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N-Terminal α-Ketoamide Peptides: Formation and Transamination

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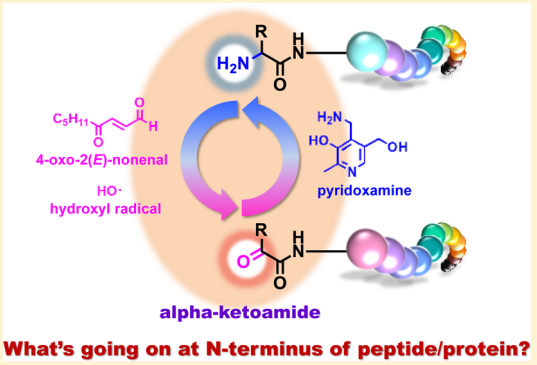
ABSTRACT: We have previously reported that N-terminal α-ketoamide peptides can be formed through 4-oxo-2(*E*)-nonenal (ONE)-derived oxidative decarboxylation of aspartic acid (Asp), which converts angiotensin (Ang) II (DRVYIHPF) to pyruvamide-Ang II (Ang P, $CH_3COCONH$-RVYIHPF). The pyruvamide group significantly inhibits Ang P binding to the Ang II type 1 receptor, which mediates the major biological effects of Ang II. In the present study, we found that ONE can also introduce an α-ketoamide moiety at the N-terminus of peptides containing N-terminal residues other than Asp. Subsequent investigation of alternative biosynthetic pathways for N-terminal α-ketoamide peptides revealed that hydroxyl radical-mediated formation is a much more efficient route. The proposed mechanism involves initial abstraction of the N-terminal α-hydrogen and hydrolysis of the ketimine intermediate. The resulting N-terminal α-ketoamide is then converted to the D- and L-amino acids by nonenzymatic transamination in the presence of pyridoxamine (PM). The formation of the epimeric N-terminus depended on the incubation time and the concentration of PM, and increased further upon the addition of Cu(II) ions. A conversion of approximately 60% after three days of incubation was observed for Ang P. We propose that the reaction intermediate contains a prochiral α-carbon and is stabilized by the chelate effect of Cu(II) ions. The ONE- and hydroxyl radical-derived formation of N-terminal α-ketoamide and its transamination in the presence of PM were also observed in amyloid β 1−11 (DAEFRHDSGYE), where the N-terminal Asp was converted to epimeric alanine. This suggests that these N-terminal modifications could occur in vivo and modulate the biological functions of peptides and proteins.



INTRODUCTION

Oxidative stress arises from an imbalance between the generation of reactive oxygen species (ROS) and the scavenging of ROS by antioxidant systems. Increased ROS production has been associated with a number of inflammatory and age-related degenerative diseases.[1,2] Highly reactive and unstable ROS react with bioactive macromolecules, including DNA, proteins, and lipids. The direct reaction of ROS with DNA forms modified DNA bases such as 7,8-dihydro-8-oxo-2′-deoxyguanosine, which has been used extensively as a biomarker of oxidative DNA damage.[3] Direct protein oxidation occurs frequently on the amino acid side chain methionine (Met) to form a sulfoxide and on cysteine (Cys) to form sulfenic, sulfinic, and sulfonic acids.[4] Oxidation of lysine (Lys), arginine (Arg), and proline (Pro) residues in the presence of metal ions and H_2O_2 causes the formation of semialdehyde amino acids and is known as direct protein carbonylation.[5] Polyunsaturated fatty acids in membrane phospholipids and other lipid-containing structures are the primary targets of ROS. The highly reactive aldehydes produced by ROS-mediated lipid peroxidation can be just as damaging as the initial ROS. 4-Oxo-2(*E*)-nonenal (ONE) and 4-hydroxy-2(*E*)-nonenal (HNE) have been identified as the most abundant, toxic, and reactive lipid-derived aldehydes.[6,7] Some of their toxic effects have been attributed to the ability to modify DNA bases and the consequent mutations.[8] The reaction between ONE and DNA bases forms exocyclic heptanone-etheno adducts.[9,10] They have been detected in cells that were subjected to oxidative stress and in the Min mouse model of colon carcinogenesis.[11−13] In contrast, HNE forms exocyclic propano adducts with 2′-deoxyguanosine, which have been identified in mammalian tissue DNA.[14] ONE and HNE are also involved in protein dysfunction and altered gene regulation through protein cross-linking and the modification of amino acid residues, respectively.[7] In ONE and HNE, C-3 is highly electron-deficient because of the α,β-unsaturated systems. As a consequence, the compounds readily undergo Michael addition with the sulfhydryl group of Cys, the imidazole nitrogen of histidine (His), and the ε-amino group of Lys.[7] In ONE, the aldehyde carbon (C-1) is another favorable reaction site for the ε-amino group of Lys, the guanidido group of Arg,[15] and the N-terminal α-amino group[16] through the formation of a Schiff base adduct. On the basis of the studies using model amino acids, extensive research has focused on ONE- and HNE-derived modifications to peptides and proteins.[7,17] As a result, a number of critical modification sites that lead to alterations of protein function and enzyme activity have been identified, such as His^{93} and His^{64} in myoglobin,[18] Cys^{120} in epithelial fatty acid-binding protein,[19] and Lys^{182} in glucose-6-phosphate dehydrogenase.[20]

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The N-terminus of peptides and proteins is also an important area for cotranslational and post-translational modifications (PTMs), which can influence the stability, localization, and activity of peptides and proteins.[21] However, in contrast to internal amino acid residues (side chains), few studies have reported lipid-derived modifications to N-terminal α-amino groups and consequent functional changes.[16,22] This may be because most modification studies have focused on HNE, which forms reversible Michael addition products with N-terminal α-amino groups. Therefore, the products can be detected only if reduction with sodium borohydride is performed prior to proteolysis.[7] Another possible reason is that the N-terminus of certain peptides and proteins can undergo cotranslational modifications, such as N-acetylation and N-myristoylation.[21] Although these modifications are incomplete or partial, they can reduce the chances of PTM by lipid-derived aldehydes. In addition, the hydrophobic properties of the N-terminus make liquid chromatography (LC) and mass spectrometry (MS) analysis after proteolysis difficult. In previous work,[16] we discovered that ONE mediates not only adduct formation but also oxidative decarboxylation of N-terminal aspartic acid (Asp) on angiotensin (Ang) II. This reaction introduces a pyruvamide (α-ketoamide) moiety at the N-terminus to form pyruvamide-Ang II (Ang P). Ang P has much lower affinity than Ang II for Ang II type 1 (AT_1) receptors, indicating that the N-terminal pyruvamide group significantly inhibits the binding of Ang P to the AT_1 receptor.[23] Recently, we also reported that epimerization of the N-terminal amino acid can occur in remarkably high yield through reactions with endogenous aldehydes, pyridoxal-5′-phosphate (PLP) and ONE.[24]

As a continuation of our study of N-terminal modifications, we demonstrate the ONE- and hydroxyl radical-mediated formation of N-terminal α-ketoamide and its transamination in the presence of pyridoxamine (PM). Bioactive Ang peptides (Ang II, DRVYIHPF; Ang A, ARVYIHPF; Ang III, RVYIHPF; Ang IV, VYIHPF; and Ang P, $CH_3COCONH$-RVYIHPF) were used as model peptides under physiologically relevant reaction conditions and concentration ratios. LC/electrospray ionization (ESI)-MS, tandem mass spectrometry (MS/MS), and selected reaction monitoring (SRM) were performed to identify, confirm, and quantify peptides formed in each reaction. We have proposed plausible mechanisms for the formation of N-terminal α-ketoamide-Ang peptides and the transamination of Ang P based on our results. Finally, these N-terminal modifications were applied to another biologically important peptide, amyloid β 1−11 ($A\beta_{1-11}$, DAEFRHDSGYE).

MATERIALS AND METHODS

Materials. Human Ang II, Ang III, Ang IV, and $A\beta_{1-11}$ were obtained from Peptide Institute, Inc. (Osaka, Japan). D-Ang A (D-Ala^1-L-Arg^2-L-Val^3-L-Tyr^4-L-Ile^5-L-His^6-L-Pro^7-L-Phe^8), L-Ang A (L-Ala^1-L-Arg^2-L-Val^3-L-Tyr^4-L-Ile^5-L-His^6-L-Pro^7-L-Phe^8), and Ang P were synthesized and supplied by Toray Research Center, Inc. (Tokyo, Japan). [$^{13}C_5$,$^{15}N_1$-Pro^7]-Ang II was obtained from Scrum, Inc. (Tokyo, Japan). ONE was purchased from Cayman Chemical Co. (Ann Arbor, MI). Copper(II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$), zinc(II) sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$), iron(II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), iron(III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), and ammonium acetate were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). L-Ascorbic acid (AA) and sodium borohydride were purchased from Sigma-Aldrich Inc. (St. Louis, MO). PM dihydrochloride was obtained from Fluka Analytical (Buchs, Switzerland). Formic acid and L-tyrosine (Tyr) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Chelex-100 chelating ion-exchange resin (100−200 mesh size) was purchased from Bio-Rad Laboratories (Hercules, CA). LC grade acetonitrile was obtained from Kanto Chemical Co. Inc. (Tokyo, Japan). Ultrapure water was obtained from a Milli-Q Integral 10 (EMD Millipore, Billerica, MA) equipped with a 0.22 μm membrane cartridge. All peptides, transition metal ions, and AA were dissolved in 50 mM Chelex-treated phosphate buffer (PB), and the final concentrations used in each experiment are described.

