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Structural analysis of *N*-glycans from human neutrophil azurocidin

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Abstract

N-glycans of human neutrophil azurocidin, enzymatic inactive homolog of serine proteinase playing important and multifunctional roles in antimicrobial defense, endotoxin binding, monocyte, and T-cell activation, were isolated by hydrazinolysis and fluorescence labeled. An ion-exchange chromatography on GlycoSep C column separated neutral, mono-, and disialylated glycans. The glycans from each group were separated subsequently on GlycoSep N and GlycoSep H columns. Sequential exoglycosidase treatment and HPLC mapping allowed determining 21 different glycan structures, majority of them being neutral (79.8%), the rest—mono- (13.1%) and disialylated (1.2%). © 2002 Elsevier Science (USA). All rights reserved.

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Azurocidin [also known as cationic antimicrobial protein 37 kDa (CAP 37) [1]] or human heparin-binding protein (HBP) [2]] is an enzymatic inactive homolog of serine proteinases residing together with elastase, proteinase 3, and cathepsin G in azurophilic granules of human neutrophils [3]. Despite high homology to neutrophil elastase (45%), azurocidin lacks serine esterase activity due to replacement of two of the three residues from the conserved catalytic triad characteristic for serine esterases [4].

Azurocidin has a broad spectrum of antimicrobial activity, mainly against gram-negative bacteria [3,5]. Another aspect of azurocidin involvement in host defense system is the ability to attract monocytes, T-, and K-cells and to induce longevity and differentiation of monocytes to macrophages [2,6]. Azurocidin is also recognized as a multifunctional inflammatory mediator through its effects on endothelial cells and fibroblasts [2,6], capacity to bind endotoxin [7], stimulation of protein kinase C activity in endothelium [8], and mediation of enhancement of LPS-induced cytokine release from monocytes [9]. Recently a major role of azurocidin in the increase of vascular permeability in inflammation was described [10].

The differences in glycosylation are probably responsible for a broad range of molecular mass (28–39 kDa) reported for azurocidin from different laboratories [11]. A glycan moiety makes up to 24% of total molecular mass and occupies three N-linked glycosylation sites located at asparagine residues 100, 114, and 145 [2,4]. The data on the possible function of azurocidin carbohydrate moiety are limited to one study in which the unchanged stabilities of nonglycosylated and glycosylated forms of the protein and the diminished ability to stimulate immunological system by nonglycosylated azurocidin were found [12]. These results are debatable, because a recombinant azurocidin used in the above study was expressed in baculovirus system that differs from human in some aspects of N-glycosylation [13]. Having this in mind we have decided to start a systematical research on native (human neutrophil-derived) azurocidin carbohydrate moiety, beginning by a full structural analysis of its glycans.

Materials and methods

Protein purification. Human neutrophil azurocidin was purified as described previously [14].

Protein estimation. Concentration of the purified protein was determined by its absorbance coefficient $A^{280} = 5.1$ calculated from amino acid sequence according to [15].

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Preparation of oligosaccharide pool. Purified azurocidin was desalted on Fast Desalting Column (Pharmacia) equilibrated with 0.1% TFA in water. The protein (about 300 μg) was lyophilized extensively overnight under high vacuum. Then the protein was immediately treated with anhydrous hydrazine [16]. The reaction was carried out at 95 °C for 8 h. Oligosaccharides were released and separated from impurities (hydrazine, salts, and peptides) using GlycoRelease Kit (Oxford GlycoSciences).

Fluorescence labeling of glycans. The reducing ends of the oligosaccharides were fluorescence labeled with 2-aminobenzamide (2-AB) [17] using a 2-AB Signal Labeling Kit (Oxford GlycoSciences) and stored at −20 °C.

