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Inhibition of L- and P-selectin by a rationally synthesized novel core 2-like branched structure containing GalNAc-Lewis^x and Neu5Acα2–3Galβ1–3GalNAc sequences

Rakesh K.Jain[2], Conrad F.Piskorz, Bao-Guo Huang, Robert D.Locke, Hui-Ling Han[1], Andrea Koenig[1], Ajit Varki[1] and Khushi L.Matta[3]

Department of Gynecologic Oncology, Roswell Park Cancer Institute, Buffalo, NY 14263, USA and [1]Glycobiology Program, Cancer Center, and the Divisions of Hematology-Oncology and Cellular and Molecular Medicine, University of California, San Diego, La Jolla, CA 92093–0687, USA

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[2]Present address: TransCell Technologies, Inc., 8 Cedar Brook Drive, Cranbury, NJ 08512

[3]To whom correspondence should be addressed at: Department of Gynecologic Oncology, Roswell Park Cancer Institute, Elm & Carlton Streets, Buffalo, NY 14263

The selectins interact in important normal and pathological situations with certain sialylated, fucosylated glycoconjugate ligands containing sialyl Lewis^x (Neu5Acα2–3Galβ1–4(Fucα1–3)GlcNAc). Much effort has gone into the synthesis of sialylated and sulfated Lewis^x analogs as competitive ligands for the selectins. Since the natural selectin ligands GlyCAM-1 and PSGL-1 carry sialyl Lewis^x as part of a branched Core 2 O-linked structure, we recently synthesized Galβ1–4(Fucα1–3)GlcNAcβ1–6(SE-3Galβ1–3)GalNAc1αOMe and found it to be a moderately superior ligand for L and P-selectin (Koenig *et al.*, *Glycobiology* 7, 79–93, 1997). Other studies have shown that sulfate esters can replace sialic acid in some selectin ligands (Yeun *et al.*, *Biochemistry,* 31, 9126–9131, 1992; Imai *et al.*, *Nature,* 361, 555, 1993). Based upon these observations, we hypothesized that Neu5Acα2–3Galβ1–3GalNAc might have the capability of interacting with L- and P-selectin. To examine this hypothesis, we synthesized Galβ1–4(Fucα1–3)GlcNAcβ1–6(Neu5Acα2–3Galβ1–3)-GalNAcα1-OB, which was found to be 2- to 3-fold better than sialyl Le^x for P and L selectin, respectively. We also report the synthesis of an unusual structure GalNAcβ1–4(Fucα1–3)GlcNAcβ1-OMe (GalNAc-Lewis^x-O-methyl glycoside), which also proved to be a better inhibitor of L- and P-selectin than sialyl Lewis^x-OMe. Combining this with our knowledge of Core 2 branched structures, we have synthesized a molecule that is 5- to 6-fold better at inhibiting L- and P-selectin than sialyl Lewis^x-OMe,

GalNAcβ1-4(Fucα1-3)GlcNAcβ1
6
Neu5Acα2-3Galβ1-3GalNAcα1-OMe

By contrast to unbranched structures, substitution of a sulfate ester group for a sialic acid residue in such a molecule resulted in a considerable loss of inhibition ability. Thus, the combination of a sialic acid residue on the primary (β1–3) arm, and a modified Le^x unit on the branched (β1–6) arm on an O-linked Core 2 structure generated a monovalent synthetic oligosaccharide inhibitor superior to SLe^x for both L- and P-selectin.

Key words: Core 2 branched structures/selectins/selectin inhibitors

Introduction

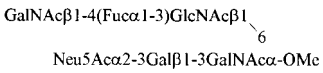
The selectins are mammalian lectins that mediate the early steps of recruitment of leukocytes from the bloodstream in a variety of normal and pathological situations (see Bevilacqua and Nelson, 1993; Rosen and Bertozzi, 1994; Varki 1994, 1997; Lasky, 1995; Lowe and Ward, 1997; McEver and Cummings, 1997, for reviews). All three members of this family of adhesion molecules (L-, P-, and E selectin) have amino terminal C-type lectin domains that originally predicted their ability to bind carbohydrates. The role of sialic acids in ligand recognition by the selectins was also evident from early studies. A landmark event in this field occurred when several groups independently reported that the selectins recognize sialylated fucosylated ligands containing the sialyl Le^x motif (SLe^x) Neu5Acα2–3Galβ1–4 (Fucα1–3)GlcNAcβ1- (structure **1**) (Phillips *et al.*, 1990; Walz, 1990; Brandley, 1990). Later it was shown that sialyl Le^a type structures can also bind with selectins (Berg *et al.*, 1991). Also, Grinell *et al.*, (1994) demonstrated that an uncommon sequence GalNAcβ1–4(Fucα1–3)GlcNAc (GalNAc-Le^x) occurred as a terminal structure on certain recombinant glycoproteins, and that it was a better inhibitor than SLe^x for E-selectin.

It has also been well documented that the sulfate group can replace sialic acid in some instances as ligands for selectins (Yuen *et al.*, 1992; Imai *et al.*, 1993). One of us (K.M.) was the first to synthesize 3-O-sulfoLe^x SE-3Galβ1–4(Fucα1–3)GlcNAc (Chandrasekaran *et al.*, 1991, 1992; Matta, 1997). Synthetic sulfo Le^x derivative SE-3Galβ1–4(Fucα1–3)GlcNAcβ1-OMe **2** is recognized by all three selectins (Koenig *et al.*, 1997). These findings stimulated interest in the chemical and enzymatic synthesis of SLe^x **1** and its isomer SLe^a, Neu5Acα2–3Galβ1–3(Fucα1–4)GlcNAcβ1-OMe (structure **3**), their sulfated analogs and other modified structures related to these motifs (for example, Nicolaou *et al.*, 1991, 1993; Tyrrel *et al.*, 1991; Maaheimo *et al.*, 1995; Bamford *et al.*, 1996; Dupre *et al.*, 1996; Marron *et al.*, 1996; Sanders *et al.*, 1996; Sprengard *et al.*, 1996; Tsuboi *et al.*, 1996; Woltering *et al.*, 1996).

Most of the efforts towards the procurement of such small molecule inhibitory ligands for selectins have been centered on the synthesis of mimics of SLe^x type structures. Of note, the affinity of selectins for all these synthetic analogs appear to be much poorer than those of the natural glycoconjugate ligands such as GlyCAM-1, CD34 (Baumheiter *et al.*, 1993; Hemmerich 1994, 1995), P-selectin glycoprotein ligand-1 (PSGL-I) (Moore *et al.*, 1994; Wilkins *et al.*, 1996), and mucosal cell adhesion molecule (MadCAM-I) (Berg *et al.*, 1993), all of which are mucin

type glycoproteins carrying large numbers of O-linked chains which include sialylated fucosylated sequences (Krall, 1997).

The complex chains of O-linked glycoproteins consist of three distinct regions: core, backbone, and nonreducing terminus. The core structures are unique to O-linked glycoproteins, while the backbone and nonreducing terminus can be found in glycopeptides and also as part of N-linked glycoproteins. Among the known core structures (Schachter, 1986; Varki and Marth, 1995) in which GalNAc is α-linked to Ser/Thr, Core 2, GlcNAc-β(1–6)[Galβ(1–3)]GalNAcα1-O Ser/Thr, appears to be the most prominent among these six structures, and our synthetic selectin inhibitor



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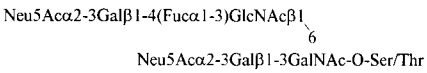
contains this same Core 2 structure. A brief account of our rationale for the synthesis of this inhibitor and its analogs follows.

Structural studies on the carbohydrate moieties of these ligands are only available for PSGL-1 and GlyCAM-1. Surprisingly, PSGL-1 is not heavily fucosylated, and the majority of O-glycans are disialylated or neutral forms of core 2 structures (Wilkins *et al.*, 1996). The following Core 2 branched SLe^x containing structure has been reported as a significant component of both PSGL-1 and GlyCAM-1 (Hemmerich *et al.*, 1995):



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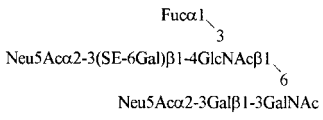
However, according to Hemmerich (1995), sulfate was reported to be present at the C-6 position of galactose, or 6 position of GlcNAc of the SLe^x moiety linked to the C-6 position of GalNAc (structures **5** and **6**). We therefore recently synthesized Neu5Acα2–3(SE-6Galβ1–4(Fucα1–3)GlcNAcβ1-OMe (structure **7**) (Jain *et al.*, 1994). However, our data indicate that this is neither a superior nor a specific ligand for L-selectin (Koenig *et al.*, 1997). The only obvious difference was that recognition by E-selectin was lost upon sulfation. The isomeric compound Neu5Acα2–3Galβ1–4(Fucα1–3)SE-6GlcNAcβ1–3Gal was synthesized by Scudder *et al.* (1994) and found to be only moderately superior to SLe^x as an inhibitor of L-selectin.



