Pergamon

Bioorganic & Medicinal Chemistry, Vol. 3, No. 12, pp. 1625–1630, 1995

0968-0896/95 $9.50 + .00

0968-0896(95)00150-6

Enzymatic Synthesis of a Sialyl Lewis X Dimer from Egg Yolk as an Inhibitor of E-Selectin

Chun-Hung Lin,[a] Makoto Shimazaki,[a] Chi-Huey Wong,[a]* Mamoru Koketsu,[b] Lekh Raj Juneja[b] and Mujo Kim[b]

[a]*Department of Chemistry, The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, CA 92037, U.S.A.*
[b]*Central Research Laboratories, Taiyo Kagaku Co., Ltd, 1-3 Takaramachi, Yokkaichi, Mie 510, Japan*

Abstract—A dimeric sialyl Lewis X (SLe^x) glycopeptide was synthesized enzymatically in three steps from an N-linked oligosaccharide prepared from egg yolk. Treatment of delipidated hen egg yolk with the protease Orientase and neuraminidase gave a dimeric *N*-acetyllactosamine-containing oligosaccharide linked to asparagine. Addition of sialic acid and fucose catalyzed by α-2,3-sialyltransferase and α-1,3-fucosyltransferase provided the dimeric SLe^x, which was shown to be as active as monomeric SLe^x as an inhibitor of E-selectin with IC_{50} 0.75 mM. The synthetic dimeric SLe^x of the mucin type (i.e. SLe^x linked to the 3- and 6-OH groups of Gal) is, however, about five times as active as the monomer. It is suggested that dimeric SLe^x glycopeptides of the mucin type would be effective ligands for E-selectin.

Introduction

Carbohydrate-mediated cell adhesion is an important event in tissue injury, infection and cancer metastasis.[1] One of these adhesion processes is the interaction between the glycoprotein E-selectin expressed on the surface of activated endothelial cells and an oligosaccharide structure distributed on the surface of neutrophils. The tetrasaccharide sialyl Lewis X (SLe^x) has been identified as the ligand recognized by E-selectin.[2]

Recently, several dimeric SLe^x analogs prepared via attachment of two SLe^x molecules to a galactose template, or a butane or pentane diol linker, have been tested as inhibitors of E-selectin.[3] The results revealed that all the galactose template-containing dimers are more active than the monomer and the diol-containing dimers are essentially the same as the monomer, indicating that the inhibition activity depends on the orientation and distance between the two monomers (Fig. 1). Although this finding suggested a possibility of multivalent ligand–receptor interactions *in vivo* (Fig. 2), it was not clear what would be the optimal linker to present the two monomers. In an effort to develop the optimal dimeric SLe^x inhibitor of E-selectin, we report here the preparation of a dimeric SLe^x from a natural source, i.e. the *N*-glycoprotein isolated from hen egg yolk,[4] and evaluation of its inhibitory activity against E-selectin.

