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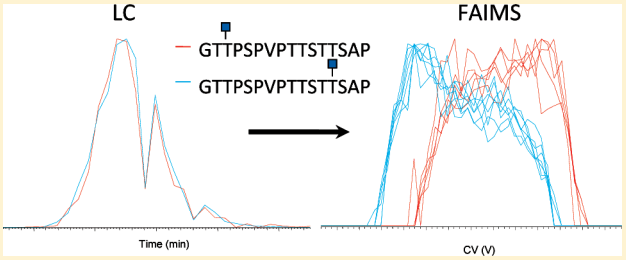
Separation and Identification of Isomeric Glycopeptides by High Field Asymmetric Waveform Ion Mobility Spectrometry

Andrew J. Creese and Helen J. Cooper*

School of Biosciences, College of Life and Environmental Sciences, University of Birmingham, Birmingham, B15 2TT, United Kingdom

ABSTRACT: The analysis of intact glycopeptides by mass spectrometry is challenging due to the numerous possibilities for isomerization, both within the attached glycan and the location of the modification on the peptide backbone. Here, we demonstrate that high field asymmetric wave ion mobility spectrometry (FAIMS), also known as differential ion mobility, is able to separate isomeric O-linked glycopeptides that have identical sequences but differing sites of glycosylation. Two glycopeptides from the glycoprotein mucin 5AC, GT(GalNAc)-TPSPVPTTSTTSAP and GTTPSPVPTTST(GalNAc)TSAP (where GalNAc is O-linked N-acetylgalactosamine), were shown to coelute following reversed-phase liquid chromatography. However, FAIMS analysis of the glycopeptides revealed that the compensation voltage ranges in which the peptides were transmitted differed. Thus, it is possible at certain compensation voltages to completely separate the glycopeptides. Separation of the glycopeptides was confirmed by unique reporter ions produced by supplemental activation electron transfer dissociation mass spectrometry. These fragments also enable localization of the site of glycosylation. The results suggest that glycan position plays a key role in determining gas-phase glycopeptide structure and have implications for the application of FAIMS in glycoproteomics.



Glycosylation of proteins is the most common post-translational modification in eukaryotic cells: It has been estimated that up to 50% of human proteins are glycosylated.[1] Glycoproteins are involved in a range of intracellular and cell−cell recognition events,[2,3] and abnormalities in glycosylation are associated with disease. For example, a decrease in O-linked glycosylation of microtubule-associated protein tau has been shown in patients with Alzheimer's disease.[4] Similarly, aberrant glycosylation of IgA1 is implicated in IgA nephropathy.[5] Proteins are glycosylated at either asparagine (N-linked) or serine, threonine, and tyrosine residues (O-linked). N-glycosylation occurs within the consensus sequence Asn-X-Ser/Thr, where X is any amino acid except Pro, whereas no consensus sequence exists for O-glycosylation. Glycoproteomics is the large scale analysis of glycoproteins and typically involves digestion of the proteins with trypsin and analysis of the resulting glycopeptides.[6] There is enormous scope for isomers within the glycopeptides: Due to the numerous possible combinations of different monosaccharide base units, glycosylation is one of the most complex post-translational modifications.[7] It is possible to have both structural and positional isomers, i.e., glycans that differ in glycosidic linkage arrangements and in branching structures. In addition, and particularly for O-glycosylation, there is the potential for localization isomers, i.e., glycopeptides with identical sequences but differing modification sites.

Glycosylation has been studied by mass spectrometry for many years but the assignment and localization of glycans on glycopeptides remains a challenge. Typically, the proteomics workflow involves online liquid chromatography coupled with tandem mass spectrometry (MS/MS). The MS/MS technique collision induced dissociation (CID) results in loss of the glycan while the peptide backbone remains intact,[6] hindering the identification of both the peptide sequence and the site of glycosylation. Electron capture dissociation and electron transfer dissociation (ETD) of glycopeptides results in retention of the glycan chain on the peptide backbone fragments, allowing localization of the modification site.[8−11] However, glycans are acidic modifications, and glycosylated peptides are commonly only observed as singly, doubly, or triply charged ions with high m/z values. These types of ions often produce ETD spectra with few fragment ions.[12] Recently, several methods have been developed which combine CID and ETD to better determine the sequence of glycopeptides.[13−15] Nevertheless, these various methods do not address the underlying issue of isomeric glycopeptides which coelute following liquid chromatography.

