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Enzymatic glycosylation of reducing oligosaccharides linked to a solid phase or a lipid via a cleavable squarate linker

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Abstract

Reducing oligosaccharides were converted into their corresponding glycosylamines, and these were reacted with 3,4-diethoxy-3-cyclobuten-1,2-dione (squaric acid diethyl ester). The resulting derivatives could be linked to amino-functionalized lipids, solids, or proteins. Treatment of the obtained lipid or solid conjugates with aqueous bromine or, alternatively, with ammonia–ammonium borate cleaved the linkage and regenerated the oligosaccharide glycosylamines, which were in turn rapidly hydrolyzed to the reducing oligosaccharides. To demonstrate the usefulness of this linkage in enzymatic oligosaccharide synthesis, lactose was linked to a lipid or a solid phase, the obtained conjugates were then subjected to two enzymatic glycosylations (either consecutively or 'one-pot'). The resulting materials were then cleaved to give, in both cases, the expected reducing tetrasaccharide (lacto-*N*-neotetraose) in good yield.

Keywords: Glycosylamines; Lipid conjugates; Glycoproteins; Enzymatic synthesis

1. Introduction

Methods for the synthesis of oligosaccharides have developed considerably during the last 20 years, but a chemical oligosaccharide synthesis is still a relatively complicated task, involving many synthetic steps. An alternative to chemical oligosaccharide syntheses is enzymatic synthesis, where nucleotide sugars are the donors and glycosyltransferases are the catalysts [1,2]. Glycosyltransferases give regio- and stereospecific glycosylations, without the complicated protecting group patterns that have to be used in chemical synthesis. Glycosyltransferase-catalyzed glycosylations in combination with solid-phase techniques offer a particularly simple way to synthesize oligosaccharides on a laboratory scale. We have previously reported [3] synthesis of a sialyl Le^x tetrasaccharide by carrying out three consecutive, high-yield enzymatic glycosylations on a starting monosaccharide (GlcNAc) attached to Sepharose via a disulfide linkage. In that case, chemical synthesis (six steps from *N*-acetylglucosamine) was used to provide a GlcNAc derivative with a thiol functionality, and this was then reacted with an activated thiol on the solid phase to obtain a reversible solid-phase disulfide linkage. In an effort to develop a simpler procedure for reversible linkage of oligosaccharides to a solid phase, we have now investigated squaric acid chemistry for the purpose. We found that reducing oligosac-

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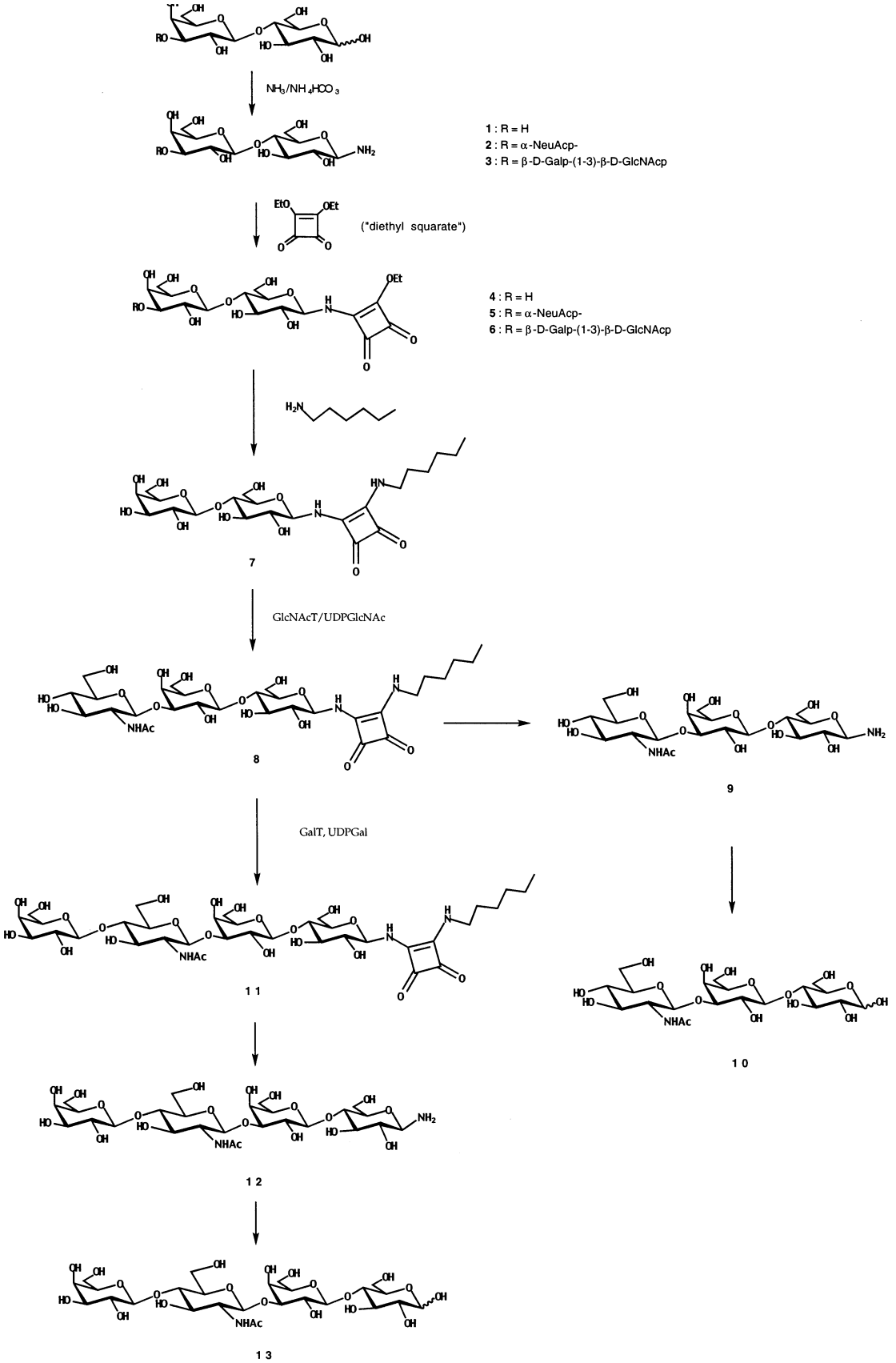

NH3/NH4HCO3
1 : R = H
2 : R = α-NeuAcp-
3 : R = β-D-Galp-(1-3)-β-D-GlcNAcp
("diethyl squarate")
4 : R = H
5 : R = α-NeuAcp-
6 : R = β-D-Galp-(1-3)-β-D-GlcNAcp
7
GlcNAcT/UDPGlcNAc
8
9
GalT, UDPGal
10
11
12
13

