Chemical-Enzymatic Synthesis and Conformational Analysis of Sialyl Lewis x and Derivatives

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Chemical-Enzymatic Synthesis and Conformational Analysis of Sialyl Lewis x and Derivatives

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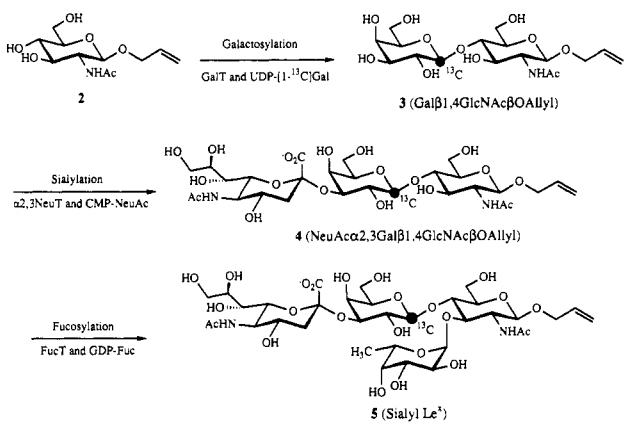
Abstract: Sialyl Lewis x and derivatives have been synthesized using β-1,4-galactosyltransferase and recombinant α-2,3-sialyltransferase and α-1,3-fucosyltransferase. The enzymatic glycosylations have been achieved on preparative scales with in situ regeneration of UDP-galactose, CMP-*N*-acetylneuraminic acid, and GDP-fucose. Additionally, galactosyltransferase and fucosyltransferases have been studied with respect to their substrate specificity and inhibition. The enzymatic procedures have also been used in the synthesis of 2′-deoxy-LacNAc, 2′-amino-2′-deoxy-LacNAc, 2-azido-Lac, Lewis x, the Lewis x analog with GlcNAc replaced with 5-thioglucose, [Gal-1-^{13}C]-LacNAc, [Gal-1-^{13}C]-sialyl Lewis x, and the corresponding terminal glycal. The synthesized ^{13}C-labeled sialyl Lewis x and intermediates (including Lewis x and sialyl LacNAc) were used for conformational study using NMR techniques combined with calculations based on GESA and MM2 programs. GESA calculation of sialyl Lewis x gave four minimum-energy conformers, and the two (A and B) consistent with NMR results were further refined with MM2 calculation. The one (A′) with lower energy was picked as the preferred conformer which had all internuclear distances and glycosidic torsional angles consistent with the NMR analysis. The glycosidic torsional angle ψ of Gal-GlcNAc, for example, was determined to be 18° on the basis of the coupling between Gal-1-^{13}C and GlcNAc, while the predicted value was 15°. The tetrasaccharide appears to form a well-defined hydrophilic surface along NeuAc-Gal-Fuc, and a hydrophobic face underneath NeuAc-Gal-GlcNAc. Comparing the conformation of sialyl Lewis x to sialyl Lewis a indicates that the recognition domain of sialyl Lewis x mainly comes from the sialic acid–galactose–fucose residues.

Introduction

Cell-surface oligosaccharides are highly diversified in their structures[1] and are associated with a variety of cell functions.[2] In an inflammatory response, for example, neutrophils or leukocytes bind to injured tissues where the adhesion process occurs. This process has been found to be mediated by the tetrasaccharide sialyl Lewis x (Le^x) (Figure 1) on neutrophils or leukocytes and the receptor ELAM-1 (endothelial leukocyte adhesion molecule 1), a glycoprotein of the selectin family.[3] High levels of sialyl Le^x-containing mucins are also found in the sera of gastrointestinal, pancreatic, and breast cancer patients.[2e,f] In order to study the function of sialyl Le^x at the molecular level and to evaluate its therapeutic potential, it is necessary to develop an efficient and practical synthetic route to this and other related novel tetrasaccharides. Although several chemical syntheses of sialyl Le^x and derivatives have been reported,[4] they require multiple protection/deprotection steps. Alternatively, enzymatic synthesis of oligosaccharides proceeds regio- and stereoselectively without protection.[5] Three major problems are, however, often encountered in the enzymatic approach. First, glycosyltransferases are not readily available; second, the donor substrates sugar nucleotides are too expensive to be used as stoichiometric reagents for the large-scale synthesis; third, the enzymatic reactions often suffer from severe production inhibition caused by the released nucleoside phosphates. As part of our interests in the development of practical enzymatic or chemoenzymatic procedures for the synthesis of oligosaccharides and analogs, we report here the total synthesis of sialyl Le^x and derivatives with in situ cofactor regeneration (to reduce the cost of sugar nucleotides and to lessen the problem of product inhibition). Our strategy for the synthesis of sialyl Le^x is based on the glycosyltransferases involved in the biosynthesis of this molecule (Scheme I); we use β-1,4-galactosyltransferase (GalT) to form Galβ1,4GlcNAc, α-2,3-sialyltransferase (NeuT) to form NeuAcα2,3Galβ1,4GlcNAc, and α-1,3-fucosyltransferase (FucT) to form NeuAcα2,3Galβ1,4-(Fucα1,3)GlcNAc. We also conducted a study of the substrate specificity and inhibition of the glycosyltransferases, particularly

Scheme I. Strategy for the Enzymatic Stoichiometric Synthesis of Sialyl Le^x and the ^{13}C-Labeled Derivatives[a]



[a] Sugars are introduced according to their biosynthetic sequence.

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(1) (a) Kobata, A. In *Biology of Carbohydrates*; Ginsburg, V., Robins, P. W., Eds.; John Wiley and Sons: New York, 1984; Vol. 2, p 87. (b) Feizi, T. *Nature* **1985**, *314*, 53. (c) Hakomori, S. *Adv. Cancer Res.* **1989**, *52*, 257.
(2) (a) Schauer, R. *Adv. Carbohydr. Chem. Biochem.* **1982**, *40*, 131. (b) Paulson, J. C. In *The Receptors*; Conn, M., Ed.; Academic Press: New York, 1985; Vol. 2, p 131. (c) Loomes, L. M.; Vemura, K.; Childs, R. A.; Paulson, J. C.; Rogers, G. N.; Scudder, P. R.; Michalski, J.; Hounsell, E. F.; Taylor-Robinson, D.; Feizi, T. *Nature* **1984**, *307*, 560. (d) Hakomori, S. *Annu. Rev. Immunol.* **1984**, *2*, 103. (e) Magnani, J. L.; Nilsson, B.; Brockhaus, M.; Zopf, D.; Steplewski, Z.; Koprowski, H.; Ginsburg, V. *Cancer Res.* **1983**, *43*, 5489. (f) Kannagi, R.; Fukushi, Y.; Tachikawa, T.; Noda, A.; Shin, S.; Shigeta, K.; Hiraiwa, N.; Fukuda, Y.; Inamoto, T.; Hakomori, S.; Imura, H. *Cancer Res.* **1986**, *46*, 2619.
(3) (a) Phillips, M. L.; Nudelman, E.; Gaeta, F. C. A.; Perez, M.; Singhal, A. K.; Hakomori, S.; Paulson, J. C. *Science* **1990**, *250*, 1130. (b) Waltz, G.; Arufto, A.; Kalanus, W.; Bevilacqua, M.; Seed, B. *Science* **1990**, *250*, 1132. (c) Lowe, J. B.; Stoolman, L. M.; Nair, R. P.; Larson, R. D.; Berhend, T. L.; Marks, R. M. *Cell* **1990**, *63*, 475.

0002-7863/92/1514-9283$03.00/0

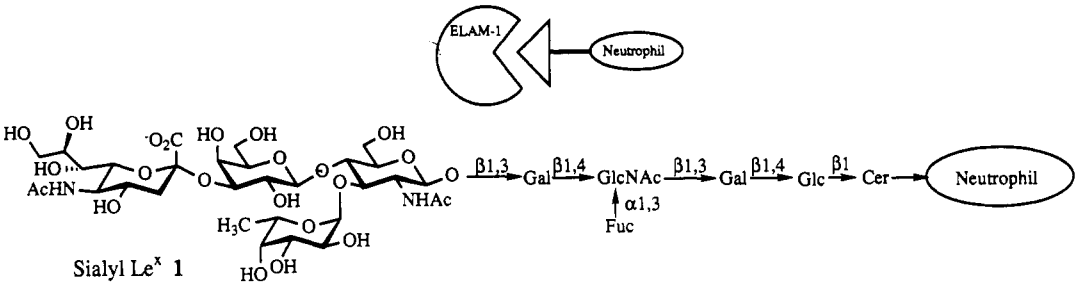


Figure 1. Structure of sialyl Le^x tetrasaccharide-containing glycolipid on neutrophils.

Table I. Disaccharides and Trisaccharides as Substrates or Inhibitors for α-1,3/4-Fucosyltransferase

entry	substrates	V_{rel}[a] (%) or IC_{50} (mM)
1	Galβ1,4GlcNAc	100
2	Galβ1,4Glc	120
3	Galβ1,4(5-thioGlc)[c]	310
4	Galβ1,3GlcNAc	580
5	GlcNAcβ1,4GlcNAc	23
6	Galβ1,4GalNAc	27
7	NeuAcα2,3Galβ1,4GlcNAc[d]	60
8	Fucα1,2Galβ1,4Glc[e]	250
9	GlcNAcβ1,6Galβ1,4Glc[e]	13
10	Galβ1,4(3-deoxy)GlcNAcβOallyl[c]	>125[g]
11	Galβ1,4deoxynojirimycin[c]	40[g]
12	Galβ1,4Glucal[e,f]	>125[g]

[a] Relative velocities with 0.20 mM GDP-Fuc, 20 mM $MnCl_2$, and 10 mM acceptor. Specific activity = 2 U/mg (1 U = 1 μmol of GDP-Fuc consumed per h). [b] Inhibitor concentration required to give 50% inhibition with 0.2 mM GDP-Fuc. [c] Gautheron-Le Narvor, C.; Wong, C.-H. *J. Chem. Soc., Chem. Commun.* **1991**, 1130. Wong, C.-H.; Ichikawa, Y.; Krach, T.; Gautheron-Le Narvor, C.; Dumas, D. P.; Look, G. C. *J. Am. Chem. Soc.* **1991**, *113*, 8137. [d] Purchased from Oxford GlycoSystems, Inc., Rosedale, New York. [e] Purchased from Sigma, St. Louis, MO. [f] Haworth, W. N.; Hirst, E. L.; Plant, M. M. T.; Reinolds, R. J. W. *J. Chem. Soc.* **1930**, 2644. [g] IC_{50} in mM.

the fucosyltransferases and galactosyltransferase, used in the synthesis. Additionally, we report a detailed conformational analysis of sialyl Le^x in aqueous solution[6] which is aimed to guide the development of sialyl Le^x mimetics with desirable biological activities.

Results and Discussion

Substrate Specificity and Inhibition Study of Glycosyltransferases. (A) α1,3/4FucT. The fucosyltransferase which is capable of transferring the Fuc moiety from GDP-Fuc to the 3- and 4-OH groups of GlcNAc to produce Le^x or Le^a is α1,3/4FucT.[7,8] As indicated in Table I, the enzyme catalyzes the fucosylation of Galβ1,3GlcNAc faster (V_{rel} 580%) than Galβ1,4GlcNAc (LacNAc) (V_{rel} 100) (Entries 1 and 4) at 10 mM concentration of acceptor. Sialylated LacNAc (Entry 7) is also a substrate for this enzyme, allowing the synthesis[8] of sialyl Le^x. Interestingly, Galβ1,4(5-thio)Glc[9] is a better substrate than the corresponding disaccharide, lactose (Entries 2 and 3) under these conditions. Galβ1,4deoxynojirimycin[9] (Entry 11), however, is an inhibitor (IC_{50} = 40 mM). Due to the limited supply of α1,3/4Fuc, no further investigation was carried out.

Table II. Disaccharides and Trisaccharides as Substrates for α-1,3-Fucosyltransferase

entry	substrates	K_m (mM)	V_{rel}[a] (%)
1	Galβ1,4GlcNAc	35	100
2	Galβ1,4Glc	500	150
3	Galβ1,4(5-thioGlc)[b]	12	51
4	Galβ1,4GlcNAcβOallyl[c]	16	64
5	Galβ1,3GlcNAc	600	130
6	Galβ1,4Glucal[b,d]	34	10
7	NeuAcα2,3Galβ1,4GlcNAc[e]	100	620
8	NeuAcα2,3Galβ1,4GlcNAcβOallyl[f]	280	380
9	NeuAcα2,3Galβ1,4Glucal[f]	64	330
10	NeuAcα2,6Galβ1,4GlcNAc[g]	70	13

[a] Relative velocities with 0.1 mM GDP-Fuc, 10 mM $MnCl_2$ and 10 mM acceptor. Specific activity = 2.6 U/mg (1 U = 1 μmol of GDP-Fuc consumed per h). [b] Gautheron-Le Narvor, C.; Wong, C.-H. *J. Chem. Soc., Chem. Commun.* **1991**, 1130. [c] Wong, C-H.; Ichikawa, Y.; Krach, T.; Gautheron-Le Narvor, C.; Dumas, D. P.; Look, G. C. *J. Am. Chem. Soc.* **1991**, *113*, 8137. [d] Haworth, W. N.; Hirst, E. L.; Plant, M. M. T.; Reinolds, R. J. W. *J. Chem. Soc.* **1930**, 2644. [e] Purchased from Oxford GlycoSystems, Inc., Rosedale, New York. [f] Prepared enzymatically using α-2,3-sialyltransferase from Cytel Co. in this study. [g] Ichikawa, Y.; Shen, G.-J.; Wong, C.-H. *J. Am. Chem. Soc.* **1991**, *113*, 4698.

(B) α1,3FucT. The enzyme responsible for sialyl Le^x production is α1,3FucT, which has been recently cloned, overexpressed,[10] and utilized in synthesis.[11] The substrate specificity indicated (Table II), as expected, the enzyme is more specific for LacNAc (V/K_m 2.9%/mM, Entry 1) than for Galβ1,3GlcNAc (V/K_m 0.22%/mM, Entry 5). Similar to the result for α1,3/4FucT (Entry 3 in Table I), Galβ1,4(5-thio)Glc is also a substrate for αFucT (Entry 3 in Table II). Unlike the α1,3/4 enzyme, lactal is a substrate for the α1,3 enzyme. The trisaccharide NeuAcα2,3Galβ1,4GlcNAc (Entry 7), a precursor to sialyl Le^x, is the best substrate with a relative maximum velocity of 620% based on LacNAc. The α2,6-linked sialoside (Entry 10) is about 50 times less active as a substrate than the α2,3-isomer. It is worth noting that the enzyme can also transfer Fuc to the glucal-containing sialylated trisaccharide (V_{rel} 330%, Entry 9).[11] With regard to binding, the enzyme has higher affinity for disaccharides (Entries 1, 3, 4, and 6) than for trisaccharides. An increase of affinity was observed when the GlcNAc moiety of LacNAc was replaced with 5-thio-Glc, glucal,[12] or GlcNAcβOallyl. Lactose has, however, a very

(4) For chemical synthesis of sialyl Le^x, see: (a) Kameyama, A.; Ishida, H.; Kiso, M.; Hasegawa, A. *Carbohydr. Res.* **1991**, *209*, C1. (b) Nicolaou, K. C.; Hummel, C. W.; Bockovich, N. J.; Wong, C.-H. *J. Chem. Soc., Chem. Commun.* **1991**, 870. (c) Nicolaou, K. C.; Hummel, C. W.; Iwabuchi, Y. *J. Am. Chem. Soc.* **1992**, *114*, 3126. For enzymatic synthesis of sialyl Le^x, see: (d) Palcic, M. M.; Venot, A.; Ratcliffe, R. M.; Hindsgaul, O. *Carbohydr. Res.* **1989**, *190*, 1. (e) Dumas, D. P.; Ichikawa, Y.; Wong, C.-H.; Lowe, J. B.; Nair, R. P. *Bioorg. Med. Chem. Lett.* **1991**, *1*, 425.

(5) (a) Sabesan, S.; Paulson, J. C. *J. Am. Chem. Soc.* **1986**, *108*, 2068. (b) Toone, E. J.; Simmon, E. S.; Bednarski, M. D.; Whitesides, G. M. *Tetrahedron* **1989**, *45*, 5365. (c) David, S.; Auge, C.; Gautheron, C. *Adv. Carbohydr. Chem. Biochem.* **1991**, *49*, 175. (d) Ichikawa, Y.; Look, G. C.; Wong, C.-H. *Anal. Biochem.* **1992**, *202*, 215.

(6) For a preliminary result of the conformational study of sialyl Le^x, see: Lin, Y. C.; Hummel, C. W.; Huang, D.-H.; Ichikawa, Y.; Nicolaou, K. C.; Wong, C.-H. *J. Am. Chem. Soc.* **1992**, *114*, 5452.

(7) For cloning, expression, and preparation of the enzyme, see: Fukowska-Latallo, J. F.; Larson, R. D.; Nair, R. P.; Lowe, J. B. *Gene Dev.* **1990**, *4*, 1288.

(8) For preliminary work on α1,3/4FucT, see: Dumas, D. P.; Ichikawa, Y.; Wong, C.-H.; Lowe, J. B.; Nair, R. P. *Bioorg. Med. Chem. Lett.* **1991**, *1*, 425.

