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Nitrite-assisted peptide iodination and conjugation

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Abstract: In this study, a simple method for selective iodination of peptides and proteins is established. Using angiotensin II as the model system, we demonstrate that nitrite catalyzed the selective iodination of the peptide at the *N*-terminus in an acidic solution. The *N*-terminal-iodinated peptides alkylated thiol-containing molecules such as *N*-acetylcysteine and glutathione to form peptide conjugates in a basic solution. Reactive species formed by increasing the pH of the reaction mixture of sodium nitrite and sodium iodide from 4 to 8 selectively iodinated peptides and proteins at tyrosine and histidine residues. These results show that nitrite is a useful catalyst for peptide and protein ligation. Copyright © 2006 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: nitrite anion; protein/peptide modifications; amino groups; nucleophilic reactions

INTRODUCTION

Specific incorporation of novel functionalities in peptides and proteins is a widely used tool in protein biochemistry and molecular biology [1–4]. Among the synthetic-chemistry-driven methodologies developed for modifying, controlling, and monitoring protein and peptide functions *in vivo* and *in vitro*, iodination has attracted widespread interest and has been extensively investigated. Protein iodination is a useful probe in crystallographic studies [5–9]. Protein iodination with radioactive iodine isotopes is a valuable tool in the monitoring of metabolic reactions both *in vitro* and *in vivo* and in studying ligand–receptor binding, ligand uptake, and clearance [10–12]. Protein iodination has also been used on the selective modification of enzyme activity [13]. Iodinated thyroid hormones and thyroglobulin play an important role in immungenicity as well as pathogenicity [14,15].

A general iodination method is performed on the aromatic side chains of Tyr and His by using sodium iodide and an oxidizing agent [16–18]. This approach may also modify amino acid residues that are sensitive to oxidation, such as Met, Trp, and Cys. The indirect method uses the amine-reactive reagents such as iodinated 3-(4-hydroxyphenyl) propionic acid *N*-hydroxysuccinimide ester, which react with the free amino groups in the protein molecule to attach the ^{125}I-labeled groups by amide bonds [19,20]. This introduces an additional organic moiety and is less specific, by reacting primarily with *N*-terminal amine, Lys, and Cys instead of Tyr and His functionalities. Peroxidase-catalyzed iodination has been widely used in the biosynthesis and it is a multiple step process including activation of peroxidase and peroxidation of iodide to iodine followed by iodination of proteins [21,22].

On the other hand, a few studies have been reported on reactions of nitrite with the amino group of amino acid and peptides [23–27]. It was found that nitrite reacted with amino acids to generate α-hydroxy acids and α-halo acids [23–27]. Using amino acid analysis, Maerker *et al.* found that reactions of nitrite with polylysine led to deamination of lysine to form hydroxynorleucines [23–27]. These results provided important information on nitrite–protein interactions. To fully characterize the reaction products of nitrite with peptides and proteins, mass spectrometry was used in our recent studies. We found that reactions of nitrite with proteins/peptides resulted in two major transformations: (i) replacement of the amino group by a hydroxyl group and (ii) formation of an alkene derivative by the loss of NH_3 from the *N*-terminus [27]. The reaction proceeds rapidly in weak acidic solutions and at 37 °C in the presence of millimolar concentrations of nitrite. On the basis of this finding, a simple method for selective iodination of peptides was established in the present work. Using angiotensin II and insulin as the model systems, we demonstrate that reactions of peptides with sodium iodide in the presence of nitrite anions generate iodinated peptides in the acidic solution. The *N*-terminal-iodinated peptides carbamidomethylated thiol-containing molecules to form peptide conjugates. The oxidative species generated by increasing the pH of the reaction mixture of $NaNO_2$ and NaI from 4 to 8 selectively iodinated peptides and proteins at tyrosine and histidine residues.

MATERIALS AND METHODS

Bovine insulin, horse heart cytochrome c, angiotensin II, bradykinin, sodium nitrite, sodium iodide, and trifluoroacetic

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acid were purchased from Sigma-Aldrich and used without further purification.

