Solid-Phase Enzymatic Synthesis of a Sialyl Lewis X Tetrasaccharide on a Sepharose Matrix

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Thiopyridyl Sepharoses with different linker arm lengths were prepared from epoxy Sepharose 6B by reaction first with 1,8-diamino-3,6-dioxaoctane and then with, sucessively, diethoxy-3-cyclobutene-1,2-dione (squaric acid diethyl ester) and 1,8-diamino-3,6-dioxaoctane in several cycles, followed by reaction of the obtained amino Sepharoses with, successively, thiobutyrolactone and 2,2′-dithiopyridine. The thiopyridyl Sepharoses were reacted with the glucosamine derivative 2-(3′-mercaptobutyrylamido)ethyl 2-acetamido-2-deoxy-β-D-glucopyranoside, giving GlcNAc Sepharoses with different linker lengths. Enzymatic galactosylation of these with β-(1−4)-galactosyltransferase and UDP-galactose gave yields varying between 70 and 98%, and there was a clear correlation between linker length and yield. A GlcNAc Sepharose with a long linker was then used in a solid-phase synthesis of a sialyl Le x tetrasaccharide. The three required enzymes (galactosyl-, sialyl, and fucosyltransferase) and nucleotide sugars were reacted consecutively with the GlcNAc Sepharose, giving, after cleavage from Sepharose with DTT, the free sialyl Le x tetrasaccharide derivative in a 57% total yield after purification.

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. Some recent attempts toward simplification have been made by applying chemical solid-phase techniques,[1−4] which are routine in peptide and oligonucleotide synthesis. However, chemical glycosylation is seldom stereospecific, and consistent high-coupling yields with respect to one anomer are therefore difficult to achieve. Furthermore, the elaborate protecting group patterns that have to be used for each monosaccharide component make their preparation tedious.

A radically different approach to oligosaccharide syntheses is to use enzymes (e.g., glycosyltransferases) as glycosylating catalysts. These give regio- and stereospecific glycosylations, without protecting groups on the monosaccharides. Here, progress has been dramatic,[5−7] mainly due to advances in enzyme production by genetic engineering. An example of what can be done with glycosyltransferases today is the reported[8] synthesis of a sialyl Lewis x derivative in kilogram amounts, using cloned glycosyltransferases for two crucial glycosylations.

Glycosyltransferase-catalyzed glycosylations in combination with solid-phase techniques should offer a particulary simple way to synthesize natural oligosaccharides on a laboratory scale (Scheme 1). Some reports on such syntheses, using different transferases and solid-phase materials, have indeed appeared.[9−17] Only few,[9−10] however, report several consecutive enzymatic glycosylations on the solid phase to produce more complicated oligosaccharides. We have previously reported[18] an enzymatic solid-phase fucosylation of a disaccharide reversibly linked to Sepharose via disulfide linkage to produce a Le a trisaccharide. We now report on the synthesis of a sialyl Le x tetrasaccharide by carrying out three consecutive, high-yield enzymatic glycosylations on a starting glucosamine monosaccharide attached to Sepharose via a disulfide linkage. In connection with this synthesis, we have also investigated how the yield of a typical enzymatic glycosylation varies with the length of the linker connecting the sugar acceptor to the Sepharose matrix.

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(1) Danishefsky, S. J.; Mcclure, K. F.; Randolph, J. T.; Ruggeri, R. B. *Science* **1993**, *260*, 1307−1309.
(2) Rademann, J.; Schmidt, R. R. *Tetrahedron Lett* **1996**, *37*, 3989−3990.
(3) Randolph, J. T.; Mcclure, K. F.; Danishefsky, S. J. *J. Am. Chem. Soc.* **1995**, *117*, 5712−5719.
(4) Yan, L.; Taylor, C. M.; Goodnow, R.; Kahne, D. *J. Am. Chem. Soc.* **1994**, *116*, 6953−6954.
(5) Ichikawa, Y.; Look, G. C.; Wong, C. H. *Anal. Biochem.* **1992**, *202*, 215−238.
(6) Wong, C. H.; Halcomb, R. L.; Ichikawa, Y.; Kajimoto, T. *Angew. Chem. Int. Ed. Engl.* **1995**, *34*, 521−546.
(7) Wong, C. H.; Halcomb, R. L.; Ichikawa, Y.; Kajimoto, T. *Angew. Chem. Int. Ed. Engl.* **1995**, *34*, 412−432.
(8) Bayer, R.; DeFrees, S.; Gaeta, F.; Jiang, C.; Norberg, T.; Ramphal, J.; Ratcliffe, M.; Walker, L. Presented at the 2nd CHI Glycotechnology Conference, LA Jolla, CA, 1994.
(9) Halcomb, R. L.; Huang, H. M.; Wong, C. H. *J. Am. Chem. Soc.* **1994**, *116*, 11315−11322.
(10) Yamada, K.; Nishimura, S. I. *Tetrahedron Lett.* **1995**, *36*, 9493−9496.
(11) Meldal, M.; Auzanneau, F.; Hindsgaul, O.; Palcic, M. *J. Chem. Soc., Chem. Commun.* **1994**, 1849−50.
(12) Nunez, H. A.; Barker, R. *Biochemistry* **1980**, *19*, 489−495.
(13) Schuster, M.; Wang, P.; Paulson, J. C.; Wong, C. H. *J. Am. Chem. Soc.* **1994**, *116*, 1135−1136.
(14) Zehavi, U.; Herchman, M. *Carbohydr. Res.* **1986**, *151*, 371−378.
(15) Zehavi, U.; Herchman, M.; Hakomori, S.; Köpper, S. *Glycoconjugate J.* **1990**, *7*, 219−28.
(16) Zehavi, U.; Herchman, M.; Schmidt, R.; Bär, T. *Glycoconjugate J.* **1990**, *7*, 229−34.
(17) Zehavi, U.; Herchman, M.; Hakomori, S.; Köpper, S. *Glycoconjugate J.* **1990**, *7*, 219−28.
(18) Blixt, O.; Norberg, T. *J. Carbohydr. Chem.* **1997**, *16*, 143.