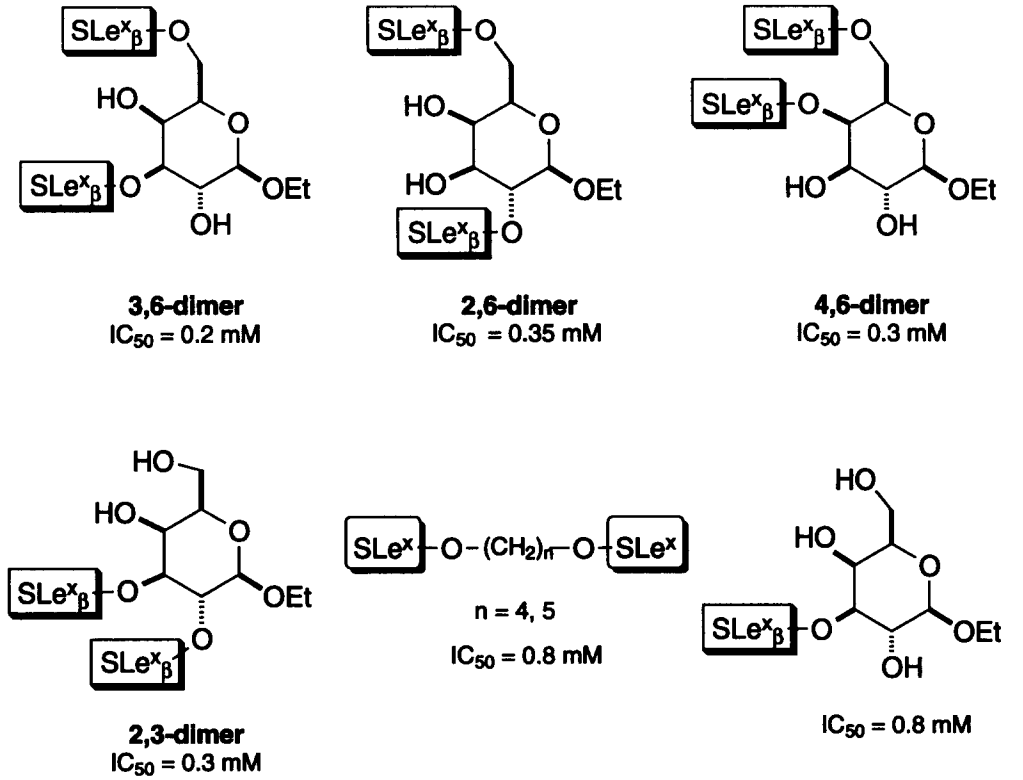
Results and Discussion

Unlike the previous chemo-enzymatic synthesis of monomeric[5] and dimeric[3] SLe^x, the synthesis of dimeric SLe^x linked to a mannose core started from compound **1**, can be easily obtained in two steps from egg yolk: delipidation (to remove unrelated lipid type compounds)[4a,6] and digestion with protease. Approximately 80 g of compound **1** can be prepared from 100 kg of egg yolk. Compound **1** is an N-linked type oligosaccharide and consists of biantennary sialyl LacNAc linked to the branched saccharide $(Man)_3(GlcNAc)_2Asn$.[4a] However, this glycoconjugate contains the α-2,6-linked sialic acids, instead of the α-2,3-linked as expected in SLe^x. Enzymatic modification of **1** was carried out to prepare the bivalent SLe^x (**4**, Fig. 3) via desialylation with neuramidase, sialylation with α-2,3-sialyltransferase and fucosylation with α-1,3-fucosyltransferase (Fig. 4).

In addition to the synthesis of bivalent SLe^x (**4**), this enzymatic process also provides the valuable intermediates **2** and **3**. The former has been shown to have a high affinity for immunoglobulin E-binding protein (IgE-binding protein),[7] while the latter is potentially an effective inhibitor against *Helicobacter pylori.*, which causes gastric inflammation.[8]

The enzymatic synthesis is quite practical and can be easily scaled up as delipidated yolk is readily available. All the enzymes involved are also readily accessible. The protease and neuraminidase can be purchased from commercial sources. Sialyl- and fucosyltransferase are now available by overexpression methods[9] and the enzymatic glycosylation reactions can be performed on a large scale when coupled with cofactor regeneration.[10]

In the inhibition analysis of E-selectin mediated cell adhesion,[3] the dimeric SLe^x glycopeptide (**4**) was, however, found as active as monomeric SLe^x (IC_{50} = 0.75 mM for the dimeric SLe^x; IC_{50} = 800 μM for



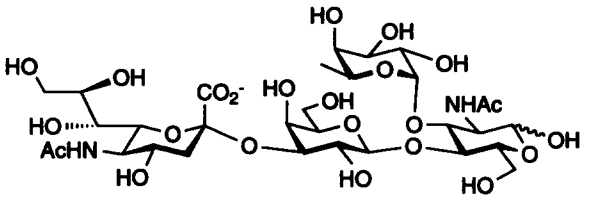


Figure 1. Dimeric sialyl Lewis X (Sle^x) and their IC_{50} in the inhibition of E-selectin.

