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Preparation of legionaminic acid analogs of sialo-glycoconjugates by means of mammalian sialyltransferases.

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Abstract

Legionaminic acids are analogs of sialic acid that occur in several bacteria. The most commonly occurring form is Leg5Ac7Ac, which differs from Neu5Ac only at the C7 (acetamido) and C9 (deoxy) positions. While these differences greatly reduce the susceptibility of Leg compounds to sialidases, several sialyltransferases have been identified that can use CMP-Leg5Ac7Ac as a donor (Watson et al. 2011). We report the successful modification with Leg5Ac7Ac of a glycolipid, GM1a, and two glycoproteins, interferon-α2b and α_1-antitrypsin, by means of two mammalian sialyltransferases, namely porcine ST3Gal1 and human ST6Gal1. The Leg5Ac7Ac form of GD1a was not recognized by the myelin-associated glycoprotein (MAG, Siglec-4), confirming the importance of the glycerol moiety in the interaction of sialo-glycans with Siglecs.

Keywords α_1-antitrypsin, GD1a, human ST6Gal1 sialyltransferase, interferon-α2b, porcine ST3Gal1 sialyltransferase

Abbreviations:

FCHASE, 6-(fluorescein-5-carboxyamido)-hexanoic acid succinimidyl ester derivative of the *p*-aminophenyl glycoside; LacNAc, Galβ1,4GlcNAc.

Introduction

Replacing sialic acid residues on the termini of glycoconjugates with the legionaminic acid Leg5Ac7Ac can result in significantly different biological properties. The two sugars have the same stereochemistry, but differ at C9 where Leg5Ac7Ac has a methyl group instead of CH_2OH and at C7 where Leg5Ac7Ac has an acetamido group instead of a hydroxyl. Biological interactions that depend upon these features of the glycerol moiety of sialic acid may therefore be compromised, for example susceptibilities to sialidases are greatly reduced [1].

Leg5Ac7Ac termini may be introduced into glycans by certain sialyltransferases that can use CMP-Leg5Ac7Ac as a donor, instead of CMP-Neu5Ac [1; 2]. These experiments were carried out with small synthetic acceptors, but we have found that bacterial sialyltransferases do not modify efficiently larger substrates such as glycoproteins with Leg5Ac7Ac (unpublished observations). Porcine ST3Gal1 is noteworthy because it retains ~60% of its activity with CMP-Leg5Ac7Ac compared to CMP-Neu5Ac, when assayed with a fluorescent derivative of Galβ1,3GalNAc (T-Ag) as the acceptor. Its specificity is for terminal Galβ1,3GalNAc groups, which occur on *O*-linked glycans of glycoproteins and on certain glycolipids such as GM1a. We show here that this enzyme can successfully attach Leg5Ac7Ac to larger substrates including GM1a, to form an analog of GD1a. One of the biological properties of GD1a is that it serves as the ligand for myelin-associated glycoprotein (MAG or Siglec-4) [3,4]. Since the recognition of sialic acid by Siglecs depends upon its glycerol moiety, we predicted that Leg5Ac7Ac analogs would not bind to Siglecs [1]. ST3Gal1 was also able to attach Leg5Ac7Ac to the *O*-glycan group on a glycoprotein, interferon-α2b, which is its only glycan [5]. We also report that a second enzyme, human ST6Gal1 can attach Leg5Ac7Ac to *N*-linked glycans on glycoproteins, as exemplified by modification of α1-antitrypsin. Natural and recombinant forms of this protein

have three *N*-glycosylation sites where the predominant glycoforms are bi-antennary sialylated structures [6-8]. Both of these glycoproteins are being produced for therapeutic purposes and modification of their glycans may alter their biological activity [9] and their pharmacodynamics [10].

