Supplementary Information

# Bioorthogonal labelling of 3-nitrotyrosine in peptides and proteins through diazotisation mediated azidation

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## Experimental Section

**Materials.** The following supplies were purchased from Sigma-Aldrich (Missouri, United States), L-Tyrosine, 3-nitro-L-tyrosine, 3-amino-L-tyrosine, angiotensin I human acetate hydrate, concentrated hydrogen peroxide, cytochrome C from *Saccharomyces cerevisiae*, sodium dithionite, sodium nitrite, sodium azide, iodoacetamide, DL-Dithiothreitol (DTT), monoclonal anti-3-nitrotyrosine and streptavidin horse radish peroxide. Biotin alkyne and $PEG_4$ carboxamide propargyl biotin was purchased from Life Technologies (California, United States). Hydrochloric acid (HCl) was obtained from AJAX fine chemicals. Biotin-PEG3-phosphine and automated high pressure liquid chromatography (HPLC) instruments were purchased from Thermo scientific (Waltham, Massachusetts). Dibenzylcyclooctyne-SS-PEG11-Biotin Conjugate (DBCO-SS-PEG11-Biotin) was purchased from Jena biosciences (Jena, Germany). Trypsin was purchased from Promega (Madison, United States).

**Nitration of tyrosine.** Tyrosine was nitrated by Fenton-type chemistry with peroxide, Cytochrome C and sodium nitrite ($NaNO_2$)[1]. Final concentrations of tyrosine in buffer were 0.1 mg/mL in amino acid analyses, 1 mg/mL in protein and lysate samples. PBS buffer was used at an adjusted pH of 6.5 ($KH_2PO_4$ 1.6 mM, NaCl 155 mM, $Na_2HPO_4.7H_2O$, pH 6.5). Cytochrome C was added to a final concentration of 7.9 μM, $NaNO_2$ 1 mM. A stock of 30% hydrogen peroxide solution (9.8 M) was quickly diluted to 3.7 mM and added quickly to sample aliquots. Samples were incubated at 37°C in a thermomixer (Eppendorf) with gentle

agitation for 15 minutes. Reactions were halted with 3.3 mM or higher molarities of methionine and samples were chilled in ice before further sample treatment. Further derivatisation of amino acids was carried out without cleanup, in peptide experiments, samples were subjected to manual solid-phase extraction (Supelco), and eluted 1-2 mL volumes of 80% Acetonitrile in 0.1% Formic acid and water. Peptides were then dried under vacuum centrifugation. Further derivatisation of protein 3-nitrotyrosine samples was performed after dialysis with 1000 MWCO dialysis tubing for two hours with protease inhibitor cocktail (Merck).

**Reduction of 3-nitrotyrosines to 3-aminotyrosines.** 3-nitrotyrosine was dissolved or exchanged into Borate buffer pH 9.4, to a concentration of 1 mg/mL. Samples were added sodium dithionite 75 mM, and incubated for 1 min. Formic acid (0.1%) was added to halt the reaction after one minute. In peptide experiments, sample was subjected to manual solid-phase extraction following reduction, in further protein derivatisation steps, reduced proteins were dialysed into the following reaction buffer for 2-4 hours with protease inhibitor cocktail.

**Diazotisation and substitution of 3-aminotyrosines with azide**. HCl (0.2 M – for amino acid experiments a range from 0.1 M to 1 M was used) and $NaNO_2$ (1 mM) was added to a solution of 3-aminotyrosine (1 mg/mL) in PBS pH 7.4. This solution was maintained at 0-4$^{o}$C, in darkness and placed on a vigorous horizontal shaker. After 20 minutes of incubation, in darkness, sodium azide (200 uM) was added. Samples were kept on ice in complete darkness and slowly agitated for 1 hour then left to return to room temperature with gentle agitation for another 30 minutes. In peptide treatments, samples were subjected to solid-phase extraction, while proteins samples were dialysed.