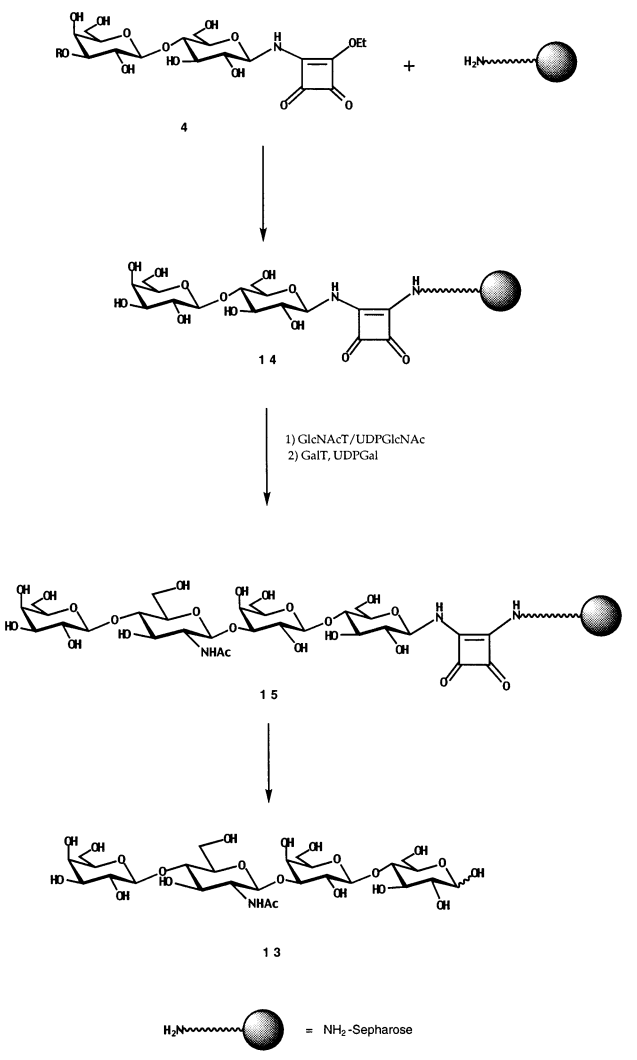
Scheme 1.

charides could be linked to and detached from a solid phase using a very simple procedure, not requiring extensive expertise in organic synthesis. Similar linkage of oligosaccharides to lipids or proteins was also possible. To demonstrate the usefulness of the new linkage type in enzymatic oligosaccharide synthesis, lactose was linked to a lipid or a solid phase, and the obtained conjugates were subjected to two enzymatic glycosylations (either consecutively or 'one-pot'), the resulting materials were cleaved to give, in both cases, the expected reducing tetrasaccharide (lacto-*N*-neotetraose) in good yield.

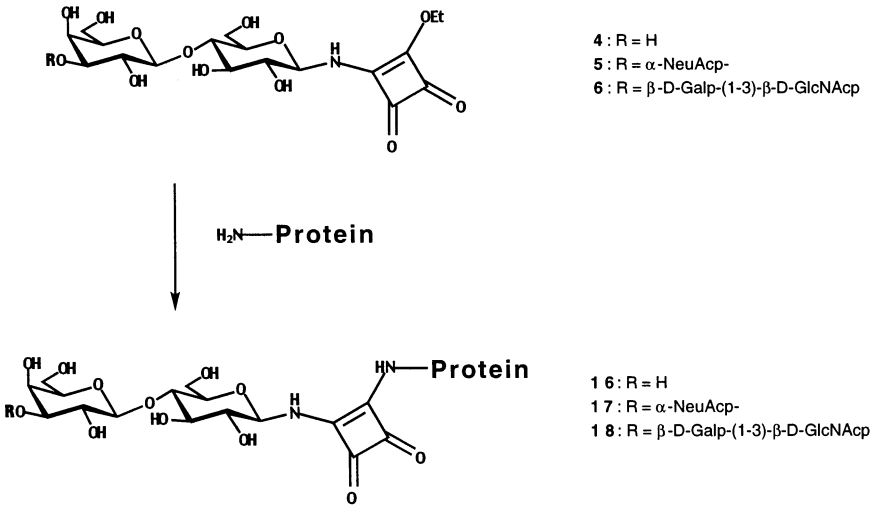
2. Results and discussion

Preparation of glycosylamine–lipid, glycosylamine–Sepharose and glycosylamine–HSA conjugates.—The reaction of 3,4-diethoxy-3-cyclobuten-1,2-dione (squaric acid diethyl ester) with molecules carrying the amine functionality was first reported by Glüsenkamp and co-workers in 1991 [4]. We used the originally reported reaction conditions, squaric acid diethyl ester, and glycosylamines **1**, **2**, and **3** to prepare the oligosaccharide squaric acid conjugates **4**, **5**, and **6** in 77, 83, and 84% yields, respectively (the oligosaccharide glycosylamines were obtained from the parent reducing oligosaccharides lactose, 3-sialyllactose, and lacto-*N*-tetraose by treatment [5,6] with ammonia/ammonium bicarbonate). The conjugates **4**, **5**, and **6** were reasonably stable and could be isolated and stored as lyophilized powders. However, they reacted smoothly with amines in aqueous pH 9 buffers to form conjugates of various types. For example, the lactose derivative **4** reacted with *n*-hexylamine to form the glycolipid **7** in 90% yield, easily isolated from the reaction mixture through solid-phase extraction with C-18 silica [7]. Under similar reaction conditions, **4** reacted with extended linker amino-Sepharose [3] to form conjugate **14** in good yield. Finally, derivatives **4**, **5**, and **6** could be reacted with proteins bearing lysine ε-amino groups, such as HSA, to give neoglycoproteins (**16**, **17**, and **18**, respectively). The obtained neoglycoprotein substitution degrees (22, 21, and 9 mol/mol HSA, respectively) were comparable to those obtained by existing methods of conjugating reducing oligosaccharides to proteins [6,8], and the present new method is therefore a viable alternative for making oligosaccharide protein conjugates (Schemes 1–3).

Enzymatic glycosylation of glycosylamine–lipid conjugates: solution-phase enzymatic oligosaccharide synthesis.—The obtained new carbohydrate–lipid conjugates had the property of being easily separated (by C-18 silica solid-phase extraction) from complex aqueous reaction mixtures, and were therefore evaluated as starting materials in enzymatic oligosaccharide synthesis. The lactose glycolipid **7** was subjected to enzymatic glycosylation, first with UDP-GlcNAc and



Scheme 2.



Scheme 3.