Purity Measurement of Synthetic Ang Peptides. Purities of synthetic Ang peptides were measured by Ultraspec 3000 (Cambridge, UK) based on the molar absorptivity of Tyr (λ_{max} = 274.6 nm, ε = 1748 M^{-1} cm^{-1} at pH 3.5, and ε = 1405 M^{-1} cm^{-1} at pH 7.0). Estimated purities of Ang P, D-Ang A, and L-Ang A were 80%, 70%, and 85%, respectively. Accordingly, their concentrations were adjusted for further reaction and quantitation.

Liquid Chromatography. Chromatography for LC system 1 was carried out using a Gulliver LC system (JASCO Co. Ltd., Tokyo, Japan) equipped with a PU-980 pump, an LG-980-02 gradient unit, a DG-980-50 degasser, and a CO-965 column oven. Solvent A was water containing 0.1% formic acid, and solvent B was acetonitrile containing 0.1% formic acid. The linear gradient was as follows: 2% B at 0 min, 50% B at 45 min, 80% B at 46 min, 80% B at 55 min, and 2% B at 56 min. Separations were performed at 40 °C. Chromatography for LC systems 2 to 4 was carried out on an Agilent 1100 LC system (Agilent Technologies, Inc., Santa Clara, CA) equipped with a G1312A bin pump, G1329A ALS autosampler, and G1379A degasser at ambient temperature. For LC system 2, solvent A was water/acetonitrile (98:2, v/v) containing 0.2% formic acid, and solvent B was acetonitrile/water (98:2, v/v) containing 0.2% formic acid. The linear gradient was as follows: 10% B at 0 min, 70% B at 30 min, 95% B at 31 min, 95% B at 34 min, 10% B at 35 min, 10% B at 50 min. For LC system 3, solvent A was water/acetonitrile (98:2, v/v) containing 0.1% formic acid, and solvent B was acetonitrile/water (70:30, v/v) containing 0.1% formic acid. The linear gradient was as follows: 19% B at 0 min, 28% B at 41 min, 90% B at 42 min, 90% B at 45 min, 19% B at 46 min, and 19% B at 60 min. For LC system 4, solvent A was 5 mM ammonium acetate in water, and solvent B was 5 mM ammonium acetate in acetonitrile/water (98:2, v/v). The linear gradient was as follows: 1% B at 0 min, 18% B at 30 min, 95% B at 31 min, 95% B at 34 min, 1% B at 35 min, and 1% B at 50 min. α-Ketoamide-Ang peptides (Ang P, Ang P_{III}, and Ang P_{IV}) and $A\beta_{1-11}$-P were isolated using LC systems 2 and 4, respectively, with a Nanospace SI-1 semimicrocolumn LC system (Shiseido Co. Ltd., Tokyo, Japan) equipped with a UV detector. All LC systems used a Jupiter C18 column (150 × 2.0 mm i.d., 5 μm, 300 Å; Phenomenex, Torrance, CA) with a flow rate of 0.2 mL/min.

Mass Spectrometry. An LCQ Deca XP ion trap mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA) equipped with an ESI source was used in positive ion mode with LC system 1 for LC/ESI-MS and MS/MS analyses of the reaction between ONE and Ang peptides. The operating conditions were as follows: heated capillary, 200 °C; source voltage, 4500 V; capillary voltage, 36.0 V; and tube lens offset, 30.0 V. Nitrogen was used as the sheath gas (80 L/min) and auxiliary gas (20 L/min). Full scanning analyses were performed in the range of m/z 300−1800. Helium was the collision gas for CID experiments coupled with MS/MS. The relative collision energy was set at 40% of the maximum (1 V). For LC systems 2 to 4, a TSQ-7000 triple quadrupole mass spectrometer (Thermo Fisher Scientific Inc.) equipped with an ESI source was used in positive ion mode. The operating conditions were as follows: the heated capillary temperature was 330 °C, and the nitrogen sheath gas and auxiliary gas pressures were 90 psi and 30 (arbitrary units), respectively. Full scanning analyses were performed in the range of m/z 300−1500. Argon was used as the collision gas in CID experiments coupled with MS/MS at 2.5−2.7 mTorr in the second (rf-only) quadrupole. Unit resolution was maintained for full scanning and MS/MS analyses. SRM analyses were conducted with LC system 3 to quantify D- and L-Ang A, and Ang III from the incubation of Ang P or Ang II with PM in the absence or presence of transition metal ions. The SRM transitions used were as follows: Ang III, m/z 466.4 → 263.0; D- and L-Ang A, m/z 501.7 → 263.0; Ang P, m/z 501.3 → 263.0; Ang II, m/z 523.9 → 263.0; and [$^{13}C_5$, $^{15}N_1$-Pro^7]-Ang II, m/z 526.8 → 268.9. A collision energy of 26 V was used for all SRM transitions.

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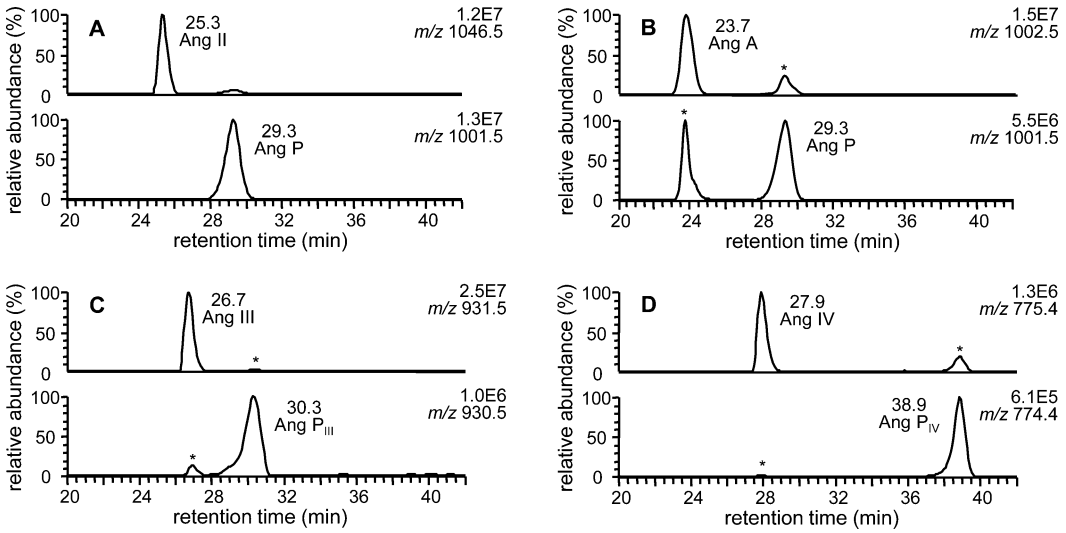

Figure 1. LC/ESI-MS analyses of the reaction of ONE (3.0 mM) with (A) Ang II (1.0 mM), (B) Ang A (1.0 mM), (C) Ang III (1.0 mM), and (D) Ang IV (1.0 mM) at 37 °C for 24 h. * indicates a cross-talk peak from a close mass ion.

Reaction of Ang Peptides with ONE. A solution of ONE (3.0 mM) in 20 μL of methylacetate/ethanol (1:1, v/v) was added to each of the Ang peptides (Ang II, Ang A, Ang III, and Ang IV; 1.0 mM) in 50 mM Chelex-treated PB (180 μL). The reaction mixture was incubated for 24 h at 37 °C. A portion of the sample (10 μL) was analyzed by LC/ESI-MS using LC system 1.

Sodium Borohydride Reduction of α-Ketoamide-Ang Peptides. ONE- or Cu(II)/AA-derived α-ketoamide-Ang peptides (Ang P, Ang P_{III}, and Ang P_{IV}) were purified using LC system 2, and $A\beta_{1-11}$-P was purified using system 4. Sodium borohydride reduction of each α-ketoamide peptide was carried out as described previously.[16]

Time Course of the Reaction of Ang Peptides with ONE. A solution of ONE (300 μM) in 20 μL of methylacetate/ethanol (1:1, v/v) was added to each of the Ang peptides (Ang II, Ang A, Ang III, and Ang IV: 100 μM) in 50 mM Chelex-treated PB (280 μL). The reaction mixture was incubated for 27 h at 37 °C. At each time point (0, 1, 2, 4, 6, and 27 h), a portion of the sample was removed, diluted with water (1:1, v/v), and analyzed (10 μL) by LC/ESI-MS using LC system 2.