Materials and Methods

Enzymes and Substrates

ST3Gal1 and CMP-Leg5Ac7Ac were produced as previously described [1] as was interferon-α2b in HEK293 cells [11]. The α_1-antitrypsin was purchased from r-Peptide (Bogart GA) and the GM1a was a gift from Dr Shawn Defrees (Neose Technologies Inc.). The Zwittergent 3-14 was purchased from Calbiochem. The codon-optimized (human codon bias) gene encoding the human ST6Gal1 protein (NP15907) intralumenal domain (aa 27-406) with a human VEGFa (P15692) signal peptide linked at its N-terminus and a C-terminal GHHHHHHHHHHG tag at its C-terminus was chemically synthesized by GenScript (Piscataway, NJ) and cloned into the pTT5 mammalian expression vector [12, 13]. The secreted ST6Gal1 enzyme was expressed in CHO-EBNA1 (CHO-3E7) cells according to previously published protocols [14,15]. The clarified culture medium supernatant was harvested at 8 days post-transfection and the secreted ST6Gal1 was purified by immobilized metal-affinity chromatography (IMAC). Its reactivity with CMP-Leg5Ac7Ac as the donor compared to CMP-Neu5Ac, with FCHASE-LacNAc as the acceptor, was measured as previously described [1].

Modification of GM1a

Reaction mixtures (3 x 1 mL) containing 2 mM GM1a, 4 mM CMP-Leg5Ac7Ac, 10 mM $MgCl_2$, 50 mM MES buffer pH 6.5, 0.3% Zwittergent 3-14 and ST3Gal1 were incubated at 30ºC for 24 h. Traces of starting material were still present, so to drive the reaction to completion a second aliquot of CMP-Leg5Ac7Ac was then added and the incubation was continued for a further 24 h. Each 1 mL reaction was chromatographed on a C18 Sep-Pak cartridge, which was washed with 50% methanol and the product was eluted with 100% methanol. A final wash with 80% acetonitrile confirmed that the product had been completely eluted. To remove the detergent, the three methanol eluates were combined and applied to a 1 mL HiTrap-Q HP column run on an Akta FPLC system (GE Healthcare). The column was washed with methanol then eluted with a gradient from 0-1.0 M of potassium acetate in methanol. The fractions containing the product were identified by TLC, the buffer was removed by C18 Sep-Pak as above, and the product was lyophilized. The identity of the product was confirmed by electrospray ionization mass spectrometry, using a Prince capillary electrophoresis system (Prince Technologies, The Netherlands) coupled to a API 3000 mass spectrometer (AB/Sciex, Concord, Canada) via a microIonspray interface. The separations were obtained on a ~90 cm long bare fused-silica capillary using 30 mM ammonium acetate in chloroform : methanol (2:1). A voltage of 20 kV was typically applied at the injection.

Binding of myelin-associated glycoprotein

An ELISA assay was used to compare the binding of MAG to the Leg form of GD1a and to the gangliosides GM1a, GD1a, GT1b and GD1b [16]. Sets of four wells were coated with 5, 25, 50 or 100 pmol of each glycolipid, a chimeric protein containing the first three Ig-like domains of

MAG conjugated to human Fc (MAG_3Fc) was added and after incubation then washing, the amount bound was determined with an anti-human Fc antibody conjugated to alkaline phosphatase.

Mass spectrometry of modified glycoproteins

The protein samples were analyzed by LC-MS using an Agilent HP1100 Capillary LC system (Agilent Technologies) coupled to a LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific) equipped with a high flow electrospray ionization source. 1 µg of protein was loaded on a Poros R2 column (2.1 x 30 mm) (Applied Biosystems) heated to 80°C. Mobile phases were 0.1% formic acid in water (A) and 75% acetonitrile, 25% tetrahydrofuran (B) (solvent B was 0.1% formic acid in 100% acetonitrile for interferon-α2b) preheated to approximately 80ºC. The following gradient was used to desalt and elute the proteins from the column: (1) 20% mobile phase B (0-3 min.), 20%-90% mobile phase B (3-6 min.), 90%-20% mobile phase B (6-7 min.). The flow rate was 1 mL/min. The HPLC eluent was split to 100 µL/min just before the electrospray source of the LTQ-Orbitrap XL and MS spectra was acquired from m/z 400 to 4,000 at a resolution of 7,500. LC-MS spectra were viewed in Xcalibur® (Thermo Fisher Scientific). Molecular weight profiles were generated with the MassLynx© MaxEnt1 deconvolution software (Waters). Databridge (Waters) was used to convert the protein LC-MS spectra into a format that was compatible with MaxEnt1.

