3$CO); ^{13}C, δ 170.5−167.4 (*C*=O), 134.3, 131.2, and 123.5 (aromatic), 100.8 and 99.5 (C-1$_B$,1$_C$), 93.5 (C-1$_A$), 76.9, 76.3, 73.5, 72.7, 71.8, 71.0, 68.9, 68.8, 67.9, 67.2, and 67.1 (C-2$_B$,2$_C$,3$_A$,3$_B$,3$_C$,4$_A$,4$_B$,4$_C$,5$_A$,5$_B$,5$_C$), 62.0, 61.4, and 60.3 (C-6$_A$,6$_B$,6$_C$), 53.9 (C-2$_A$), and 21.0−20.5 (*C*H$_3$CO); FABMS *m*/*z* 994 [(M + H-$C_2H_2Cl_3NO$)$^+$], 934 [994 − AcOH]$^+$. Anal. ($C_{46}H_{53}Cl_3N_2O_{26}$) C,H,N,Cl.

*8-(Benzyloxy)octyl O-(2,3,4,6-Tetra-O-acetyl-α-D-galactopyranosyl)-(1→4)-O-(2,3,6-tri-O-acetyl-β-D-galactopyranosyl)-(1→4)-3,6-di-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranoside (**28**).* To a stirred mixture of **27** (2.05 g, 1.8 mmol), 8-(benzyloxy)octanol (10) (1.1 g, 4.7 mmol), and 4A molecular sieves (2 g) in CH_2Cl_2 (20 mL) was added $BF_3{\cdot}Et_2O$ (100 μL, 0.8 mmol) at −30 °C. The mixture was allowed to reach 25 °C in 1 h. Removal of the solids by filtration followed by extractive workup ($CHCl_3$/aqueous $NaHCO_3$) and concentration under vacuum afforded a syrup which was crystallized from EtOH−diisopropyl ether to give **28** (1.75 g, 82%): mp 143−145 °C; $[\alpha]_D$ +49° (*c* 0.4); NMR ($CDCl_3$) ^{1}H, 5.737 (m, 1 H, $J_{2,3}$ = 10.5 Hz, $J_{3,4}$ = 7.7 Hz, H-3$_A$), 5.573 (br d, 1 H, H-4$_C$), 5.365 (dd, 1 H, $J_{2,3}$ = 11 Hz, $J_{3,4}$ = 3.4 Hz, H-3$_C$), 5.356 (d, 1 H, $J_{1,2}$ = 8.4 Hz, H-1$_A$), 5.160 (dd, 1 H, $J_{1,2}$ = 3.5 Hz, H-2$_C$), 5.116 (dd, 1 H, $J_{1,2}$ = 7.8 Hz, $J_{2,3}$ = 10.6 Hz, H-2$_B$), 4.971 (d, 1 H, $J_{1,2}$ = 3.4 Hz, H-1$_C$), 4.735 (dd, 1 H, $J_{3,4}$ = 2.4 Hz, H-3$_B$), 4.566 (d, 1 H, H-1$_B$), 3.991 (br d, 1 H, H-4$_B$), 2.134−1.944 (8 s, 27 H, 9 C*H*$_3$CO); ^{13}C, δ 170.6−168.8 (*C*=O of Ac), 167.7 (*C*=O of Phth), 138.6−123.4 (aromatic), 101.0, 99.6, and 97.9 (C-1$_A$,1$_B$,1$_C$), 76.6, 76.2, 72.83, 72.5, 71.7 (2C), 69.0, 68.8, 67.9, and 67.1 (2C) (C-2$_B$,2$_C$,3$_A$,3$_B$,3$_C$,4$_A$,4$_B$,4$_C$,5$_A$,5$_B$,5$_C$), 72.77 (*C*H$_2$ of Bn), 70.4 and 70.1 (C-1′,8′), 62.5, 61.2, and 60.2 (C-6$_A$,6$_B$,6$_C$), 55.0 (C-2$_A$), 29.7, 29.3 (2C), 29.1, 26.0, and 25.7 (C-2′,3′,4′,5′,6′,7′), and 20.9−20.5 (*C*H$_3$CO); FABMS *m*/*z* 1252 [(M + Na)$^+$], 1228 [(M + H − H$_2$)$^+$], 934 {[M + H − AcOH − HO(CH$_2$)$_8$OBn]$^+$}. Anal. ($C_{59}H_{75}NO_{29}$) C,H,N.