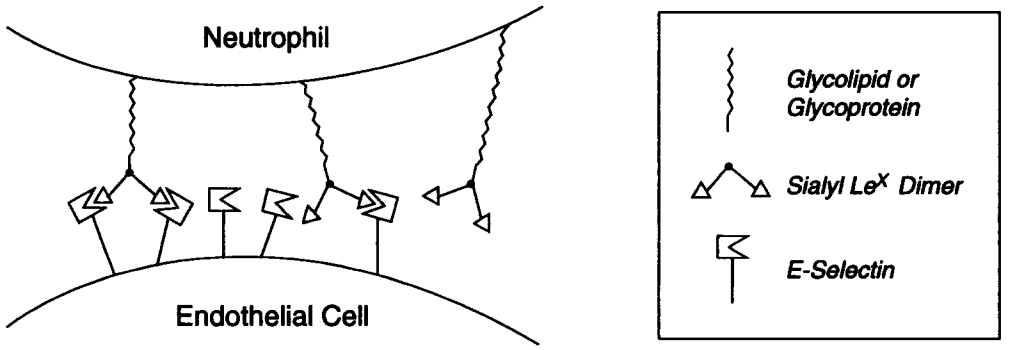


Figure 2. Proposed action of dimeric sialyl Lewis X.

monomeric SLe^x). This result suggests that the relative orientation and distance between the two monomeric SLe^x domains in this type of *N*-glycopeptide structure are not ideal for multivalent interaction with E-selectin. The principle and strategy described here for enzymatic remodeling of N-linked glycoproteins from egg yolk is, however, applicable to the synthesis of other dimeric ligands. Since the synthetic 3,6-dimer reported previously[5c] is about five times as active as the monomer and the 3,6-dimer contains the core structure