Modification of asialo-interferon-α2b

Sialic acid residues were removed from the glycoprotein by treatment with *Micromonospora viridifaciens* sialidase. MES buffer pH 6.5 (0.5 mL of 1 M), was added to 5 mL of interferon-α2b solution (0.92 mg/ml) along with 50 mU of the sialidase, and the mixture was incubated for 2 h at 37°C. The removal of the sialic acid residues was confirmed by SDS-PAGE, with a 15% gel. The digest was diluted to 100 mL with sodium acetate buffer, 25 mM pH 5.0, and applied to a HiTrap SP HP column. After washing with the buffer, the asialo-interferon-α2b was eluted with a gradient of 0-0.8 M NaCl in buffer.

The asialo-interferon-α2b was modified with Leg5Ac7Ac by incubation of 4 mL of the protein (2.13 mg/mL) with 0.5 mL of sodium acetate buffer, 0.5 M pH 5.5, 0.5 mL of 100 mM $MgCl_2$, 45 µl of 10 mM CMP-Leg5Ac7Ac and 200 µl of ST3Gal1, for 3 h at 30°C; further aliquots of the CMP-Leg5Ac7Ac were added (75 µl) after 1 h and 2 h. The reaction's completeness was checked by SDS-PAGE. The product was then purified by ion exchange chromatography in the same manner as the asialo-interferon-α2b above. The removal of sialic acid residues and the addition of Leg5Ac7Ac were confirmed by mass spectrometry.

Modification of α_1-antitrypsin

The glycoprotein (6 mg) was desialylated in MES buffer pH 6.0 (1 mL of 0.5 M) with 25 mU of *M. viridifaciens* sialidase, at 37°C for 4 h. Removal of the sialic acid residues was confirmed by iso-electric focussing (IEF), carried out with a Pharmacia (GE Healthcare) Phastgel apparatus, using precast PhastGel pH 3-9, and manual staining with Coomassie Blue. The product was diluted to 50 mL with 20 mM Tris buffer pH 7.5 and purified by ion-exchange chromatography

on a 5ml HiTrap Q HP column, run in the same Tris buffer and eluted with a 0-0.3M NaCl gradient.

The asialo-α_1-antitrypsin was modified with Leg5Ac7Ac by incubation of 0.5 mL of the protein (3.03 mg/mL) with 100 µl of 0.5 M MES buffer, pH 6.5, 100 µL of 100 mM $MgCl_2$, 300 µL of 10 mM CMP-Leg5Ac7Ac and 100 µL of ST6Gal1, for 20 h at 30ºC; a second 300 µL aliquot of the CMP-Leg5Ac7Ac was added after 4 h. The reactions were checked by IEF. The reaction mixture was diluted with 100 mL of 20 mM Tris buffer pH 7.5, and the product was purified by ion exchange as described above. The removal of sialic acid residues and the addition of Leg5Ac7Ac were quantified by mass spectrometry.

Results and Discussion

Mono-Leg5Ac7Ac analog of GD1a

The structure of the Leg5Ac7Ac derivative is shown in **Figure 1**. The GM1a substrate is considerably larger than the fluorescent T-Ag substrate used previously with this enzyme, with which the CMP-Leg5Ac7Ac reaction was ~60% that of the CMP-Neu5Ac [1]. With GM1a and CMP-Leg5Ac7Ac, the reactivity of the ST3Gal1 was lower than with CMP-Neu5Ac, requiring an overnight reaction whereas the reaction with CMP-Neu5Ac appeared complete in a few hours. Complete modification could be achieved however by adding a further aliquot of CMP-Leg5Ac7Ac. The final yield of purified Leg-GD1a was 6.8 mg, from a total of 9.4 mg of GM1a starting material. The identity of the product was confirmed by electrospray mass spectrometry, which gave a mass of 1862.1 Da for the $[M]^{-1}$ ion compared to the mass of 1546.2 Da for the

$[M]^{-1}$ ion of the GM1a starting material (**Figure 2**). This is consistent with the 316 Da increase expected for the addition of one Leg5Ac7Ac residue to GM1a.