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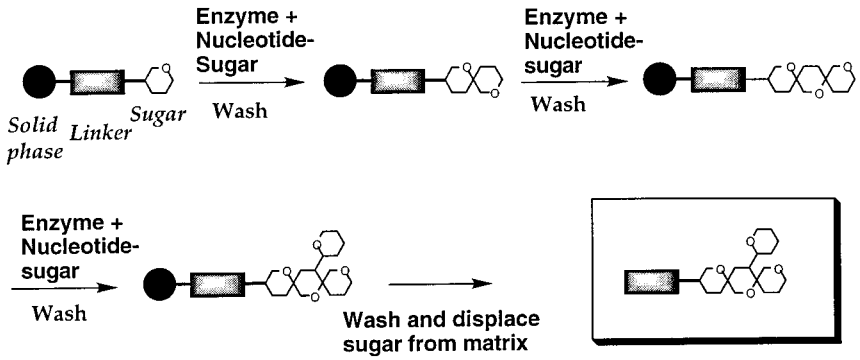
Scheme 1. Typical Solid-Phase Enzymatic Oligosaccharide Synthesis



Results and Discussion

In our previous work[18] on solid-phase enzymatic synthesis, a disaccharide (Gal1-3β-GlcNAc) acceptor was used, linked to Sepharose via a disulfide linkage and a linker arm with a total length of 12 atoms. The yield in the enzymatic fucosylation seldom exceeded 70%, despite attempts with increases in enzyme and nucleotide sugar amounts, or reaction time. Even washing of the gel after coupling and repeated coupling with fresh enzyme and nucleotide sugar ("double-coupling") did not lead to better yields. Obviously, there were unreactive acceptor sites present on the gel. This is a well-known phenomenon in chemical solid-phase synthesis, caused by, e.g., capping by an undesired chemical group during the coupling procedure or unfavorable conformational changes when the polymer chain length increases (steric factors). In our case, it was reasonable to assume that the low reactivity was caused not by capping but rather by steric factors. The relatively short length of the linker (12 atoms, approximately 15 Å in the most extended conformation) connecting the acceptor disaccharide to the Sepharose matrix and the size of the fucosyltransferase enzyme (diameter approximately 60 Å, assuming a globular form) should make at least some acceptor sites "unapproachable" by the enzyme. A longer linker between the Sepharose matrix and the acceptor sugar should give a better result because of less steric interference between enzyme and solid phase and also because of greater conformational flexibility. To test this hypothesis, we prepared Sepharose gels with different lengths of the linker. We chose a PEG-type linker and 1,4-β-galactosylation with galactosyltransferase of an *N*-acetylglucosamine acceptor as a model reaction. To prepare the *N*-acetylglucosamine acceptor, 2-azidoethyl 3,4,6-tri-*O*-acetyl-2-acetamido-2-deoxy-β-D-glucopyranoside[19] was deacetylated (sodium methoxide) and hydrogenated (Pd/C) to give 2-aminoethyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**1**), which was further reacted with thiobutyrolactone to give a monosaccharide (**2**) with a thiol group on the aglycon. This monosaccharide was then coupled to several thiopyridyl Sepharoses having linkers differing in length. The thiopyridyl Sepharoses were prepared in the following way (Scheme 2):

