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Fluoroglycoproteins by copper-free strain-promoted azide–alkyne cycloaddition

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Abstract

This chapter describes a reliable two-step, metal-free protocol for the preparation of well-defined fluoroglycoproteins. It starts with a first alkylation step to chemoselectively install strain alkyne handles at cysteine residues followed by a second strain-promoted azide–alkyne cycloaddition using an inverse electron-demand Diels–Alder reaction. This proof-of-principle study that uses the apoptotic protein marker Annexin V enables the efficient metal-free incorporation of 2-deoxy-2-fluoro-glycopyranosyl azides into proteins and complements previous methods using Cu(I)-mediated azide–alkyne cycloadditions and thiol chemistry.

Keywords

Selective protein modification; bioconjugation; bioorthogonal reaction; covalent conjugation chemistry; homogeneous glycoconjugates; fluor; fluorosugars; fluoroglycoproteins; strain-promoted azide–alkyne cycloaddition; inverse electron-demand Diels–Alder reaction

1. Introduction

Rational OH-to-F replacements in carbohydrate antigens represent a key strategy for the design of biologically active (glyco)peptides and proteins with improved pharmacological parameters that simultaneously carry a powerful ^{19}F spectroscopic label ***(1–3)***. Alternatively, the use of ^{18}F provides new opportunities for non-invasive imaging and diagnosis using this positron-emitting isotope ***(4)***. The unique physical and chemical properties of the fluorine atom, such as its small size and high electronegativity, allows the modulation of physical and pharmacological properties of the biomolecule to which is attached (*e.g.*, increased chemical/enzymatic stability, hydrophobicity, etc.) ***(5)***. However, despite the increasing interest in fluorinated pharmaceuticals, including those derived from carbohydrates, the biological consequences of fluorination on the structure and activity of (glyco)peptides and proteins (*e.g.*, how fluorine interacts with protein recognition sites) are still being discussed and rationalized ***(6, 7)***. Recent advances in selective chemical protein modification ***(8–11)*** has stimulated the selective incorporation of such synthetic, unnatural F-oligosaccharide mimetics into peptides and proteins mainly using Cu(I)-mediated azide–alkyne cycloadditions ***(12–16)*** and thiol chemistries ***(17–21)***, which have emerged as versatile and powerful synthetic tools for the preparation of well-defined F-carbohydrate-based vaccines and other detection/diagnosis elements ***(22–32)***. However, avoiding the use of detrimental reaction conditions that may lead to toxicity ***(33–35)*** and/or reactivity ***(36)*** problems are important issues still to be addressed.

This chapter reports the utilization of a two-step protein-modification protocol for the development of synthetic homogeneous fluorinated glycoproteins *via* the incorporation of strained alkynes into proteins by either α-haloacetamide or maleimide *S*-alkylation ***(37, 38)*** and subsequent *copper-free* strain-promoted azide–alkyne cycloaddition (SPAAC) ***(39, 40)*** using the apoptotic marker Annexin V (AnxV) and 2-deoxy-2-fluoro-β-D-glucopyranosyl azide (FDGN$_3$) pair as model systems. Studying the reactivity of novel partners and conditions to access fluorinated (glyco)peptides and proteins will provide valuable information to further explore the biological implications of these emerging therapeutics and imaging agents *in vivo*.

2. Materials

All reagents and solvents (Analytical or HPLC grade) are used as received from commercial suppliers without prior purification. Milli–QR purified water is used for protein manipulations. Follow all waste disposal regulations when disposing waste materials.

1. 20 mM Tris HCl buffer, pH 8.
2. *N*,*N*-dimethylformamide (DMF)
3. DBCO-Maleimide was purchased from Sigma–Aldrich.
4. DBCO-Bromoacetamide was prepared according to Liang *et al* ***(41)***
5. Ellman's reagent: 14 mg mL^{-1} stock solution in H_2O.
6. Eppendorf tube: 0.5 mL.
7. Zeba™ Spin desalting column: ThermoFisher Scientific, 7K MWCO, 0.5 mL.
8. Vivaspin® 500 centrifugal concentrator: Sigma–Aldrich, 5K MWCO, 0.5 mL.
9. Bradford reagent: Sigma–Aldrich.
10. Laboratory centrifuge.
11. Vortex shaker.
12. Eppendorf incubator shaker.
13. MS vials with 300 μL inserts.
14. Standard biological pipettes (various sizes).

2.1. Protein Mass Spectrometry Equipment

Liquid chromatography-mass spectrometry (LC–MS) is performed on a Xevo G2-S TOF mass spectrometer coupled to an Acquity UPLC system (Acquity UPLC BEH300 C4 column, 1.7 µm, 2.1 mm × 50 mm) (*see* **Note 1**). Water with 0.1% formic acid (solvent A) and 70% acetonitrile and 29.925% water with 0.075% formic acid (solvent B), are used as the mobile phase at a flow rate of 0.2 mL min^{-1}. The gradient is programmed as follows: From 72% A to 100% B for 25 min then 100% B for 2 min and 72% A for 18 min. The electrospray source is operated with a capillary voltage of 2.0 kV and a cone voltage of 40 V. Nitrogen is used as the desolvation gas at a total flow of 850 L h^{-1}. Total mass spectra are reconstructed from the ion series using the MaxEnt algorithm preinstalled on MassLynx software (v. 4.1 from Waters) according to the manufacturer's instructions.

2.2. Protein LC–MS/MS Equipment

All LC–MS/MS experiments are performed using a nanoAcquity UPLC (Waters Corp., Milford, MA) system and an LTQ Orbitrap Velos hybrid ion trap mass spectrometer (Thermo Scientific, Waltham, MA). Separation of peptides is performed by reverse-phase chromatography using a Waters reverse-phase nano column (BEH C18, 1.7 µm, 75 µm x 250 mm) at flow rate of 300 nL min^{-1}. Peptides are initially loaded onto a pre-column (Waters UPLC Trap Symmetry C18, 5 µm, 180 µm x 20 mm) from the nanoAcquity sample manager with 0.1% formic acid for 3 min at a flow rate of 10 µL min^{-1}. After this period, the column valve is switched to allow the elution of peptides from the pre-column onto the analytical column. Solvent A is water + 0.1% formic acid and solvent B is acetonitrile + 0.1% formic acid. The linear gradient employed is 5–40% B in 60 min. The LC eluent is sprayed into the mass spectrometer by means of a New Objective nanospray source. All *m/z* values of eluting ions are measured in the

Orbitrap Velos mass analyzer, set at a resolution of 30000. Data dependent scans (Top 20) are employed to automatically isolate and generate fragment ions by collision-induced dissociation in the linear ion trap, resulting in the generation of MS/MS spectra. Ions with charge states of 2+ and above are selected for fragmentation. Post-run, the data is processed using Protein Discoverer (version 1.3., ThermoFisher Scientific).

2.3. Protein of Interest Containing a Unique Cysteine

In this protocol, we use Annexin-V (AnxV) that is expressed and purified according to Salvadó *et al* ***(17)***.