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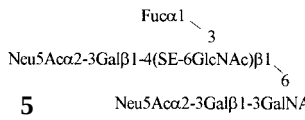

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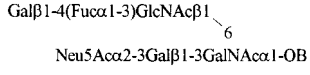
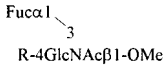
One explanation of the lack of high affinity of 6 and 6′-sulfo $Lewis^x$ for L-selectin (compared to native ligand of GLYCAM-1) could be that the whole carbohydrate moiety represented in **5** and **6** is involved in interaction with L-selectin. In other words, the Neu5Acα2–3Galβ1–3GalNAc- moiety of the Core 2 mucin structure might exert an appreciable impact on binding of L-selectin along with sulfated sialyl $Lewis^x$ linked at C-6 position in structures **5** and **6**. Based on these data, we decided to examine the role of the Neu5Acα2–3Galβ1–3GalNAc sequence by synthesizing Core 2 branched structures. For example, we synthesized structure **34**:



which was better than Sle^x.

We have now synthesized GalNAcLe^x-β-OMe and studied its inhibitory properties towards all three selectins. We have combined this with our knowledge of the properties of the sialylated Core 2 structures to rationally design a better inhibitor **27** for L- and P-selectin.

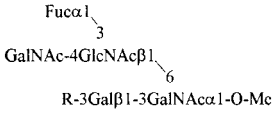
Table I. Relative inhibitory properties of SLe^x and GalNAcLe^x against recombinant selectins binding to immobilized SLe^x



		IC_{50} values (±SD) (μM) against SLe^x		
Compound	**R**	E-Selectin	P-Selectin	L-Selectin
1	Neu5Acα2–3Galβ	550 ± 40	500 ± 25	600 ± 30
18	GalNAcβ	500 ± 120	400 ± 100	300 ± 100

The compounds shown were tested in an ELISA competition assay against the binding of recombinant selectins as described (*Materials and methods*). IC_{50} values (μM) against immobilized SLe^x are the mean values of three separate experiments, and were calculated as described under *Materials and methods*.

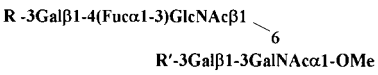
Table II. Relative inhibitory properties of branched chains including Neu5Ac and/or GalNAcLe^x against recombinant selectins binding to immobilized Sle^x



		IC_{50} values (±SD) (μM) against SLe^x		
Compound	**R**	E-Selectin	P-Selectin	L-Selectin
24	OH	>500	400 ± 60	300 ± 80
27	Neu5Ac	>500	85 ± 55	105 ± 75
31	3-SE	>500	>500	500 ± 70

These compounds were tested in an ELISA competition assay against the binding of recombinant selectins as described (*Materials and methods*). IC_{50} values (μM) against immobilized SLe^x are the mean values of two or three experiments, and were calculated as described under *Materials and methods*. Some examples of inhibition curves are shown in Figure 2.

Table III. Relative inhibition data of branched chains containing sulfate at one arm and sialic acid at the other arm of core 2 branched structures



		IC_{50} values (±SD) (μM) against SLe^x		
		E	L	P
8	R = H, R′ = SE	600 ± 100	300 ± 50	270 ± 50
32	R = SE, R′ = Neu5Ac	590 ± 90	500 ± 10	585 ± 25
33	R = Neu5Ac, R′ = SE	200 ± 40	620 ± 90	450 ± 70
34	R = H, R′ = Neu5Ac, α-benzyl analog GalNAc	500 ± 50	200 ± 50	300 ± 55

Results and discussion

A series of branched structures have been examined for inhibition of these three selectins. Synthetic strategies for the synthesis of oligosaccharides represented by structures **32** and **33** (Table III) have been described by Huang *et al.* (unpublished observations),

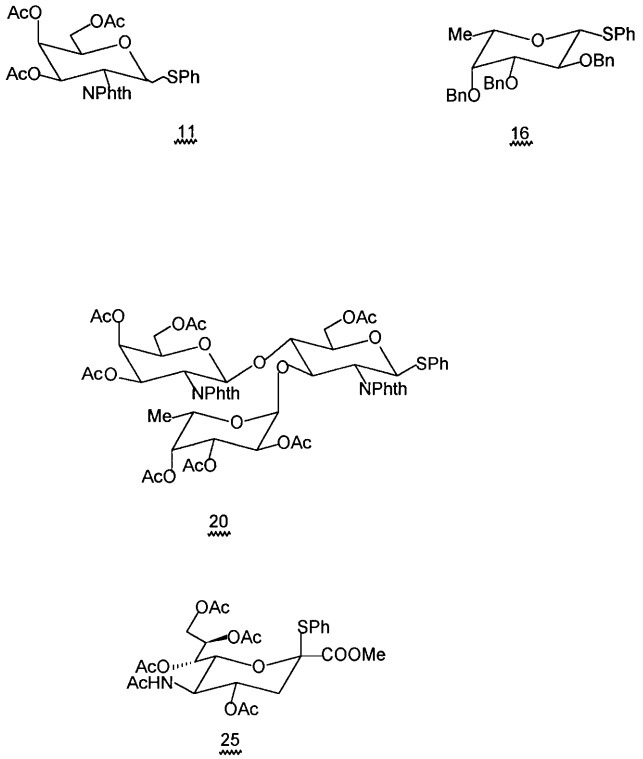


Fig. 1. Key glycosyl donors involved in the synthesis of target compounds.

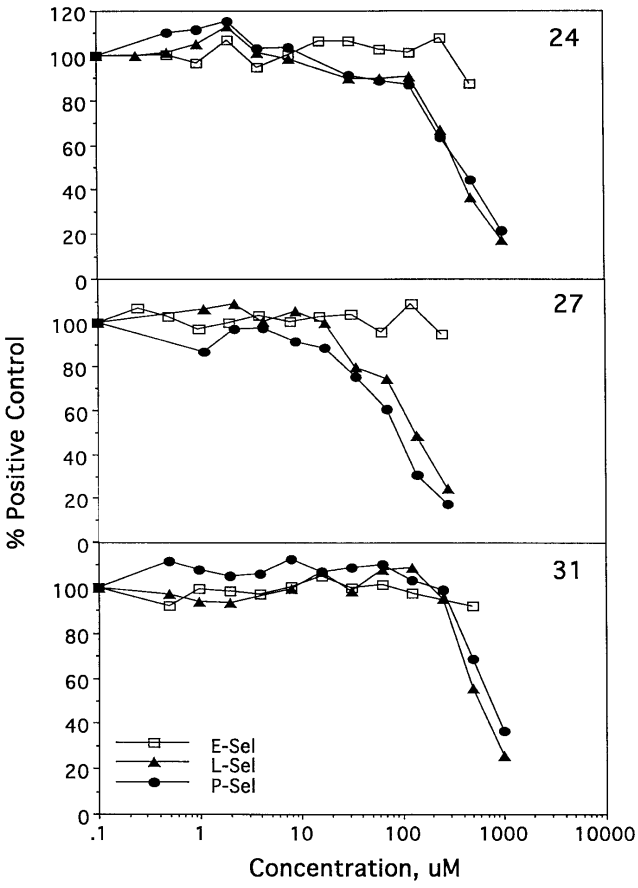


Fig. 2. Inhibitory properties of Compounds **24**, **27**, and **31** against E-, L-, and P-selectin against immobilized SLe^x. Compounds **24**, **27**, and **31** were tested for their ability to inhibit the binding of each of the recombinant selectins to immobilized SLe^x in ELISA inhibition experiments, as described under *Materials and methods*. The IC_{50} values from such studies and the structures of the compounds **24**, **27**, and **31** are represented in Table III.

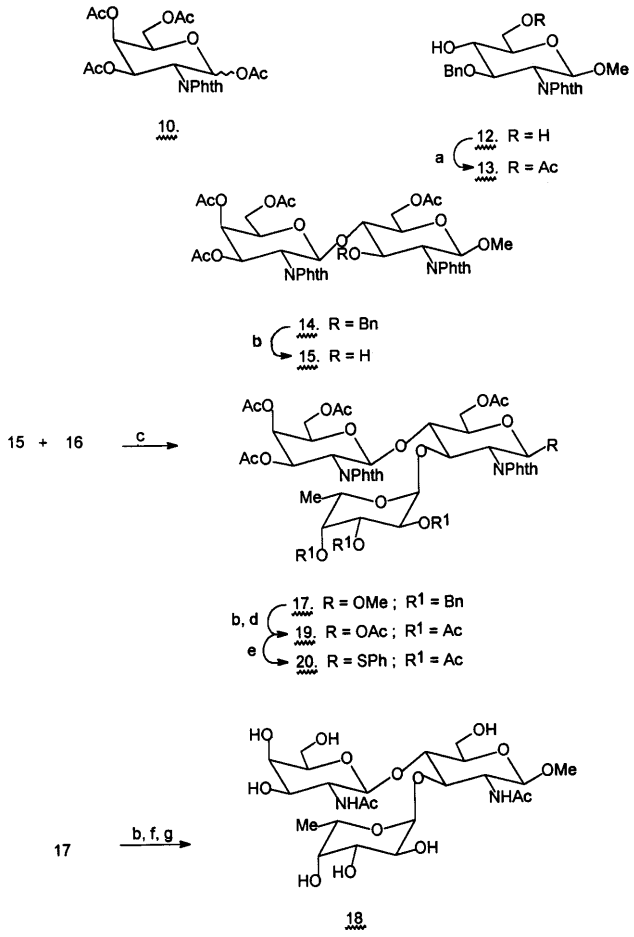
whereas synthesis of **34** will be published elsewhere. In the present studies, under *Materials and methods* we have described the synthesis of **18** (Table I), **24**, **27** and **31** (Table II). Figure 1 represents the four key 1-thio glycosyl donors employed in our synthetic schemes **1–4** depending upon the target structures. For introduction of GalNAcβ- linkage, we have utilized a key glycosyl donor phenyl 3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido-1-thio-α/β-D-galactopyranoside (**11**). Compound **11** was prepared by treatment of known 1,3,4,6-tetra-O-acetyl-2-deoxy-2-phthalimido-α/β-D-galactopyranose (**10**) (Ogawa, 1992) with thiophenol in dichloromethane and in the presence of borontrifluoride-ethearate. Compound (**11**) existed largely as the β-anomer (α/β ratio 1:4) as judged by its ^{1}H NMR spectrum.

