1. Materials & Methods

Methods

Optimised $NaIO_4$ based oxidation: Sia specific glycan oxidation with $NaIO_4$ was optimised previously on GD HEK293 derived, sialylated GBP-R86N nanobody, which is a mixture of non-glycosylated and LacNAc-Sia glycosylated GBP.[1] The non-sialylated nanobody species in the mixture served as internal control for undesired side-oxidation/conjugation. Optimal conditions for Sia specific oxidation on GBP-R86N (160 µg, 12 nmol) were found at 10 mM $NaIO_4$, 0.1 M NaPi buffer pH 7 (or lower pH) (total reaction volume 200 µl), on ice, in the dark for 30 min. The reaction was then quenched with 50 eq. of glycerol, with respect to $NaIO_4$, for 15 min. on ice in the dark before undergoing a buffer exchange purification (using Pierce™ Polyacrylamide Spin Desalting Columns, 7K MWCO, 0.7 mL) to remove unreacted $NaIO_4$, glycerol and formaldehyde by-product.

Optimised Ugi conjugation conditions: Ugi conjugation was performed using the aldehyde from the oxidised glycan as a starting point (20 µg, ca. 1.5 nmol of oxidised GBP-R86N nanobody in 20 µl of 0.1 M NaAc buffer). 200 equivalents of amine (PPD; 1.54 µl of 100 mM PPD in MQ stock), AcOH (0.85 µl of 1% AcOH in MQ solution) and t-Bu Isocyanide (1.60 µl of 1% tBuNC in MQ stock solution) were always calculated relative to protein content. The reaction was performed in 0.1 M NaAc buffers at pH 5 (40 µl total reaction volume), at room temperature (20-25°C), overnight (ca. 12 h).

Materials

All products including $NaIO_4$, p-Phenylenediamine (PPD, 98% pure, solid), Aniline (99.5%, liquid), benzylamine (99% pure, liquid), cyclohexylamine (99% pure, liquid) and tert-butyl carbazate (98% pure, solid) were acquired for Sigma-Aldrich unless otherwise stated.

0.1 M sodium phosphate (NaPi) buffers were made by mixing 0.5 M $Na_2HPO_4.2H_2O$ and 0.5 M $NaH_2PO_4.H_2O$ solutions until the desired pH was reached. Then, this solution was diluted and the pH was checked.

0.1 M sodium acetate (NaAc) buffer was obtained by mixing 0.2 M solutions of sodium acetate and acetic acid until de desired pH was reached. Then this solution was diluted and the pH was checked.

Aminooxy label biotin-PEG_3-ONH_2 (EZ-Link™ Alkoxyamine-PEG4-Biotin) and biotin were obtained from Thermo Fisher ScientificTM.

FabRICATOR enzyme was purchased from Genovis.

[1] Breedam, W. V.; Thooft, K.; Santens, F.; Vanmarcke, S.; Wyseure, E.; Laukens, B.; Moer, B. V.;Nerinckx, W.; Devos, S.; Madder, A.; Callewaert, N. GlyConnect: A Glycan-Based Conjugation Extension of the GlycoDelete Technology. *bioRxiv* **2021**, 2021.06.02.446789. https://doi.org/10.1101/2021.06.02.446789.

LC-MS Measurements

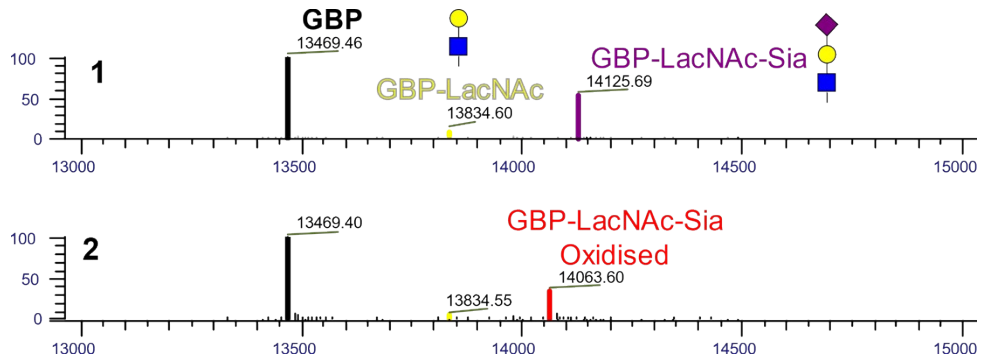
Protein samples (10 µg; ca. 10-20 µl injection volume depending on protein concentration) were injected on a Poroshell 300SB-C8 column (5 µm, 300Å, 1x75mm IDxL; Agilent Technologies) at a flow rate of 100 µl/min using solvent A (0.1% formic acid and 0.05% trifluoroacetic acid in water) and solvent B (0.1% formic acid and 0.05% trifluoroacetic acid in acetonitrile) as mobile phases. After on-column desalting for 2.5 min at 10% solvent B, the proteins were separated using a 6.5 min gradient from 10% to 90% solvent B. Column temperature was maintained at 60°C. Eluting proteins were directly sprayed in the mass spectrometer with an ESI source using the following parameters: spray voltage of 4.2 kV, surface-induced dissociation of 30 V, capillary temperature of 325 °C, capillary voltage of 35 V and a sheath gas flow rate of 7 (arbitrary units). The mass spectrometer was operated in MS1 mode using the orbitrap analyzer at a resolution of 60,000 (at m/z 400) and a mass range of 600-4000 m/z, in profile mode.

The resulting MS spectra were deconvoluted with the BioPharma FinderTM 3.0 software (Thermo Fischer Scientific) using the Xtract deconvolution algorithm (isotopically resolved spectra). The deconvoluted spectra were manually annotated.

Antibody samples were pre-treated with FabRICATOR enzyme at 37°C for 2h (10 µg of Antibody was incubated with 10 U of FabRICATOR) and then incubated with TCEP at 37°C for 1h (final TCEP concentration at 10 mM).

All protein species (non-glycosylated, glycosylated, non-conjugated and conjugated) within a sample, as run on the described LC system, eluted at the same retention time and were detected via UV (214 nm). Thus, only a single UV absorption signal was observed. Simultaneously, the total MS ion count (TIC) also showed but a single peak. The protein mixture's ion trace was extracted from the TIC and subsequently deconvoluted to yield the separate protein masses. Because all protein species eluted together, were ionized together and were extracted together, the intensities of the different deconvoluted protein could be compared relative to each other (relative abundance).