High field asymmetric wave ion mobility spectrometry[16,17] (FAIMS), or differential ion mobility, separates gas-phase ions at atmospheric pressure. Ions are transferred between two heated electrodes by a carrier gas. Voltages are applied to the electrodes via an asymmetric waveform producing alternate high and low electric fields perpendicular to the direction of the ions. As a result of their differential mobility, ions are attracted toward one of the electrodes to a greater extent than the other. In the absence of intervention, the ions will collide with the

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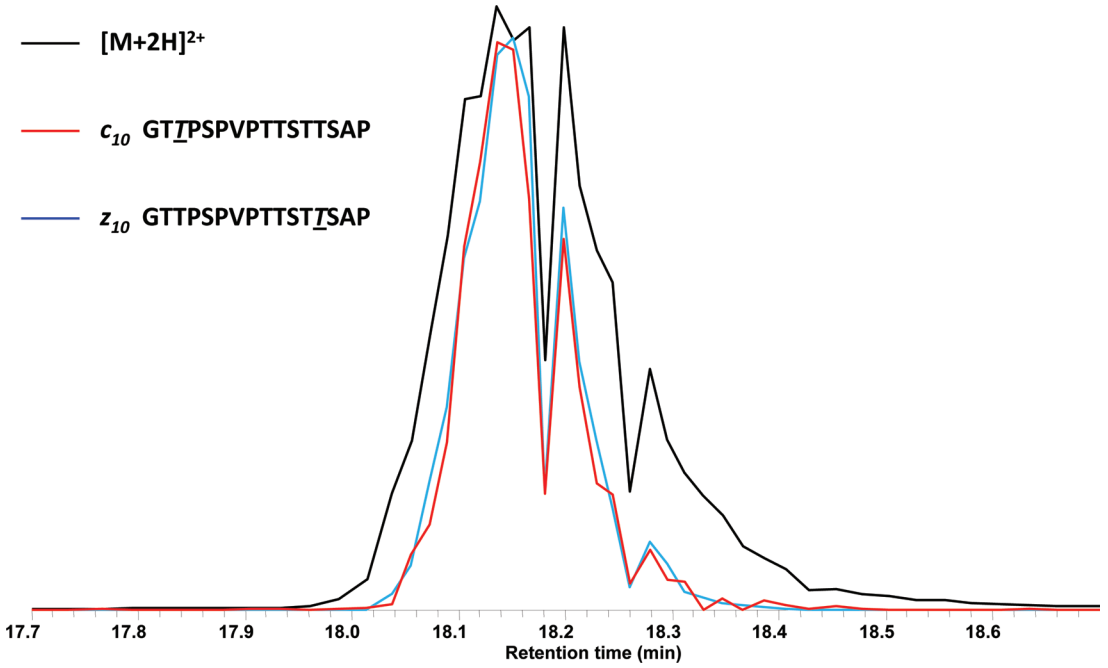

Figure 1. The extracted ion chromatograms for the doubly charged precursor ion m/z 852.91 (black), fragment c_{10} (red) from the glycopeptide GT***T***PSPVPTTSTTSAP, and fragment z_{10} (blue) from the glycopeptide GTTPSPVPTTST***T***SAP. ***T*** represents the threonine amino acid residue modified with N-acetylgalactosamine, GalNAc.

electrode. To prevent that from happening, a dc compensation voltage (CV) is superimposed. By tuning the CV, it is possible to selectively transmit particular ions through the FAIMS device into the mass spectrometer. The FAIMS device can therefore be used as an ion filter, effectively reducing signal-to-noise,[18] increasing sensitivity,[19,20] and increasing dynamic range.[21]

As FAIMS separates ions according to their differential ion mobility, rather than m/z, it may be applied for the analysis of isobaric species: The amino acids leucine and isoleucine can be separated by FAIMS,[22] as can the ortho, meta, and para forms of phthalic acid.[23] We have shown previously that FAIMS can separate isobaric phosphopeptides, i.e., those with identical sequence but differing sites of phosphorylation.[24,25] In further work, we have shown that FAIMS may be used to separate sequence isomers of nitrated peptides.[26] Fenn and McLean have separated structural and positional isomeric glycans[27] by drift tube ion mobility spectrometry and traveling wave ion mobility spectrometry; however, to date, there have been no reports of ion mobility separation of intact glycopeptides.