Chromatographic separations of labeled glycans. Oligosaccharide fractionation was performed at 30 °C by HPLC on a Knauer apparatus with a Shimadzu RF-551 fluorescence detector. (λ_{max} excitation = 330 nm and λ_{max} emission = 420 nm). Partially hydrolyzed dextran (Oxford GlycoSciences), labeled with 2-AB, was used as an external standard. The values of the elution positions of separated AB-oligosaccharides were expressed in glucose units in reference to the AB-isomaltosooligosaccharides from dextran hydrolyzate. The neutral, mono-, and disialylated pools of 2-AB glycan derivatives were separated on an ion exchange column (GlycoSep C, 100 × 4.6 mm, Oxford GlycoSciences) in charge profile mode, following manufacturer's protocol. Each glycan pool was subsequently chromatographed on normal-phase amide column (GlycoSep N, 250 × 4.6 mm, Oxford GlycoSciences). Gradient was constructed with a high salt solvent system (HSSS) as described by [18]. Fractions corresponding to fluorescence peaks were collected, dried, redissolved in a minimal volume (up to 6 μl) of water, and injected onto a GlycoSep H column (150 × 4 mm, Oxford GlycoSciences) for the final purification. Glycans were eluted with a 10–30% acetonitrile gradient containing 0.1% TFA, following manufacturer's protocol. Homogenous, desalted peaks were collected and dried.

Exoglycosidase sequencing. Table 1 presents the specificity and concentration of exoglycosidases used in sequential digestions. All enzymes were from Oxford GlycoSciences except for α1-2,3-mannosidase, α1-6-mannosidase, and α1-3,4-fucosidase, which were from New England Biolabs. Before glycosidase treatment, purified and fluorescence labeled glycans (0.081–1 nmol of each) were dissolved in 100 mM phosphate/citrate buffer, pH 4.5 containing 100 mM NaCl and 0.2 mM $ZnCl_2$. Then the first exoglycosidase was added to obtain a final concentration as described in Table 1 and a total volume of 15–25 μl. Incubation buffer, reaction vials, and pipette tips were autoclaved before the experiment. After 16–24 h of incubation at 37 °C, the reaction was stopped by filtration through a 5 kDa membrane (UltrafreeMC, Millipore). The membrane was rinsed twice with 100 μl of water. Both filtrates were combined. A small aliquot from the filtrate (usually less than 1/8 of total volume) was dried on a rotary evaporator, redissolved in 7 μl of 70% acetonitrile in water (v/v), and injected onto a GlycoSep N column. The GU values of glycans were determined as described above. The rest of the filtrate was dried, dissolved in water and the next exoglycosidase was added to obtain the required concentration of the enzyme in 15–25 μl of total volume.

In case of glycans making less than 1% of total glycan pool the whole volume of the filtrate was dried and separated on a GlycoSep N column. Fractions containing glycan peaks were collected, dried, and dissolved in 15–25 μl of the incubation buffer and the next enzyme was added. The same procedure was performed prior to hydrolysis with α1-2,3-mannosidase, when a different incubation buffer (100 mM citrate–phosphate, pH 6.0 with 5 mM $CaCl_2$ and 0.1 mg/ml BSA) was used.

Standard oligosaccharides were separated on the same column in the same conditions and their GU values were determined. Some standard native glycans (β-mannosyl core, conserved trimannosyl core, conserved trimannosyl core substituted with α1-6 fucose, trimannosyl core with two GlcNAc and trimannosyl core with two GlcNac and two Gal attached) were purchased from Oxford GlycoSciences, then fluorescence labeled as described above.

Results and discussion

Isolation of oligosaccharides

Glycans released by hydrazinolysis from human neutrophil azurocidin were separated on GlycoSep C column into three pools (neutral, mono-, and disialylated) (Fig. 1). Each pool was subsequently chromatographed on GlycoSep N column. As a result of this chromatography 11 neutral (I–XI), 9 monosialylated (MS I–MS IX), and 3 disialylated (DS I–DS III) glycan peaks were obtained (Figs. 2–4, respectively). All peaks were subsequently repurified on GlycoSep H column. This step has resulted in a separation of neutral glycan peaks V, VI, VII, X, and XI into additional peaks containing homogeneous glycans (Va, Vb, VIa, VIb, VIIa, VIIb, VIIc, Xa, Xb, XIa, and XIb).