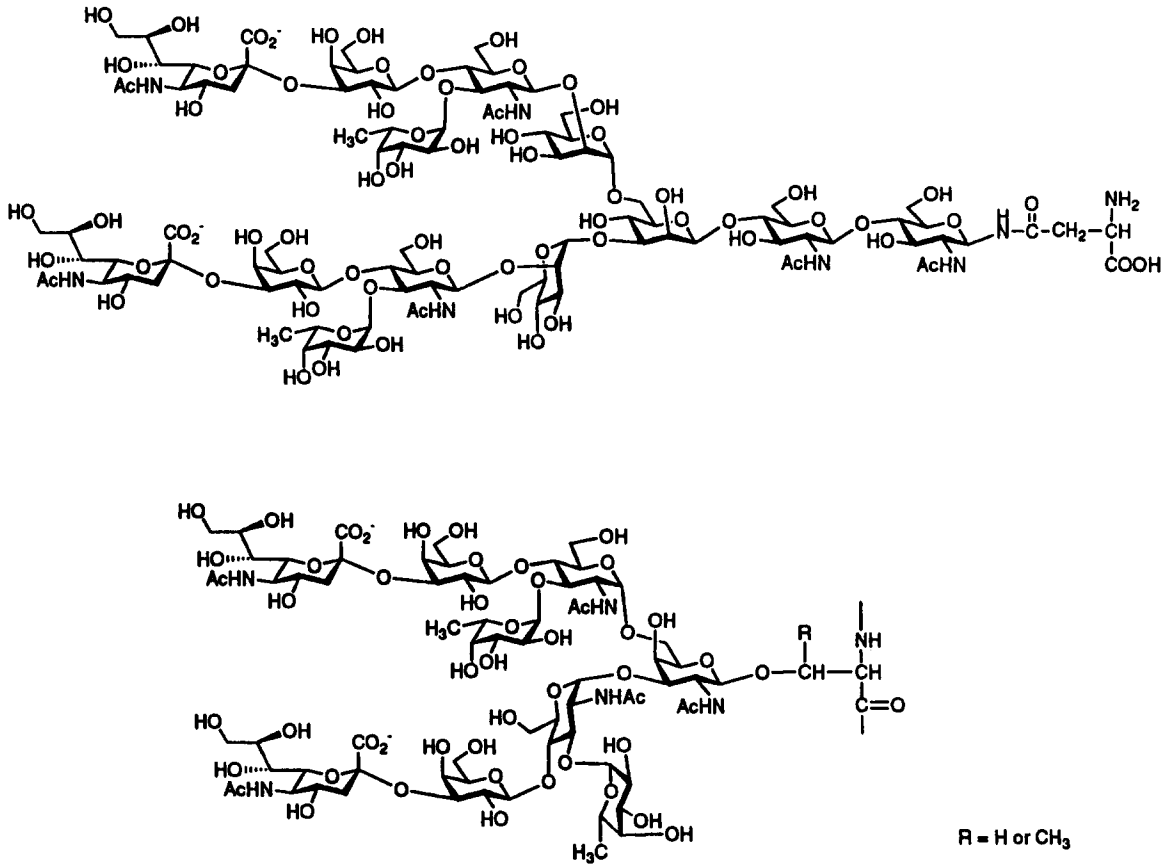


Figure 3. (a) Structure of dimeric SLe^x glycopeptide **4**; (b) structure of dimeric SLe^x in *O*-glycan.

identical to that of the mucin-type carbohydrate, i.e. GlcNAc-β-1,3 (GlcNAc-β-1,6) Gal, it is suggested that dimeric SLe^x glycopeptides of the mucin type would be effective ligands for E-selectin. Work is in progress to use this strategy in the synthesis of other SLe^x dimers from natural source.

Experimental

Materials

Neuraminidase (*Clostridium perfringens*, Type V) was purchased from Sigma. α-2,3-Sialyltransferase (α-2,3-NeuAcT) and α-1,3-fucosyltransferase (α-1,3-FucT) were obtained from Cytel Co. All other enzymes and biochemicals were purchased from Sigma Co. unless otherwise indicated.

Digestion of dilipidated egg yolk and preparation of sialylglycopeptides

Sialylglycopeptides were prepared from the dilipidated egg yolk via treatment with Orientase and ion-exchange chromatography as described previously.[4d]

Gel filtration of glycopeptides mixture (fraction A)

A sample of the sialylglycopeptides (10 g) was further purified by passing through a column (5.0 × 200 cm) of Sephadex G-50, equilibrated with 50 mM Tris-HCl buffer (pH 7.5). The column was eluted with the same buffer and 10 mL of each fraction were collected. Peptides were located by subjecting appropriate aliquots of every fraction to UV absorbance (230 nm). The glycopeptides and sialylglycopeptides were detected by the phenol–H_2SO_4 method[11] and the resorcinol–$CuSO_4$ method,[12] respectively. The effluent pattern was divided into regions and the positive fractions were combined and lyophilized.

Ion exchange column chromatography

A subfraction obtained by the previous gel filtration was dissolved in 50 mM Tris-HCl buffer (pH 7.5) and applied to a column (2.5 × 80 cm) of Sephadex A-25 (Cl^- form), equilibrated with 50 mM Tris-HCl buffer (pH 7.5). Linear gradient elution (0–0.5 M NaCl; 7 mL each fraction) gave the effluent pattern and the positive fractions were combined and lyophilized.

Desalt by gel filtration

A subfraction obtained by the previous ion exchange column chromatography was dissolved in H_2O and applied to a column (2.5 × 50 cm) of Sephadex G-25. A linear gradient elution (0–5% EtOH, 7 mL per fraction)