Sequence of AnxV C315 (modified residue underlined and bold)

AQVLRGTVTDFPGFDERADAETLRKAMKGLGTDEESILTLLTSRSNAQRQEISA
AFKTLFGRDLLDDLKSELTGKFEKLIVALMKPSRLYDAYELKHALKGAGTNEK
VLTEIIASRTPEELRAIKQVYEEEYGSSLEDDVVGDTSGYYQRMLVVLLQANRD
PDAGIDEAQVEQDAQALFQAGELKWGTDEEKFITIFGTRSVSHLRKVFDKYMTI
SGFQIEETIDRETSGNLEQLLLAVVKSIRSIPAYLAETLYYAMKGAGTDDHTLIR
VMVSRSEIDLFNIRKEFRKNFATSLYSMIKGDTSGDYKKALLLL**C**GEDD

Calculated average isotopic mass = 35805.58 (*N*-terminal Met cleaved)

A typical analysis of a conjugation reaction by LC–MS is described below (Section 3.1, Fig. 1). The total ion chromatogram, combined ion series, and deconvoluted spectra are shown for AnxV. Identical analyses are carried out for all the conjugation reactions performed in this chapter.

3. Methods

All manipulations are carried out at room temperature unless otherwise indicated.

3.1. Control Reaction of AnxV with Ellman's Reagent

1. Transfer a 10 μL aliquot of AnxV (1 mg mL^{-1}, 0.616 nmol) in 20 mM Tris HCl buffer at pH 8 to a 0.5 mL eppendorf tube.
2. Add Ellman's reagent (2 μL of a 14 mg mL^{-1} stock solution in H_2O, 70.65 nmol) and vortex the resulting mixture for 10 seconds (*see* **Note 2**).
3. Incubate the reaction mixture at room temperature (shaking at 500 r.p.m.).
4. Monitor reaction progress by LC–MS until no starting material is detected (*see* **Note 3**). After 1 h of additional shaking, a 3 μL aliquot is analyzed by LC–MS (3 μL aliquot diluted by 7 μL of 20 mM Tris HCl buffer at pH 8) and complete conversion to the expected Ellman's product (calculated mass, 36003; observed mass, 36003) is observed (Fig. 2).

3.2. Reaction of AnxV with DBCO-Maleimide (*S*-Alkylation Method 1)

1. In a 0.5 mL eppendorf tube, dilute 8 μL of a 25 μM solution of AnxV with 28 μL of 20 mM Tris HCl buffer at pH 8 and 3.5 μL of DMF (*see* **Note 4**).
2. Add dibenzocyclooctyne-maleimide (DBCO-Maleimide) (*see* **Note 5**) (0.5 μL of a 10 mM stock solution in DMF) and vortex the resulting mixture for 10 seconds (*see* **Note 6**).
3. Incubate the reaction mixture at room temperature (shaking at 500 r.p.m.).
4. Monitor reaction progress by LC–MS until no starting material is detected (*see* **Note 3**). After 5 days of additional shaking, a 3 μL aliquot is analyzed by LC–MS (3 μL aliquot diluted by 7 μL of 20 mM Tris HCl buffer at pH 8) and complete

conversion to AnxV-*S*-Maleimide-DBCO (calculated mass, 36233; observed mass, 36232) is observed (Fig. 3) (*see* **Note 8**).

5. Remove small molecules from the reaction mixture by loading the sample onto a Zeba™ Spin desalting column (ThermoFisher Scientific, 7K MWCO) previously equilibrated with 20 mM Tris HCl buffer at pH 8. The sample is eluted by centrifugation (2 min, 1500xg) (*see* **Note 7**).
6. Concentrate the protein sample to 10 µM (by Bradford assay) ***(42)*** using a Vivaspin® 500 centrifugal concentrator (Sigma–Aldrich, 5K MWCO).
7. Flash frozen the sample with liquid nitrogen and store at –20 ºC.

3.3. LC–MS/MS Analysis

1. A 10 µM solution of the purified AnxV-*S*-Maleimide-DBCO is enzymatically digested by trypsin overnight and subjected to LC–MS/MS analysis according to Cal *et al* ***(43)***. No reduction/alkylation steps are performed.
2. Briefly, all MS/MS data is converted to mgf files and these are submitted to the Mascot search algorithm (Matrix Science, London UK) and searched against a custom database containing the corresponding protein and applying variable modifications of oxidation (M), deamination (NQ) and a custom modification (C), using a peptide tolerance of 25 ppm (MS) and 0.8 Da (MS/MS). Peptide identifications are accepted if they could be established at greater than 95.0% probability.
3. Significant hits, which suggested that the Maleimide-DBCO modification is bound to peptides, are then verified by manual inspection of the MS/MS data. Mascot search shows that ALLLL**C**GEDD is detected with the expected

modification at cysteine. The MS/MS spectrum is depicted in the following figure with the majority of the sequence ions assigned (Fig. 4) (*see* **Note 9**).

3.4. Reaction of AnxV with DBCO-Bromoacetamide (*S*-Alkylation Method 2)

1. In a 0.5 mL eppendorf tube, dilute 8 μL of a 25 μM solution of AnxV with 28 μL of 20 mM Tris HCl buffer at pH 8.
2. Add dibenzocyclooctyne-α-bromoacetamide (DBCO-Bromoacetamide) (4 μL of a 10 mM stock solution in DMF) and vortex the resulting mixture for 10 seconds (*see* **Note 6**).
3. Incubate the reaction mixture at room temperature (shaking at 500 r.p.m.).
4. Monitor reaction progress by LC–MS until no starting material is detected (*see* **Note 3**). After 5 days of additional shaking, a 3 μL aliquot is analyzed by LC–MS (3 μL aliquot diluted by 7 μL of 20 mM Tris HCl buffer at pH 8) and complete conversion to AnxV-*S*-Acetamide-DBCO (calculated mass, 36122; observed mass, 36121) is observed (Fig. 5) (*see* **Note 8**).
5. Remove small molecules from the reaction mixture by loading the sample onto a Zeba™ Spin desalting column (ThermoFisher Scientific, 7K MWCO) previously equilibrated with 20 mM Tris HCl buffer at pH 8. The sample is eluted by centrifugation (2 min, 1500xg) (*see* **Note 7**).
6. Concentrate the protein sample to 10 μM (by Bradford assay) ***(42)*** using a Vivaspin® 500 centrifugal concentrator (Sigma–Aldrich, 5K MWCO).
7. Flash frozen the sample with liquid nitrogen and store at –20 ºC.