β-(1→3)-*N*-acetylglucosaminyltransferase [9], which gave the trisaccharide glycolipid **8** in 99% yield after C-18 silica solid-phase extraction purification. Further reaction of **8** with UDP-Gal and β-(1→4)-galactosyltransferase gave the tetrasaccharide glycolipid **11** in 46% yield (from **8**). The same tetrasaccharide glycolipid could also be obtained, in a similar yield (66% from **7**), by carrying out the enzymatic glycosylations in a one-pot reaction mixture, without purification of the intermediate trisaccharide glycolipid. The enzymatic reactions were rapid (1–3 h), and the reaction mixtures (according to TLC analysis) contained very little by-product. The low yields of **11** (46 and 66%, respectively) were probably a result of loss occurring during the last solid-phase extraction procedure. Preliminary experiments showed that although disaccharide and trisaccharide glycolipids like **7** and **8** are almost quantitatively extracted from an aqueous buffer solution, a tetrasaccharide glycolipid like **11**, being more polar, is not. A longer lipid on **11** would probably have given better solid-phase extraction yields, but, on the other hand, that would have required a more lipophilic starting disaccharide glycolipid, which would have been less water-soluble and more prone to form micelles (a complicating factor). For example, the benzhydrylamine analog of **7** is only sparingly soluble in water. Therefore, in any case where solid-phase extractions are used to purify glycolipid-type products from aqueous enzymatic glycosylations mixtures, the choice of the lipid part has to be made carefully, considering solubility properties of both starting and product glycolipid.

The trisaccharide glycolipid **8** was cleaved by treatment with aqueous bromine, giving first the trisaccharide glycosylamine **9**. If the reaction mixture was pH-adjusted to 5–6, complete hydrolysis to the reducing trisaccharide **10** was observed within a few hours (as expected from what is known about the pH dependence of glycosylamine hydrolysis rates [10,11]). We found, however, that the glycosylamine could be more conveniently hydrolyzed, and then even at above neutral pH, by addition of sodium borate to the reaction mixture. The scope and generality of this new borate-catalyzed hydrolysis is currently under further investigation. The obtained trisaccharide **10** was purified by gel-filtration; the yield was 91%.

In an analogous way, tetrasaccharide glycolipid **11** was cleaved, to give first the glycosylamine **12**, then, without isolation, the tetrasaccharide **13** (lacto-*N*-neotetraose, 85% yield).

An alternative method for cleavage of the oligosaccharide–lipid conjugates was also investigated. It was found that treatment of the conjugates at room temperature with concentrated aqueous ammonia containing ammonium borate resulted in cleavage, giving first the glycosylamines, then the parent free sugars in good yield (reaction with only aqueous

ammonia gave a much slower cleavage rate). For example, treatment of **7** with aqueous ammonia containing ammonium borate gave lactose in 88% yield.

The NMR spectra of the squaric-acid-derived oligosaccharides all showed the characteristics of compounds with restricted rotation around a C–N bond [4,12]. For example, the ^{1}H spectrum of **7** at 27 °C (Fig. 1(a)) showed a broad signal in the anomeric region that was assigned to H-1. The other glucose proton and carbon signals were also, to various degrees, broadened. The phenomenon was ascribed to the presence of two C–N rotamers of **4**, interconverting at an intermediate rate (on the NMR time scale). As

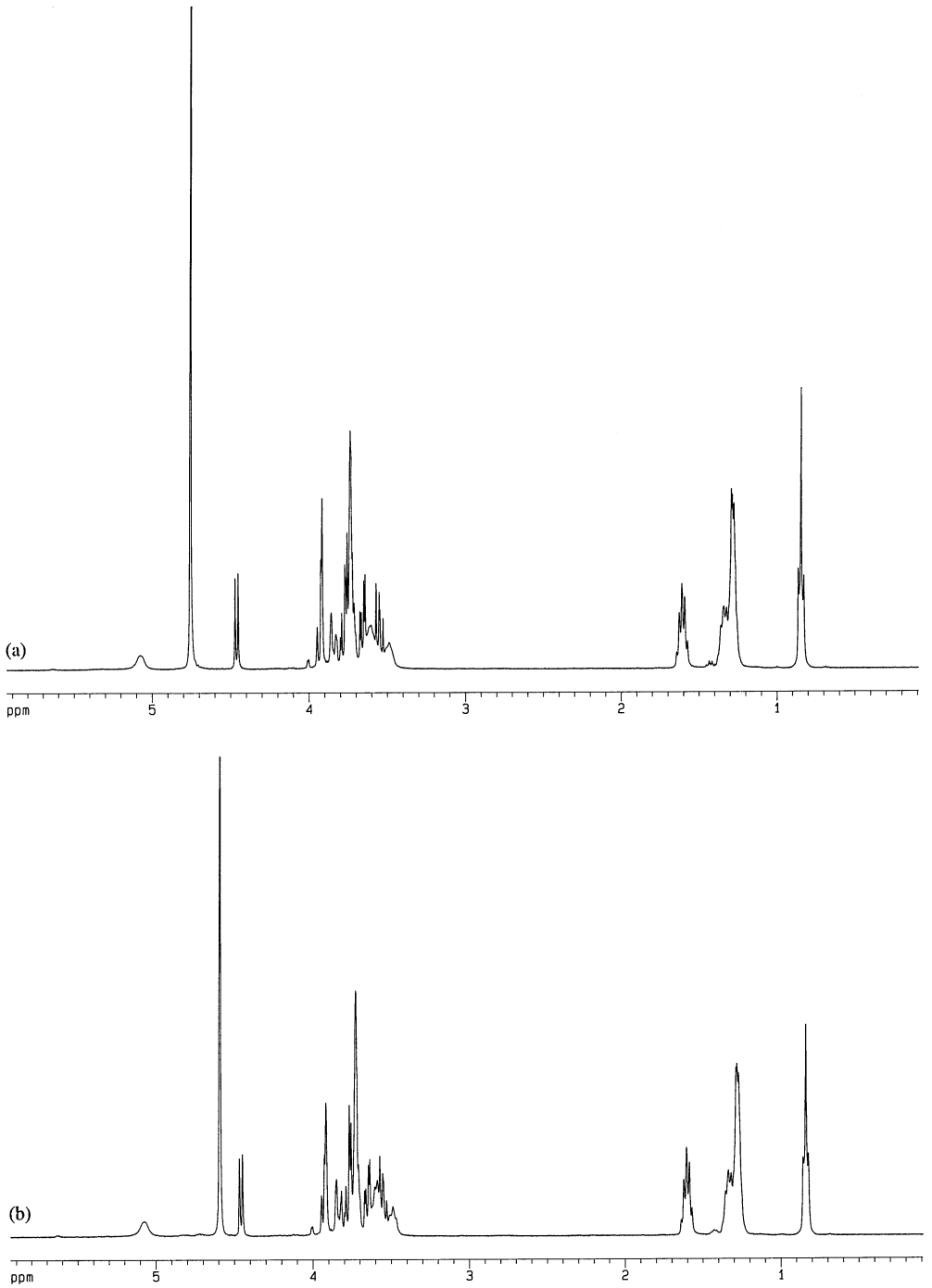


Fig. 1. 400 MHz ^{1}H NMR spectra of compound **7** in D_2O at (a) 27 °C; (b) 40 °C; (c) 60 °C; and (d) 80 °C.