Here, we demonstrate FAIMS separation of two glycopeptide localization isomers from the glycoprotein mucin 5AC: GT(GalNAc)TPSPVPTTSTTSAP and GTTPSPVPTTST-(GalNAc)TSAP, where GalNAc is O-linked N-acetylgalactosamine. These glycopeptides coelute following reversed-phase liquid chromatography. FAIMS separation was confirmed by supplemental activation (sa) ETD mass spectrometry. As the differential ion mobility of an ion is intrinsically linked to its structure, our finding suggests that the position of the glycan affects the gas-phase structure of the glycopeptides. Our findings have implications for the field of glycoproteomics: The ability to separate isomeric glycopeptides will further enable identification of isoforms and ultimately improve glycoproteome coverage.

METHODS

Sample Preparation. The glycopeptides from Mucin 5AC with the sequences, GT***T***PSPVPTTSTTSAP and GTTPSPVPTTST***T***SAP (where ***T*** represents the threonine amino acid residue modified with N-acetylgalactosamine, GalNAc), herein referred to as peptides **A** and **B**, respectively, were supplied by Anaspec (Fremont, CA) and used without further purification. For the LC-ETD MS/MS experiments, the samples were resuspended in water (J. T. Baker, The Netherlands) to a final concentration of 20 fmol/μL. For the FAIMS experiments, the samples were resuspended in water and diluted in water/methanol (both J. T. Baker, The Netherlands) (30:70) containing 2% formic acid (Sigma Aldrich, Poole, Dorset), to a final concentration of 2 pmol/μL.

LC-ETD MS/MS. Online liquid chromatography was performed by use of a Dionex Ultimate 3000 HPLC system (Sunnyvale, USA). A 1:1 mixture comprising 100 fmol of each peptide was loaded onto a 75 μm (internal diameter) Acclaim PepMap100 (LC Packings, Sunnyvale, USA) C_{18} column (length 10 cm) and separated over a 30 min gradient from 3.2% to 44% acetonitrile (J. T. Baker, The Netherlands) (0.1% formic acid) at a flow rate of 350 nL/min. Eluting peptides were infused by use of an Advion Triversa Nanomate (Ithaca, USA) electrospray ionization source directly into a Thermo Scientific LTQ-Orbitrap Velos ETD mass spectrometer (Bremen, Germany). The mass spectrometer alternated between a full FT-MS scan (m/z 380−1600) and a sa ETD scan (conditions as below) of the most abundant precursor ion. Survey scans were acquired in the Orbitrap with a resolution of 60 000 at m/z 400. Precursor ions were isolated and subjected to ETD in the linear ion trap and recorded in the Orbitrap at a resolution of 7500 at m/z 400. Isolation width was 3 Th. Automatic gain control (AGC) was used to accumulate sufficient precursor ions (target value 1×10^5 charges, maximum fill time 100 ms). Dynamic exclusion was not used.