3.5. Reaction of AnxV-*S*-Maleimide-DBCO with 2-Deoxy-2-fluoro-β-D-glucopyranosyl Azide

1. Prepare AnxV-*S*-Maleimide-DBCO as a 5 μM solution in 20 mM Tris HCl buffer at pH 8 and transfer 30 μL to a 0.5 mL eppendorf tube.
2. Add 2-deoxy-2-fluoro-β-D-glucopyranosyl azide ($FDGN_3$) (0.4 μL of a 48.3 mM stock solution in DMF) and vortex the resulting mixture for 10 seconds (*see* **Note 10**).
3. Incubate the reaction mixture at room temperature (shaking at 500 r.p.m.).
4. Monitor reaction progress by LC–MS until no starting material is detected (*see* **Note 3**). After 19 h of additional shaking, a 3 μL aliquot is analyzed by LC–MS (3 μL aliquot diluted by 7 μL of 20 mM Tris HCl buffer at pH 8) and complete conversion to AnxV-*S*-Maleimide-DBCO-FDG (calculated mass, 36439; observed mass, 36440) is observed (Fig. 6) (*see* **Note 11**).
5. Remove small molecules from the reaction mixture by loading the sample onto a Zeba™ Spin desalting column (ThermoFisher Scientific, 7K MWCO) previously equilibrated with 20 mM Tris HCl buffer at pH 8. The sample is eluted by centrifugation (2 min, 1500xg).
6. Flash frozen the sample with liquid nitrogen and store at –20 ºC (*see* **Note 12**).
7. The hydrolysis of the maleimide conjugate is evaluated upon incubation at room temperature for 2 weeks. After this period, *ca.* 30% maleimide hydrolysis (calculated mass, 36457; observed mass, 36457) and *ca.* 30% retro-Michael to AnxV (calculated mass, 35806; observed mass, 35805) is observed (Fig. 7).

3.6. Reaction of AnxV-*S*-Acetamide-DBCO with 2-Deoxy-2-fluoro-β-D-glucopyranosyl Azide

1. Prepare AnxV-*S*-Acetamide-DBCO as a 5 μM solution in 20 mM Tris HCl buffer at pH 8 and transfer 20 μL to a 0.5 mL eppendorf tube.

2. Add 2-deoxy-2-fluoro-β-D-glucopyranosyl azide (FDGN_3) (2.1 µL of a 48.3 mM stock solution in DMF) and vortex the resulting mixture for 10 seconds (*see* **Note 10**).
3. Incubate the reaction mixture at room temperature (shaking at 500 r.p.m.).
4. Monitor reaction progress by LC–MS until no starting material is detected (*see* **Note 3**). After 14.5 h of additional shaking, a 3 µL aliquot is analyzed by LC–MS (3 µL aliquot diluted by 7 µL of 20 mM Tris HCl buffer at pH 8) and complete conversion to AnxV-*S*-Acetamide-DBCO-FDG (calculated mass, 36329; observed mass, 36329) is observed (Fig. 8) (*see* **Note 11**).
5. Remove small molecules from the reaction mixture by loading the sample onto a Zeba™ Spin desalting column (ThermoFisher Scientific, 7K MWCO) previously equilibrated with 20 mM Tris HCl buffer at pH 8. The sample is eluted by centrifugation (2 min, 1500xg).
6. Flash frozen the sample with liquid nitrogen and store at –20 ºC (*see* **Note 12**).

4. Notes

1. The use of liquid chromatography–mass spectrometry (LC–MS) analysis is recommended for monitoring the reaction progress on peptides and proteins with accurate resolution.
2. The use of chemical controls such as the cysteine-specific Ellman’s reagent allows for determining not only the presence of free cysteine residues but also their relative reactivity under the conditions tested, taking into consideration their native residue microenvironment.

3. Reaction monitoring by LC–MS analysis determines the minimum reaction time necessary to achieve full conversion. Extended reaction times are typically associated to a decrease of final protein concentration.
4. Same reaction was attempted on a larger scale (x10) but the results were not reproducible and extra 13 equiv of Maleimide-DBCO was required to achieve full conversion after 11 days at room temperature. Importantly, the amount of organic solvent used should be adjusted to up to 10% to avoid protein precipitation and/or denaturing.
5. A number of structurally varied cyclooctynes (*e.g.*, DIFO, BCN, and ADIBO, among others) have been developed featuring different reaction kinetics and hydrophobicity/philicity ***(39, 40)***. Among them, dibenzocyclooctyne (DBCO)-based reagents combine high reactivity and sufficient hydrophilicity thus reducing non-specific binding while having greater efficiency than common copper-mediated azide–alkyne cycloadditions (CuAAC).
6. We freshly prepare this solution prior to each experiment.
7. This desalting step is essential to remove excess alkyne reagent that will react with FDGN$_3$ in the next step.
8. Optimization of assay conditions described above is required (protein concentration/reaction scale, buffer composition and pH, amount of alkylating agent, % of organic co-solvent, and reaction time and temperature) ***(43)***.
9. Proteolytic digest followed by peptide mapping by LC–MS/MS should confirm the desired site of modification and the absence of additional modifications at other residues.
10. The FDGN$_3$ solution can be stored at 4 °C for several months.

11. No catalyst or accessory reagents and thus no extensive optimization of assay conditions required ***(44)*** unlike for CuAAC reactions where optimization is typically required (type and concentration of copper source, reduction reagent, ligands, and co-solvents) ***(45)***.
12. Following the two consecutive reactions described above, proteins should be observed by LC–MS as a single species with >95% conversion to the desired final product, as judged by calculation from peak intensities in the deconvoluted spectrum.

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Glossary

ADIBO	Azadibenzobicyclooctyne
AnxV	Annexin V
BCN	Bicyclo[6.1.0]nonyne
CuAAC	Copper-mediated azide–alkyne cycloadditions
DBCO	Dibenzocyclooctyne
DIFO	Difluorinated cyclooctyne
DMF	*N*,*N*-dimethylformamide

ESI	Electrospray ionization
FDG	2-Deoxy-2-fluoro-β-D-glucopyranose
$FDGN_3$	2-Deoxy-2-fluoro-β-D-glucopyranosyl azide
HPLC	High performance liquid chromatography
LC	Liquid chromatography
MS	Mass spectrometry
MWCO	Molecular weight cut-off
SPAAC	Strain-promoted azide–alkyne cycloaddition
TOF	Time of flight
UPLC	Ultra performance liquid chromatography

References

1. Uhrig ML, Lantaño B, Postigo A (2019) Synthetic strategies for fluorination of carbohydrates. Org Biomol Chem 17:5173–5189.
2. Gillis EP, Eastman KJ, Hill MD, Donnelly DJ, Meanwell NA (2015) Applications of fluorine in medicinal chemistry. J Med Chem 58:8315–8359.
3. Purser S, Moore PR, Swallow S, Gouverneur V (2008) Fluorine in medicinal chemistry. Chem Soc Rev 37:320–330.
4. Maschauer S, Prante O (2014) Sweetening pharmaceutical radiochemistry by ^{18}F-fluoroglycosylation: a short review. Biomed Res Int 2014:1–16.
5. Hunter L (2010) The C–F bond as a conformational tool in organic and biological chemistry. Beilstein J Org Chem 6.
6. Ardá A, Jiménez-Barbero J (2018) The recognition of glycans by protein receptors. Insights from NMR spectroscopy. Chem Commun 54:4761–4769.