Results



SI-Figure 1A: *Deconvoluted ESI-MS spectra of the $NaIO_4$-based, selective Sia oxidation (annotated spectra). HEK293-GlycoDelete derived GBP-R86N nanobody, which is a mixture of non-glycosylated nanobody (GBP, black, MW = 13469 Da, 65%), LacNAc carrying (yellow, MW =13834, 1%) and LacNAc-Sia carrying glycan (purple, MW = 14125 Da, 34%), was used (**1**). The non-sialylated fraction serves as internal control for unspecific side oxidation/conjugation. Sia selective oxidation was performed using 10 mM $NaIO_4$ in a 0.1M NaPi buffer pH 7, for 30 min. at 4°C. Oxidation was successful, complete and selective for Sia as al the Sia glycan signal (purple) has disappeared and only oxidised Sia glycan (red, MW = 14063 Da) is left (**2**).*

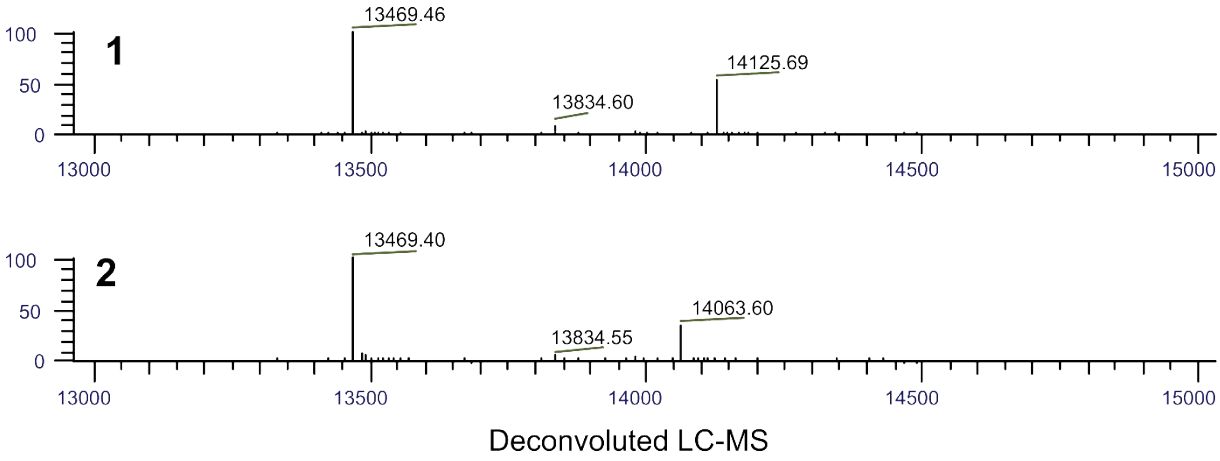
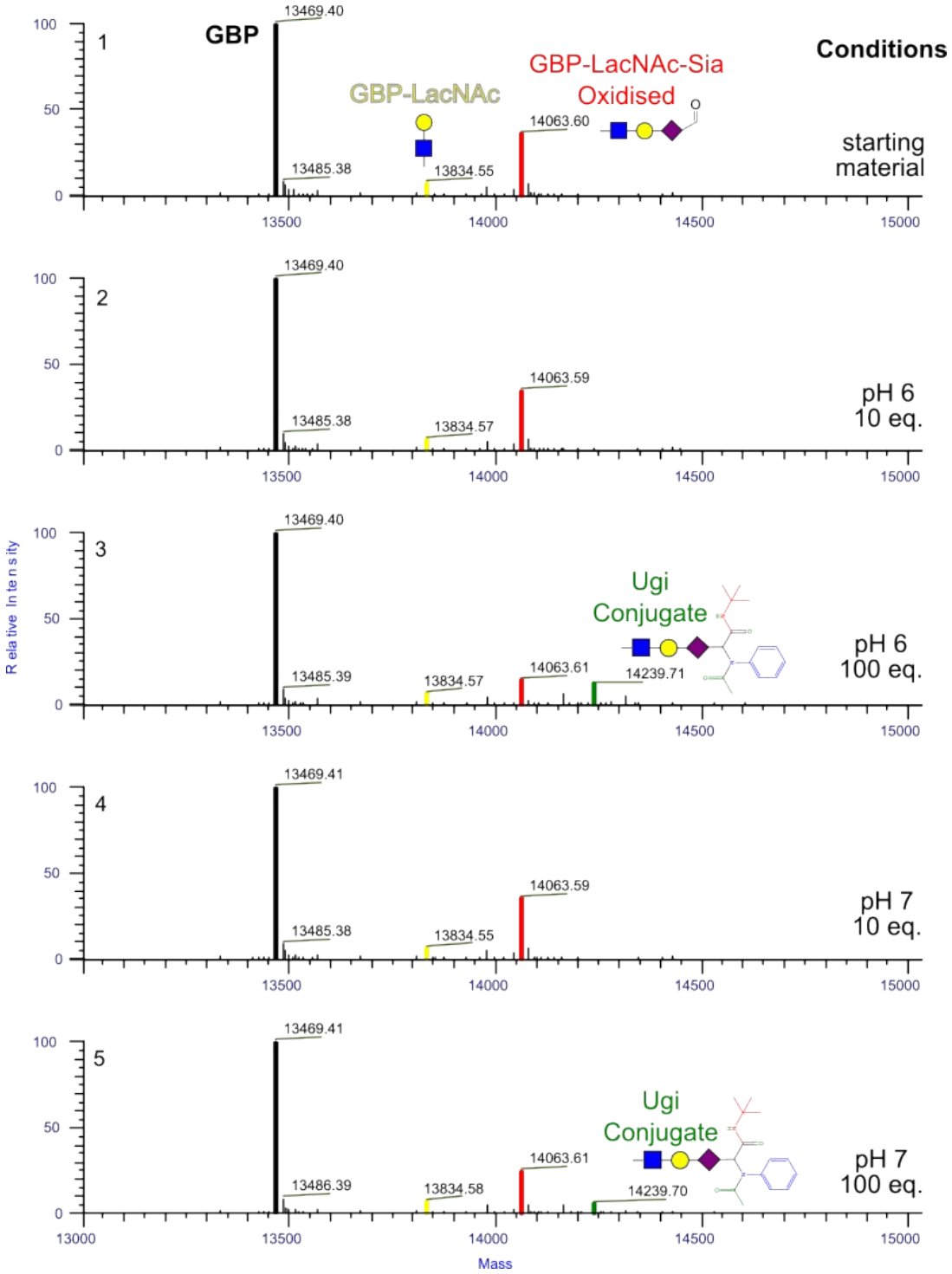