ELISA assays with the Leg-GD1a analog and other gangliosides demonstrated that MAG was completely unable to bind to the Leg5Ac7Ac compound, in contrast to its strong binding to GD1a and to GT1a (**Figure 3**), as previously observed [3,4]. This behavior could not be reliably predicted from the role of the glycerol moiety of sialic acids in Siglec recognition, as revealed by the X-ray crystal structures of Siglecs such as Siglec 1 [17] and Siglec 7 [18]. These showed significant H-bond interactions of the C9 $–CH_2OH$ group and a sequence/structure alignment [18] shows that the CD22 and CD33 subgroups of Siglecs share nearly all the sialic-acid contacting residues. The C9 $–CH_2OH$ is replaced by $–CH_3$ in Leg5Ac7Ac, abrogating any H-bonding, but hydrophobic interactions with nearby Trp residues (the homologous residue is Tyr in the case of MAG [18]) were still possible. The C7 –OH groups projected away from the protein surface, hence it appeared that the C7 acetamido group on Leg5Ac7Ac could be readily accommodated. Thus the loss of activity of the Leg-GD1a analog demonstrates the critical importance of the C9 – CH_2OH in Siglec binding of sialic acids.

Modification of interferon-α2b

Interferon-α2b has only a single glycan, an *O*-linked T-antigen structure which is disialylated [11]. The linkage of the Galβ1,3GalNAc disaccharide to the inner region of the ganglioside in GM1a is β1,4 whereas in the *O*-linked structure (T-Ag) on glycoproteins it is attached through an α1,*O* linkage. Unlike lectins such as jacalin [19], the ST3Gal1 enzyme does not discriminate between the two linkages. **Figure 4** shows mass spectra confirming the addition of Leg to the

asialo-interferon-α2b by means of ST3Gal1. The sialidase removed both sialic acids from the T-Ag glycan, changing its mass from 20,213 Da [11] to 19,628 Da. After treatment with ST3Gal1 and CMP- Leg5Ac7Ac, SDS-PAGE analysis of the product indicated that the modification was nearly complete. The mass of the main protein peak was 19,943 Da, confirming the addition of a single Leg5Ac7Ac residue.

Modification of α_1-antitrypsin

In contrast to the ST3Gal1 enzyme used above, the ST6Gal1 enzyme will form α2,6 bonds to the terminal galactoses. It has been used to produce fully sialylated forms of IgG [20], but it displayed significant sialidase activity. We found it modified the fluorescent substrate FCHASE-LacNAc with CMP-Leg5Ac7Ac as the donor at only approximately 4% of its reactivity with CMP-Neu5Ac, in a 60 min reaction, while after 20 h the conversion was 22%. Since this enzyme prefers a larger substrate, consistent with its natural role, we tested it with α_1-antitrypsin.

This glycoprotein shows more heterogeneity in its glycoforms than the interferon-α2b; approximately six Neu5Ac residues per molecule are expected, since the predominant *N*-glycan forms present are bi-antennary structures, along with some tri-antennary structures [6-8]. It also occurs in several genetic variants, which are probably responsible for the appearance of pairs of ions separated by 33-35 Da for the major pairs of peaks in the mass spectra **(Figure 5)**. In the spectrum of the native protein, the pair of peaks around 51,700 Da is attributed to glycoproteins with tri-antennary components, and the pairs around 51,200 and 51,850 Da to the addition of fucose to the bi-antennary and tri-antennary components respectively. The second and third spectra show the removal of the α_1-antitrypsin's sialic acid residues with *M. viridifaciens*

sialidase and the addition of Leg5Ac7Ac residues to the asialo-protein by ST6Gal1. The mass of the main component of the glycoprotein of 51,062 Da was reduced to 49,314 Da by sialidase treatment. This mass difference of 1748 is consistent with six Neu5Ac residues being removed. Treatment with ST6Gal1 and two aliquots of CMP-Leg5Ac7Ac resulted in the addition of three Leg5Ac7Ac residues to the *N*-glycan structures as shown by the increase in mass to 50,262 Da, i.e. by 948 Da. Thus as expected the ST6Gal1 enzyme was more active with the larger substrate.