7. Arntson KE, Pomerantz WCK (2015) Protein-observed fluorine NMR: a bioorthogonal approach for small molecule discovery. J Med Chem 59:5158–5171.

8. Li C, Wang, LX (2018) Chemoenzymatic methods for the synthesis of glycoproteins. Chem Rev 118:8359–8413.

9. Boutureira O, Bernardes GJL (2015) Advances in chemical protein modification Chem Rev 115:2174–2195.

10. Krall N, da Cruz FP, Boutureira O, Bernardes GJL (2015) Site-selective protein-modification chemistry for basic biology and drug development. Nat Chem 8:103–113.

11. Spicer CD, Davis, BG (2014) Selective chemical protein modification. Nat Commun 5.

12. Boutureira O, D'Hooge F, Fernández-González M, Bernardes GJL, Sánchez-Navarro M, Koeppe JR, Davis BG (2010) Fluoroglycoproteins: ready chemical site-selective incorporation of fluorosugars into proteins. Chem Commun 46:8142–8144.

13. Fernández-González M, Boutureira O, Bernardes GJL, Chalker JM, Young MA, Errey JC, Davis BG (2010) Site-selective chemoenzymatic construction of synthetic glycoproteins using endoglycosidases. Chem Sci 1:709–715.

14. Vala C, Chrétien F, Balentova E, Lamandé-Langle S, Chapleur Y (2011) Neoglycopeptides through direct functionalization of cysteine. Tetrahedron Lett 52:17–20.

15. Maschauer S, Prante O (2009) A series of 2-*O*-trifluoromethylsulfonyl-D-mannopyranosides as precursors for concomitant ^{18}F-labeling and glycosylation by click chemistry. Carbohydr Res 344:753–761.

16. Maschauer S, Einsiedel J, Haubner R, Hocke C, Ocker M, Hübner H et al (2010) Labeling and glycosylation of peptides using click chemistry: a general approach to ^{18}F-glycopeptides as effective imaging probes for positron emission tomography. Angew Chem Int Ed 49:976–979.

17. Salvadó M, Amgarten B, Castillón S, Bernardes GJL, Boutureira, O (2015) Synthesis of fluorosugar reagents for the construction of well-defined fluoroglycoproteins. Org Lett 17:2836–2839.

18. Boutureira O, Bernardes GJL, Fernández-González M, Anthony DC, Davis, BG (2012) Selenenylsulfide-linked homogeneous glycopeptides and glycoproteins: synthesis of human "hepatic Se metabolite A". Angew Chem Int Ed 51:1432–1436.

19. Boutureira O, Bernardes GJL, D'Hooge F, Davis, BG (2011) Direct radiolabelling of proteins at cysteine using [^{18}F]-fluorosugars. Chem Commun 47:10010–10012.

20. Fröhlich RFG, Schrank E, Zangger K (2012) 2,2,2-Trifluoroethyl 6-thio-β-D-glucopyranoside as a selective tag for cysteines in proteins. Carbohydr Res 361:100–104.

21. Prante O, Einsiedel J, Haubner R, Gmeiner P, Wester H-J, Kuwert T et al (2007) 3,4,6-Tri-O-acetyl-2-deoxy-2-[^{18}F]fluoroglucopyranosyl phenylthiosulfonate: a thiol-reactive agent for the chemoselective ^{18}F-glycosylation of peptides. Bioconjugate Chem 18:254–262.

22. Collet C, Maskali F, Clément A, Chrétien F, Poussier S, Karcher G et al (2015) Development of 6-[^{18}F]fluoro-carbohydrate-based prosthetic groups and their conjugation to peptides via click chemistry. J Label Compd Radiopharm 59:54–62.

23. Fischer CR, Müller C, Reber J, Müller A, Krämer SD, Ametamey SM et al (2012) [^{18}F]Fluoro-deoxy-glucose folate: a novel PET radiotracer with improved in vivo properties for folate receptor targeting. Bioconjugate Chem 23:805–813.

24. Wuest F, Berndt M, Bergmann R, van den Hoff J, Pietzsch J (2008) Synthesis and application of [^{18}F]FDG-maleimidehexyloxime ([^{18}F]FDG-MHO): a [^{18}F]FDG-based prosthetic group for the chemoselective ^{18}F-labeling of peptides and proteins. Bioconjugate Chem 19:1202–1210.

25. Namavari M, Cheng Z, Zhang R, De A, Levi J, Hoerner JK et al (2009) A novel method for direct site-specific radiolabeling of peptides using [^{18}F]FDG. Bioconjugate Chem 20:432–436.

26. Hultsch C, Schottelius M, Auernheimer J, Alke A, Wester H-J (2009) ^{18}F-Fluoroglucosylation of peptides, exemplified on *cyclo*(RGDfK). Eur J Nucl Med Mol Imaging 36:1469–1474.

27. Yang F, Zheng X-J, Huo C-X, Wang Y, Zhang Y, Ye X-S (2011) Enhancement of the immunogenicity of synthetic carbohydrate vaccines by chemical modifications of STn antigen. ACS Chem Biol 6:252–259.

28. Huo C-X, Zheng X-J, Xiao A, Liu C-C, Sun S, Lv Z et al (2015) Synthetic and immunological studies of *N*-acyl modified *S*-linked STn derivatives as anticancer vaccine candidates. Org Biomol Chem 13:3677–3690.

29. Lee H-Y, Chen C-Y, Tsai T-I, Li S-T, Lin K-H, Cheng Y-Y et al (2014) Immunogenicity study of globo H analogues with modification at the reducing or nonreducing end of the tumor antigen. J Am Chem Soc 136:16844–16853.

30. Orwenyo J, Huang W, Wang L-X (2013) Chemoenzymatic synthesis and lectin recognition of a selectively fluorinated glycoprotein. Bioorg Med Chem 21:4768–4777.

31. Oberbillig T, Mersch C, Wagner S, Hoffmann-Röder A (2012) Antibody recognition of fluorinated MUC1 glycopeptide antigens. Chem Commun 48:1487–9.

32. Hoffmann-Röder A, Kaiser A, Wagner S, Gaidzik N, Kowalczyk D, Westerlind U et al (2010) Synthetic antitumor vaccines from tetanus toxoid conjugates of MUC1 glycopeptides with the Thomsen-Friedenreich antigen and a fluorine-substituted analogue. Angew Chem Int Ed 49:8498–503.

33. Hong V, Presolski SI, Ma C, Finn MG (2009) Analysis and optimization of copper-catalyzed azide-alkyne cycloaddition for bioconjugation. Angew Chem Int Ed 48:9879−9883.

34. Besanceney-Webler C, Jiang H, Zheng T, Feng L, Soriano del Amo D, Wang W et al (2011) Increasing the efficacy of bioorthogonal click reactions for bioconjugation: a comparative study. Angew Chem Int Ed 50:8051−8056.