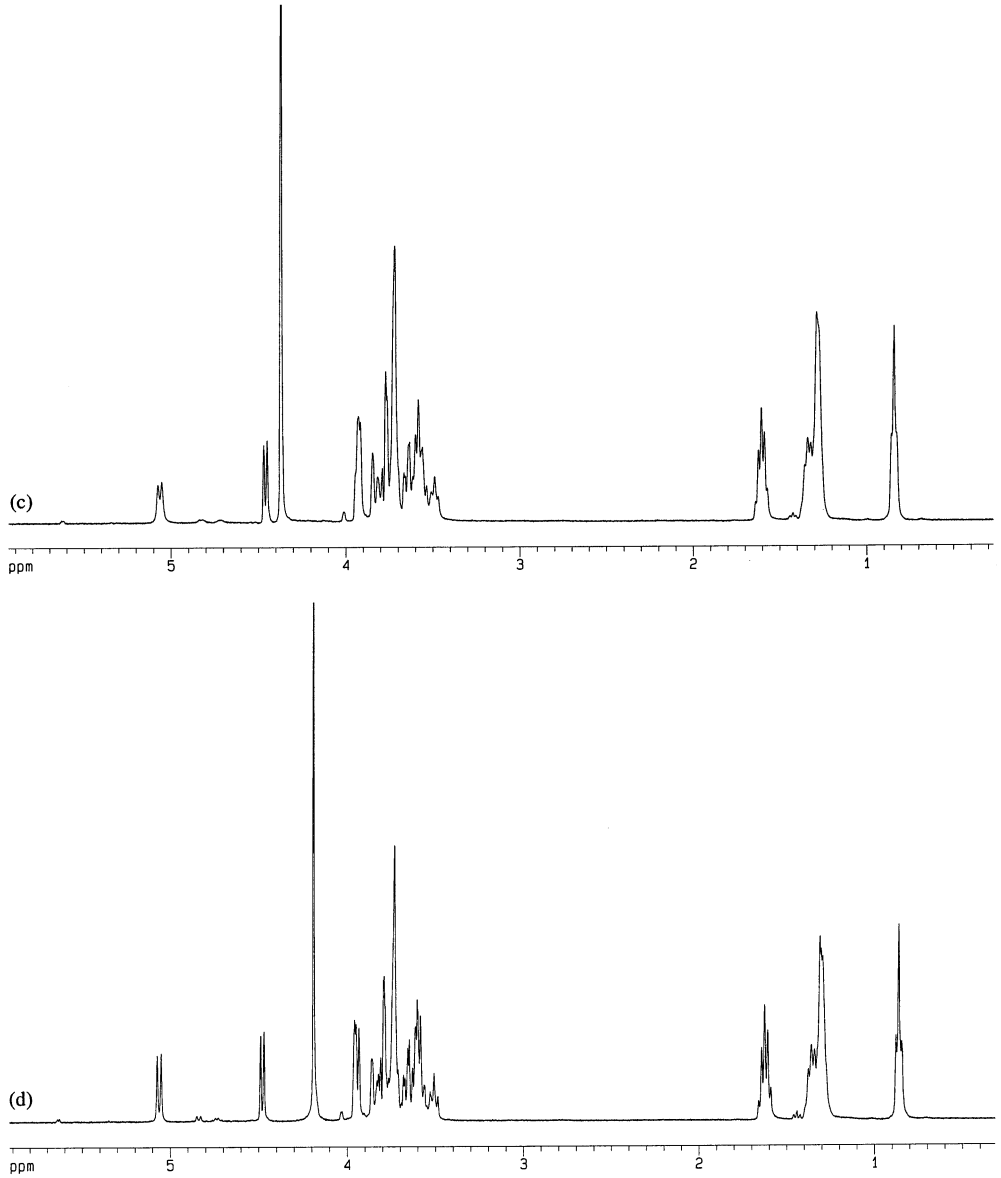


Fig. 1. (*Continued*)

expected in such a situation, heating of **7** to 80 °C gave a ^{1}H spectrum (Fig. 1(d)) with sharp signals for all glucose nuclei.

Enzymatic glycosylation of glycosylamine–Sepharose conjugates: solid-phase enzymatic oligosaccharide synthesis.—The lactose–Sepharose conjugate **14**, having an extended linker, was investigated in enzymatic synthesis (the reported procedure [3] for preparation and quantification of **14** was considerably improved, and these details are therefore given in Section 3). Lactose–Sepharose conjugate **14** was treated with the same two enzymes under similar reaction conditions as with the glycolipids described above (two separate glycosylation steps were carried out in this case). As before [3,13], in solid-phase enzymatic glycosylations, longer reaction times (18 h as compared to 3 h), slightly larger excesses of nucleotide sugar (2.0 equivalents as compared to 1.3–1.5 equivalents) and larger excesses of enzyme (80–100 mU/μmol substrate as compared to 17–30 mU) were used to drive the reactions to completion, as compared to the solution case with the glycolipids. This can be seen as a disadvantage if the availability of enzymes is limiting. However, on the important advantage side, filtration purification after each reaction step was much faster, simpler and more quantitative than the corresponding

glycolipid solid-phase extraction, and this is an advantage that becomes increasingly important with increasing number of steps in the synthesis. The obtained Sepharose-bound tetrasaccharide derivative **15** was cleaved, analogous to the above with the glycolipids, by treatment with aqueous bromine, followed by neutralization and addition of sodium borate to complete the hydrolysis of the formed glycosylamine to the reducing oligosaccharide. The yield of lacto-*N*-neotetraose **13** (after gel-filtration purification to remove salts and traces of shorter oligosaccharides) was 89% (calculated from **14**). The alternative cleavage method using concentrated aqueous ammonia/ammonium borate was also demonstrated on the same material **15**, giving a yield of lacto-*N*-neotetraose **13** (after gel-filtration purification to remove salts and traces of shorter oligosaccharides) of 75% (calculated from **14**).

In conclusion, using squaric acid chemistry for reversible coupling of oligosaccharides to lipids or solids, simple protocols were developed, which should be highly suitable for use in enzymatic oligosaccharide synthesis with reducing oligosaccharides as starting materials.