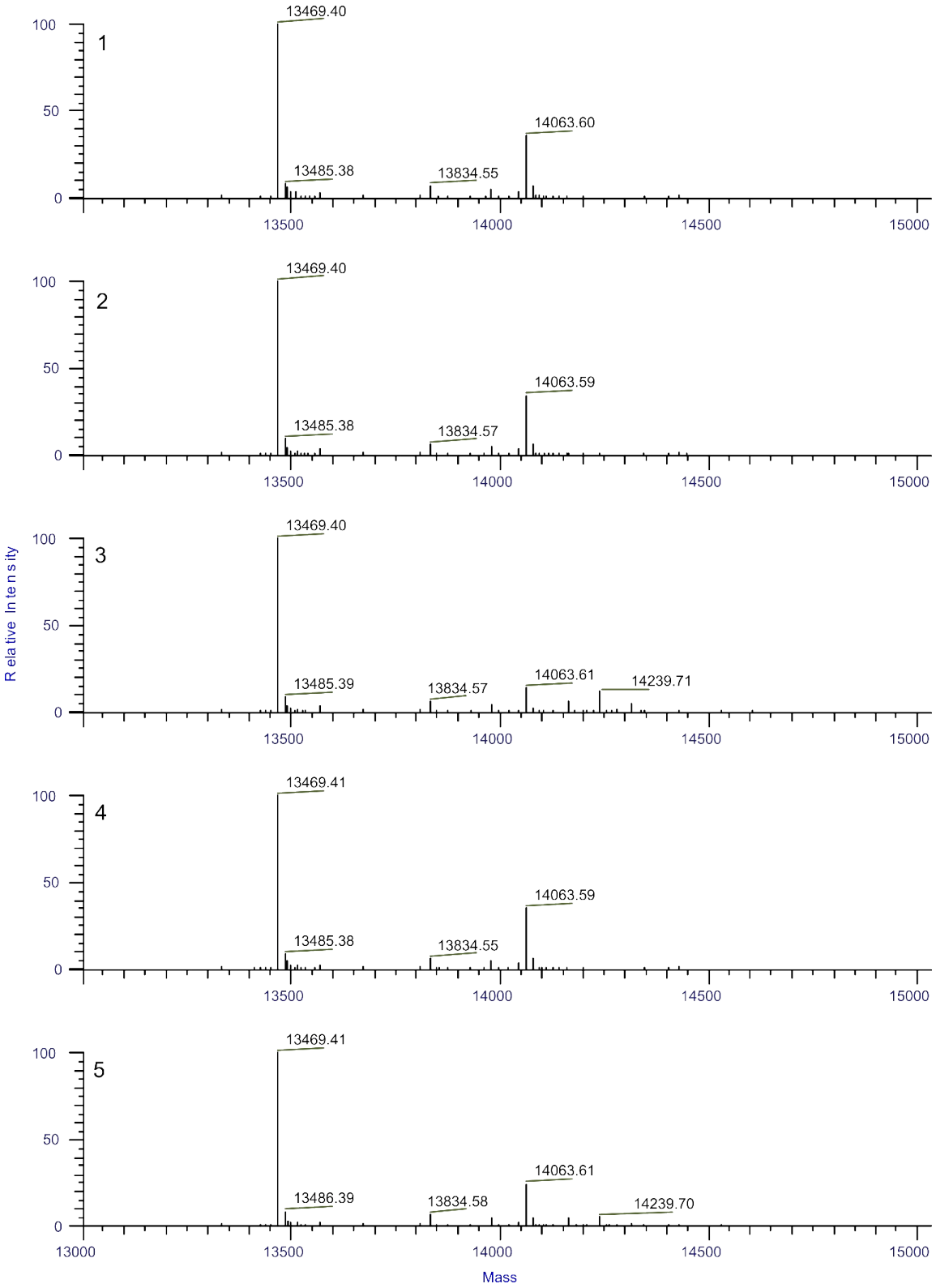
Figure 1B: *Deconvoluted ESI-MS spectra of the $NaIO_4$-based, selective Sia oxidation (raw data).*



SI-Figure 2A: *Deconvoluted ESI-MS spectra of the Ugi conjugation attempt using aniline together with AcOH and t-Bu Isocyande (annotated spectra). HEK293-GlycoDelete derived GBP-R86N nanobody, which is a mixture of non-glycosylated nanobody (GBP, black, MW = 13469 Da), LAcNAc (yellow, MW =13834 Da) and LacNAc-Sia (oxidised, red, MW = 14063 Da) carrying glycan, was used. The non sialylated fraction serves as internal control for unspecific side oxidation/conjugation. Ugi conjugation was performed at pH 6 or 7 (0.1M NaPi buffers), using 10 or 100 eq. of aniline together with 10 to 100 eq. of AcOH and t-Bu Isocyande at room temperature (20-25°C), overnight (ca. 12h).*

With the use of 100 eq. of reagents, both at pH 7 and pH 6 the relative abundance of the oxidised Sia glycan had decreased from 35% relative abundance to 24% at pH 7 and 14% at pH 6. The relative abundance of the species of interest were calculated using the abundance of the non-glycosylated GBP, which does not participate in the reactions, as internal reference. In addition, a mass corresponding to the formed conjugate after loss of CO_2, could be seen (green, MW = 14239

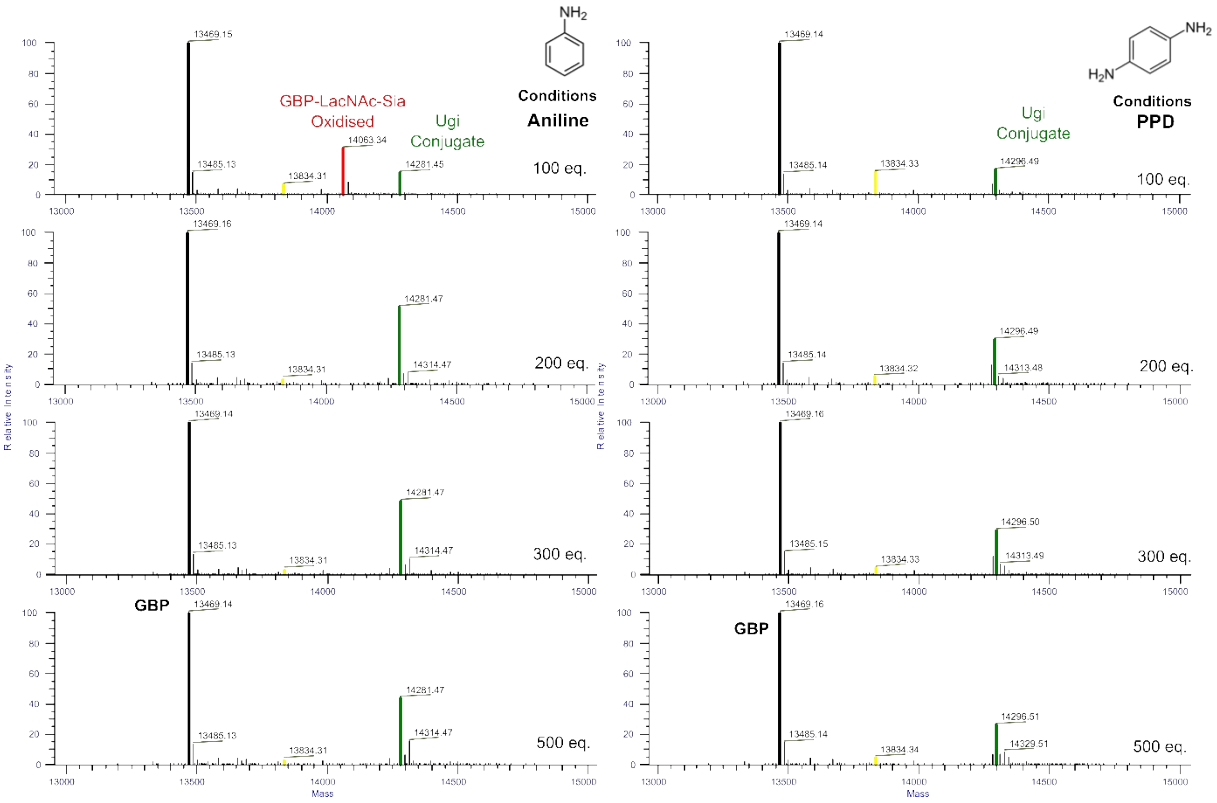
Da, calculated as follows: 14063 (oxidized GBP-LacNac) + 60 (for CH_3COOH) + 83 (for tBuNC) + 93 (aniline) -18 (for loss of water during initial imine formation) = 14281 (conjugate mass), followed by - 42 for the loss of CO_2 = 14239). Lower pH seemed to be beneficial for conjugation (15% conjugate yield at pH 7 vs 35% yield at pH 6, see SI-Table 1). With only 10 eq., no conjugation was observed.