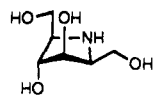
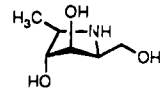
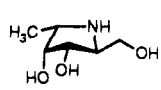
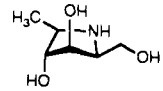
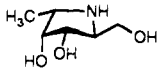
(9) (a) Gautheron-Le Narvor, C.; Wong, C.-H. *J. Chem. Soc., Chem. Commun.* **1991**, 1130. (b) Wong, C.-H.; Ichikawa, Y.; Krach, T.; Gautheron-Le Narvor, C.; Dumas, D. P.; Look, G. C. *J. Am. Chem. Soc.* **1991**, *113*, 8137–8145.

(10) Weston, B. W.; Nair, R. P.; Larsen, R. D.; Lowe, J. B. *J. Biol. Chem.* **1992**, *267*, 4152.

(11) For a preliminary result on fucosylation, see: Wong, C.-H.; Dumas, D. P.; Ichikawa, Y.; Weston, B. W.; Lowe, J. B. *J. Am. Chem. Soc.* **1992**, *114*, 7321.

(12) Haworth, W. N.; Hirst, E. L.; Plant, M. M. T.; Reinolds, R. J. W. *J. Chem. Soc.* **1930**, 2644.

Table III. Inhibition of α1,3FucT

entry	inhibitors	IC_{50}[a] (mM)
1	Galβ1,4Glucal[b]	NI[c]
2	Galβ1,3GlcNAc	NI
3	Galβ1,3GalNAc	>100
4	Galβ1,4deoxynojirimycin[b]	8
5	Galβ1,4(3-deoxy)GlcNAcβOallyl[b]	710
6	GlcNAcβ1,4GlcNAc	NI
7	GDP-Man	2
8	 [d]	52
9	 [d]	34[e]
10	 [f]	80
11	GDP	0.05[g]
12	GDP (0.05 mM) +  (34 mM)	synergistic inhibition[h]
13	GDP (0.05 mM) +  (80 mM)	synergistic inhibition[i]

[a] Inhibitor concentration required to give 50% inhibition with 0.1 mM GDP-Fuc, 10 mM Mn^{2+}, and 10 mM LacNAc at pH 6.2 and 37 °C. [b] Gautheron-Le Narvor, C.; Wong, C-H. *J. Chem. Soc., Chem. Commun.* **1991**, 1130. [c] No inhibition observed up to 50 mM of inhibitor concentration. [d] Kajimoto, T.; Chen, L.; Liu, K. K.-C.; Wong, C.-H. *J. Am. Chem. Soc.* **1991**, *113*, 6679. [e] K_i = 19 ± 3 mM. [f] See ref 11. [g] K_{ii} = 0.13 ± 0.05 mM, K_{is} = 0.16 ± 0.06 mM. [h] ~90% of the enzyme activity is inhibited. [i] 80% of the enzyme activity is inhibited.

low affinity although the relative rate at V_{max} is quite high (150%).

In our study of the inhibition of α1,3FucT (Table III), the observation that 3'-deoxy-LacNAc[9] is a weak inhibitor (Entry 5) is consistent with the previous report on deoxygenated oligosaccharides for glycosyltransferases.[13] Among the acceptor substrate analogs examined, Galβ1,4deoxynojirimycin is the most potent inhibitor (Entry 4, IC_{50} = 8 mM).

Three aza sugars[14,15] known to be potent α-fucosidase inhibitors were tested as the acceptor analogs (Entries 8–10), and they were found to be moderate inhibitors versus LacNAc for FucT (IC_{50} = 34–80 mM). Deoxynojirimycin was, however, a substrate for β1,4GalT.[9b] The two five-membered aza sugars (Entries 12 and 13) showed a strong synergistic inhibition in the presence of GDP; at the IC_{50} of the individual inhibitors, more than 80% of the enzyme activity was inhibited, suggesting a possible interaction between GDP and the aza sugar in the active site to mimic the transition-state structure of the fucosyl-transfer reaction.[11] GDP-Man is also a potent inhibitor of α1,3FucT (IC_{50} = 2 mM).

For the product inhibition study, we focused our attention on the released nucleoside diphosphate. GDP is a byproduct of enzymatic fucosylation and is a very potent noncompetitive inhibitor versus LacNAc (K_{ii} = 0.13 mM, K_{is} = 0.16 mM, Entry 11) (Figure 2). Another nucleoside diphosphate, UDP, released from the enzymatic galactosylation is also a very potent inhibitor of GalT (K_i = 0.46 mM) (Figure 3). GDP-Fuc is a potent inhibitor of α1,3FucT at concentrations above 0.2 mM in the presence of 10 mM LacNAc (Figure 4). It is, however, not an inhibitor of α1,3/4FucT (Figure 5).

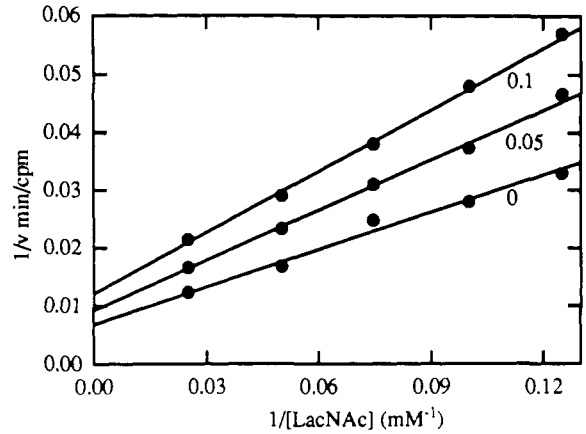


Figure 2. Inhibition kinetics of α1,3FucT with GDP versus LacNAc in the presence of 0.1 mM ^{14}C-GDP-Fuc at pH 6.2. Concentrations of GDP in mM are indicated. K_{ii} = 0.13 ± 0.05 mM; K_{is} = 0.16 ± 0.06 mM.

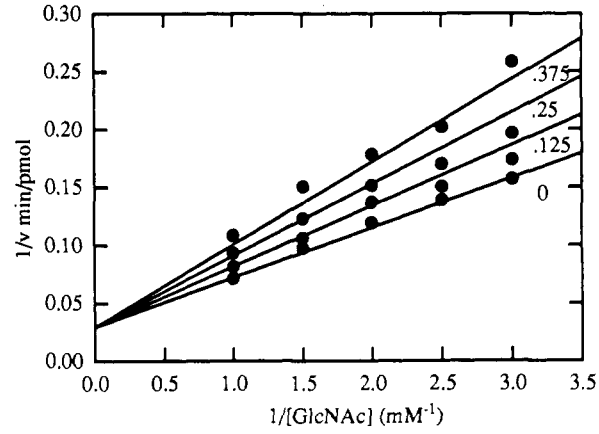


Figure 3. Inhibition kinetics of β1,4GalT with UDP versus GlcNAc in the presence of 0.1 mM ^{14}C-UDP-Gal at pH 7.5. Concentrations of UDP in mM are indicated. K_m for GlcNAc = 1.5 ± 0.3 mM; K_i for UDP = 0.57 ± 0.07 mM.

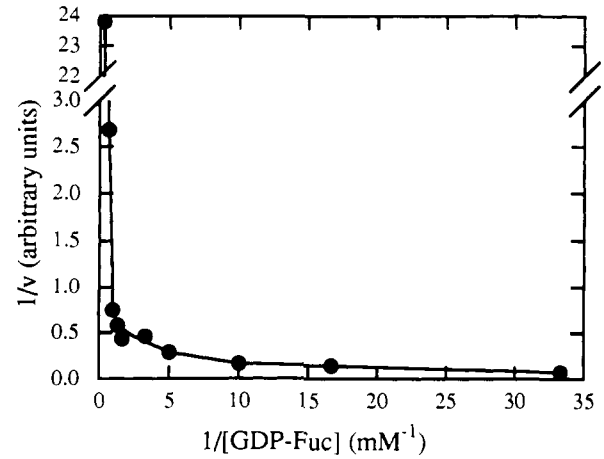


Figure 4. Effect of GDP-Fuc concentration on α1,3FucT in the presence of 20 mM LacNAc and 20 mM $MnCl_2$ at pH 6.2.

Synthesis of Sialyl Le^x and Derivatives. Since nucleoside diphosphates inhibit GalT and FucT reactions at very low concentrations, the problem of such a product inhibition prohibits the use of glycosyltransferases in large-scale synthesis.

One solution to this problem is to use calf alkaline phosphatase to decompose the released nucleoside di-(UDP) and mono-

(13) Hindsgaul, O.; Kaur, K. J.; Srivastava, G.; Blaszczyk-Thurin, M.; Crawley, S. C.; Heerze, L. D.; Palcic, M. M. *J. Biol. Chem.* **1991**, *266*, 17858.
(14) Kajimoto, T.; Chen, L.; Liu, K. K.-C.; Wong, C.-H. *J. Am. Chem. Soc.* **1991**, *113*, 6679.
(15) Wang, Y.-F.; Dumas, D. P.; Wong, C.-H. *J. Am. Chem. Soc.* Submitted.
(16) Dumas, D. P.; Kajimoto, T.; Liu, K. K.-C.; Berkowitz, D. B.; Danishefsky, S. J. *Bioorg. Med. Chem. Lett.* **1992**, *2*, 33.

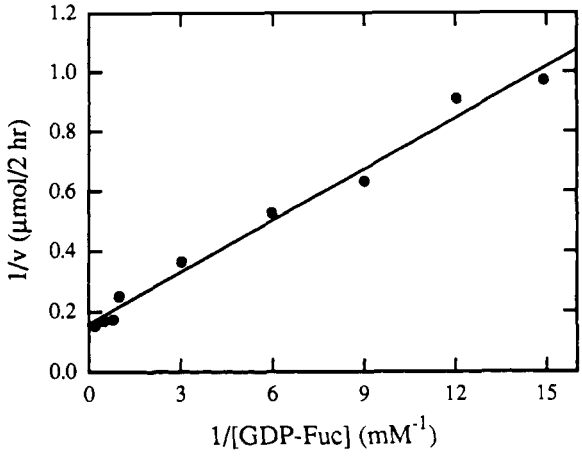


Figure 5. Effect of GDP-Fuc concentration on α1,3/4FucT. Conditions are the same as those for Figure 4. K_m = 0.27 ± 0.02 mM.

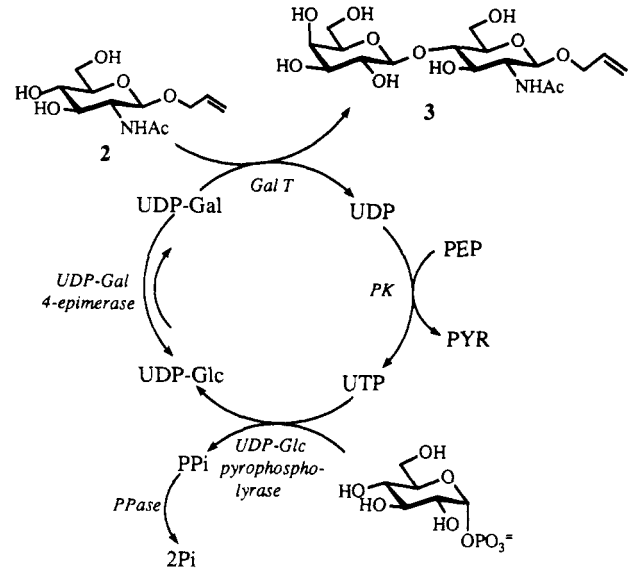
phosphate (CMP) to uridine and cytidine, respectively, which are no longer inhibitors.[17] However, to ensure a complete reaction, the sugar nucleotide must be used in excess. Furthermore, the donor substrate (sugar nucleotide) is quite expensive and must be prepared separately. To solve these problems, a multiple-enzyme system with in situ cofactor regeneration[18-20] was used for the synthesis of LacNAc and sialyl LacNAc. In the cofactor regeneration system, the concentration of nucleoside phosphate is kept sufficiently low to avoid inhibition and the sugar nucleotide is continuously regenerated by sequential enzyme reactions.

On the basis of the synthetic strategy shown in Scheme I, both unlabeled and ^{13}C-labeled sialyl Le^x were synthesized in the following order: (1) Galactosylation coupled with UDP-Gal regeneration to give LacNAc (**3**), (2) sialylation coupled with CMP-NeuAc regeneration to give sialyl LacNAc (**4**), and (3) fucosylation coupled with GDP-Fuc regeneration to give sialyl Le^x (**5**).

Galactosylation. Two multienzyme systems for the synthesis of LacNAc have been developed with in situ cofactor regeneration. One starts with Glc-1-P and uses UDP-Glc pyrophosphorylase (EC 2.7.7.9, UDPGP) and UDP-Gal 4-epimerase (EC 5.1.3.2, UDPGE) (Scheme II).[18] UDP-galactose is generated from UDP-Glc with UDPGE; however, this equilibrium favors the formation of UDP-Glc, and Glc-1-P has to be prepared separately. The other procedure uses Gal instead of Glc-1-P, as a donor precursor, and UDPGP, galactokinase (EC 2.7.1.6, GK), and Gal-1-P uridyltransferase (EC 2.7.7.12, Gal-1-P UT) (Scheme III).[19] GK is specific for galactose, allowing the direct production of Gal-1-P, which is converted to UDP-Gal with Gal-1-P UT and UDP-Glc. The latter system was proven to be suitable for the preparation of [Gal-1-^{13}C]LacNAc.

The multienzyme system (Scheme III) started with 1-^{13}C-Gal,[21] GlcNAcβOallyl,[22] phosphoenolpyruvate (PEP), and catalytic amounts of Glc-1-P, ATP, and UDP. UDP was converted into UTP with pyruvate kinase (EC 2.7.1.40, PK) and PEP, and UTP reacted with Glc-1-P catalyzed by UDPGP to produce UDP-Glc. The byproduct inorganic pyrophosphate (PPi) was decomposed by inorganic pyrophosphatase (EC 3.6.1.1, PPase). With Gal-1-P UT, UDP-Glc reacted with ^{13}C-Gal-1-P, generated from ^{13}C-Gal

Scheme II. Synthesis of LacNAc with in Situ Cofactor Regeneration Employing UDP-Glc Pyrophosphorylase and UDP-Gal 4-Epimerase



and ATP in the presence of GK, to give UDP-^{13}C-Gal and Glc-1-P. The ^{13}C-Gal of UDP-^{13}C-Gal was transferred to the acceptor (GlcNAcβOallyl) by GalT to give [Gal-1-^{13}C]-containing LacNAc. The produced UDP was again converted to UTP with PK and PEP, which reacted with the released Glc-1-P to regenerate UDP-Glc. Using this multienzyme system, [Gal-1-^{13}C]LacNAc was obtained in 54% yield. The same procedure was also used in the preparation of unlabeled LacNAc and analogs **3a–3c**. As indicated in Scheme III, this system also allows the regeneration of UDP-2-deoxy-D-galactose and UDP-galactosamine.

Sialylation. The multienzyme system for sialylation was similar to that reported previously[20] except that α-2,3-sialyltransferase was used in the sialylation. It starts with NeuAc, [Gal-1-^{13}C]-LacNAc, PEP, and catalytic amounts of ATP and CMP (Scheme IV). CMP was converted to CDP by nucleoside monophosphate kinase (EC 2.7.4.4, NMK) in the presence of ATP, which was regenerated from its byproduct ADP catalyzed by PK in the presence of PEP, then to CTP with PEP by PK. The CTP formed then reacted with NeuAc catalyzed by CMP-NeuAc synthetase (EC 2.7.7.43) to produce CMP-NeuAc. The byproduct PPi was hydrolyzed to Pi by PPase. Sialylation of LacNAc was accomplished with CMP-NeuAc and α-2,3-sialyltransferase (EC 2.4.99.6). The released CMP was again converted to CDP, to CTP, and finally to CMP-NeuAc. Using this system, [Gal-1-^{13}C]NeuAcα2,3Galβ1,4GlcNAcβOallyl (**4**) as well as the unlabeled trisaccharide was prepared. Interestingly, lactal (Galβ1,4Glucal) was also a good substrate for α2,3NeuT, allowing NeuAcα2,3Galβ1,4Glucal (**6**) to be synthesized in 21% yield. Lactal was prepared either chemically[12] or enzymatically using GalT and glucal.[9] The glycal-containing oligosaccharides such as **6** may be converted to other sialyl Le^x derivatives.[23]

Fucosylation. The cloned human enzyme was used for stoichiometric fucosylation with GDP-Fuc[21] (Scheme V). Fucosylation of sialyl LacNAc (**4**) gave sialyl Le^x (**5**) after silica gel and BioGel P-2 column chromatographies. LacNAc (**3**) and the sialyl glycal **6** were also fucosylated to give Le^x trisaccharide (**7**) and sialyl Le^x glycal (**8**), respectively. These labeled and unlabeled fucosylated oligosaccharides were used for the conformational study employing NMR techniques (see below). One interesting

(17) Unverzagt, C.; Kunz, H.; Paulson, J. C. *J. Am. Chem. Soc.* **1990**, *112*, 9308.

(18) Wong, C.-H.; Haynie, S. L.; Whitesides, G. M. *J. Org. Chem.* **1982**, *47*, 5416. For other GalT-catalyzed syntheses with *in situ* cofactor regeneration, see: (a) Auge, C.; Mathieu, C.; Merienne, C. *Carbohydr. Res.* **1986**, *151*, 147. (b) Thiem, J.; Wiemann, T. *Angew. Chem., Int. Ed. Engl.* **1991**, *30*, 1163. (c) Thiem, J.; Wiemann, T. *Synthesis* **1992**, 141.

(19) Wong, C.-H.; Wang, R.; Ichikawa, Y. *J. Org. Chem.* **1992**, *57*, 4343.