**'Click chemistry' biotinylation reaction.** To a solution of 3-azidotyrosine peptides (0.5mg/mL) the following reagents were added, Triton X-100 (0.01%), tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (2 mM), $PEG_4$ carboxamide-Propargyl Biotin (Alkyne-Biotin) (0.4 mM or excess molarity), copper wire and copper sulphate (0.1 mM). Samples were incubated for 1 hour at 30$^{o}$C. Samples were then subjected to solid-phase extraction as described. In protein and lysate samples Phosphine-PEG3-Biotin (Php-Biotin) was added to a final concentration of 1 mM. In copper free click reactions final concentrations of 1 mM Dibenzylcyclooctyne-SS-PEG11-Biotin (DBCO-Biotin) or 50 times molar substrate

equivalent of azides was added and incubated for 4-16 hours at room temperature with protease inhibitors.

**High Pressure Liquid Chromatography for amino acid analysis.** HPLC analysis was performed on a Thermoscientific auto sampler, pump and UV detection system (Thermo Electron, Bremen, Germany). A C-18 hydrophilic/hydrophobicity column was used to separate amino acids and peptides over a 17 minute solvent gradient in acetonitrile (ACN) and MilliQ water ($H_2O$). A four step gradient was utilised, beginning with 95:5 ($H_20$:ACN) for 3 minutes followed by 8 minute gradient from 5%-80% ACN, a clearing wash 80-95% for 1 minute followed by column re-equilibration to 95:5 for 5 minutes. Absorbance was monitored at 274 nm.

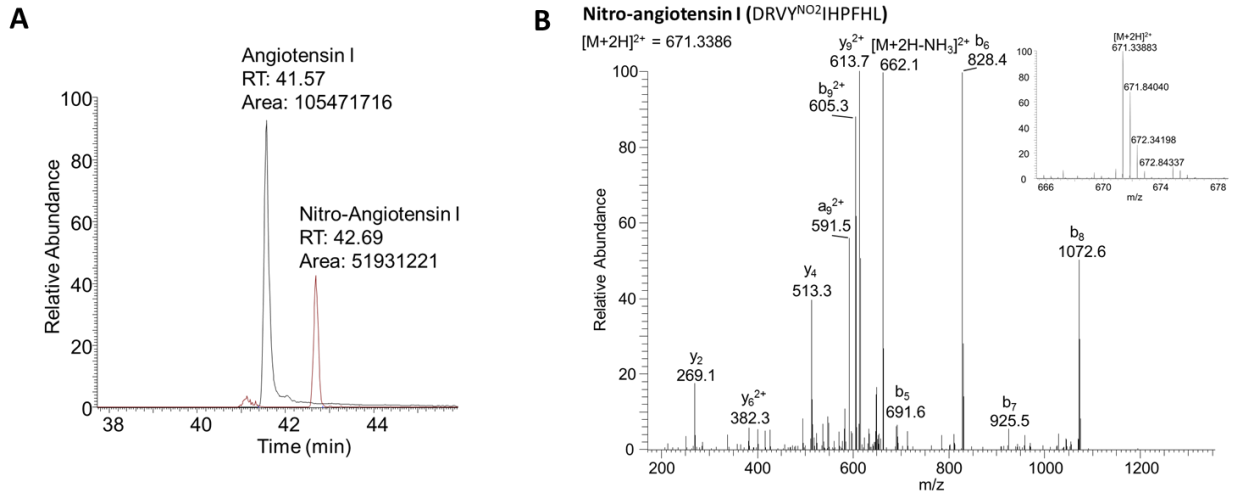
**Liquid Chromatography-tandem mass spectrometry**. Peptides were separated by nano-LC using an Ultimate 3000 HPLC and autosampler system (Dionex, Amsterdam, Netherlands). Peptides were eluted using a linear gradient of $H_2O$:$CH_3CN$ (98:2, 0.1 % formic acid) to $H_2O$:$CH_3CN$ (64:36, 0.1 % formic acid) at 250 nl/min over 30 min. 2000 Volts were applied to a low volume tee (Upchurch Scientific) and the column tip positioned ~ 0.5 cm from the heated capillary (T=280°C) of an Orbitrap Velos (Thermo Electron, Bremen, Germany) mass spectrometer. A survey scan of range m/z 350-1750 was acquired, under collision induced dissociation (CID) and electron transfer dissociation fragmentation (ETD). For CID, the normalised collision energy was set at 25% with an activation time of 20 ms. For ETD, the reaction time was set to 67 ms.