Scheme I represents the synthesis of **18** and key glycosyl donor **20** for the synthesis of GalNAc Le^x linked compounds. Regioselective acylation of methyl 3-O-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranoside **12** (Alais and David, 1990) with acetyl chloride in pyridine afforded the 6-O-acetyl derivative (**13**) in 79% yield. Glycosylation (catalyzed by N-iodosuccinimide-triflic acid (Veeneman *et al.*, 1990) of **13** with donor **11** gave (**14**) in 52% yield. Hydrogenolytic cleavage of the benzyl group of **14** in glacial acetic acid and in the presence of 10% palladium-on-carbon furnished the partially protected disaccharide (**15**). α-L-Fucosylation of **15** with the tri-O-benzyl thiophenyl donor (**16**) in the presence of N-iodosuccinimide-triflic acid (Scheme I) afforded the fully protected trisaccharide derivative (**17**) in 68% yield. Compound **17** was converted, in 76% yield, into the diphthaloyl peracetate (**19**) by hydrogenolysis (glacial acetic acid-10% Pd-C), followed by acetolysis (acetic anhydride–acetic-acid–sulfuric acid). Compound **19** was, in turn, converted (49% yield) into the key glycosyl donor (**20**) by treatment with thiophenol and boron-trifluoride ethearate. A portion of **17** was converted to free trisaccharide GalNAcβ1–4(Fucα1–3)GlcNAc-β1-OMe **18** in three steps; removal of O-benzyl group by hydrogenolysis, and phthalamido removal followed by N-acetylation. The enzymatic synthesis of **18** has been reported (Bergwerff *et al.*, 1993).

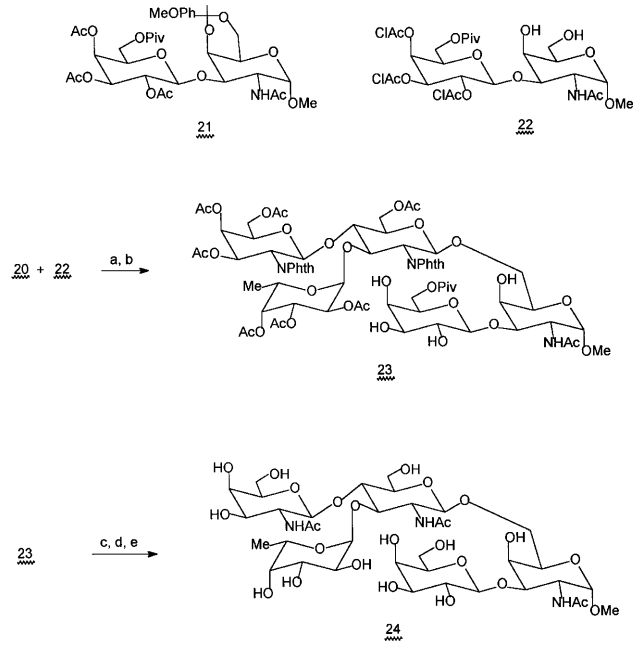
Selective deacylation of methyl O-(2,3,4-tri-O-acetyl-6-O-trimethylacetyl-β-D-galactopyranosyl)-(1–3)-2-acetamido-2-deoxy-4,6-O-(4-methoxybenzylidene)-α-D-galactopyranoside (**21**) in 1:1 dichloromethane-methanol (0.5 M NaOMe, pH ~11), followed by chloroacetylation and cleavage of the 4-methoxybenzylidene acetal with 70% aqueous acetic acid afforded compound **22** (unpublished observations) (Scheme II).

N-Iodosuccinimide-triflic acid glycosylation of compound (**22**) with glycosyl donor **20**, followed by removal of the chloroacetyl groups in the β-galactopyranosyl residue afforded the partially-protected pentasaccharide intermediate (**23**) in 35% yield (based on **22**). The ^{1}H NMR spectrum of **23** was in conformity with the overall structure expected.

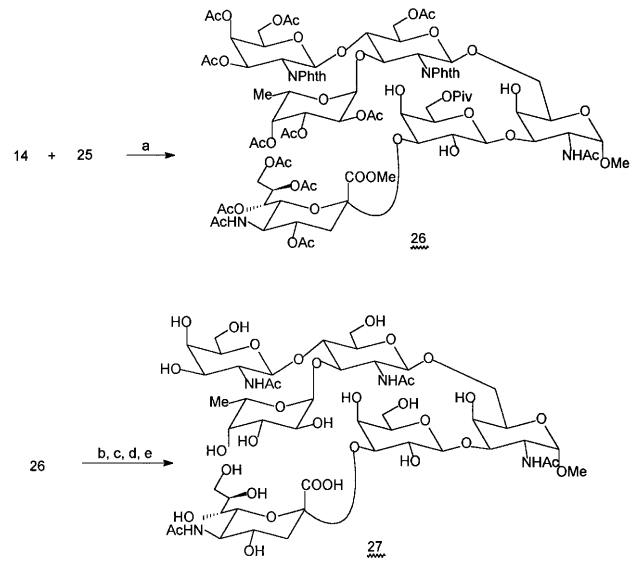
Compound **23** is the key intermediate for obtaining the desired inhibitors **24** and **27**. Thus, for the production of compound **24** the partially-protected **23** was subjected to complete removal of the blocking groups in three successive steps (see *Materials and methods*), whereas for obtaining the sialylated compound **27**, the same intermediate **23** was allowed to react with known (Marra and Sinay, 1989) sialyl donor (**25**) to give, in 47% yield, the



Scheme I. (a) Acetyl chloride-pyridine-CH_2Cl_2, -30°C, 2 h; (b) 10% Pd-C, acetic acid; (c) NIS-triflic acid; CH_2Cl_2-ether (1:1, v/v), 0°C; (d) acetic acid- H_2SO_4-acetic anhydride, 5°C, 16 h; (e) BF_3-ethereate-thiophenol, CH_2Cl_2, 5 h, 30°C; (f) hydrazine hydrate ethanol (4:1, v/v), 100°C, 16 h; (g) methanol triethylamine-acetic anhydride (4:2:1, v/v).



Scheme II. (a) NIS-triflic acid; CH_2Cl_2, -65°C; (b) thiourea-lutidine, ethanol- CH_2Cl_2 (1:1, v/v), 6 h, 80°C; (c) hydrazine hydrate-methanol (4:1, v/v); (d) methanol-triethylamine-acetic anhydride (4:2:1, v/v); (e) MeOH-MeONa.



Scheme III. (a) NIS-triflic acid, propionitrile, -65°C, 3 h; (b) LiI-pyridine, 120°C, 6 h; (c) methanol-hydrazine hydrate (4:1, v/v), 80°C, 16 h; (d) methanol-CH_2Cl_2 (1:1, v/v), acetic anhydride, 0°C, 1 h; (e) MeOH-MeONa, 48 h.

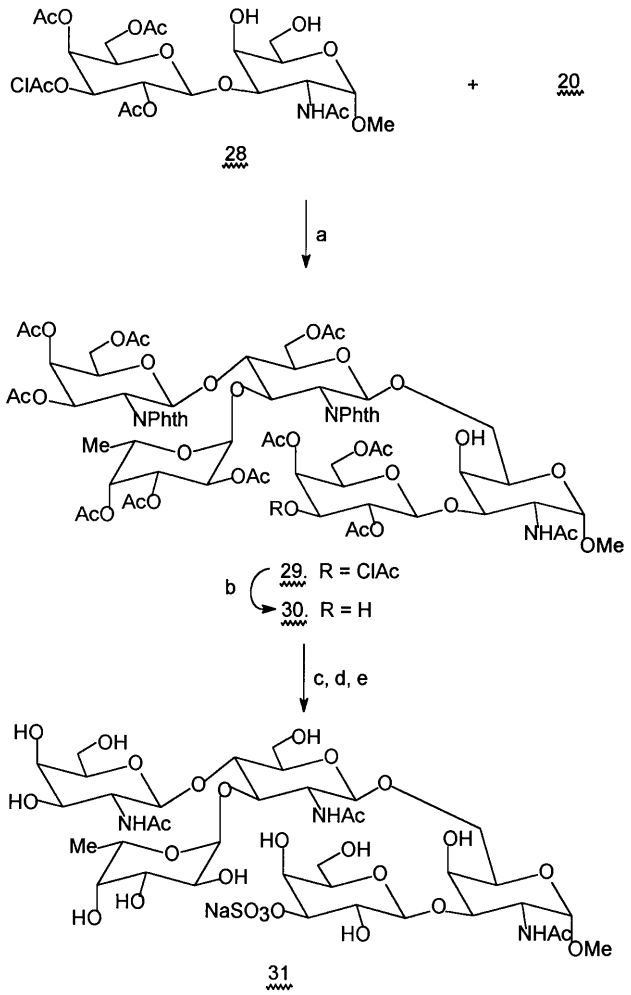
hexasaccharide derivative (**26**) (Scheme III). The α-configuration for the sialic acid residue was confirmed by the ^{1}H NMR of **26** which exhibited a double doublet at δ 2.67 (J = 4.6 Hz), attributable to H-3e of this residue. The conversion of **26** into the target compound (**27**) was performed in four successive steps: (1) lithium iodide-pyridine (Nicolaou *et al.*, 1992) (methyl ester to free acid), (2) methanol-hydrazine hydrate (removal of the phthalimido group), (3) acetic anhydride-methanol-dichloromethane (N-acetylation), and (4) methanolic sodium methoxide (O-deacetylation). The ^{1}H and ^{13}C NMR spectra of **27** were in accord with the structure assigned.