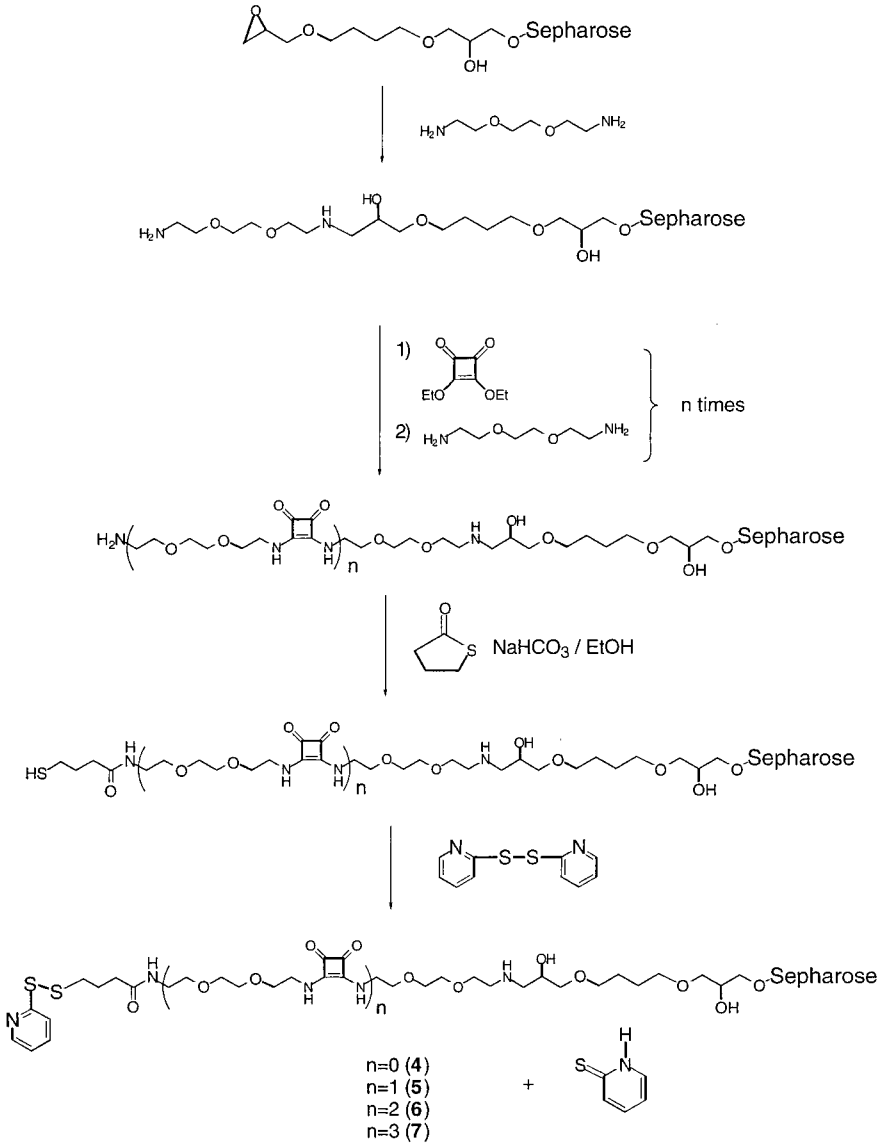
First, epoxy Sepharose 6B was reacted with 1,8-diamino-3,6-dioxaoctane; this gave the first (21 linker atoms) of a series of amino Sepharoses. Next, a portion of this first amino Sepharose was reacted with, successively, 3,4-diethoxy-3-cyclobutene-1,2-dione (squaric acid diethyl ester[20]) and 1,8-diamino-3,6-dioxaoctane. This gave a second amino Sepharose (33 linker atoms). Further repetition of this elongation cycle gave Sepharoses with linker length of 45 and 57 atoms. The obtained amino Sepharoses were then reacted with, successively, thiobutyrolactone and 2,2′-dithiopyridine, giving thiopyridyl Sepharoses with linker lengths of 26, 38, 50, and 62 atoms. Coupling (Scheme 3) of these with monosaccharide **2** gave GlcNAc Sepharoses **4**, **5**, **6**, and **7** with linker lengths of 35, 47, 59, and 71 atoms, respectively. For reference purposes, monosaccharide **2** was also coupled (Scheme 3) with commercial thiopyridyl Sepharose 6B to give GlcNAc Sepharose **3** with 12 linker atoms; this was the linker used in our previous work. All the chemical reactions performed on Sepharose proceeded in excellent yields, as measured by *p*-toluenesulfonic acid titration of the gel amino groups after each step in the elongation cycles. The degree of functionalization on the gels was 7.5−12.4 μmol/ml drained gel, as measured by NMR quantification of released sugar after coupling.