Reactions of Angiotensin II with Iodide in the Presence of Sodium Nitrite

The reaction was studied by mixing the appropriate quantity of the solutions of peptides or proteins with sodium nitrite and sodium iodide at different concentrations. In most reactions reported here, the concentrations of angiotensin II, sodium nitrite, and sodium iodide were 100 μM, 10 mM, and 100 mM, respectively. For iodination of the *N*-terminus of peptides and proteins, the pH of the reaction mixture was adjusted to 4 by stepwise addition of 1% TFA aqueous solution. The mixture was incubated at 37 °C under air or N_2 for a desired time. For iodination of tyrosine and histidine, a mixture of 10 mM sodium nitrite and 100 mM sodium iodide was incubated at 37 °C and pH 4 for 30 min, and then the pH of the mixture was increased to 8 by stepwise addition of 100 mM sodium hydroxide for the reactions with peptides and proteins. The pH values of the reaction mixture were monitored by pH paper or a pH meter. The conjugations of the *N*-terminal-iodinated peptides with glutathione and *N*-acetylcysteine were studied by mixing the semipurified peptide with 10 mM GSH or *N*-acetylcysteine at 37 °C and pH 8 for 1 h. All reaction products were analyzed using MALDI-TOF or LC-MS/MS.

Analysis of Reaction Products by Mass Spectrometry

The reaction products were analyzed by MALDI-TOF mass spectrometry. Briefly, samples were mixed 1 : 1 (vol/vol) with a solution of α-cyno-4-hydroxycinnamic acid in 50% aqueous acetonitrile/0.1% aqueous trifluoroacetic acid (1 : 1, vol/vol), and a 1 μl sample was spotted onto a stainless steel MALDI target plate and allowed to dry before analysis by MALDI-TOF MS. The resulting spectra were analyzed by the DataExplorer (Applied Biosystems, Inc.) software. The instrument was calibrated using an external standard, and the resulting spectra were not Gaussian-smoothed and baseline-corrected.

For LC-MS/MS analysis, each reaction product was separated by gradient elution with a Dionex capillary/nano-HPLC system and analyzed by an Applied Biosystems QSTAR XL mass spectrometer using information-dependent, automated data acquisition. The peptide mixtures were separated on a 15 cm × 75 μm i.d. reversed-phase C18 column (Micro-Tech Scientific) using a 35 min linear gradient of 5–50% acetonitrile in 0.1% formic acid and at a flow rate of 200 nl/min.

RESULTS AND DISCUSSIONS

Formation of N-terminal-iodinated Angiotensin II in the Acidic Solution

For the present study, angiotensin II was chosen as the model system for studying the nitrite-assisted peptide iodination and conjugation. Angiotensin II is a polypeptide in the blood, that causes vasoconstriction, increased blood pressure, and aldosterone release from the adrenal cortex [28,29]. Iodinated angiotensin has been used as the pharmacological tool to study its biological functions [30,31]. Figure 1 (b) shows the MALDI-TOF mass spectrum of reaction products from incubation of angiotensin II (DRVYIHPF) (100 μM) and $NaNO_2$ (10mM) in a weak acidic solution at 37 °C for 1 h. Three major reaction products were observed and their masses corresponded to (M − 61), (M − 17), and (M + 1), respectively. M stands for the molecular mass of the protonated angiotensin II. These products were identified in previous work [27]: product 3 was one mass unit higher than that of angiotensin II corresponding to the replacement of the NH_2 group by an OH group at the *N*-terminus; the product 2 at (M − 17) was an alkene derivative by the loss of NH_3 from the peptide. The product 1 at (M − 61) was a residue-dependent product and was observed only from peptides that have an acidic amino acid residue at the *N*-terminus. This product is proposed to be an alkene derivative formed by losing both the *N*-terminal NH_2 group and the HCO_2 group of the carboxyl side chain.