A similar glycosylation of compound (Jain and Matta, 1994) (**28**) with **20** gave, in 76% yield, the pentasaccharide derivative (**29**), the chloroacetyl group of which was removed to give, in 71% yield, intermediate (**30**) (Scheme IV).

For the production of the target compound (**31**), intermediate **30** was treated with five molar equivalents of sulfur trioxide–pyridine complex in N,N-dimethylformamide at 0°C (Scheme IV), followed by customary removal of the protecting groups. Column chromatographic purification on silica gel then furnished the desired compound **31** in 37% yield. The structure and purity of our synthetic compounds was established by TLC, NMR, and FAB mass spectroscopy.

Inhibition studies

To ascertain the importance of GalNAc in the GalNAcLe^x structure, we synthesized the two compounds shown in Table I, and compared their inhibitory properties against all three selectins. As shown in the table, the presence of a β-linked GalNAc residue at the 4 position of GlcNAc(Fucα1–3) was clearly superior to having the α2–3sialylated Gal residue



Scheme IV. (a) NIS-triflic acid; CH_2Cl_2, -65°C; (b) thiourea-lutidine, ethanol-CH_2Cl_2 (1:1, v/v), 6 h, 80°C; (c) SO_3-pyridine complex-DMF, 0°C, 5 h; (d) methanol-hydrazine hydrate (4:1, v/v), 90°C, 5 h; (e) Methanol-triethylamine-acetic anhydride (4:2:1, v/v), 0°C→ room temperature, 1 h.

(Neu5Ac2–3Gal) of SLe^x, structure **1**. These data confirm the previous report for E-selectin (Grinnell *et al.*, 1994), and extend them to show that GalNAcLe^x is an even better inhibitor than SLe^x of L- and P-selectin.

Based upon this finding and the rationale discussed in the introduction, we felt that Neu5Acα2–3Galβ1–3GalNAc sequence in Core 2 structure ligands could combine with GalNAcLe^x to obtain even better binding to L- and P-selectin. We have therefore synthesized an oligosaccharide containing both of these sequences. The results of inhibition studies shown in Table II support the value of this approach. It can be seen that putting the GalNAc-Le^x into a Core 2 branched structure made no difference when compared to GalNAc-Le^x. However, adding a sialic acid residue to the Galβ1–3GalNAc sequence of this structure potentiated the inhibitory properties to the point where it is ~6-fold better as an inhibitor of L- and P-selectin. The improvement in binding to E-selectin was not as great. Interestingly, a sulfate ester at the 3-position of Galβ1–3GalNAc could not substitute for the sialic acid residue. These data suggest that L- and P-selectin are recognizing a specific clustered motif involving components of both branches of the Core 2 structure.

Our interest in the synthesis of branched ligands containing Neu5Acα2-Galβ1–3GalNAcα- arm has been also due to the following findings. We recently identified a 3-O-sulfotransferase from ovarian, breast, and other tissues capable of incorporating sulfate esters at C-3 of Gal in the Galβ1–3GalNAc sequence (Kuhns *et al.*, 1995; Chandrasekaran *et al.*, 1996, 1997). Recently, reports of a native oligosaccharide carrying SE-3Galβ1–3GalNAc have appeared (Chance and Mawhinney, 1996; Florea *et al.*, 1997). We synthesized a branched derivative Core 2 structure **8**, containing a 3-O-sulfate ester group instead of sialic acid moiety and found it to be inhibitory for L- and P- selectin (Koenig *et al.*, 1997). This encouraged us to synthesize **34** having the Neu5Acα2–3Galβ1–3GalNAc sequence.

Galβ1-4(Fucα1-3)GlcNAcβ1
6
SE-3Galβ1-3GalNAcα1-OZ **8**

We also synthesized structure **9** wherein the sulfate ester is located at C-6 of Gal in the Galβ1–3GalNAc moiety

Galβ1-4(Fucα1-3)GlcNAcβ1
6
SE-6Galβ1-3GalNAcα1-OMe **9**

In Table III, we have given the inhibition data for compounds **8**, **32–34** and sialyl Lewis^x. The carbohydrate sequence represented by structure **32** has been reported to be part of respiratory mucin (Lo-Guidice *et al.*, 1994).

Compound **32** was found to behave similar to sialyl Lewis^x. Compound **33** was 2-fold better than sialyl Lewis^x against E-selectin and slightly better against P-selectin. Comparative inhibition data of **8**, **32**, and **33** suggest that in core 2 structures, sialyl Lewis^x arm is a preferred site for interacting with E-selectin. However, we have observed an interesting phenomena for L- and P-selectin ligands containing sulfate at the C-3 position of galactose on one arm and sialic acid at C-3 of galactose in the other arm of Core 2 structure. When sulfate is present at C-3 of Gal in Galβ1–3GalNAc the presence of sialic acid at C-3 of Gal in the Le^x moiety further linked to C-6 of GalNAc (as in compound **33)** has a negative impact toward binding, as our previous studies showed that Galβ1–4(Fucα1–3)GlcNAcβ1–6(SE-3Galβ1–3)GalNAcα1-OMe **8** is a 2-fold better ligand than sialyl Lewis^x for L- and P-selectin (Koenig *et al.*, 1997). It is also surprising that when sialic acid is linked to C-3 of Gal in Galβ1–3GalNAc, the presence of a 3-O-sulfo Le^x moiety on C-6 of GalNAc (as in compound **32**) also has a negative impact. Our compound Galβ1–4(Fucα1–3)GlcNAcβ1–6(Neu5Acα2–3Galβ1–3)GalNAcα1-OBn **34** (R.K.Jain and K.L.Matta, unpublished observations) is almost 3-fold better than sialyl Lewis^x for L-selectin. It is apparent that in an approach for the construction of a library of analogs of the Galβ1–4(Fucα1–3)GlcNAcβ1–6(Galβ1–3)GalNAcα1- sequence if sialic acid (or replacement of sialic acid by a carboxy alkyl group) (Allanson, 1993) is kept at the C-3 position of galactose in one arm it will be advisable to introduce sulfate at a position other than C-3 of galactose in the other arm. Thus, it is not surprising to see a sulfate ester group located on C-6 of galactose or GlcNAc, as shown in **5** and **6** which represent portions of GlyCAM-1. Once again, our recent inhibition studies of these selectins with branched Core 2 structures warrant mention here. Interestingly, our synthetic compound **9** having SE-6Galβ1–3GalNAc is found to be a ligand for both L and P selectin. It is striking that 6′-sulfo Le^x or 6-sulfo Le^x type structures without a sialic acid moiety do not act as inhibitors of selectin, whereas the presence of a 6-O-sulfo group on the galactose moiety of Galβ1–3GalNAc showed inhibitory

effects. It should be noted that the SE-6Galβ1–3GalNAc sequence has been reported to be a part of various O-linked mucin glycoproteins (Chance *et al.*, 1996). Also, the Neu5Acα2–3(SE-6Gal)β1–3GalNAc sequence has been suggested to comprise a part of O-linked glycoprotein (Mawhinney *et al.*, 1992). Thus, it is quite possible that earlier mentioned naturally occurring selectin ligands such as CD34 or other unidentified sialo mucin type ligands (Kraal and Mebius, 1997) may contain 6′-sulfo Gal in the Galβ1–3GalNAc chain contained in a core 2 structure in the form of SE-6Galβ1–3GalNAc or SE-6(Neu5Acα2–3)Galβ1–3GalNAcα sequences. In fact, the present studies demonstrating the ability of Neu5Acα2–3Galβ1–3GalNAc to bind to selectins, combined with our previous finding of inhibitory impact of SE-6Galβ1–3GalNAc, enhances our interest in the preparation of branched core 2 structure with SE-6(Neu5Acα2–3)Galβ1–3GalNAc having sialyl Le^x or Le^x moiety O-linked at C-6 position of GalNAc.

