Distinct Substrate Specificities of Bacterial Heparinas against N-Unsubstituted Glucosamine Residues in Heparan Sulfate*

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Heparin and heparan sulfate (HS)† are complex, sulfated copolymers of alternating α1→4-linked glucosamine (GlcN), and α/β1→4-linked hexuronic acid. They have a number of important biological activities in developmental processes (1, 2), angiogenesis (3), blood coagulation (4), cell adhesion (5), and tumor metastasis (6). These involve interactions between HS proteoglycans and proteins, such as enzymes, cytokines, growth factors, and extracellular matrix proteins (7–12).

HS and heparan are initially synthesized as a common non-sulfated precursor structure composed of repeating disaccharides of glucuronic acid (GlcA) and N-acetylglucosamine (GlcNAc) (8, 10, 13, 14). This precursor is first modified by coordinated N-deacetylation and N-sulfation of GlcNAc residues forming N-sulfoglucosamine (GlcNS). The N-sulfated polymer then undergoes a further series of modifications, i.e. C5-epimerization of GlcA to iduronic acid (IdoA), O-sulfation at C2 of hexuronic acid (primarily IdoA), and O-sulfation at C6 of GlcNS or GlcNAc. Additionally, a rare but functionally important step, 3-O-sulfation of the GlcN residue, can also occur late in the modification process. These modifications create a complex sulfated domain structure in HS, whereas heparin is extensively and uniformly sulfated.

Because of the varied structure of mature HS chains and their extensive repertoire of interactions with different proteins, the rare structural components presumably contribute to selective protein binding. Recently, the N-unsubstituted Glcn (GlcNH3) unit was found in native HS structures (15, 16) and was implicated in important cell biological and pathophysiological phenomena. Levels of GlcNH3 residues were found to correlate with the ability of bovine and human endothelial HS to bind L-selectin (17). GlcNH3 residues were also identified as targets for an HS 3-O-sulfotransferase isoform (HS 3-OST-3A) that generates a sequence in cell surface HS that is utilized as a binding site by the herpes simplex virus glycoprotein D, thus making cells susceptible to herpes simplex virus-1 entry (18–20). Moreover, two monoclonal antibodies that recognize GlcNH3 units in HS were reported, and they highlighted distinctive localizations of their respective epitopes in tissues. One monoclonal antibody bound to extracellular tissue components in rat kidney (21), whereas the other reacted with scapie lesions in murine brain (22, 23). Finally, it has been proposed that GlcNH3 residues provide cleavage sites in HS chains for endogenous NO-derived nitroxy anion and thus contribute to recycling of glypican-1 (24).

The content of GlcNH3 residues is low but variable between HS species. Values from 1.2 to 7.5 wt% of total GlcN were calculated for a range of porcine and bovine HS species based on reaction with o-phthalaldehyde (15). However, GlcNH3 residues are mostly analyzed by treatment with high pH (pH 3.9) nitric acid, leading to chain cleavage at the GlcNH3-hexosaminidic linkage. By this method the content in human aortic and kidney HS was shown to be 2–4% (16). Quantitation of GlcNH3 by this approach requires either the analysis of metabolically radiolabeled HS, or post-cleavage, reducing end chemical radiolabeling of the cleavage products. So far there has been no identification and characterization of GlcNH3 residues in HS by the use of degradative enzymes that could obviate the need for radiolabeling. Moreover, a better understanding of the substrate specificities of heparin/HS-degrading enzymes could be of value in sequencing GlcNH3-containing oligosaccharides and in the isolation of fragments with either intact internal or reducing end GlcNH3 residues.