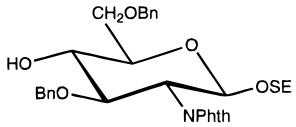
*8-(Benzyloxy)octyl O-(2,3,4,6-Tetra-O-acetyl-α-D-galactopyranosyl)-(1→4)-O-(2,3,6-tri-O-acetyl-β-D-galactopyranosyl)-(1→4)-2-acetamido-3,6-di-O-acetyl-2-deoxy-β-D-glucopyranoside (**29**).* A solution of **28** (1.76 g, 1.5 mmol) and hydrazine hydrate (1.8 g, 56 mmol) in EtOH was stirred under reflux for 2 days. More hydrazine hydrate (0.65 g) was added, and stirring under reflux was continued for an additional day. The volatiles were removed under the vacuum of a water aspirator. The residual syrup was dissolved in pyridine (15 mL, 185 mmol) to which Ac_2O (15 mL, 160 mmol) and a catalytic amount of 4-(dimethylamino)pyridine were added. After 2 days, the solution was concentrated. Extractive workup ($CHCl_3/H_2O$) followed by chromatographic purification (4:1 EtOAc−hexane) afforded a syrup which was homogeneous by TLC when visualized by charring with H_2SO_4, but contained additional compounds as detected by I_2 vapor. A solution of this syrup in MeOH was treated with a catalytic amount of NaOMe at 25 °C for 24 h. The solution was neutralized (Dowex 50 × 2, H$^+$) and concentrated. Column chromatographic purification of the residue (2:1 EtOAc−MeOH) afforded a syrup which was dissolved in pyridine (10 mL, 124 mmol) to which Ac_2O (10 mL, 106 mmol) and a catalytic amount of 4-(dimethylamino)pyridine were added. The usual workup followed by chromatographic purification (4:1 EtOAc−hexane) afforded **29** as a syrup (1.07 g, 63%): $[\alpha]_D$ +30° (*c* 0.2); NMR ($CDCl_3$) ^{1}H, 5.645 (d, 1 H, $J_{H\text{-}2,NH}$ = 9.4 Hz, *H*N), 5.575 (br d, 1 H, H-4$_C$), 5.378 (dd, 1 H, $J_{2,3}$ = 11 Hz, $J_{3,4}$ = 3.4 Hz, H-3$_C$), 5.187 (dd, 1 H, $J_{1,2}$ = 3.6 Hz, H-2$_C$), 5.115 (dd, 1 H, $J_{1,2}$ = 7.8 Hz, $J_{2,3}$ = 10.8 Hz, H-2$_B$), 5.080 (d, 1 H, $J_{2,3}$ + $J_{3,4}$ = 18 Hz, H-3$_A$), 4.989 (d, 1 H, $J_{1,2}$ = 3.5 Hz, H-1$_C$), 4.754 (dd, 1 H, $J_{3,4}$ = 2.5 Hz, H-3$_B$), 4.538 (d, 1 H, H-1$_B$), 4.418 (d, 1 H, H-1$_A$), and 2.129−1.948 (10 s, 30 H, 10 C*H*$_3$CO); ^{13}C, δ 170.5−169.0 (*C*=O), 128.2−127.4 (aromatic), 101.0 (2C) and 99.6 (C-1$_A$,1$_B$,1$_C$), 76.9, 75.9, 72.6 (2C), 72.5, 71.8, 68.9, 68.7, 67.8, and 67.1 (2C) (C-2$_B$,2$_C$,3$_A$,3$_B$,3$_C$,4$_A$,4$_B$,4$_C$,5$_A$,5$_B$,5$_C$), 72.8 (*C*H$_2$ of Bn), 70.4 and 69.6 (C-1′,8′), 62.5, 61.3, and 60.3 (C-6$_A$,6$_B$,6$_C$), 53.3 (C-2$_A$), 29.7, 29.4 (2C), 29.2, 26.1, and 25.8 (C-2′,3′,4′,5′,6′,7′), 23.2 (*C*H$_3$CON), and 20.9−20.6 (*C*H$_3$COO); FABMS *m*/*z* 1142 [(M + H)$^+$], 906 {[M + H − HO(CH$_2$)$_8$OBn]$^+$}, 846 [(906 − AcOH)$^+$]. Anal. ($C_{53}H_{75}NO_{26}$) C,H,N.

*8-Hydroxyoctyl O-(2,3,4,6-Tetra-O-acetyl-α-D-galactopyranosyl)-(1→4)-O-(2,3,6-tri-O-acetyl-β-D-galactopyranosyl)-(1→4)-2-acetamido-3,6-di-O-acetyl-2-deoxy-β-D-glucopyranoside (**30**).* A mixture of **29** (430 mg, 0.38 mmol), 10% Pd−C (100 mg), EtOH (10 mL), and AcOH (2 mL) was stirred under H_2 at 25 °C at 345 kPa for 1 day. Removal of the catalyst by filtration and the volatiles under vacuum afforded amorphous **30** (380 mg, 96%): $[\alpha]_D$ +45° (*c* 0.2); NMR ($CDCl_3$) ^{1}H, 5.983 (d, 1 H, $J_{H\text{-}2,NH}$ = 9.3 Hz, *H*N), 5.574 (br d, 1 H, H-4$_C$), 5.382 (dd, 1 H, $J_{2,3}$ = 11 Hz, $J_{3,4}$ = 3.3 Hz, H-3$_C$), 5.186 (dd, 1 H, $J_{1,2}$ = 3.3 Hz, H-2$_C$), 5.115 (dd, 1 H, $J_{1,2}$ = 7.8 Hz, $J_{2,3}$ = 10.8 Hz, H-2$_B$), 5.058 (dd, 1 H, $J_{2,3}$ + $J_{3,4}$ = 18 Hz, H-3$_A$), 4.987 (d, 1 H, H-1$_C$), 4.751 (dd, 1 H, $J_{3,4}$ = 2.5 Hz, H-3$_B$), 4.537 (d, 1 H, H-1$_B$), 4.404 (d, 1 H, H-1$_A$), 2.129−1.948 (9 s, 30 H, 10 C*H*$_3$CO); ^{13}C, δ 171.0−169.2 (*C*=O), 101.1, 101.0, and 99.7 (C-1$_A$,1$_B$,1$_C$), 76.9, 76.0, 72.7, 72.6, 72.5, 71.9, 68.9, 68.7, 67.8, 67.1, and 67.0 (C-2$_B$,2$_C$,3$_A$,3$_B$,3$_C$,4$_A$,4$_B$,4$_C$, 5$_A$,5$_B$,5$_C$), 69.3 (C-1′), 62.8, 62.4, 61.3, and 60.2 (C-6$_A$,6$_B$,6$_C$,8′), 53.2 (C-2$_A$), 32.4, 28.9, 28.8, 28.6, 25.2, and 25.1 (C-2′,3′,4′,5′,6′,7′), 23.1 (*C*H$_3$CON), and 20.8−20.5 (*C*H$_3$COO); FABMS *m*/*z* 1052 [(M + H)$^+$], 906 {[M + H

Chart 2



4



5

Bn = benzyl
NPhth = phthalimido
Ph = phenyl
SE = 2-(trimethylsilyl)ethyl

$-(CH_2)_8(OH)_2]^+$}, 846 [(906 − AcOH)$^+$]. Anal. ($C_{46}H_{69}NO_{26}$) C,H,N.