It is not clear at this time why an oligosaccharide containing GalNAcβ1–4(Fucα1–3)GlcNAcβ1-OMe sequence, which lacks a negative charge, appears to be a better ligand for E-selectin. However, Grinnell *et al.* (1994) reported SLe^x and GalNAcβ1–4(Fucα1–3)GlcNAc appear to be structurally similar through modeling studies. We became interested in the synthesis of oligosaccharides containing GalNAcβ1–4(Fucα1–3)GlcNAc determinant partly because GalNAc as a starting material is cheaper than sialic acid. Moreover, the chemical synthesis of an oligosaccharide containing the GalNAcβ1–4(Fucα1–3)GlcNAc determinant is not as time consuming as an oligosaccharide with sialyl Lewis x moiety. GalNAcβ1–4(Fucα1–3)GlcNAcβ- sequence has been found to be a part of various glycoproteins, especially N-linked glycoproteins (Dell *et al.*, 1995; Manzella *et al.*, 1996). The disaccharide GalNAcβ1–4GlcNAcβ- is part of certain O-linked glycoproteins (Hirano *et al.*, 1993), some containing fucose (Siciliano, 1994). The sequence GalNAcβ1–4(Fucα1–3)GlcNAc has been reported to be a part of oligosaccharides isolated from sea squirt H antigen, an O-linked glycoprotein (Ohata *et al.*, 1991). The sequence shown in structure 24 has been reported recently (Strecker *et al.*, 1994).

Thus, our present studies provide a new avenue toward the synthesis of GalNAc-Le^x containing branched ligands for the inhibition of L- and P-selectin that is considerably better than SLe^x. Furthermore, we have clearly demonstrated that the Neu5Acα2–3Galβ1–3GalNAc sequence of the core 2 structure contributes to the binding of L- and P- selectin.

Materials and methods

Materials

Sources of several chemicals used are indicated below. Most of the other materials used were obtained from the Sigma Chemical Co. The following materials were obtained from the sources indicated

All other chemicals were of reagent grade or better, from commercial sources. The recombinant L-selectin Ig-fusion chimeric protein was prepared as described (Norgard *et al.*, 1993), and the E- and P-selectin Ig-fusion chimeric constructs were produced using the pcDM8 vectors (Nelson *et al.*, 1993).

ELISA inhibition assays

ELISA inhibition assays were done as previously reported (Koenig *et al.*, 1997). Sterile polystyrene 96 well ELISA plates (no. 25801, Corning) were coated with 200 ng of polyacrylamide-SLe^x (no. 18205PA, Syntesome) by overnight incubation at 4°C in 100 µl of 50 mM sodium carbonate/bicarbonate buffer, pH 9.5. Plates were then blocked with 200 µl per well of assay buffer: 20 mM Hepes (no. 16926, U.S. Biochemical), 125 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 1% protease-free BSA (no. 82–045–1, Pentex, Miles, Inc.), pH 7.45 (osmolality 290 milliosmoles, determined with a Vapor Pressure Osmometer, model 5500XR, Wescor, Inc.) for a minimum of 2 h at 4°C. During the blocking step, the selectin chimeras were separately preincubated at 4°C with a secondary antibody, peroxidase-conjugated goat anti-human IgG (No. 109–035–098, Jackson Immunoresearch Laboratories, Inc.) in assay buffer for ~1 h. Final selectin-Rg concentration was 20 nM, and the optimal secondary antibody dilution was determined to be 1:1000 for the particular serum used. Potential inhibitors were serially diluted in assay buffer, at 2× the final required concentration. The selectin-Rg/secondary antibody stock was aliquoted into tubes containing an equivalent volume of inhibitor solution; buffer alone for the positive control, or buffer with 10 mM Na_2 EDTA, pH 7.5, for the negative control (giving a final concentration of 5 mM EDTA). These tubes were preincubated at 4°C for 30 min, and then added to ELISA plates, in duplicates, at final well volume of 100 µl. After 4 h of plate incubation at 4°C, plates were washed three times with 200 µl per well of assay buffer at 4°C, followed by development with 150 µl per well of OPD solution at room temperature: 0.002 mg *o*-phenylenediamine dihydrochloride (OPD)/ml in 50 mM sodium citrate, 50 mM disodium phosphate buffer, pH 5.2 containing 1 µl/ml 30% H_2O_2. Using a timer, each well was sequentially quenched with 40 µl of 4 M H_2SO_4 after a fixed time of peroxidase reaction. Softmax software and a microplate reader (Molecular Devices, Inc.) determined and recorded absorbance at 492 nM. Prior to curve fitting, the data was changed into percentages for comparative purposes, using the formula: ([average of duplicates) - (negative control)]/[(positive control) - (negative control)] × 100) again with the Softmax software.

General methods

Optical rotations were measured at ~25°C with a Perkin-Elmer 241 Polarimeter. Thin layer chromatography (TLC) was conducted on glass plates precoated with 0.25 mm layers of silica gel 60F-254 (Analtech GHLF uniplates). The compounds were located by exposure UV light or by spraying with 5% H_2SO_4 in ethanol and charring, or by both techniques. The silica gel used for column chromatography was Baker Analyzed (60–200 mesh). NMR spectra were recorded at ~25°C, ^{1}H-spectra with a Varian EM-390 at 90 MHz and with a Bruker AM-400 at 400 MHz, and the ^{13}C-spectra with a Bruker AM-400 at 100.6 MHz. All chemical shifts are referenced to tetramethylsilane. Solutions in organic solvents were generally dried with anhydrous sodium sulfate. Dichloromethane, N,N-dimethylformamide, 1,2-dichloroethane, benzene, and 2,2-dimethoxypropane were kept dried over 4 Å molecular sieves. Elemental analyses were performed by Robertson Laboratory, Madison, New Jersey, USA.

General procedure for glycosidation

Using 1-thiophenyl glycoside as glycosyl donor, a solution of the acceptors (1.0–1.2 mmol) and donor (1.0–1.5 mmol) and N-iodosuccinimide (2.5–3.0 mmol) in dichloromethane (20 ml, for preparation of compound **14**, **23**, and **29**) or 1:1 dichloromethane-ether (30 ml, for compound **17**), propionitrile (15 ml, for compound **26**) was stirred for 0.5 h with 4 Å molecular sieves

(2 g) under an argon atmosphere at 0°C (compound **14**), or -40°C (compound **26**) or -65°C (compound **23** and **29**). Then a dilute solution of trifluoromethanesulfonic acid (triflic acid, 0.2 ml in 10 ml dichloromethane or propionitrile) was added dropwise. Stirring was continued at the same temperature for an additional hour, and the acid was neutralized with aqueous sodium bicarbonate solution. The mixture was filtered (Celite bed), and the solids thoroughly washed with water, saturated sodium bicarbonate solution, 10% sodium thiosulfate solution, dried and concentrated under diminished pressure.

*Phenyl 3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido-1-thio-α/β-D-galactopyranoside (**11**)*

To a stirred solution of **10** (6.4 g, 13.4 mmol) in dichloromethane (70 ml) was added thiophenol (4.0 ml, 36.4 mmol) and BF_3 ethereate (4.0 ml, 28.4 mmol). Stirring was continued for 4 h at room temperature. The reaction mixture was then washed with aqueous sodium bicarbonate solution, water, dried, and concentrated. The residue was purified on a column of silica gel with a solvent gradient consisting of hexane-ethyl acetate 3:2 → 1:1 (v/v) to afford **11** (6.1 g, 84%); $[\alpha]_D$ +28° (c 1.0, $CHCl_3$); ^{1}H NMR (CD_2Cl_2): δ 7.88–7.26 (m, 9 H, arom.), 5.79 (dd, J = 9.1 Hz and 10.1 Hz, 1 H, H-3), 5.70 (d, J = 10.5 Hz, 0.8 H, H-1), 5.49 (d, J = 3.5 Hz, 1 H, H-4), 2.2, 2.06, and 1.98 (each s, 3 × OAc-α), 2.18, 2.04, and 1.81 (each s, 3 × OAc-β).

Anal Calc. for $C_{26}H_{25}NO_9S$: C, 59.19; H, 4.78; N, 2.66. Found: C, 59.21; H, 4.91; N, 2.54.

*Methyl 6-O-acetyl-3-O-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranoside (**13**)*

To a cold (-30°C), stirred solution of methyl 3-O-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranoside **12** (4.8 g, 11.6 mmol) in pyridine (50 ml) was added, dropwise, a solution of acetyl chloride (0.97 ml, 12.4 mmol) in pyridine-dichloromethane (1:2, 15 ml). Stirring was continued for 2 h at the same temperature, and then the mixture was kept overnight at 5–6°C. It was then cooled to O°C and methanol (5 ml) was added to decompose excess reagent. The solvents were removed under diminished pressure and the residue dissolved in dichloromethane. The organic layer was successively washed with 10% aqueous hydrochloric acid, water, saturated sodium bicarbonate solution, dried, and concentrated. The crude product was purified on a column of silica gel with a solvent gradient consisting of 40–50% ethyl acetate in hexane to give **13** (4.2 g, 79%); $[\alpha]_D$ +23° (c 1.0, $CHCl_3$); ^{1}H NMR (CD_2Cl_2): δ 7.73–6.98 (m, 9 H, arom.), 5.04 (d, J = 8.3 Hz, 1 H, H-1), 4.04 (dd, J = 8.6 Hz, H-6), 3.37 (s, 3 H-OMe), 2.14 (s, 3 H, OAc).

Anal Calc. for $C_{24}H_{25}NO_8$: C, 63.29; H, 5.53; N, 3.08. Found: C, 63.31; H, 5.60; N, 3.01.