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† The abbreviations used are: HS, heparan sulfate; GlcA, α-D-glucuronic acid; IdoA, α-L-iduronic acid; GlcN, glucosamine; GlcNAc, β-D-N-acetylglucosamine; GlcNS, β-D-N-sulfoglucosamine; GlcNH3, N-unsubstituted glucosamine; HexA, Δ-5-sulfated hexuronic acid; NS-K5, N-sulfated K5 polysaccharide; S, sulfate; HS 3-OST-3A, heparan sulfate 3-O-sulfotransferase-3A isoform; dp, degree of polymerization (i.e. dp2 corresponds to a disaccharide); SAX-HPLC, strong anion-exchange high performance liquid chromatography.
Glycosaminoglycan-degrading bacterial enzymes are useful analytical tools for investigating the composition and sequence of various glycosaminoglycans (25). Three heparinases (I, II, and III) have been isolated from Flavobacterium heparinum that differ from one another in terms of their substrate specificities (26). These have been fairly well defined. Heparinase I has high specificity for highly sulfated disaccharides containing iduronate-2-O-sulfate and thus cleaves at 4-GlcNS(3-6)1→IdoA(2S)1→ linkages (27–29). Heparinase III has highest activity toward non-sulfated or N-sulfated disaccharides (GlcNAc/GlcNS-HexA) with a preference for GlcA over IdOa. It will tolerate 6-O-sulfation of the amino sugar but is inhibited by 2-O-sulfation of IdoA (30, 31). Heparinase II has a broad substrate specificity, being able to cleave a wide range of disaccharide repeat units (30, 32, 33). It was reported that heparinase II has two distinct active sites, one of which is heparinase-I-like, whereas the other is heparinase-III-like (34). Moffat et al. reported that heparinase II has activity on N-unsubstituted disaccharides in chemically modified heparin (32, 35). However, we are not aware of any detailed reports about the comparative substrate specificities of heparinase I, II, and III toward sulfated and non-sulfated disaccharides containing GlcNH3+ residues. Because the GlcNH3+ content is generally very low in HS, we have, in this study, used the partially or fully de-N-sulfated heparin preparations, partial and complete, were digested with a mixture of heparinase I, II, and III (24 milliunits of each enzyme) at 37 °C for 48 h, and then applied to a Bio-Gel P2 column (1.7 × 115 cm) eluted with 0.2 M NaHCO3. Fractions of 1.5 ml were collected, and their absorbance was measured at 232 nm. The disaccharide peak was collected, and the NH4HCO3 removed, as described above.

**RESULTS**

Preparation of De-N-sulfated Heparins—In this study, two de-N-sulfated heparin preparations, partial and complete, were prepared. The degrees of de-N-sulfation, calculated from the increase in N-acetylated disaccharides obtained after re-N-acetylation and digestion with heparinases, were 50 and 98% for the two preparations, respectively.

Digestion of Partially De-N-sulfated Heparin with Heparinases—Partially de-N-sulfated heparin was digested with a mixture of heparinases I, II, and III, and separated by Bio-Gel P2 gel filtration chromatography (Fig. 1). Only two major peaks were obtained, which were identified as dp4 (minor peak) and dp2 by comparing their elution positions with those of standard oligosaccharides. From the peak areas it was calculated that 86% of the linkages in partially de-N-sulfated heparin were cleaved by the heparinases. The excess of degradation (86%) over the content of N-unsubstituted residues (50%) indicated that (i) heparinase I, II, or III must have activity toward N-unsubstituted disaccharides and (ii) N-unsubstituted oligosaccharides (probably disaccharides) can be obtained by this procedure.

Characterization of Disaccharides from Partially De-N-sulfated Heparin—The pooled disaccharide fraction from Bio-Gel P2 chromatography (Fig. 1) of enzyme-digested, partially de-N-sulfated heparin was analyzed by SAX-HPLC, and compared with that from unmodified heparin and the elution positions of eight known disaccharides (Fig. 2). In contrast to the unmodi-
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Fig. 2. SAX-HPLC analysis of disaccharides obtained enzymatically from modified heparin. Heparin (A), partially de-N-sulfated heparin (B), and re-N-acetylated, partially de-N-sulfated heparin (C) were digested with a mixture of heparinases I, II, and III. Disaccharide fractions obtained from Bio-Gel P2 chromatographies were analyzed by SAX-HPLC (see "Experimental Procedures"). Arrows indicate the elution positions of standard disaccharides: ΔHexA-GlcNac (1), ΔHexA-GlcNS (2), ΔHexA-GlcA (3) and ΔHexA(2S)-GlcNac (4). Unidentified disaccharide peaks in B are labeled a–d.