(20) (a) Ichikawa, Y.; Shen, G.-J.; Wong, C.-H. *J. Am. Chem. Soc.* **1991**, *113*, 4698. (b) Ichikawa, Y.; Liu, J. L.-C.; Shen, G.-J.; Wong, C.-H. *J. Am. Chem. Soc.* **1991**, *113*, 6300.

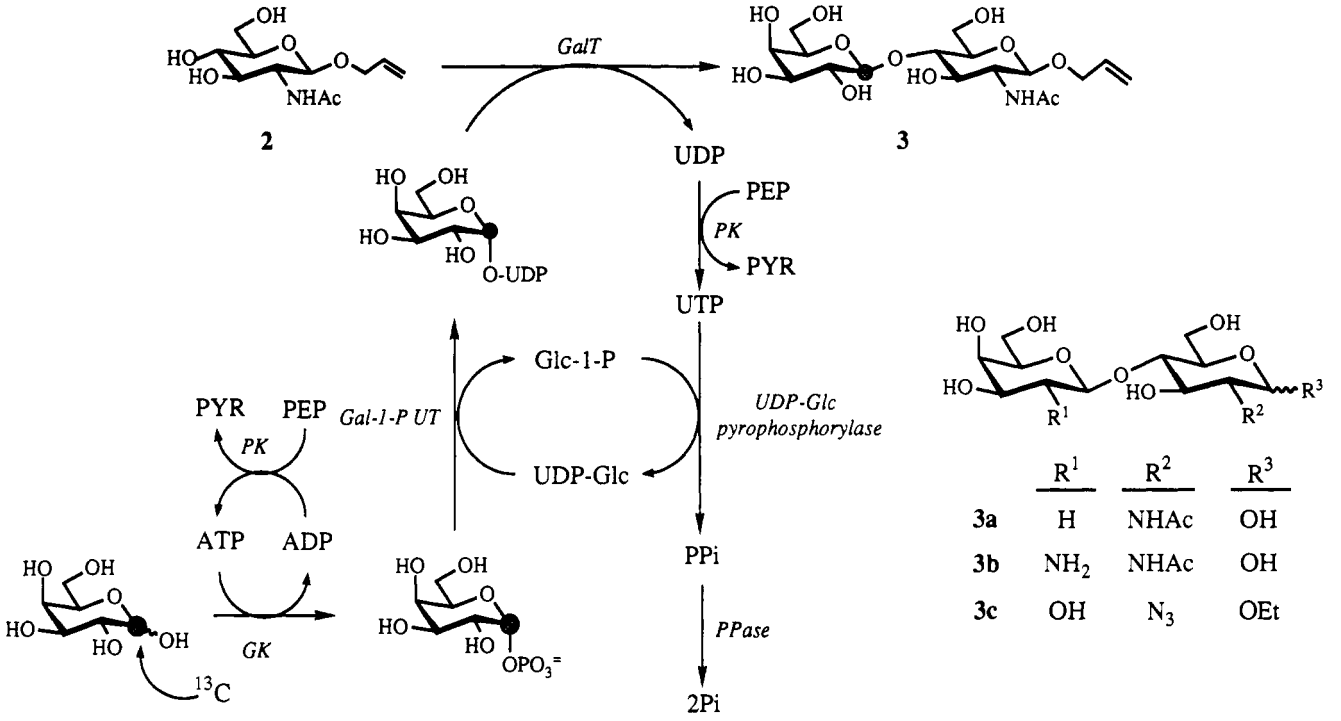
(21) 99 Atom %, purchased from Isotec Inc., Miamisburg, OH.

(22) Lee, R. T.; Lee, Y. C. *Carbohydr. Res.* **1974**, *37*, 193.

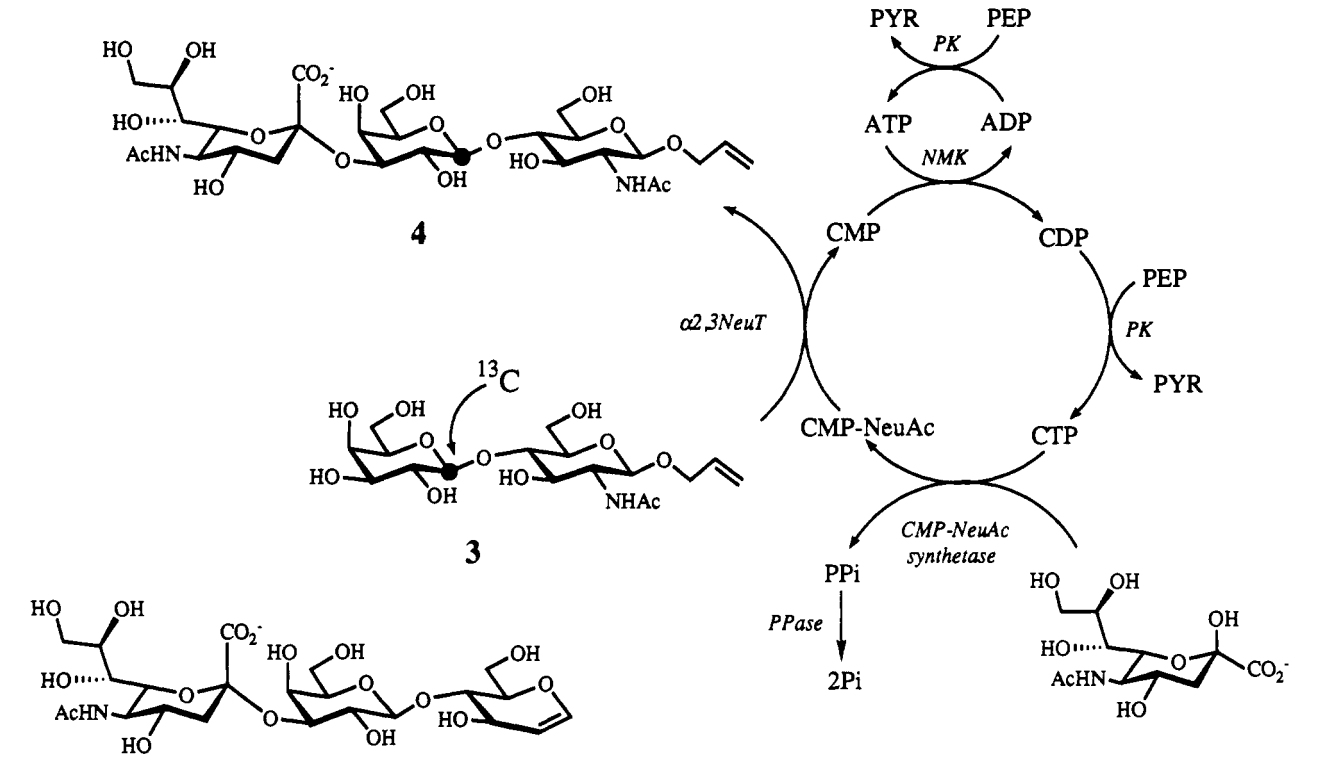
(23) (a) Griffith, D. A.; Danishefsky, S. J. *J. Am. Chem. Soc.* **1990**, *112*, 5811. (b) Halcomb, R. L.; Danishefsky, S. J. *J. Am. Chem. Soc.* **1989**, *111*, 6661. (c) Kessler, H.; Kling, A.; Kottenhahm, M. *Angew. Chem., Int. Ed. Engl.* **1990**, *29*, 425. (d) Thiem, J.; Schwentner, H. K.; Schwentner, J. *Synthesis* **1978**, 696. For chemical syntheses of **6** and **8**, see: Danishefsky, S. J.; Gervay, J.; Peterson, J. M.; McDonald, F. E.; Koeski, K.; Oriyama, T.; Griffith, D. A.; Wong, C.-H.; Dumas, D. P. *J. Am. Chem. Soc.* Submitted.

(24) Ichikawa, Y.; Sim, M. M.; Wong, C.-H. *J. Org. Chem.* **1992**, *57*, 2943.

Scheme III. Synthesis of LacNAc and Analogs with in Situ Cofactor Regeneration Employing Galactokinase (GK) and Gal-1-P Uridyltransferase (Gal-1-P UT)



Scheme IV. Synthesis of NeuAcα2,3LacNAc and the Terminal Glycal with in Situ Cofactor Regeneration



6 (NeuAcα2,3Galβ1,4Glucal)

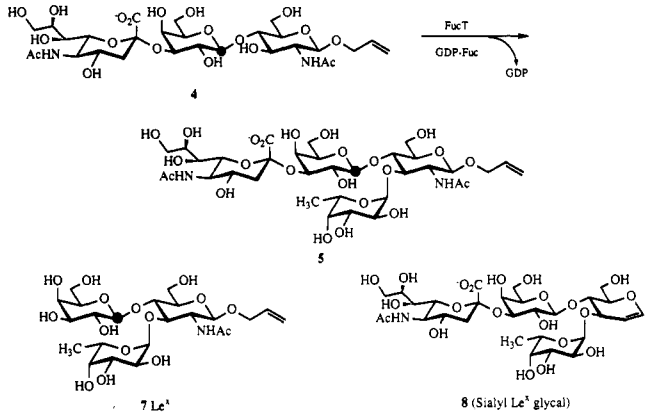
observation is that α1,3-FucT and α1,3/4FucT accept Galβ1,4-(5-thioGlc) to give a (5-thioGlc)-Le^x analog. Galβ1,4deoxynojirimycin is, however, an inhibitor of both enzymes (Scheme VI).

As for the in situ regeneration of GDP-Fuc, we first examined the conversion of Man-1-P to GDP-Fuc via GDP-Man based on the biosynthetic pathway of GDP-Fuc in microorganisms (A in Scheme VII). We used microbial enzymes because of the ease of access. Furthermore, this system allows regeneration of GDP-Man. GDP-Man pyrophosphorylase (GDP-Man PP) has been found in yeast,[25] and GDP-Fuc-generating enzymes are known to exist in the bacterium[26] *Klebsiella pneumonia.* In this

(25) (a) Munch-Peterson, A. *Methods Enzymol.* **1962**, *5*, 171. (b) For synthesis of GDP-Man, see: Simon, E. S.; Grabowski, S.; Whitesides, G. M. *J. Org. Chem.* **1990**, *55*, 1834.

(26) (a) Ginsburg, V. *J. Biol. Chem.* **1960**, *235*, 2196. (b) Ginsburg, V. *Methods Enzymol.* **1966**, *8*, 293.

Scheme V. Fucosylation with FucT and GDP-Fuc: Synthesis of Le^x, Sialyl Le^x, the ^{13}C-Labeled Oligosaccharide, and Sialyl Le^x Glycal



regeneration, GTP was generated from GDP in the presence of PEP and PK. Man-1-P reacted with GTP to give GDP-Man by GDP-Man PP from dried yeast cells. GDP-Man was transformed to GDP-Fuc in the presence of NADPH and GDP-Fuc-generating enzymes partially purified from the bacterium. The oxidized NADP was converted back to NADPH by *Thermoanaerobium brockii* alcohol dehydrogenase (TADH) (EC 1.1.1.2) and 2-propanol. The productions of GDP-Man and GDP-Fuc were confirmed by HPLC, and fucosylation of LacNAc and **4** to give **7** and **5** in 5–10 mg was accomplished. A preparative synthesis of sialyl Le^x with in situ regeneration of GDP-Fuc using purified enzymes is in progress.

An alternative method was to start with Fuc-1-P, which was converted to GDP-Fuc catalyzed by GDP-Fuc pyrophosphorylase (GDP-Fuc P, Scheme VIIB).[27] We have partially purified GDP-Fuc P from porcine thyroids[27] and have demonstrated that the regeneration system depicted in Scheme VIIB is functional on an analytical scale for the synthesis of sialyl Le^x.

Conformational Analysis of Sialyl Le^x and Derivatives. Conformational study of carbohydrates is of general interest as the molecular shape of carbohydrates is essential for recognition and function.[28] Our interest in studying the conformation of sialyl Le^x is to gain some information regarding the binding of this ligand to the receptor and to provide information for the design of sialyl Le^x analogs or mimetics as new antiinflammatory agents.

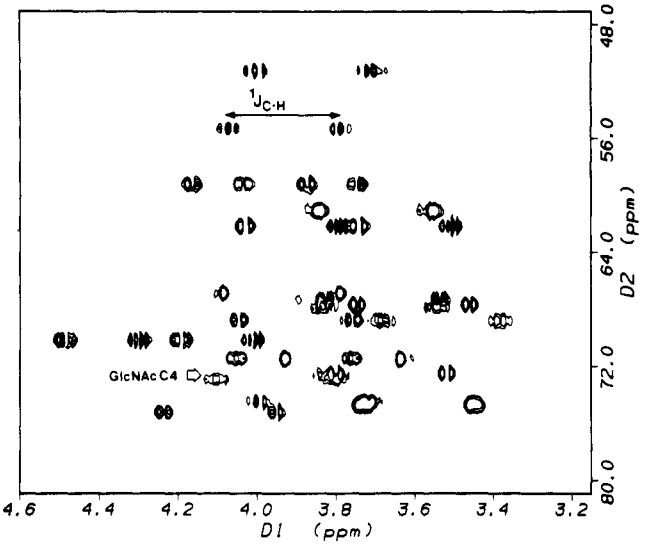
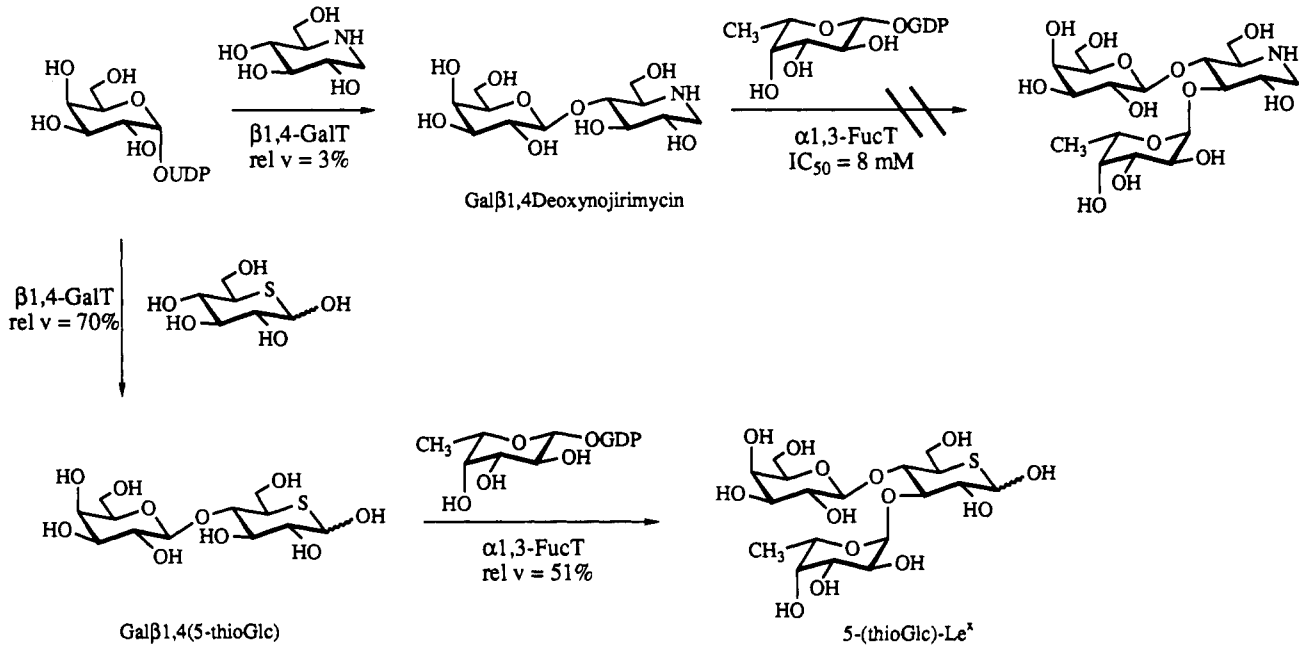


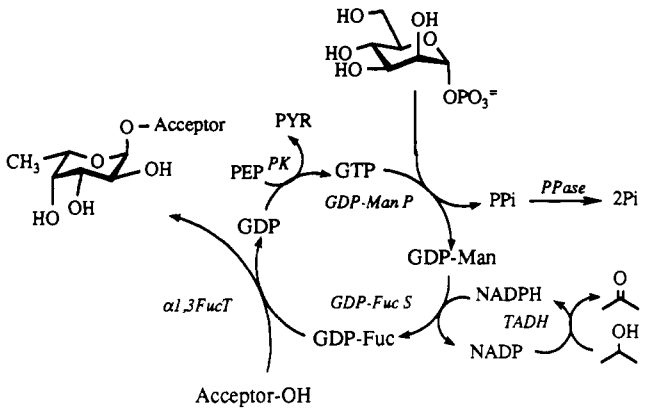
Figure 6. ^{13}C–^1H correlation of Gal-1-^{13}C-labeled sialyl Lewis x obtained from the HMQC experiment with no ^{13}C decoupling during acquisition.