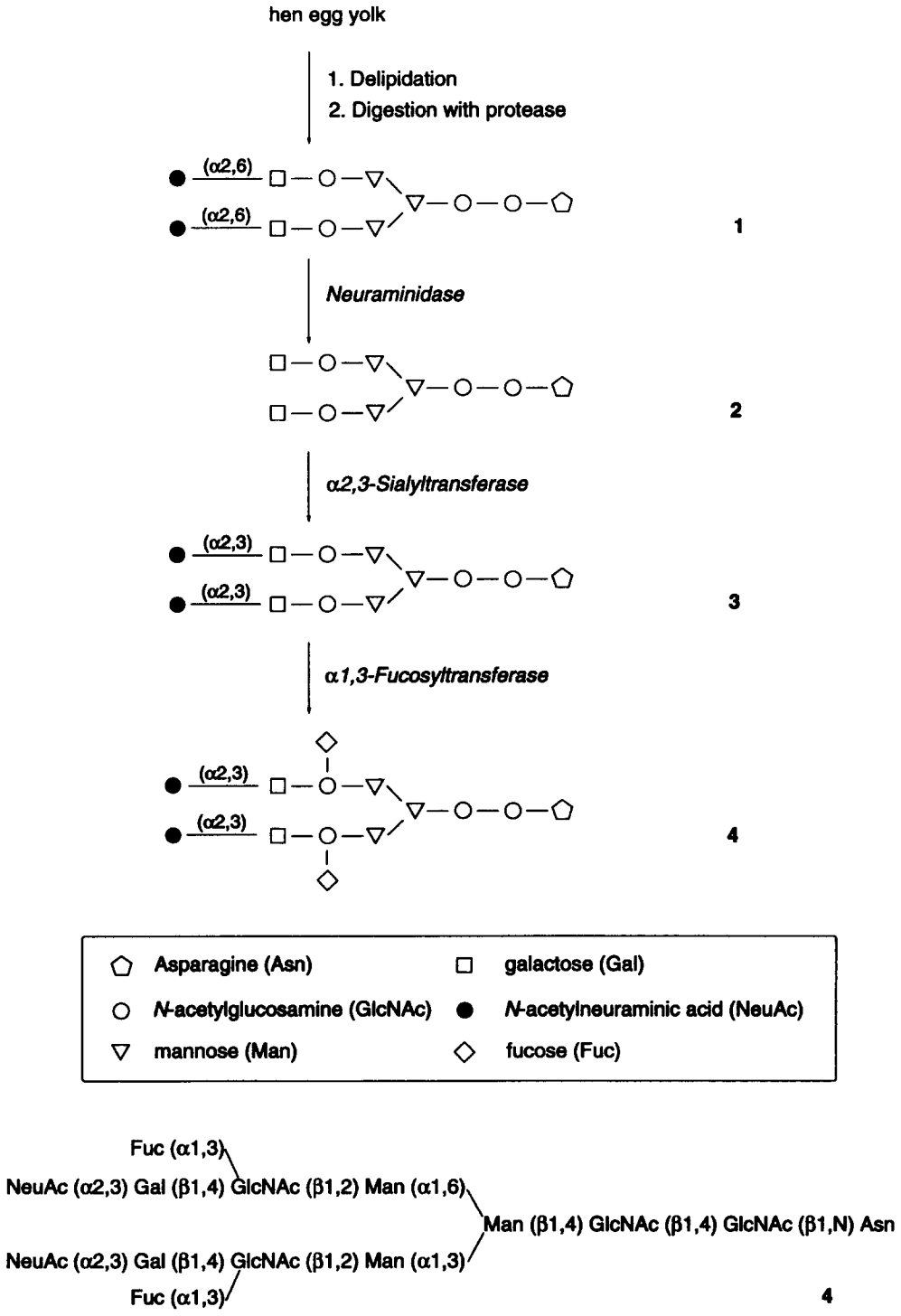


Figure 4. Enzymatic synthesis of dimeric SLe^x glycopeptide **4**.

was performed and the fractions positive to the resorcinol–$CuSO_4$ test were combined and lyophilized to obtain 1.7 g of product as a colorless solid. The structure of this compound was determined by comparison with the authentic ^1H NMR data[13] and confirmed to be compound **1**.

Desialylation with neuraminidase

The sialylglycopeptide **1** (1.7 g) obtained by the previous procedure was dissolved in 50 mM acetate buffer (pH 4.5, 20 mL) and neuraminidase (5 U) was added to the solution. The mixture was stirred at room temperature for two days. After concentration *in vacuo*, the residue was purified by column chromatography using Sephadex A-25 and G-25, as mentioned above, to obtain the corresponding asialo glycopeptide **2** (1.1 g) as colorless plates. The characteristic signals of ^1H NMR are consistent with those reported previously.[13] ^1H NMR (400 MHz, HOD): δ 5.101 (1H, *s*, *J* = 2.5 Hz, H-1 of **D**), 5.030 (1H, *d*, *J* = 9.3 Hz, H-1 of **A**), 4.911 (1H, *d*, *J* = 0.5 Hz, H-1 of **D'**), 4.594 (1H, *d*, *J* = 7.6 Hz, H-1