3. Experimental

General methods.—Concentrations were performed under reduced pressure at < 40 °C bath temperature. NMR spectra were recorded, if not otherwise indicated, at 30 or 80 °C using Bruker 400 and 600 MHz instruments (data recorded at 80 °C are marked with an asterisk). The following reference signals were used: acetone δ 2.225 (^{1}H in D_2O), acetone δ 30.7 (^{13}C in D_2O). FABMS were recorded in the positive-ion mode with a Jeol JMS-SX/SX-102A mass spectrometer using glycerol as matrix. MALDI-TOF MS of protein conjugates were recorded in the positive-ion mode with a LDI-1700-XP instrument, using sinapic acid (3,5-dimethoxy-4-hydroxycinnamic acid) as matrix. Thin-layer chromatography (TLC) was performed on Kieselgel 60 F_{254} Fertigplatten (E. Merck, Darmstadt, Germany). After elution with appropriate eluants, spots were visualized by UV light and/or by dipping in 5% sulfuric acid, followed by charring. For solid-phase extractions, reversed-phase C-18 Silica Gel (International Sorbent Technology Ltd, UK) or Isolute cartridges (International Sorbent Technology) were used. Water for all solutions was from a MilliQ water purification system (Millipore Corp., Bedford, Mass., USA), and was degassed by vacuum treatment before use. Gel-filtrations were performed on Sephadex columns using MilliQ-purified water with added n-butanol (5%, to prevent microorganism contamination). The solid-phase enzymatic reactions were carried out in small, silanized (by brief treatment with 5% dichlorodimethylsilane in hexane) columns (5–15 mL) with a fritted glass filter and a Teflon stopcock at the bottom end. After charging with the appropriate reagents, the column was sealed at the top with a ground glass stopper, the Teflon stopcock was closed, and the column was slowly rotated (by attachment to a rotavapor glass tube) at room temperature (rt) for the specified time. The quantitative ninhydrin determinations of amino groups on Sepharose were carried out essentially as described [14]. Briefly, a soln of ninhydrin in Me_2SO (0.5 M, 50 μL) and an aq sodium acetate soln (0.5 M, pH 5.2, 50 μL) were added to a weighed amount of drained gel (40–50 mg, approx. 50 μL, resuspended in 50 μL of water), then the mixture was heated to 100 °C for 10 min. The resulting mixture was diluted to 50 mL with 1:1 EtOH–water, and the absorbance of the supernatant liquid was then measured at 570 nm. The original Sepharose amino group content was calculated from this adsorbance value and the extinction coefficient ($\varepsilon_{570} = 8750$) of the formed purple dye (diketohydrindylidenediketohydrindamine).

Uridine 5′-diphosphogalactose (UDP-Gal), uridine 5′-diphospho-*N*-acetylglucosamine (UDP-GlcNAc), bovine milk β-(1→4)-galactosyltransferase, BSA, HSA, and 1,2-*O*-isopropylidene-α-D-glucofuranose were from Sigma Chemical Co. (St. Louis, IL, USA), 3,4-diethoxy-3-cyclobuten-1,2-dione (squaric acid diethyl ester) and 1,8-diamino-3,6-dioxaoctane were from Acros Chimica (Geel,

Belgium), 3′-sialyllactose, lacto-*N*-tetraose, and lacto-*N*-neotetraose were from IsoSep AB (Tullinge, Sweden) and recombinant *Neisseria meningitidis* β-(1→3)-*N*-acetylglucosaminyltransferase was obtained as previously described [9]. One unit of this enzyme is defined [9] as the amount required to glycosylate (with UDP-GlcNAc) 1 μmol of lactose in 1 min. Epoxy Sepharose 6B was from Amersham Pharmacia Biotech AB (Uppsala, Sweden).

General procedure for preparation of oligosaccharide–squaric acid conjugates.—A soln of reducing oligosaccharide (0.2 M) and ammonium bicarbonate (0.2 M) in concd aq ammonia was stirred and heated to 35 °C for 24 h. The mixture was then concentrated and co-concentrated twice with water, the residue was dissolved in a 1:1 sodium bicarbonate–EtOH (0.1 M, pH 9) mixture to a concn of 0.13 M. 3,4-Diethoxy-3-cyclobuten-1,2-dione was added to a concn of 0.4 M, and the pH was kept at 9–10 by dropwise addition of aq NaOH. After 1 h at rt, the mixture was concentrated and purified by gel-filtration on a Sephadex G-15 column. Fractions containing (the UV-absorbing) product were pooled and lyophilized.

Lactose–squaric acid conjugate **4**.—This derivative was prepared essentially as described in the previous paragraph. Thus, lactose (1.60 g, 4.68 mmol) was converted to the glycosylamine **1** using ammonium bicarbonate (0.5 g, 5.8 mmol) and concd aq ammonia (30 mL). An NMR analysis of the crude glycosylamine intermediate revealed signals at 4.12/85.3 ppm (H-1/C-1) from the glycosylamine **1**. From the ^{1}H NMR spectrum, it was estimated that 80% of the original amount of lactose had been converted into **1**. The crude, concd glycosylamine was reacted with 3,4-diethoxy-3-cyclobuten-1,2-dione (1.77 mL, 12 mmol) in a mixture of aq sodium bicarbonate (0.1 M, pH 9, 10 mL) and EtOH (10 mL). The pH was adjusted to 9–10 with 1 M aq NaOH soln. After 3 h, the mixture was concentrated and purified by gel-filtration (Sephadex G-15, 5 × 100 cm column). Appropriate fractions were pooled and lyophilized giving **4** (1.68 g, 77%). NMR data: ^{1}H, δ 1.45 (t, 2 H, C***H***$_3$–CH$_2$–O–) 3.54 (t, 1 H, H-2), 3.56 (t, 2 H, H-2′), 3.67 (dd, H-3′), 3.73 (1 H, H-3), 3.78 (1 H, H-4), 3.93 (d, 1 H, H-4′), 4.47 (d, 1 H, $J_{1,2}$ 7.8 Hz, H-1′), 4.77 (m, 2 H, CH$_3$–C***H***$_2$–O–), 4.90, 5.16 (2 broad s, 1 H, H-1), 4.97* ($J_{1,2}$ 8.8* Hz, H-1*); ^{13}C, δ 15.5 (***C***H$_3$–CH$_2$–O–), 69.0 (C-4′), 71.5, (C-2′), 71.5 (CH$_3$–***C***H$_2$–O–), 72.7 (C-2), 73.0 (C-3′), 75.8 (C-3), 76.8 (C-4), 83.9 (broad s, C-1), 103.4 (C-1′). FABMS: Anal. Calcd for $C_{18}H_{28}O_{13}N$ [M + H]: 466.1561. Found: 466.1559.

Sialyllactose–squaric acid conjugate **5**.—3-Sialyllactose (100 mg) was converted, essentially as previously described, into **5** (100 mg, 83%) using first concd ammonia/ammonium bicarbonate (0.8 mL/13.5 mg), then 3,4-diethoxy-3-cyclobuten-1,2-dione (0.11 mL) in 1:1 EtOH–aq sodium bicarbonate (4 mL). NMR data: ^{1}H, δ 1.45 (t, 2 H, C***H***$_3$–CH$_2$–O–), 1.81, 2.88 (2 H, H-3″), 2.03 (NAc), 3.54 (1 H, H-2), 3.59 (t, 2 H, H-2′), 3.60 (1 H, H-7″), 3.64 (1 H, H-9″), 3.66 (1 H, H-6″), 3.71 (1 H, H-4″), 3.74 (1 H, H-3), 3.78 (1 H, H-4), 3.85 (1 H, H-5″), 3.91 (1 H, H-8″), 3.96 (d, 1 H, H-4′), 4.12 (dd, H-3′), 4.56 (d, 1 H, $J_{1,2}$ 7.8 Hz, H-1′), 4.77 (m, 2 H, CH$_3$–C***H***$_2$–O), 4.91, 5.16 (2 broad s, H, H-1); ^{13}C, δ 15.5 (***C***H$_3$–CH$_2$–O–), 22.5 (NAc), 40.1 (C-3″), 52.1 (C-5″), 63.0 (C-9″), 68.0 (C-4′), 68.5 (C-4″), 68.7 (C-7″), 69.8 (C-2′), 71.5 (CH$_3$–***C***H$_2$–O–), 72.1 (C-8″), 72.7 (C-2), 73.3 (C-6″), 75.6 (C-3), 75.9 (C-3′), 76.9 (C-4), 83.9 (broad s, C-1), 103.4 (C-1′). FABMS: Anal. Calcd for $C_{29}H_{43}O_{21}N_2$ (M + H): 755.2358. Found: 755.2390.