**Western Blotting analysis**. Protein and cell lysate samples were loaded and run on bis-tris 4-12 % SDS-PAGE gel (Invitrogen) and transferred to PVDF membrane. The membrane was blocked with 2.5% skim milk and phosphate buffered saline tween (PBS-t) and incubated with Streptavidin HRP for 2 hours. Membrane was washed thrice with 5 minute intervals and visualised by ECL chemiluminescence and radiography film.

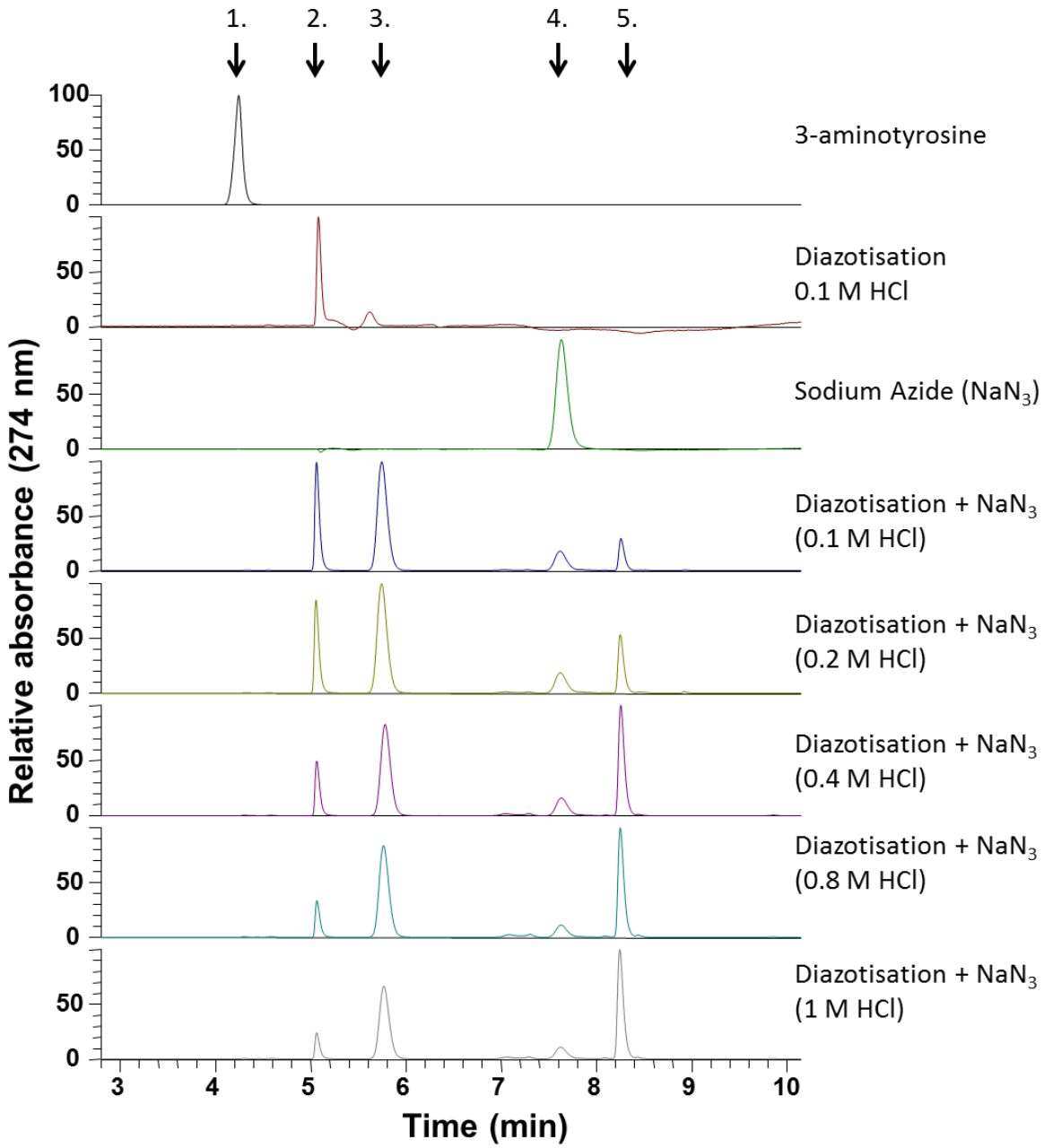
**Proteolytic digestion of derivatised HSA and LC-MS/MS data analysis.** Dialysed DBCO-biotin labelled HSA (~0.1 mg/mL) was incubated with 5 mM DTT in 25 mM $NH_4CO_2$ for 1 h at 25 $^{o}$C. Subsequently 10 mM iodoacetamide was added and the solution incubated at room temperature in darkness for 30 minutes. 5 mM DTT was then added to quench the alkylation reaction. Trypsin was finally added at 20 ng.$\mu L^{-1}$ and the protein was digested overnight at 30°C. Following digestion, the tryptic peptides were analysed by LC-MS/MS as described above with the tandem mass spectrum generated by CID.