The GlcNAc Sepharoses **3**−**7** were next subjected to enzymatic galactosylation using a total of 3 equiv of UDP−galactose and 1 U of β-1,4-galactosyltransferase/10 μmol gel and a total reaction time of 90 h (Scheme 4). After galactosylation, the gels were treated with dithiothreitol (DTT) to release the bound carbohydrates into solution, and the obtained solutions were mixed with a known amount of internal standard and analyzed by ^{1}H NMR spectroscopy. The results are shown in Table 1. In all cases, the recovery of material (monosaccharide plus disaccharide) exceeded 95%. The gel with the shortest linker (**3**, 12 atoms) gave a moderate galactosylation yield (70%), in accordance with the results obtained in our previous work[18] using the same linker in an enzymatic fucosylation reaction. The other gels gave significantly better yields; thus, there was a significant increase in yield (70−89%) on going from gel **3** (12 linker atoms) to gel **4** (35 linker atoms). The best yield (98%) was obtained with the gel (**7**) with the longest (71 linker atoms) linker; however, the increase in yield is less significant here, and the variations between gel **5**−**6** and **7** are within the limits of the estimated experimental error (±5%). The reason for the better yield obtained on changing the linker can only be speculated at this point. Other laboratories have studied the influence on solid-phase synthesis yields of PEG-type linkers grafted to polyamide[11] or polystyrene,[22,23] and the general observation, at least in regard to the yields in chemical synthesis,

(19) Eklind, K.; Gustafsson, R.; Tiden, A. K.; Norberg, T.; Aberg, P. M. *Carbohydr. Chem.* **1996**, *15 (9)*, 1161−1178.

(20) Tietze, L.; Arlt, M.; Beller, M.; Glusenkamp, K. H.; Jahde, E.; Rajewsky, M. F. *Chem. Ber.* **1991**, *124*, 1215.

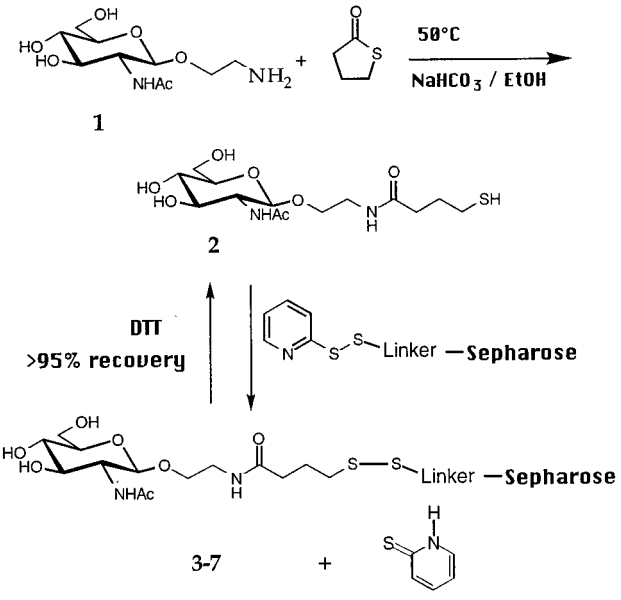
Scheme 2. Preparation of Dithiopyridyl Sepharoses with Different Linker Lengths



is that the presence of such a linker is beneficial (which is also evidenced by the fact that PEG-modified resins have become commercially available).

Whatever the cause is of the yield improvement in our case, it can be concluded that the yields now achieved (90−98%) are in the same high range as is common in repetetive chemical solid-phase synthesis. With such yields, it should be possible to construct more complicated oligosaccharide structures by carrying out several consecutive enzymatic glycosylations on the solid phase, without losing much material or ending up with inseparable mixtures at the end of the synthesis. We decided to use gel **7** to synthesize the sialyl Lewis x tetrasaccharide **8**, since this is a biologically interesting molecule, and because of the fact that all three enzymes needed are reasonably accessible. The synthesis was carried out as shown in Scheme 5.

Scheme 3. Attachment of Monosaccharide 2 to Sepharoses with Linkers of Different Length



(21) Lin, Y. C.; Hummel, C. W.; Huang, D. H.; Ichikawa, Y.; Nicolaou, K. C.; Wong, C. H. *J. Am. Chem. Soc.* **1992**, *114*, 5452−5454.

(22) Vagner, J.; Barany, G.; Lam, K. S.; Krchnak, V.; Sepetov, N. F.; Ostrem, J. A.; Strop, P.; Lebl, M. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 8194−8199.

(23) McGuinness, B. F.; Kates, S. A.; Griffin, G. W.; Herman, L. W.; N. A. Sole, Vagner, J.; Albericio, F.; Barany, G. *Proc. Am. Pept. Symp., 14th* 1995.

Scheme 4. Galactosylation of *N*-Acetylglucosamine Sepharoses 3−7 and Release of the Attached Sugar

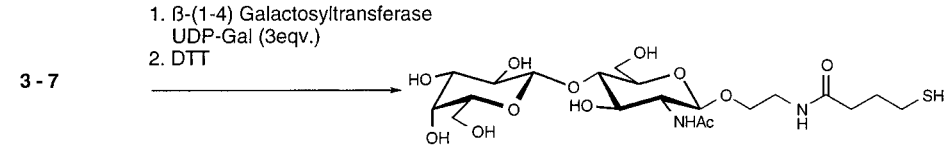


Table 1. Enzymatic Galactosylation of Gels with Different Linker Lengths

gel	linker no. atoms	bound GlcNAc (μmol/mL)	enzymatic gal. transfer[a] (%)
3	12	7.5	70
4	35	12.4	89
5	47	12.4	89
6	59	12.4	90
7	71	9.0	98

[a] By ^{1}H NMR (Gal/GlcNAc H-1). All experiments at 1.0 mL drained gel volume.

