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Nitropeptide profiling and identification illustrated by Angiotensin II

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Cover Letter

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December 20, 2018

Jaydev Upponi, Ph.D.
Editorial Office of JoVE
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Dear Dr. Upponi,

We are submitting our revised manuscript entitled "Nitropeptide profiling and identification illustrated by Angiotensin II" to be considered for publication in the *Journal of Visualized Experiments*.

We really appreciate the editorial suggestions and reviewers' comments, which together tremendously helped us improve our manuscript. In the revised manuscript, we have addressed the raised points, which are detailed in the Rebuttal Letter.

Thank you very much for your consideration of our manuscript. We look forward to hearing from you.

Sincerely yours,

Xin Lu, Ph.D.
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TITLE:

Nitropeptide Profiling and Identification, Illustrated by Angiotensin II

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KEYWORDS:

Nitropeptide, Angiotensin II, nitrotyrosine, dimethyl labeling, sodium dithionate, biotin labeling, enrichment

SUMMARY:

Proteomic profiling of tyrosine-nitrated proteins has been a challenging technique due to the low abundance of the 3-nitrotyrosine modification. Here we describe a novel approach for nitropeptide enrichment and profiling by using Angiotensin II as the model. This method can be extended for other *in vitro* or *in vivo* systems.

ABSTRACT:

Protein nitration is one of the most important post-translational modifications (PTM) on tyrosine residues and it can be induced by chemical actions of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in eukaryotic cells. Precise identification of nitration sites on proteins is crucial for understanding the physiological and pathological processes related to protein nitration, such as inflammation, aging, and cancer. Since the nitrated proteins are of low abundance in cells even under induced conditions, no universal and efficient methods have been developed for the profiling and identification of protein nitration sites. Here we describe a protocol for nitropeptide enrichment by using a chemical reduction reaction and biotin labeling, followed by high resolution mass spectrometry. In our method, nitropeptide derivatives can be

identified with high accuracy. Our method exhibits two advantages compared to the previously reported methods. First, dimethyl labeling is used to block the primary amine on nitropeptides, which can be used to generate quantitative results. Second, a disulfide bond containing NHS-biotin reagent is used for the enrichment, which can be further reduced and alkylated to enhance the detection signal on a mass spectrometer. This protocol has been successfully applied to the model peptide Angiotensin II in the current paper.

INTRODUCTION:

Nitration of tyrosine residues in proteins to form 3-nitrotyrosine regulates many biological processes. Due to the different chemical properties between tyrosine and 3-nitrotyrosine, a nitrated protein may have perturbed signaling activity[1-2]. Therefore, it is important to develop methods that can enrich and identify nitration sites on proteins efficiently. As 3-nitrotyrosine is a low abundance modification on proteins compared to other forms of PTM, such as phosphorylation and acetylation, it is challenging to identify endogenous nitration sites directly from cell lines or tissue samples. Nevertheless, the methodology of using mass spectrometry (MS) to characterize the fragmentation pattern of nitrotpeptide has been developed (for example, Zhan & Desiderio[3]), which lays the foundation for new methods of nitroproteomics.

Currently, an enrichment step followed by MS is the most powerful strategy for nitropeptide profiling[4-5]. The enrichment methods can be classified into two classes. One class is based on antibodies that can recognize 3-nitrotyrosine specifically, whereas the other class is based on the chemical derivation that reduces a nitro group to an amine group[4-5]. For the antibody-based method, nitrotyrosine affinity column is used for the enrichment, from which the eluted material is further resolved and analyzed by high resolution MS[6-7]. For the chemical derivation-based method, the amine groups at the N-terminus of the peptide or lysine should be blocked in the first step either by acetylation, isobaric tags for relative and absolute quantitation (iTRAQ), or tandem mass tags (TMT) reagents. Next, a reducer is used to reduce nitrotyrosine to aminotyrosine followed by modifying the newly formed amine group, which includes biotin ligation, sulphydryl peptide conversion, or other types of tagging systems[8-11]. Most of the protocols established so far are based on *in vitro* over-nitrated proteins, instead of endogenously nitrated proteins.

In the present study, a modified procedure of the chemical derivation of nitrotyrosine is developed for the nitropeptide enrichment and identification, which shows enhanced sensitivity during MS detection and is suitable for quantification purpose. Our recent study employing this method in biological systems identified that nitration of lymphocyte-specific protein tyrosine kinase (LCK) at Tyr394 by RNS produced from myeloid-derived suppressor cells (MDSCs) plays an important role in the immunosuppression of the tumor microenvironment[12]. Therefore, our method of nitropeptide identification can be applied to complex biological samples as well. Here, we describe our protocol by using the model peptide Angiotensin II, of which the fragmentation pattern is known and widely used in nitroproteomic studies[8-11], as an example.

PROTOCOL:

1. Nitration of Angiotensin II

1.1. To generate nitrated peptide, dilute 10 µL of Angiotensin II (DRVYIHPF) parent solution (2 mM in water) in 390 µL PBS solution (10 mM NaH_2PO_4, 150 mM NaCl, pH 7.4) at the final concentration of 50 µM.

1.2. Add 10 µL of peroxynitrite (200 mM in 4.7% NaOH) to the Angiotensin II solution to make the final concentration of peroxynitrite 5 mM.

NOTE: Peroxynitrite is unstable and easy to resolve when it is in acid form, so parent solution of peroxynitrite is kept in basic solution and stored in -80 °C.

1.3. Adjust the pH value of the solution to 7-8 by 1 M HCl. To initiate the nitration reaction, shake the tube at 400 rpm for 5 min.

1.4. Use a commercially available reverse phase SPE column (see **Table of Materials**) for the desalting.

1.4.1. Prepare 2 solutions for the desalting, 5% methanol in water for the washing and 80% methanol in water for the elution.

1.4.2. Add 500 µL of methanol, followed by 500 µL of water to pre-condition the column, put 1 mL pipettor with the tip on the top of the column, press the pipettor to accelerate the flow.

1.4.3. Load 410 µL of the sample in step 1.3 on the column, discard the flow through.

1.4.4. After washing with 500 µL of 5% methanol, use 300 µL of 80% methanol to elute the nitropeptide, which is then fully dried by speed-vac in the default setting at room temperature.

NOTE: Desalting is very important for the reactions, since the solvent left in the previous step may have a negative effect on the next steps.