For the LC-MS/MS data analysis, Maxquant (version 1.5.0.3) was used. Briefly, the data was searched against the Uniprot-SwissProt Human database including common contaminant proteins. Default mass error tolerance for the Orbitrap instrument was used. For post-translation modification, variable modification was set for Acetyl (Protein N-term), Oxidation (M, Y), Nitro (Y) and DBCO-S-CAM (Y) (+462.14741 Da).

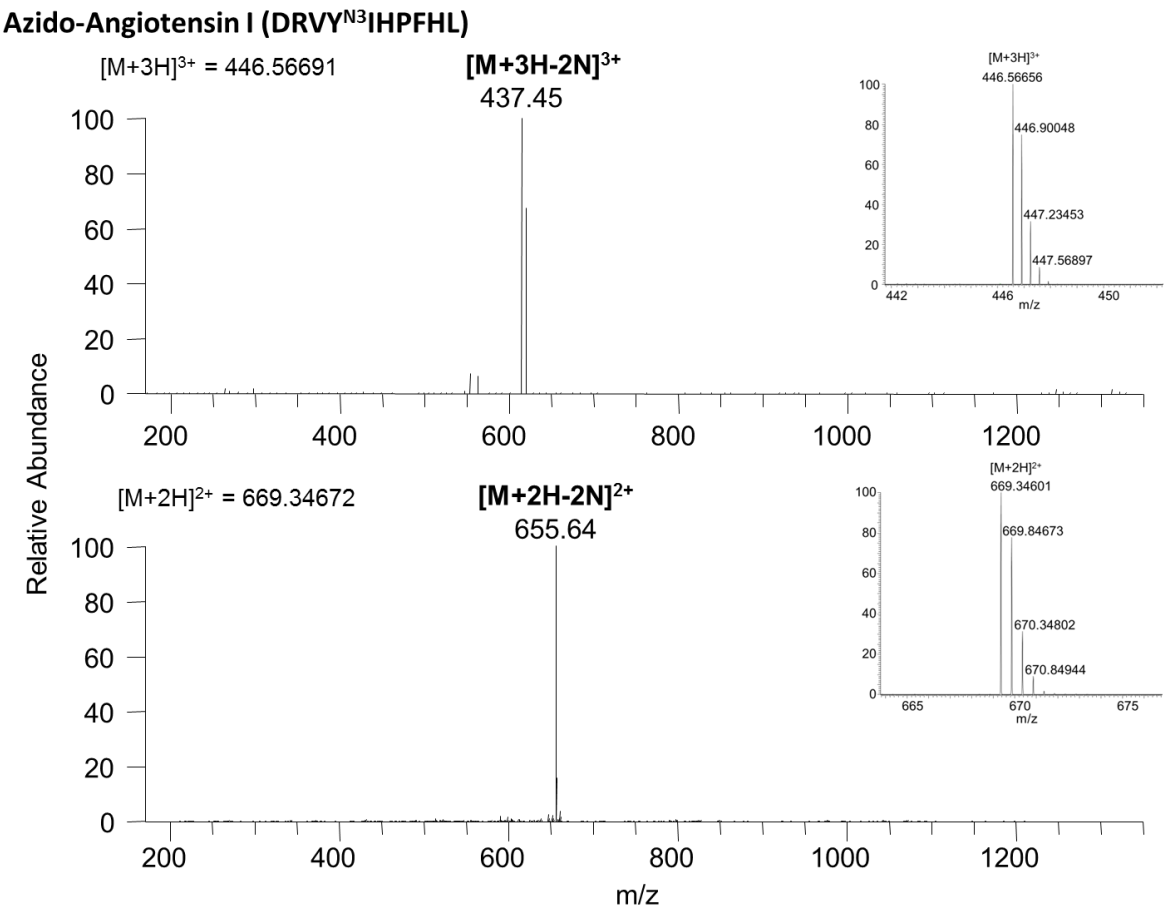
# Supplementary Figures




**Supplementary Fig 1.** (A) Extracted ion chromatograms showing angiotensin I (black) and nitro-angiotensin I (red) with retention time and area under chromatograms for each peptide. (B) Tandem mass spectra for nitro-angiotensin I. The accurate mass spectrum of the doubly charge ion of the intact peptide is shown in the inset.

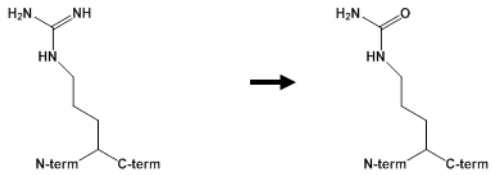