Reaction of Ang Peptides with Cu(II)/AA. A solution of $CuSO_4 \cdot 5H_2O$ (10 μM) was added to a solution of each Ang peptide (Ang II, Ang A, Ang III, and Ang IV; 100 μM). The reaction was initiated by adding 100 μM AA, and the mixture was incubated for 24 h at 37 °C. A portion of the sample (10 μL) was analyzed by LC/ESI-MS using LC system 2.

Reaction of Ang II with Zn(II)/AA, Fe(II)/AA, and Fe(III)/AA. A solution of each transition metal ion ($ZnSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$, and $FeCl_3 \cdot 6H_2O$: 10 μM) was added to a solution of Ang II (100 μM). The reaction was initiated by adding 100 μM AA, and the mixture was incubated for 24 h at 37 °C. A portion of the sample (10 μL) was analyzed by LC/ESI-MS using LC system 2.

Reaction of Ang P or Ang II with PM. Ang P or Ang II (10 μM) was reacted with PM (0, 50, 500, or 5000 μM) for 7 days. Aliquots of the reaction mixture were periodically withdrawn and stored at −20 °C until use. The aliquots were diluted to 50% with a mixture of 50 mM Chelex-treated PB and acetonitrile (4:1, v/v) containing an internal standard (IS), [$^{13}C_5$, $^{15}N_1$-Pro^7]-Ang II (30 μM) prior to LC/ESI-MS analysis (10 μL) using LC system 3. All samples were analyzed within 2 days.

Reaction of Ang P with PM in the Presence of Transition Metal Ions. Ang P (10 μM) was reacted with PM (5000 μM) in the presence of various transition metal ions (Cu(II), Zn(II), Fe(II), Fe(III); 0, 1, 10 μM) for 3 days. Aliquots of the reaction mixture were periodically withdrawn and stored at −20 °C until use. The aliquots were diluted to 50% with a mixture of 50 mM Chelex-treated PB and acetonitrile (4:1, v/v) containing an IS ([$^{13}C_5$, $^{15}N_1$-Pro^7]-Ang II, 30 μM) prior to LC/ESI-MS analysis (10 μL) using LC system 3. All samples were analyzed within 2 days.

Quantitation of D- and L-Ang A, and Ang III from the Reaction of Ang P with PM. To quantify the amount of D- and L-Ang A, and Ang III produced from the reaction of Ang P with PM in the absence or presence of transition metal ions, 50 mM Chelex-treated PB was spiked with authentic D- and L-Ang A (0.25−5.0 μM), and Ang III (0.15−3.0 μM). Each standard solution was diluted to 50% with a mixture of 50 mM Chelex-treated PB and acetonitrile (4:1, v/v) containing an IS ([$^{13}C_5$, $^{15}N_1$-Pro^7]-Ang II, 30 μM) prior to LC/ESI-SRM MS analysis (10 μL) using LC system 3. Calibration curves were obtained by linear regression analysis of the peak area ratios of the analytes against the IS. Concentrations of D- and L-Ang A, and Ang III in the reaction mixture at each time point were calculated by interpolation from the regression lines.

Preparation of Standard Solutions and Quality Control (QC) Samples. Standard solutions and QC samples were prepared as follows. Standard solutions containing the highest concentration of Ang peptides (3 μM for Ang III and 5 μM for D- and L-Ang A) were prepared. The standards were diluted sequentially with 50 mM Chelex-treated PB. QC samples were prepared separately from the highest concentration standard solutions.

Validation. Precision and accuracy studies were performed (n = 3) on the lower (LQC, 0.5 μM for D- and L-Ang A, and 0.3 μM for Ang III), medium (MQC, 2.0 μM for D- and L-Ang A, and 1.2 μM for Ang III), and higher (HQC, 5.0 μM for D- and L-Ang A, and 3.0 μM for Ang III) concentration QC samples on three separate days. Assay accuracy was assessed by comparing the mean of the study samples with the theoretical concentration of the QC samples and expressed as a percentage. Intraday precision expressed as the coefficient of variance (% CV), and accuracy (%) of the three replicates were assessed. Interday precision (% CV) and accuracy (%) on the validation runs conducted on three separate days were also assessed.

Reaction of Lower Concentrations of Ang P with PM and Cu(II) Ions. Ang P (1 or 0.1 μM) was incubated with PM (500 or 50 μM) in the presence of Cu(II) ions (0, 1, or 0.1 μM). Aliquots of the reaction mixture were withdrawn after 14 days of incubation and analyzed by LC/ESI-SRM MS using LC system 3 as described above.

Reaction of $A\beta_{1-11}$ with ONE or Cu(II)/AA. $A\beta_{1-11}$ (100 μM) was reacted with ONE (300 μM) or $CuSO_4 \cdot 5H_2O$ (10 μM)/AA (100 μM) as described above for Ang peptides. The reaction mixture was incubated for 24 h at 37 °C, and a portion of the sample (10 μL) was analyzed by LC/ESI-MS and MS/MS using LC system 4 after dilution (1:1, v/v) with acetonitrile.

Reaction of $A\beta_{1-11}$-P with PM. $A\beta_{1-11}$-P was isolated from the incubation of $A\beta_{1-11}$ in the presence of Cu(II)/AA by LC-UV (280 nm) using LC system 4 and reacted with PM (0, 50, or 500 μM) for 7 days.

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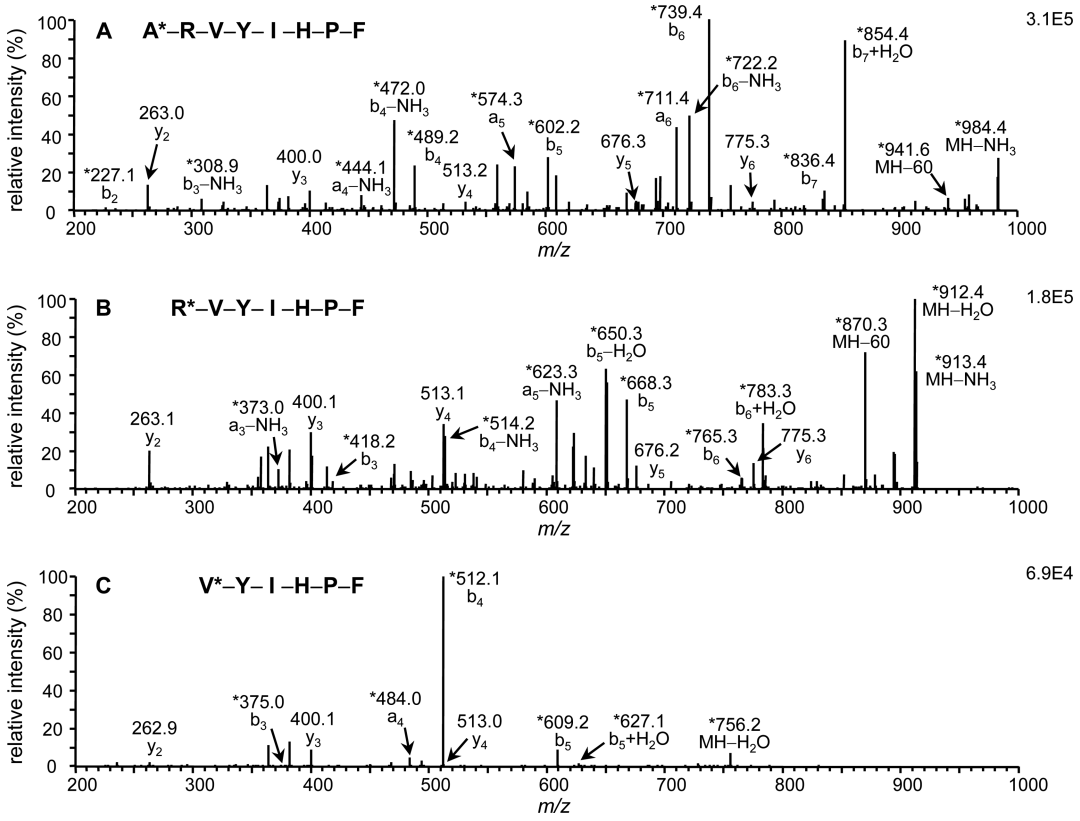

Figure 2. LC/ESI-MS/MS analyses of α-ketoamide-Ang peptides formed from the reactions of ONE (3.0 mM) with (A) Ang A (1.0 mM), (B) Ang III (1.0 mM), and (C) Ang IV (1.0 mM) at 37 °C for 24 h. * indicates a modified ion (−1 Da). The m/z value of each peak indicates the monoisotopic mass observed.

Aliquots of the reaction mixture were diluted with acetonitrile (1:1, v/v) and then analyzed by LC/ESI-MS and MS/MS using LC system 3.