In conclusion, our results show it is possible to produce Leg5Ac7Ac analogs of both glycolipids and glycoproteins with the two mammalian sialyltransferases. These are all considerably larger substrates than the ones we examined previously [1, 2] so their successful modification extends the scope of these reactions to more biologically interesting substances, which was not possible with the bacterial sialyltransferases. However the recent finding of natural antibodies to legionaminic acid in human sera [21] rules out their use as therapeutics, though its known derivatives such as the 5-acetamidino form [22] may not be as reactive.

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Figure legends

Fig. 1. Structure of the mono-Leg5Ac7Ac form of GD1a.

Fig. 2. Negative ion mass spectra of the Leg5Ac7Ac product from the reaction with ST3Gal1 (upper panel) and the GM1a starting material (lower panel). In the GM1a spectrum, the 802.5 ion is a doubly-charged acetate complex.

Fig.3. ELISA assay of the binding of MAG_3Fc to Leg-GD1a and four reference gangliosides. From left to right, the sets of bars represent 5, 25, 50 and 100 pmol loads of each ganglioside.

Fig. 4. Mass spectral analysis of the modification of asialo-interferon-α2b by ST3Gal1. Upper panel, the desialylated glycoprotein showing its major glycoforms, and lower panel, the Leg5Ac7Ac reaction product.

Fig. 5. Mass spectral analysis of the modification of α_1-antitrypsin by ST6Gal1. Top panel, the native glycoprotein, middle panel, the desialylated glycoprotein, and bottom panel, the Leg5Ac7Ac product.

Fig. 1

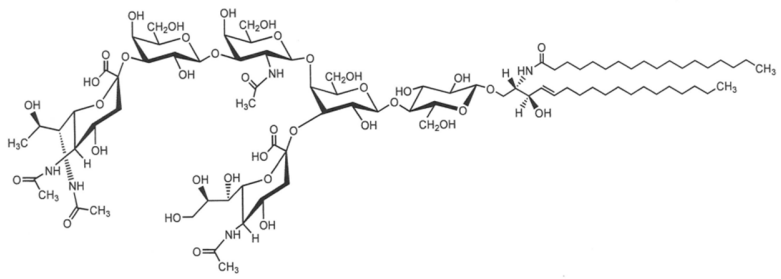

Fig. 2

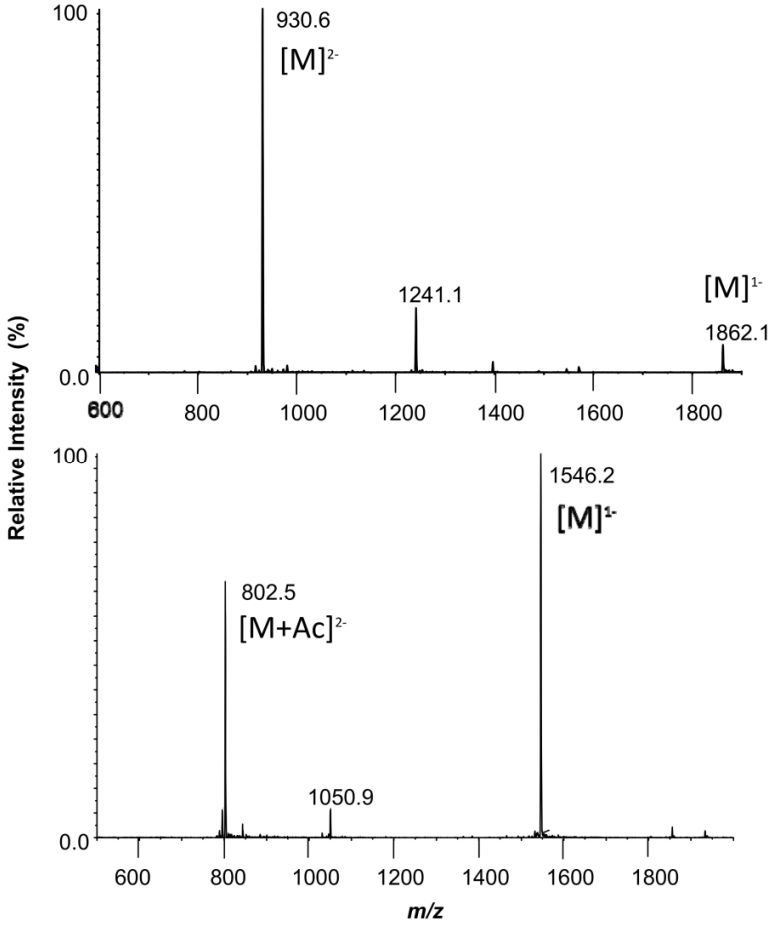
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Fig. 3