*8,8-Dimethoxyoctyl O-(2,3,4,6-Tetra-O-acetyl-α-D-galactopyranosyl)-(1→4)-O-(2,3,6-tri-O-acetyl-β-D-galactopyranosyl)-(1→4)-2-acetamido-3,6-di-O-acetyl-2-deoxy-β-D-glucopyranoside (**31**).* $(CH_3)_2SO$ (70 μL, 1 mmol) was added through a microsyringe to a stirred solution of $(COCl)_2$ (58 μL, 0.66 mmol) in CH_2Cl_2 at −50 °C over a period of 1 min (*23*). To this solution was added a solution of **30** (240 mg, 0.23 mmol) in CH_2Cl_2 (2 mL) through a syringe. The vial containing **30** was rinsed with CH_2Cl_2 (2 × 0.5 mL), and the washing liquids were transferred to the reaction mixture. Stirring was continued for 10 min at −25 to −30 °C, and then the reaction mixture was allowed to reach −10 °C and was treated with *N*-ethyldiisopropylamine (300 μL, 1.72 mmol). The solution was concentrated. The residue was dissolved in 2,2-dimethoxypropane (3 mL, 24.4 mmol). To this solution was added a catalytic amount of *p*-toluenesulfonic acid monohydrate at 5 °C. After 15 min, the solution was treated with Et_3N (1 mL, 7.2 mmol) and the mixture was concentrated under vacuum. The residue was equilibrated between $CHCl_3$ and H_2O. The organic layer was concentrated. Column chromatographic purification of the residue (9:1 EtOAc−hexane) gave **31** (165 mg, 66%) as a syrup: $[\alpha]_D$ +28° (*c* 0.6); NMR ($CDCl_3$) ^{1}H, 5.577 (br d, 1 H, H-4_C), 5.383 (dd, 1 H, $J_{2,3}$ = 11 Hz, $J_{3,4}$ = 3.3 Hz, H-3_C), 5.188 (dd, 1 H, $J_{1,2}$ = 3.6 Hz, H-2_C), 5.119 (dd, 1 H, $J_{1,2}$ = 7.8 Hz, $J_{2,3}$ = 10.8 Hz, H-2_B), 5.084 (dd, 1 H, $J_{2,3} + J_{3,4}$ = 18 Hz, H-3_A), 4.990 (d, 1 H, H-1_C), 4.752 (dd, 1 H, $J_{3,4}$ = 2.7 Hz, H-3_B), 4.535 (d, 1 H, H-1_B), 2.132−1.958 (9 s, 30 H, 10 C*H*$_3$CO); ^{13}C, δ 170.6−169.0 (*C*=O), 104.5 (C-8′), 101.0 (2C) and 99.7 (C-1_A,1_B,1_C), 76.0, 72.7 (2C), 72.6, 71.9, 69.7, 68.9, 68.7, 68.1, 67.9, 67.13, and 67.09 (C-1′,2_B,2_C,3_A,3_B,3_C,4_A,4_B,4_C,5_A,5_B,5_C), 62.6, 61.4, and 60.3 (C-6_A,6_B,6_C), 53.3 (C-2_A), 32.5, 29.4 (2C), 29.2, 25.7, and 24.5 (C-2′,3′,4′,5′,6′,7′), 23.3 (*C*H$_3$CON), and 20.9−20.7 (*C*H$_3$COO); FABMS *m*/*z* 1094 [(M + H − H_2)$^+$], 1064 [(M + H − CH_3OH)$^+$]. Anal. ($C_{48}H_{71}NO_{27}$) C,H.

*7-Formylheptyl O-α-D-Galactopyranosyl-(1→4)-O-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside (**32**).* A solution of **31** (180 mg, 0.165 mmol) in MeOH (10 mL) was treated with NaOMe as described for **13**. After 12 h the solution was carefully neutralized (Dowex 50 × 2 H$^+$) and then concentrated under vacuum. The solution of the residue in H_2O was applied to a Sep Pak 18 cartridge (*24*), which was eluted with a gradient of H_2O−MeOH until the concentration of MeOH reached 20%. The fractions giving a positive phenol−sulfuric acid reaction (*26*) were combined and concentrated to a volume of ∼20 mL. To this solution was added at 25 °C AcOH (200 μL, 3.5 mmol). After 24 h, the solution was freeze-dried to give amorphous **32** (95 mg, 86%): $[\alpha]_D$ +24° (*c* 0.4, H_2O); NMR (D_2O) ^{1}H, 9.669 (br t, H-8′), 5.019 (t, *J* = 5.8 Hz, *H-8′*), 4.946 (d, 1 H, $J_{1,2}$ = 4.0 Hz, H-1_C), 2.525 (dt, $J_{7',8'}$ = 1.6 Hz, $J_{6',7'}$ = 7 Hz, H-7′), 2.03 (CH_3-CON); ^{13}C, δ 210.2 (C-8′), 175.0 (*C*=O of Ac), 104.0, 101.7, and 101.0 (C-1_A,1_B,1_C), 91.9 (*C-8′*), 79.6, 78.0. 76.2, 75.5, 73.2, 72.9, 71.7, 71.6, 69.9, 69.7, and 69.3 (C-2_B,2_C,3_A,3_B,3_C,4_A,4_B,4_C,5_A,5_B,5_C), 71.2 (C-1′), 61.3, 61.1, and 60.8 (C-6_A,6_B,6_C,), 56.0 (C-2_A), 43.9 (C-7′), 37.7 (*C-7′*), 29.3, 29.0, and 28.8 (C-2′,3′,4′), 25.73 (*C-6′*), 25.64 (C-6′), 24.7 (*C-5′*), 23.0 (CH_3CON), and 22.0 (C-5′); CIMS *m*/*z* 672 [(M + H)$^+$].