Enzymatic sequencing of the released glycans

Sequential degradation with exoglycosidases listed in Table 1, HPLC analysis of the degradation products on GlycoSep N and GlycoSep H columns, and comparison with retention times of reference glycans allowed us to determine structures of the separated glycans. Proposed

Table 1
Concentration and specificity of exoglycosidases used in sequential digestion of *N*-glycans from human neutrophil azurocidin

Enzyme	Concentration	Specificity
β-Galactosidase from *Streptococcus pneumoniae*	80 mU/ml	Gal(β1-4)
β-*N*-Acetylhexosaminidase from jack bean	10 U/ml	GlcNAc(β1-2,3,4,6)
β-*N*-Acetylhexosaminidase from *S. pneumoniae*	10 mU/ml	GlcNAc(β1-2)
α-Mannosidase from *Xanthomonas manihotis*	20 mU/ml	Man(α1-2,3)
α-Mannosidase from *X. manihotis*	400 U/ml	Man(α1-6)[a]
α-Mannosidase from jack bean	10 U/ml	Man(α1-2,3,6)
β-Mannosidase from *Helix pomatia*	2.5 U/ml	Man(β1-4)
α-Fucosidase from *X. manihotis*	50 U/ml	Fuc(α1-3,4)
α-Fucosidase from bovine epididymis	100 mU/ml	Fuc(α1-2,3,4,6)
Sialidase from *Arthrobacter ureafaciens*	2 U/ml	Neu(α2-3,6,8)
Sialidase from Newcastle disease virus (NDV)	0.2 U/ml	Neu(α2-3,8)

[a] The enzyme was able to release α1-6-mannose residues only when they are linked to a nonbranched sugar.

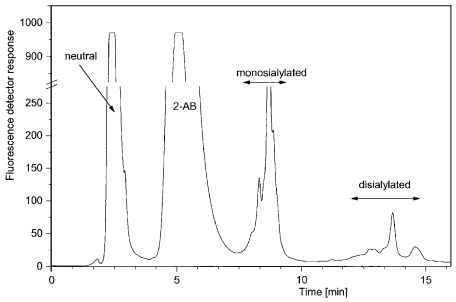

Fig. 1. Separation of fluorescence labeled with 2-aminobenzamide (2-AB) human neutrophil azurocidin *N*-glycans on ion-exchange GlycoSep C column.

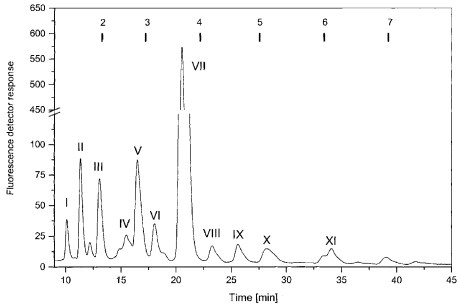

Fig. 2. Separation of fluorescence labeled human neutrophil azurocidin neutral *N*-glycans on GlycoSep N column. The assigment of peaks was made using GU values by comparison with a standard dextran hydrolysate ladder.

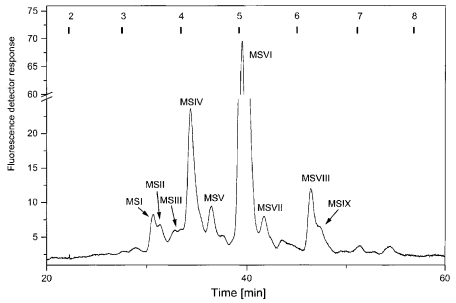

Fig. 3. Separation of fluorescence labeled human neutrophil azurocidin monosialylated *N*-glycans on GlycoSep N column. The assigment of peaks was made using GU values by comparison with a standard dextran hydrolysate ladder.

structures of the oligosaccharides isolated from human neutrophil azurocidin are presented in Table 2.

Glycan I peak has a GU value equal to 1 (on GlycoSep N and GlycoSep H columns) and is composed of one *N*-acetylglucosamine residue.

Glycan II (1.43 GU) was resistant to all the applied exoglycosidases with the exception of unspecific α-fucosidase. The action of this enzyme resulted in a removal of one fucose residue and a final 1 GU product (*N*-acetylglucosamine).

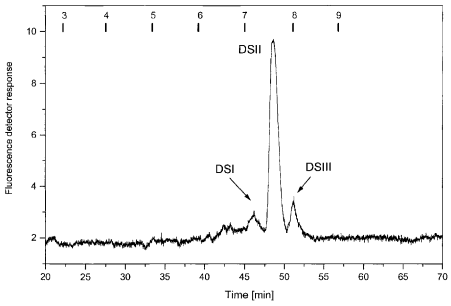

Fig. 4. Separation of fluorescence labeled human neutrophil azurocidin disialylated *N*-glycans on GlycoSep N column. The assigment of peaks was made using GU values by comparison with a standard dextran hydrolysate ladder.