35. Kennedy DC, McKay CS, Legault MCB, Danielson DC, Blake JA, Pegoraro AF et al (2011) Cellular consequences of copper complexes used to catalyze bioorthogonal click reactions. J Am Chem Soc 133:17993–8001.

36. Moorman RM, Collier MB, Frohock BH, Womble MD, Chalker JM. Halide inhibition of the copper-catalysed azide–alkyne cycloaddition (2015) Org Biomol Chem 13:1974–1978.

37. Renault K, Fredy JW, Renard PY, Sabot C (2018) Covalent modification of biomolecules through maleimide-based labeling strategies. Bioconjugate Chem 29:2497–2513.

38. Ravasco JMJM, Faustino H, Trindade A, Gois PMP (2018) Bioconjugation with maleimides: a useful tool for chemical biology. Chem Eur J 25:43–59.

39. Pickens CJ, Johnson SN, Pressnall MM, Leon MA, Berkland CJ. Practical considerations, challenges, and limitations of bioconjugation via azide–alkyne cycloaddition (2018) Bioconjugate Chem 29:686–701.

40. Oliveira BL, Guo Z, Bernardes GJL (2017) Inverse electron demand Diels–Alder reactions in chemical biology. Chem Soc Rev 46:4895–950.

41. Liang Y, Jiang X, Tang, N, Yang L, Chen H, Wang, Q (2015) Quantification and visualization of glutathione *S*-transferase omega 1 in cells using inductively coupled plasma mass spectrometry (ICP-MS) and fluorescence microscopy. Anal Bioanal Chem 407:2373–2381.

42. Bradford, MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 72:248–254.

43. Cal PMSD, Sieglitz F, Santos FMF, Parente Carvalho C, Guerreiro A, Bertoldo J et al (2017) Site-selective installation of BASHY fluorescent dyes to Annexin V for targeted detection of apoptotic cells. Chem Commun 53:368–371.

44. Davis DL, Price EK, Aderibigbe SO, Larkin MX-H, Barlow ED, Chen R et al (2016) Effect of buffer conditions and organic cosolvents on the rate of strain-promoted azide–alkyne cycloaddition. J Org Chem 81:6816–6819.

45. Van Kasteren SI, Kramer HB, Gamblin DP, Davis BG (2007) Site-selective glycosylation of proteins: creating synthetic glycoproteins. Nat Protoc 2:3185–3194.

Figure Captions

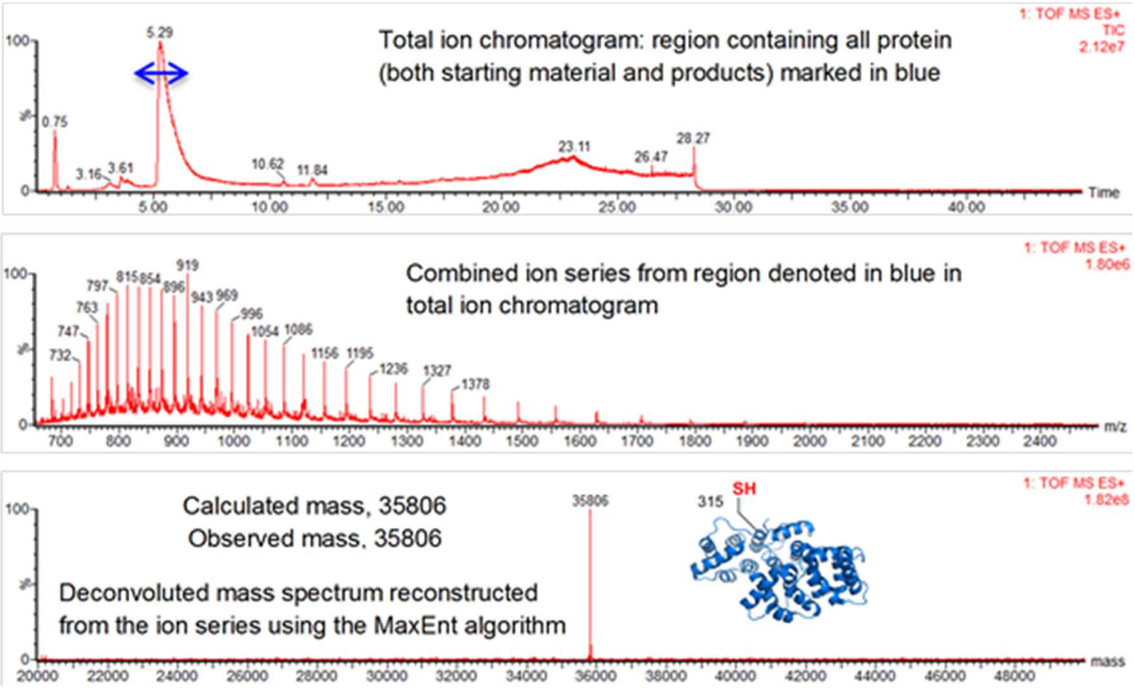


Fig. 1 ESI–MS spectrum of AnxV.

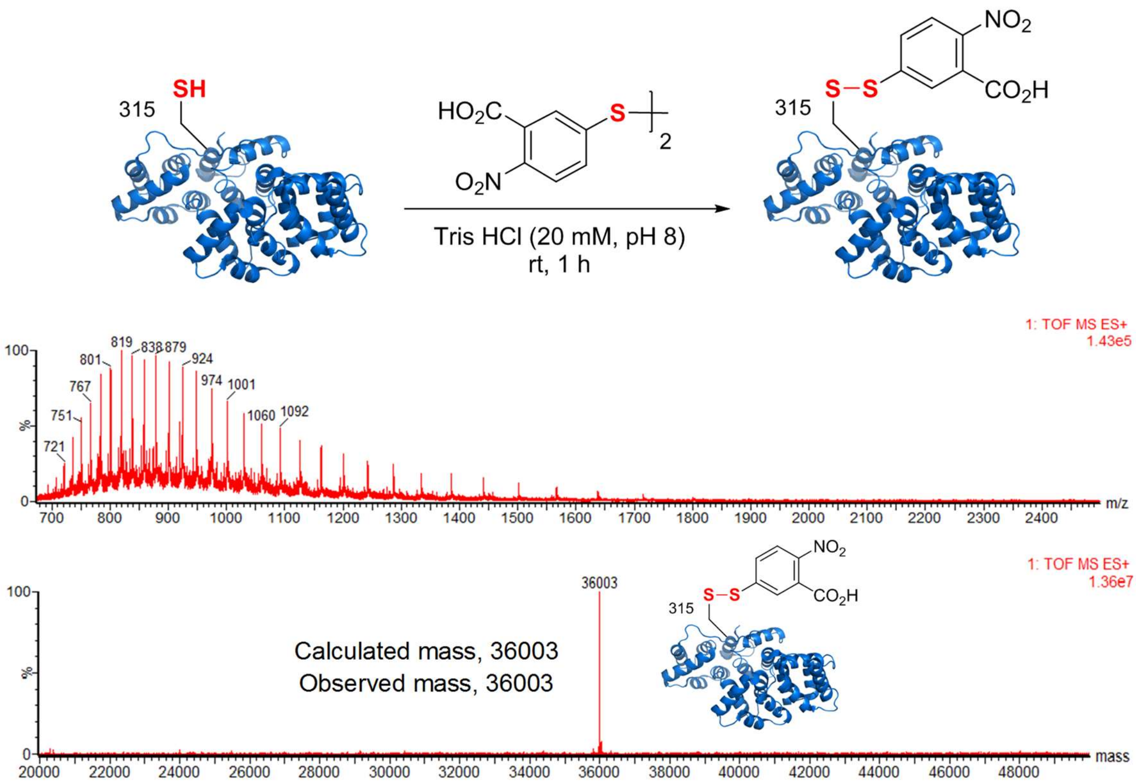


Fig. 2 ESI–MS of the reaction of AnxV with Ellman's reagent.