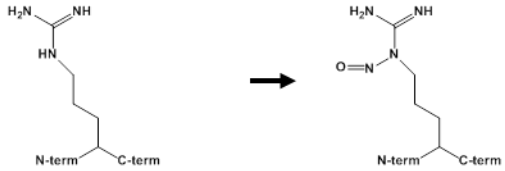
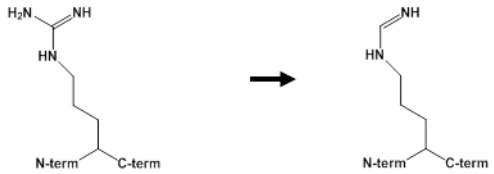
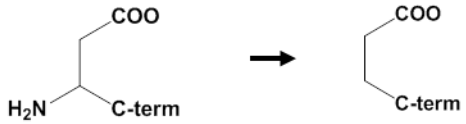
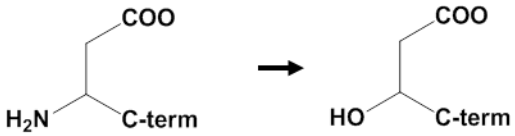


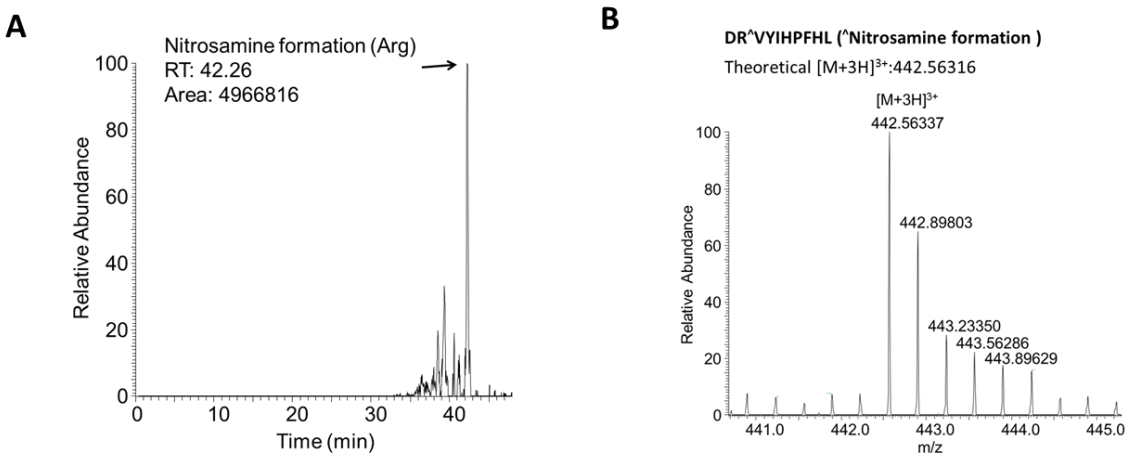

**Supplementary Fig 2.** Azido substitution of tyrosine by diazonium intermediate and sodium azide. HPLC analysis showing (top to bottom): 3-aminotyrosine (sigma), diazonium tyrosine intermediate, sodium azide, and concentration dependent increases in HCl addition, with concomitant increases in 3-azidotyrosine formation. Peaks are numbered as follows: 1. aminotyrosine, 2. diazonium intermediate, 3. hydroxyl-tyrosine, 4. sodium azide and 5. azido-tyrosine.