RESULTS

LC/ESI-MS Analyses of Reactions between ONE and Ang Peptides. The reactions were carried out in millimolar scale for the better characterization of modifications to Ang peptides. LC/ESI-MS analysis of the reaction between ONE and Ang II at 37 °C for 24 h (Figure 1A) revealed the presence of Ang P at a retention time (t_R) of 29.3 min, residual Ang II (t_R = 25.3 min), and other products. The ESI mass spectrum of Ang P showed an expected $[M + H]^+$ ion at m/z 1001.5, corresponding to a loss of 45 Da from Ang II ($[M + H]^+ = m/z$ 1046.5). The formation of Ang P was also observed as a major product of the reaction between ONE and Ang A at 37 °C for 24 h (Figure 1B). The retention time and mass spectrum were identical to those of Ang P formed in the Ang II reaction. Thus, the $[M + H]^+$ ion corresponded to a loss of 1 Da from Ang A ($[M + H]^+ = m/z$ 1002.5). LC/ESI-MS analysis of the reaction between ONE and Ang III at 37 °C for 24 h revealed the presence of Ang P_{III} (t_R = 30.3 min) (Figure 1C). The LC/ESI-MS spectrum of Ang P_{III} contained an $[M + H]^+$ ion at m/z 930.5 corresponding to a loss of 1 Da from Ang III ($[M + H]^+ = m/z$ 931.5). Similarly, Ang P_{IV} (t_R = 38.9 min) was formed by the reaction between ONE and Ang IV (Figure 1D). The LC/ESI-MS spectrum of Ang P_{IV} showed an $[M + H]^+$ ion at m/z 774.4 corresponding to a loss of 1 Da from Ang IV ($[M + H]^+ = m/z$ 775.4).

LC/ESI-MS/MS Analyses of ONE-Derived α-Ketoamide-Ang Peptides. Ang P (t_R = 29.3 min, $[M + H]^+ = m/z$ 1001.5) formed from the reaction of ONE and Ang II has been characterized in a previous study.[16] Its product ion spectrum showed that all of the a- and b-ions (a_3 to a_7 and b_2 to b_7) lost 45 Da compared with Ang II and all of the y-ions (y_2 to y_7) were unmodified. Upon reaction with sodium borohydride, Ang P gained two hydrogen atoms on the N-terminal pyruvamide (α-ketoamide) ketone.[16] The product ion spectra of ONE/Ang A-derived Ang P before (Figure 2A) and after sodium borohydride reduction were identical to those of ONE/Ang II-derived Ang P. The MS/MS analysis of Ang P_{III} (t_R = 30.3 min) at m/z 930.5 ($[M + H]^+$) revealed that a modification occurred at the N-terminus of Ang III (Figure 2B). Thus, all of the a- and b-ions that were detected (a_3 to a_5 and b_3 to b_6) had lost 1 Da compared with the corresponding a- and b-ions of Ang III. In contrast, all of the y-ions (y_2 to y_6) remained unmodified. The product ion spectrum of m/z 774.4, the $[M + H]^+$ ion of Ang P_{IV} (t_R = 38.9 min), was also consistent with the modification at the N-terminus of Ang IV (Figure 2C). In the spectrum, all of the a- and b-ions (a_4 and b_3 to b_5) lost 1 Da from Ang IV and all of the y-ions (y_2 to y_4) were unmodified. Ang P_{III} and Ang P_{IV} were then subjected to the sodium borohydride reduction. LC/ESI-MS analyses of reduced Ang P_{III} and Ang P_{IV} revealed $[M + H]^+$ ions at m/z 932.5 and m/z 776.4, respectively, which corresponded to the addition of two hydrogen atoms. Subsequent MS/MS analyses confirmed that the hydrogen atoms were added to the N-terminal α-ketoamide moieties of Ang P_{III} and Ang P_{IV}.

ONE-Mediated Formation of α-Ketoamide-Ang Peptides. For the time course experiments, reactions were conducted for 27 h with more physiologically relevant concentrations (Ang peptides, 100 μM; ONE, 300 μM) that produce detectable levels of all α-ketoamide Ang peptides especially at early time points. The formation of each α-ketoamide-Ang peptide was plotted (Figure 3A) using the MS

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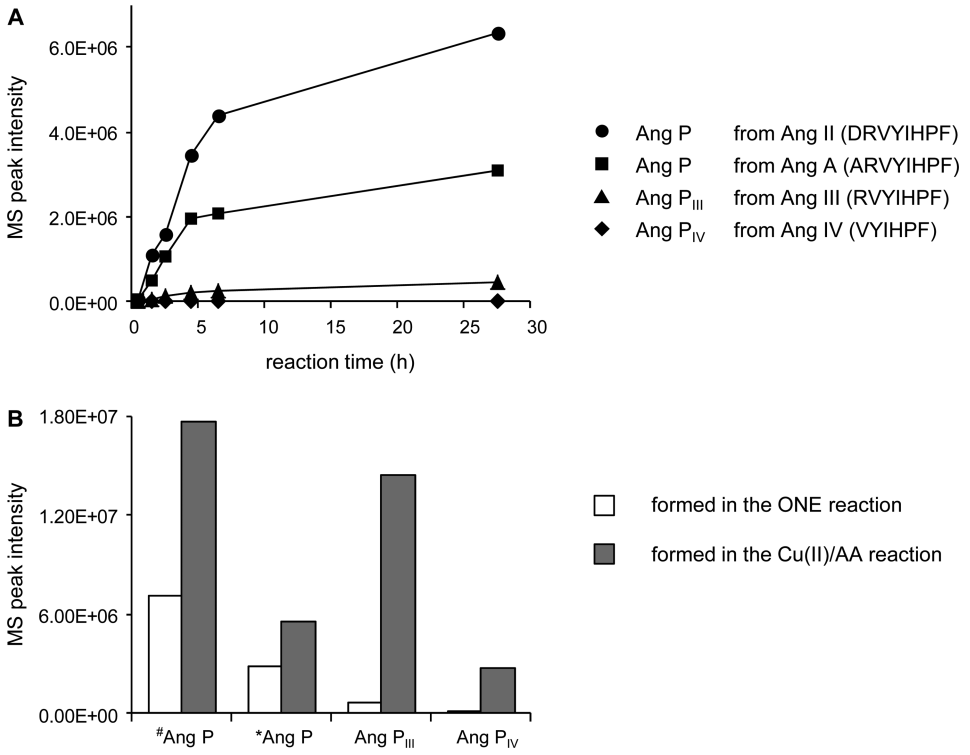

Figure 3. (A) Formation of α-ketoamide-Ang peptides from the reaction of ONE (300 μM) with Ang II (100 μM, closed circles), Ang A (100 μM, closed squares), Ang III (100 μM, closed triangles), and Ang IV (100 μM, closed diamonds) at 37 °C for 27 h. (B) Formation of α-ketoamide-Ang peptides from the reaction with ONE (300 μM, white bars) and with Cu(II)/AA (10/100 μM, gray bars) at 37 °C for 24 h. # indicates Ang P formed from Ang II, and * indicates Ang P formed from Ang A.

Table 1. Typical Calibration Curve Regressions, Accuracy, and Precision of QC Samples Used to Quantify D- and L-Ang A, and Ang III

	D-Ang A			L-Ang A			Ang III		
typical regression	$y = 0.162x - 0.011$, $r^2 = 0.995$			$y = 0.197x - 0.017$, $r^2 = 0.997$			$y = 0.435x - 0.017$, $r^2 = 0.995$		
QC (μM)	LQC (0.50)	MQC (2.00)	HQC (5.00)	LQC (0.50)	MQC (2.00)	HQC (5.00)	LQC (0.30)	MQC (1.20)	HQC (3.00)
intraday mean (μM)	0.54	2.00	5.20	0.56	1.96	5.18	0.32	1.20	3.13
% CV (n = 3)	3.3	7.0	1.9	8.6	3.4	4.4	4.6	8.8	2.8
accuracy (%)	109.5	98.5	103.9	112.4	98.2	103.6	109.2	99.6	104.5
interday mean (μM)	0.49	1.91	5.43	0.52	1.89	5.40	0.29	1.15	3.29
% CV (n = 9)	13.7	10.1	7.5	15.5	8.6	5.6	15.7	10.5	7.7
accuracy (%)	99.6	95.6	108.6	104.0	94.7	108.0	98.3	96.6	109.9

peak intensity from the extracted ion chromatogram of the most intense ion ($[M + H]^+$ or $[M + 2H]^{2+}$). In the reaction of ONE with Ang II, the formation of Ang P was observed from the earliest time point (1 h) and increased during the incubation. After 27 h, Ang P reached a maximum level of 8.8 μM, which was determined by the standard curve constructed by using authentic Ang P. The levels of Ang P at each time point in the reaction between ONE and Ang A were about 50% greater than those in the Ang II reaction. Therefore, the Ang P level after 27 h was calculated to be 4.3 μM. Similar patterns were observed for the formation of Ang P_{III}, Ang P_{IV}, and Ang P, although the levels of Ang P_{III} and Ang P_{IV} were much lower than that of Ang P when estimated by their UV responses at 274.6 nm (λ_{max} of Tyr).