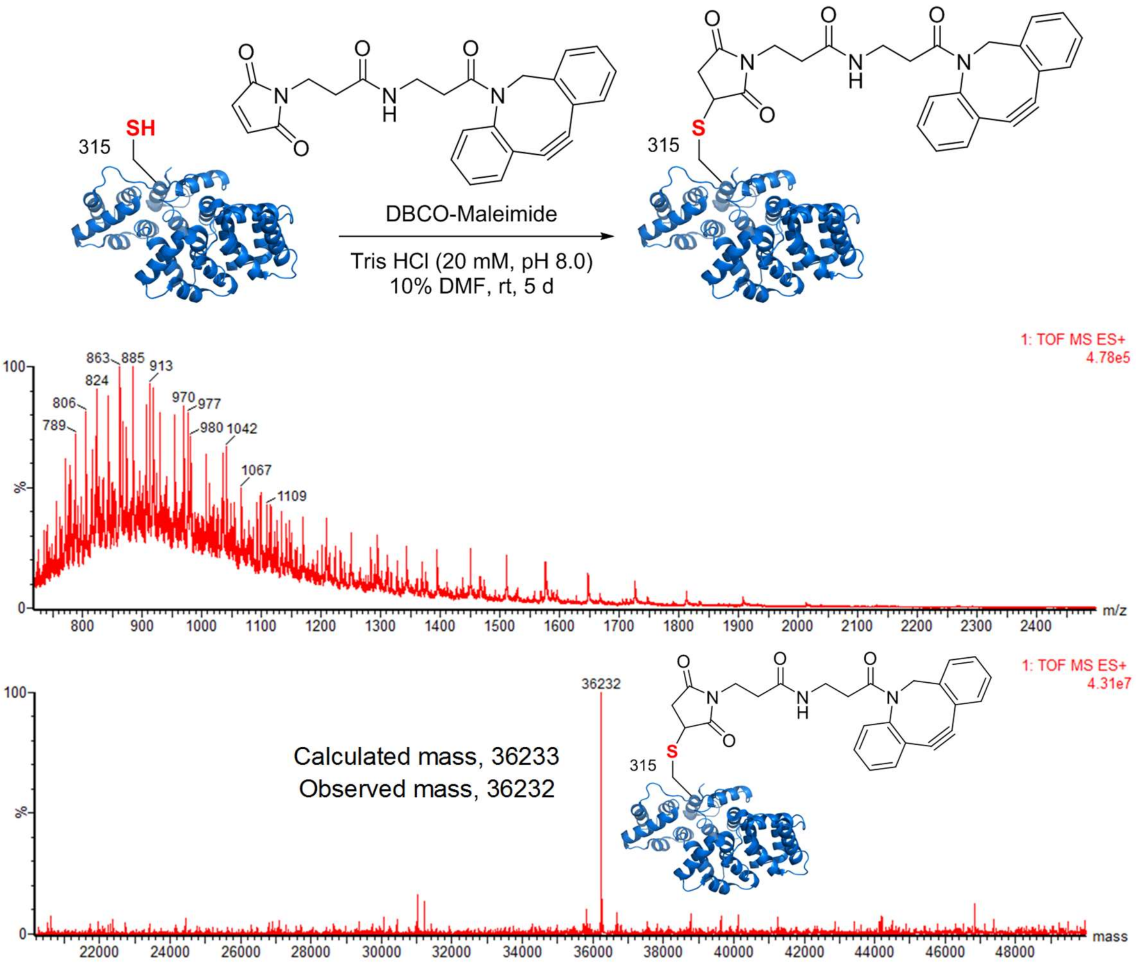


Fig. 3 ESI–MS of the reaction of AnxV with DBCO-Maleimide.