A number of NMR techniques have been developed in recent years for the determination of primary and secondary structures of oligosaccharides.[29] Since there are extensively overlapped resonances for the skeleton protons of oligosaccharides, it is generally necessary to apply several NMR techniques in tandem to sort out the assignments thoroughly. The experimental methods generally used for the analysis of oligosaccharides include phase-sensitive DQF-COSY (double quantum filtered correlational spectroscopy), TOCSY (total correlational spectroscopy), HMQC (heteronuclear multiple quantum correlation), NOE (nuclear Overhauser enhancement), and ROESY (rotating frame NOE spectroscopy). A complete assignment of the NMR spectrum is generally required for detailed structural analysis. NMR techniques and hard-sphere energy calculations have been used in the study of human ABH and Lewis group determinants.[30] Previous NMR and conformational studies of a variety of oligosaccharides containing sialic acid residues indicate that the aglycon attached to the sialic acid preferred to adopt an antiperiplanar-like orientation to the anomeric carboxylate group, thus enabling an inter-residual carbohydrate–carbohydrate interaction to stabilize the conformation.[31] Two recent review articles focus attention

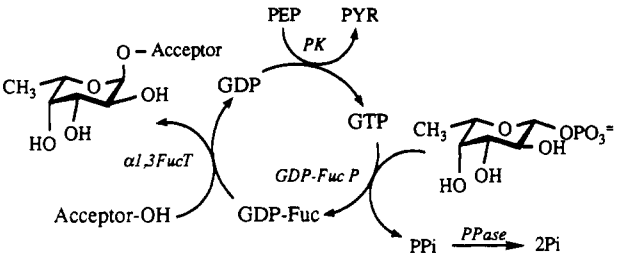
Scheme VI. Glycosyltransferase-Catalyzed Incorporation of Thiosugar and Azasugar into Oligosaccharides



Scheme VII. Fucosylation with in Situ Cofactor Regeneration



A. Regeneration of GDP-Fuc from Man-1-P *via* GDP-Man: GDP-Man P, GDP-Man pyrophosphorylase; GDP-Fuc S, GDP-Fuc synthesizing enzymes.



B. Regeneration of GDP-Fuc from Fuc-1-P: GDP-Fuc P, GDP-Fuc pyrophosphorylase.

on the conformational study of oligosaccharides by NMR spectroscopy and theoretical calculations.[32]

For calculation of conformations of hydrocarbons or moderately polar molecules, the most widely used calculation is the MM2 program.[33] In this calculation, the full optimization of the geometry with consideration of inter-residual H-bonding interactions was used, leading to a demand for relatively long computing time for large molecules. The disadvantage of this program for the analysis of oligosaccharide conformation is that the anomeric center has not until recently been properly defined. A simplified force field HSEA (hard-sphere exoanomeric) effect developed by Lemieux and his co-workers[34] had been used successfully to establish the secondary structures of oligosaccharides on the basis of the nonbonded interactions between the monosaccharide constituents of a disaccharide. To account for the exoanomeric effect, an additional term that rules the rotameric distribution at the O1–C1 bond of a glycosidic linkage is included in the force field calculation. The GESA (geometry of saccharide) program[35] (an extension of HSEA) uses the same potential energy function but allows the simultaneous relaxation of all relevant parameters at the same time. The method of choice for the evaluation of conformational energies depends to some extent upon the size of the structure (number of degrees of freedom) to be investigated. The disadvantage of the program is that inter-residual H-bonding interactions are not considered. In this study, both GESA and MM2 calculations were used to investigate the conformation of sialyl Le^x and its two trisaccharide intermediates. It is worth noting that the possibility of conformational averaging about a given glycosidic linkage can be assessed by computing a potential surface with respect to the glycosidic torsional angles ϕ and ψ. In the absence of conformational averaging, the conformation corresponding to the computed energy minimum should agree closely with the NMR-derived conformation. In the presence of conformational averaging, however, the precise conformational dynamics and equilibrium positions are difficult to verify experimentally.

Assignment of Proton Resonances. For comparison, NMR spectra of three oligosaccharides, namely, Le^x, sialyl-2,3-LacNAc, and sialyl Le^x were examined. A complete assignment of sialyl-2,3-LacNAc had been reported.[36] Although the proton spectra of all these oligosaccharides were very complex in the region of 3.5–4.0 ppm, the resolution obtained with new NMR techniques at 500 MHz was sufficient to make a complete assignment. Although the following discussion deals with sialyl Le^x in particular, similar data were obtained and similar conclusions could be drawn for all the oligosaccharides.

In the 1D NMR spectrum of sialyl Le^x, on the basis of the intensity and coupling constant, the two doublets at δ 4.51 (J_{H1-H2} = 8.35 Hz) and 4.52 (J_{H1-H2} = 8.06 Hz) were assigned to the H1 atoms of GlcNAc and Gal (the H1 of Gal has a larger coupling constant and higher intensity), respectively, leaving the well-resolved doublet at δ 5.09 (J_{H1-H2} = 3.98 Hz) to be assigned to the H1 atom of the Fuc residue. The J_{H1-H2} value of the fucose indicates an α linkage. Since the signals for all these anomeric hydrogens, the C6 methyl hydrogens of fucose, and the C3 methylene hydrogens of NeuAc residues were well resolved, total correlation spectroscopy (TOCSY) as well as DQF-COSY was used to relay spin–spin information from these hydrogens within a pyranose ring to establish their chemical shifts. In TOCSY, the spin–spin information could be relayed in glucose from H1 to H6, whereas, in galactose and fucose, it extended only to H4 from the anomeric hydrogen. Furthermore, since these hydrogens are located around the sialoside linkage, some of these ^{1}H signals

(27) Prohaska, R.; Schenkel-Brunner, H. *Anal. Biochem.* **1975**, *69*, 536. For porcine liver, see: Ishihara, H.; Heath, E. C. *J. Biol. Chem.* **1968**, *243*, 1110.

(28) Berg, E. L.; Robinson, M. K.; Mansson, O.; Butcher, E. C.; Magnani, J. L. *J. Biol. Chem.* **1991**, *266*, 14869.

(29) (a) Lemieux, R. U.; Bock, K.; Delbaere, L. T. J.; Koto, S.; Rao, V. S. *Can. J. Chem.* **1980**, *58*, 631. (b) Bock, K.; Josephson, S.; Bundle, D. R. *J. Chem. Soc., Perkin Trans. II* **1982**, 59. (c) Homans, S. W.; Mehlert, A.; Turco, S. J. *Biochemistry* **1992**, *31*, 654. (d) Kessler, H.; Matter, H.; Genmecker, G.; Kling, A.; Kottenhahm, M. *J. Am. Chem. Soc.* **1991**, *113*, 7550. (e) Pease, J. H. B.; Wemmer, D. E. *Biochemistry* **1988**, *27*, 8491. (f) Sabesan, S.; Bock, K.; Paulson, J. C. *Carbohydr. Res.* **1991**, *218*, 27. (g) Choe, B.-Y.; Ekborg, G. C.; Roden, L.; Harvey, S. C.; Krishna, N. R. *J. Am. Chem. Soc.* **1991**, *113*, 3743. (h) Skelton, M. A.; Cherniak, R.; Poppe, L.; van Halbeek, H. *Magn. Reson. Chem.* **1991**, *29*, 786. (i) Poppe, L.; Stuike-Prill, R.; Meyer, B.; van Halbeek, H. *J. Biomol. NMR* **1992**, *2*, 109. (j) Neuhaus, D.; Williamson, M. P. *The Nuclear Overhauser Effect in Structural and Conformational Analysis*; VCH Publisher: New York, 1989. (k) Alvarado, E.; Nukada, T.; Ogawa, T.; Ballou, C. E. *Biochemistry* **1991**, *30*, 881. (l) Christofides, J. C.; Davies, D. B. *J. Chem. Soc., Chem. Commun.* **1982**, 560. (m) St-Jacques, M.; Sundararajan, P. R.; Taylor, K. J.; Marchessault, R. H. *J. Am. Chem. Soc.* **1976**, *98*, 4386. (n) Vliegenthart, J. F. G.; Lambertus, D.; van Halbeek, H. *Adv. Carbohydr. Chem. Biochem.* **1983**, *41*, 209. (o) Rivera-Sagredo, A.; Solis, D.; Diaz-Maurino, T.; Jimenez-Barbero, J.; Martin-Lomas, M. *Eur. J. Biochem.* **1991**, *197*, 217. (p) Homans, S. W.; Forster, M. *Glycobiology* **1992**, *2*, 143. (q) Homans, S. W. *Glycobiology* **1992**, *2*, 153.

(30) Lemieux, R. U. *Chem. Soc. Rev.* **1989**, *18*, 347 and references cited therein.

(31) Sabesan, S.; Paulson, J. C. *J. Am. Chem. Soc.* **1986**, *108*, 2068.

(32) (a) Homans, S. W. *Prog. Nucl. Magn. Reson. Spectrosc.* **1990**, *22*, 55. (b) Meyer, B. *Top. Curr. Chem.* **1990**, *154*, 141.

(33) (a) Allinger, N. L. *J. Am. Chem. Soc.* **1977**, *99*, 8127. (b) Novskov, L.; Allinger, N. L. *J. Comput. Chem.* **1984**, *5*, 326. (c) Weiner, S. J.; Kollman, P. A.; Nguyen, D. T. *J. Comput. Chem.* **1986**, *7*, 230.

(34) (a) Kitagorodsky, A. I. *Tetrahedron* **1961**, *14*, 230. (b) Thogersen, H.; Lemieux, R. U.; Bock, K.; Meyer, B. *Can. J. Chem.* **1982**, *60*, 44. (c) Bock, K. *Pure Appl. Chem.* **1983**, *55*, 605.

(35) (a) Paulsen, H.; Peters, T.; Sinnwell, V.; Lebuhn, R.; Meyer, B. *Liebigs Ann. Chem.* **1984**, 489; **1985**, 489. (b) Stuike-Prill, R.; Meyer, B. *Eur. J. Biomol.* **1990**, *194*, 903. (c) Levy, S.; York, W. S.; Stuike-Prill, R.; Meyer, R.; Staehelin, L. A. *Plant J.* **1991**, *1*, 195.

(36) (a) Breg, J.; Kroon-Batenburg, L. M. J.; Strecker, G.; Montreuil, J.; Vliegenthart, J. F. G. *Eur. J. Biochem.* **1989**, *178*, 727. (b) Poppe, L.; Dabrowski, J.; von der Lieth, C. W.; Numata, M.; Ogawa, T. *Eur. J. Biochem.* **1989**, *180*, 337.

Table IV. ^1H and ^{13}C Chemical Shift Assignments of β-O-Allyl Sialyl Lewis x and Its Trisaccharide Intermediates

	NeuAc		Gal		GlcNAc		Fuc		aglycon (allyl)	
	H	C	H	C	H	C	H	C	H	C
					Sialyl Le^x					
1		173.5	4.52	101.2	4.51	100.6	5.09	98.3	4.17, 4.33	70.5
2		99.3	3.52	68.9	3.89	55.5	3.68	67.3	5.90	133.3
3	1.79	39.4	4.08	75.3	3.83	74.6	3.90	68.8	5.26, 5.31	118.2
	2.76									
4	3.69	68.0	3.93	66.9	3.93	73.0	3.78	71.5		
5	3.86	51.3	3.58	74.5	3.58	74.9	4.83	66.3		
6	3.65	72.6	3.69	61.1	4.00	59.3	1.16	14.9		
					3.88					
7	3.59	67.7								
8	3.90	71.4								
9	3.64	62.2								
	3.87									
CH_3	2.03	21.7			2.01	21.9				
C=O		174.7				173.8				
					Sialyl-2,3-*N*-Acetyllactosamine					
1		174.8	4.56	103.3	4.58	100.8			4.17, 4.34	71.2
2		100.6	3.56	70.1	3.77	55.8			5.91	134.1
3	1.81	40.4	4.12	76.2	3.83	73.2			5.26, 5.32	118.9
	2.76									
4	3.68	69.1	3.96	68.2	3.73	79.0				
5	3.85	52.4	3.70	75.9	3.58	75.5				
6	3.65	73.6	3.73	61.8	4.00, 3.86	60.8				
7	3.60	68.8								
8	3.91	72.5								
9	3.63	63.3								
	3.85									
CH_3	2.03	22.9			2.03	22.9				
C=O		175.9				175.5				
					Lewis x					
1			4.46	101.7	4.59	99.9	5.11	98.6	4.16, 4.34	70.5
2			3.50	71.0	3.92	55.7	3.68	67.7	5.91	133.2
3			3.64	72.4	3.86	74.9	3.91	69.2	5.27, 5.31	118.2
4			3.90	68.3	3.93	73.3	3.80	71.9		
5			3.58	74.9	3.57	75.3	4.84	66.7		
6			3.72	61.5	4.00, 3.87	59.7	1.17	14.9		
CH_3					2.03	22.2				
C=O						174.3				

could be used for measuring the nuclear Overhauser enhancement to estimate internuclear distances.

In the 1D TOCSY experiment using a low-power selective excitation at the anomeric proton of Gal and GlcNAc, two multispin systems are revealed each of which starts with a doublet, due to an anomeric proton of a sugar residue. The large (ca. 7–10 Hz) coupling constants between all of the vicinal H1 to H6 protons of one of these residues are from the GlcNAc residue, with its exclusively axial protons in the $^4C_1$ conformation.[37] For the other residues, $J(1,2)$ and $J(2,3)$ are also large, but $J(3,4)$ and $J(4,5)$ are small (ca. 3 and 1 Hz, respectively), indicating the presence of the equatorial H4 of the galactose residue. Owing to the deshielding effect of the amido group, the resonances of protons at amido carbon (GlcNAc H-2 and NeuAc H-5) are strongly shifted toward lower field. The TOCSY subspectra through the anomeric signals of the Fuc as well as Gal residues showed clearly observable connectivities only to the H4 resonances. Apparently, the small value of J_{H4-H5} in Fuc and Gal hampered propagation of magnetization transfer beyond H4. From DQF-COSY, it is easy to identify the Fuc H-6 (δ 1.17 ppm) and Fuc H-5 (δ 4.84 ppm). The rather low-field resonance of Fuc H-5 indicates the location of this residue in vicinal position to Gal as both Fuc and Gal are connected to GlcNAc.[38] For sialic acid, selective excitation of H3 of NeuAc at 2.76 ppm resulted in the spin–spin information being transferred to signals at 3.86 (triplet H5), 3.69 (multiplet H4), and 3.65 (H6). The spin–spin transfer from $H3_{ax}$ at 1.79 ppm was not suitable for chemical shift assignment. From pertinent J values, it is confirmed that the NeuAc residue adopts the $^2C_5$ chair conformation. The proton chemical shifts of sialic acid reported here are similar to those of terminal NeuAc found in other sialosides.[39] These methods allowed approximately 85% of the resonances in the ^1H spectrum of the oligosaccharides to be assigned. The rest of the resonances were assigned with the aid of 2D heteronuclear correlation spectroscopy described below. The proton chemical shift values thus obtained for the sialyl Le^x tetrasaccharide correspond well with those previously published data, which were only partially assigned.[40]

Heteronuclear Correlation Spectroscopy. It is known that the number of NOE cross peaks in oligosaccharides, especially those inter-residual ones, is notoriously few when compared with that of oligopeptides.[32] Therefore, use of complementary information in addition to the use of NOE constraint is always preferable for the 3D structure determination of oligosaccharides. The application of homonuclear and heteronuclear coupling constants to obtain structural information using the Karplus equation[41] in peptides has been reported.[42] Via a similar relation, knowledge of a long-range heteronuclear coupling across the glycosidic linkage in oligosaccharides provides useful information about the conformation. Therefore, it becomes desirable to make a complete assignment of the ^{13}C resonances, from which useful coupling

(37) Hassnoot, C. A. G.; De Leeuw, F. A. A. M.; Altona, C. *Tetrahedron* **1980**, *36*, 2783.
(38) Lemieux, R. U.; Bundle, D. R.; Barker, D. A. *J. Am. Chem. Soc.* **1975**, *97*, 4076.
(39) Sabesan, S.; Bock, K.; Paulson, J. C. *Carbohydr. Res.* **1991**, *218*, 27.
(40) Palcic, M. M.; Venot, A. P.; Ratcliffe, R. M.; Hindsgaul, O. *Carbohydr. Res.* **1989**, *190*, 1.
(41) Karplus, M. J. *J. Chem. Phys.* **1956**, *30*, 11.
(42) (a) Bystrov, V. F. *Prog. Nucl. Magn. Reson. Spectrosc.* **1976**, *10*, 41. (b) Guntert, P.; Braun, W.; Billeter, M.; Wuthrich, K. *J. Am. Chem. Soc.* **1989**, *111*, 3997. (c) Driscoll, P. C.; Gronenborn, A. M.; Clore, G. M. *FEBS Lett.* **1989**, *243*, 223.

Table V. Torsional Angle ψ of the Gal–GlcNAc Linkage Calculated from $^3J_{C-H}$

	Lewis x	sialyl-2,3-*N*-acetyllactosamine	sialyl Le^x
$^3J_{C-H}$ (Hz)	5.4 ± 0.4	4.9 ± 0.6	5.0 ± 0.6
ψ	11	21	18

constants can be extracted to derive the conformation. In our study, the more sensitive proton detection HMQC experiment was conducted to obtain the C–H correlation for oligosaccharides. A partial 2D HMQC spectrum of sialyl Le^x is shown in Figure 6. For the NeuAc residue of sialyl Le^x, from HMQC and the assignment of H4 through H7 described above, the carbon chemical shifts of C3 through C7 were established. The chemical shifts of hydrogens at C8 and C9 were established by first establishing the carbon chemical shift; namely, the ^{13}C resonances at 71.4 and 62.2 ppm were assigned to C8 and C9, respectively, by comparing with the published data.[39] The chemical shifts of hydrogens at C8 and C9 were then established from the HMQC spectrum. The ^{13}C chemical shifts for other monosaccharide residues were similarly established and listed in Table IV. In the ^{13}C-labeled compound, the coupling constants $^1J_{C1-C2}$, $^2J_{C1-C3}$, and $^2J_{C1-C5}$ have the same magnitudes (46, 3.7, and 0 Hz, respectively) as those observed in the reducing sugar for the β anomer.[43] Thus there appears to be no distortion of the galactopyranosyl ring in the oligosaccharide. The ^{13}C resonance of GlcNAc C4 is approximately 1-Hz broader than that of the unlabeled compound.