of **B**), 4.579 (2H, *d*, *J* = 4.8 Hz, H-1 of **E** and H-1 of **E'**), 4.455 (1H, *d*, *J* = 7.7 Hz, H-1 of **F'**), 4.449 (1H, *d*, *J* = 7.8 Hz, H-1 of **F**), 4.369 (1H, m, H-α of Asn), 4.232 (1H, *d*, *J* = 1.2 Hz, H-2 of **C**), 4.176 (1H, *d*, *J* = 3.0 Hz, H-2 of **D**), 4.093 (1H, *d*, *J* = 3.0 Hz, H-2 of **D'**), 2.061 (3H, s, *N*-acetyl of **B**), 2.034 (3H, s, *N*-acetyl of **E**), 2.029 (3H, s, *N*-acetyl of **E'**), 1.990 (3H, s, *N*-acetyl of **A**). MS (electrospray mass): calcd for $C_{66}H_{109}N_6O_{48}$ ($M - H^+$) 1754, found 1754.

Compound 3

A solution of LacNAc dimer **2** (28.9 mg, 0.0152 mmol) and CMP-sialic acid (20 mg, 0.3037 mmol) in Tris-HCl buffer (100 mM, pH 7.5; 1.5 mL) containing 20 mM $MgCl_2$ and 5 mM $MnCl_2$ was added to α-2,3-NeuAcT (0.1 U, 0.4 mL). The mixture was gently stirred under an argon atmosphere at room temperature (25 °C) for three days. The reaction solution was concentrated and purified twice with BioGel P-4, eluted with water, to give **3** (26.3 mg, 74%). The characteristic signals of ^1H NMR are consistent with those reported previously.[13] ^1H NMR (500 MHz, HOD): δ 5.075 (1H, *s*, *J* = 2.5 Hz, H-1 of **D**), 5.030 (1H, *d*, *J* = 9.5 Hz, H-1 of **A**), 4.889 (1H, *s*, H-1 of **D'**), 4.845 (1H, *s*, H-1 of **C**), 4.569 (1H, *d*, *J* = 8.0 Hz, H-1 of **B**), 4.529 (2H, *d*, *J* = 8.0 Hz, H-1 of **E** and H-1 of **E'**), 4.505 (2H, *d*, *J* = 8.0 Hz, H-1 of **F** and H-1 of **F'**), 4.427 (1H, *d*, *J* = 7.5, 2.5 Hz, H-α of Asn), 4.211 (1H, *d*, *J* = 0.5 Hz, H-2 of **C**), 4.151 (1H, *d*, *J* = 3.0 Hz, H-2 of **D**), 4.084 (1H, *d*, *J* = 2.5 Hz, H-2 of **D'**), 4.075 (2H, *dd*, *J* = 2.5, 9.5 Hz, H-3 of **F** and H-3 of **F'**), 2.714 (2H, *dd*, *J* = 12.5, 5.0 Hz, H-3eq of **G** and H-3eq of **G'**), 2.037 (3H, *s*, *N*-acetyl of **B**), 2.015 (3H, *s*, *N*-acetyl of **E**), 2.007 (3H, *s*, *N*-acetyl of **E'**), 1.995 (3H, *s*, *N*-acetyl of $\mathbf{G}^a$), 1.987 (3H, *s*, *N*-acetyl of $\mathbf{G'}^a$), 1.965 (3H, *s*, *N*-acetyl of **A**), 1.758 (2H, *t*, *J* = 12.5 Hz, H-3ax of **G** and H-3ax of **G'**); a: assignment may be reversed. MS (electrospray mass): calcd for $C_{88}H_{143}N_8O_{64}$ ($M - H^+$) 2337, found 2337; calcd for $C_{44}H_{71}N_4O_{32}$ ($M_{1/2}$–H^+) 1168, found 1168.