Unspecific β-*N*-acetylhexosaminidase was able to release one *N*-acetylglucosamine residue from *glycan III*with a final product (1.43 GU) identical with glycan II.

β-Mannosidase released one mannose residue (0.9 GU) from *glycan Vb* leaving α1-6-fucosyl chitobiose (2.03 GU) as the final result.

Glycan VIa was resistant to α1-2,3-mannosidase. Unspecific mannosidase and α1-6-mannosidase were able to remove one mannose residue from this glycan giving 2.38 GU product sensitive to β-mannosidase action. A release of β-mannose residue resulted in a 1.47 GU glycan (chitobiose) degraded further by unspecific β-*N*-acetylhexosaminidase to a 1 GU final product (*N*-acetylglucosamine).

Glycan VIIc constituting more than 50% of the total glycan pool was resistant to α1-2,3-mannosidase but sensitive to unspecific mannosidase or α1-6-mannosidase actions. The release of one mannose residue by these enzymes resulted in a GU 2.92 product identical with glycan Vb.

β-*N*-Acetylhexosaminidase released one *N*-acetylglucosamine residue from *glycan VIII* with a 3.76 GU product identical with a major glycan VIIc.

Glycan IX was sensitive to α1-2,3-mannosidase and resistant to α1-6-mannosidase. Unspecific mannosidase removed two mannose residues from this glycan giving a 2.94 GU product identical with glycan Vb. A digestion with unspecific fucosidase yielded in a "trimannosyl core" structure (GU = 4.26).

Glycan Xa was resistant to all mannosidases from Table 1 but was sensitive to β1-4-galactosidase. A removal of one galactose residue resulted in a 4.29 GU product identical with glycan VIII.

The release of one mannose residue with unspecific mannosidase or α1-2,3-mannosidase (with a concomitant resistance to α1-6-mannosidase) proved that in *glycan Xb* α1-6-mannose arm is substituted. The product of the above reactions (4.27 GU) was identical with a glycan VIII.

Table 2
Postulated structures of *N*-glycans isolated from human neutrophil azurocidin

Glycan	Size (GU)	% of total	Postulated structure
I	1.00	1.5	GlcNAc
II	1.43	4.1	Fucα1→6GlcNAc
III	2.05	4.3	GlcNAcβ1→4GlcNAc 6 ↑ Fucα1
Vb	2.88	7.2	Manβ1→4GlcNAcβ1→4GlcNAc 6 ↑ Fucα1
VIa	3.29	3.0	Manα1 ↘ 6 Manβ1→4GlcNAcβ1→4GlcNAc
VIIc	3.76	53.2	Manα1 ↘ 6 Manβ1→4GlcNAcβ1→4GlcNAc 6 ↑ Fucα1
VIII	4.29	1.3	GlcNAcβ1→2Manα1 ↘ 6 Manβ1→4GlcNAcβ1→4GlcNAc 6 ↑ Fucα1
IX	4.73	1.5	Manα1 ↘ 6 Manβ1→4GlcNAcβ1→4GlcNAc 3 6 Manα1↗ ↑ Fucα1
Xa	5.13	0.8	Galβ1→4 GlcNAcβ1→2Manα1 ↘ 6 Manβ1→4GlcNAcβ1→4GlcNAc 6 ↑ Fucα1
Xb	5.13	1.1	GlcNAcβ1→2Manα1 ↘ 6 Manβ1→4GlcNAcβ1→4GlcNAc 3 6 Manα1↗ ↑ Fucα1
XIa	6.15	0.6	Manα1 ↘ 6 Manβ1→4GlcNAcβ1→4GlcNAc 3 6 Galβ1→4GlcNAcβ1→2Manα1↗ ↑ Fucα1
XIb	6.15	1.2	Galβ1→4GlcNAcβ1→2Manα1 ↘ 6 Manβ1→4GlcNAcβ1→4GlcNAc 3 6 Manα1↗ ↑ Fucα1

Table 2 (*continued*)