In addition to assisting in the assignment of all the ^1H and ^{13}C resonances, the much higher chemical shift dispersion in the heteronuclear dimension of the HMQC provides good resolutions for the overlapped proton resonances of oligosaccharides in the region 3.5–4.0 ppm. Also, another major advantage over the conventional ^{13}C-detected experiment is the much improved digital resolution in the ^1H dimension of the spectrum. With enough digital resolution in this dimension, one can even get a good estimate of homonuclear coupling constants for part of those overlapped resonances. The signals for GlcNAc H4 for all oligosaccharides studied were all overlapped in regular ^1H spectra but were well resolved and clearly visible in the HMQC spectrum. With the synthesis of Gal-1-^{13}C-labeled sialyl Le^x and its trisaccharide intermediates, the long-range heteronuclear coupling constant $^3J_{C1-H4'}$ was easily obtained from the HMQC experiment with no heteronuclear decoupling during acquisition (Figure 7). The long-range coupling constants measured are listed in Table V and were used to determine the torsion angle ψ of the Gal–GlcNAc linkage using the equation $^3J_{C-H} = 0.5 - 0.6 \cos \psi + 5.7 \cos^2 \psi$[44] (Table V). By applying the 1D and 2D methods, we were able to obtain complete assignment of the resonances as established in Table IV.

Glycosidic Linkage and Nuclear Overhauser Enhancement. No sequence information was available from the TOCSY/COSY spectra. However, the availability of a complete set of assignments paves the way for the determination of the 3D structure. The method basically consists of the application of NOESY to generate short-range through-space (<4 Å) connectivities within the molecule. Ideally, the measurement of NOE together with the use of intra-residue NOE should allow a 3D structure determination. Unfortunately, the laboratory frame NOESY spectrum of sialyl Le^x showed very weak intra- as well as inter-residual NOE, indicating a condition where the correlation time of the molecule at 500 MHz approaches the inverse of the Larmor frequency, $\tau_c\omega_0 \sim 1$, resulting in near to zero cross peak, whereas in rotating-frame experiments the effective Larmor frequency is low compared to the molecular reorientation rates.[45] ROESY was therefore used

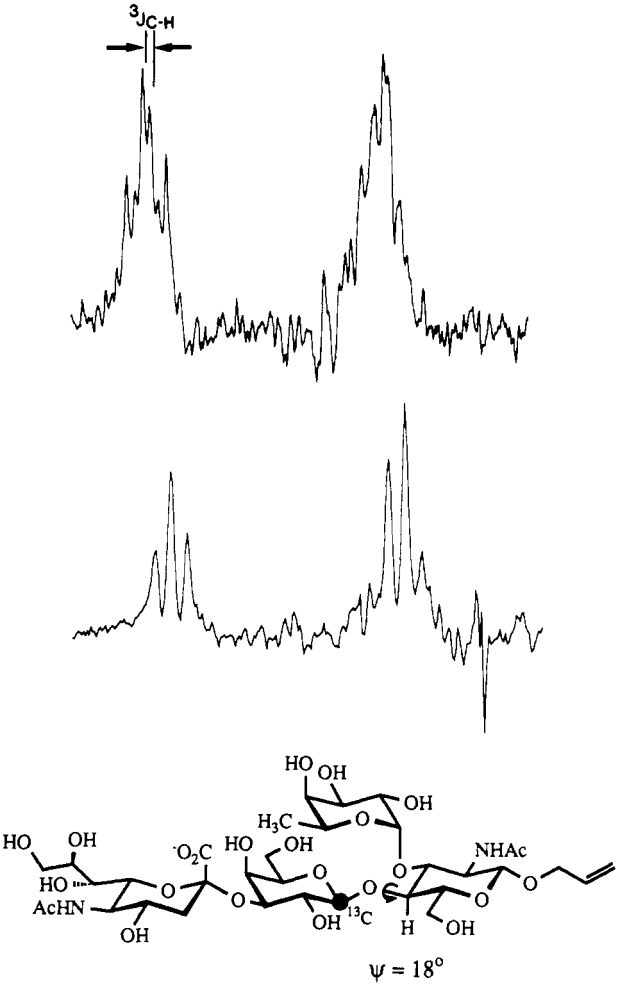


Figure 7. GlcNAc H4 proton subspectra of ^{13}C-labeled (upper trace) and unlabeled (lower trace) sialyl Lewis x. The long-range coupling constant $^3J_{C-H}$ (5.0 ± 0.6 Hz), of Gal C1 and GlcNAc H4, was used to calculate the torsion angle ψ of the Gal–GlcNAc linkage using the equation $^3J_{C-H} = 0.5 - 0.6 \cos \psi + 5.7 \cos^2 \psi$.

Table VI. Cross Peaks Observed in ROESY Spectra of Sialyl Le^x

	intra-residue	inter-residue	scalar coupling
NeuAc $H3_{ax}$	NeuAc H5	Gal H3	NeuAc H3, H4
NeuAc $H3_{eq}$	NeuAc H4		
Gal H1	Gal H3	GlcNAc H4	
Gal H3	Gal H4, H5		
GlcNAc H1	GlcNAc H3, H5	OCH_2	
Fuc H1	Fuc H2, H3	GlcNAc H3	
Fuc H4	Fuc H3, H5		
Fuc H5	Fuc H3, H4	Gal H2	
Fuc H6	Fuc H4	Gal H2	

for the determination of NOE. A distinctive disadvantage of ROESY is the presence of both homonuclear Hartmann–Hahn (HOHAHA) and ROESY cross peaks.[46] Fortunately, cross relaxation in the rotating frame shows only weak dependence on resonance offsets, whereas homonuclear Hartmann–Hahn transfer is strongly dependent on resonance effect. Thus by judicious choice of the carrier frequency and field strength of the rf spin-lock irradiation, it is possible to suppress Hartmann–Hahn transfer.

With the carrier frequency set at 6.2 ppm and spin-lock power set at ca. 0.5 W, some antiphase cross peak derived from coherent transfer was still visible, as indicated in Figure 8. The conformationally important NOE data are listed in Table VI. Of clear

(43) (a) King-Morris, M. J.; Serianni, A. S. *J. Am. Chem. Soc.* **1987**, *109*, 3501. (b) Krivdin, L. B.; Della, E. W. *Prog. Nucl. Magn. Reson. Spectrosc.* **1991**, *23*, 301.

(44) (a) Parfondry, A.; Cyr, N.; Perlin, A. S. *Carbohydr. Res.* **1977**, *59*, 299. (b) Hansen, P. E. *Prog. Nucl. Magn. Reson. Spectrosc.* **1981**, *14*, 175. (c) Tvaroska, I.; Hricovini, M.; Petrakova, E. *Carbohydr. Res.* **1989**, *189*, 359. (d) Lemieux, R. U.; Nagabhushan, T. L.; Paul, B. *Can. J. Chem.* **1972**, *50*, 773. (e) Morat, C.; Taravel, F. R. *Tetrahedron Lett.* **1990**, *31*, 1413.

(45) (a) Bothner-By, A. A.; Stephens, R. L.; Lee, J. M. *J. Am. Chem. Soc.* **1984**, *106*, 811. (b) Bax, A.; Sklenar, V.; Summers, M. F. *J. Magn. Reson.* **1986**, *70*, 327.

(46) (a) Neuhaus, A.; Keeler, J. *J. Magn. Reson.* **1986**, *68*, 568. (b) Farmer, B. T., II; Brown, L. R. *J. Magn. Reson.* **1987**, *72*, 197.

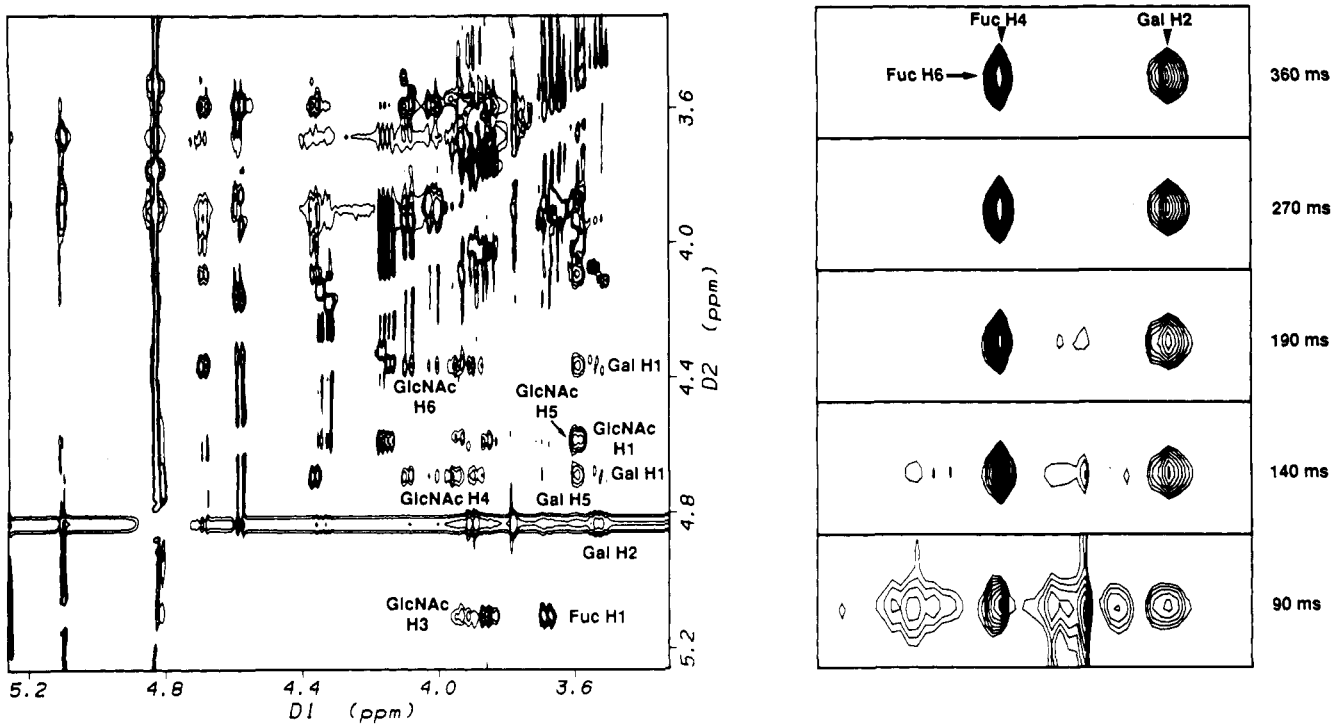


Figure 8. Left, ROESY spectra of sialyl Le^x. Right, variations of cross peaks of Gal H2–Fuc H6 and Fuc H4–Fuc H6 with respect to the mixing time.

significance for the primary structure are the dipolar correlations; the cross peak between H1 of Gal and H4 of GlcNAc is at δ (3.93, 4.52). The close spatial proximity of these protons is consistent with a β1,4 linkage between the two sugar units. Although the latter resonance occurs at the same chemical shift as that of H4 of Gal, a close inspection of the higher resolution 1D subspectrum shows that splitting of the enhanced resonance is a triplet for GlcNAc H4 but not a doublet, as expected, for Gal H4. For the Fuc–GlcNAc linkage, NOE was observed between Fuc H1 and GlcNAc H3. An accurate volume integration of the NOE was difficult due to the overlap of signals from other protons. Gal H3 has inter-residual polar interactions with NeuAc $H3_{ax}$, instead of NeuAc $H3_{eq}$, a characteristic NOE pattern for the antiperiplanar conformation. The most unusual cross peaks in ROESY are those between Fuc H5 and Gal H2 and between Fuc H6 and Gal H2. Unfortunately, the H5 and H6 groups of Fuc overlap with deuterated water and make the volume estimate difficult. As shown in Figure 8, variation of spin-lock mixing time from 90 to 360 ms clearly reveals the dipolar cross peaks between Fuc H6 and Gal H2 (right cross peak) and between Fuc H6 and Fuc H4 (left cross peak). In the unlabeled sialyl Le^x, the anomeric protons of Gal and GlcNAc are overlapped, as are their cross peaks with Gal H5 and GlcNAc H5 in the 2D ROESY spectra. Using the [Gal-1-^{13}C]sialyl Le^x, in which the $^1J_{C-H}$ coupling is present for Gal H1 and absent for GlcNAc H1, these two protons can be well resolved and their correlations through dipolar interaction are separated. An estimated volume of the ROESY cross peaks between NeuAc $H3_{ax}$ and GalH3 relative to the GlcNAc H1–H5 correlation gave an estimated internuclear distance of 2.1 ± 1.0 Å.

The site of the glycosidic linkage can also be deduced from the chemical shift data. Inspection of Table IV shows that the Gal H3 resonance has been shifted significantly downfield from its position in Le^x upon addition of the NeuAc residue. The attachment of Fuc via an α1,3 linkage to GlcNAc C3 in sialyl Le^x and Le^x is revealed in a downfield shift of the H3 signal of the GlcNAc residue in comparison to that of sialyl-2,3-LacNAc. These shifts are not as significant as that observed for the attachment of NeuAc to the Gal residue. It is also known that the sensitivity of ^{13}C chemical shift toward changes in substitution renders ^{13}C-NMR spectroscopy very useful for the determination of secondary structure of oligosaccharides. The signals of alkoxylated carbon atoms (e.g. C5 of Gal, Fuc, and GlcNAc, C6 of NeuAc, C3 of Gal, and C3 and C4 of GlcNAc for sialyl Le^x) are shifted 5–10 ppm to lower field when compared with those of the corresponding hydroxy-substituted carbon atoms.

Conformational Analysis. For an independent assessment of conformation, the GESA and MM2 calculations were performed. In the GESA calculation, the atomic coordinates of the constituent monosaccharides were from either X-ray or neutron diffraction studies and the glycosidic angles were set at 117° for the calculation.[35] The glycosidic dihedral angles, measured in degrees, are defined as follows. The dihedral angle ϕ is defined by the four atoms H–C1–O1–Cx′ (in the case of NeuAc, by the four atoms C1–C2–O2–Cx′) and ψ is defined by C1–O1–Cx′–Hx′. In this calculation, each monosaccharide ring is treated as a rigid entity and the conformational energy is calculated from different angles of ϕ and ψ by taking into account van der Waals interactions of the substituents and the contribution of the exoanomeric effect to the torsional movement of the glycosidic linkage. In MM2, the force field includes hydrogen bond and electrostatic interactions, and the geometry of the molecule is fully optimized.

For comparison, the GESA potential energy minimization program was also applied to all three oligosaccharides.[30,32] In addition to global energy minimization, two-parameter grid searches were done for ϕ and ψ angles vs internal energy for the NeuAc–Gal, Gal–GlcNAc, and Fuc–GlcNAc linkages and for each of these linkages while holding the others constant at their minima. Calculations of the tetrasaccharide predict essentially a single minimum for the Le^x moiety as illustrated in Figure 9. According to the calculations, the orientation of the Galβ1,4 linkage is not influenced by the presence of NeuAc or Fuc and the orientation of the NeuAcα2,3Gal linkage is not influenced by GlcNAc. The conformations of Le^x and the Le^x part of sialyl Le^x derived from the calculation show short distances between Gal H2 and Fuc H5 and between Gal H2 and Fuc H6, consistent with the cross peaks observed in the NMR study. These two studies thus suggest a single conformation for the Le^x moiety. Using the Karplus equation for the derivation of ψ, the observed values of $^3J_{C-H}$ between Gal C1 and GlcNAc H4 (Table V) could be interpreted as being indicative of a similar conformation of these lactosamine analogs around the glycosidic linkage. For the sialoside, the energy maps show various conformers about the NeuAc–Gal linkage. The global minimum at −170°/−8° (con-

Table VII. Comparison of Torsional Angles (deg) of Three Oligosaccharides

	NeuAc–Gal	Gal–GlcNAc	Fuc–GlcNAc	energy (relative)	d1[a]	d2[a]
		GESA Program				
sialyl Le^x	163/−57 (A)	54/8	48/25	−3.70		
	−170/−8 (B)	54/9	48/24	−8.48		
	−79/7 (C)	55/7	48/25	−7.77		
	68/−20 (D)	54/9	48/24	−5.86		
sialyl LacNAc	162/−56	55/2		2.98		
	−169/−7	55/2		−2.08		
	−78/8	54/0		−1.55		
	68/−20	55/2		0.53		
Lewis x		54/9	48/24			
		MM2 Program				
sialyl Le^x	167/−63 (A′)	48/15	23/30	79.42	1.93	2.77
	−171/−6 (B′)	46/16	24/30	80.87	2.03	3.47
sialyl LacNAc	170/−63	44/4				
Lewis x		46/16	24/30			

[a] d1 and d2 are separations (Å) for Gal H3–NeuAc H3$_{ax}$ and Gal H3–NeuAc H3$_{eq}$, respectively.