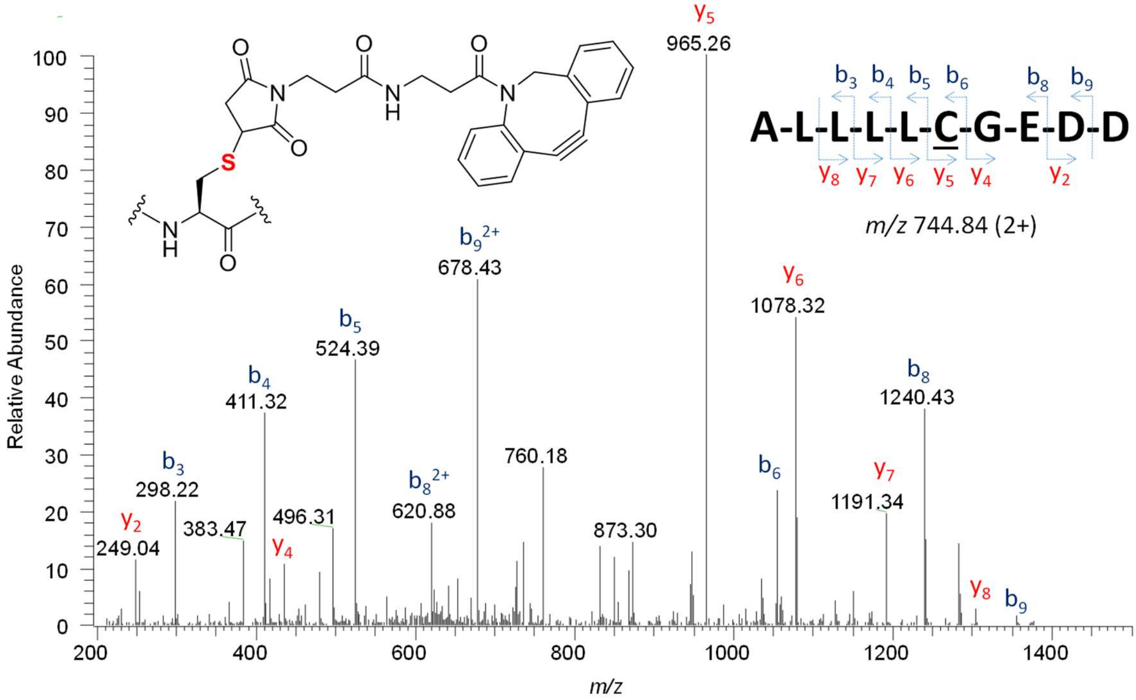
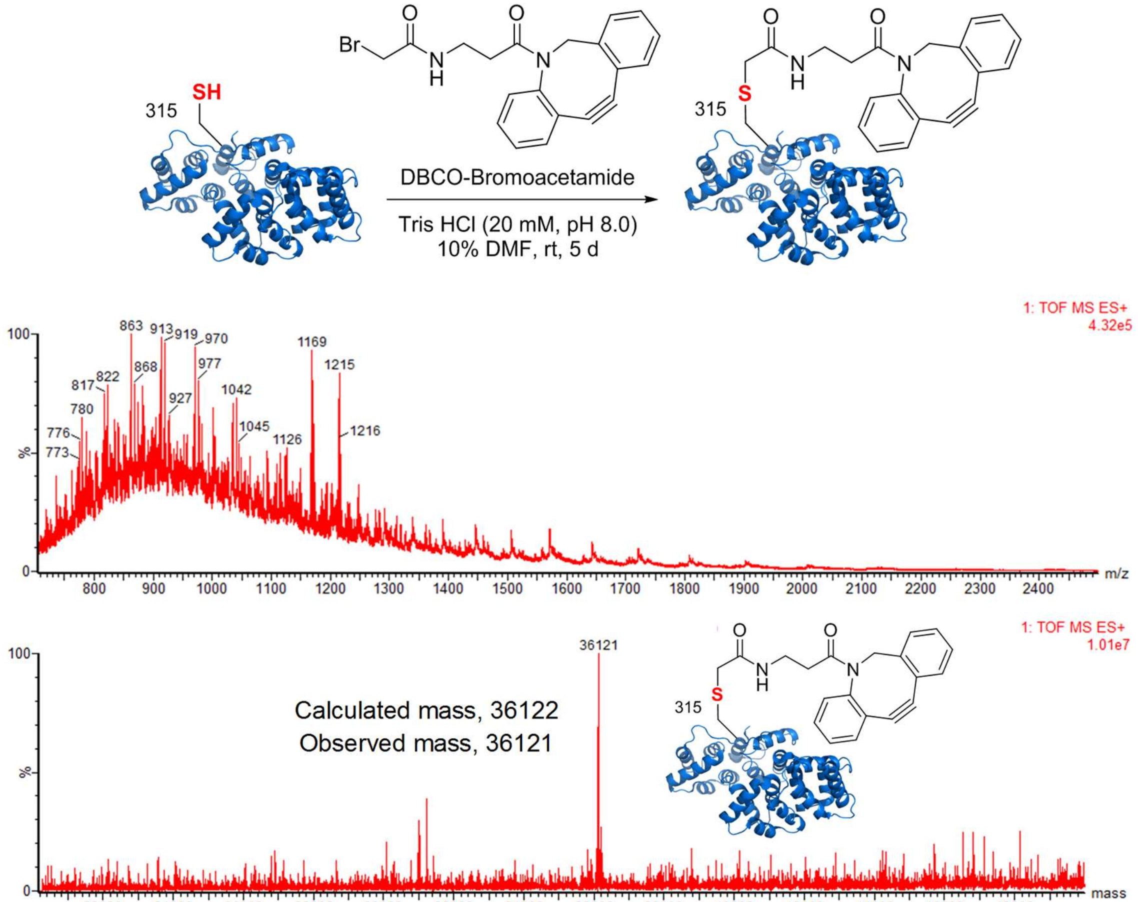


Fig. 4 MS/MS spectrum of the *m/z* 744.84 (2+) ion of the tryptic peptide ALLLL**C**GEDD from AnxV-*S*-Maleimide-DBCO, containing the Maleimide-DBCO modification at the original cysteine residue. The generated fragment ions are consistent with the mass of the modification.



Calculated mass, 36122
Observed mass, 36121

Fig. 5 ESI–MS of the reaction of AnxV with DBCO-Bromoacetamide.

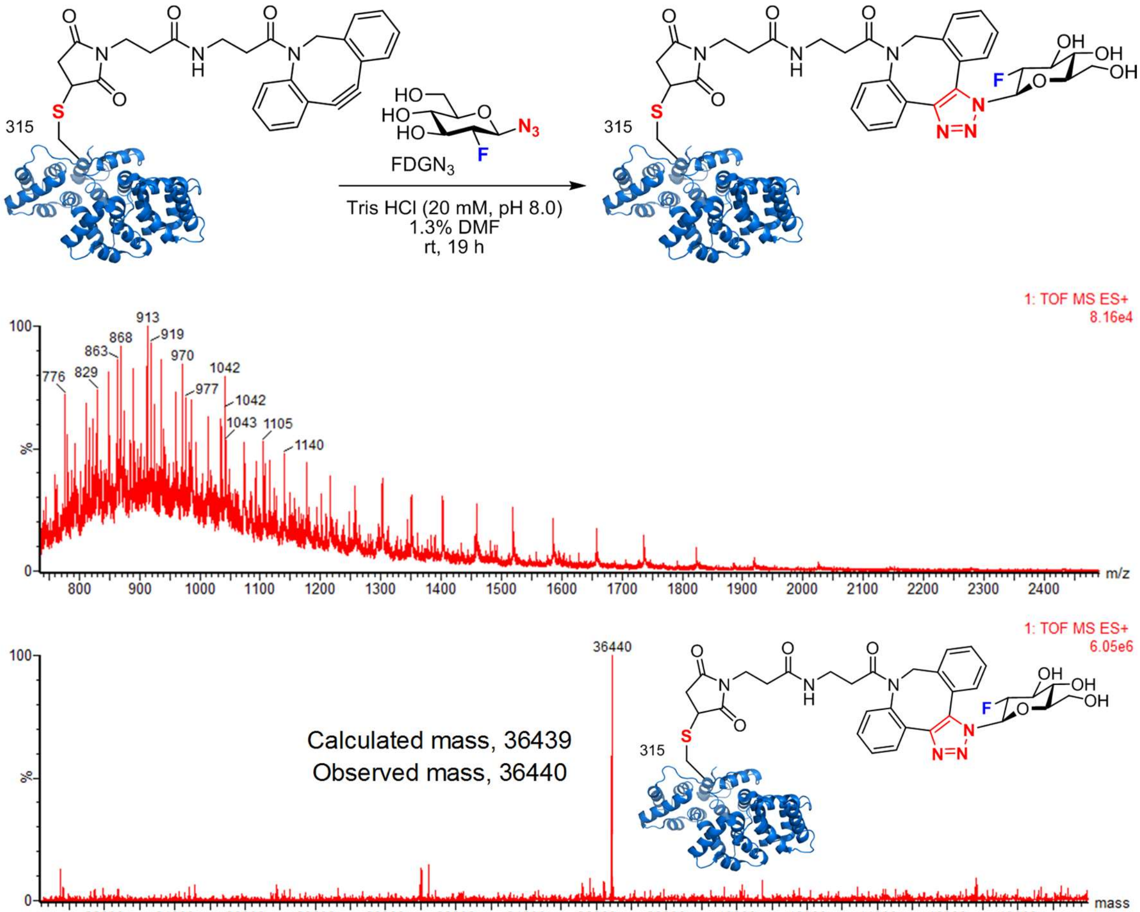


Fig. 6 ESI–MS of the reaction of AnxV-*S*-Maleimide-DBCO with 2-deoxy-2-fluoro-β-D-glucopyranosyl azide (FDGN$_3$).