2. Alkylation of primary amines by dimethyl labeling

2.1. Reconstitute the powdered nitro-Angiotensin II in 100 µL of 100 mM triethylammonium bicarbonate (TEAB) solution.

NOTE: The pH value of the solution should be between 7 and 9, and make sure no primary amine is added in the solution.

2.2. Add 4 µL of 4% formaldehyde to the solution, mix briefly.

CAUTION: Formaldehyde vapors are toxic; perform the experiments in a fume hood.

2.3. Add 4 µL of 0.6 M $NaBH_3CN$ to the solution, shake the tube at 400 rpm for 1 h at room temperature.

2.4. Quench the labeling reaction by adding 16 µL of 1% ammonia solution, incubate at room temperature for 5 min.

2.5. Add 8 µL of formic acid to acidify the solution and perform desalting using reverse phase SPE column as described in step 1.4.

3. Reduction of nitrotyrosine to aminotyrosine

3.1. Reconstitute the dimethyl labeled nitro-Angiotensin II in 500 µL of PBS (10 mM NaH_2PO_4, 150 mM NaCl, pH 7.4).

3.2. Add 10 µL of 1 M sodium dithionate to the solution and incubate at room temperature for 1 h. After the reduction reaction, the yellow color of the solution becomes clear.

NOTE: Weigh 174.1 mg sodium dithionate and dissolve it in water to make the final volume is 1 mL. This solution can be stored at -20 °C no longer than a week.

3.3. Use the reverse phase SPE column for the desalting as described in step 1.4.

4. Biotinylation, enrichment, and detection

4.1. Reconstitute the dimethyl labeled amino Angiotensin II in 500 µL of PBS (10 mM NaH_2PO_4, 150 mM NaCl, pH 7.4).

4.2. Add 5 µL of 40 nM NHS-S-S-biotin, dissolved in DMSO, to the solution, after 2 h incubation at room temperature, use 1 µL of 5% hydroxylamine to quench the reaction.

4.3. In the meantime, equilibrate streptavidin agarose beads by adding 500 µL of PBS (10 mM NaH_2PO_4, 150 mM NaCl, pH 7.4), centrifuging at 580 x g for 5 min at room temperature and discarding the supernatant. Perform the equilibration 3 times.

4.4. Add 100 µL streptavidin agarose beads to the reaction system, incubate 1 h on a rotary shaker at room temperature. Wash 4 times with PBS (10 mM NaH_2PO_4, 150 mM NaCl, pH 7.4). For each washing step, add 500 µL of PBS to the beads, mix well, then centrifuge at 580 x g for 5 min at room temperature and discard the supernatant.

4.5. Use 400 µL of 10 mM dithiothreitol (DTT) to incubate with agarose beads at 50 °C for 45 min, spin down at 580 x g for 5 min at room temperature and discard the beads, add 20 µL of 0.5 M idoacetamide (IAM) to the supernatant for 20 min in darkness.

NOTE: DTT is used to break the disulfide bond between the link of biotin and peptide, the latter

is released from beads and further alkylated by IAM.

4.6. Use reverse phase SPE column for the desalting shown in step 1.4.

4.7. After resolving the dry peptides in 20 µL of 0.1% formic acid (FA), subject the product of each section to liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis.

4.7.1. Separate the modified Ang II peptides by a 60 min gradient elution (0-8 min, 2% B; 8-10 min, 5% B; 10-35 min, 18% B; 35-50 min, 28% B; 50-52 min, 80% B; 52-58 min, 80% B; 58-59 min, 2% B; 59-60 min, 2% B) at a flow rate 0.300 µl/min with nano-HPLC system which is directly interfaced with the high resolution mass spectrometer.

NOTE: The analytical column is in 75 µm ID, 150 mm length, packed with C-18 resin. Mobile phase A consisted of 0.1% formic acid, and mobile phase B consisted of 100% acetonitrile and 0.1% formic acid.

4.7.2. Operate the mass spectrometer in the data-dependent acquisition mode, set a single full-scan mass spectrum (300-1800 m/z, 60 000 resolution) followed by 20 data-dependent MS/MS scans at 28% normalized collision energy.

4.7.3. Open mass spectra data and identify the peaks of the product in each step to confirm the chemical reaction has been successfully performed.

5. Quantification of nitropeptide

5.1. Prepare 3 sets of nitro-Angiotensin II solutions from step 1.4, each set containing 2 concentrations of nitro-Angiotensin II: Set #1, 20 nmol in tube A and 20 nmol in tube B, each in 100 µl TEAB solution; Set #2, 10 nmol in tube C and 20 nmol in tube D, each in 100 µl TEAB solution; Set #3, 40 nmol in tube E and 10 nmol in tube F, each in 100 µl TEAB solution.

Note: The two concentrations of nitro-Angiotensin II in each set are used for dimethyl labeling, to react with formaldehyde (light) and formaldehyde-D2 (heavy), respectively.

5.2. For each set, add 4 µL of 4% formaldehyde and formaldehyde-D2 to the sample to be labeled as light and heavy, respectively. Follow the steps 2.3 to 2.5 to finish dimethyl labeling.

5.3. Mix the light and heavy labeled samples.

5.4. Follow the steps from 3.1 to 4.6 for the reduction, biotinylation, enrichment, and detection.

REPRESENTATIVE RESULTS:

The flowchart for nitropeptide profiling in this manuscript is shown in **Figure1**. **Figure 2**, **3**, **4** and **5** show the mass spectra of Angiotensin II, nitro-Angiotensin II, dimethyl labeled nitro-Angiotensin II and dimethyl labeled amino Angiotensin II, respectively. The molecular weight of

the compound can be reflected by the m/z values of the mono-isotope peak in each figure, indicating the chemical modification on Angiotensin II was successfully achieved for each step. **Figure 6** shows the final product in step 4.7 detected and characterized by LC-MS/MS. **Figure 7** shows the quantitative results of nitropeptide by dimethyl labeling. The relative amounts of light and heavy were determined by the comparison of the intensity of the mono-isotope peak in each group, which allows us to quantify the enriched nitropeptides from different groups.

FIGURE AND TABLE LEGENDS:

Figure 1. The workflow of the chemical derivation method for enriching and detecting nitro-Angiotensin II. First, Angiotensin II is nitrated by peroxynitrite, after desalting, dimethyl labeling is used to block the primary amine on the peptide. Then Sodium dithionate is used to reduce nitrotyrosine to aminotyrosine, which is further reacted with NHS-S-S-biotin. After enriched by streptavidin beads, the peptide is cut by DTT followed by alkylation and detected by LC-MS/MS. This figure has been modified from[12], Figure 2.