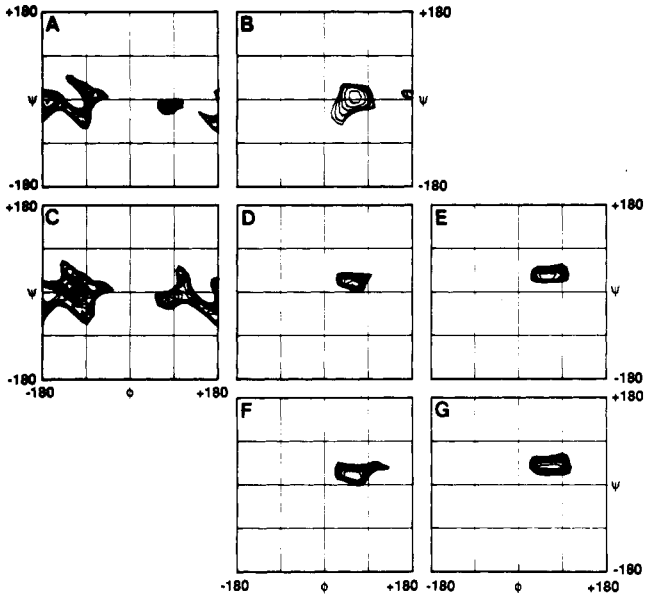


Figure 9. Two-parameter grid search for ϕ and ψ of glycosidic bonds: A, NeuAc–Gal; B, Gal–GlcNAc linkage of sialyl lactosamine; C, NeuAc–Gal; D, Gal–GlcNAc; E, Fuc–GlcNAc linkage of sialyl Lewis x; F, Gal–GlcNAc; G, Fuc–GlcNAc linkage of Lewis x.

former B) for this linkage is close to that found for a ganglioside.[47] The observation that one (163°/−57°, conformer A) of the four pseudominima for the NeuAc2,3Gal linkage is calculated to be of higher energy than that for conformer B should call for some degree of attention. Conformers A and B show short distances between NeuAc H3$_{ax}$ and Gal H3. Two other conformers (C and D) with separation between H3$_{ax}$ of NeuAc and H3 of Gal were not in agreement with the NMR data, even though the energies of these conformers calculated by GESA are lower than those of conformer A. In conformers A and B, the bulky carboxylate group of the sialic acid and the aglycon (i.e., the C3 of Gal) are separated from each other (anti conformation), whereas, in conformers C and D, they are syn to each other. Based on the HSEA calculation where only conformers B, C, and D were considered, it was predicted that >99% of the total population would have a solution structure within the energy well containing the lowest energy conformer B.[31] Only little contribution was estimated from conformers C and D on the basis of the calculation and of the NMR results where an NOE was observed between NeuAc H3$_{ax}$ and Gal H3. An estimated volume of the ROESY cross peaks between NeuAc H3$_{ax}$ and Gal H3 relative to the GlcNAc H1–H5 correlation gave an estimated internuclear distance of 2.1 ± 1.0 Å. Considering this distance constraint would therefore exclude substantial contributions from conformers C and D (Figure 9).

Only small shifts in the minimum-energy linkage position of sialyl Le^x relative to those in trisaccharides are expected, since the addition of one sugar unit (NeuAc, Fuc) would be expected to have virtually no effect on the other sugar's conformation-dependent potential energy. These differences are presumably the result of nonbonded (hard-sphere) interactions between the two vicinally linked terminal sugars. The potential wells for each linkage in sialyl Le^x are steeper than those for their trisaccharide intermediates and the conformationally more restricted disaccharide Gal–GlcNAc linkage. The conformations of the Galβ1,4GlcNAc and Fucα1,3GlcNAc linkages cannot be calculated quantitatively from the NOE data, even though the cross peaks were observed, because GlcNAc H4–Gal H4 and GlcNAc H3–Fuc H3 nearly coincide, precluding separate measurement of the NOEs between these protons and Gal H1 and Fuc H1.

The MM2 calculations were then carried out by using torsional angles of the conformers A and B as the initial settings to yield correspondingly two new conformers A′ and B′, the torsion angles and energies of which are listed in Table VII. Interestingly, conformer A′ gave a lower energy minimum by about 1.4 kcal/mol. The reason that conformer A predicted by the GESA program is energetically more favorable in the MM2 calculation may be due to the consideration of inter-residual H-bonding interactions and the full optimization of the geometry used in the MM2 program. The torsional angles of the glycosidic linkage between Gal and GlcNAc from MM2 are close to that obtained from the GESA calculation (48°/15° vs 54°/8°), whereas the torsional angle ϕ of the Fuc–GlcNAc linkage from MM2 differs by 24°. The torsional angle ψ of the Gal–GlcNAc linkage is consistently greater in MM2 calculations for all three oligosaccharides and seems to be in better agreement with the angle derived from $^3J_{C-H}$ via the Karplus equation. Since sialyl Le^a (Figure 10) binds to ELAM-1[28] as well as sialyl Le^x, it is of interest to compare the solution conformations of both tetrasaccharides. Since the glycosidic torsional angles of sialyl Le^a were not reported, we used the same computational methods as described above to calculate the glycosidic torsional angles and to determine the conformation. The results (Table VII, Figure 10) are generally consistent with the NMR data reported by Bechtel et al.[48a] The minimum-energy conformations of the three oligosaccharides studied and sialyl Le^a along with their space filling models obtained from MM2 are shown in Figure 10. As indicated, the two tetrasaccharides have very similar conformations except that the GlcNAc residue in sialyl Le^x flips ~180° compared to that in sialyl Le^a. This difference suggests that the recognition domain in sialyl Le^x is mainly from the NeuAc-Gal-Fuc residues, consistent

(47) Sabesan, S.; Duus, J. Ø.; Fukunaga, T.; Bock, K.; Ludvigsen, S. *J. Am. Chem. Soc.* **1991**, *113*, 3236.

(48) (a) Bechtel, B.; Wang, A. J.; Wroblewski, K.; Koprowski, H.; Thurin, J. *J. Biol. Chem.* **1990**, *265*, 2028. (b) Tyrrell, D.; James, P.; Rao, N.; Foxall, C.; Abbas, S.; Dasgupta, F.; Nashed, M.; Hasegawa, A.; Kiso, M.; Asa, D.; Kidd, J.; Brandley, B. K. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 10372.

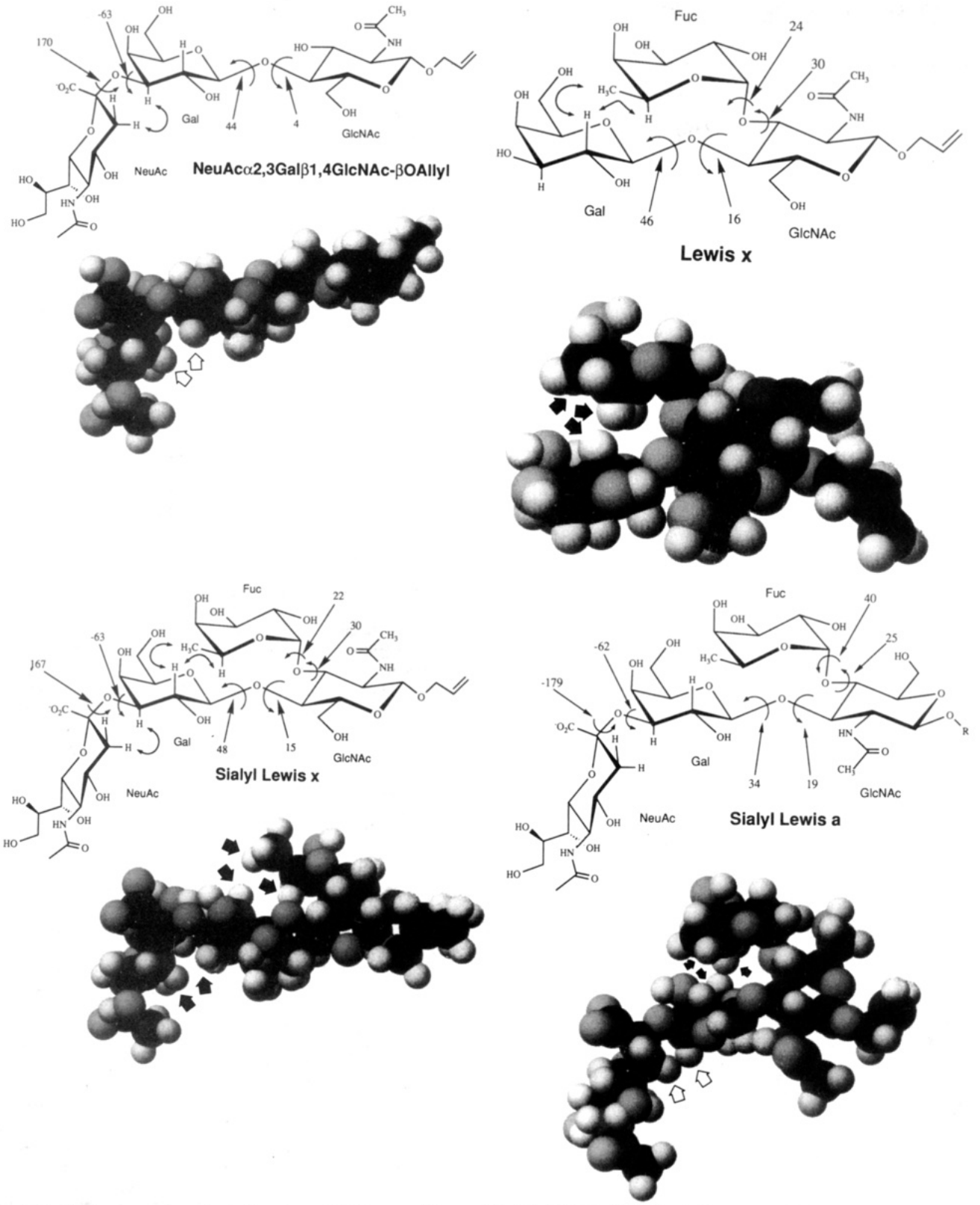


Figure 10. Minimum-energy conformations along with the space-filling models of sialyl 2,3 LacNAc (top left), Le^x (top right), sialyl Le^x (bottom left), and sialyl Le^a (bottom right). The conformations were determined based on NMR analysis and GESA-MM2 energy minimization. Glycosidic torsional angles and NOE are indicated. The conformation of sialyl Le^a and the glycosidic torsional angles were determined in our laboratory with the GESA and MM2 programs.

with the predicted result.[28] Another interesting observation is that replacement of the GlcNAc residue of sialyl Le^x with Glc increases the activity by ~15%.[48b] The sialic acid and fucose residues in sialyl Le^x and sialyl Le^a are in approximately the same orientation, resulting in properly aligned hydroxy groups on the surface.

The structure of the oligosaccharide-containing Le^x moiety is unusual in that there is very close stacking of the fucose and galactose rings.[49] Both the experimental and theoretical results are in agreement with the conformation and dynamics of the blood group trisaccharide derivative Fucα1,2(GalNAcα1,3)-GalβOmethyl, which was shown to have a single stereoconformer

(49) Wormald, M. R.; Edge, C. J.; Dwek, R. A. *Biochem. Biophys. Res. Commun.* **1991**, *180*, 1214.

over a wide range of temperature and solvent conditions due to the linkage stereochemistry.[50] In contrast, the more elongated structures in small oligosaccharides[51] normally exist in several conformations, and conformation averaging should be considered.

In both sialyl Le^x and Le^a, the interaction between the Gal and Fuc rings provides a very rigid structure for the Le^x part of the molecule[49] and leads to the formation of a well-defined hydrophilic surface along the NeuAc-Gal-Fuc residues and a hydrophobic surface underneath the NeuAc-Gal-GlcNAc residues. The conformation of the Le^x determinant seems not to be altered by addition of sialic acid.

The early work[52] on polysaccharide geometry indicates that some polysaccharides, irrespective of composition or linkage, are capable of adopting ordered conformations. Such ordered conformations would place sugar unit and functional groups at regular and predictable spatial dispositions. The potential energy surfaces for the glycosidic linkages indicate that the conformation of the Le^x must be relatively rigid although molecular dynamic simulations are necessary to confirm it. The calculation for the sialyl Le^x tetrasaccharide predicts essentially a single minimum for the Le^x moiety and some restriction of the flexibility of the NeuAc residue.

Conclusion

In summary, we have developed efficient enzymatic methods for the synthesis of sialyl Le^x and its related oligosaccharides using β1,4GalT, α2,3NeuT, and α1,3FucT. The in situ cofactor regeneration systems have been proven to be effective for the glycosyltransferase-mediated oligosaccharide synthesis. The substrate specificity studies of FucT indicate that the enzymes, like GalT, are flexible for certain types of acceptor structures. This study also illustrates that, for large-scale synthesis of oligosaccharides using glycosyltransferases, regeneration of sugar nucleotides is necessary to lessen the problem of product inhibition caused by sugar nucleotides and to reduce their cost. The conformational study of sialyl Le^x and derivatives using NMR techniques and molecular mechanics calculations based on MM2 and GESA suggested that one stable conformer of sialyl Le^x exists in aqueous solution. With the solution conformations of sialyl Le^x and intermediates available, development of sialyl Le^x mimetics with desirable biological activities may be possible.

Experimental Section

Unless otherwise indicated, enzymes and biochemicals were from Sigma. The α1,3FucT and α1,3/4FucT were prepared essentially as described.[7,8,10,11]

Expression of Galβ1,3/4GlcNAc α-2,3-Sialyltransferase (Scheme VIII). High-yield expression of a soluble Galβ1,3/4GlcNAc α-2,3-sialyltransferase (α2,3NeuT) was accomplished in a baculovirus expression system using cDNA encoding a fusion protein between the pre-insulin signal peptide and the catalytic domain of the sialyltransferase. The cDNA encoding the fusion protein was constructed by Wen et al.[53] in the plasmid vector pGIR199.[54] To isolate a DNA fragment containing the entire coding sequence, the unique EcoRI site at the 3′ end of the chimera was first digested, the overhang was made blunt, and synthetic linkers containing an NheI site were ligated. The resulting plasmid was digested with NheI to release the fusion protein cDNA, and this fragment was cloned at the unique NheI site in pBlueBac, a baculovirus expression system transfer vector, under the control of the baculovirus polyhedron promoter (Invitrogen; San Diego, CA). All recombinant DNA manipulations were performed under the conditions recommended by the enzyme manufacturers' instructions using standard protocols.[55] Creation of recombinant baculovirus was done using the MaxBac expression system (Invitrogen) following exactly the protocols recommended by the manufacturer. Briefly, plasmid and wild type virus DNA were mixed and used to transfect Sf-9 cells by the calcium phosphate method. Recombinant virus produced by the transfected cells was shed into the culture medium and was isolated by repetitive plaque purification at limiting dilution. Several clonal plaques were isolated and analyzed for the ability to cause secretion of α2,3NeuT into the infected cell medium by testing an aliquot of the media directly for α2,3NeuT activity in a sialyltransferase assay. The isolate that directed the highest levels of α2,3NeuT secretion was designated rBv2,3ST and was expanded to 500 mL by infection of fresh Sf-9 cells. α2,3NeuT activity was assayed using a modification of the published assay[56] with 0.9 mM Galβ1,3GlcNAcβ1,3Galβ1,4Glc as acceptor.

To produce large amounts of α2,3NeuT, rBv2,3ST was used to infect Sf-9 cells in monolayer culture and generally yielded 2–3 units of α2,3NeuT activity secreted per 10^8 infected cells when grown in Excell-400 media (JRH Biosciences, Lenexa, KS). Units of activity (μmol/min) are defined by multiplication of the assay results by a factor of 1.6 to give activity at V_{max}. Conditioned media from rBv2,3ST-infected cells were collected 72 h postinfection, and the recombinant α2,3NeuT was partially purified in one chromatography step. Three liters of media containing α2,3NeuT was filtered and concentrated to approximately 250 mL in an Amicon CH2PRS spiral cartridge system equipped with an S1Y10 cartridge. The unit was then run in diafiltration mode to desalt to the concentrated supernatant with 3 volumes of 10 mM cacodylic acid, 25 mM NaCl, 25% glycerol, pH 5.3 (buffer A). Samples were then applied to a column (2.5 × 17 cm) of S-Sepharose Fast Flow (Pharmacia) equilibrated with buffer A at a flow rate of 2 mL/min. After all of the sample had been loaded, the column was washed with buffer A until the OD_{280} of the column effluent had returned to baseline (1.6 column volumes). α2,3NeuT was then eluted from the column with 50 mM cacodylic acid, 1 M NaCl, 25% glycerol pH 6.5. Fractions containing α2,3NeuT were pooled and dialyzed overnight against 1 L of 50 mM cacodylic acid, 0.5 M NaCl, 50% glycerol, pH 6.0 and then stored at −20 °C.