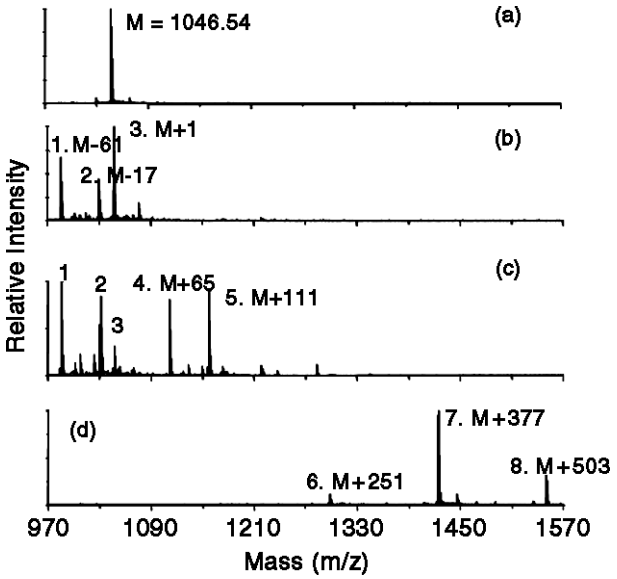

Figure 1 MALDI mass spectra of the reaction products of angiotensin II with nitrite and sodium iodide. (a) angiotensin II alone; (b) angiotensin II and 10 mM $NaNO_2$ at pH 4 for 1 h at 37 °C; (c) angiotensin II and 10 mM $NaNO_2$ and 100 mM NaI at pH 4 for 1 h at 37 °C; and (d) increase of the pH of the mixture of angiotensin II and 10 mM $NaNO_2$ and 100 mM NaI from pH 4–8 for 5 min.

Addition of NaI (100 mM) to the mixture of angiotensin II and $NaNO_2$ resulted in two more reaction products, labeled as 4 and 5 in Figure 1(c). The mass of the product 5 was observed at *m/z* 1157.5 (M + 111) corresponding to the replacement of the NH_2 group by an iodine atom. Using MS/MS analysis, it was found that iodination occurred at the peptide *N*-terminus. The mass of the product 4 observed at 1111.5 (M + 65) matched the addition of one iodine atom to the *N*-terminus of the product 1 as confirmed by MS/MS analysis (data not shown). Formation of

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the *N*-terminal-iodinated peptide was pH dependent: the reaction was slow in the neutral solution and no products were observed in the basic solution. Similar products were observed for reactions conducted under N_2, indicating that O_2 was not involved in the reaction. The ratio of the product 3 to 5 was dependent on the concentrations of water and sodium iodide. Using an organic solvent such as aqueous acetonitrile enhances the yield of the product 5. However, the *N*-terminal-iodinated peptide was unstable in the basic solution (pH > 7.5) to be rapidly hydrolyzed by the replacement of the iodine with a hydroxyl group to form the product 3.

Nitrite-assisted Iodination of Tyrosine and Histidine Residues in the Basic Solution

Increase of pH of the reaction mixture from 4 to 8 not only resulted in the hydrolysis of the *N*-terminal-iodinated peptides but also led to formation of multiple iodinated peptides at tyrosine and histidine residues, as shown in Figure 1(d). The masses of products 6, 7, and 8 observed at 1297.5, 1423.6, and 1549.6 amu in Figure 1(d) correspond to addition of 2, 3, and 4 iodines to the peptide, respectively. LC-MS/MS analysis was employed to probe the structures of these products. The MS/MS spectrum of the product 7 observed at MH_2^{2+} 713.2 is displayed in Figure 2. The fourth and sixth residues of angiotensin II (DRVYIHPF) are tyrosine and histine, respectively. The b_2 to b_3 fragment ions are 1 amu higher than those from the unmodified peptide indicating that the amino group at the *N*-terminus was replaced by an OH group. The b_4–b_5 fragment ions are 253 amu higher than those from the unmodified peptide, indicating that tyrosine 4 was di-iodinated. The b_6 ion is 379 amu higher than that from the unmodified peptide, indicating that histidine 6 was monoiodinated. This is confirmed by the y-ion series in which the y_3 and y_4 ions are 126 amu higher than those of unmodified peptide. Using MS/MS, we also demonstrated that the product 8 had a di-iodinated tyrosine and a di-iodinated histidine. By increasing the incubation time, the tri-iodinated peptide was converted into the tetraiodinated peptide. It is worth noting that direct incubation of angiotensin II with NaI and $NaNO_2$ at pH 8 did not generate any products. But similar products to those shown in Figure 1(d) were observed when angiotensin II was added to a mixture of $NaNO_2$ and NaI at pH 8 that was preincubated at pH 4 for 30 min. This suggested that reactive species were formed by increasing the pH of the mixture of NaI and $NaNO_2$.

The reactive species generated by increasing the pH of the mixture of $NaNO_2$ and NaI from 4 to 8 also iodinated proteins such as insulin and cytochrome *c*. The MS spectra of the reaction products of insulin with NaI and $NaNO_2$ are displayed in Figure 3. After 1 h incubation, four more peaks were observed in the mass spectrum (Figure 3(b)). The mass difference between any consecutive peaks is 126 Da corresponding to the mass of one iodine atom. Increasing the incubation time led to a shift of the reaction products to higher masses, and attachments of up to 8 iodine atoms to the insulin were observed after 2 h incubation as shown in Figure 3(c). Similar reaction products were observed for cytochrome *c*.