Fig. 3. SAX-HPLC analysis of N-unsubstituted disaccharides derived from partially de-N-sulfated heparin NS-K5. As the content of disaccharide a is low in partially de-N-sulfated heparin, and it was eluted from the SAX-HPLC column very early at low salt concentration, it was difficult to identify its structure conclusively using heparin. According to its elution position, and the known identities of the three other species (b–d), we might expect disaccharide a to be a non-sulfated, N-unsubstituted disaccharide. To possibly prepare a larger amount of this disaccharide, we chose de-N-sulfated NS-K5 as a potential substrate. The K5 polysaccharide has the same sequence as completely unmodified HS. NS-K5 is thus comprised mainly of GlcA-GlcNS disaccharides (37).

NS-K5 was partially de-N-sulfated to generate a significant proportion of GlcA-GlcNH₃ disaccharide units. The degree of de-N-sulfation achieved was 45%. This material was then digested with a mixture of heparinases I, II, and III and separated by Bio-Gel P2 gel filtration chromatography. The dp2 fraction obtained was analyzed by SAX-HPLC. Compared with control NS-K5, which yielded almost entirely ΔHexA-GlcNS (Fig. 4A), one additional peak was obtained from the partially de-N-sulfated NS-K5, which eluted very early at 3.1 min (Fig. 4B). Its elution position is the same as that of disaccharide a from partially de-N-sulfated heparin (Fig. 2B). This suggests that this disaccharide is likely to be ΔHexA-GlcNH₃. To confirm this, disaccharide a was collected, re-N-acetylated, desalted on Bio-Gel P2, and then re-analyzed by SAX-HPLC. As shown in Fig. 4C, after re-N-acetylation, disaccharide a shifted its retention time to a later position corresponding to the known elution position of ΔHexA-GlcNac, unequivocally confirming that the original structure of disaccharide a is ΔHexA-GlcNH₃.

Specificity of Individual Heparinases toward N-Unsubstituted Glucosamine Residues—To identify the specific heparinases (s) responsible for the activity toward GlcNH₃-containing residues, de-N-sulfated heparins and partially de-N-sulfated NS-K5 were digested with heparinases I, II, or III individually. Enzymatic activities were monitored by following the increase in UV absorbance at 232 nm with time (Fig. 5A). Heparinase I
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showed no activity toward the fully de-N-sulfated heparin, and heparinase III acted only to a small extent. In contrast, heparinase II demonstrated significant activity against this substrate.

A comparison of the kinetics of heparinase II degradation of heparin to those of both partially and fully de-N-sulfated heparins showed that the presence of increasing levels of GlcNH₃/H₁¹⁰⁰₁ did slow the rate of degradation (Fig. 5B), although an extensive, but incomplete, level of degradation can be achieved with time with both de-N-sulfated substrates.

Heparinase II also showed activity toward partially de-N-sulfated NS-K5 (45% de-N-sulfated) (Fig. 5C). However, this polysaccharide was a much better substrate for heparinase III (Fig. 5C). This demonstrates the likely activity of heparinase III against non-sulfated GlcNH₃/H₁¹⁰⁰₁ residues.

To further analyze the differential activities of individual heparinas toward N-unsubstituted disaccharides, the digestion products from fully de-N-sulfated heparin incubated for 48 h with either heparinase I, II, or III were separated by Bio-Gel P2 gel filtration chromatography (Fig. 6A). The disaccharide fractions were then analyzed by SAX-HPLC (Fig. 6B, C). No disaccharides were released by treatment with heparinase I (data not shown). After heparinase III digestion,
only a small amount of disaccharides and longer oligosaccharides were detected (Fig. 6A). However, heparinase II digestion generated a large amount of disaccharides, a small amount of tetra- and hexasaccharides, and nothing larger (Fig. 6A), suggesting almost quantitative breakdown of this substrate. These results confirmed that heparinase II displays much more activity toward O-sulfated, N-unsubstituted sequences than does heparinase III, and heparinase I has no activity. Also, both heparinases II and III can liberate disaccharides containing GlcNH₃⁺.