Galactosylation. LacNAcβOallyl from Scheme II. A mixture of **2**[22] (2.0 g, 7.65 mmol), Glc-1-P (2.74 g, 7.65 mmol), PEP (K salt, 1.6 g, 7.65 mmol, 0.95%), NAD^+ (193 mg, 0.25 mmol), $MnCl_2{\cdot}4H_2O$ (79.2 mg, 0.4 mmol), $MgCl_2{\cdot}6H_2O$ (162.6 mg, 0.8 mmol), DTT (306 mg, 2 mmol), KCl (1.04 g, 15 mmol), NaN_3 (20 mg, 0.31 mmol), and UDP (90 mg, 0.19 mmol) in HEPES buffer (100 mM, pH 7.5; 200 mL) was adjusted with 10 N and 1 N NaOH to pH 7.5, and the enzymes UDPGE (10 U), UDPGP (20 U), PK (100 U), GalT (5 U), and PPase (100 U) were added to the solution. The mixture was gently stirred under an argon atmosphere at room temperature (25 °C) for 5 days. The mixture was concentrated and chromatographed on silica gel with $CHCl_3$–EtOAc–MeOH (5:2:2 to 5:2:3) to give a disaccharide, which was further purified with Sephadex G-25, with water, to give LacNAcβOallyl (1.7 g, 50%): ^{1}H NMR (D_2O) δ 2.00 (3 H, s, NHAc), 3.49 (1 H, dd, J 7.84, 9.97 Hz, H2 of Gal), 3.52–3.57 (1 H, m, H5 of GlcNAc), 3.63 (1 H, dd, J 3.31, 10.04 Hz, H3 of Gal), 3.65–3.75 (8 H, m), 3.79 (1 H, dd, J 5.10, 12.27 Hz, H6a of GlcNAc), 3.88 (1 H, br d, J 3.32 Hz, H4 of Gal), 3.95 (1 H, dd, J 2.14, 12.27 Hz, H6b of GlcNAc), 4.43 (1 H, d, J 7.81 Hz, H1 of Gal), 4.55 (1 H, d, J 8.28 Hz, H1 of GlcNAc), 5.21–5.29 (2 H, m, allylic), 5.83–5.90 (1 H, m, allylic); ^{13}C NMR (D_2O) δ 22.6, 55.5, 60.5, 61.5, 69.0, 70.9, 71.4, 72.9, 75.2, 75.8, 78.8, 100.4, 103.3, 118.6, 133.7.

Compound 3 (Scheme III). A solution of **2** (1.15 g, 4.4 mmol), 1-^{13}C-Gal[21] (800 mg, 4.4 mmol), PEP K salt (1.82 g, 8.8 mmol; 95%), UDP (90 mg, 0.19 mmol), ATP (100 mg, 0.18 mmol), cysteine (116 mg, 0.96 mmol), DTT (183 mg, 1.2 mmol), $MgCl_2{\cdot}6H_2O$ (244 mg, 1.2 mmol), $MnCl_2{\cdot}4H_2O$ (118 mg, 0.6 mmol), KCl (179 mg, 2.4 mmol), and Glc-1-P (77 mg, 0.22 mmol) in HEPES buffer (100 mM, pH 7.5; 120 mL) was adjusted with 10 N and 1 N NaOH to pH 7.5, and the enzymes GK (10 U), PK (200 U), PPase (10 U), Gal-1-P UT (10 U), UDPGP (10 U), and GalT (10 U) were added to the solution. The mixture was gently stirred under an argon atmosphere at room temperature (ca. 25 °C) for 3 days. The mixture was concentrated in vacuo, and the residue was chromatographed on silica gel, with EtOAc–MeOH (2:1), to give a disaccharide, which was further purified with a column of Sephadex G-25, with water, to give **3** (106 g, 57%): ^{1}H NMR (D_2O) δ 2.00 (3 H, s, NHAc), 3.48–3.52 (1 H, m, H2 of Gal), 4.43 (1 H, dd, $J_{H1,H2}$ 8.32, $J_{H1,^{13}C1}$ 162.33 Hz, H1 of Gal), 4.54 (1 H, d, J 8.32 Hz, H1 of GlcNAc); HRMS calcd for $^{12}C_{16}{}^{13}CH_{29}NO_{11}Na$ (M + Na^+) 447.1672, found 447.1681.

2-Deoxy-D-galactopyranosyl-β(1,4)-2-acetamido-2-deoxyglucopyranose (3a) (36%). Both the ^{1}H NMR spectrum of its heptaacetate

(50) (a) Yan, Z. Y.; Busch, C. A. *Biopolymers* **1990**, *29*, 799. (b) Cagas, P.; Busch, C. A. *Biopolymers* **1990**, *30*, 1123.

(51) Homans, S. W.; Dwek, R. A.; Rademacher, T. W. *Biochemistry* **1987**, *26*, 6571.

(52) Lemieux, R. U.; Koto, S.; Voisin, D. *ACS Symposium Series 87*; American Chemical Society: Washington, DC, 1979; p 17.

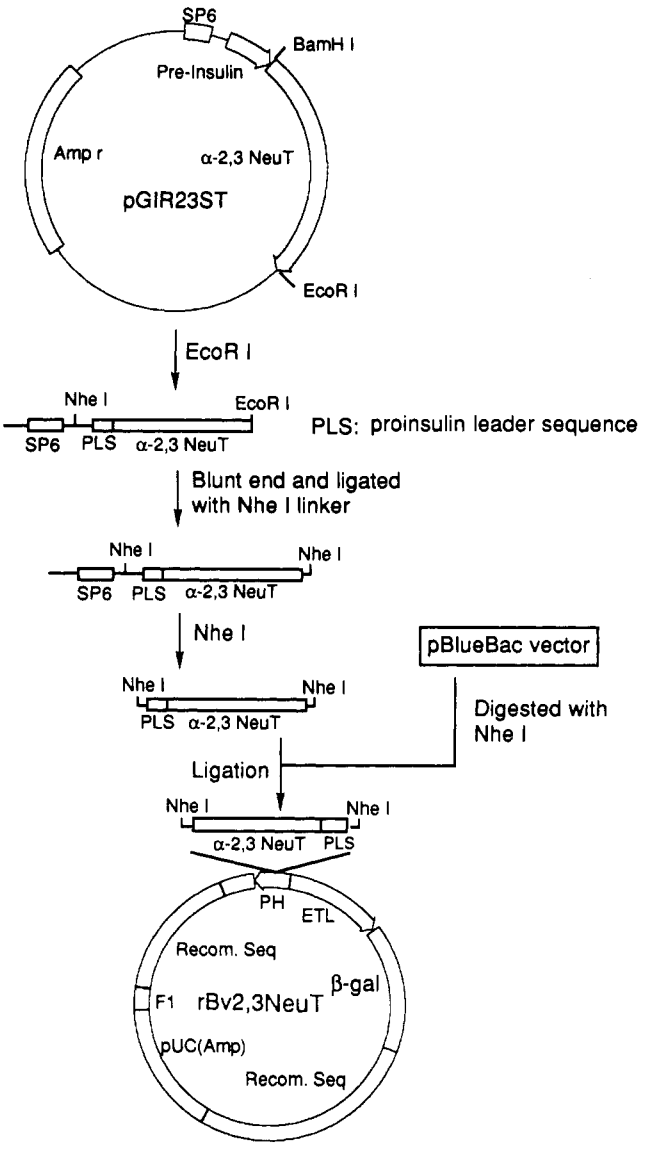
(53) Wen, D. X.; Livingston, B. D.; Medzihradsky, K. F.; Kelm, S.; Burlingame, A. L.; Paulson, J. C. *J. Biol. Chem.* Submitted.

(54) Huseh, E. C.; Holland, E. C.; Carrerra, G. M., Jr.; Drickamer, K. *J. Biol. Chem.* **1986**, *261*, 4940.

(55) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning: A Laboratory Manual*, 2nd ed.; Cold Spring Harbor Laboratory Press: Plainview, NY, 1989.

(56) Sadler, J. E.; Rearick, J. I.; Paulson, J. C.; Hill, R. L. *J. Biol. Chem.* **1979**, *254*, 4434.

Scheme VIII. Construction of Galβ1,3/4GlcNAc α-2,3-Sialyltransferase Expression Vector for Baculovirus Expression System



and the ^{13}C NMR spectrum of **3a** are in good agreement with those reported.[57]

2-Amino-2-deoxy-D-galactopyranosyl-β(1,4)-2-acetamido-2-deoxy-glucopyranose (3b) (12%): ^1H NMR for HCl salt (D_2O) δ 2.022, 2.024 (s, NHAc of α and β anomer of GlcNAc), 3.17–3.23 (1 H, m, H2 of GalN), 4.67 (d, *J* 7.53 Hz, H1β of GlcNAc), 5.13 (d, *J* 1.54 Hz, H1α of GlcNAc); HRMS calcd for $C_{14}H_{26}N_2O_{10}Na$ (M + Na^+) 405.1485, found 405.1489. ^1H NMR of its acetate form is in good agreement with that reported.[58]

Ethyl D-Galactopyranosyl-β(1,4)-2-azido-2-deoxy-D-glucopyranoside (3c). In this case DTT was eliminated, since the 2-azido group was reduced to the corresponding amine with DTT (15%): ^1H NMR β anomer (D_2O) δ 1.22 (1 H, t, *J* 7.80 Hz, OCH_2CH_3), 3.27 (1 H, *J* 8.33, 9.64 Hz, H2 of $GlcN_3$), 4.40 (1 H, d, *J* 7.81 Hz, H1 of Gal), 4.55 (1 H, *J* 8.24 Hz, H1 of $GlcN_3$); HRMS calcd for $C_{14}H_{25}N_3O_{10}Na$ (M + Na^+) 418.1438, found 418.1438.

The acceptor ethyl 2-azido-2-deoxy-D-galactopyranoside was prepared as follows: Triacetyl-D-glucal was azidonitrated[59] (NaN_3 and $Ce(NH_4)_2(NO_3)_6$ in CH_3CN) and acetolyzed (NaOAc in AcOH) to give 2-azido-1,3,4,6-tetra-*O*-acetyl-2-deoxy-D-glucopyranose, which was treated with $TiBr_4$ in CH_2Cl_2 and EtOAc, giving a glycosyl bromide, and then glycosylated with EtOH in the presence of AgOTf and MS4A in CH_2Cl_2 to give, after O-deacetylation with NaOMe in MeOH, ethyl 2-azido-2-deoxy-D-glucopyranoside (22% overall yield) as a 1:1.5 mixture of α and β: ^1H NMR (D_2O) δ 1.21 (t, *J* 7.80 Hz, OCH_2CH_3 of β anomer), 1.22 (t, *J* 7.80 Hz, OCH_2CH_3), 2.99 (dd, *J* 7.43, 9.83 Hz, H2 of β anomer), 5.11 (d, *J* 3.58 Hz, H1 of α anomer); HRMS calcd for $C_8H_{15}N_3O_5Cs$ (M + Cs^+) 366.0066, found 366.0066.

Sialylation. Compound 4. A solution of **3** (210 mg, 0.50 mmol), NeuAc (160 mg, 0.52 mmol), PEP Na_3 salt (120 mg, 0.51 mmol), $MgCl_2 \cdot 6H_2O$ (20 mg, 0.1 mmol), $MnCl_2 \cdot 4H_2O$ (4.9 mg, 0.025 mmol), KCl (7.5 mg, 0.10 mmol), CMP (16 mg, 0.05 mmol), ATP (2.7 mg, 0.005 mmol), and mercaptoethanol (0.34 μL) in HEPES buffer (200 mM, pH 7.5; 3.5 mL) was adjusted with 1 N NaOH to pH 7.5, and the enzymes NMK (5 U), PK (100 U), PPase (10 U), CMP-NeuAc synthetase (0.4 U), and α2,3NeuT (0.1 U) were added to the solution. The mixture was gently stirred under an argon atmosphere at room temperature (25 °C) for 3 days. The mixture was concentrated, and the residue was chromatographed on silica gel, with EtOAc–iPrOH–H_2O (2:2:1), to give a trisaccharide, which was further purified with BioGel P-2, with water, to give **4** (88 mg, 24%): ^1H NMR (D_2O) δ 1.81 (1 H, br t, *J* 12.02 Hz, $H3_{ax}$ of NeuAc), 2.04 (6 H, s, NHAc of GlcNAc and NeuAc), 2.76 (1 H, dd, *J* 4.57, 12.33 Hz, $H3_{eq}$ of NeuAc), 3.96 (1 H, br d, *J* 3.10 Hz, H4 of Gal), 4.13 (1 H, dd, *J* 3.09, 9.94 Hz, H3 of Gal), 4.56 (1 H, dd, $J_{H1,H2}$ 7.83, $J_{H1,^{13}C1}$ 162.78 Hz, H1 of Gal), 4.58 (1 H, d, *J* 8.32 Hz, H1 of GlcNAc); HRMS calcd for $C_{27}H_{44}N_2O_{19}Cs_2$ (M − H^+ + 2Cs^+) 980.0759, found 980.0720.

NeuAcα2,3'Lactal (6) (82 mg): ^1H NMR (D_2O, 320 K) δ 1.84 (1 H, br t, *J* 12.18 Hz, $H3_{eq}$ of NeuAc), 2.08 (3 H, s, NHAc of NeuAc), 2.82 (1 H, dd, *J* 4.46, 12.32 Hz, $H3_{eq}$ of NeuAc), 4.01 (1 H, br d, *J* 2.50 Hz, H4 of Gal), 4.16 (1 H, dd, *J* 2.50, 9.50 Hz, H3 of Gal), 4.43 (1 H, dt, *J* 1.18, 6.46 Hz, H3 of Glucal), 4.65 (1 H, d, *J* 7.86 Hz, H1 of Gal), 4.88 (1 H, dd, *J* 2.63, 6.07 Hz, H2 of Glucal), 6.51 (1 H, dd, *J* 1.45, 6.08 Hz, H1 of Glucal); HRMS calcd for $C_{23}H_{35}NO_{17}NaCs_2$ (M − H^+ + 2Cs^+) 864.0092, found 864.0066.

Fucosylation. Compounds 5, 7, and 8. A solution of FucT (0.02 U; 2 mL) was added to a solution of **4** (23 mg, 0.031 mmol) and GDP-Fuc[24] (24 mg, 0.036 mmol) in HEPES buffer (3 mL; 200 mM, pH 7.5) containing 5 mM ATP, 20 mM Mn^{2+}, and the mixture was gently stirred under an argon atmosphere for 5 days at room temperature (25 °C). The mixture was concentrated and chromatographed on silica gel, with EtOAc–iPrOH–H_2O (2:2:1), to give a tetrasaccharide, which was further purified with BioGel P-2, with water. The eluant was passed through a column of Dowex 50W-X8 [H^+], eluted with water, neutralized with 1 N NaOH, and lyophilized to give **5** (18 mg). Similarly, compounds **7** (42 mg) and **8** (51 mg) were prepared. For complete NMR (^1H and ^{13}C) assignments for **5** and **7**, see Table I. Compound **8**: ^1H NMR (D_2O) δ 1.15 (3 H, d, *J* 6.61 Hz, 6-CH_3 of Fuc), 1.76 (1 H, br t, *J* 12.00 Hz, $H3_{ax}$ of NeuAc), 1.98 (3 H, s, NHAc of NeuAc), 2.71 (1 H, dd, *J* 4.52, 12.38 Hz, $H3_{eq}$ of NeuAc), 4.44 (1 H, br q, *J* 7.50 Hz, H5 of Fuc), 4.58 (1 H, d, *J* 8.0 Hz, H1 of Gal), 4.95 (1 H, dd, *J* 2.5, 6.0 Hz, H2 of Glucal), 5.00 (1 H, d, *J* 3.98 Hz, H1 of Fuc), 6.45 (1 H, d, *J* 6.0 Hz, H1 of Glucal).

Galβ1,4(Fucα1,3)-5-thioGlc. A solution of Galβ1,4(5-ThioGlc) (30 mg, 84 μmol), GDP-Fuc (60 mg, 84 μmol), and α1,3/4FucT (0.5 U) in sodium cacodylate buffer (5.4 mL; 50 mM, pH 6.2) containing 5 mM ATP and 20 mM $MnCl_2$ was stirred for 2 days at room temperature. The R_f values of the starting material and the product were 0.39 and 0.31, respectively, in EtOAc–AcOH–H_2O 3:2:1 on silica TLC. The reaction mixture was applied directly to a column of Sephadex G-25 Superfine (1.5 × 30 cm) and eluted with water. The fractions containing the product were pooled and successively passed through columns of QAE-Sephadex and Dowex 50-X8 [H^+] with water. The effluent was pooled and lyophilized (21 mg): ^1H NMR (D_2O) δ 1.13 (3 H, d, *J* 6.7 Hz, 6-CH_3 of Fuc), 3.40 (1 H, dd, *J* 6.4 and 11.7 Hz), 3.60 (1 H, dd, *J* 3.6 and 11.7 Hz), 4.52 (1 H, d, *J* 7.9 Hz), 4.95 (1 H, *J* 2.6 Hz), 5.34 (1 H, d, *J* 3.8 Hz).

Kinetic Study of Enzymes. (A) FucT. The assay procedure was essentially the same as described previously[7] with some modifications. A stock mixture containing 0.25 mM GDP-^{14}C-Fuc (5000 cpm/μL), 6.25 mM ATP, 25 mM $MnCl_2$, and 62.5 mM sodium cacodylate buffer, pH 6.2, was mixed freshly and kept on ice. To this solution was added FucT immediate before use, and the reaction was initiated by combining 16 μL of this mixture and 4 μL of 100 mM acceptor (total incubation solution was 20 μL). The incubation was carried out at 37 °C for 30–240 min depending upon the acceptor under study. Separate assays in the absence of acceptor were used to correct for background hydrolysis of GDP-Fuc. Upon completion of incubation, 400 μL of a 25% (v/v) suspension of QAE-Sephadex was added. These suspensions were gently mixed at room temperature for 10 min before centrifugation at 13 000 rpm for 1 min. From the supernatant fluid, 200 μL was extracted and mixed with 10 mL of scintillation coctail. The radioactivity was counted on a scintillation counter. Care was taken to be sure less than 10% of the enzy-

(57) Thiem, J.; Wiemann, T. *Angew. Chem., Int. Ed. Engl.* **1991**, *30*, 1163.
(58) Palcic, M. M.; Hindsgaul, O. *Glycobiology* **1991**, *1*, 205.
(59) Lemieux, R. U.; Ratcliffe, R. M. *Can. J. Chem.* **1979**, *57*, 1244.

matic reaction had taken place over the incubation period.