Figure 2. The mass spectrum of Angiotensin II. This figure showed that the mono-isotope peak at m/z 523.78 matched the duple charged peptide (up) and the MS/MS fragmentation pattern of Angiotensin II (bottom). This figure has been modified from[12], Figure S2.

Figure 3. The mass spectrum of nitro-Angiotensin II. This figure showed that the mono-isotope peak at m/z 546.28 matched the duple charged peptide (up) and the MS/MS fragmentation pattern of nitro-Angiotensin II (bottom). This figure has been modified from[12], Figure S2.

Figure 4. The mass spectrum of dimethyl labeled nitro-Angiotensin II. This figure showed that the mono-isotope peak at m/z 560.29 matched the duple charged peptide (up) and the MS/MS fragmentation pattern of dimethyl labeled nitro-Angiotensin II (bottom). This figure has been modified from[12], Figure S2.

Figure 5. The mass spectrum of dimethyl labeled amino Angiotensin II. This figure showed that the mono-isotope peak at m/z 545.31 matched the duple charged peptide (up) and the MS/MS fragmentation pattern of dimethyl labeled amino Angiotensin II (bottom). This figure has been modified from[12], Figure S2.

Figure 6. The mass spectrum of the final product. This figure showed that the mono-isotope peak at m/z 617.82 matched the duple charged peptide (up) and the MS/MS fragmentation pattern of the final product (middle) and the zoom-in spectrum from 400 to 1000 m/z exhibited more detailed fragmentation pattern (bottom). This figure has been modified from[12], Figure S2.

Figure 7. The mass spectra of the quantification results of the nitropeptides by dimethyl labeling. The light to heavy ratios are 1:1 (up), 1:2 (middle) and 4:1 (bottom), respectively.

DISCUSSION:

The protocol here describes the nitropeptide enrichment and profiling. Using Angiotensin II as

the model peptide, we illustrated the procedure shown in **Figure 1**. After obtaining the nitro-Angiotensin II, the primary amine on the peptide should be blocked to avoid the further amine conjugation, which is one of the most critical steps within the protocol. In the current protocol, dimethyl labeling is used to block the primary amines for two reasons: first, it enables the acquisition of quantitative results; second, the cost for this reaction is lower than NHS-based reaction and the blocking efficiency is high[13]. For the quantification, the measured light to heavy ratios are very close to the expected ratios (**Figure 7**), the errors are calculated less than 10%.
Another critical step of the protocol is the reduction of the 3-nitrotyrosine on dimethyl labeled peptide to aminotyrosine by sodium dithionate, which reacts fast and efficiently. The newly generated amine group is further labeled with 4-5 folds excess of NHS-S-S-biotin. Less or more NHS-S-S-biotin would lead to incomplete labeling reaction or incomplete enrichment, respectively. DTT can fully break the disulfide bond and release the enriched peptide from streptavidin beads, which are alkylated by IAM and analyzed by LC-MS/MS.

This protocol is suitable for identifying and quantifying nitropeptides in both biochemical system and biological materials such as cells or tissues. Since biotin enrichment and IAM alkylation can significantly enhance the sensitivity, the lowly abundant nitropeptides become detectable by MS. For example, we used this protocol to profile endogenous nitropeptides of isolated infiltrating T cells from the murine prostate and lung carcinoma models[12]. We identified nitro-Tyr294 of the protein LCK and performed functional studies to show that this modification may play a critical role in the process of immunosuppression by MDSCs[12].

The application of our technique is limited by two factors: first, the low abundance of nitropeptides in cells and tissues dictates that few nitropeptides can be identified; second, the inherent low detection sensitivity of MS compared with the highly sensitive methodologies such as single cell genomic and transcriptomic sequencing. We envision that the application of this protocol to other pathological conditions will help define more protein nitration PTMs with previously unrecognized functions in disease progression.

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DISCLOSURES:
The authors have nothing to disclose.

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Academy of Sciences of the United States of America. **101**, 4003-4008 (2004).
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Figure 1