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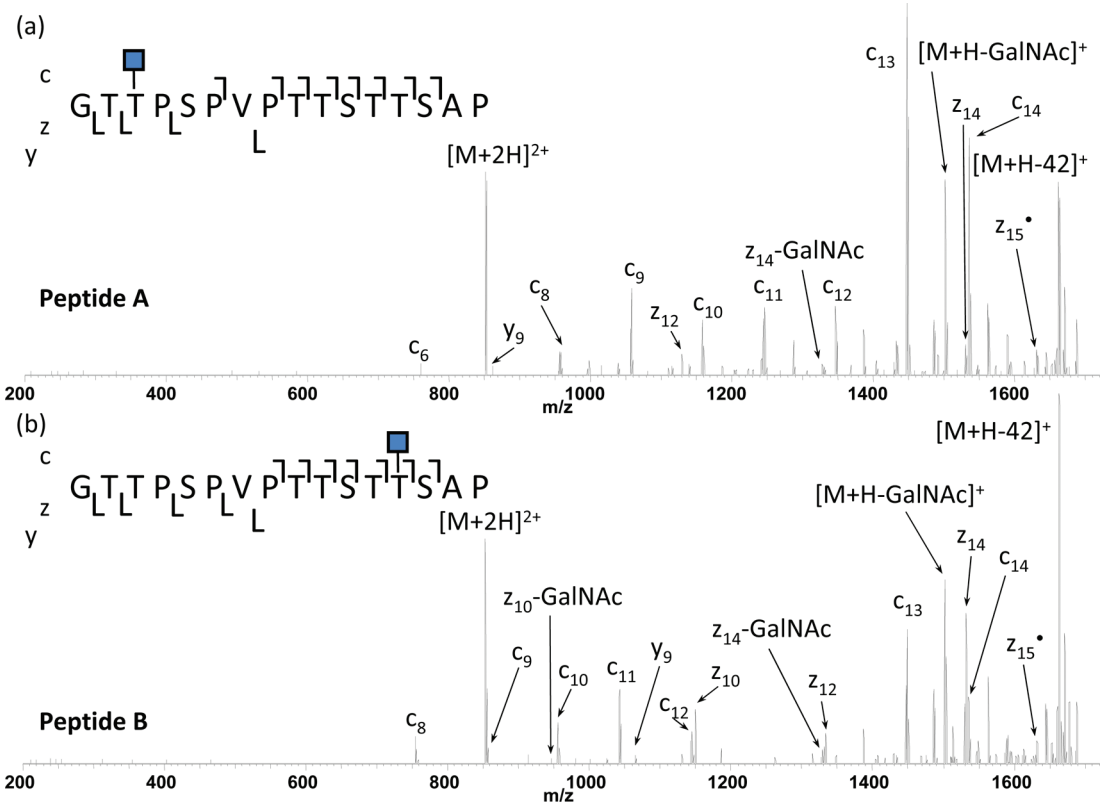

Figure 2. (a) Supplemental activation ETD mass spectrum of $[M + 2H]^{2+}$ ions of peptide **A**, recorded at a CV of −23.7 V. (b) Supplemental activation ETD mass spectrum of $[M + 2H]^{2+}$ ions of peptide **B**, recorded at a CV of −25.5 V. The CV values represent the maximum transmission of the precursor ions through the FAIMS device.

ESI-FAIMS-ETD MS/MS. The peptides were introduced into the mass spectrometer by direct infusion heated electrospray ionization (HESI) at a flow rate of 3 μL/min and a temperature of 40 °C. All FAIMS experiments were performed on an LTQ Orbitrap Velos ETD hybrid mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The FAIMS conditions were: dispersion voltage, −5 kV; inner and outer electrode temperatures, 70 and 90 °C, respectively; carrier gas flow rate, 2 L/min with a composition of 50:50 helium to nitrogen. The electrospray voltage was 5 kV with a capillary temperature of 275 °C and a sheath gas flow rate of 5 L/min. During analysis, the compensation voltage was scanned from −59.7 to −5 V in 0.3 V steps. The precursor ion of interest was fragmented by supplemental activation ETD at each CV interval, resulting in 180 saETD spectra. All mass spectra were acquired in the Orbitrap. An automated method was created: The first scan event was a full scan mass spectrum at CV of −25 V. This was followed by 180 sa ETD mass spectra of the doubly charged precursor (m/z 852.91) with CV scanning as described above. The automatic gain control (AGC) target for full scan and subsequent MS/MS spectra was 1×10^6, with a maximum injection time of 1000 ms for MS spectra and 300 ms for MS/MS spectra. Both MS and MS/MS scans were recorded with a resolution of 7500 at m/z 400. MS spectra were the result of 1 microscan, and MS/MS were 5 summed microscans. Precursor ions were isolated with a window of 3 Th. Supplemental activation (sa) ETD was performed in the ion trap, with an ETD activation time of 140 ms and a normalized collision energy (sa) of 25%.

RESULTS AND DISCUSSION

A 1:1 mixture of the two glycopeptides were analyzed by online liquid chromatography saETD MS/MS. Figure 1 shows the extracted ion chromatograms of the doubly charged precursor (m/z 852.91; black) and the reporter ions c_{10} from glycopeptide **A** (m/z 1158.58; red) and z_{10} from glycopeptide **B** (m/z 1149.55; blue). Reporter ions are those ETD fragments which are unique to a particular modification site within a peptide. These results show that it is not possible to separate these glycopeptides by reversed-phase liquid chromatography.