(B) GalT. Initial velocities of the enzyme reaction were determined by measuring the rate of LacNAc formation with a slight modification of the assay by Pierce et al.[60] All the reactions were carried out in 100 mM cacodylate buffer (pH 7.5) with fixed concentrations of Mn^{2+} (9.3 mM) and UDP-Gal (0.1 mM; 58.5 cpm/pmol of UDP-^{14}C-Gal) in 100 μL of solution. The reaction was initiated by the addition of GalT (0.05 U, 120 μg protein; from Sigma) and allowed to stand at 20 °C for 30 min. Nonspecific hydrolysis of UDP-Gal was measured by the control reaction in the absence of GalT. The reaction was stopped by passing the reaction mixture through a column of QAE-Sephadex (700 μL), and the mixture was eluted by gentle air pressure to remove the unreacted UDP-Gal. The reaction vial was rinsed twice with 400 μL of water each, and the rinses were passed through the resin column. The filtrates were collected and directly transferred to a scintillation vial. The scintillation fluid was added to the vial, and then radioactivity was counted by a liquid scintillation counter. The data were analyzed by a double-reciprocal plot to obtain K_m (1.5 mM for GlcNAc)[61] and K_i (0.46 ± 0.06 mM) for UDP. Similarly, the IC_{50} value of UDP for GalT was determined using a different concentration of UDP.

GDP-Fuc-Generating Enzyme for Scheme VIIA. Bacterium *K. pneumonia* ATCC 12658 was grown in 2 L of the medium containing 10 g of casamino acid (Difco), 5 g of yeast extract, 3 g of K_2HPO_4, 1 g of KH_2PO_4, and 5 g of D-glucose per liter (pH 7.0). After incubation at 37 °C for 18 h, the cells were harvested by centrifugation (10 000 × *g*, 50 min, 4 °C) and resuspended in 50 mM tris buffer containing 0.5 mM DTT. The cells were disrupted by a French press at 16 000 lb/in. The cell debris was removed by centrifugation at 23 000 × *g* for 60 min, and the supernatant (cell free extract) was used for enzyme purification. The cell free extract (50 mL) for 2 L of culture was treated with 60 mg of protamine sulfate, and the resulting precipitate was removed after centrifugation. Solid ammonium sulfate was then added with slow stirring until 70% saturation was reached (0.436 g/mL at 0 °C). After the centrifugation, the precipitate was collected and resuspended in 20 mL of the buffer (50 mM tris containing 0.5 mM DTT, pH 7.5) and dialyzed overnight at 4 °C in 4 L of the same buffer. The remaining solution (20 mL) was then passed through a DEAE-Sepharose CL-6B column (Pharmacia) (3 × 30 cm) preequilibrated with the same buffer. The enzyme was eluted with a linear gradient of NaCl from 0 to 1 M in the same buffer (total 400 mL). The active fractions were pooled and dialyzed in 2 L of 50 mM tris buffer containing 0.5 mM DTT (pH 7.5). This enzyme preparation was used for the preparation of GDP-Fuc. The activity was estimated to be about 0.05 U/mL on the basis of HPLC and NADH oxidation assay.

Enzymatic Preparation and Regeneration of GDP-Fuc from Man-1-P (Scheme VIIA). A solution of Man-1-P (5 mM), GDP (2 mM), PEP (20 mM), KF (5 mM), Mg^{2+} (20 mM), KCl (20 mM), NADP (2 mM), EDTA (6 mM), iPrOH (2%), PK (680 U), TBDH (60 U), yeast cells (*S. cerevisae*, 150 mg, freeze-dried from 50 mM tris buffer, pH 7.5), PPase (100 U), L-homoarginine (10 mM), and GDP-Fuc-generating enzyme (1 mL) in HEPES buffer (0.1 M, pH 7.5) (the total solution volume was 10 mL) was incubated at 37 °C under an argon atmosphere for 18 h. The HPLC column partisil 5 SAX (Whatman Co.), 0.46 × 12.5 cm, with particle size 5 μm was used. The mobile phase was 0.1 M phosphate buffer (pH 3.5) with flow rate 0.5 mL/min (pressure 600 psi). The compounds were detected by a UV detector at 254 nm. The retention times for GDP-Man and GDP-Fuc were 9.92 and 13.4 min, respectively. GDP-Fuc (5%) and GDP-Man (30%) were formed on the basis of the HPLC analysis. A solution of **3** or **4** (10 mM in 2 mL 100 mM HEPES buffer pH 7.5, containing 2 mM ATP and α1,3FucT (25 mM) was then added, and the mixture was stirred for 5 days. TLC on silica gel plate: R_f = 0.28 for **7** and 0.50 for **3** with EtOAc–AcOH–H_2O 4:2:1 (v/v) and 0.56 for **5** and 0.63 for **4** with 1 M NH_4OH–iPrOH 1:2:4 (v/v). Compounds **7** and **5** (8 and 4 mg each) were isolated and purified as described above.

Purification of GDP-Fucose Pyrophosphorylase for Scheme VIIB. Porcine thyroid glands (376 g) were homogenized in ice cold 10 mM MOPS (752 mL), pH 7.5, with 1 μg/mL each antipain, aprotinin, chymotrypsin, leupeptin, and pepstatin, in a Waring blendor (five 15-s bursts on high setting). Cell debris was removed by centrifugation of 8000 × *g* for 20 min at 4 °C. To the supernatant fraction was added 188 mL of a 2% solution of protamine sulfate. The mixture was stirred for 15 min, and the precipitate was removed by centrifugation as above. Solid ammonium sulfate was slowly added to the supernatant fraction to 50% saturation (0.291 g/mL at 0 °C). After centrifugation as described above, the precipitate was collected and resuspended in 500 mL of 1.2 M ammonium sulfate. The sample was mixed with a slurry of phenyl Sepharose (50 mL) that had been equilibrated in 1.2 M ammonium sulfate. The resin with the bound enzyme was washed with 1.2 M ammonium sulfate (200 mL) and the enzyme activity eluted with 0.4 M ammonium sulfate (200 mL). Throughout the purification, GDP-Fuc pyrophosphorylase was assayed according to the method of Ishihara and Heath.[27] One unit of activity is defined as the incorporation of 1 μmol of inorganic ^{32}P-pyrophosphate into GTP per minute.

GDP-Fucose Regeneration Employing GDP-Fucose Pyrophosphorylase (Scheme VIIB). Synthesis of Sialyl Lewis x. A solution of MOPS, pH 7.5 (50 mM), Fuc 1-P (10 mM), GDP (1 mM), PEP (10 mM), KF (5 mM), Mg^{2+} (10 mM), Mn^{2+} (10 mM), PK (5 U), sialyl-[^{3}H]-LacNAcβ-O-$(CH_2)_6CO_2Me$ (10 mM), α1,3FucT (0.1 U), inorganic pyrophosphatase (5 U), and GDP-Fuc pyrophosphorylase (0.1 U) was mixed in a volume of 100 μL. The reaction was incubated on a tube turner at 37 °C for 64 h. The sample was extracted with ten volumes of methanol, dried by evaporation under reduced pressure, resuspended in water, and analyzed by thin layer chromatography on silica gel plates with 2-propanol–1 M ammonium acetate (6:1) as solvent. Sialyl Lewis-x was formed with a yield of 47%, as determined by scintillation counting.

NMR Study. Proton and carbon NMR spectra in D_2O at 21 °C were assigned by a combination of 1D and 2D techniques using a Bruker AMX-500 NMR spectrometer equipped with an X-32 computer and an ASPECT-3000 process controller. A broad band inverse probe was used for all 2D experiments, and the sample was not spun. All NMR data were processed, analyzed, and plotted with the Felix program (Hare Research, Woodinville, WA) run on a Silicon Graphic 4D/35 Personal Iris workstation.

The proton double quantum filtered (DQF) COSY experiment[62] was performed in the phase-sensitive mode using the time proportional phase incrementation (TPPI)[63] at a spectral width of 2500 Hz. The evolution time was incremented in steps at 200 μs to obtain 512 FIDs each acquired in 2K data points in 32 scans. The relaxation delay was 1.5 s. A square sine-bell function shifted by $\pi/2$ was applied for processing in the t_2 dimension. The same window function was applied in the t_1 dimension, and zero filling was used to expand the data matrix to 1K in this dimension. TOCSY spectra[64] were recorded using the MLEV-17 phase scheme described by Bax and Davis,[65] with a total mixing time of 125 ms. A total of 512 TPPI experiments were collected with 32 transients per t_1 and a relaxation delay of 1.5 s. The data were transformed as a 1K × 1K matrix with $\pi/2$ phase-shifted sine-bell apodiation applied in both dimensions. Rotating-frame NOE spectroscopy involved the pulse sequence 90–t_1–SL–FID,[66] where SL stands for a continuous spin-lock pulse of 200 ms at a field strength corresponding to a 90-deg pulse width between 90–100 μs. The carrier frequency was placed at the left side of the spectrum at 6.2 ppm in order to minimize HOHAHA type magnetization transfer. The {^{1}H,^{13}C} one-bond shift correlation spectrum was obtained in the ^{1}H detection mode by an HMQC experiment.[67] The ^{1}H spectral width was 2500 Hz, and the ^{13}C spectral width was 17 500 Hz. No ^{13}C decoupling was applied during data acquisition. By increasing t_1 in steps, 400 FIDs were collected, each consisting of 4K data points. The relaxation delay was 1.5 s. A sine-bell function shifted by $\pi/2$ was applied in the t_2 dimension, and a Gaussian window (line broadening 5 Hz) was applied in the t_1 dimension. Zero filling to 1K was used in the t_1 dimension before Fourier transformation.

Conformational Analysis. The conformational analysis was first performed on a Silicon Graphics Iris workstation 4D/35, using the GESA program in the GEGOP (geometry of glycoproteins) software package[35] kindly provided by Drs. B. Meyer and K. Bock. In addition to energy minimization, two-parameter grid searches were carried out for ϕ and ψ angles vs internal energy for each glycosidic linkage while holding the others constant at its minimum. The local minima obtained from GESA

(60) Pierce, M.; Cummings, R. D.; Roth, S. *Anal. Biochem.* **1980**, *102*, 441.

(61) Palcic et al. reported K_m 1.3 ± 0.1 mM for GlcNAc. Palcic, M. M.; Srivastava, O. P.; Hindsgaul, O. *Carbohydr. Res.* **1987**, *159*, 315.

(62) (a) Piantinis, U.; Sorensen, O. W.; Ernst, R. R. *J. Am. Chem. Soc.* **1982**, *104*, 6800. (b) Rance, M.; Sorensen, O. W.; Bodenhausen, G.; Wagner, G.; Ernst, R. R.; Wuthrich, K. *Biochem. Biophys. Res. Commun.* **1983**, *117*, 479.

(63) Marion, D.; Wuthrich, K. *Biochem. Biophys. Res. Commun.* **1983**, *113*, 967.

(64) (a) Braunschweiler, L.; Ernst, R. R. *J. Magn. Reson.* **1983**, *53*, 521. (b) Subramanian, S.; Bax, A. *J. Magn. Reson.* **1987**, *71*, 325.

(65) (a) Bax, A.; Davis, D. G. *J. Magn. Reson.* **1985**, *63*, 207. (b) Davis, D. G.; Bax, A. *J. Am. Chem. Soc.* **1985**, *107*, 2820.

(66) Bax, A.; Davis, D. G. *J. Magn. Reson.* **1985**, *65*, 355.

(67) (a) Muller, L. *J. Am. Chem. Soc.* **1979**, *101*, 4481. (b) Cassels, F. J.; Fales, H. M.; London, J.; Carlson, R. W.; van Halbeek, H. *J. Biol. Chem.* **1990**, *265*, 14127. (c) Bax, A.; Subramanian, S. *J. Magn. Reson.* **1986**, *67*, 565. (d) Lerner, L.; Bax, A. *Carbohydr. Res.* **1987**, *166*, 35. (e) Poppe, L.; van Halbeek, H. *Magn. Reson. Chem.* **1991**, *29*, 848. (f) Cavanagh, J.; Keeler, J. *J. Magn. Reson.* **1988**, *77*, 356.

were further minimized through molecular mechanics using the MM2 program in a Tektronik CAChe workstation. The energies given in the tables are calculated from a vacuum and are to be considered as qualitative only to support the experimental observation.

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Genetic Engineering of Surface Attachment Sites Yields Oriented Protein Monolayers†

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Abstract: There has been considerable interest in the use of self-assembly and Langmuir-Blodgett techniques to generate ordered macromolecular monolayers. We describe a general method of using genetic engineering to produce a unique chemical group on the surface of a protein at a predefined site which can be used to orient the macromolecule in a self-assembled film. Using the heme protein cytochrome b_5, produced from a totally synthetic gene, allows direct determination of orientation through measurement of absorbance linear dichroism.

There is considerable current interest in the use of biologically derived macromolecules for materials applications.[1] However, while the naturally occurring variety of protein structure and function is impressive, biomaterial fabrication is inherently limited by the availability and inflexibility of the native protein pool. Protein engineering techniques offer, in principle, the means to circumvent these limitations by allowing the equivalent of synthetic flexibility. For example, genetic engineering techniques represent a potential means to achieve the defined assembly of protein thin films. The construction of close-packed monolayers of oriented cytochrome b_5 on optical substrates has been achieved through the introduction of unique thiol functionalities at defined surface sites. These protein thin films are self-assembled through the covalent linkage of cysteine mutants with a sulfhydryl specific silane layer, and linear dichroism measurements of the resultant heme orientation show that non-random monolayer assemblies are formed. Most importantly, differential heme orientation can be achieved through the choice of attachment site, demonstrating a striking means to manipulate molecular orientation in protein monolayers.

Cytochrome b_5 is a small, 13 000 daltons, bis-imidazole-ligated heme protein for which high resolution crystal and solution structures are available.[2,3] Heme proteins are attractive candidates for biomaterial fabrication due to the unique optical and electronic properties associated with the heme prosthetic group.[4] Because the heme orientation is constrained by the protein scaffolding, orientation of the prosthetic group relative to the substrate should be controlled by the stereochemistry of the protein-surface interactions. We reasoned that a first-order manipulation of protein-surface interactions could be accomplished by genetically engineering unique attachment sites on the cytochrome b_5 surface for covalent linkage with a derivatized surface.

Site-directed mutagenesis techniques were utilized to produce two mutants by introducing a unique thiol functional group on the protein surface at two independent points, replacing threonine 8 (mutant T8C) and, separately, threonine 65 (mutant T65C).[5] Glass substrates were prepared by treatment with a silane coupling agent, (3-iodopropyl)trimethoxysilane.[6] Surface coverage measurements utilized the pyridine hemochrome assay for measuring the heme content on derivatized slides.[7] Typical measurements yielded ca. $1.1-1.4 \times 10^{13}$ molecules/cm^2, corresponding to 700-900 Å^2 per protein molecule. The crystallographic dimensions of cytochrome b_5 are approximately $30 \times 25 \times 25$ Å, which suggests that nearly complete monolayer coverage has been achieved. Covalent attachment of cytochrome b_5 to the surface through specific thioether linkages was verified by extensive buffer washings to remove physisorbed protein and comparisons to control experiments with wild-type b_5.

A low-noise single-pass linear dichroism method was utilized to provide the necessary sensitivity for measuring the heme orientation in cytochrome b_5 monolayer assemblies.[8] Because the

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(1) (a) McGirth, K. P.; Tirrell, D. A.; Kawai, M.; Mason, T. L.; Fournier, M. *J. Biotechnol. Prog.* **1990**, *6*, 188. (b) Birge, R. R. *Annu. Rev. Phys. Chem.* **1990**, *41*, 683. (c) Hong, F. *Biosystems* **1986**, *19*, 223.

(2) (a) Mathews, F. S.; Levine, M.; Argos, P. *J. Mol. Biol.* **1972**, *64*, 449. (b) Pochapsky, T. C.; Sligar, S. G.; McLachlan, S. J.; La Mar, G. N. *J. Am. Chem. Soc.* **1990**, *112*, 5258.

(3) Beck von Bodman, S.; Schuler, M. S.; Jollie, D. R.; Sligar, S. G. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 9443.

(4) Gouterman, M. In *The Porphyrins*; Dolphin, D., Ed.; Academic Press, Inc.: New York, 1978; pp 1-165.

(5) Stayton, P. S.; Fisher, M. T.; Sligar, S. G. *J. Biol. Chem.* **1988**, *263*, 13544.

(6) Ti:Zn glass microscope cover slips (60 × 24 mm, Corning) were acid/base washed (3 hot H_2SO_4 × 3 min/hot $NH_4OH-H_2O_2$ (4:1) × 3 min) and derivatized by refluxing 1% solutions of (3-iodopropyl)trimethoxysilane (Petrarch) in dried chloroform (3 h). Siloxane coverage was estimated by spectrophotometric titration of (3-mercaptopropyl)trimethoxysilane treated substrates with dithionitrobenzoic acid and contact angle measurements. Free thiol quantitation yielded ca. 64 A^2/SH group, and water contact angles of 55-60° were measured. Protein modification was executed in Teflon tanks allowing for submersion of half the slide in 50 mM phosphate buffer, pH 7.5-8.0, 5 mM EDTA, with overnight incubation followed by consecutive buffer and distilled H_2O rinses, and final drying under N_2. Control pyridine hemochrome assays and linear dichroism measurements were conducted on slides exposed to wild-type cytochrome b_5 and buffer alone. No heme was measureable and no linear dichroism relative to the blank was observed in either control.

(7) Berry, E. A.; Trumpower, B. L. *Anal. Biochem.* **1987**, *161*, 1.

0002-7863/92/1514-9298$03.00/0