*Methyl O-(3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido-β-D-galacto-pyranosyl)-(1→4)-6-O-acetyl-3-O-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranoside (**14**)*

Glycosidation of **13** (4.0 g, 8.6 mmol) with **11** (6.0 g, 11.1 mmol) followed by silica gel column chromatography (solvent gradient consisting of hexane-ethyl acetate 3:2 → 1:1) afforded **14** (6.1 g, 52%); $[\alpha]_D$ +10° (c 1.0, $CHCl_3$); ^{1}H NMR (CD_2Cl_2): δ 7.90–6.92 (m, 13 H, arom.), 5.79 (dd, 1 H, H-3′), 5.48 (d, J = 8.7 Hz, 1 H, H-1), 5.44 (d, J = 3.1 Hz, 1 H, H-4′), 4.91 (dd, 1 H, H-2′), 4.46 (d, J = 8.0 Hz, 1 H, H-1′), 3.27 (s, 3 H, OMe), 2.10, 2.03, 2.01, and 1.81 (each s, 12 H, 4 × OAc).

Anal Calc. for $C_{44}H_{44}N_2O_{17}$: C, 60.54; H, 5.08; N, 3.21. Found: C, 60.35; H, 5.11; N, 3.16.

*Methyl O-(3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido-β-D-galactopyranosyl)-(1→4)-6-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranoside (**15**)*

A mixture of compound **14** (1.0 g) and 10% Pd-C (1.0 g) in glacial acetic acid (20 ml) was shaken at ~345 kPa. The suspension was then filtered (Celite bed), the solids were thoroughly washed with glacial acetic acid, and the combined filtrate and washings were concentrated under reduced pressure. The crude product was applied to a column of silica gel and eluted with hexane-ethyl acetate 2:3 → 1:4 (v/v). The fractions corresponding to **15** were pooled and concentrated to give an amorphous solid (0.55 g, 62%); $[\alpha]_D$ -11° (c 1.5, $CHCl_3$); ^{1}H NMR (CD_2Cl_2): δ 7.89–7.74 (m, 8 H, arom.), 5.79 (dd, 1 H, H-3′), 5.06 (d, J = 9.1 Hz, 1 H, H-1), 4.54 (dd, 1 H, H-2′), 3.34 (s, 3 H, OMe), 2.17, 1.91, 1.82 and 1.81 (each s, 12 H, 4 × OAc).

Anal Calc. for $C_{37}H_{38}N_2O_{17}$: C, 56.77; H, 4.89; N, 3.58. Found: C, 56.82; H, 4.91; N, 3.49.

*Methyl O-(2,3,4-tri-O-acetyl-2-deoxy-2-phthalimido-β-D-galactopyranosyl)-(1→4)-O-[2,3,4-tri-O-benzyl-α-L-fucopyranosyl)-(1→3)-O]-6-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranoside (**17**)*

Glycosidation of **17** (3.9 g, 5.0 mmol) with **16** (10.8 g, 20.0 mmol) in dichloromethane-ether (1:1, 100 ml) and purification of the crude product mixture by silica gel column chromatography [solvent gradient consisting of hexane-ethyl acetate 3:2 → 1:1 (v/v) furnished compound **18** (3.0 g, 68%); $[\alpha]_D$ +3° (c 1.5, $CHCl_3$); ^{1}H NMR (CD_2Cl_2): δ 7.88–6.99 (m, 23 H, arom.), 5.79 (dd, 1 H, H-3′), 5.31 (d, J = 4.1 Hz, 1 H, H-1′′), 4.89 (d, J = 8.6 Hz, 1 H, H-1), 4.82 (d, J = 10.0 Hz, 1 H, H-1′), 3.27 (s, 3 H, OMe), 2.07, 2.03, 2.02, and 1.78 (each s, 12 H, 4 × OAc), 1.30 (d, J = 6.5, Hz, 3 H, CMe).

Anal Calc. for $C_{64}H_{66}N_2O_{21}$: C, 64.10; H, 5.55; N, 2.34. Found: C, 64.31; H, 5.51; N_2, 2.16.

*Methyl O-(2-acetamido-2-deoxy-β-D-galactopyranosyl)-(1→4)-O-[α-L-fucopyranosyl)-(1→3)-O]-2-acetamido-2-deoxy-β-D-glucopyranoside (**18**)*

A mixture of **17** (0.3 g) and 10% Pd-C (0.8 g) in glacial acetic acid (20 ml) was shaken under hydrogen at ~345 kPa for 16 h at room temperature. After processing as described for the preparation of **16** (from **12**) and followed by phthalamido removal with hydrazine hydrate-ethanol (1:4; v/v) at 100°C for 16 h and N-acetylation with methanol-triethylamine-acetic anhydride (4:2:1, v/v) afforded **18**. After purification over a silica gel column with $CHCl_3$-MeOH-water (13:6:1→5:4:1) as the eluent, **18** (0.07 g, 51%); $[\alpha]_D$ -88° (c 0.5, H_2O); ^{1}H NMR (D_2O): δ 5.12 (d, J = 3.9 Hz, 1H, H-1′′), 4.55 (d, J = 8.4 Hz, 1H, H-1), 4.49 (d, J = 7.8 Hz, 1H, H-1′), 3.52 (s, 3H, OMe), 2.09 and 2.05 (each s, 6 H, 2×NAc), 1.28 (d, J = 6.6 Hz, 3 H, CMe); ^{13}C-NMR: GalNAc-β-(1→4) residue: 100.69 (C-1), 51.34 (C-2), 70.97 (C-3), 66.50 (C-4), 72.36 (C-5), 60.40 (C-6), 21.15 (NAc); Fuc-α-(1→3) residue: 97.41 (C-1), 66.68 (C-2), 68.16 (C-3), 69.79 (C-4), 65.90 (C-5), 14.33 (C-6); GlcNAc-β-OMe residue: 99.72 (C-1), 54.38 (C-2), 74.44 (C-3), 73.81 (C-4), 73.70 (C-5), 59.01 (C-6), 56.09 (OMe), 21.18 (NAc). ES-MS: *m/z* = 583.22 $[M-1]^-$ (584.58).

Anal Calc. for $C_{23}H_{40}N_2O_{15}$: C, 47.25; H, 6.90; N, 4.79. Found: C, 47.09; H, 6.85; N, 4.67.

*O-(3,4,6-Tri-O-acetyl-2-deoxy-2-phthalimido-β-D-galactopyranosyl)-(1→4)-O-[(2,3,4-tri-O-acetyl-α-L-fucopyranosyl)-(1→3)-O]-1,6-di-O-acetyl-2-deoxy-2-phthalimido-D-glucopyranose (**19**)*

A solution of compound **17** (6.0 g) in glacial acetic acid (60 ml) was treated with 10% Pd-C (4.0 g) and the mixture was shaken for 16 h at room temperature under hydrogen (~345 kPa). The suspension was then filtered (Celite bed) and the solids were thoroughly washed with methanol. The filtrate and washings were combined and concentrated and the residue was directly utilized in the next step. A solution of this residue in acetic acid (80 ml) and acetic anhydride (96 ml) containing conc. H_2SO_4 (8.4 ml) was stirred for 16 h at 5 °C. The mixture was then diluted with dichloromethane (700 ml), and successively washed with water, saturated aqueous sodium bicarbonate solution, water, dried, evaporated to dryness, and redissolved in dichloromethane. Addition of ether-hexane caused the precipitation of **19** as an amorphous solid (4.0 g, 76%); $[\alpha]_D$ -63° (c 1.0, $CHCl_3$); ^1H NMR (CD_2Cl_2): δ 7.92–7.78 (m, 8 H, arom.), 6.00 (d, J = 8.5 Hz, 0.6 H, H1β), 5.94 (d, J = 3.2 Hz, 0.4 H, H-1α), 5.82 (dd, 1H, H-3′) 5.51 (d, J = 3.8 Hz, 1 H, H-4″), 5.48 (d, J = 3.6 Hz, 1 H, H-4′), 5.40 (d, J = 4.6 Hz, 1 H, H-1″), 4.83 (d, J = 10.6 Hz, 1 H, H-1′) 2.22–1.76 (cluster of s, 24 H, 8 × OAc), 1.41 (d, J = 6.7 Hz, 1.8 H, CMe-β), 1.36 (d, J = 6.5 Hz, 1.2 H, CMe-α).

Anal Calc. for $C_{50}H_{54}N_2O_{25}$: C, 55.48; H, 5.03; N, 2.59. Found: C, 55.29; H, 5.11; N, 2.58.

*Phenyl O-(3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido-β-D-galactopyranosyl)-(1→4)-O-[(2,3,4-tri-O-acetyl-α-L-fucopyranosyl)-(1→3)-O]-6-O-acetyl-2-deoxy-2-phthalimido-1-thio-α/β-d-glucopyranoside (**20**)*

To a stirred solution of **19** (2.0 6, 1.8 mmol) in dichloromethane (40 ml) was added thiophenol (2.0 ml, 18 mmol) and BF_3-etherate (0.8 ml, 5.6 mmol). Stirring was continued for 5 h at room temperature. The reaction mixture was washed with aqueous sodium bicarbonate solution, water, dried, and concentrated. The residue was purified on a column of silica gel with a solvent gradient consisting of hexane-ethyl acetate 1:1 → 1:4 to afford **20** (1.1 g, 49%); $[\alpha]_D$ -72° (c 1.1, $CHCl_3$); ^1H NMR (CD_2Cl_2): δ 7.91–7.17 (m, 13 H, arom.), 5.84 (dd, 1 H, H-3′), 5.51 (d, J = 3.8 Hz, 1 H, H-4″), 5.41 (d, 2.8 Hz, 1H, H-4′) 5.36 (d, J = 8.4 Hz, 1 H, and H-1), 5.34 (d, J = 4.0 Hz, 1 H, H-1″), 5.28 (d, J = 10.5 Hz, 1 H, H-1′), 5.19 (dd, 1 H, H-2′), 2.20, 2.12, 2.11, 2.08, 2.07, 1.93, and 1.80 (each s, 21 H, 7 × OAc), and 1.35 (d, J = 6.7 Hz. 3 H, CMe).