GlcNAc Sepharose **7** (24 μmol), was incubated with β-(1−4)-galactosyltransferase and UDP galactose over an incubation period of 4 days, with succesive additions at intervals of UDP galactose (a total of 3 equiv). This, according to NMR analysis of the mixture released from an aliquot of the gel, gave a 90% galactosylation yield. Washing of the gel and subsequent sialylation with α-(2−3)-sialyltransferase and CMP-Neu5Ac using similar amounts of enzyme and nucleotide sugar gave a 75% glycosylation yield; the lower yield here was probably due to nonoptimized conditions. Washing of the gel and subsequent fucosylation with partially purified α-(1−3/4)-fucosyltransferase from human milk and GDP-fucose gave an approximately 95% fucosylation yield. Finally, treatment of the gel with DTT and purification of the released mixture by gel filtration gave the sialyl Le x tetrasaccharide (**8**) in a 57% yield (calculated from the amount of starting GlcNAc on the gel). The purity of the material in terms of tetrasaccharide content was better than 90%. The ratio of thiol to disulfide form of the product varied, the latter dominating after exposure to air for an extended time. The thiol form could be recovered quantitatively from any thiol-disulfide mixture by brief treatment with dithiothreitol.

In conclusion, using GlcNAc linked via a new linker to Sepharose, the sialyl Le x tetrasaccharide **8** was synthesized in good (57%) yield by sequential treatment of the Sepharose gel with the three necessary enzymes and their corresponding monosaccharide nucleotide sugars. Further investigations with other sugars and transferases using the described solid-phase technique are in progress.

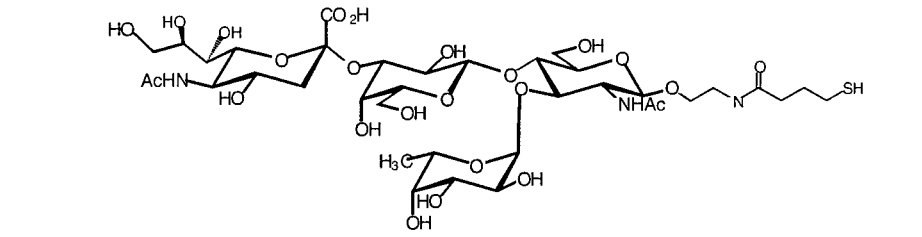
Experimental Section

General Methods. Concentrations were performed under reduced pressure at <40 °C bath temperature. NMR spectra were recorded at 30 °C using Bruker 400 and 600 MHz instruments. The following reference signals were used: acetone δ 2.225 (^{1}H in D_2O), acetone δ 30.7 (^{13}C in D_2O). FAB MS was recorded with a JEOL JMS-SX/SX-102A mass spectrometer using thioglycerol as matrix. Thin-layer chromatography was performed on Kieselgel 60 F_{254} Fertigplatten (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. Water for all solutions was from a MilliQ water purification system (Millipore Corp., Bedford, MA) and was degassed by vacuum treatment before use. 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 being charged 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 in a 37 °C water bath for the specified time. Epoxy Sepharose 6B and thiopyridyl Sepharose 6B were from Pharmacia Biotechnology AB (Uppsala, Sweden). β-(1−4)-Galactosyltransferase, 1,2-*O*-isopropylidene-α-D-glucofuranose, dithiothreitol (DTT), uridine 5′-diphosphogalactose, calf intestine alkaline phosphatase (CIAP), α-lactalbumin, and cytidine monophospho-*N*-acetylneraminic acid (CMP-NeuAc) were from Sigma Chemical Co. (St. Louis, MO), and 2,2′-dithiopyridine, 3,4-diethoxy-3-cyclobutene-1,2-dione, and 1.8-diamino-3.6-dioxaoctane were from Acros Chimica (Geel, Belgium). Recombinant α-(2−3)-sialyltransferase was a gift from Cytel Corp., San Diego, CA. Milk α-(1−3/4)-fucosyltransferase and guanosine 5′-(β-L-fucopyranosyl)diphosphate were prepared as previously described.[18] Buffer A had the following composition: 25 mM sodium cacodylate, 5 mM manganese(II) chloride, 0.1% TritonX, pH 7.5. Buffer B had the following composition: 10 mM sodium cacodylate, 5 mM manganese(II) chloride, 2% glycerol, pH 6.5.