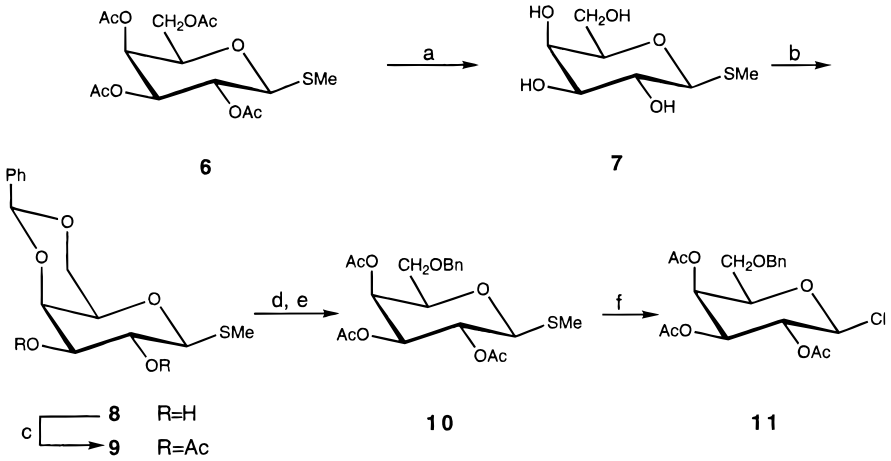
Coupling of **32** to Polyacrylhydrazinoagarose. Approximately 10 mL (bed volume) of a commercial polyacrylhydrazino-agarose gel (Sigma, cat. no. P9282) was washed with 50 mM phosphate buffer, pH 7.5 (3 × 15 mL) and then was mixed with a solution of **32** (20 mg, 0.03 mmol) in 15 mL of the same buffer. The mixture was gently stirred at 25 °C for 5 h and then was treated with $NaCNBH_3$ (22 mg, 0.35 mmol) (*25*). Stirring was continued for 48 h. The gel was transferred to a plastic column (∼10 × 1 cm) which was subsequently eluted with H_2O (50 mL). Phenol−sulfuric acid assay (*26*) of the combined washings indicated that ∼15 mg of **32** is bound to the solid matrix.

Production and Isolation of Subunit B of Shiga Toxin. Fermentation of *V. cholerae* (Strain 0395-N1, pSBC32), containing the gene of subunit B of Shiga toxin but lacking in the gene of subunit A, was carried out in a computer-controlled 10.0 L Magnaferm fermenter (New Brunswick Scientific, Edison, NJ) as described previously (*9*). After an overnight growth, the culture was harvested with an air-driven, continuous flow centrifuge (Alfa Laval Sharples, Warminster, PA). The cell pellet was washed once with PBS to remove residual medium and treated with polymyxin B to 2 mg/mL final concentration. The suspension was incubated at 4 °C for 20 min and centrifuged at 13200*g* for 20 min. Supernatant was collected as periplasmic material (360 mL) and stored at −70 °C. The total protein content of the periplasmic extract was 2.63 g. Twenty mL of the periplasmic material was loaded on a column of the affinity sorbent (2.6 × 10 cm) at 2 mL/min flow rate. The column was washed with 130 mL of PBS and then eluted with 3.5 M guanidine·HCl. In preliminary experiments, fractions containing subunit B were identified by their reaction with the anti-subunit B antibody. These fractions were subsequently identified by photometry at 280 nm. The fractions containing subunit B of Shiga toxin were pooled and dialyzed in a 3500 D molecular weight cutoff tubing against several changes of 4 L PBS for 2 days. Protein assay (*27*) indicated that 10 mg of subunit B was recovered in each run. The combined yield of pure subunit B from one fermentation was 175 mg.

RESULTS AND DISCUSSION

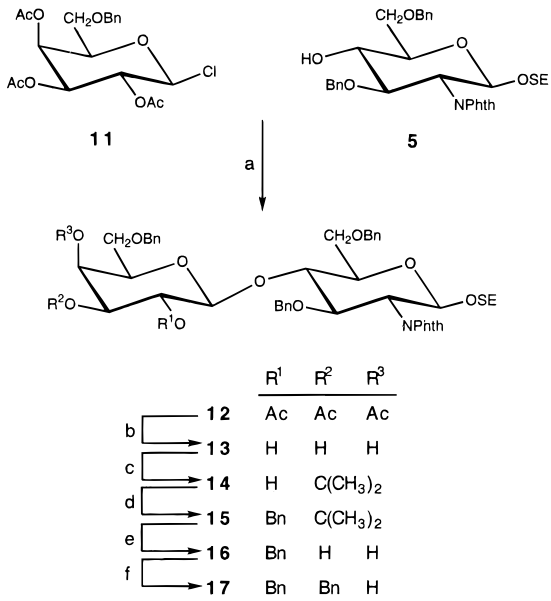
Stepwise syntheses of the P_1 trisaccharide (*28*) and its propyl glycoside have been described by the groups of Anderson and Sinaÿ (*28, 29*). Using block-synthetic approaches, preparation of spacer-linked P_1 trisaccharide

Scheme 1[a]



[a] Abbreviations, reagents and conditions: Ac = acetyl, Bn = benzyl, Me = methyl, Ph = phenyl; (a) MeOH, cat NaOMe, 23 °C, 2 h, 92%; (b) $C_6H_5CH(OMe)_2$ (excess), DMF, CSA, 23 °C, 12 h, 76%; (c) Ac_2O, C_5H_5N, 0 °C, 3 h, 85; (d) $NaCNBH_3$, HCl, Et_2O, 0 °C; (e) Ac_2O, C_5H_5N, 25 °C, 12 h, 74% for two steps; (f) Cl_2, CH_2Cl_2, 0 °C, 20 min, 88%.