Hydroxyl Radical-Mediated Formation of α-Ketoamide-Ang Peptides. LC/ESI-MS analyses for the reaction of Ang peptides with Cu(II)/AA at 37 °C for 24 h revealed the presence of α-ketoamide-Ang peptides, such as Ang P from Ang II and Ang A, Ang P_{III} from Ang III, and Ang P_{IV} from Ang IV. The identity of each α-ketoamide-Ang peptide was confirmed by LC/ESI-MS/MS analysis before and after the reduction with sodium borohydride. Levels of α-ketoamide-Ang peptides were then compared with those for the reaction with ONE at 37 °C for 24 h (Figure 3B). The Cu(II)/AA reaction (Figure 3B, gray bars) produced more of all the α-ketoamide-Ang peptides than the reaction with ONE (Figure 3B, white bars): approximately 2-fold more Ang P from Ang II and Ang A, and 20- to 50-fold more Ang P_{III} and Ang P_{IV}. As for the UV response (274.6 nm), Ang P formed from Ang II was the most intense, followed by Ang P_{III}, Ang P from Ang A, and Ang P_{IV}. The formation of Ang P was also observed in the reaction of Ang II with other transition metal ions (Zn(II), Fe(II), and Fe(III)) in the presence of AA. However, the levels of Ang P were 30- to 40-fold lower than that produced in Cu(II)/AA reaction.

LC/ESI-MS Analysis of the Reaction between Ang P and PM. LC/ESI-MS analysis using LC system 2 of the reaction between Ang P and PM at 37 °C at various time points revealed the presence of D- and L-Ang A (t_R = 27.9 and 29.3 min, respectively), Ang III (t_R = 26.9 min), and residual Ang P (t_R =

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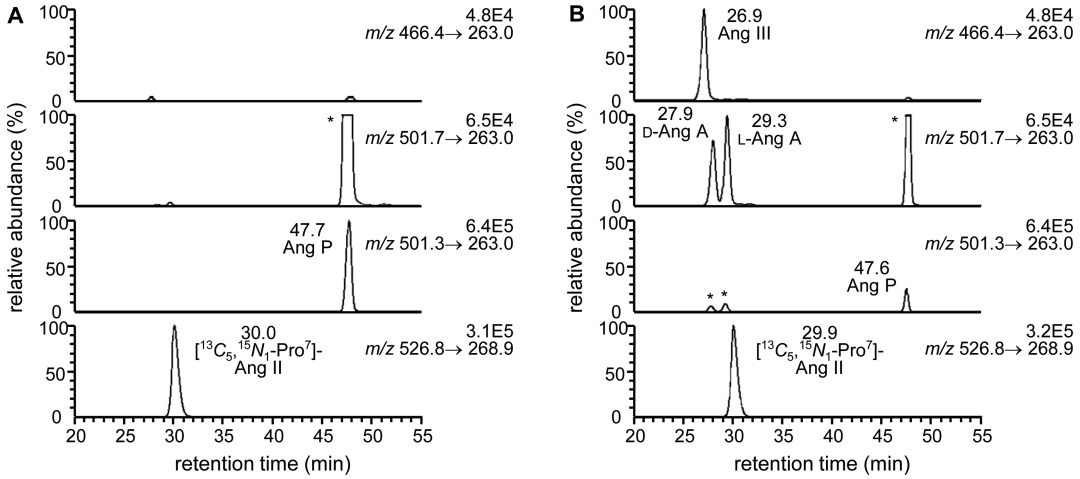

Figure 4. LC/ESI-SRM MS analysis of the reaction between Ang P (10 μM) and PM (5000 μM) for (A) 0 days and (B) 7 days. * indicates a cross-talk peak from a close mass precursor ion.

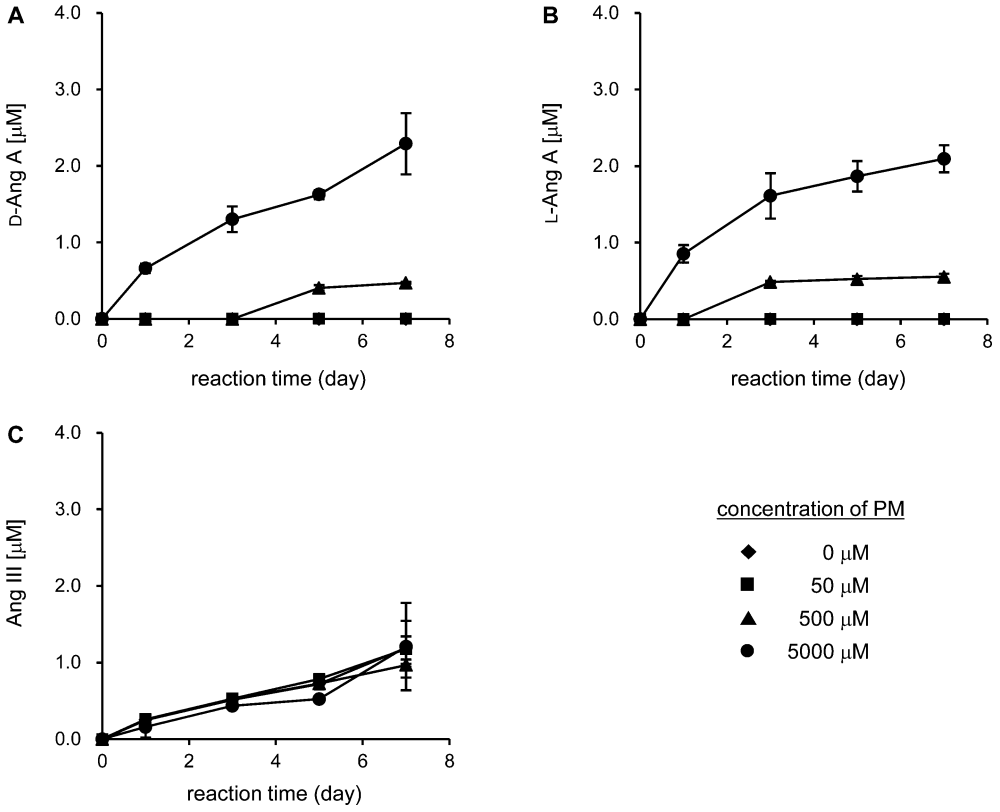

Figure 5. Formation of (A) D-Ang A, (B) L-Ang A, and (C) Ang III from the reaction between Ang P (10 μM) and PM (0 μM, closed diamonds; 50 μM, closed squares; 500 μM, closed triangles; 5000 μM, closed circles).

47.6 min). Identities of D- and L-Ang A and Ang III were confirmed by comparison against authentic standards. LC/ESI-MS spectra of D- and L-Ang A showed an identical $[M + H]^+$ ion at m/z 1002.5 ($[M + 2H]^{2+} = m/z$ 501.7), which corresponded to an increase in mass of 1 Da ($-$ O + NH_3) from Ang P. The LC/ESI-MS spectrum of Ang III contained an $[M + H]^+$ ion at m/z 931.5 ($[M + 2H]^{2+} = m/z$ 466.4).

Validation of the LC/ESI-SRM MS Method for D- and L-Ang A, and Ang III. Collision-induced dissociation (CID) and MS/MS analysis were performed on each $[M + 2H]^{2+}$ ion for D- and L-Ang A, and Ang III. The most intense product ion in their product ion spectra was at m/z 263.0, corresponding to the y_2 ions. Therefore, the transitions of the $[M + 2H]^{2+} \rightarrow y_2$ ion were selected for each Ang peptide for the LC/ESI-SRM MS analysis. [$^{13}C_5$,$^{15}N_1$-Pro^7]-Ang II was employed as an IS, and its optimal SRM transition was m/z 526.8 → 268.9 ($[M + 2H]^{2+} \rightarrow y_2$ ion). The calibration standards were analyzed in the range of 0.25−5.0 μM for D- and L-Ang A, and 0.15−3.0 μM for Ang III. Typical regressions for each calibration curve are listed in Table 1. The LC/ESI-SRM MS method was then validated by using replicate QC samples on three separate days (Table 1). Intraday (n = 3) precision (% CV) ranged from 1.9% to 8.8%. Deviation from the nominal concentration (accuracy) ranged from 98.2% to 112.4%.

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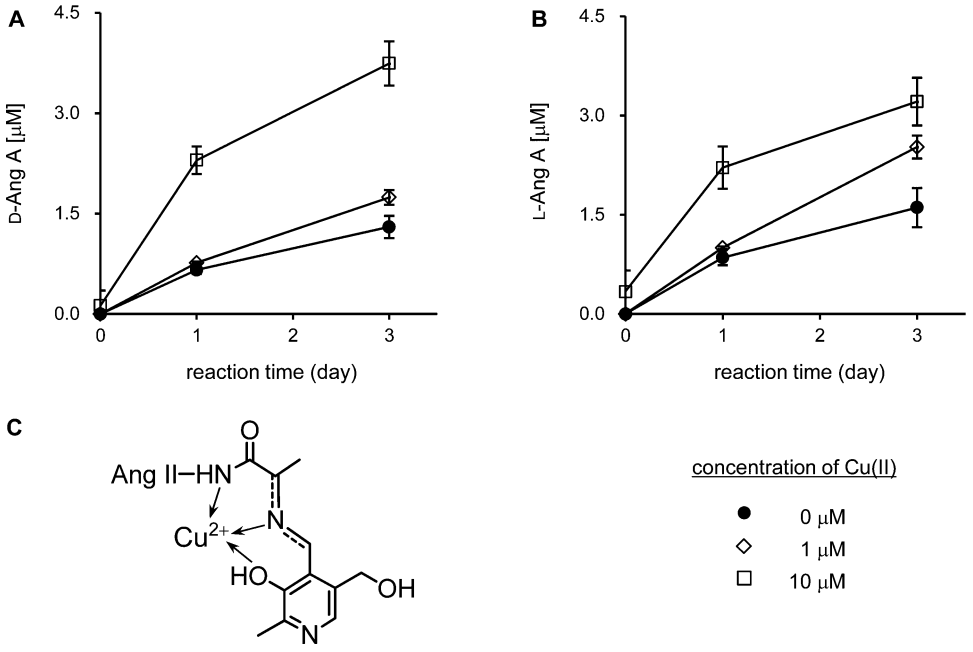

Figure 6. Formation of (A) D-Ang A, and (B) L-Ang A from the reaction between Ang P (10 μM) and PM (5000 μM) in the presence of Cu(II) ions (0 μM, closed circles; 1 μM, open diamonds; and 10 μM, open squares). (C) Proposed intermediate stabilized by the chelate effect of Cu(II) ion.