The glycopeptides were individually infused and analyzed by FAIMS-saETD to assess the level of fragmentation observed, the potential number of unique reporter ions, and the CV range over which the peptides were transmitted. As the reporter ions are unique to a particular modification site within a peptide, their presence can be used to confirm the success, or otherwise, of FAIMS separation.[25] For both glycopeptides **A** and **B**, singly and doubly charged ions were observed. Doubly charged glycopeptide ions were subjected to saETD. Figure 2a shows the saETD spectrum for peptide **A** recorded with at a CV of −23.7 V, approximately the midpoint in the CV range over which the doubly charged peptide was observed. All but three N−Cα bonds were cleaved. (Cleavage N-terminal to proline is rare due to the cyclic nature of the side chain.[28]) Two peaks (m/z 1501.73 and m/z 1328.62) in the spectrum show the loss of the GalNAc from the peptide backbone: $[M + 2H\text{-GalNAc}]^{+\bullet}$ and z_{14}-GalNAc. No other neutral losses of GalNAc were observed. The probable cause of the losses of the glycan is the use of supplemental activation with the ETD. Similarly, the presence of the y_9 ion may be the result of supplemental activation, although production of y ions is known to be a

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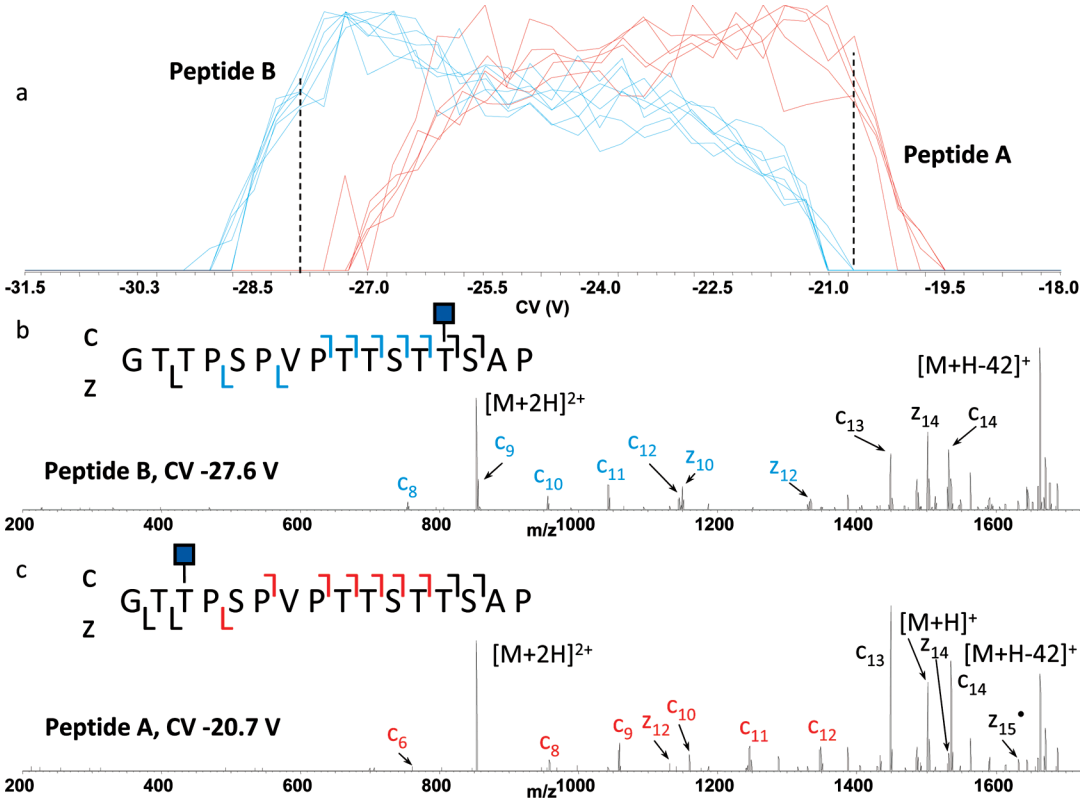