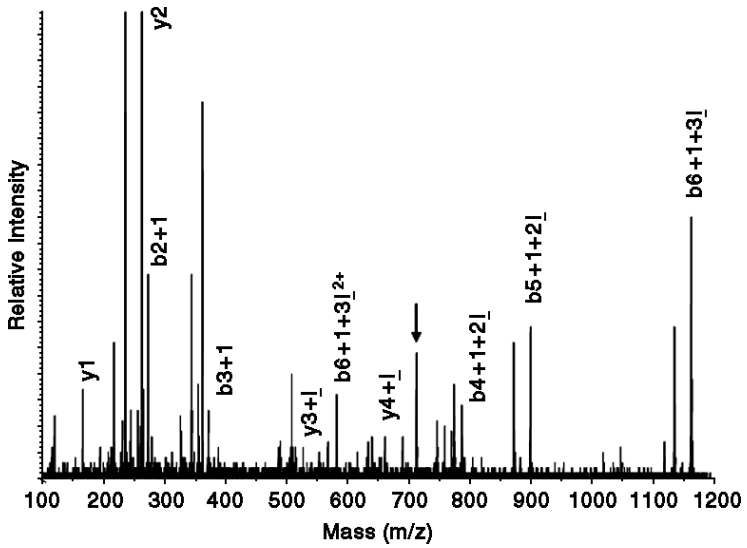

Figure 2 MS/MS of the product 7 observed at MH_2^{2+} 713.2. The b_2 to b_3 fragment ions are 1 amu higher than those from the unmodified peptide, indicating that the amino group at the *N*-terminus was replaced by an OH group. The b_4 to b_5 fragments ions are 253 amu higher than those from the unmodified peptide indicating that tyrosine 4 was di-iodinated. The b_6 ion is 379 amu higher than that from the unmodified peptide indicating that histidine 6 was monoiodinated. This is confirmed by the y-ion series in which y_3 and y_4 ions are 126 amu higher than those of the unmodified peptide.

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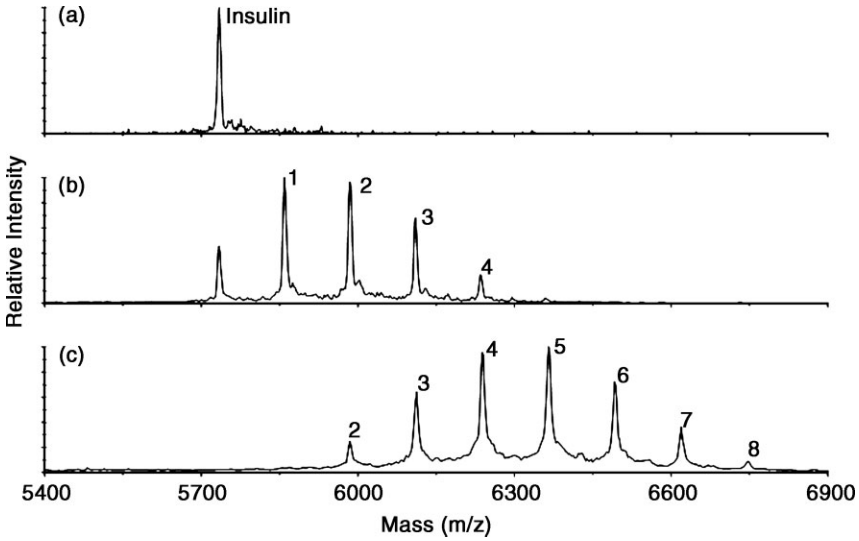

Figure 3 MALDI mass spectra of reaction products of insulin with the mixture of sodium nitrite and sodium iodide that was preincubated at pH 4 for 30 min. (a) insulin alone; (b) insulin and 10 mM $NaNO_2$ and 100 mM NaI at pH 8 for 1 h at 37 °C; (c) insulin and 10 mM $NaNO_2$ and 100 mM NaI at pH 8 for 2 h at 37 °C.