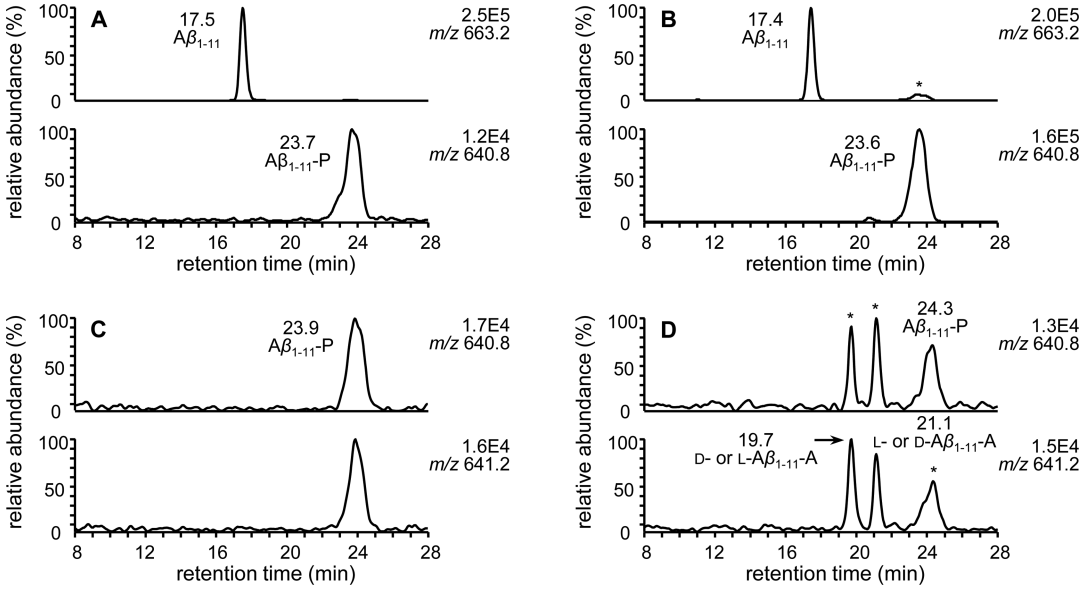

Figure 7. LC/ESI-MS analyses of the reactions of (A) Aβ_{1-11} (100 μM) with ONE (300 μM) at 37 °C for 24 h, (B) Aβ_{1-11} (100 μM) with Cu(II)/AA (10/100 μM) at 37 °C for 24 h, (C) Aβ_{1-11}-P with PM (0 μM) for 7 days, and (D) Aβ_{1-11}-P with PM (500 μM) for 7 days. * indicates a cross-talk peak from a close mass ion.

Interday (n = 9) precision (% CV) and accuracy ranged from 5.6% to 15.7% and from 94.7% to 109.9%, respectively.

Formation of D- and L-Ang A and Ang III from the Reaction of Ang P with PM. Levels of D- and L-Ang A, and Ang III formed from the reaction of Ang P (10 μM) with PM (0, 50, 500, and 5000 μM) were quantified using the validated LC/ESI-SRM MS method described above. Figure 4 shows representative chromatograms of the reaction with 5000 μM PM after 0 days (A) and 7 days (B). Results are presented as the mean ± standard error of the mean (SEM) from duplicate samples (Figure 5). In the absence of PM, D-Ang A was not formed when Ang P was incubated for 7 days (Figure 5A, closed diamonds). After 5 days of incubation with 50 μM PM, the peak for D-Ang A was detected, but it was below the quantitation limit of our method (closed squares). A quantifiable amount of D-Ang A (0.41 μM) was produced after 5 days of incubation with 500 μM PM (closed triangles). When Ang P was incubated with 5000 μM PM, 0, 0.66, 1.30, 1.63, and 2.29 μM D-Ang A was formed after 0, 1, 3, 5, and 7 days, respectively (closed circles). Patterns for the formation of L-Ang A (Figure 5B) were similar to those for the formation of D-Ang A. The levels of L-Ang A were almost identical to those of D-Ang A at all of the time points (Figure 4B, second panel from the top). Thus, the levels of L-Ang A produced from the reaction of Ang P with 5000 μM PM were 0, 0.85, 1.61, 1.87, and 2.09 μM after 0, 1, 3, 5, and 7 days, respectively (Figure 5B, closed circles). Ang III was also formed (Figure 4B, the top panel). In contrast to

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D- and L-Ang A, Ang III was formed irrespective of the presence or the concentration of PM, and its concentration increased in a time-dependent manner (Figure 5C). However, D- and L-Ang A, and Ang III were not formed in the reaction of Ang II (10 μM) with PM (0, 50, 500, and 5000 μM) for 7 days.

Increased Formation of D- and L-Ang A in the Presence of Cu(II) Ions. The reaction of Ang P (10 μM) with PM (5000 μM) in the presence of Cu(II) ions (1 μM) for 3 days produced slightly increased levels (1.2- to 1.6-fold) of D- and L-Ang A at each time point (Figure 6A and B, open diamonds) compared with the levels for the reaction without Cu(II) ions (closed circles). Levels of D- and L-Ang A were further increased to 2.0- to 3.5-fold from the basal level in the presence of 10 μM Cu(II) ions (Figure 6A and B, open squares). However, there were no significant changes in the D- and L-Ang A levels when other transition metal ions (Zn(II), Fe(II), and Fe(III); 0, 1, 10 μM) were added to the reaction.

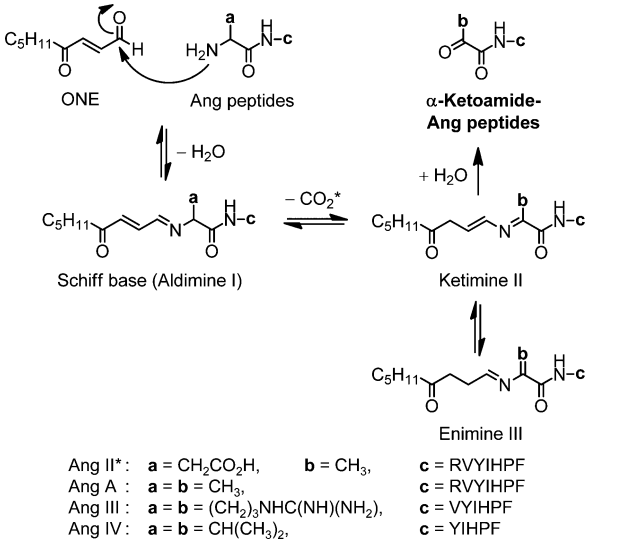
Formation of D- and L-Ang A from the Reaction of Lower Concentrations of Ang P with PM and Cu(II) ions. The formation and Cu(II) ion-dependent increase in levels of D- and L-Ang A were observed in the reaction with Ang P (1 μM) and PM (500 μM) in the absence and presence of Cu(II) ions (1 μM), respectively. After 14 days of incubation in the presence of Cu(II) ions, levels of D- and L-Ang A were 0.14 and 0.11 μM, respectively. When the reaction was performed with Ang P (0.1 μM), PM (50 μM), and Cu(II) ions (0.1 μM), peaks for D- and L-Ang A were also detected. However, they were below the quantitation limit of the MS method.