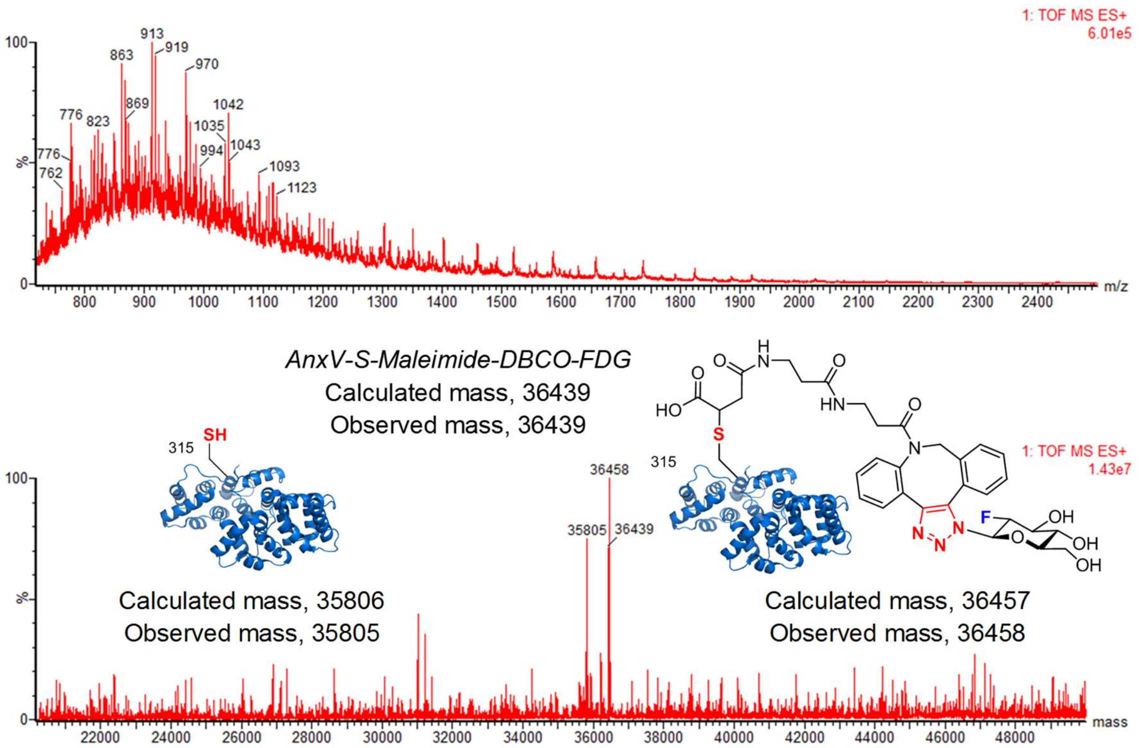


Fig. 7 ESI–MS of AnxV-*S*-Maleimide-DBCO-FDG upon incubation at room temperature for 2 weeks.

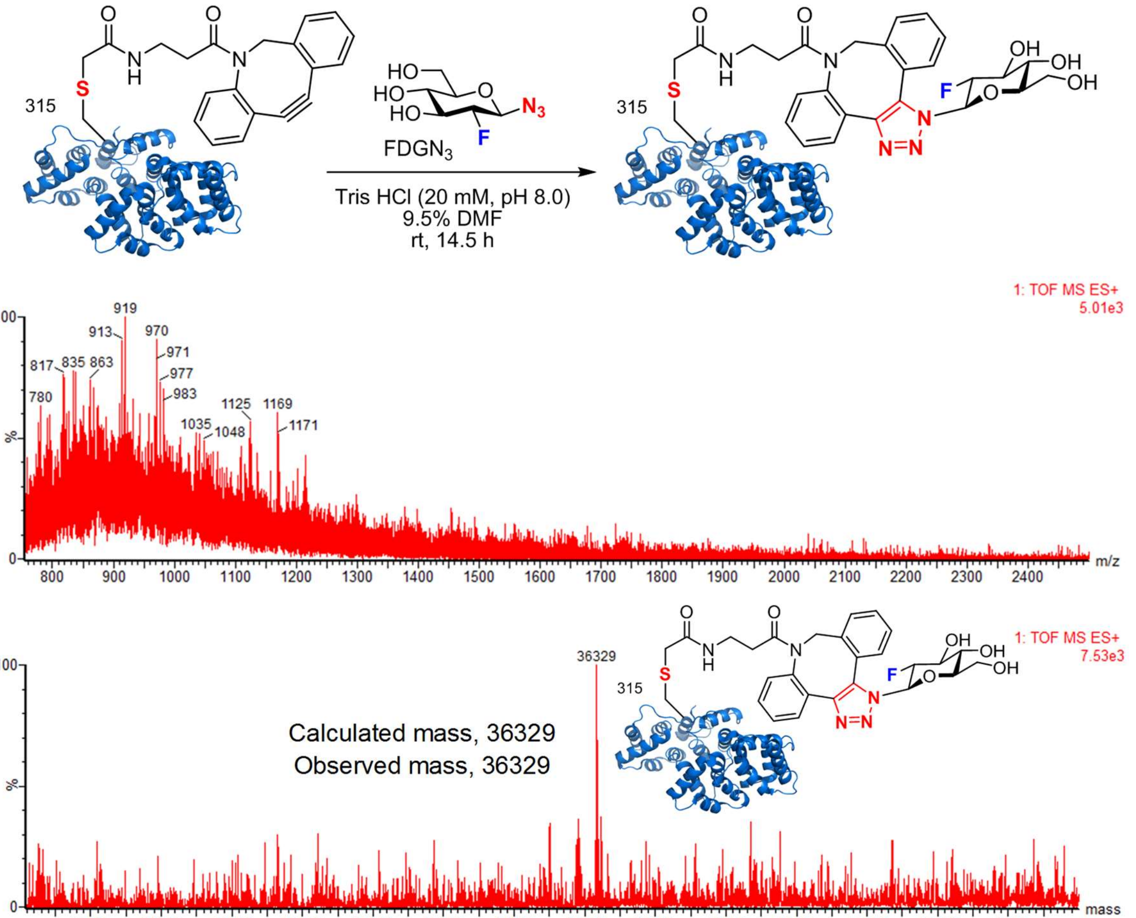


Fig. 8 ESI–MS of the reaction of AnxV-*S*-Acetamide-DBCO with 2-deoxy-2-fluoro-β-D-glucopyranosyl azide (FDGN$_3$).