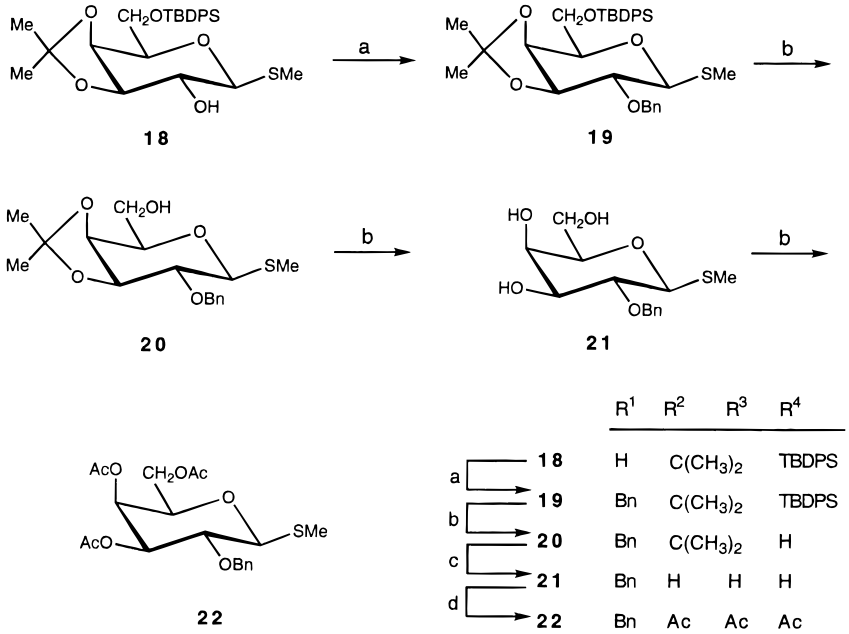
Scheme 2[a]



[a] Abbreviations, reagents, and conditions: Ac = acetyl, Bn = benzyl, SE = 2-(trimethylsilyl)ethyl; Phth = phthalyl; (a) 2.7 equiv of **11**, 2.2 equiv of 2,6-di-*tert*-butyl-4-methylpyridine, 5.5 equiv of CF_3SO_2OAg, CH_2Cl_2, 4A molecular sieves, −40 → +25 °C, 1 h 77%; (b) MeOH, cat. NaOMe, 25 °C, 96%; (c) $Me_2C(OMe)_2$ (excess), CSA, 25 °C, 2 h, 94%; (d) 3.2 equiv of Bn−Br, DMF, NaH, 0 °C, 82%; (e) AcOH−H_2O, 80 °C, 2 h, 78%; (f) 1.05 equiv of Bu_2SnO, PhMe, reflux 2 h, then 1.04 equiv of Bu_4NBr 1.35 equiv of Bn−Br, 40 °C, 3 h, 98%.

derivatives has also been reported (*30, 31*), which enables the covalent attachment of the P_1 trisaccharide to proteins (*31*). Our approach targeted the glycotriosyl donor **27** as the key intermediate which can be linked to a heterobifunctional aglycon suitable for covalent coupling to macromolecular matrices and also can be used as a building block for the synthesis of extended oligosaccharides. Retrosynthetic analysis indicated that the 2-(trimethylsilyl)ethyl (SE) glycoside **25** can be a suitable precursor to the glycotriosyl imidate **27**, according to a well-documented sequence (*32*) involving acid-catalyzed removal of the SE group (*33*) followed by base-catalyzed reaction with CCl_3CN (*21, 22*). SE glycosides are particularly suitable for temporary protection of the anomeric hydroxyl group since the glycosidic linkage of such compounds is stable in glycosylation reactions and under numerous transformations commonly used in protecting group manipulations and can be selectively cleaved under conditions that leave most protecting groups and the interglycosidic linkages intact (*33*). Exceptions to this were recently reported by Pozsgay and Pannell (*32*) and Zhiyuan and Magnusson (*34*), who observed that the interglycosidic linkage of deoxysugar residues is unstable under attempted, direct conversion of oligosaccharide SE glycosides into glycosyl chlorides. Since the target in this study contains no such residue, the SE glycoside-based approach was followed. The starting material was the known alcohol **5** (*11*) obtained from the benzylidene derivative **4** (*11*) (Chart 1). The precursor to the interchain galactose residue was methyl 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-galactopyranoside (**6**) (*13*), which was prepared from galactose pentaacetate and MeSH under $BF_3{\cdot}Et_2O$ catalysis (*12*). This approach complements earlier syntheses of **6** that employed tributyltin (*13b*) and trimethylsilyl derivatives of MeSH (*13c*) and is applicable on a 100 g scale without chromatography. It appeared that a temporary protecting group at O-6 of the interchain galactose synthon could be advantageous to prevent side reactions at a later conversion in the synthetic sequence. The benzyl group was selected for this purpose. Thus, **6** was deacetylated [→ **7** (*13*)] and then benzylidenated according to Evans (*14*) ($C_6H_5CH(OMe)_2$/ H^+/DMF) to afford **8** (*12*) in 76% yield, which was acetylated (Ac_2O/ C_5H_5N, → **9**) (Scheme 1). Reductive opening of the benzylidene ring according to Garegg *et al.* (*16*) ($NaCNBH_3$/ H^+/THF) followed by acetylation afforded compound **10** in 74% yield. Chlorinolysis of the thioglycoside **10** with a solution of Cl_2 in CCl_4 (*17*) afforded the β chloride **11** ($J_{H\text{-}1,H\text{-}2}$ = 8.7 Hz) as a crystalline compound. Condensation of the β-galactosyl donor **11** with the nucleophile **5** using silver trifluoromethanesulfonate as promoter in the presence of the non-nucleophilic base 2,6-di-*tert*-butyl-4-methylpyridine (*18*) gave the disaccharide **12** in 77% yield (Scheme 2). Compound **12** was converted to the triol **13** by treatment with sodium methoxide in MeOH (96%). Subsequent reaction with 2,2-dimethoxypropane under catalysis by camphorsulfonic acid (*19*) afforded the 3,4-*O*-isopropylidene derivative **14** in 94% yield. Such high yields of 3,4-isopropylidene derivatives of galactopyranosides cannot usually be achieved without previous protection of the HO-6 group due to side reactions that lead to 4,6-*O*-isopropylidene and 6-*O*-(1-methoxy-1-methylethyl) derivatives (*13c, 19, 35*). After the completion of this work, Nishida and Thiem (*36*) reported that reaction of β-galactosides with acetone and