The SAX-HPLC analyses of the dp2 fractions released by heparinase II or III were very different. Heparinase II (Fig. 6B) released mainly three N-unsubstituted disaccharides, i.e. ΔHexA-GlcNH₃⁺(6S), ΔHexA(2S)-GlcNH₃⁺, and ΔHexA(2S)-GlcNH₃⁺(6S), together with small amounts of four N-acetylated disaccharides, i.e. ΔHexA-GlcNAc, ΔHexA-GlcNAc(6S), ΔHexA(2S)-GlcNAc, and ΔHexA(2S)-GlcNAc(6S). This suggests that heparinase II cleaves all the mono- or di-O-sulfated, N-unsubstituted disaccharide units. The presence of small amounts of N-acetylated disaccharides reflects their low presence (13% of total) in the original heparin preparation. In contrast, the main disaccharide released by heparinase III (Fig. 6C) was ΔHexA-GlcNAc, together with minor amounts of ΔHexA-GlcNH₃⁺(6S) and ΔHexA-GlcNAc(6S). This shows that with the de-N-sulfated heparin as substrate heparinase III is cleaving mostly at native N-acetylated residues, although it does appear to be capable of generating some N-unsubstituted disaccharide units of low overall sulfation, but with low yield.

To confirm the different activities of heparinases II and III toward sulfated and non-sulfated, N-unsubstituted units, the digestion products from partially de-N-sulfated NS-K5, after incubation with heparinase II or III, were also separated by Bio-Gel P2 gel filtration chromatography (Fig. 7A). Heparinase II yielded a very small amount of disaccharides and longer fragments. However, heparinase III generated a large amount of disaccharides and some tetrasaccharides, but nothing larger, implying almost quantitative cleavage of this substrate. These results suggested that heparinase III might display preferential activity toward non-sulfated, N-unsubstituted sequences.

The disaccharide fractions from Bio-Gel P2 column chromatography (Fig. 7A) were then analyzed by SAX-HPLC. Three species, i.e. ΔHexA-GlcNH₃⁺, ΔHexA-GlcNAc, and ΔHexA-GlcNNS, were identified in the heparinase III digest (Fig. 7C). This clearly demonstrates that heparinase III cleaves at non-sulfated, N-unsubstituted disaccharides. However, in the heparinase II digest (Fig. 7B), only ΔHexA-GlcNS and a very small amount of ΔHexA-GlcNAc were detected, with no presence of GlcNH₃⁺-containing disaccharides, suggesting that heparinase II is not capable of cleaving at non-sulfated, N-unsubstituted disaccharide units.

Identification of N-Unsubstituted Disaccharides in HS Species—To assay for the presence of natural GlcNH₃⁺ residues in HS using heparinases, bovine kidney and porcine intestinal mucosal HS were chosen. The GlcNH₃⁺ content of these two HS species had previously been shown to be very different (15, 16). Bovine kidney HS was exhaustively digested with a mixture of heparinases I, II, and III, and the disaccharide fraction was recovered by Bio-Gel P2 chromatography and analyzed by SAX-HPLC (Fig. 8A). Eight components were identified that correspond to known N-substituted disaccharide standards (labeled I–8). Four additional peaks were also present (comprising 12% of total disaccharides), corresponding to the known N-unsubstituted units (labeled a–d). To confirm these latter structural assignments, bovine kidney HS was first N-acetylated before enzymatic digestion and disaccharide analysis. The four N-unsubstituted disaccharides (a–d) now disappeared, but the corresponding N-acetylated derivatives (peaks 1, 3, 4, and 7) increased by 10% in total (including the significant appearance of disaccharide 7 (HexA(2S)-GlcNAc(6S)), which was barely present in the native HS (Fig. 8B). These results show that HexA-GlcNH₃⁺, HexA-GlcNH₃⁺(6S), HexA(2S)-GlcNH₃⁺, and HexA(2S)-GlcNAc(6S) disaccharides are present in bovine kidney HS at levels of 5.1%, 3.4%, 2.2%, and 1.9% of total disaccharides, respectively (calculated as an average of two analyses). In the same way, three N-unsubstituted disaccharides, HexA-GlcpNH₃⁺, HexA-GlcpNH₃⁺(6S), and HexA(2S)-GlcNH₃⁺, were detected in porcine intestinal mucosal HS at 0.4%, 0.4%, and 0.3% of total disaccharides, respectively, i.e. a total of 1.1% (data not shown). The more highly sulfated HexA(2S)-GlcNH₃⁺(6S) disaccharide was not detected in this HS. This compares with a total GlcNH₃⁺ content of 0.7% determined by pH3.9 nitric acid treatment and chromatographic analysis (16).