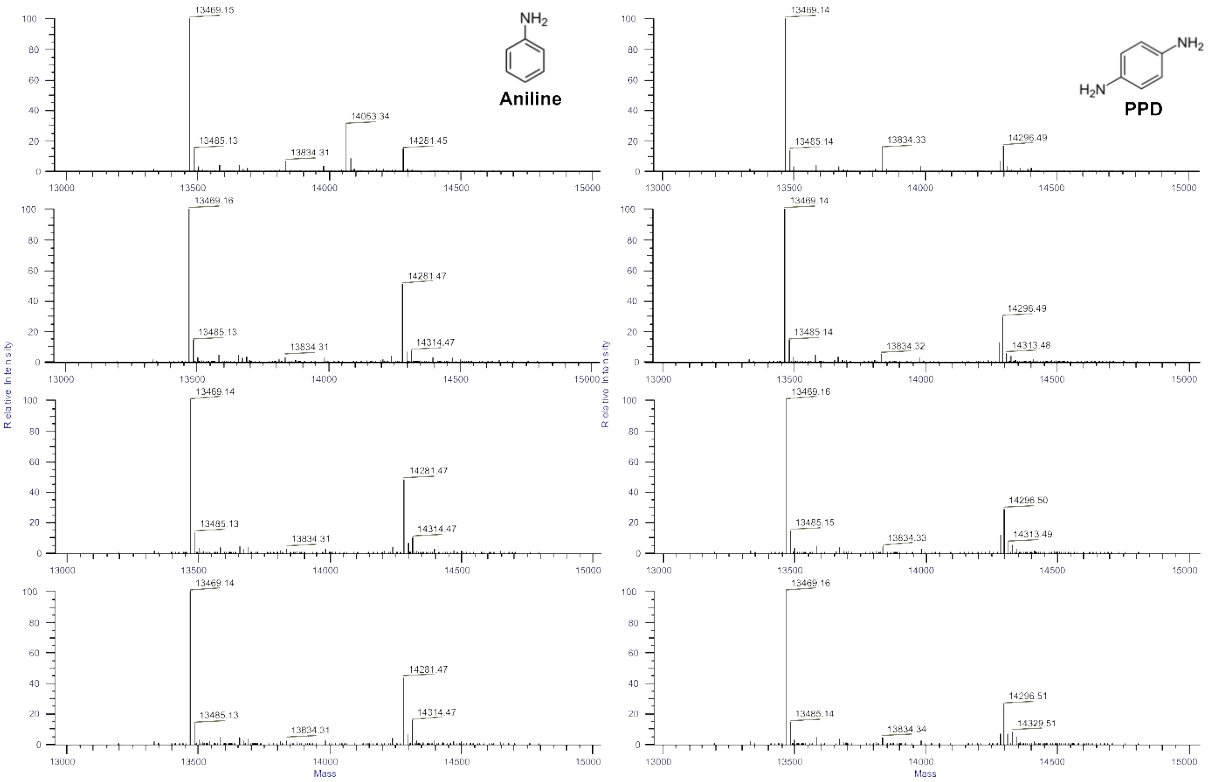
SI-Figure 2B: *Deconvoluted ESI-MS spectra of the Ugi conjugation attempt using aniline together with AcOH and t-Bu Isocyande (raw data).*

		Relative Abundance with respect to the non-glycosylated GBP species		
	Equivalents	Oxidised LAcNAc-Sia	Ugi conjugate	**Yield**
Ugi Starting Mixture	0	35,73	0	0%
Ugi conjugation pH 7	10 eq.	35,21	0	0%
Ugi conjugation pH 7	100 eq.	24,40	5,48	15%*
Ugi conjugation pH 6	10 eq.	34,57	0	0%
Ugi conjugation pH 6	100 eq.	13,88	12,58	35%*

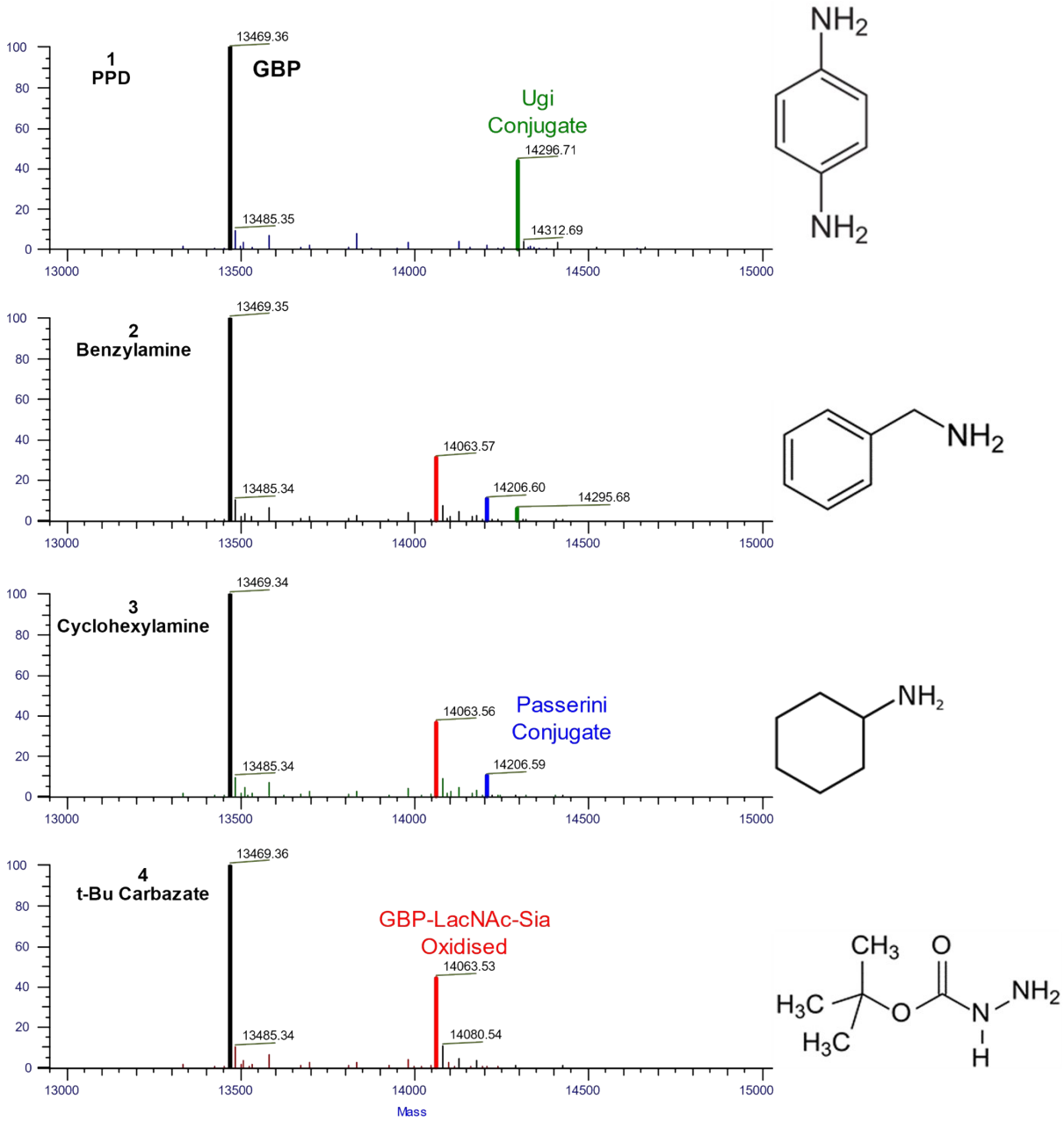
SI-Table 1: *Relative abundances and yield calculations of the initial Ugi conjugation experiment. Relative abundances were extracted from the deconvoluted ESI-MS spectra (See Fig SI 1). All protein species eluted together, were ionized together and were extracted and deconvoluted together. Therefore, the intensities of the different deconvoluted protein species could be compared relative to each other (relative abundance). Relative abundances are calculated as a percentage of peak intensity, with respect to the most intense peak, in this case the peak of the non-glycosylated nanobody. The relative abundance of the oxidised Sia glycan had decreased from 35% to ca. 24% at pH 7 and to ca. 14% at pH 6, hinting towards conjugation. Also, an Ugi conjugate mass was observed. *Note; The presumed Ugi conjugate mass was 42 Da short of the theoretical mass. This could correspond to the loss of CO_2 from the glycan, during ionisation (see also Palmisano et al. 2013).*[1]