Scheme 3[a]

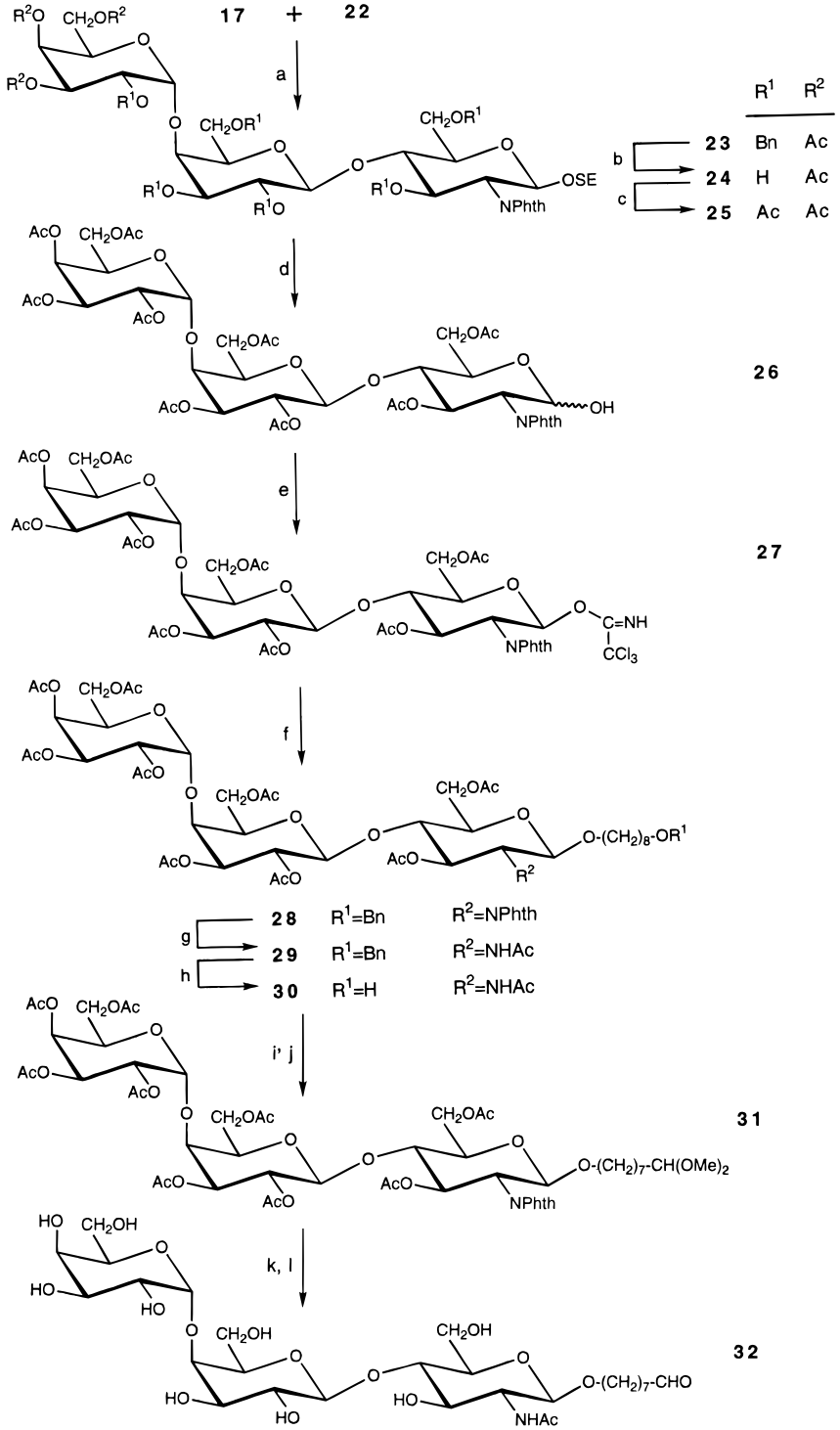


[a] Abbreviations, reagents, and conditions: Ac = acetyl, Bn = benzyl, Me = methyl, TBDPS=*tert*-butyldiphenylsilyl;(a) 2.7 equiv of 11, 2.2 equiv of 2,6-di-*tert*-butyl-4-methylpyridine, 5.5 equiv of CF_3SO_2OAg, CH_2Cl_2, 4A molecular sieves, −40 → +25 °C, 1 h, 77%; (b) MeOH, cat. NaOMe, 25 °C, 96%; (c) $Me_2C(OMe)_2$ (excess), CSA, 25 °C, 2 h, 94%; (d) 3.2 equiv of Bn−Br, DMF, NaH, 0 °C, 82%; (e) AcOH−H_2O, 80 °C, 2 h, 78%; (f) 1.05 equiv of Bu_2SnO, PhMe, reflux 2 h, then 1.04 equiv of Bu_4NBr 1.35 equiv of Bn−Br, 40 °C, 3 h, 98%.