Lacto-N-*tetraose–squaric acid conjugate* **6**.—Lacto-*N*-tetraose (150 mg) was converted, essentially as previously described, into **6** (155 mg, 84%) using first concd ammonia/ammonium bicarbonate (1.0 mL/17 mg), then 3,4-diethoxy-3-cyclobuten-1,2-dione (0.13 mL) in 1:1 EtOH–aq sodium bicarbonate (5 mL). NMR data: ^{1}H, δ 1.45 (t, 2 H, C***H***$_3$–CH$_2$–O), 4.44 (d, 1 H, $J_{1,2}$ 7.8 Hz, H-1‴), 4.47 (d, 1 H, $J_{1,2}$ 7.8 Hz, H-1′), 4.74 (d, 1 H, $J_{1,2}$ 8.4 Hz, H-1″), 4.77 (m, 2 H, CH$_3$–C***H***$_2$–O–), 4.91, 5.16 (2 broad s, 1 H, H-1); ^{13}C, δ 15.5 (***C***H$_3$–CH$_2$–O–), 71.5 (CH$_3$–***C***H$_2$–O–), 83.9 (broad s, C-1), 103.0 (C-1‴), 103.4 (C-1′), 103.9 (C-1″). FABMS: Anal. Calcd for $C_{32}H_{51}O_{23}N_2$ [M + H]: 831.2882. Found: 831.2910.

Lactose–hexylamine conjugate **7**.—A soln of lactose squaric acid conjugate **4** (0.80 g, 1.72 mmol) in a mixture of aq sodium bicarbonate (5.0 mL, 0.1 M, pH 9.0), EtOH (5.0 mL) and 2-propanol (1.0 ml) was stirred while n-hexylamine (0.5 mL, 3.8 mmol) was added. The mixture was further stirred at rt for 12 h, then evaporated to dryness and resuspended in water (50 mL). The soln was filtered, and the filtrate was slowly passed through a C-18 silica gel column (100 mL, previously washed with first 300 mL of MeOH, then 2000 mL of water). After washing of the column with water (200 mL), the desired material was eluted with MeOH (200 mL). Appropriate fractions were pooled, concd and lyophilized to give compound **7** (0.80 g, 1.55 mmol, 90%). NMR data: ^{1}H, δ 0.86, 1.29, 1.30, 1.35, 1.62, 3.62 (CH_3–CH_2–CH_2–CH_2–CH_3–CH_2–NH–), 3.50 (1 H, H-2), 3.56 (t, 1 H, H-2′), 3.67 (dd, H-3′), 3.74 (1 H, H-3), 3.78 (1 H, H-4), 3.93 (d, 1 H, H-4′), 4.47 (d, 1 H, $J_{1,2}$ 7.8 Hz, H-1′), 5.08 (broad s, 1 H, H-1), 5.06* ($J_{1,2}$ 8.8* Hz, H-1*); ^{13}C, δ 13.6, 22.3, 31.1, 25.6, 30.5, 44.9 (CH_3–CH_2–CH_2–CH_2–CH_3–CH_2–NH), 69.2 (C-4′), 71.6 (C-2′), 73.1 (C-2), 73.2 (C-3′), 75.2 (C-3), 76.1 (C-4), 84.1 (C-1), 103.4 (C-1′). FABMS: Anal. Calcd for $C_{22}H_{37}O_{12}N_2$ (M + H): 521.2346. Found: 521.2337.

Enzymatic solution glycosylation of **7** *with β-(1→3)-*N*-acetylglucosaminyltransferase to give* **8**.—Conjugate **7** (20 mg, 38.5 μmol) was added to a 4 mL aq buffer (15 mM in manganese(II)chloride and 0.5 M in sodium cacodylate) soln containing UDP-GlcNAc (38 mg, 58.4 μmol), BSA (20 mg), and recombinant β-(1→3)-*N*-acetylglucosaminyltransferase (20 mU, 0.2 mL of a 50% glycerol stock soln). The reaction mixture was stirred at rt for 5 h, after which TLC analysis (6:3:3:2 EtOAc–AcOH–MeOH–water) showed complete consumption of starting material. The mixture was diluted with water to 20 mL, and was then slowly passed through a C-18 Isolute cartridge (10 g, previously washed with first 50 mL of MeOH, then 200 mL of water). After washing of the column with water (50 mL), the desired material was eluted with MeOH (20 mL). Appropriate fractions were pooled and lyophilized to give compound **8** (27 mg, 38 μmol, 99%). NMR data: ^{1}H, δ 0.86, 1.29, 1.30, 1.35, 1.62, 3.62 (CH_3–CH_2–CH_2–CH_2–CH_3–CH_2–NH–), 2.04 (s, 3 H, NAc), 3.48 (1 H, H-2), 3.59 (t, 1 H, H-2′), 3.71 (dd, H-3′), 3.73 (1 H, H-3), 3.74 (1 H, H-2″), 3.77 (1 H, H-4), 3.88 (1 H, H-4″), 3.90 (1 H, H-3″), 4.15 (d, 1 H, H-4′), 4.45 (d, 1 H, $J_{1,2}$ 7.8 Hz, H-1′), 4.68 (1 H, $J_{1,2}$ 8.4 Hz, H-1″), 5.08 (broad s, 1 H, H-1); 13C, d 13.6, 22.3, 31.1, 25.6, 30.5, 44.9 (CH_3–CH_2–CH_2–CH_2–CH_3–CH_2–NH–), 22.7 (NAc), 59.8 (C-4″), 60.6 (C-3″), 61.1 (C-2″), 68.6 (C-4′), 70.2 (C-2′), 72.7 (C-2), 74.8 (C-3), 75.1 (C-3′), 76.4 (C-4), 83.5 (C-1), 103.0 (C-1″), 103.1 (C-1′). FABMS: Anal. Calcd for $C_{30}H_{50}O_{17}N_3$ [M + H]: 724.3140. Found: 724.3178.