SI-Figure 3A: *Deconvoluted ESI-MS spectra of the Ugi conjugation optimisation using aniline or PPD together with AcOH and t-Bu Isocyande at pH 5 (0.1 M NaAc buffers) (annotated spectra). NaIO4-oxidised HEK293-GlycoDelete derived GBP-R86N nanobody, which is a mixture of non-glycosylated nanobody (GBP, black, MW = 13469 Da), and LacNAc-Sia carrying (oxidised, red, MW = 14063 Da), was used. The non-glycosylated fraction serves as internal control for unspecific side oxidation/conjugation. Ugi conjugation was performed using 500 to 100 eq. of aniline or PPD together with 500 to 100 eq. of AcOH and t-Bu Isocyande. The reaction was incubated at room temperature (20-25°C), overnight (ca. 12h). The difference in reactivity between aniline and PPD was investigated. Ugi conjugate is formed successfully (green, MW = 14281 Da (aniline) or 14296 Da (PPD)). With aniline, at 100 eq. incomplete reaction is observed, however with PPD Ugi formation is complete with 100 eq.*



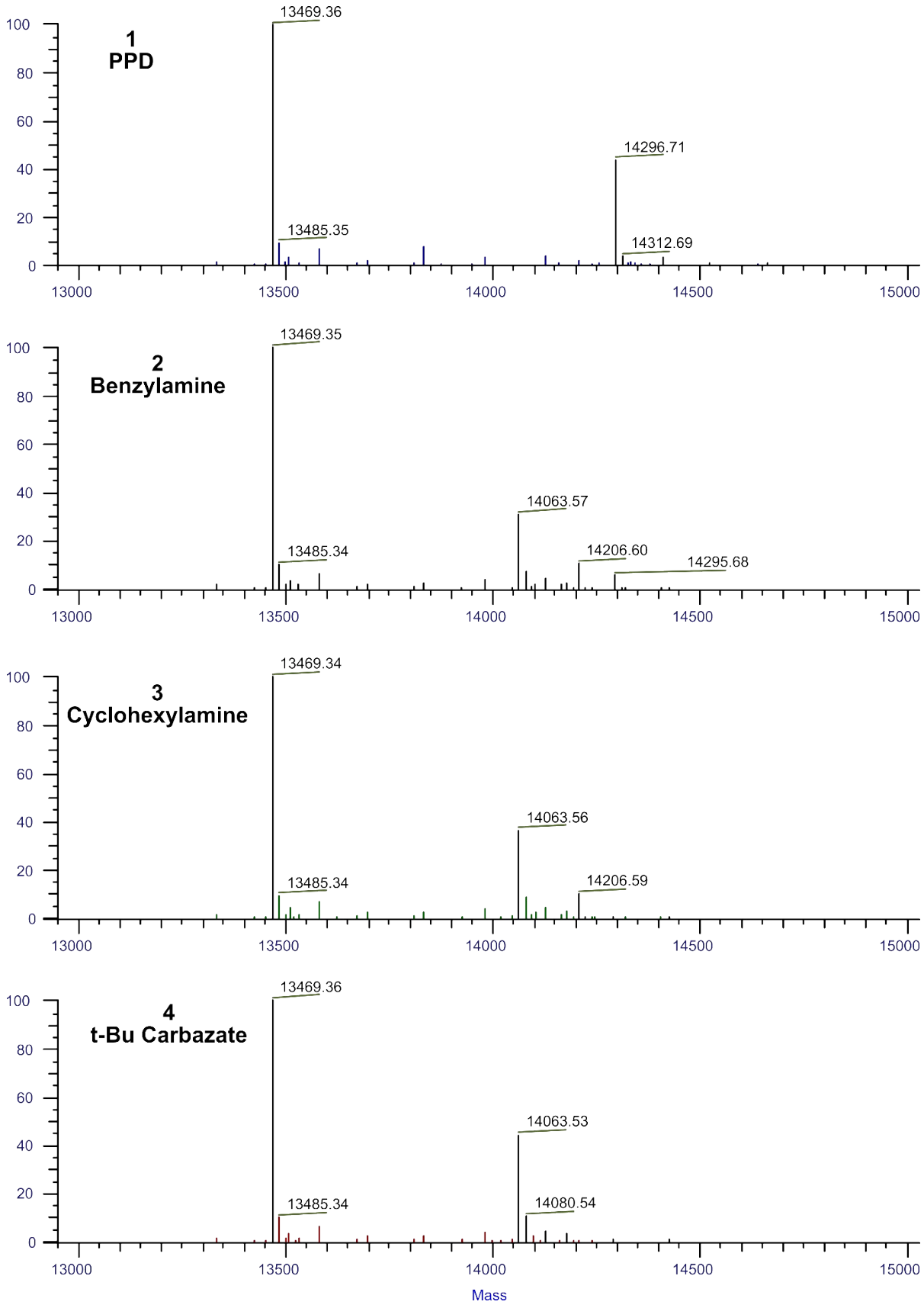
SI-Figure 3B: *Deconvoluted ESI-MS spectra of the Ugi conjugation optimisation using aniline or PPD together with AcOH and t-Bu Isocyande at pH 5 (0.1 M NaAc buffers) (raw data).*