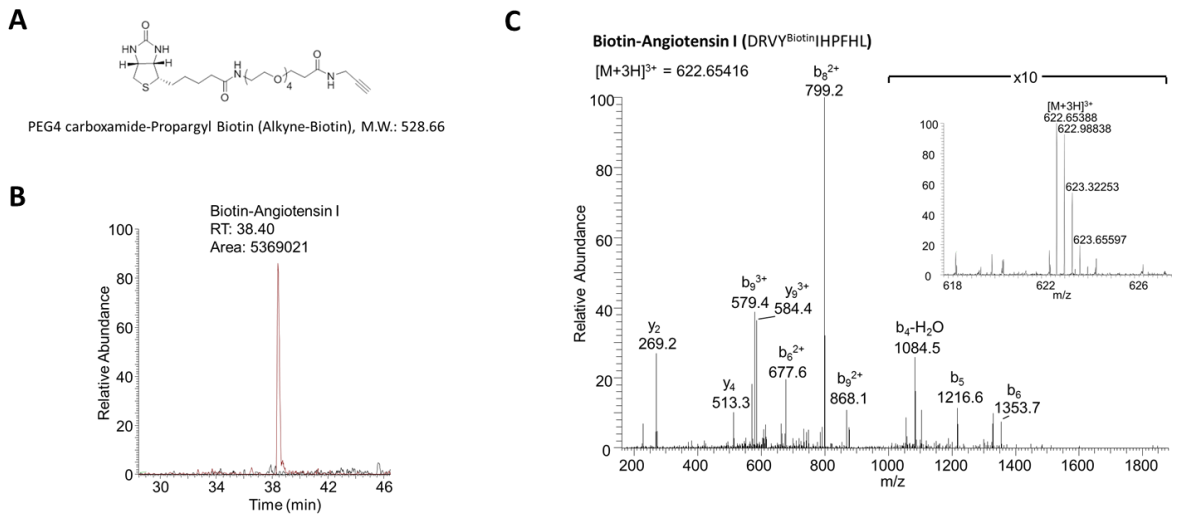

**Supplementary Fig 3.** CID tandem mass spectra of the 2+ and 3+ ions of azido-angiotensin I illustrating the inability of CID to fragment the azidated peptide.

**Supplementary Table 1.** Potential diazotisation reactions of primary amines and secondary amines searched for from the LC-MS/MS analysis of diazotised amino-angiotensin I.