**DISCUSSION**

The content of GlcNH₃⁺ residues is low and variable between HS species, ranging from 1.2% to 7.5 wt%, as determined using o-phthalaldehyde (15). Because of this apparently low (15, 16) natural content in HS, we have used chemical modification of heparin and the K5 polysaccharide to increase the content of GlcNH₃⁺ residues to test the susceptibilities of N-unsubstituted disaccharides to the heparinases, which are widely used for analysis of HS and heparin.

Unexpectedly, we found that the majority of hexosaminidic linkages in a partially (50%) de-N-sulfated heparin were cleaved to disaccharides by a combination of heparinases I, II,
and III (Fig. 1). This led us to surmise that (i) the heparinases could be used as alternative tools for the quantitation and analysis of GlcNH$_3^+$ residues in HS, and (ii) that N-unsubstituted disaccharides could be obtained by this procedure. SAX-HPLC analyses of the disaccharides obtained from de-N-sulfated heparin revealed four additional disaccharide peaks (a–d) (Fig. 2B), that were not present in digests of the parent heparin (Fig. 2A), and did not correspond to any of the known N-substituted disaccharide standards. Re-N-acetylation of the partially de-N-sulfated heparin, prior to enzymatic digestion, did not give these four disaccharide peaks (Fig. 2C), but the content of the four N-acetylated disaccharides increased, suggesting that the four novel disaccharides (a–d) are indeed N-unsubstituted ones. Individual recovery of three of these disaccharides (b–d), followed by their N-acetylation, allowed their structures to be unequivocally determined (Fig. 3). These were variously O-sulfated, N-unsubstituted species, namely HexA-GlcNH$_3^+$ (6S), HexA(2S)-GlcNH$_3^+$, and HexA(2S)-GlcNH$_3^+$ (6S). The content of one further putative N-unsubstituted disaccharide a in partially de-N-sulfated heparin was low (Fig. 2B), but from its elution position on SAX-HPLC we surmised that it might be the non-sulfated, N-unsubstituted disaccharide. To confirm this, we prepared a disaccharide with identical elution position, but in greater abundance, from a similar enzyme digest of partially de-N-sulfated NS-K5 (Fig. 4B). Collection and N-acetylation of this disaccharide yielded the structure HexA-GlcNAc (Fig. 4C), confirming that disaccharide a was indeed the non-sulfated disaccharide HexA-GlcNAc.

N-unsubstituted disaccharides are normally identified and quantified in HS by chemical assay or by specific pH 3.9 nitrous acid degradation and chromatographic separation of the cleavage products. However, both methods destroy the GlcNH$_3^+$ residues. In this study we describe the generation of intact, unsaturated N-unsubstituted disaccharides by digestion with a combination of heparinases followed by SAX-HPLC. By their identification, and the characterization of their SAX-HPLC elution positions, it is now possible for heparinases to be conveniently used for analyzing the content of N-unsubstituted disaccharides in HS as part of a standard disaccharide analysis (Fig. 8A).