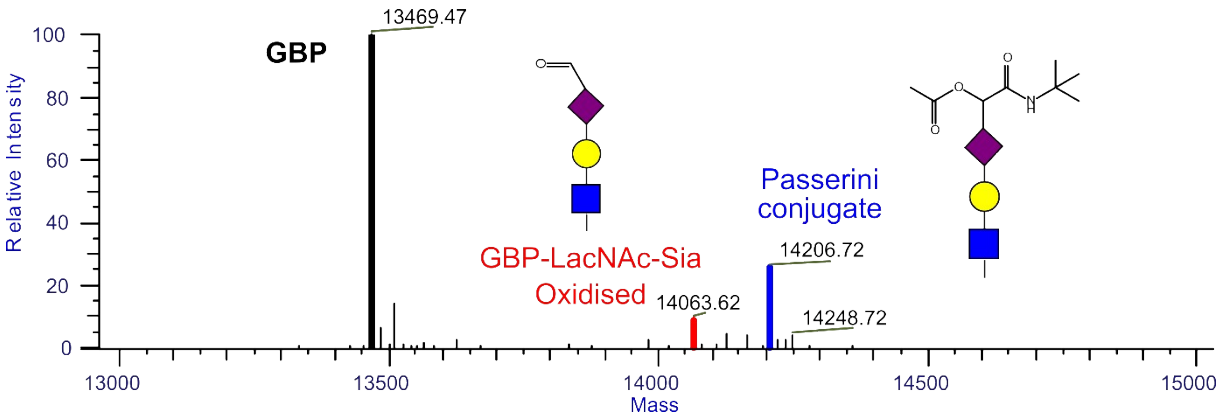
SI-Figure 4A: *Deconvoluted ESI-MS spectrum of competition experiment between different amines in Ugi formation at pH 5 (0.1 M NaAc buffers) (annotated spectra)*

*$NaIO_4$ oxidised HEK293-GlycoDelete derived GBP-R86N nanobody, which is a mixture of non-glycosylated nanobody (GBP, black, MW = 13469 Da) and LacNAc-Sia carrying (oxidised, red, MW = 14063 Da) nanobody, was used. The non-glycosylated fraction serves as internal control for unspecific side oxidation/conjugation. Conjugation was performed at pH 5 with 200 eq. of different amines, namely PPD (**1**), benzylamine (**2**), cylcohexylame (**3**) and t-Bu carbazate (**4**). These were combined with 200 eq. of AcOH and t-Bu Isocyande and incubated overnight (ca. 12h) at room temperature (20-25°C).*

*For PPD, successful and complete Ugi conjugation was observed (green, MW = 14296 Da) (**1**). With Benzylamine, Ugi conjugation was observed albeit only partially. Next to the desired Ugi product also the Passerini conjugate (blue, MW = 14206 Da) is observed next to unreacted, oxidised LacNAc-Sia glycan (red, MW = 14063 Da) (**2**). When cyclohexylamine is used, only Passerini conjugation is observed (**3**). In the presence of t-Bu carbazate, no conjugation at all is observed.*

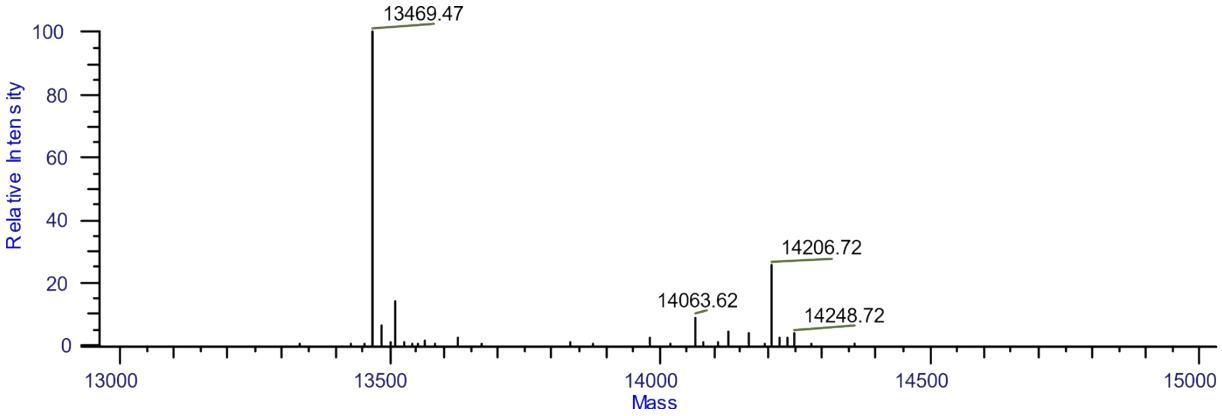


SI-Figure 4B: *Deconvoluted ESI-MS spectrum of competition experiment between different amines in Ugi formation at pH 5 (0.1 M NaAc buffers) (raw data)*