2-(3′-Mercaptobutyrylamido)ethyl 2-Acetamido-2-deoxy-β-D-glucopyranoside (2). 2-Azidoethyl 3,4,6-tri-*O*-acetyl-2-acetamido-2-deoxy-β-D-glucopyranoside[19] (360 mg, 0.87 mmol) in methanolic sodium methoxide (3 mL, 0.1 M) was stirred at room temperature until TLC indicated complete reaction, and then the mixture was neutralized with Dowex-50 (H^+) ion-exchange resin and concentrated. The residue was taken up in ethanol/water/acetic acid (5:5:1, 11 mL), Pd/C (10%, 0.3 g) was added, and the mixture was hydrogenated at atmospheric pressure. When ready (TLC: ethyl acetate/methanol/acetic acid/water, 3:3:3:1), the mixture was filtered, concentrated, and lyophilized to give 2-aminoethyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**1**, acetate form, 280 mg, 0.87 mmol, 98%). This material was dissolved in a mixture of sodium bicarbonate (0.5 M, 5 mL) and ethanol (4 mL), and then γ-thiobutyrolactone (0.75 mL, 8.6 mmol) and DTT (0.67 g, 4.3 mmol) were added and the mixture was stirred at 50 °C under a nitrogen atmosphere overnight (TLC: ethyl acetate/methanol/acetic acid/water, 3:3:3:1 by volume). The mixture was then neutralizied to pH 7 with 1 M HCl and concentrated, and the residue was dissolved in water (3 mL), filtered, and applied onto a BioGel P-2 column (2.5 × 75 cm, packed and eluted with 5% 1-butanol in water). The appropriate fractions were pooled and lyophilized to give **2** (227 mg, 0.62 mmol, 71%). NMR data: ^{1}H, δ 1.88 (quintet, 2H, $CH_2CH_2CH_2SH$), 2.04 (s, 3H, CH_3CONH), 2.37 (t, 2H, $CH_2CH_2CH_2SH$), 2.55 (t, 2H, $CH_2CH_2CH_2SH$), 3.37 (t, 2H, OCH_2CH_2NH), 3.33−3.50 (m, 2H, H-4, H-5), 3.55 (t, 1H, H-3), 3.66−3.79, 3.87−3.96 (2 m, 1H each, OCH_2CH_2NH), 3.69 (1H, H-2), 3.66−3.79, 3.87−3.96 (2 m, 1H each, H-6a,b), 4.53 (d, 1H, 8.3 Hz, H-1); ^{13}C, δ 22.9, 23.5, 29.8, 34.8 (CH_3CONH-, $-CH_2CH_2CH_2-$), 39.7 (OCH_2CH_2NH-), 56.0 (C-2), 61.2 (C-6), 68.5 (OCH_2CH_2NH-), 74.2, 70.3, 76.3 (C-3, C-4, C-5), 101.5 (C-1), 175.0 (CH_3CONH-), 176.6 ($-NHCOCH_2-$); HRMS calcd for $C_{14}H_{26}O_7N_2SNa$ 389.1358, found 389.1410 (M + Na).

Solid-Phase Preparations. Dithiopyridyl Sepharoses 6B with Extended Linker Arm: (1) Amino Derivatization of Epoxy Sepharose 6B. Dry epoxy Sepharose 6B (7 g) was swollen in water, washed (water, 200 mL), transferred to a glass filter column (2.5 × 40 cm), and drained. A solution of 1.8-diamino-3,6-dioxaoctane (3.0 mL, 4.4 g, 30 mmol) in aqueous sodium bicarbonate buffer (0.1 M, pH 11.0, 50 mL)

Scheme 5. Solid-Phase Enzymatic Synthesis of a Lewis x Tetrasaccharide



Sialyl Le x derivative **8** (57% yield)

was added, and the column was sealed and rotated for 48 h at room temperature. The resin was transferred to a glass filter funnel, filtrated, and washed with water (4 L). An aliquot of the gel (0.25 mL, drained) was reacted with aqueous *p*-toluenesulfonic acid (1mM, 5.0 mL) for 10 min with gentle stirring. The absorbance at 264 nm of the supernatant was recorded before and after gel treatment, and from this difference the amount of amino groups on the gel was calculated to be approximately 14 μmol/mL drained gel.

(2) Amino-Linker Elongation Cycle. A portion of the amino-derivatized gel (2.0 mL drained) was set aside, and the rest of the material (approximately 23 mL drained gel) was reacted with 3,4-diethoxy-3-cyclobutene-1,2-dione (1.3 mL, 1.5 g, 8.8 mmol) dissolved in a mixture of ethanol (95%, 50 mL) and bicarbonate buffer (0.1M, pH 9.5, 50 mL) and rotated for 6 h at room temperature. The gel was filtered off and washed with ethanol (50 mL) and water (4 L). An aliquot of the gel (0.25 mL, drained) was reacted with *p*-toluenesulfonic acid as described above. The absorbance of the supernatant at 264 nm before and after reaction with resin did not differ notably, indicating complete disappearance of amino groups on the gel.