Conjugation of *N*-terminal-iodinated Angiotensin II with Thiol-containing Molecules

Hydrolysis of the *N*-terminal-iodinated angiotensin is a nucleophilic reaction. Therefore, other nucleophiles may also react with the *N*-terminal-iodinated angiotensin. In this study, the *N*-terminal-iodinated angiotensin II was semipurified, and its reactions with the thiol-containing molecules were studied. The reactions of the *N*-terminal-iodinated angiotensin II with *N*-acetylcysteine or glutathione (GSH) were carried out at pH 8 for 60 min. The mass spectra of the reaction products are displayed in Figure 4(a) and (b). Two major reaction products observed at 1192.55 and 1336.59 correspond to replacement of iodine by the *N*-acetylcysteine and glutathione molecule, respectively. To confirm that the conjugation was at the *N*-terminus of angiotensin II, an MS/MS analysis of these two products was conducted. The MS/MS spectrum of the conjugation product of angiotensin II and *N*-acetylcysteine (M + 146) observed at MH_2^{2+} 596.8 is displayed in Figure 5. The b_2 to b_6 fragment ions are 146 amu higher than those from the unmodified peptide indicating that the amino group at the *N*-terminus was replaced by an *N*-acetylcysteine group.

On the basis of the above mass spectrometric analyses, the reaction process is outlined in Scheme 1. The reaction of nitrous acid with the amino group at the *N*-terminus resulted in formation of a diazonium ion that is unstable to give nitrogen gas and a carbocation. The carbocation proceeds either to nucleophilic substitution or to elimination. Both hydroxyl and iodine are nucleophiles that attack the carbocation to form products 3 (M + 1), 4, and 5 (M + 111) by substitution. In a basic solution, the *N*-terminal-iodinated peptides either hydrolyze to form the nitrosative de-iodination product

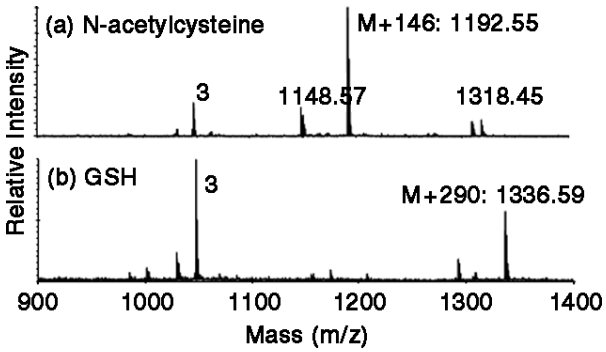

Figure 4 MALDI mass spectra of reaction products of the *N*-terminal-iodinated peptide with *N*-acetylcysteine and glutathione: (a) *N*-acetylcysteine 100 mM; (b) glutathione 100 mM at pH 8 for 30 min at 55 °C.

or react with other nucleophiles. By alkylating the thiol-containing molecules, the *N*-terminal-iodinated peptide is proven to be an efficient reagent for peptide conjugation.

The increase of pH of the reaction mixture also resulted in selective iodination of tyrosine and histidine residues of peptides. Although the reaction mechanism is still under study, it is proposed that the oxidative species generated from the reaction mixtures in the acidic conditions activate aromatic side chains of tyrosine and histidine residues to the quinone form at the higher pH, leading to iodination of tyrosine and histidine residues.

CONCLUSIONS

A simple method to selectively iodinate peptides and proteins was established in the present study.