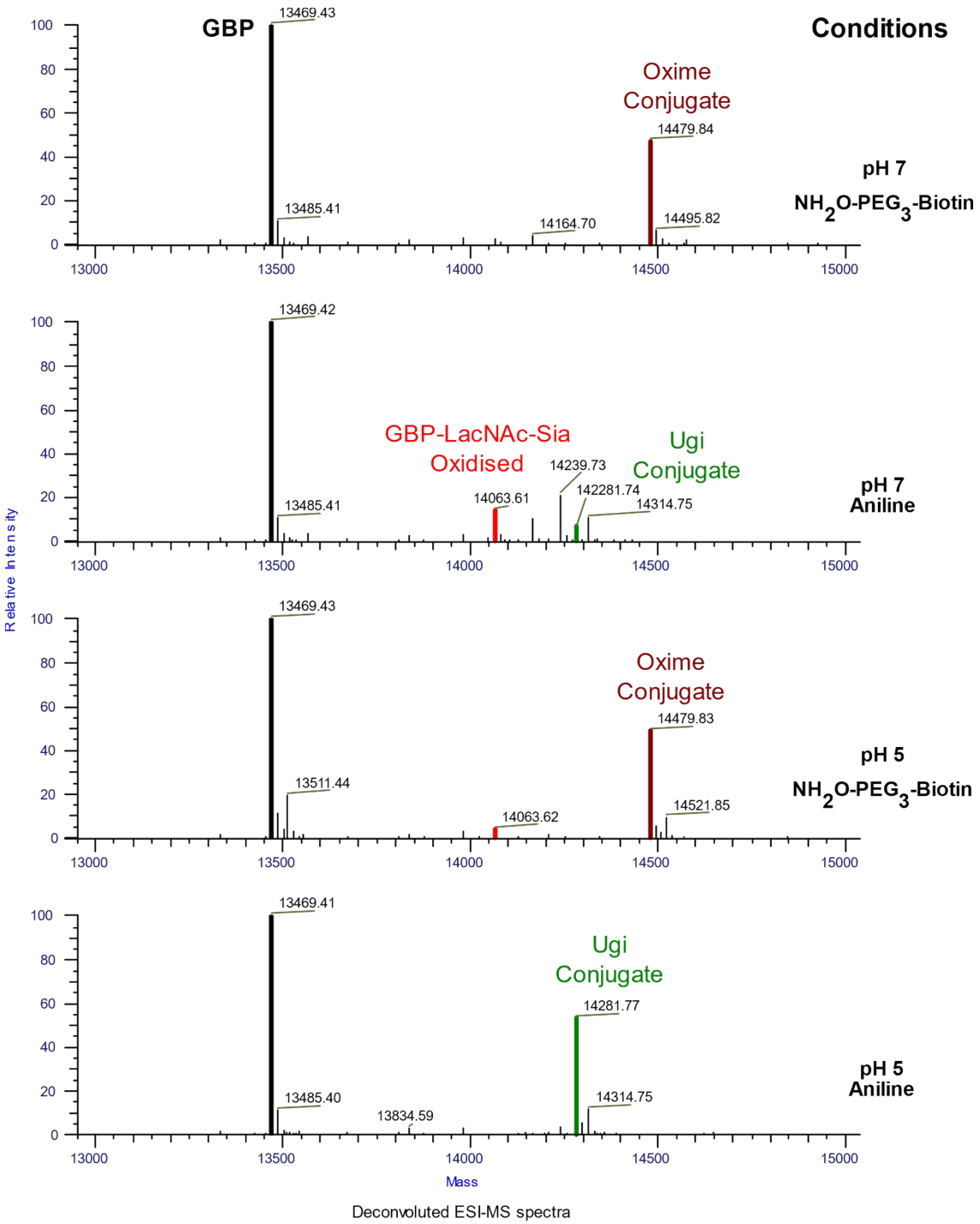


***SI-Figure 5A**: Deconvoluted ESI-MS spectrum of Passerini-based conjugation pH 5 (0.1 M NaAc buffers) (annotated spectrum).*

$NaIO_4$ oxidised HEK293-GlycoDelete derived GBP-R86N nanobody, which is a mixture of non-glycosylated nanobody (GBP, black, MW = 13469 Da) and LacNAc-Sia carrying (oxidised, red, MW = 14063 Da) nanobody, was used. The non-glycosylated fraction serves as internal control for unspecific side oxidation/conjugation. Conjugation was performed at pH 5 with 200 eq. of AcOH and t-Bu Isocyande, incubated 12h at room temperature (20-25°C).



***SI-Figure 5B**: Deconvoluted ESI-MS spectrum of Passerini-based conjugation pH 5 (0.1 M NaAc buffers) (raw data).*



SI-Figure 6A: *Deconvoluted ESI-MS spectrum of competition experiment between oxime and Ugi formation at different pH values.*

$NaIO_4$ oxidised HEK293-GlycoDelete derived GBP-R86N nanobody, which is a mixture of non-glycosylated nanobody (GBP, black, MW = 13469 Da) and LacNAc-Sia carrying (oxidised, red, MW = 14063 Da) nanobody, was used. The non-glycosylated fraction serves as internal control for unspecific side oxidation/conjugation. Conjugation was performed with using 500 eq. of aniline or biotin-PEG3-ONH2 label together with 500 eq. of AcOH and t-Bu Isocyande at pH 5 (0.1 M NaAc buffer) of pH 7 (0.1 M NaPi buffer). The reaction was incubated at room temperature (20-25°C), overnight (ca. 12h). When aminooxy label is present, an oxime conjugate (brown, MW = 14477 Da) is formed both at PH5 and pH 7. Ugi conjugation (green, MW = 14281 Da) occurs at pH 5, but does not seem to produce ('stable') Ugi conjugate at neutral pH, and is incomplete as there

remains unreacted, oxidised Sia glycan (red, MW = 14063 Da). Purification was achieved using an Agilent Biosec 3 column (3µm, 150A, 4.6x300mm), eluting with 10 mM NH4PO4 at 0,4 ml/min. Elution time for the desired conjugate was 15 min.