Enzymatic galactosylation of **8** *to give* **11**.—Conjugate **8** (10 mg, 13.8 μmol) was added to a 4 mL aq buffer (20 mM in manganese(II)chloride and 0.5 M in sodium cacodylate) soln containing UDP-Gal (11 mg, 18.0 μmol), and bovine milk β-(1→4)-galactosyltransferase (500 mU, 0.05 mL of a 50% glycerol stock soln). The reaction mixture was stirred at rt for 3 h, after which TLC analysis (6:3:3:2 EtOAc–AcOH–MeOH–water) showed complete consumption of starting material. The mixture was diluted with water to 20 mL, and was then slowly passed through a C-18 Isolute cartridge (10 g, previously washed with first 50 mL of MeOH, then 200 mL of water). After washing of the column with water (50 mL), the desired material was eluted with MeOH (20 mL). Appropriate fractions were pooled and lyophilized to give compound **11** (5.7 mg, 6.4 μmol, 46%). NMR data: ^{1}H, δ 0.86, 1.29, 1.30, 1.35, 1.62, 3.62 (CH_3–CH_2–CH_2–CH_2–CH_3–CH_2–NH–), 2.04 (s, 3 H, NAc), 4.46 (d, 1 H, $J_{1,2}$ 7.8 Hz, H-1′), 4.48 (1 H, H-1‴), 4.72 (1 H, $J_{1,2}$ 8.4 Hz, H-1″), 5.08 (broad s, 1 H, H-1); ^{13}C, δ 13.6, 22.3, 31.1, 25.6, 30.5, 44.9 (CH_3–CH_2–CH_2–CH_2–CH_3–CH_2–NH–), 22.7 (NAc), 83.7 (C-1), 103.0 (C-1‴), 103.2 (C-1′, C-1″). FABMS: Anal. Calcd for $C_{36}H_{60}O_{22}N_3$ [M + H]: 886.3668. Found: 886.3669.

One-pot enzymatic glycosylation of **7** *to give* **11**.—Conjugate **7** (15 mg, 29.0 μmol) was added to a 4 mL aq buffer (15 mM in manganese(II)chloride and 0.5 M in sodium cacodylate, pH 7.2) soln containing UDP-Gal (23 mg, 37.7 μmol), UDP-GlcNAc (25.0 mg,

38 μmol), BSA (20 mg), β-(1-4)-galactosyltransferase (500 mU, 0.05 mL of a 50% glycerol stock soln), and recombinant β-(1-3)-*N*-acetylglycosamintransferase (60 mU, 0.3 mL of a 50% glycerol stock soln). The reaction mixture was stirred at rt for 3 h, after which TLC analysis (6:3:3:2 EtOAc–AcOH–MeOH–water) showed complete consumption of starting material. The mixture was diluted with water to 20 mL, and was then slowly passed through a C-18 Isolute cartridge (10 g, previously washed with first 50 mL of MeOH, then 200 mL of water). After washing of the column with water (50 mL), the desired material was eluted with MeOH (20 mL). Appropriate fractions were pooled and lyophilized to give compound **11** (17 mg, 19 μmol, 66% yield). NMR data were as previously shown.

General procedure for cleavage of oligosaccharide–lipid conjugates to give reducing sugars

Method A (using bromine). A freshly prepared soln of bromine in water (100 mM, 1.0 ml) was added, at rt, to an aq soln of oligosaccharide–lipid conjugate (40 mM, 1.0 mL). The orange bromine color decreased rapidly on mixing. If necessary, more bromine soln was added until a slight yellow color persisted. The reaction mixture was then made slightly basic (pH 7–8) by the addition of an aq 1 M ammonium bicarbonate soln. Aq sodium borate (0.2 M, 1.0 mL, pH 8.0) was then added, and the mixture was stirred at rt until TLC indicated complete conversion of the glycosylamine to the reducing sugar (1 h). The resulting mixture was purified by gel-filtration.

Method B (using ammonia/ammonium borate). Oligosaccharide–lipid conjugate (0.040 mmol) was dissolved in a mixture of aq boric acid (2.0 mL, 0.2 M) and concd aq ammonia (2.0 mL). The mixture was stirred at 30 °C for 3 h, then the mixture was filtered, and the filtrate was concentrated and purified by gel-filtration.

Cleavage of compound **8** *(method A) to give reducing trisaccharide* **10**.—A soln of compound **8** (10 mg, 0.038 mmol) in water (1.0 mL) was treated, essentially as described above (method A), with bromine (1 mL of a 100 mM aq soln). Adjustment of pH and treatment with sodium borate as described above gave a mixture that was purified by gel-filtration (Sephadex G-15, 1.8 × 80 cm). Appropriate fractions were pooled and lyophilized, giving trisaccharide **10** (6.6 mg, 0.0125 mmol, 91%). NMR data: ^{1}H, δ 2.04 (s, 3 H, NAc), 3.28 (t, 1 H, H-2β), 3.58 (t, 1 H, H-2α), 3.64 (t, 1 H, H-3β), 3.65 (t, 1 H, H-4), 3.73 (t, 1 H, H-3α), 3.73 (dd, 1 H, H-3′), 3.59 (t, 1 H, H-2′), 3.76 (1 H, H-2″), 3.84 (1 H, H-4″), 3.90 (1 H, H-3″), 4.15 (d, 1 H, H-4′), 4.44 (d, 1 H, $J_{1,2}$ 7.8 Hz, H-1′), 4.66 (1 H, $J_{1,2}$ 7.8 Hz, H-1β) 4.70 (1 H, H-1, $J_{1,2}$ 8.1 Hz, H-1″), 5.22 (1 H, $J_{1,2}$ 3.8 Hz, H-1α); ^{13}C, δ 22.7 (NAc), 56.0 (C-2″), 60.6 (C-4″), 61.0 (C-3″), 68.8 (C-4′), 70.2 (C-4), 70.5 (C-2′), 70.9 (C-2α), 74.3 (C-2β), 74.9 (C-3β), 75.3 (C-3α), 75.4 (C-3′), 92.2 (C-1α), 96.1 (C-1β), 103.2 (C-1″), 103.3 (C-1′). FABMS: Anal. Calcd for $C_{20}H_{36}O_{16}N$ [M + H]: 546.2034. Found: 546.2029.

Cleavage of compound **11** *(method A) to give lacto*-N-*neotetraose* (**13**).—Compound **11** (5.0 mg, 5.8 μmol) was cleaved with bromine as described (method A) for compound **8** to give, after purification by gel-filtration, material (4.0 mg, 5.7 μmol, 85%) whose NMR data were identical to those of an authentic sample [15] of lacto-*N*-neotetraose (**13**).

Cleavage of **7** *(method B) to give lactose.*—The lactose–lipid conjugate **7** (21 mg, 0.040 mmol) was cleaved with ammonia/ammonium borate as described (method B) to give, after purification by gel-filtration, a material (12 mg, 88%) whose NMR data were identical to those of an authentic sample of lactose.

Simplified preparation of long-chain amino Sepharose 6B

Initial amino derivatization of Sepharose 6B. Epoxy Sepharose 6B (5 g) was swollen in water, washed (water, 100 mL) and mixed (in drained form) with a soln of 1,8-diamino-3,6-dioxaoctane (1.0 g, 6.8 mmol) in aq sodium bicarbonate buffer (0.1 M, pH 9.0, 30 mL). The mixture was shaken for 12 h at rt, then filtered and washed with water (500 mL total volume). The first filtrate was saved and used later. An aliquot of the gel (50 μL drained) was removed and analyzed with a quantitative ninhydrin test (see above). An amino group content of 55 μmol/mL drained gel was found.