Anal Calc. for $C_{54}H_{56}N_2O_{23}S$: C, 57.24; H, 4.98; N, 2.47. Found: C, 57.37; H, 5.01; N, 2.29.

*Methyl O-(3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido-β-D-galactopyranosyl-(1→4)-O-[(2,3,4-tri-O-acetyl-α-L-fucopyranosyl)-(1→3)-O]-)6-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-(1→6)-O-[(6-O-trimethylacetyl-β-D-galactopyranosyl)-(1→3)-O]-2-acetamido-2-deoxy-α-D-galactopyranoside (**23**) and Methyl O-(2-acetamido-2-deoxy-β-D-galactopyranosyl)-(1→4)-O-[(α-L-fucopyranosyl)-(1→3)-O]-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→6)-O-[(β-D-galactopyranosyl)-(1→3)-O]-2-acetamido-2-deoxy-α-D-galactopyranoside (**24**)*

Glycosidation of **22** (0.9 g, 1.26 mmol) with **20** (1.0 g, 0.88 mmol) followed by processing in the usual manner gave a crude product mixture which was directly employed in the next step without further purification. A solution of the crude product in 1:1 ethanol-dichloromethane (30 ml) containing thiourea (2.8 g, 37.8 mmol) and lutidine (2.0 ml, 18.72 mmol) was stirred for 6 h at 80 °C. The solvents were evaporated under reduced pressure and the residue redissolved in dichloromethane. The organic layer was washed with water, dried, and concentrated under diminished pressure. The residue was purified on a column of silica gel by elution with a solvent gradient consisting of 10–15% MeOH in dichloromethane to give **23** (0.44 g, 37%; based on **22**); $[\alpha]_D$ -40° (c 0.5, $CHCl_3$); ^1H NMR (CD_2Cl_2): δ 7.92–7.77 (m, 8 H, arom.), 5.83 (dd, 1 H, H-3‴), 5.76 (d, J = 9.6 Hz, 1 H, H-1″), 5.51 (d, J = 3.5 Hz, 1H, H-4⁗), 5.41 (d, J = 2.9 Hz, 1 H, H-4‴), 5.38 (d, J = 8.6 Hz, 1 H, H-1‴), 5.22 (d, J = 3.3 Hz, 1 H, H-1⁗), 5.20 (d, J = 2.9 Hz, 1 H, H-1), 2.83 (s, 3 H, OMe), 2.20–1.81, (cluster of s, 24 H, 7 × OAc and NAc), 1.36 (d, J = 6.4 Hz, 3 H, CMe), and 1.14 (s, 9 H, CMe_3).

Anal Calc. for $C_{68}H_{85}N_3O_{35}$: C, 54.29; H, 5.69; N, 2.79. Found: C, 54.03; H, 5.69; N, 2.79.

A portion of compound **23** was treated with hydrazine hydrate in methanol to cleave the phthalimido group, followed by N-acetylation (MeOH-Et_3N-Ac_2O) chromatographed and finally O-deacetylation in furnish in 66% yield, amorphous **24**; $[\alpha]_D$ -12° (c 1.0, H_2O); ^1H NMR (D_2O): δ 5.11 (d, J = 4.0 Hz, 1 H, H-1⁗), 4.74 (d, J = 3.8 Hz, 1 H, H-1), 4.52 (d, J = 8.3 Hz, 1 H, H-1″), 4.46 (d, J = 7.8 Hz, 1 H, H-1‴), 4.44 (d, J = 7.0 Hz, 1 H, H-1′), 3.35 (s, 3 H, OMe), 2.04, 2.00 and 1.99 (each s, 9 H, 3 × NAc), and 1.26 (d, J = 6.6 Hz, 3 H, CMe); ^{13}C NMR: GalNAc-β-(1→4) residue: 100.36 (C-1), 51.44 (C-2), 69.79 (C-3), 66.76 (C-4), 73.71 (C-5), 60.00 (C-6), 21.23 (NAc); Fuc-α-(1→3): 97.49 (C-1), 67.97 (C-2), 68.24 (C-3), 69.67 (C-4), 65.94 (C-5), 14.40 (C-6); GlcNAc-β-(1→6) residue: 99.76 (C-1), 53.97 (C-2), 74.45 (C-3), 74.00 (C-4), 72.43 (C-5), 59.06 (C-6), 21.30 (NAc); Gal-β-(1→3) residue: 103.68 (C-1), 69.03 (C-2), 71.06 (C-3), 67.63 (C-4), 73.88 (C-5), 60.48 (C-6); GalNAc-α-OMe residue: 97.24 (C-1), 47.56 (C-2), 76.11 (C-3), 66.41 (C-4), 71.58 (C-5), 68.19 (C-6), 54.60 (OMe), 21.06 (NAc). ES-MS: *m/z* = 948.39 [M-1]-.

Anal. Calc. for $C_{37}H_{63}N_3O_{25}.H_2O$: C, 45.91; H, 6.77; N, 4.34. Found: C, 46.08; H, 6.63; N, 4.29.

*Methyl O-(3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido-β-D-galactopyranosyl)-(1→4)-O-[(2,3,4-tri-O-acetyl-α-L-fucopyranosyl)-(1→3)-O]-6-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-(1→6)-O-[methyl-(5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonate-(2→3)-O-(6-O-trimethylacetyl-β-D-galactopyranosyl-(1→3)-O]-2-acetamido-2-deoxy-α-D-galactopyranoside (**26**)*

Compound **23** (0.2 g, 0.13 mmol) was treated with donor **25** (0.6 g, 1.1 mmol) in propionitrile (15 ml) at -65 °C for 3 h. The reaction mixture was then processed as described above, and the crude product subjected to column chromatography on silica gel with 10% MeOH in dichloromethane as the eluent to give **26** (0.12 g, 46%); $[\alpha]_D$ -28° (c 0.5, $CHCl_3$); ^1H NMR (CD_2Cl_2): δ 7.88–7.76 (m, 8 H, arom.), 5.48 (d, J = 9.2 Hz; 1 H, NH), 5.84 (dd, 1 H, H-3‴), 5.49 (d, J = 3.3 Hz, 1H, H-4⁗), 5.40 (d, J = 3.0 Hz, 1 H, H-4‴), 5.37 (d, J = 8.6 Hz, 1 H, H-1″), 5.28 (d, J = 3.3 Hz, 1 H, H-1⁗), 5.18 (d, J = 3.3 Hz, 1 H, H-1), 5.14 (d, J = 9.4 Hz, 1 H, H-1‴), 3.78 (s, 3 H, OMe), 2.79 (s, 3 H, OMe), 2.67 (dd, J = 4.6 Hz, H-3e⁗), 2.18–1.77, (cluster of s, 39 H, 12 × OAc and NHAc), 1.34 (d, J = 6.5 Hz, 3 H, CMe) and 1.15 (s, 9 H, Cme_3).

Anal Calc. for $C_{88}H_{112}N_4O_{47}$: C, 53.44; H, 5.71; N, 2.83. Found: C, 53.09; H, 5.89; N, 2.93.

Methyl O-(2-acetamido-2-deoxy-β-D-galactopyranosyl-(1→4)-O-[α-L-fucopyranosyl)-(1→3)-O]-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→6)-O-[(5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-(2→3)-O-(β-D-galactopyranosyl)-(1→3)-O]-2-acetamido-2-deoxy-α-D-galactopyranoside ***(27)***