Heparinases are very important tools in studying the composition and role of biologically relevant HS/heparin sequences. It was reported that heparinase I has specificity for relatively highly sulfated disaccharides containing both N- and 2-O-sulfation, whereas heparinase III cleaves at non- and low sulfated disaccharides predominantly containing GlcA. Heparinase II has a broader specificity, cleaving at both high sulfation and low sulfation sites, but not at all linkages. Which heparinases are responsible for cleaving at GlcNH$_3^+$ residues? We have investigated the individual activities of heparinases I, II, and III toward substrates with either a high content of O-sulfate groups (i.e. partially or fully de-N-sulfated heparin) or deficient in O-sulfation (i.e. partially de-N-sulfated NS-K5) (Fig. 5). This revealed that heparinase II was primarily responsible for the specificity of cleavage at GlcNH$_3^+$ in the de-N-sulfated heparins, although the rate and extent of depolymerization was reduced by the presence of the primary amine (Fig. 5A). Heparinase II excises N-unsubstituted disaccharides that are O-sulfated at C-2 of uronic acid, or C-6 of GlcNH$_3^+$, or both (Fig. 6B); it is inactive against the non-sulfated counterpart (Fig. 7B). This is consistent with the broad sulfation specificity of heparinase II. However, with the same substrates, heparinase III specifically excises non-sulfated, N-unsubstituted disaccharides (Fig. 7C), but has almost no activity toward mono- or di-O-sulfated ones in either fully de-N-sulfated heparin (Fig. 6C) or native HS (date not shown). By contrast, heparinase I showed no propensity to cleave at any sites of N-unsubstitution. The differences in activity of heparinase enzymes on GlcNH$_3^+$ residues in HS are illustrated in Fig. 9.

Chai et al. (38) reported that, using controlled conditions for partial heparinase III digestion of partially de-N-acetylated K5 polysaccharide, the enzyme was not able to cleave the GlcNH$_3^+$-GlcA linkage within a HexA-GlcNH$_3^+$-GlcA-GlcNAc sequence; even under forced conditions for exhaustive digestion, the GlcNH$_3^+$-GlcA linkages were still only partly cleaved. In our study, using the partially de-N-sulfated NS-K5 as a substrate, we reached a different conclusion, because this alternative substrate was highly degraded into disaccharides by heparinase III (Fig. 7A). This suggests that heparinase III has higher activity toward the GlcNH$_3^+$-GlcA linkage within HexA-GlcNH$_3^+$-GlcA-GlCNSequences. It is unlikely that this could be due to contaminating enzyme activities, because neither hepa-
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Since heparin II shows specificity to GlcNH$_3$-unsubstituted disaccharides, for identifying other proteins that require the unsubstituted primary amine. For example, by using a partially de-N-sulfated heparin as substrate, heparinase II would yield oligosaccharides with internal and reducing terminal GlcNH$_3$ residues (Fig. 9). Such oligosaccharides would also be valuable substrates for testing the activities of HS polymer-modifying enzymes.