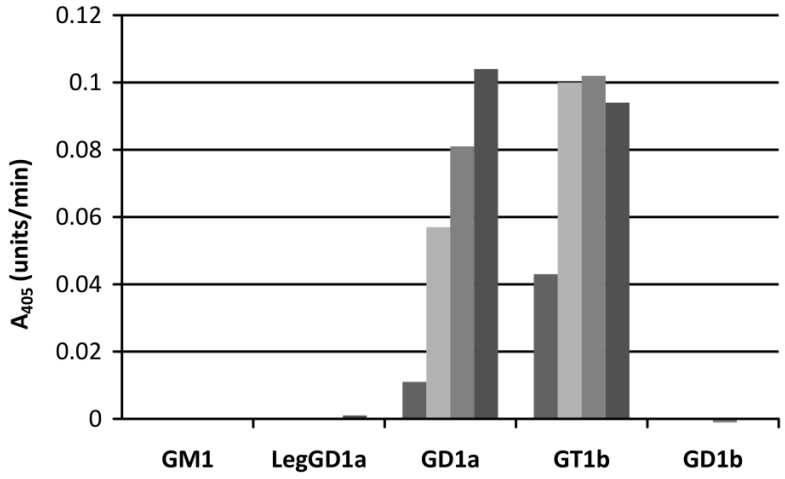
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Fig. 4