chlorotrimethylsilane (*37*) leads exclusively to 3,4-*O*-isopropylidene derivatives. Their discovery may make the multistep process that was necessary to avoid side reactions at the HO-6 hydroxyl group during isopropylidenation unnecessary in future work. Conventional benzylation of **14** (BnBr/NaH/DMF → **15**) followed by acid hydrolysis afforded the diol **16** which was regioselectively benzylated at HO-3′ according to David *et al.* (*38*) (Bu_2-SnO/BnBr/Bu_4NBr/toluene) to afford the acceptor **17** for coupling with the nonreducing end terminal galactose moiety. 1-Thiogalactoside **22** was selected as the synthetic equivalent for the nonreducing terminal unit in which the *O*-benzyl group next to the anomeric center was expected to facilitate the 1,2-*cis* coupling of this moiety to the disaccharide **17**. Precursor to **22** was the known alcohol **18** (*20*), which was benzylated at O-2 (BnBr/NaH/DMF) to provide **19** in 83% yield (Scheme 3). Sequential removal of the silyl group by Bu_4NF in THF (*39*) (→ **20**, 97%) and the isopropylidene group by acidolysis afforded the triol **21** in 95% yield. Conventional acetylation of **21** (Ac_2O/C_5H_5N) afforded the triacetate **22** in 97% yield. Condensation of the 1-thiogalactoside donor **22** with the disaccharide acceptor **17** under promotion by methyl trifluoromethanesulfonate according to Lönn (*40*), in the presence of the hindered base 2,6-di-*tert*-butyl-4-methylpyridine, afforded the trisaccharide **23** in 73% yield (Scheme 4). The benzyl groups in **23** were replaced by acetyl groups [(i) H_2/Pd−C → **24**; (ii) Ac_2O/C_5H_5N → **25**] to make the protecting group scenario compatible with the subsequent transformations. Sequential treatment of **25** with CF_3CO_2H (*11*) (→ **26**, 77%) and then with trichloroacetonitrile and 1,8-diazabicyclo[5.4.0]undec-1-ene (*21, 22*) furnished the key trisaccharide donor **27** in 82% yield. Condensation of compound **27** with 8-(benzyloxy)octanol (*10*) in the presence of $BF_3{\cdot}Et_2O$ afforded the glycoside **28** in 82% yield. The aglycon employed was used previously as a spacer for covalent attachment of mono- and disaccharides to macromolecular matrices (*10*). Replacement of the phthalyl group in **28** by acetyl [(i) NH_2NH_2/EtOH; (ii) Ac_2O/C_5H_5N) afforded compound **29** in 63% yield. Liberation of the ω-hydroxyl group of the aglycon by hydrogenolytic removal of the *O*-benzyl protecting group furnished the alcohol **30**. Swern-oxidation [Me_2SO, $(COCl)_2$] (*23*) of **30** to an aldehyde that was treated *in situ* with 2,2-dimethoxypropane afforded the dimethyl acetal **31** in 66% yield. The *O*-acetyl groups in **31** were removed by treatment with NaOMe in MeOH. Purification of the intermediate so obtained through a Sep Pak 18 cartridge (*24*) followed by exposure to mild acid afforded the spacer-equipped P_1 trisaccharide **32** in 86% yield. The purity and the structure of **32** and those of the intermediates described in this work were verified by ^1H and ^{13}C NMR spectroscopy at 300 and 75.5 MHz, respectively, and by chemical ionization and fast atom bombardment mass spectroscopy that gave data consistent with the proposed structures (Experimental Procedures). Thus, the presence of the formyl group in **32** is indicated by the resonance at 9.669 ppm in its ^1H NMR spectrum in D_2O (*H*C=O), which is corroborated by the corresponding signal in the ^{13}C NMR spectrum at 175 ppm (H*C*=O). The hydrated form is also present as indicated by a triplet at 5.019 ppm in the ^1H NMR spectrum [C*H*$(OH)_2$] and by a signal at 91.9 ppm in the ^{13}C NMR spectrum [*C*H$(OH)_2$]. Compound **32** is therefore suitable for covalent attachment to proteins and other, amino group-containing matrices by reductive amination (*25*). Indeed, coupling of compound **32** with commercial polyacrylhydrazinoagarose in the presence of $NaCNBH_3$ afforded the P_1 trisaccharide-containing affinity sorbent. Carbohydrate analysis (*26*) indicated that approximately 75% of the trisaccharide was bound under the conditions reported in the Experimental Procedures.

Subunit B of Shiga toxin was produced by fermentation of a nonvirulent *V. cholerae* strain (Strain 0395-N1) containing the gene of subunit B but lacking in the gene of subunit A as described previously (*9*). An aliquot of the periplasmic extract containing ∼150 mg of protein was loaded directly on the affinity column. This was the maximum amount that could be applied while maintainig complete adsorption of subunit B. Subsequent washing

Scheme 4[a]