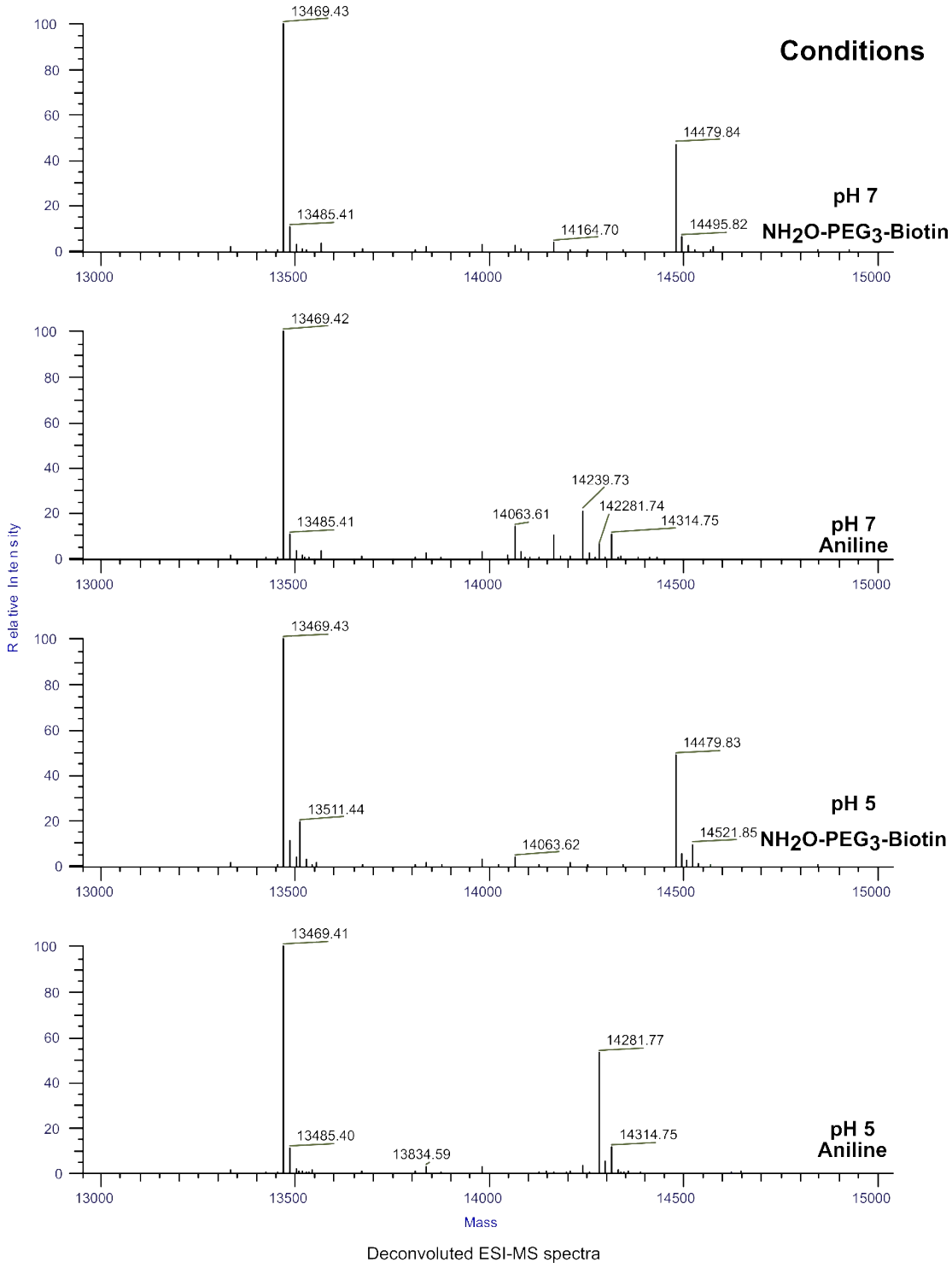
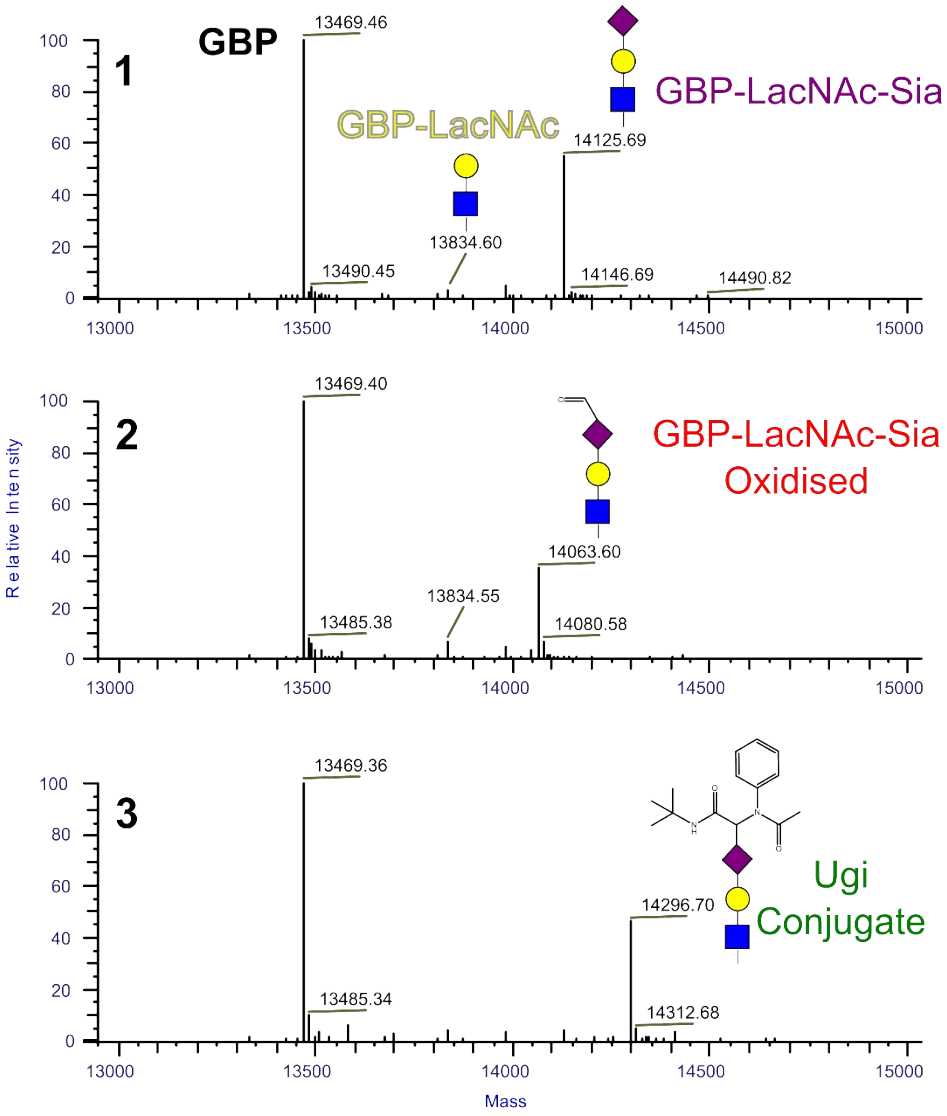
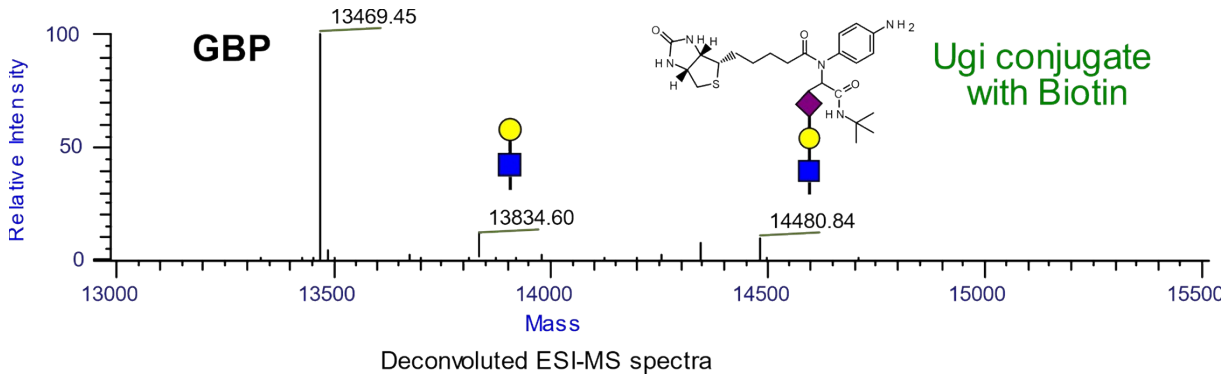


Figure 6B: *Deconvoluted ESI-MS spectrum of competition experiment between oxime and Ugi formation at different pH values. (raw data).*



SI-Figure 7: *Deconvoluted ESI-MS spectrum of the Ugi-based conjugation towards the LacNAc-Sia glycan of the HEK293-SiaHigh GlycoDelete derived GBP-R86N nanobody. (raw spectra)*



SI-Figure 8: *Deconvoluted ESI-MS spectrum of the Ugi-based conjugation of biotin towards the LacNAc-Sia glycan of the HEK293-SiaHigh GlycoDelete derived GBP-R86N nanobody. (raw data)*

A side note about the overall stability of the Ugi product in the conjugation mixture can be made. Through this work, we have noticed that Ugi conjugation at pH 5 gives the best and most stable results. Ugi conjugation at pH 7 is possible as we have shown, however it seems less reliable. Especially upon longer reaction times or prolonged storage without purification e.g. awaiting analysis, at neutral pH, side reactions and precipitation might occur.