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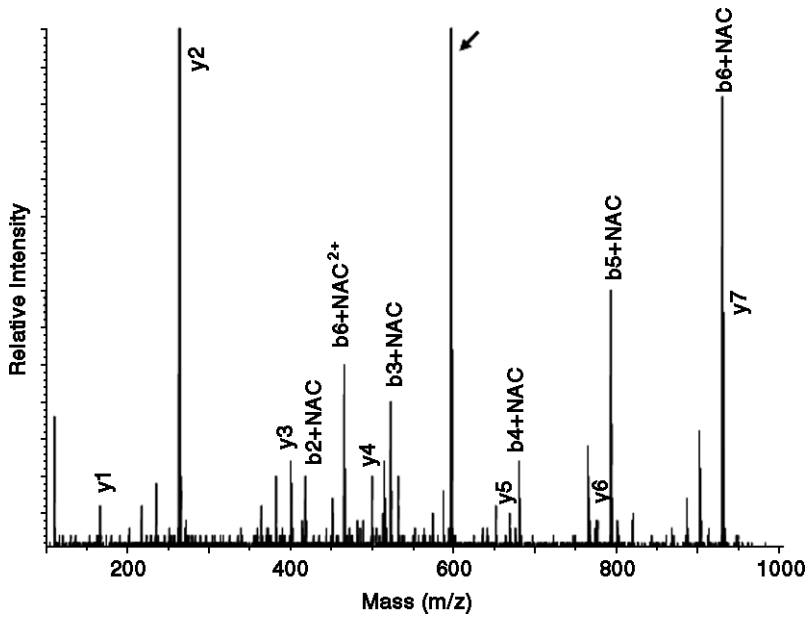
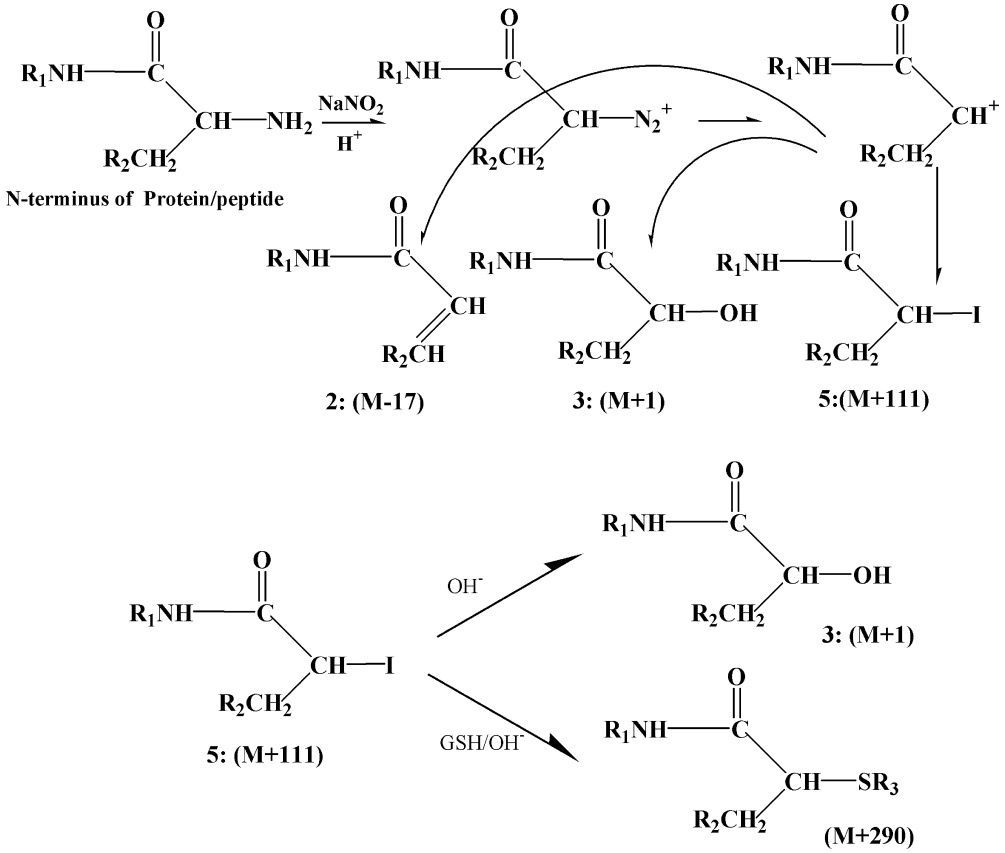


Figure 5 MS/MS of the conjugation product of angiotensin II and *N*-acetylcysteine (M + 146) observed at MH_2^{2+} 596.8. The b_2 to b_6 fragment ions are 146 amu higher than those from the unmodified peptide indicating that the amino group at the *N*-terminus was replaced by an *N*-acetylcysteine group.



Scheme 1 Reaction Process of nitrite-assisted Peptide *N*-terminal iodination and conjugation.

Nitrite-assisted iodination of peptide *N*-terminus in the acidic solution and the *N*-terminal-iodinated peptides alkylated thiol-containing molecules to form peptide conjugates. The reactive species generated by mixing NaI and $NaNO_2$ iodinated the tyrosine and histidine residues of peptides and proteins in basic solutions. The results demonstrate that nitrite is a useful catalyst for peptide and protein iodination and conjugation.

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