The gel was mixed with a solution of 1.8-diamino 3,6-dioxaoctane (3.0 mL) in carbonate buffer (0.1 M, 50 mL, pH 9.5) rotated for 24 h at room temperature, and then filtered and washed with water (4 L). An aliquot of the gel (0.25 mL, drained) was reacted with *p*-toluenesulfonic acid as described above. The absorbance at 264 nm of the supernatant was recorded before and after gel treatment; from this difference the amount of amino groups on the gel was calculated to be approximately 14 μmol/mL drained gel.

A portion of this amino-derivatized gel (2.0 mL drained) was set aside, and the rest of the material was subjected to two more elongation cycles as described above. For each elongation, the amount of amino groups on the gel, as measured by *p*-toluenesulfonic acid titration, did not decrease significantly.

(3) Conversion of Gel Amino Groups to Dithiopyridyl Groups. Amino-derivatized gels from the above-described preparations were each subjected to the following procedure:

Drained gel (2 mL) was mixed, in a glass column (1.8 × 10 cm), with a solution of DTT (62 mg) and thiobutyrolactone (70 μL) in ethanol (95%, 4.5 mL) and aqueous sodium bicarbonate (0.5 M, 5.0 mL). The column was rotated in a water bath at 50 °C for 20 h, and then the gel was washed with water (200 mL), and treated with phosphate buffer (0.1 M, pH 8.0, 10 mL), followed by treatment with DTT (77 mg) in phospate buffer (0.1 M, 10 mL) for 2 h. After being washed with water (200 mL), the gel was rotated overnight at room temperature with a solution of 2,2′-dithiopyridine (35 mg) in phosphate buffer (0.1 M, pH 8.0, 5.5 mL) and ethanol (2.5 mL). The absorbance at 343 nm (proportional to released thiopyridone) was determined for the supernatant, giving indirect estimates of dithiopyridyl substitution degrees (in order of increasing linker length) of 14, 13, 14, and 9 μmol/mL drained gel. The gels were filtered off and washed with water (200 mL) and were stored until used under 30% ethanol at 4 °C.

Reaction of Amino Gels with the GlcNAc Derivative 2 To Give Gels 3−7. GlcNAc thiol **2** (3 equiv) in citric acid buffer (0.2 M, pH 4.0, 7 mL) was added to the Sepharose gels obtained as described above (conditioned in citric acid buffer), and the mixtures were rotated in columns overnight at room temperature. The amount of released thiopyridone (as determined by supernatant absorbance at 343 nm) indicated substitution degrees of 13, 12, 12, and 10 μmol/drained gels for **4**, **5**, **6**, and **7**, respectively. The gels were filtered and washed with water (200 mL), and then three bed volumes of a 50 mM solution of mercaptoethanol in citric acid buffer (0.2 M, pH 4.0) was added to the gels and the columns were rotated for 2 h at room temperature, followed by filtering and washing the gels with water (200 mL). The amount of bound sugar was determined by treating gel (0.5 mL) with DTT in phosphate buffer, as described previously, and quantifying the released sugar with ^{1}H NMR against an added internal standard. This gave values of 12.4, 12.4, 12.4, and 9.0 for gels **4**, **5**, **6**, and **7**, respectively, in good agreement with the values obtained above with spectrophotometry.

Using the same procedure as above, commercial thiopyridyl Sepharose 6B was reacted with **2** to give gel **3**, with a substitution degree of 7.5 μmol/drained gel.

General Enzymatic Galactosylation Procedure. Drained gel (**3**−**7**, 1 mL) conditioned in buffer (50 mM cacodylate, 5mM $MnCl_2$, 0.1% TritonX, pH 7.5, 5 mL) was transferred to a column (1 × 5 cm), buffer (3 mL), β-(1−4)-galactosyltransferase (1U), CIAP (4U), α-lactalbumin (0.6 mg), and UDP-galactose (1 equiv) were added, and the column was rotated in a water bath at 37 °C. After 6, 18, 24, and 48 h more UDP-galactose (0.5 equiv) was added. Incubation was continued for 90 h, and the gel was then filtered and washed with water (200 mL). To release the carbohydrates, the gels were treated for 2 h at room temperature with DTT (23 mg) in phosphate buffer (0.1 M, pH 8.0, 3 mL) containing 5 μmol of 1,2-*O*-isopropylidene-α-D-glucofuranose (as internal standard). After filtration and washing with water (10 mL), the filtrate was lyophilized and the amount of galactosylation was determined by ^{1}H NMR (Table 1).