REFERENCES

1. Perrimon, N., and Bernfield, M. (2000) Nature 404, 725–728
2. Lander, A. D., and Selleck, S. B. (2000) J. Cell Biol. 148, 227–232
3. Robinson, C. J., and Stringer, S. E. (2001) J. Cell Sci. 114, 853–865
4. Marcum, J. A., McKenny, J. B., Galli, S. J., Jackman, R. W., and Rosenberg, R. D. (1986) Am. J. Pathol. 125, H579–H588
5. Couchman, J. R. (2003) Nat. Rev. Mol. Cell. Biol. 4, 926–937
6. Liu, D., Shriver, Z., Venkataraman, G., El-Shabrawi, Y., and Sasisekharan, R. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 568–573
7. Bernfield, M., Cotte, M., Park, P. W., Reizes, O., Fitzgerald, M. L., Linacero, J., and Zako, E. (1999) Annu. Rev. Biochem. 68, 729–777
8. Casu, B., and Lindahl, U. (2001) Adv. Carbohydr. Chem. Biochem. 57, 159–206
9. Henson, R. G. (1998) Heparin Binding Proteins, p. 10, Academic Press, San Diego and London
10. Esko, J. D., and Selleck, S. B. (2002) Annu. Rev. Biochem. 71, 435–471
11. Li, J., and Thorp, S. C. (2002) Med. Res. Rev. 1, 1–25
12. Schiavon, M., Loidl, R., and Lindahl, U. (1996) FASEB J. 10, 1270–1279
13. Lindahl, U., Kusche-Gullberg, M., and Kjellen, L. (1998) J. Biol. Chem. 273, 24979–24982
14. Esko, J. D., and Lindahl, U. (2001) J. Clin. Invest. 108, 169–173
15. Toida, T., Yoshida, H., Toyoda, H., Koshishii, I., Imanari, T., Hileman, R. E., Fromm, J. R., and Linhardt, R. J. (1997) Biochem. J. 322, 499–506
16. Westermark, C., and Lindahl, U. (2002) J. Biol. Chem. 277, 49247–49255
17. Norgard-Sumnicht, K., and Varki, A. (1995) J. Biol. Chem. 270, 12014–12024
18. Liu, J., Shriver, Z., Blaiklock, P., Yoshida, K., Sasisekharan, R., and Rosenberg, R. D. (1999) J. Biol. Chem. 274, 38155–38162
19. Liu, J., Shriver, Z., Pope, M. F., Thorp, S. C., Duncan, M. B., Copeland, R. J., and Sasisekharan, R. (2002) J. Biol. Chem. 277, 33456–33467
20. Shukla, D., Liu, J., Blaiklock, P., Shworer, N. W., Bai, X., Esko, J. D., Cohen, G. H., Eisenberg, R. J., Rosenberg, R. D., and Spear, P. G. (1999) Cell 99, 14–22
21. Van den Born, J. B., Junnarsson, K., Bakker, M. A. H., Kjellen, L., Kusche-Gullberg, M., Gacarana, M., Berden, J. H. M., and Lindahl, U. (1995) J. Biol. Chem. 270, 31305–31309
22. Leteux, C., Chai, W., Ngai, K., Herbert, C. G., Lawson, A. M., and Feizi, T. (2001) J. Biol. Chem. 276, 12539–12545
23. McIlrath, P. A., Wilson, M., Ekerenbohm, P., Tunstall, A., and Bruce, M. E. (1998) Exp. Neurol. 149, 447–454
24. Ding, K., Mani, K., Feng, F., Belting, M., and Fran worm, L.-Å. (1998) J. Biol. Chem. 273, 33353–33360
25. Lyon, M. (1997) in A Laboratory Guide to Glycoconjugate Analysis (Jackson, P., and Gallagher, J. T., eds) pp. 61–76, Birkhäuser Verlag, Basel
26. Lindhardt, R. J., Rice, K. G., Kim, Y. S., Loose, D. L., Wang, H. M., and Loganathan, D. S. (1988) Biochim. Biophys. Acta 957, 731–747
27. Linker, A., and Sampson, P. (1960) Biochem. Biophys. Acta 43, 366–368
28. Hoving, P., and Linker, A. (1970) J. Biol. Chem. 245, 6170–6175
29. Perlín, A. S., Mackie, D. M., and Dietrich, C. P. (1971) Carbohydr. Res. 18, 185–194
30. Nader, H. B., Porcionatto, M. A., Tersariol, I. L. S., Oliveira, F. W., Moraes, C. T., and Dietrich, C. P. (1999) J. Biol. Chem. 274, 16077–16083
31. Desai, U. R., Pinhal, M. A. S., Oliveira, M. A. S., and Gallagher, J. T., eds) pp. 61–76, Birkhäuser Verlag, Basel
32. Moffat, C. F., McLean, M. W., Long, W. F., and Williamson, F. B. (1991) Eur. J. Biochem. 205, 531–541
33. Linhardt, R. J., Turnbull, J. E., Wang, H. M., Loganathan, D., and Gallagher, J. T. (1990) Biochem. J. 261, 2611–2617
34. Blomberg, A. J., Shriver, Z., Bismann, K., and Sasisekharan, R. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 12232–12237
35. Moffat, C. F., McLean, M. W., Long, W. F., and Williamson, F. B. (1991) Eur. J. Biochem. 197, 449–459
36. Nagasawa, K., Inoue, Y., and Kamata, T. (1977) Carbohydrate Res. 58, 47–55
37. Murphy, K. J., Merry, C. L. R., Lyon, M., Thompson, J. E., Roberts, I. S., and Gallagher, J. T. (2004) J. Biol. Chem. 279, 37339–37345
38. Chai, W. G., Leteux, C., Westling, C., Lindahl, U., and Feizi, T. (2004) Biochemistry 43, 8590–8599
39. Wang, S., Xu, X., Freeman, S. D., Pownall, M. E., Lu, Q., Kessler, D. S., and Gallagher, J. T. (2003) J. Biol. Chem. 278, 21301–21311