First elongation cycle. The bulk of the gel (in drained form) was mixed with a freshly prepared soln of 3,4-diethoxy-3-cyclobutene-1,2-

dione (1.0 g, 5.9 mmol) in EtOH (15 mL) and bicarbonate buffer (0.1 M, pH 9.0, 15 mL). The mixture was shaken for 2 h at rt, then filtered and washed with 1:1 EtOH–water (50 mL total) and water (200 mL). An aliquot of the gel (50 μL drained) was removed and analyzed with a quantitative ninhydrin test (see above). A negligible amino group content was found at this stage. The bulk of the gel (in drained form) was mixed with the saved 1,8-diamino-3,6-dioxaoctane filtrate from above. The mixture was shaken for 12 h at rt, then filtered and washed with water (500 mL total). The first filtrate was saved and used later.

Second elongation cycle. The elongation cycle described above was repeated once more, giving a final long-chain amino Sepharose (45 chain atoms) that, according to the ninhydrin test, had an amino group content of 36 μmol/mL.

Coupling of lactose–squaric acid conjugate **4** *to long-chain amino Sepharose to give lactose–Sepharose derivative* **14**.—A soln of lactose conjugate **4** (11 mg, 23 μmol) in bicarbonate buffer (2.0 mL, 0.1 M, pH 9.0) was rotated for 12 h at rt with long-chain amino Separose prepared as described above (1.0 mL, 36 μmol amino groups). The solid was filtered off and washed with water (30 mL). An aliquot of the gel (50 μL drained) was removed and analyzed with a quantitative ninhydrin test (see above). An amino group content of 23 μmol/mL was now found, which, assuming a clean derivatization reaction, corresponds to a lactose incorporation of 13 μmol/mL. This value was verified by cleaving off the bound lactose (bromine method, for general conditions, see below) and analyzing the contents of the supernatant by quantitative ^{1}H NMR analysis (1,2-*O*-isopropylidene-α-D-glucofuranose was added as internal standard). This gave a value of 12 μmol/mL for the lactose incorporation.

In another experiment, lactose conjugate **4** (15 mg, 32 μmol) was mixed with long-chain amino Separose (1.0 mL, 30 μmol amino groups, a different batch than above) in bicarbonate buffer (2.0 mL, 0.1 M, pH 9.0). After 2 h, the solid was filtered off and washed with water (30 mL). An aliquot of the gel (50 μL drained) was removed and analyzed with a quantitative ninhydrin test (see above), An amino group content of 15 μmol/mL was now found, which, assuming a clean derivatization reaction, corresponds to a lactose incorporation of 15 μmol/mL. This value was verified by cleaving off the bound lactose (ammonia/ammonium borate method, for general conditions, see below) and analyzing the contents of the supernatant by quantitative ^{1}H NMR analysis (1,2-*O*-isopropylidene-α-D-glucofuranose was added as internal standard). This gave a value of 13 μmol/mL for the lactose incorporation.

Enzymatic glycosylation of lactose–Sepharose derivative **14** *to give* **15**.—The Sepharose derivative **14** from above (0.9 mL, 12 μmol) was resuspended in sodium cacodylate buffer soln (2.0 mL, 0.5 M, pH 7.2) containing manganese(II)chloride (15 mM), BSA (10 mg), UDP-*N*-acetylglucosamine (15 mg, 23 μmol), and recombinant β-(1→3)-*N*-acetylglycosamintransferase (100 mU, 0.5 mL of a 50% glycerol stock soln). The reaction mixture was rotated for 18 h at rt, filtered, and washed with water (20 mL). The solid was resuspended in sodium cacodylate buffer soln (2.0 mL, 0.5 M, pH 7.2) containing manganese(II)chloride (20 mM), UDP-galactose (15 mg, 25 μmol), and β-(1→4)-galactosyltransferase (1.0 U, 0.05 mL of a 50% glycerol stock soln). The reaction mixture was rotated for 18 h at rt, then filtered and washed with water (50 mL). The resulting gel **15** was used as such in the next step.

Cleavage of gel **15** *(with bromine) to give lacto*-N-*neotetraose*.—Gel **15** (0.9 mL) was resuspended in water (4.0 mL) containing bromine (15 μL, 0.29 mmol), and the mixture was rotated for 10 min at rt. The gel was then filtered and washed with water (10 mL). The combined filtrate and washings was adjusted to pH 7.0–8.0 with aq ammonium bicarbonate (1 M), then aq sodium borate (0.5 mL, 0.2 M, pH 8.0) was added. The mixture was stirred at rt for 1 h. Analysis by TLC (4:3:3:2 EtOAc–AcOH–MeOH–water) of the reaction mixture at this stage showed the presence of a major compound (lacto-*N*-neotetraose) and trace amounts of the corresponding di- and trisaccharide. The mixture was concentrated and purified by gel-filtration (Sephadex G-15, 1.8 × 80 cm column). Appropriate frac-

tions were pooled, mixed with 1,2-*O*-isopropylidene-α-D-glucofuranose (1.1 mg, 5.0 μmol, internal standard) and lyophilized. The residue, according to ^{1}H NMR analysis, contained pure lacto-*N*-neotetraose (**13**, 12 μmol, 92%).

Cleavage of gel **15** *(with ammonia/ammonium borate) to give lacto*-N-*neotetraose.*—Gel **15** (0.80 mL, 11 μmol) was resuspended in aq boric acid (0.2 M, 1.0 mL) and concd ammonia (1.0 mL) was added. The mixture was rotated overnight at rt. The gel was filtered and washed with water (10 mL). The combined filtrate and washings was concentrated and purified by gel-filtration (Sephadex G-15, 1.8×80 cm column). Appropriate fractions were pooled, the obtained soln was mixed with 1,2-*O*-isopropylidene-α-D-glucofuranose (0.44 mg, 2.0 μmol, internal standard) and lyophilized. The residue, according to NMR analysis, contained lacto-*N*-neotetraose (8.2 μmol, 75%).

Coupling of **4**, **5** *or* **6** *to HSA to give oligosaccharide–protein conjugates* **14**, **15**, *and* **16**.—HSA (50 mg, 0.75 μmol, 1/33 equivalent) was dissolved in carbonate buffer (7.0 mL, pH 9.0). When all protein had dissolved, oligosaccharide derivative (25 μmol, i.e., 11.7 mg of **4**, 18.9 mg of **5**, or 20.8 mg of **6**) was added, and the soln was slowly stirred at rt for 18 h. The reaction mixture was ultrafiltrated down to 2 mL, water (10 mL) was added, and ultrafiltration was repeated. After four ultrafiltration cycles, the retentate was lyophilized to give the glycoconjugates: **14** (52 mg), **15** (48 mg), and **16** (53 mg). These were analyzed by MALDI-TOF mass spectroscopy. The substitution degrees were 22, 21, and 9 mol/mol of HSA, respectively, corresponding to coupling yields of 70, 68 and 25%, respectively.

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