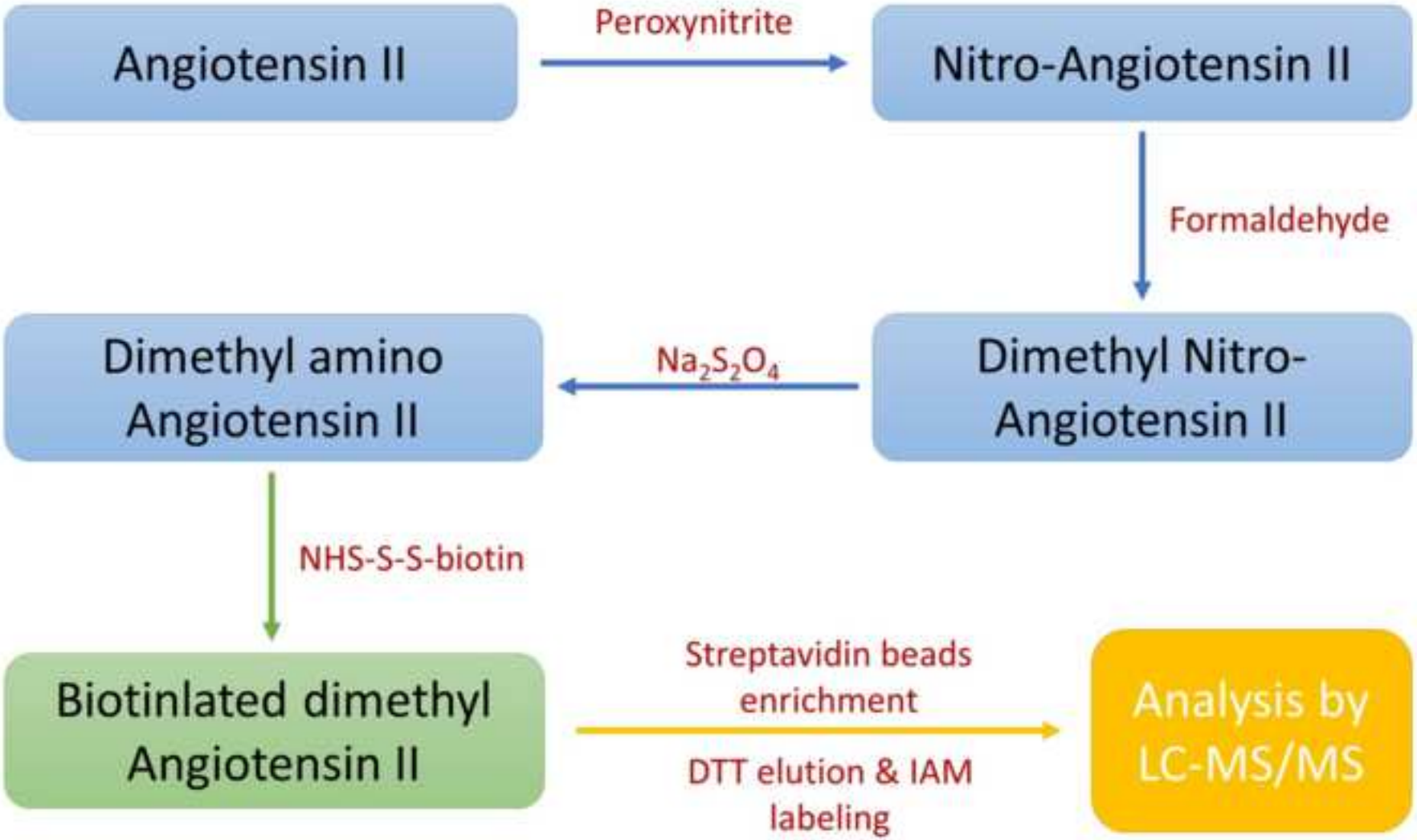


Figure 2

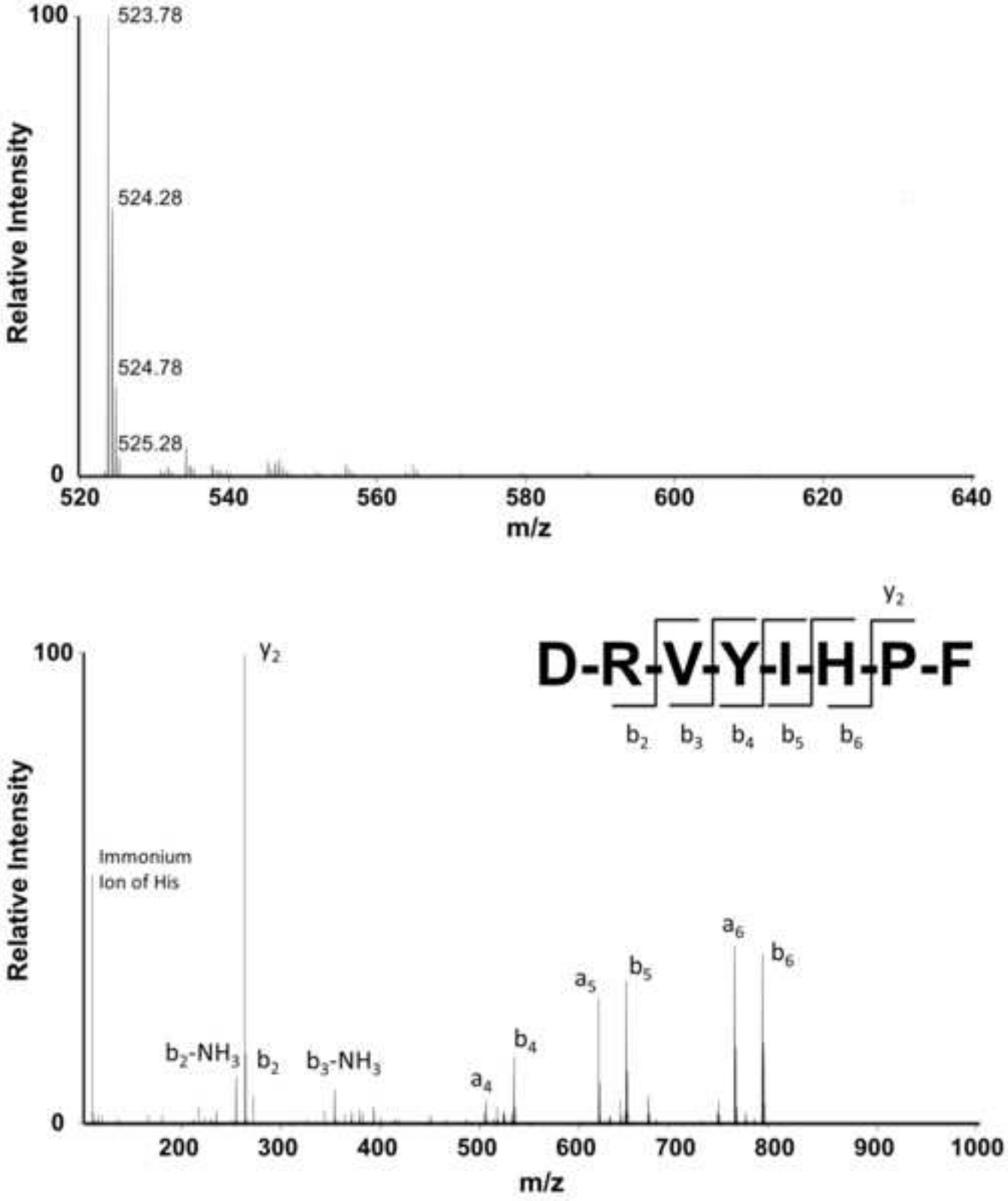
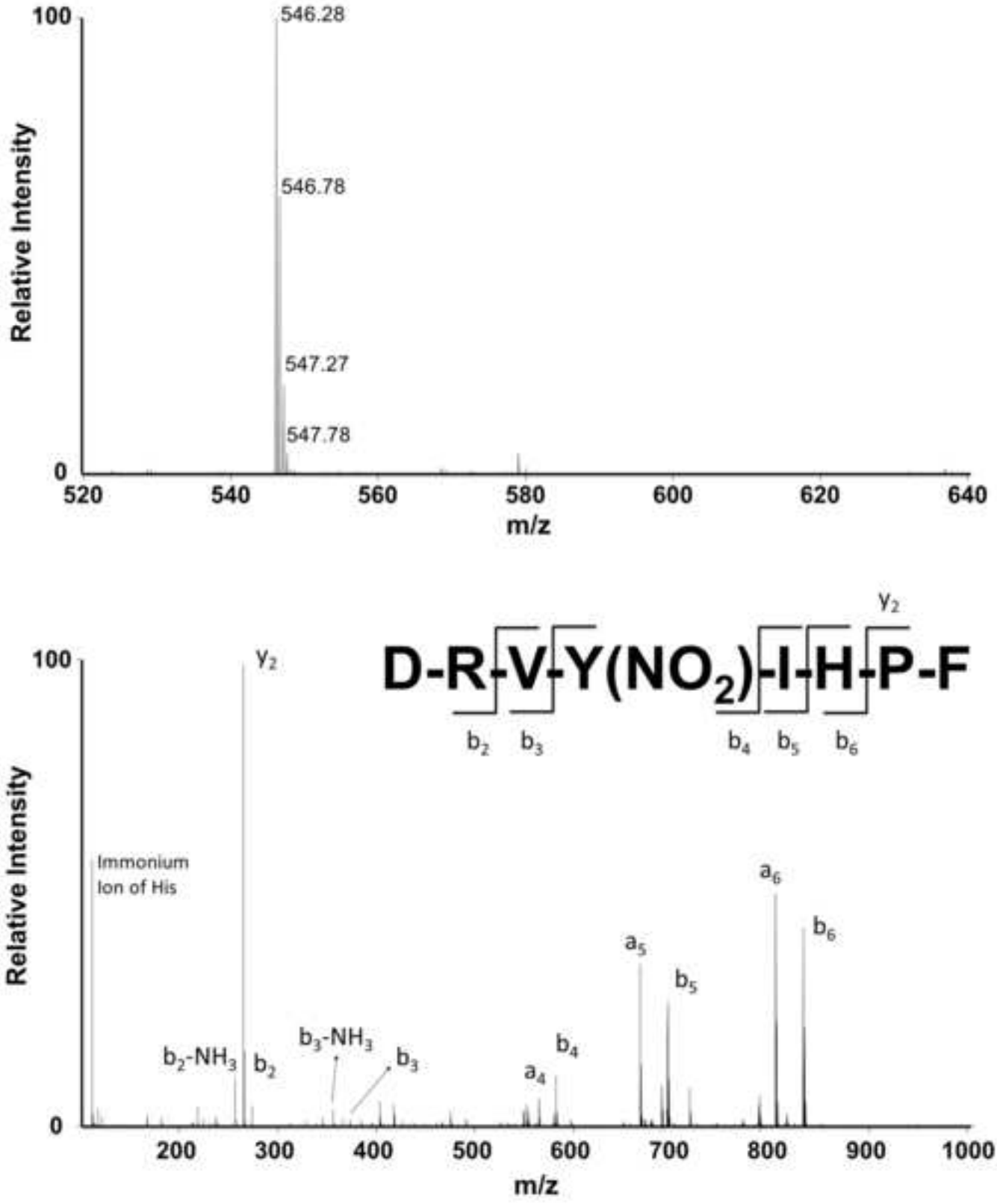