Formation and Transamination of Aβ_{1-11}-P. LC/ESI-MS analysis of the reaction between ONE and Aβ_{1-11} at 37 °C for 24 h showed Aβ_{1-11}-P at a retention time of 23.7 min and residual Aβ_{1-11} (t_R = 17.5 min) (Figure 7A). The LC/ESI-MS spectrum of Aβ_{1-11}-P contained an $[M + H]^+$ ion at m/z 1280.5 ($[M + 2H]^{2+} = m/z$ 640.8) corresponding to a loss of 45 Da from Aβ_{1-11} ($[M + H]^+ = m/z$ 1325.5, $[M + 2H]^{2+} = m/z$ 663.2). Upon reaction with sodium borohydride, Aβ_{1-11}-P gained two hydrogen atoms. MS/MS analyses of Aβ_{1-11}-P before and after the reduction confirmed that the modification occurred at the N-terminus of Aβ_{1-11}. Therefore, Aβ_{1-11}-P was identified as pyruvamide-Aβ_{1-11}. The formation of Aβ_{1-11}-P increased approximately 14-fold when Aβ_{1-11} was incubated in the presence of Cu(II)/AA (Figure 7B). Aβ_{1-11}-P was then isolated and incubated with PM (0, 50, 500 μM) for 7 days. LC/ESI-MS analysis of the reaction without PM showed that only Aβ_{1-11}-P was present (Figure 7C). After incubation with 50 μM PM, LC/ESI-MS analysis detected the formation of D (or L)-des[Asp^1]-[Ala^1]-Aβ_{1-11} (Aβ_{1-11}-A) and L (or D)-Aβ_{1-11}-A, eluting at 19.7 and 21.1 min, respectively. The MS spectra of the two peaks exhibited an identical $[M + H]^+$ ion at m/z 1281.5 ($[M + 2H]^{2+} = m/z$ 641.2), which corresponded to an increase in mass of 1 Da ($-$ O + NH_3) from Aβ_{1-11}-P. The amount of epimeric Aβ_{1-11}-A increased further when Aβ_{1-11}-P was incubated with 500 μM PM (Figure 7D).

DISCUSSION

PTMs play a vital role in modulating the activity of proteins. To elucidate various modification pathways, chemical transformations, including site-specific protein modification, have been used to create and manipulate protein PTMs.[25] The N-terminus of peptides and proteins is a major target of site-specific modifications because of its unique chemical properties. PLP-mediated transamination is a site-selective reaction that can introduce an α-ketoamide moiety to the N-terminus of peptides and proteins.[26] ONE, which is a major bifunctional electrophile derived from lipid hydroperoxides, also converts N-terminal Asp to pyruvate efficiently.[16] In the reaction of ONE with Ang II, Ang P was the main product, formed via oxidative decarboxylation of N-terminal Asp.[27] To test whether ONE can also introduce an α-ketoamide moiety to peptides containing N-terminal residues other than Asp, Ang peptides (Ang A, Ang III, and Ang IV) were reacted with ONE. LC-MS analyses showed that α-ketoamide-Ang peptides (Ang P, Ang P_{III}, and Ang P_{IV}) were formed and they were identified by LC-MS/MS analyses before and after sodium borohydride reduction. We propose a mechanism (Scheme 1) where a Schiff base (aldimine I) is formed through

Scheme 1. Proposed Mechanism for the Formation of α-Ketoamide-Ang Peptides from the Reaction between ONE and Ang Peptides[a]



[a]*, decarboxylation occurs in only the Ang II reaction.

the initial reaction of the α-amino group on the N-terminus of Ang peptides at C-1 of ONE. Intermediate aldimine I undergoes tautomerization to form ketimine II, which exists in equilibrium with enimine III. Ketimine II is subsequently hydrolyzed to yield the ketone moiety at the N-terminus of Ang peptides (α-ketoamide-Ang peptides). Upon sodium borohydride reduction, two hydrogen atoms were added to the N-terminal ketone group. Two main factors affecting the rate of ketimine hydrolysis are tautomerism between the ketimine and enimine, and steric hindrance.[28] Thus, the most unstable enimine is formed in the methyl side chain (Ang II and Ang A), and this allows rapid hydrolysis of ketimine to Ang P. In the Ang II reaction, decarboxylation can accelerate the production of the ketimine, which yields approximately double the amount of Ang P compared with the Ang A reaction (Figure 3A). The stability of the enimines in guanidino (Ang III) and isopropyl groups (Ang IV) should be similar. However, the two methyl groups near the ketimine carbon could hinder hydrolysis, explaining why Ang IV produced the smallest amounts of Ang P_{IV} (Figure 3A).

Zhu and Sayre[29] have demonstrated that 4-ketoamides are major products of the reaction between ONE and Lys residues. In the present study, N-terminal 4-ketoamide was also detected as one of the main products from the reactions of ONE with each Ang peptide (data not shown). In the reaction with Ang II or Ang A, α-ketoamides (Ang P) were more abundant than 4-

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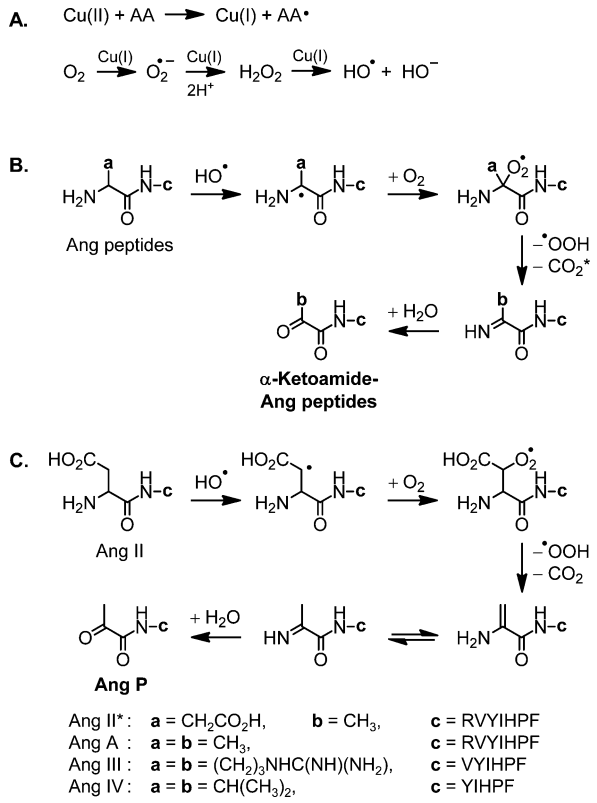
ketoamides of [N-ONE]-Ang II or [N-ONE]-Ang A as reported in our previous study.[27] In contrast, 4-ketoamide forms were more abundant than α-ketoamides in Ang III and Ang IV reactions where low levels of α-ketoamides were produced because of an unfavorable ketimine hydrolysis as described above.

Free radical-medicated oxidation of amino acid residues forms a large group of PTMs that can result in structural and functional changes in proteins. These protein oxidation-induced modifications include hydroxylation, nitration, sulfoxidation, carbonylation of certain amino acid residues, and cleavage of peptide bonds.[5,30] Studies of backbone cleavage pathways have proposed that protein peroxyl radicals can undergo cleavage by an α-amidation pathway to form amide and α-ketoacyl fragments.[30,31] However, radicals form predominantly on side chains in larger peptides and proteins because of the steric hindrance of the main chain α-carbon.[31,32] This suggests that an α-amidation-like reaction should preferentially occur at the less hindered N-terminus and form an α-ketoamide instead of cleaving the bond. We used the Cu(II)/AA system as a source of hydroxyl radicals (Scheme 2A). α-Ketoamide-Ang peptides were formed in the reaction of each Ang peptide with hydroxyl radicals generated by the Cu(II)/AA system (Figure 3B). The reaction is thought to be initiated by the hydroxyl radical-dependent abstraction of a hydrogen atom from the N-terminal α-carbon (Scheme 2B). In the presence of oxygen, the carbon-centered radical is rapidly converted to the peroxyl radical.[31] The loss of $HO_2^•$ from the peroxyl radical with the concomitant decarboxylation of Asp in Ang II, followed by hydrolysis of the ketimine intermediate, yields α-ketoamide-Ang peptides. In the case of Ang II, the β-carbon on the side chain (Asp) is another favorable site for hydrogen abstraction.[31] Therefore, Ang P can be also produced via the formation of peroxyl radicals from the β-carbon-centered radical (Scheme 2C), which could explain why the conversion of Ang II to Ang P was the highest (Figure 3B). The formation of Ang P from Ang II was also observed in other transition metal ion/AA systems, although the levels were much lower than those Ang P produced in the Cu(II)/AA reaction. This suggests that Cu(II) may coordinate with the N-terminal amine and carbonyl oxygen of Asp^1, which would allow for the localized production of the hydroxyl radical. It is also conceivable that other radial species such as Cu(I)(OOH) can initiate the reaction. For the Ang III reaction, the higher yield of Ang P_{III} may be due to the strong binding of Cu(II) to Arg^1 and/or the specific conformation of Ang III. The electrostatic and hydrogen bonding between the Arg and Tyr side chains[27] may allow the facile abstraction of a hydrogen atom from the less hindered N-terminal α-carbon. In all of the Cu(II)/AA reactions, α-ketoamide-Ang peptides were the major products, although oxidized-Ang peptides (+16 Da) were also detected. Products of peptide backbone cleavage were not observed. Figure 3B shows that the formation of α-ketoamide-Ang peptides was higher in the Cu(II)/AA (10 μM/100 μM) reactions compared with the ONE (300 μM) reaction. These results suggest that a significant amount of α-ketoamide-Ang peptides could be formed in biological systems.