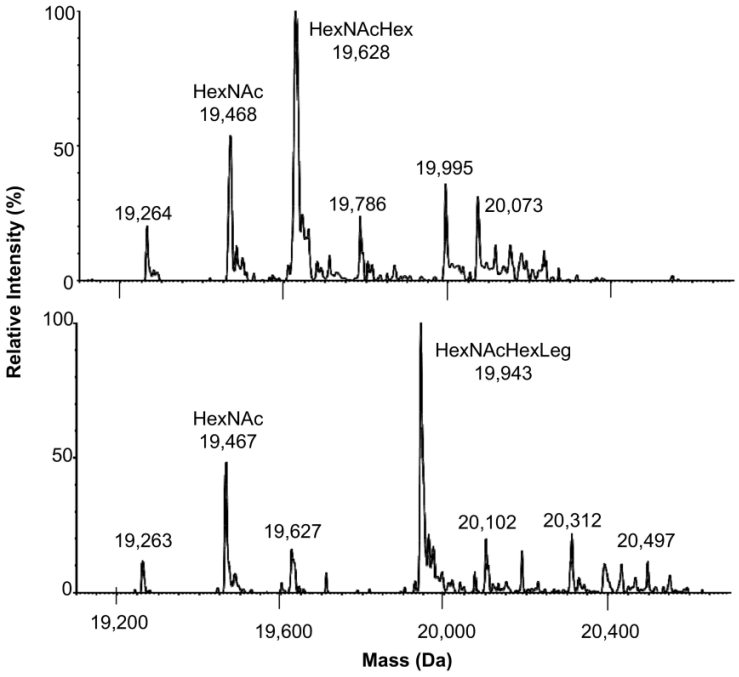
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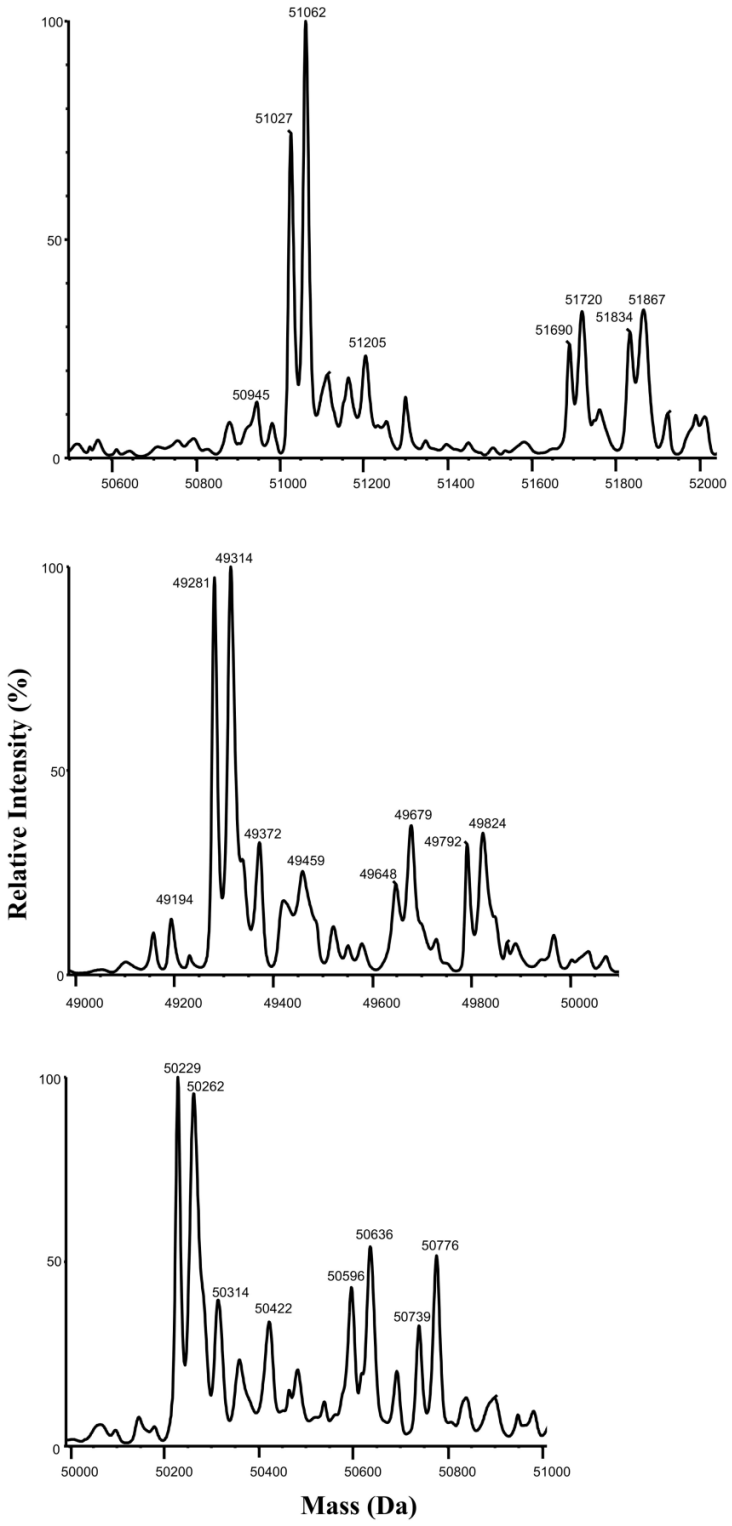
Fig. 5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Relative Intensity (%)
Mass (Da)