Compound 4

α-1,3-FucT (0.2 U, 0.5 mL; immobilized on a resin) was added to a solution of sialyl LacNAc dimer **3** (15 mg, 0.0064 mmol) and GDP-fucose (11.2 mg, 0.0141 mmol) in HEPES buffer (100 mM, pH 7.5; 5 mL) containing 20 mM $MnCl_2$. The mixture was gently stirred under an argon atmosphere at room temperature (25 °C) for two days The reaction solution was centrifuged to remove the immobilized enzyme and the supernatant was concentrated and purified twice with Sephadex G-25, eluted with water, to give **4** (12.9 mg, 77%). ^1H NMR (500 MHz, HOD): δ 5.177 (1H, *J* = 4.1 Hz, H-1 of **H'**), 5.073 (1H, *s*, *J* = 2.5 Hz, H-1 of **D**), 5.039 (1H, *d*, *J* = 9.3 Hz, H-1 of **A**), 4.866 (1H, *s*, H-1 of **D'**), 4.824 (1H, *s*, H-1 of **C**), 4.725 (1H, *d*, *J* = 7.8 Hz, H-1of **B**), 4.544 (2H, *m*, H-1 of **E** and H-1 of **E'**), 4.478 (2H, *d*, *J* = 7.5 Hz, H-1 of **F** and H-1 of **F'**), 4.420 (1H, *m*, H-α of Asn), 4.225 (1H, *s*, H-2 of **C**), 4.154 (1H, *d*, *J* = 2.5 Hz, H-2 of **D**), 4.064 (1H, *d*, *J* = 2.5 Hz, H-2 of **D'**), 4.054 (2H, *dd*, *J* = 2.5, 10.0 Hz, H-3 of **F** and H-3 of **F'**), 2.728 (2H, *dd*, *J* = 12.0, 5.4 Hz, H-3eq of **G** and H-3eq of **G'**), 2.047 (3H, *s*, *N*-acetyl of **B**), 2.022 (3H, *s*, *N*-acetyl of **E**), 2.007 (3H, *s*, *N*-acetyl of **E'**), 2.007 (3H, *s*, *N*-acetyl of $\mathbf{G}^a$), 1.994 (3H, *s*, *N*-acetyl of $\mathbf{G'}^a$), 1.994 (3H, *s*, *N*-acetyl of **A**), 1.761 (2H, *t*, *J* = 12.5 Hz, H-3ax of **G** and H-3ax of **G'**) 1.130 (6H, *d*, *J* = 5.5 Hz, CH_3 of **H** and CH_3 of **H'**) a: assignment may be reversed. MS (electrospray mass): calcd for $C_{100}H_{163}N_8O_{72}$ (M–H^+) 2629, found 2629; calcd for $C_{50}H_{81}N_4O_{36}$ ($M_{1/2}$–H^+) 1314, found 1314.

References and Notes

1. (a) Paulson, J. C. In *The Receptors*; M. Conn, Ed.; Academic Press: New York, 1985; Vol. 2; pp 131–219; (b) Paulson, J. C. In *Adhesion: Its Role In Inflammatory Disease*; Harlan, J.; Liu, D., Eds; W.H. Freeman: New York, 1992; Ch. 2, p. 19; (c) Springer, T. A.; Lasky, L. A. *Nature* **1991**, *349*, 196; Lasky, L. A. *Science* **1992**, *258*, 964; (d) Rice, G. E.; Bevilacqua, M. P. *Science* **1989**, *246*, 1303; (e) Bevilacqua, M. P.; Pober, J. S.; Hendrick, D. L.; Cotran, R. S.; Gimbrone, Jr M. A. *Proc. Natl. Acad. Sci. USA* **1987**, *84*, 9238; (f) For cDNA of E-selectin: Bevilacqua, M. P.; Stengeling, S.; Gimbrone, Jr M. A..; Seed, B. *Science* **1989**, *243*, 1160.

2. (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. P.; 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.

3. (a) DeFrees, S. A.; Gaeta, F. C. A.; Lin, Y. C.; Ichikawa, Y.; Wong, C. H. *J. Am. Chem. Soc.* **1993**, *115*, 7549; (b) DeFrees, S. A.; Kosch, W.; Way, W.; Paulson, J. C.; Sabesan, S.; Halcomb, R. L.; Huang, D. H.; Ichikawa, Y.; Wong, C. H. *J. Am. Chem. Soc.* **1995**, *117*, 66.