Figure 3



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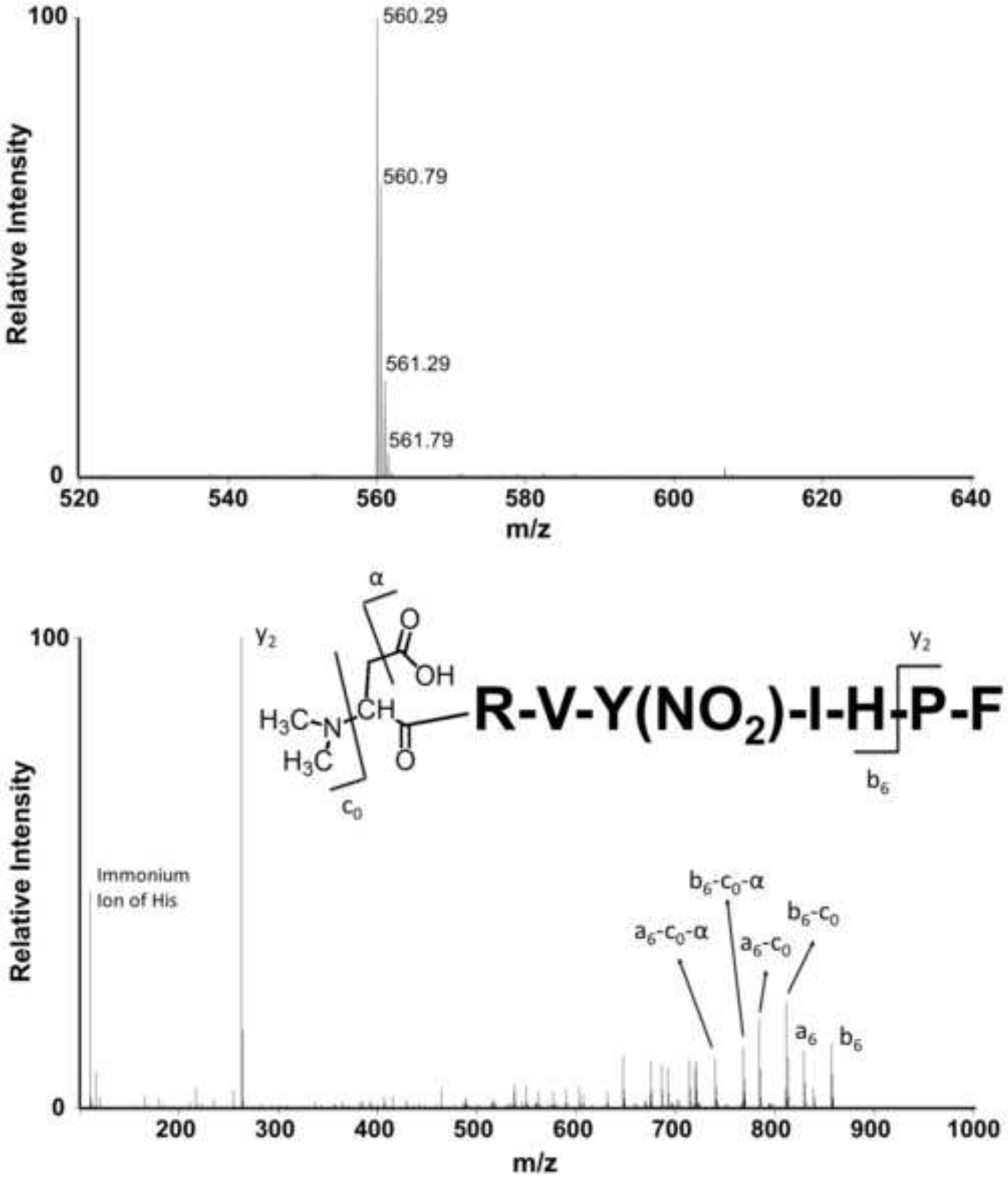