[a] Abbreviations, reagents, and conditions: Bn = benzyl, SE = 2-(trimethylsilyl)ethyl; Phth = phthalyl; (a) 2.1 equiv of **22**, 2.1 equiv of 2,6-di-*tert*-butyl-4-methylpyridine, 5.1 equiv of CF_3SO_2OMe, Et_2O, 4A molecular sieves, +25 °C, 96 h 73%; (b) H_2/Pd−C, EtOH, AcOH, 23 °C, 16 h 92%; (c) Ac_2O, C_5H_5N 25 °C, 12 h, 94%; (d) CF_3CO_2H, CH_2Cl_2, 25 °C, 4 h, 77%; (e) CCl_3CN, 1,8-diazabicyclo[5.4.0]undec-7-ene, CH_2Cl_2, −20 → +20 °C, 2 h, 82%; (f) 2.6 equiv of $C_6H_5CH_2OH$, cat. $BF_3{\cdot}Et_2O$, CH_2Cl_2, −30 → +25 °C, 1 h, 82%; (g) 57 equiv of NH_2NH_2, EtOH, reflux, 48 h, Ac_2O, C_5H_5N, 25 °C, 63%; (h) H_2/Pd−C, EtOH, AcOH, 23 °C, 24 h 96%; (i) 4.3 equiv of Me_2SO, 2.9 equiv of $(COCl)_2$, CH_2Cl_2, −25 °C, 10 min; (j) $Me_2C(OMe)_2$, PTS, 5 °C, 66% for two steps; (k) MeOH, cat. NaOMe, 25 °C, 12 h; (l) MeOH, AcOH, H_2O, 24 h, 86% for two steps.

with PBS eluted the accompanying proteins (Figure 1). Our initial attempts to elute subunit B with 1 M galactose in saline, 1.7 M NaCl, or 3 M $MgCl_2$ were unsuccessful. On the other hand, 3.5 M guanidine·HCl (*9*) completely eluted subunit B from the affinity matrix. (Figure 1). Bound subunit B could also be eluted with 4.5 M $MgCl_2$. In preliminary experiments, the fractions containing subunit B were identified by their reaction with a monoclonal antisubunit B antibody. After the conditions for elution were established, the process was performed in a semiautomatic mode in a Pharmacia liquid chromatograph, using photometry at 280 nm for detection. Following elution, subunit B was further purified by dialysis against PBS to give 10 mg of pure subunit B of shiga toxin in each run. The affinity sorbent was used for over 20 cycles with unchanged capacity for subunit B. The identity and purity of the subunit B so prepared was assessed by SDS−PAGE electrophoresis

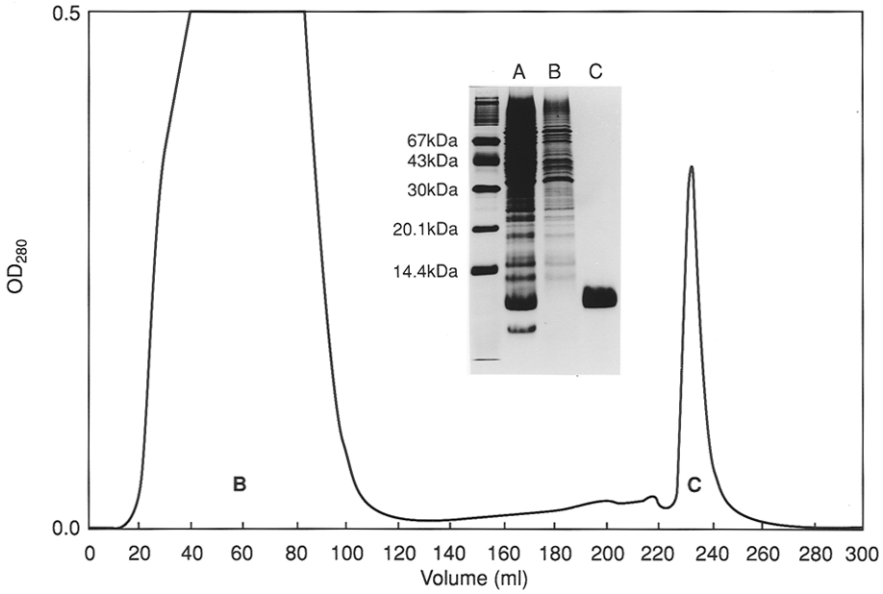


Figure 1. Elution of the proteins in the periplasmic extract from the P_1 trisaccharide−hydrazinoagarose column. The periplasmic extract (20 mL) was loaded on the affinity column (2.6 × 10 cm) equilibrated with PBS buffer. The column was washed with 150 mL of PBS and then was eluted with 3.5 M guanidine·HCl. Fraction B: proteins eluted from the column with the buffer. Fraction C: subunit B of Shiga toxin. Inset shows the sodium dodecyl sulfate−polyacrylamide gel electrophoresis of the proteins in the periplasmic extract. Left lane: molecular weight markers. Lane A: periplasmic extract. Lane B: proteins eluted from the column with the buffer. Lane C: subunit B of Shiga toxin eluted with 3.5 M guanidine·HCl.

(Figure 1) and its antigenicity was demonstrated by its reaction with a monoclonal antibody directed against subunit B. The monoclonal antibody has been described in ref 9.

CONCLUSION

We have described a method for the preparative-scale isolation of subunit B of Shiga toxin, using a P_1 trisaccharide-containing affinity matrix. The approach presented here is based on a chemically synthesized derivative (**27**) of the P_1 trisaccharide that permits its covalent attachment to a variety of linking arms. We selected an ω-aldehydoalkyl spacer (*10*) for covalent attachment of the P_1 trisaccharide to the insoluble matrix under the mild conditions of reductive amination. The geometry of the linking arm provided a sufficient distance of the hapten from the polymeric support that can avoid possible unfavorable steric interactions between the lectin and the supportive matrix. Our protocol represents an improvement over existing methods since it obviates the need for laborious, multiple chromatographic procedures (*9*) and maintains the simplicity of the one-step, receptor analog affinity chromatography (*8*). The ability of the affinity matrix to be used repeatedly in a semiautomatic fashion makes this approach suitable for the isolation of subunit B on the 100 mg scale. Furthermore, our method avoids the reliance on affinity material isolated form biological sources (*8*), which may be objectionable if the subunit B so purified is intended for human or animal experiments. The pure subunit B is currently being evaluated as a possible protein component of saccharide-protein conjugate vaccines (*41*). This work will be the subject of a future publication.

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