Sialyl Lewis x Derivative 8 and Its Disulfide Isomer. GlcNAc Sepharose derivative **7** (2.7 mL, 24 μmol) was transferred to a 15 mL glass column, washed with water (200 mL), conditioned with buffer A, and drained. Buffer A (8.0 mL) was added, followed by UDP-galactose (17 mg, 28 μmol, dissolved in 0.4 mL of buffer A), β-(1−4)-galactosyltransferase (5 U, dissolved in 0.8 mL of 1:1 buffer A/glycerol), α-lactalbumin (2.7 mg), and CIAP (25 mg, 30 U), and the pH was adjusted to 7.35. The mixture was rotated in a water bath at 37 °C. After 6, 24, and 48 h, new additions of UDP-galactose (17, 13, and 10 mg, respectively) were made, and the pH was adjusted to to 7.35 with 1 M aqueous cacodylic acid. After 5 days, the gel was filtered off and washed with water (300 mL). An aliquot (0.50 mL, 4.5 μmol) was taken out, washed with phosphate buffer (0.1 M, pH 8.0, 10 mL), drained, and mixed with DTT (11 mg) in phosphate buffer (1.5 mL) containing 1,2-*O*-isopropylidene-α-D-glucofuranose (as internal standard, 5.0 μmol). After rotation for 2 h at room temperature, the gel was filtered off and washed with water (10 mL). The filtrate was lyophilized, and the ^{1}H NMR spectrum was recorded. Integra-

Table 2. NMR Data for 8 and Its Disulfide Isomer[a]

X	Neu5Ac	Gal	GlcNAc	Fuc	CH_2	CH_2	CH_2	CH_2	CH_2
1		4.54	4.51	5.09	3.66−3.96	3.37	2.37	1.99 (1.88)	2.75 (2.55)
		101.9	102.0	99.4	68.5	39.7	34.6	24.6 (29.6)	37.3 (22.2)
2		3.56	3.90	3.69					
		69.0	56.0	68.5					
3	1.79, 2.75	4.08	3.84	3.90					
	39.8, 37.3	75.4	75.0	69.0					
4	3.70	3.94	3.93	3.78					
	68.2	67.0	73.5	72.0					
5	3.84	3.59	3.59	4.81					
	52.0	75.0	75.1	67.0					
6	3.66	3.71	4.01	1.16					
	73.4	61.5	59.0	14.5					
7	3.58								
	68.0								
8	3.90								
	72.1								
9	3.65								
	62.9								
CH_3	2.04		2.02						
	21.1		21.1						
C=O			175.0	spacer−$NHCOCH_2$− 176.6					

[a] CH_2 groups in order of increasing distance from the GlcNAc C-1. Numbers in parentheses refer to the thiol form, if different from the disulphide form.

tion of appropriate analyte and internal standard peaks gave a recovery estimate of >95%, and a di-monosaccharide ratio of 90%.

The bulk part of the gel was conditioned with buffer A and drained, and then buffer A (5 mL), α-(2−3)-sialyltransferase (3.5 U), CMP-Neu5Ac (15 mg, 1.2 equiv, 24 μmol, dissolved in 1 mL of buffer A), and CIAP (15 mg, 18 U) were added. The pH was adjusted to 7.45. The mixture was rotated in a water bath at 37 °C. After 6, 24, and 48 h new additions (0.5 equiv each) of CMP-Neu5Ac were made, and pH was adjusted to 7.35 by addition of 1 M aqueous cacodylic acid. After 5 days, the gel was washed with water (300 mL). An aliquot (0.50 mL) was taken out and was treated and analyzed with NMR as described above; this indicated a >95% recovery and a 75% sialylation yield.

The bulk part of the gel was finally fucosylated with α-(1−3/4)-fucosyltransferase from human milk (0.2 U in buffer B, 11 mL), GDP-fucose (15 mg, 1.4 equiv, 24 μmol), and CIAP (4.0 mg, 5U); and the pH was 6.5. The mixture was rotated in a water bath at 37 °C. After 15, 26, 42, 62, and 84 h, new additions of GDP-fucose (15, 10, 8, 8, and 8 mg, respectively) were made, and the pH was adjusted to 6.5 with 1 M aqueous cacodylic acid. After 5 days, the gel was washed with water (300 mL) and 1 M NaCl (50 mL), and then treated with DTT (77 mg) in phosphate buffer (0.1 M, pH 8.0, 10 mL) containing internal standard (10 μmol) and analyzed by NMR. This analysis indicated a >95% recovery and a 95% fucosylation yield. The crude reaction mixture was dissolved in 3.0 mL of 20 mM ammonium formate buffer (pH 4.7) and loaded on a BioGel P2 column (1.8 × 100 cm), conditioned in 20 mM ammonium formate buffer (pH 4.7) containing 5% *n*-BuOH. Elution with the same buffer gave, after pooling of appropriate fractions and lyophilization, tetrasaccharide derivative **8** (8.2 mg, 8.5 μmol, 57% total yield) as a white fluffy solid. NMR data are given in Table 2. For all assigned sugar atoms, there was excellent agreement with previously published data on sialyl Le x tetrasaccharide β methyl glycoside.[21] The proportion between **8** and its disulfide form in the NMR sample was estimated to be 1:9, from the relative intensities of the respective CH_2S methylene signals. The FAB positive-ion mass spectrum showed a peak at *m*/*z* 966, corresponding to M + H for **8**, and a peak at *m*/*z* 1929, corresponding to M + H of the disulfide isomer of **8** (proportions varied between runs).

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