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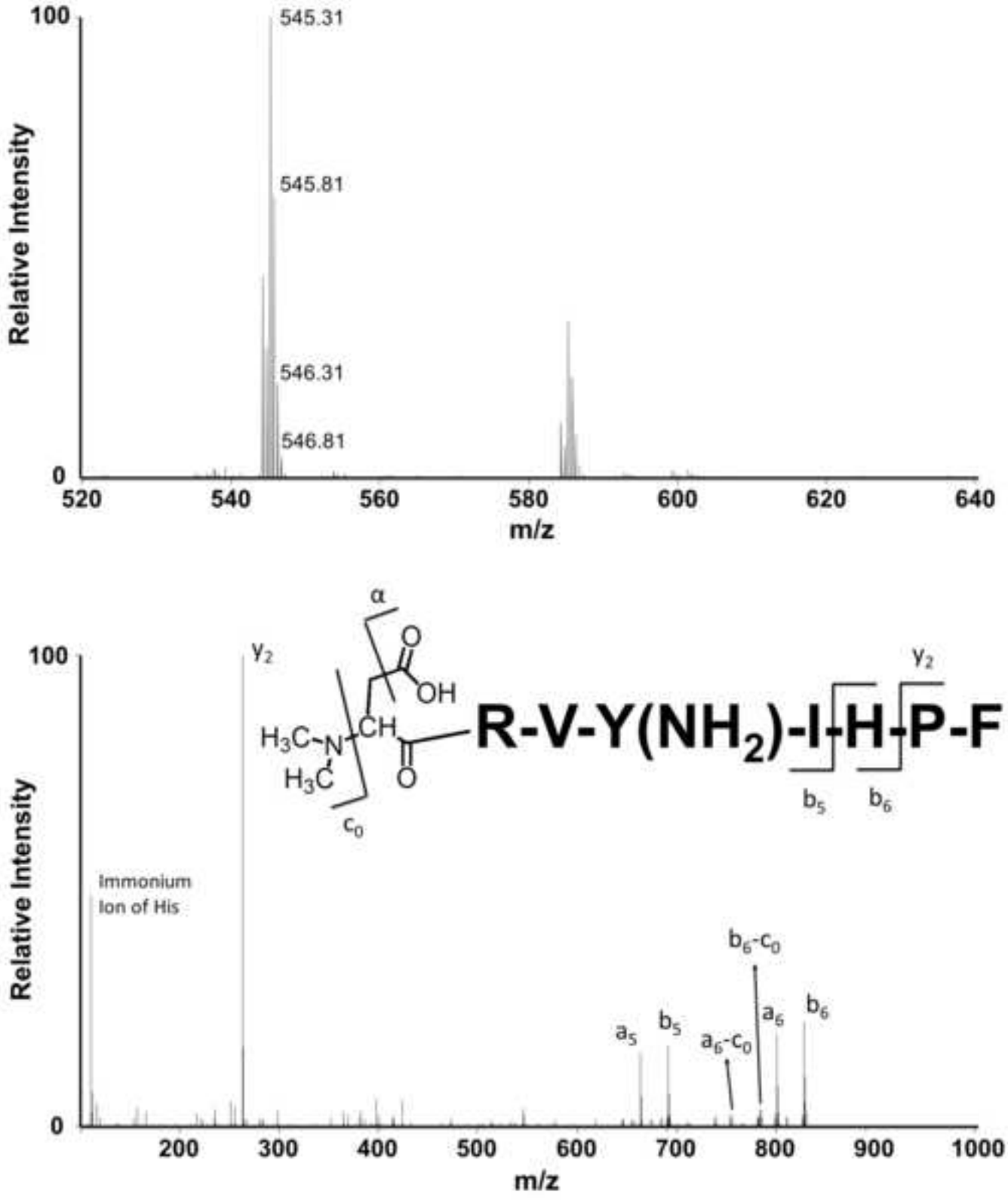