4. (a) Shainkin, R.; Perlmann, G. *J. Biol. Chem.* **1971**, *246*, 2248; (b) Shainkin, R.; Perlmann, G. *Arch. Biochem. Biophys.* **1971**, *145*, 693; (c) Koketsu, M.; Juneja, L. R.; Kawanami, H.; Kim, M.; Yamamoto, T. *Glycoconjugate J.* **1992**, *9*, 70; (d) Koketsu, M.; Seko, A.; Juneja, L. R.; Kim, M. *J. Carbohydr. Chem.*, in press.

5. (a) Lin, Y. C.; Hummel, C. W.; Huang, D. H.; Ichikawa, Y.; Nicolaou, K. C.; Wong, C. H. *J. Am. Chem. Soc.* **1992**, *114*, 5452; (b) Ichikawa, Y.; Liu, J. L.; C.; Shen, G. J.; Wong, C.-H. *J. Am. Chem. Soc.* **1991**, *113*, 6300; (c) Ichikawa, Y.; Lin, Y.-C.; Dumas, D. P.; Shen, G. J.; Garcia-Junceda, E.; Williams, M. A.; Bayer, R.; Ketcham, C.; Walker, L. E.; Paulson, J. C.; Wong, C.-H. *J. Am. Chem. Soc.* **1992**, *114*, 9283.

6. Koketsu, M.; Juneja, L. R.; Ohta, M.; Matsuura, F.; Kim, M.; Yamamoto, T. *J. Food Sci.* **1993**, *58*, 743.

7. The sialyl LacNAc dimer **2** has been proved to have activity towards IgE-binding protein and the result will be published in due course. Regarding the adhesive specificity of IgE-binding protein, see Feizi, T.; Solomon, J. C.; Yuen, C.-T.; Jeng, K.-C. G.; Frigeri, L. G.; Hsu, D. K.; Liu, F.-T. *Biochemistry* **1994**, *33*, 6342.

8. See representative reviews: Boren, T.; Falk, P. *Sci. Am. Sci. Med.* **1994** 29; Garratty, G. *Immunol. Invest.* **1995**, *24*, 213.

9. (a) For fucosyltransferase, see Fukowska-Latallo, J. F.; Larson, R. D.; Nair, R. P.; Lowe, J. B. *Gene Dev.* **1990**, *4*, 1288; Weston, B. W.; Nair, R. P.; Larson, R. D.; Lowe, J. B. *J.*

Biol. Chem. **1992**, *267*, 4152; (b) For sialyltransferase, see Sasaki, K.; Watanabe, E.; Kawashima, K.; Sekine, S.; Dohi, T.; Oshima, M.; Hanai, N.; Nishi, T.; Hasegawa, M. *J. Biol. Chem.* **1993**, *268*, 22782; Kitagawa, H.; Paulson, J. C. *Biochem. Biophys. Res. Commun.* **1993**, *194*, 375; Gillespie, W.; Kelm, S.; Paulson, J. C. *J. Biol. Chem.* **1992**, *267*, 21004.

10. Both enzymatic sialylation and fucosylation have been carried out on large scales using sialyltransferase and fucosyltransferase, respectively. The sialylation couples with *in situ* regeneration of CMP-NeuAc. The step of fucosylation currently utilizes the stoichiometric amount of GDP-Fuc which can be made chemically in five steps. Ichikawa, Y.; Sim, M.-M., Wong, C.-H. *J. Org. Chem.* **1992**, *57*, 2943. Work is in progress to prepare recombinant GDP-Fuc pyrophosphorylase for regeneration of GDP-Fuc.

11. Svennerholm, L. *Biochem. Biophys. Acta* **1957**, *24*, 604.

12. Dubois, M.; Gilles, K.; Hamilton, J. K.; Rebers, P. A.; Smith, F. *Nature* **1951**, *168*, 167.

13. Vliegenthart, J. F. G.; Dorland, L.; van Halbeek, H. *Adv. Carbohydr. Chem. Biochem.* **1983**, *41*, 209.

(*Received in U.S.A.* 20 *July* 1995; *accepted* 28 *August* 1995)