Scheme 2. Proposed Mechanism for the Hydroxyl Radical-Mediated Formation of α-Ketoamide-Ang Peptides[a]



[a](A) Formation of hydroxyl radicals in the Cu(II)/AA system. (B) Formation of α-ketoamide-Ang peptides by H-abstraction at the α-carbon site. *, decarboxylation occurs in only the Ang II reaction. (C) Formation of Ang P by H-abstraction at the Asp residue of Ang II.

In addition to Ang II, other Ang peptides that contribute to vascular regulation have been identified, including Ang 1−7, Ang III, and Ang IV.[33] More recently, a new Ang peptide, Ang A, has been identified in human plasma.[34] It shows a strong vasoconstrictive effect and is present at higher concentrations in patients with end-stage renal failure. Interestingly, Ang A differs from Ang II in containing an alanine (Ala^1) residue instead of Asp^1, and it has been proposed that Ang A is formed enzymatically through the action of an unknown decarboxylase in human mononuclear leukocytes. We reasoned that Ang A could be generated through transamination of Ang P in the presence of a reactive primary amine. PM is a vitamin B_6 vitamer and functions as a coenzyme in enzymatic transaminations in vivo.[35,36] PM is also a promising pharmacological agent for the treatment of diabetic complications and other multifactorial chronic conditions.[36] The plasma concentration of PM in normal human subjects is in the nanomolar range,[36,37] which is approximately 3 orders of magnitude higher than that of Ang II.[16,27] It has been reported that rat plasma PM levels increased to about 100 μM upon pharmacological supplementation.[38] According to this information, we have used 5- to 500-fold molar excess of PM for the reaction with Ang P. In the reaction of Ang P with PM, the formation of epimeric Ang A was observed at retention times identical to those of authentic standards (Figure 4B). The identities of D- and L-Ang A were also confirmed by LC/ESI-MS and MS/MS analyses. The amounts of D- and L-Ang A were then quantified using a validated LC/ESI-SRM MS method. The conversion of Ang P to epimeric Ang A increased as a function of time and PM concentration (Figure 5A and B). When 10 μM Ang P was incubated with 5000 μM PM, 2.29 μM D-Ang A and 2.09 μM L-Ang A were produced, which corresponds to about 40% conversion of Ang P. Formation of epimeric Ang A was further increased when Cu(II) ions were added to the reaction of Ang P and PM (Figure 6A and B). Scheme 3 shows

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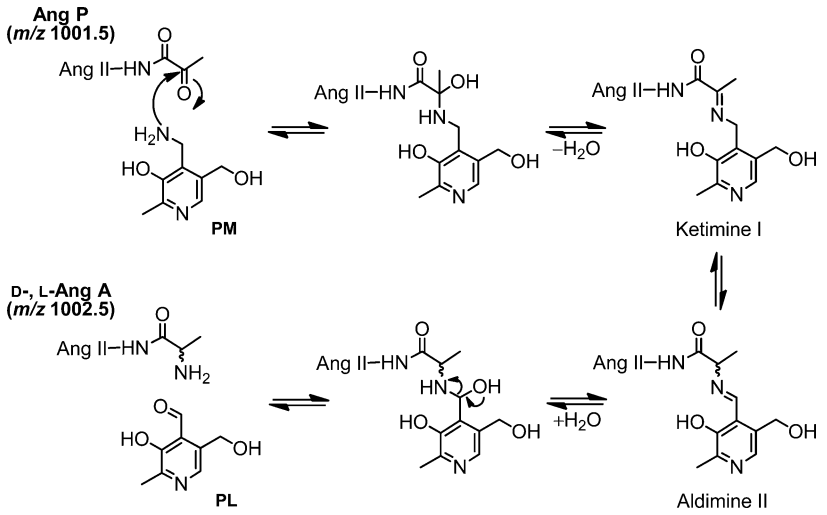
Scheme 3. Proposed Mechanism for the Transamination of Ang P with PM to Form D- and L-Ang A



our proposed mechanism, in which the initial reaction of the PM primary amino group at the Ang P carbonyl carbon yields a carbinolamine that is dehydrated to a Schiff base (ketimine I). This intermediate has a prochiral α-carbon and can undergo tautomerization to form a epimeric aldimine II because the protonation occurs in a nonstereospecific manner. The subsequent hydrolysis of the resulting aldimine II produces D- and L-Ang A. The chelate effect of Cu(II) ions can stabilize intermediates I and II (Figure 6C),[39] which increases the yield of D- and L-Ang A. When the reactions were conducted with lower concentrations of Ang P, PM, and Cu(II) ions in order to mimic physiological conditions, D- and L-Ang A were also formed, indicating that Ang P is a potential precursor of Ang A in vivo. Furthermore, the N-terminal ketone group of Ang P withdraws electrons from the adjacent carbon, which facilitates the hydrolysis of the amide bond to generate Ang III during the reaction (Figure 5C). In addition to the nonenzymatic transamination reaction described above, an enzymatic transamination was also examined for the conversion of Ang P to Ang A using the alanine transaminase (ALT). However, the formation of Ang A was not observed in various incubation conditions, suggesting that Ang P cannot reach the active site of ALT where PLP is located.

Aβ peptides are the main component of the amyloid plaques associated with Alzheimer's disease. The membrane-bound amyloid precursor protein is cleaved by β-and γ-secretases to generate Aβ peptides with N-terminal Asp. Their propensity for self-aggregation and potential for PTMs play a critical role in plaque formation.[40,41] In the reaction between ONE and Aβ_{1-11}, we confirmed the formation of Aβ_{1-11}-P (Figure 7A). However, the 4-ketoamide form of the [N-ONE]-Aβ_{1-11} adduct was not detected under the current LC/ESI-MS conditions. The amount of Aβ_{1-11}-P produced was 14-fold greater during the incubation of Aβ_{1-11} with Cu(II)/AA (Figure 7B). Aβ_{1-11}-P was then converted to epimeric Aβ_{1-11}-A (Figure 7D) in the presence of PM. These novel modifications of Aβ peptides could contribute to their self-aggregation and thus to neuronal cell death.

In summary, we demonstrated that ONE can mediate the formation of N-terminal α-ketoamide on peptides containing not only N-terminal Asp but also other amino acid residues, such as Ala, Arg, and valine. Hydroxyl radical-mediated reactions in which the N-terminus was converted to an α-ketoamide had higher yields for all of the peptides we examined. Ang P (N-terminal pyruvamide) derived from Ang II (N-terminal Asp) was the most abundant product in both the ONE- and hydroxyl radical-mediated reactions, indicating that decarboxylation accelerates the formation of the ketimine intermediate. We also showed that D- and L-Ang A were generated from Ang P through a nonenzymatic transamination reaction catalyzed by PM. In the presence of Cu(II) ions, approximately 60% of the Ang P was converted to epimeric Ang A after 3 days of incubation. The detection of Aβ_{1-11}-P and epimeric Aβ_{1-11}-A under the same reaction conditions used for the Ang peptides also indicates that these N-terminal modifications could occur in vivo. We are currently developing better analytical methods and examining the biological effects of N-terminal α-ketoamide peptides and proteins.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

AA, L-ascorbic acid; Aβ_{1-11}, amyloid beta 1–11; D-Aβ_{1-11}-A, D-Ala^1-L-Ala^2-L-Glu^3-L-Phe^4-L-Arg^5-L-His^6-L-Asp^7-L-Ser^8-L-Gly^9-L-Tyr^{10}-L-Glu^{11}; L-Aβ_{1-11}-A, L-Ala^1-L-Ala^2-L-Glu^3-L-Phe^4-L-Arg^5-L-His^6-L-Asp^7-L-Ser^8-L-Gly^9-L-Tyr^{10}-L-Glu^{11}; Aβ_{1-11}-P, pyruvamide-Aβ_{1-11}; Ala, alanine; ALT, alanine transaminase; Ang, angiotensin; D-Ang A, D-Ala^1-L-Arg^2-L-Val^3-L-Tyr^4-L-Ile^5-L-His^6-

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L-Pro^7-L-Phe^8; L-Ang A, L-Ala^1-L-Arg^2-L-Val^3-L-Tyr^4-L-Ile^5-L-His^6-L-Pro^7-L-Phe^8; Ang P, pyruvamide-Ang II; Ang P_{III}, α-ketoamide-Ang III; Ang P_{IV}, α-ketoamide-Ang IV; Arg, arginine; Asp, aspartic acid; AT_1, angiotensin II type 1; CID, collision-induced dissociation; ESI, electrospray ionization; HNE, 4-hydroxy-2(*E*)-nonenal; Cys, cysteine; His, histidine; IS, internal standard; LC, liquid chromatography; Lys, lysine; Met, methionine; $[M + H]^+$, protonated molecule; MS, mass spectrometry; MS/MS, tandem mass spectrometry; ONE, 4-oxo-2(*E*)-nonenal; PB, phosphate buffer; PLP, pyridoxal 5′-phosphate; PM, pyridoxamine; PTM, post-translational modification; QC, quality control; ROS, reactive oxygen species; SEM, standard error of the mean; SRM, selected reaction monitoring; t_R, retention time; Tyr, tyrosine

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