A solution of **26** (0.1 g, 0.05 mmol) and lithium iodide (0.3 g, 2.2 mmol) in pyridine (10 ml) was stirred for 6 h at ~120°C. The solvent was then removed under diminished pressure and the residue was passed through a small column of silica gel by elution with 20–30% methanol in dichloromethane to give the protected free acid derivative. This compound was taken in methanol-hydrazine hydrate (4:1, 20 ml) and heated at ~80°C for 16 h. After evaporation to dryness, the residue was redissolved in methanol-dichloromethane (1:1, 20 ml) and treated with acetic anhydride (6 ml) for 1 h at 0°C. The mixture was then evaporated to dryness and the residue so obtained was deacetylated by stirring in methanolic sodium methoxide (0.05 N; 20 ml) for 2 days at room temperature. The crude product was purified by column chromatography on silica gel by using chloroform/methanol/water 13:6:1 and 4:5:1 (v/v/v) as the eluent, to give the target compound **27** (0.015 g, 24%); $[\alpha]_D$ -8° (c 0.15, H_2O); ^{1}H NMR (D_2O): δ 5.11 (d, J = 3.9 Hz, 1 H, H-1‴′), 4.76 (d, J = 3.9 Hz; 1 H, H-1), 4.52 (d, J = 8.2 Hz, 1 H, H-1″), 4.51 (d, J = 7.7 Hz, 1 H, H-1‴), 4.46 (d, J = 7.0 Hz, 1 H, H-1′), 3.34 (s, 3 H, OMe), 2.75 (dd, $J_{3'''''e,4'''''}$ = 4.6 Hz, 1 H, H-3‴′e), 2.04, 2.03, 2.01 and 1.99 (each s, 12 H, 4 × NHAc), 1.81 (t, $J_{3'''''a,4'''''} = J_{3'''''a}$, 3‴′e = 12.1 Hz, 1 H, H-3‴′a), and 1.26 (d, J = 6.5 Hz, 3 H, CMe); ^{13}C NMR; D_2O; GalNAc-β-(1→4) residue: 100.36 (C-1), 51.43 (C-2), 69.79 (C-3), 66.77 (C-4), 73.72 (C-5), 60.00 (C-6), 21.23 (Nac); Fuc-α-(1→3) residue: 97.49 (C-1), 67.83 (C-2), 68.24 (C-3), 69.12 (C-4), 65.94 (C-5), 14.41 (C-6); GlcNAc-β-(1→6) residue: 99.76 (C-1), 53.97 (C-2), 74.44 (C-3), 73.87 (C-4), 72.44 (C-5), 59.05 (C-6), 21.30 (NAc); Gal-β-(1→3) residue: 103.46 (C-1), 68.22 (C-2), 76.22 (C-3), 66.42 (C-4), 73.81 (C-5), 60.49 (C-6); NeuAc-α-(2→3) residue: 174.05 (C-1), 98.75 (C-2), 38.82 (C-3), 67.16 (C-4), 50.73 (C-5), 71.85 (C-6), 67.40 (C-7), 70.86 (C-8), 61.59 (C-9), 21.12 (NAc); GalNAc-α-OMe residue: 97.21 (C-1), 47.46 (C-2), 74.71 (C-3), 66.42 (C-4), 71.07 (C-5), 68.09 (C-6), 54.62 (OMe), 21.09 (NAc). ES-MS: *m/z* = 1239.8 $[M-1]^-$.

Anal Calc. for $C_{48}H_{80}N_4O_{33}.1.5\ H_2O$: C, 45.46; H, 6.60; N, 4.42. Found: C, 45.37; H, 6.62; N, 4.40.

Methyl O-(3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido-β-D-galactopyranosyl)-(1→4)-O-[(2,3,4-tri-O-acetyl-α-L-fucopyranosyl)-(1→3)-O]-(6-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-(1→6)-O-[(2,4,6-tri-O-acetyl-3-O-chloroacetyl-β-D-galactopyranosyl-(1→3)-O]-2-acetamido-2-deoxy-α-D-galactopyranoside ***(29)***

Compound **28** (0.92 g, 1.5 mmol) was treated with **20** (1.5 g, 1.3 mmol) as described in the general glycosidation methods. After the customary processing, the crude product was purified by silica gel column chromatography with a solvent gradient consisting of 20–25% acetone in dichloromethane to give **29** (1.0 g, 74%); $[\alpha]_D$ -36° (c 1.0, $CHCl_3$); ^{1}H NMR (CD_2Cl_2): δ 7.90–7.78 (m, 8 H, arom.), 5.82 (dd, 1 H, H-3‴), 5.50 (d, J = 3.5 Hz, 1 H, H-4‴′), 5.40 (d, J = 3.4 Hz, 1 H, H-4‴) 5.38 (d, J = 7.9 Hz, 1 H, H-1′), 5.21 (d, J = 3.5 Hz, 1 H, H-1‴), 5.19 (d, J = 3.2 Hz, 1 H, H-1), 5.14 (d, J = 8.2 Hz, 1 H, H-1″), 4.11–4.07 (bs, 2 H, CH_2Cl), 2.83 (s, 3 H, OMe), 2.19–1.80 (cluster of s, 33 H, 10 × OAc and NHAc), 1.35 (d, J = 6.8 Hz, 3 H, CMe).

Anal Calc. for $C_{71}H_{84}N_3O_{38}Cl$: C, 52.55; H, 5.22; N, 2.59. Found: C, 52.31; H, 5.16; N, 2.38.

Methyl O-(3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido-β-D-galactopyranosyl)-(1→4)-O-[(2,3,4-tri-O-acetyl-α-L-fucopyranosyl)-(1→3)-O]-(6-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-(1→6)-O-[(2,4,6-tri-O-acetyl-β-D-galactopyranosyl)-(1→3)-O]-2-acetamido-2-deoxy-α-D-galactopyranoside ***(30)***

Compound **29** (0.96 g, 0.58 mmol) was de-O-chloroacetylated in a manner analogous to that described for the preparation of **14.** After customary processing, silica gel column chromatographic purification (5–10% MeOH in dichloromethane), gave **30** (0.65 g, 71%); $[\alpha]_D$ -40° (c 1.0, $CHCl_3$); ^{1}H NMR (CD_2Cl_2): δ 7.91–7.78 (m, 8 H, arom.), 5.82 (dd, 1 H, H-3‴), 5.50 (d, J = 3.7 Hz, 1 H, H-1‴′), 5.40 (d, J = 3.3 Hz, 1H, H-4‴), 5.38 (d, J = 2.9 Hz, 1 H, H-4‴′), 5.38 (d, J = 9.0 Hz, 1 H, H-1″), 5.25 (d, J = 3.5 Hz, 1 H, H-4′), 5.23 (d, J = 3.4 Hz, 1 H, H-4), 5.19 (d, 2.9 Hz, 1 H, H-1), 2.81 (s, 3 H, OMe), 2.20–1.58, (cluster of s, 33 H, 10 × OAc and NHAc), 1.35 (d, J = 6.2 Hz, 3 H, CMe).

Anal Calc. for $C_{69}H_{83}N_3O_{37}$: C, 53.59; H, 5.41; N, 2.72. Found: C, 53.39; H, 5.63; N, 2.59.

Methyl O-(2-acetamido-2-deoxy-β-D-galactopyranosyl-(1→4)-O-[α-L-fucopyranosyl-(1→3)-O]-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→6)-O-[(3-O-sulfo-β-D-galactopyranosyl sodium salt)-(1→3)-O]-2-acetamido-2-deoxy-α-D-galactopyranoside ***(31)***

Compound **30** (0.45 g, 0.29 mmol) in N,N-dimethylformamide (20 ml) was treated with sulfur trioxide-pyridine complex (0.25 g, 1.6 mmol) at 0°C for 5 h. Excess reagent was destroyed by the addition of methanol (~5 ml), followed by pyridine (~5 ml). The mixture was then concentrated under diminished pressure and the residue was passed through a small column of silica gel by using 15–20% methanol in dichloromethane as the eluent. The fractions corresponding to product were pooled and concentrated and the residue taken in methanol-hydrazine hydrate (4:1, 50 ml) and heated at ~90°C for 5 h. The mixture was then concentrated and the crude product mixture was taken in methanol-triethylamine (2:1, 24 ml), cooled (0°C) and treated with acetic anhydride (5 ml). It was allowed to gradually attain room temperature and kept for an additional 1 h at same temperature. The mixture was concentrated, and the residue applied to a column of silica gel and eluted with chloroform/methanol/water 13:6:1 and 4:5:1 (v/v/v). Fractions corresponding to product were pooled and concentrated and the residue redissolved in water and passed through a small column of Amberlite IR-120 (Na^+) cation exchange resin. Lyophilization of the eluate then furnished **31** (0.11 g, 37%), $[\alpha]_D$ (c 1.0, H_2O); ^{1}H NMR (D_2O): δ 5.12 (d, J = 3.9 Hz, 1 H, H-1‴′), 4.77 (d, J = 3.7 Hz; 1 H, H-1), 4.57 (d, J = 7.9 Hz, 1 H, H-1″), 4.54 (d, J = 8.3 Hz, 1H, H-1‴), 4.48 (d, J = 8.2 Hz, 1 H, H-1′), 3.37 (s, 3 H, OMe), 2.07, 2.03 and 2.02 (each s, 9 H, 3× NAc), and 1.27 (d, J = 6.6 Hz, 3 H, CMe); ^{13}C NMR (D_2O); GalNAc-β-(1→4) residue: 100.37 (C-1), 51.44 (C-2), 69.79 (C-3), 66.77 (C-4), 73.57 (C-5), 59.90 (C-6), 21.14 (NAc); Fuc-α-(1→3) residue: 97.50 (C-1), 67.76 (C-2), 68.24 (C-3), 69.11 (C-4), 65.85 (C-5), 14.41 (C-6); GlcNAc-β-(1→6) residue: 99.76 (C-1), 53.99 (C-2), 74.45 (C-3), 73.88 (C-4), 72.44 (C-5), 59.06 (C-6), 21.31 (NAc); 3-O-SO_3Na Gal-β-(1→3) residue: 103.37 (C-1), 68.21 (C-2), 79.29 (C-3), 66.42 (C-4), 73.73 (C-5), 60.48 (C-6); GalNAc-α-OMe residue: 97.25 (C-1), 47.48 (C-2), 76.55 (C-3), 65.40 (C-4), 71.07 (C-5), 67.87 (C-6), 54.62 (OMe), 21.06 (NAc). ES-MS: *m/z* = 1028.38 $[M-Na]^-$.

Anal Calc. for $C_{37}H_{62}N_3O_{28}$.SNa. 2 H_2O: C, 40.84; H, 6.11; N, 3.86. Found: C, 40.73; H, 6.15; N, 3.73.

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