Figure 3. (a) Extracted ion chromatograms for the reporter c and z ions of peptide **A** (c_8, c_9, c_{10}, c_{11}, and c_{12}, red) and peptide **B** (c_8, c_9, c_{10}, c_{11}, c_{12}, z_{10}, and z_{12}, blue). (b) Supplemental activation ETD mass spectrum recorded at a CV of −27.6 V. Reporter ions for peptide **B** are labeled in blue. (c) Supplemental activation ETD mass spectrum recorded at a CV of −20.7 V. Reporter ions for peptide **A** are labeled in red.

minor channel in ETD. These results suggest that fragments c_6, $c_8 - c_{12}$, z_{12}, and y_9 may be used as reporter ions for peptide **A**. The saETD spectrum for peptide **B** is shown in figure 2b. The spectrum was recorded at a CV of −25.5 V, the approximate midpoint of the CV range over which the doubly charged peptide was observed. The fragmentation pattern observed is nearly identical to that seen for peptide **A**; the only differences being the absence of the c_6 fragment (m/z 558.29) and the presence of a peak corresponding to z_{10}-GalNAc (m/z 946.46). These results suggest that the fragment ions $c_8 - c_{12}$, z_{10}, z_{12}, and y_9 may be used as reporter ions for peptide **B**.

The glycopeptides were combined in a 1:1 mixture and analyzed using the same method as above. The peptides "eluted" from the FAIMS device in the compensation voltage range of −29.7 V to −19.5 V, and ETD reporter ions were observed in the CV range of −29.1 V and −19.8 V. (At CV points −29.7, −29.4, and −19.5 V, the ETD mass spectra contained peaks corresponding to the charge-reduced species and the more abundant fragments common to both glycopeptides only.) Figure 3a shows the extracted ion chromatograms for seven of the reporter ions for peptide **B** (fragments c_8, c_9, c_{10}, c_{11}, c_{12}, z_{10}, and z_{12}, shown in blue) and five of the reporter ions for peptide **A** (fragments c_8, c_9, c_{10}, c_{11}, and c_{12}, shown in red). The additional reporter ions for peptide **A** (c_6 and z_{12}) and the y_9 fragments for both peptides are not shown here because they were not observed in every spectrum. The overlaid extracted ion chromatograms of the reporter ions for the two glycopeptides (Figure 3a) show compensation voltage ranges where (a) only peptide **B** is observed (−29.1 to −27.3 V), (b) both glycopeptides are observed (−27.0 to −20.7 V), and (c) only peptide **A** is observed (−20.4 to −19.8 V). Figure 3b shows the saETD mass spectrum obtained at CV of −27.6 V. All of the fragments in this mass spectrum originate from glycopeptide **B**. Figure 3c shows the saETD spectrum recorded at CV −20.7 V. All the fragments in this mass spectrum derive from glycopeptide **A**.

As FAIMS separates ions on the basis of their differential ion mobility, which in turn is linked to ion structure, these findings suggest that glycopeptides have gas-phase structures dependent on the site of glycosylation. The glycopeptides studied here are modified by a single GalNAc unit. It is possible that steric hindrance gives rise to differing gas-phase structures. An alternative explanation for the separation of the two glycopeptides by FAIMS is the presence of salt bridges between a carboxylate of the GalNAc and the amine group of the N-terminus.[29,30] We postulate that such a salt-bridge would only be present in glycopeptide **B**, giving the peptide a more compact structure, and that the proximity of the GalNAc on peptide **A** to the N-terminus and the lack of other potential binding sites would hinder the formation of such a noncovalent bond.

■ CONCLUSION

We have shown that it is possible to use FAIMS to separate localization isomers of O-linked glycopeptides and confirmed the separation by saETD mass spectrometry. Glycosylation is unlike any other post-translational modification. The complexity and heterogeneity of glycosylation can have a dramatic effect on the structure and folding of peptides and proteins.[31] Our results suggest that structural differences can also be observed in the gas phase and that those differences can be exploited for

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the analysis of glycosylation, with potential applications in the field of glycoproteomics.

AUTHOR INFORMATION

Corresponding Author

*Tel: +44 (0)121 414 7527. Fax: +44 (0)121 414 5925. E-mail h.j.cooper@bham.ac.uk.

Notes

The authors declare no competing financial interest.

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