Figure 6

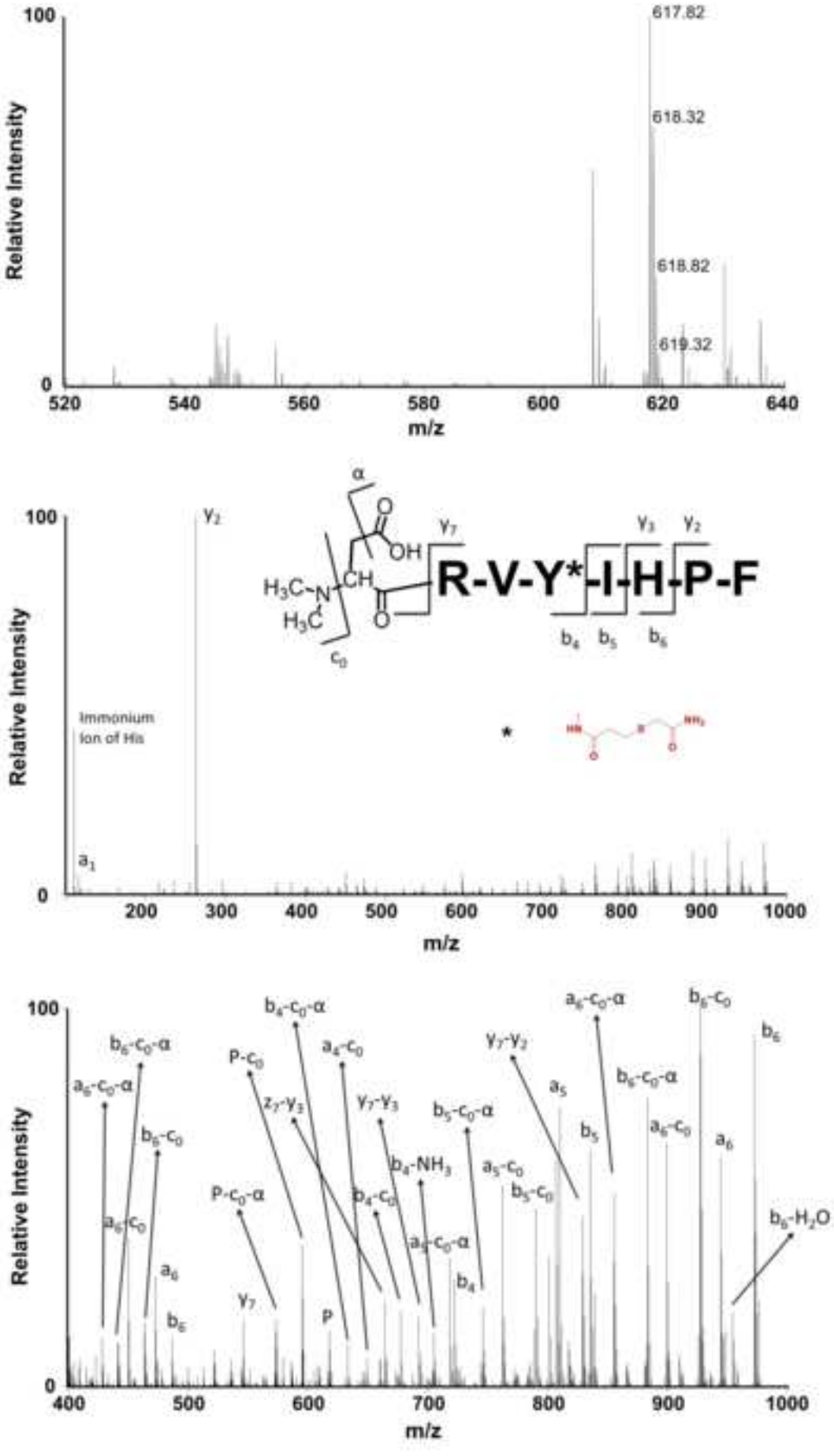
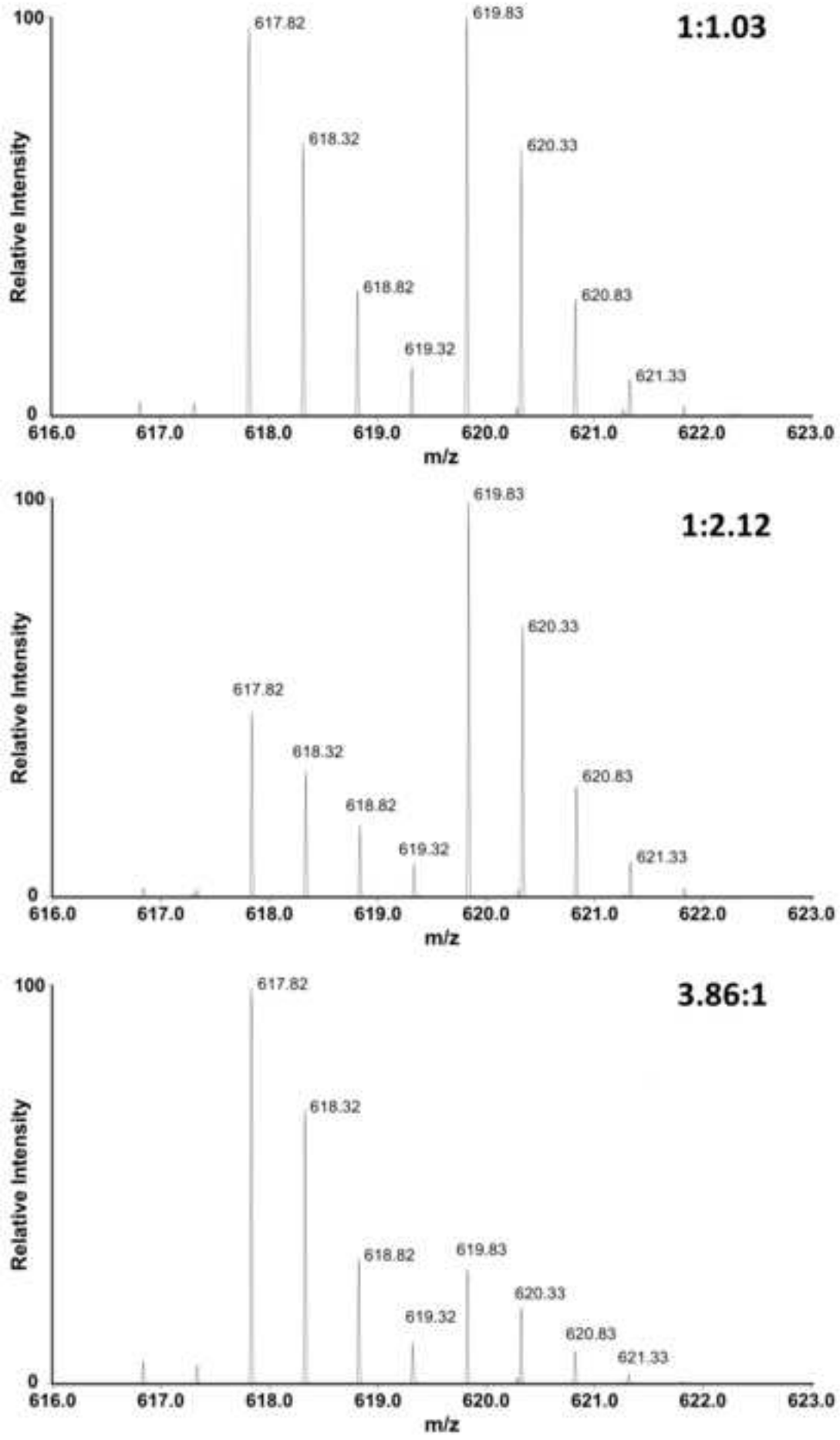


Figure 7



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Acclaim pepmap 100 C18 column	Thermo-Fisher	164534	
1 M TEAB solution	Sigma-Aldrich	T7408	
50% hydroxylamine	Thermo-Fisher	90115	
Acetonitrile	Thermo-Fisher	A955	MS Grade
dithiothreitol	Sigma-Aldrich	43819	
formaldehyde	Sigma-Aldrich	F8775	Molecular Biology Grade
formaldehyde-D2	Toronto Research Chemicals	F691353	
formic acid	Sigma-Aldrich	695076	ACS reagent
Fusion Lumos mass spectrometer	Thermo		
isoacetamide	Sigma-Aldrich	I1149	
Methanol	Thermo-Fisher	A456	MS Grade
NHS-S-S-bition	Thermo-Fisher	21441	
Oasis HLB column (10 mg)	Waters	186000383	
peroxynitrite	Merck-Millipore	516620	
sodium cyanoborohydrite	Sigma-Aldrich	42077	PhamaGrade
sodium dithionate	Sigma-Aldrich	157953	Technical Grade
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Rebuttal Letter for JoVE59391 "Nitropeptide profiling and identification illustrated by Angiotensin II"

Editorial comments:

Changes to be made by the author(s) regarding the manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We thank the editor's kind reminder. We have tried our best to correct any spelling and grammar errors.

2. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

3. 1.5: Please specify how to use HPLC for the purification of nitro-Angiotensin II. Alternatively, add relevant references.

Since step 1.5 is the alternative step, we have deleted this part in revised manuscript.

4. 4.3: Please describe how this step is actually done.

Following the editor's suggestion, we have added the detailed information for this step.

5. 4.5: Please specify centrifugation parameters for spinning down the beads.

Following the editor's suggestion, we have specified centrifugation parameters.

6. 4.6: Please provide the parameters for LC-MS/MS analysis.

The detailed information for LC-MS/MS has been provided in the revised manuscript.

7. Please expand to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc.

We have expanded the Representative Results to include content along the comments given by the editor in the revised manuscript. More details are described in the figure legends.

8. JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

We have added a few more sentences in Discussion and ensured that all the five points are now explicitly covered. In particular, we added sentences in paragraph 1 and 2 to highlight critical steps. The limitation of the technique is described in the last paragraph.

9. Figures 2-7: Please include a title in the figure legend for each figure.

We revised all the figure legends of Figure 2-7.

10. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, .pdf, .svg, .eps, .psd, or .ai file.

We have uploaded as instructed.

11. Table of Equipment and Materials: Please sort the items in alphabetical order according to the name of material/equipment.
We have reordered the sequence of the items.

12. References: If there are six or more authors, list the first author and then “et al.”.
According the editor’s suggestion, we have corrected the format for all reference.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
Due to the challenges of detecting low abundance nitroproteins, enrichment may be necessary. The goal of this manuscript is to establish a novel method of nitropeptide enrichment by using angiotensin II as a model. This technique could be expanded to in vitro and in vivo models.

Major Concerns:
I have no major concerns regarding this work.

Minor Concerns:
The authors should also include the Zhan and Desiderio Int. J Mass Spectrometry citation from 2009 as it is applicable. The in vivo application is solely based on the authors work. It would be nice to see broader array, but this may not be possible due to the novelty of this work.
We appreciate the positive comments from the reviewer, and agree that the *in vivo* application in broader contexts will require further investigations in follow-up studies. We have now added Zhan & Desiderio as Reference#3 in Introduction.

Reviewer #2:

Manuscript Summary:

The proposal is useful and will help the scientific community dealing with this challenging PTM. It has some details to be adjusted:

We appreciate the reviewer’s positive comments on our work.

Under 1.

The composition of 150 mM PBS solution should be described in detail.
We have described the composition of PBS solution in the revised manuscript.

The concentration of peroxynitrite should be measured.Since peroxynitrite is highly unstable in neutral to acidic solution (t½ about 10 ms) the mixture with the protein should be done fast (addition in the whirl generated by a vortex). Because of that the incubation for 30 min after pH adjustment is unnecessary.
We thank the reviewer’s great suggestions. We agree with the reviewer that the nitration reaction is fast, and we confirmed by experiments that there were no significant differences in the productivity between 5

and 60 min of the reaction time. Therefore, we have shortened the reaction time to 5 min in the revised manuscript. Regarding the concentration of peroxynitrite, we have tested the amounts of peroxynitrite used in this reaction from 50 to 500 folds excess of the substrate peptide and observed no significant differences in the products. According to the product information of peroxynitrite (Millipore – Calbiochem, 516620), freshly received product will decrease its activity by ~2% /day at -20°C. Since we follow the instruction to aliquot and store at -80°C, and only use it within 4 weeks after receiving it, we think the level of drop in concentration and activity of peroxynitrite would not affect the reaction products. Therefore, measuring concentration may not be particularly useful from the user's point of view.

It would be advisable to evaluate the level of nitration obtained (Absorbance at 420/350 nm (Crow and Beckman, 1995).

Regarding evaluating the level of nitropeptide, we agree that the mentioned method of absorbance measurement is feasible. In fact, in the current protocol, we use MS to detect the nitration product of Angiotensin II, therefore we could use mass spectrum to evaluate the nitration reaction. Based on our preliminary result, over 50% of Angiotensin II was converted to nitro-Angiotensin II, and some side products formed by oxidation rather than nitration. Because our protocol is focused on derivation of the nitrated peptide and its MS characterization, which is not affected by the efficiency of nitration in the first step, we hope the reviewer could allow the performance of nitration measurement in a separate study.

Under 5.1.

Is the sentence "Prepare 3 sets of nitro-Angiotesin II solutions, each set contains 2 concentrations of the solvent: 1st set, 20 and 20 nmol; 2nd set, 10 and 20 nmol; 3rd set, 40 and 10 nmol" correct? Please revise.
We have revised it to "Prepare 3 sets of nitro-Angiotensin II solutions, each set contains 2 concentrations of nitro-Angiotensin II: Set #1, 20 nmol in tube A and 20 nmol in tube B, each in 100 ul TEAB solution; Set #2, 10 nmol in tube C and 20 nmol in tube D, each in 100 ul TEAB solution; Set #3, 40 nmol in tube E and 10 nmol in tube F, each in 100 ul TEAB solution."

Editorial comments:

1. The editor has formatted the manuscript to match the journal's style. Please retain the same.

We thank the kind effort from the editor, and keep the same style.

2. Please address all the specific comments marked in the manuscript.

Following the editor's suggestion, we have addressed all the comments marked in the manuscript.

3. For the protocol section, please write exactly how you perform the experiment with all necessary details in imperative tense throughout as if telling someone how to perform your experiment.

We appreciate the editor's great suggestion, and have tried our best to make everything clear in detail in the revised manuscript.

4. Once done, please highlight 2.75 pages of the protocol section in yellow including headings and spacings that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

We have highlighted from step 2 to 4 for the video.

5. Please cite the figures appropriately in Figure Legend, i.e. "This figure has been modified from [citation]."

Following the editor's comment, Figure 1 to 6 have been cited from ref 12 in the revised edition.

6. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file

We have supplied all the information of reagents and equipment used in the protocol.

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