Glycan	Size (GU)	% of total	Postulated structure
MS I	5.99	0.5	Neu2→6Galβ1→4 GlcNAcβ1→2Manα1 ↘ 6 Manβ1→4GlcNAcβ1→4GlcNAc 6 ↑ Fucα1
MS IV	6.51	2.4	Manβ1→4GlcNAcβ1→4GlcNAc Neu2→6Galβ1→4 GlcNAcβ1→2Manα1 ↗ 3 6 ↑ Fucα1
MS V	6.98	0.6	Manα1 ↘ 6 Manβ1→4GlcNAcβ1→4GlcNAc 3 6 Neu2→3Galβ1→4GlcNAcβ1→2Manα1 ↗ ↑ Fucα1
MS VI	7.49	8.2	Manα1 ↘ 6 Manβ1→4GlcNAcβ1→4GlcNAc 3 6 Neu2→6Galβ1→4GlcNAcβ1→2Manα1 ↗ ↑ Fucα1
MS VII	7.83	0.5	GlcNAcβ1→2Manα1 ↘ 6 Manβ1→4GlcNAcβ1→4GlcNAc 3 6 Neu2→6Galβ1→4 GlcNAcβ1→2Manα1 ↗ ↑ Fucα1
MS VIII	8.63	0.9	Galβ1→4 GlcNAcβ1→2Manα1 ↘ 6 Manβ1→4GlcNAcβ1→4GlcNAc 3 6 Neu2→6Galβ1→4 GlcNAcβ1→2Manα1 ↗ ↑ Fucα1
DS I	9.21	0.5	Neu2→3Galβ1→4GlcNAcβ1→2Manα1 ↘ 6 Manβ1→4GlcNAcβ1→4GlcNAc 3 6 Neu2→6Galβ1→4GlcNAcβ1→2Manα1 ↗ ↑ Fucα1
DS II	9.29	0.5	Neu2→6Galβ1→4GlcNAcβ1→2Manα1 ↘ 6 Manβ1→4GlcNAcβ1→4GlcNAc 3 6 Neu2→3Galβ1→4GlcNAcβ1→2Manα1 ↗ ↑ Fucα1
DS III	9.59	0.2	Neu2→6Galβ1→4GlcNAcβ1→2Manα1 ↘ 6 Manβ1→4GlcNAcβ1→4GlcNAc 3 6 Neu2→6Galβ1→4GlcNAcβ1→2Manα1 ↗ ↑ Fucα1

β1-4-Galactosidase was able to remove one galactose residue from *glycan XIa*. A further digestion with β-*N*-acetylhexosaminidase has removed one *N*-acetylglucosamine residue giving a 4.72 GU product identical with a glycan IX. A resistance to α1-2,3-mannosidase and a very slow action of unspecific mannosidase are a proof of a substitution of α1-3-mannose arm with *N*-acetylglucosamine and galactose in this glycan.

Glycan XIb was sensitive to unspecific mannosidase or α1-2,3-mannosidase. A removal of one mannose residue gave 5.15 GU product identical with a glycan Xa.

Monosialylated *glycan MS I* was resistant to specific α2-3,8-sialidase and to all other exoglycosidases used in this study, except for unspecific α2-3,6,8-sialidase. A decrease of 0.82 GU after a desialylation proves that sialic acid residue is terminating α1-6-mannose arm in this glycan. If α1-3 arm would be sialylated a decrease of a glycan hydrodynamic volume after a desialylation should be equal to 1.15 GU [17]. A resistance to NDV sialidase and sensitivity to α2-3,6,8-sialidase indicate that sialylation is α2-6 type. A further sequencing showed that after a desialylation MS I glycan is identical with a glycan Xa.

Glycan MS IV was resistant to unspecific mannosidase, α1-2,3-mannosidase, and NDV sialidase. After the action of α2-3,6,8-sialidase the decrease in GU value of the product was equal to 1.1 units, showing that α1-3-glycan arm was sialylated. This was also confirmed by a sequential action of β1-4-galactosidase (−1.02 GU) and β-*N*-acetylhexosaminidase (−0.56 GU) leading to the product becoming sensitive to unspecific mannosidase (−0.89 GU) with a final GU value equal to a neutral glycan Vb.