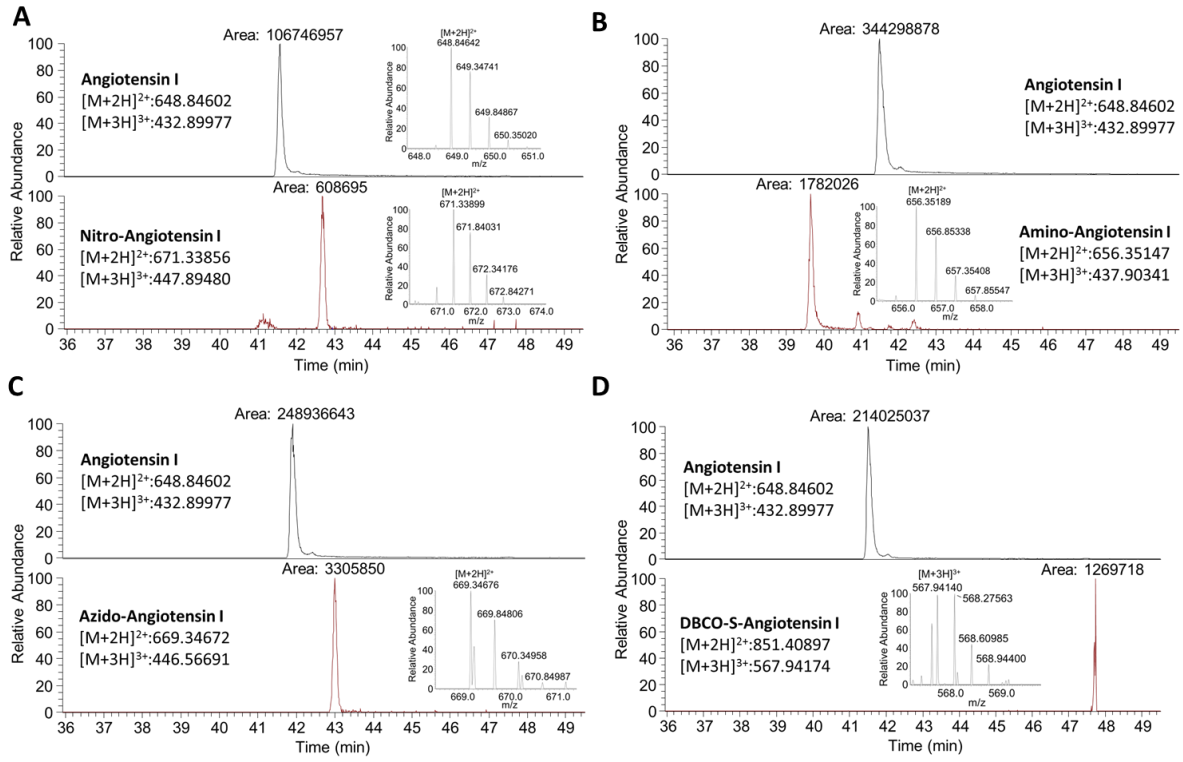
| Structural conversions | Reaction | Mass change |
|---|---|---|
|  | Alcohol formation from Arginine | -0.02381 |
|  | Nitrosamine formation from Arginine | +28.99016 |
|  | Alkene formation from Arginine | -15.01090 |
|  | Alkene formation from N-terminus | -15.01090 |
|  | Alcohol formation from N-terminus | -0.02381 |



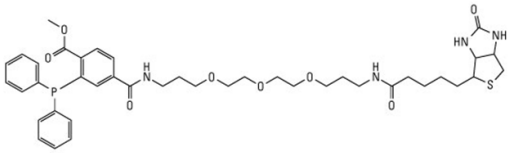

**Supplementary Fig 4.** (A) Extracted ion chromatogram for the angiotensin I with the conversion of secondary amine on arginine to nitrosamine. (B) Accurate mass spectrum of the triply charged ion of angiotensin I with nitrosamine formation at arginine.



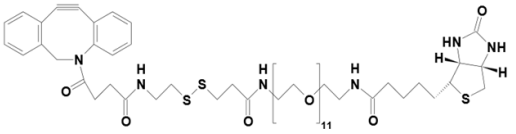

**Supplementary Fig 5.** (A) Structure of PEG4 carboxamide-Propargyl Biotin (Alkyne-Biotin). (B) Extracted ion chromatograms showing biotin-angiotensin I (red) with retention time and area under chromatograms for the peptide. (C) Tandem mass spectra for biotinylated-angiotensin I. The accurate mass spectrum of the triply charge ion of the intact peptide is shown in the inset.




**Supplementary Fig 6.** Extracted ion chromatogram for unmodified angiotensin I and expected product at each step of the derivatisation procedure. (A) Starting sample showing the ion abundance of angiotensin I and nitro-angiotensin I. (B) Ion abundance of angiotensin I and amino-angiotensin I following $Na_2S_2O_4$ reduction. (C) Ion abundance of angiotensin I and azido-angiotensin I following azidation. (D) Ion abundance of angiotensin I and DBCO-S-angiotensin I following ligation of azido-angiotensin and DBCO-biotin. The accurate MS1 spectrum is shown as an inset for the respective products.



Phosphine-PEG3-Biotin (PhP-Biotin), M.W.: 792.92



Dibenzylcyclooctyne-SS-PEG11-Biotin (DBCO-Biotin), M.W.: 1221.54

**Supplementary Fig 7.** Structures of Phosphine-PEG3-Biotin (PhP-Biotin) and Dibenzylcyclooctyne-SS-PEG11-Biotin (DBCO-Biotin).

## References

1. B. Balabanli, Y. Kamisaki, E. Martin and F. Murad, *Proc Natl Acad Sci U S A*, 1999, **96**, 13136-13141.