A sensitivity of *glycan MS V* to NDV sialidase (a decrease of 0.82 GU) proves that sialic acid residue is α2-3 bound to α1-3-mannose arm. After a removal of galactose and *N*-acetylglucosamine residues from this arm by β1-4-galactosidase and β1-2-*N*-acetylhexosaminidase, respectively, the product became sensitive to unspecific mannosidase. The enzyme has removed two mannose residues (−1.82 GU) from 1-3 and 1-6 arm giving the product identical in GU value with a neutral glycan Vb.

Glycan MS VI is a major (8.2% of a total glycan content) monosialylated oligosaccharide in azurocidin. A resistance to an NDV sialidase (α2-3,8 specific) and a sensitivity to unspecific sialidase (a decrease of 0.82 GU after a desialylation) indicate that sialic acid residue is α2-6 glycosidically bound to 1-3-mannose arm. After desialylation glycan MS VI has an identical GU value with desialylated glycan MS V and behaves identically in a further sequencing.

β1-2-*N*-Acetylhexosaminidase is able to release one *N*- acetylglucosamine residue from *glycan MS VII* with a product identical in GU value to glycan MS VI and with the identical sequencing pattern.

Glycan MS VIII is initially sensitive to β1-4-galactosidase. A digestion product is identical in GU value with a glycan MS VII.

Glycans MS II, *MS III*, and *MS IX* were not sequenced because they constituted less than 0.2% of the total glycan content.

A digestion of disialylated *glycan DS I* with NDV sialidase resulted in a release of one residue of α2-3 bound sialic acid and a glycan identical in GU value and with the same sequencing pattern as monosialylated glycan MS VIII.

Sensitivity of *glycan DS II* to NDV sialidase and a susceptibility of the desialylated product to a further sequential action of β1-4-galactosidase, β1-2-*N*-acetylhexosaminidase, and α1-2,3-mannosidase indicate that α2-3 bound sialic acid was located on 1-3-mannose arm. A further sequencing with unspecific sialidase (release of α2-6 bound sialic acid residue), β1-4-galactosidase, and β1-2-*N*-acetylhexosaminidase resulted in a product identical with a major neutral glycan VIIc.

Glycan DS III was resistant to NDV sialidase. Unspecific sialidase was able to remove two α2-6 bound sialic acid residues. Desialylated product became sensitive to the consecutive action of β1-4-galactosidase (two galactose residues removed) and β1-2-*N*-acetylhexosaminidase (two *N*-acetylglucosamine residues released) resulting in a product identical with a neutral glycan IX.

As a result of applied sequencing procedure 94.1% of a total glycan pool content was sequenced (12 neutral, 6 mono-, and 3 disialylated structures). Among a small amount of unsequenced glycans were glycans constituting less than 0.2% of a total glycan pool content (the amount below a sensitivity of the applied sequencing method) or artifacts (glycans IV, Va, VIb, VIIa, and VIIb) resulting from a partial destruction of fucosylated glycans occurring during hydrazinolysis. It was known that the bond between two core *N*-acetylglucosamines is partially broken during hydrazinolysis if terminal *N*-acetylglucosamine is α1-3 fucosylated [19,20]. Our present data show that α1-6 fucosylation also causes a partial destruction of core chitobiose during hydrazinolysis.

Azurocidin is synthesized in the early stages of neutrophil maturation process [21] and stored in mature neutrophil azurofilic granules together with a set of glycosidases (sialidase, α-mannosidase and β-*N*-acetylhexosaminidase) [22]. Broad heterogeneity of azurocidin glycans (more than 20 structures occupying three N-glycosylation sites) is probably a result of intensive trimming occurring with time of storage in azurophilic granules. Recombinant azurocidin expressed in insect cells shows a very limited degree of glycan heterogeneity with only two glycan structures: $Man_3GlcNAc_2$ (Fuc) and $Man_3GlcNAc_2$ [9,23].

Major azurocidin neutral, mono-, and disialylated glycans (VIIc, MS VI, DS I, and DS II) are also char-

acteristic for neutrophil elastase and cathepsin G—the other homologous azurophilic granules proteins analyzed by us previously [24]. In both proteins three to four discrete subsets of glycoforms were found [24]. A similar pattern was also observed in case of azurocidin (our unpublished data). The detailed analysis of azurocidin glycoforms carbohydrate moiety is now in progress.

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