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ARTICLE *in* CHEMICAL RESEARCH IN TOXICOLOGY · OCTOBER 2010

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Mass Spectrometric Characterization of Modifications to Angiotensin II by Lipid Peroxidation Products, 4-Oxo-2(*E*)-nonenal and 4-Hydroxy-2(*E*)-nonenal

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Received July 4, 2010

The octapeptide angiotensin II (Ang II; Asp[1]-Arg[2]-Val[3]-Tyr[4]-Ile[5]-His[6]-Pro[7]-Phe[8]) is the primary active hormone of the renin/angiotensin system (RAS) and has been implicated in various cardiovascular diseases. Numerous structure−activity relationship studies have identified Asp[1], Arg[2], and His[6] of Ang II to be critical for its biological activity and receptor binding. From the reactions of Ang II with lipid peroxidation-derived aldehydes, 4-oxo-2(*E*)-nonenal (ONE) or 4-hydroxy-2(*E*)-nonenal (HNE), we have identified the major modifications to the N-terminus, Asp[1], Arg[2], and His[6] of Ang II by liquid chromatography/mass spectrometry (LC/MS) and matrix-assisted laser desorption ionization-time-of-flight/MS (MALDI-TOF/MS). The identities of ONE- and HNE-modified Ang II were confirmed by tandem mass spectrometry (MS/MS) and postsource decay (PSD)-TOF/MS before and after the reaction with sodium borohydride. In the reaction with ONE, a pyruvamide-Ang II that formed via oxidative decarboxylation of N-terminal Asp was detected as the most abundant product after 48 h of incubation. It was followed by Arg-modified [Arg[2](ONE−H_2O)]-Ang II and the N-terminal-modified 4-ketoamide form of [N-ONE]-Ang II. The Michael addition products of [His[6](HNE)]-Ang II were the most abundant products in the beginning of the reaction with HNE, followed by the dehydrated Michael addition products of [His[6](HNE−H_2O)]-Ang II. [His[6](HNE)]-Ang II was dehydrated to [His[6](HNE−H_2O)]-Ang II during the prolonged incubation, and [His[6](HNE−H_2O)]-Ang II became the major products after 7 days. The model reactions of N^{α}-*tert*-butoxycarbonyl (*t*Boc)-Arg with ONE and *t*Boc-His with HNE were performed and compared with the Ang II reaction. *t*Boc-Arg readily reacted with ONE to produce a compound analogous to [Arg[2](ONE−H_2O)]-Ang II, which confirmed Arg as one of the important target nucleophiles of ONE. However, *t*Boc-His exclusively formed a Michael addition product upon the reaction with HNE. The unexpected formation of [His[6](HNE−H_2O)]-Ang II can be explained by the proximity of His[6] to C-terminal carboxylate in the specific conformation of Ang II, which facilitates the dehydration of Michael addition products. Therefore, our results suggest a possible discrepancy in the adduction chemistry of ONE and HNE for model amino acids and endogenous bioactive peptides, which is governed by the microenvironment of peptides, such as the specific amino acid sequence and conformation. Such stable ONE- and HNE-derived modifications to Ang II could potentially modulate its functions *in vivo* by disrupting the interaction with Ang II type 1 (AT_1) receptor and/or inhibiting the enzyme activity of aminopeptidase A (APA), which cleaves the N-terminal Asp residue of Ang II to generate Ang III.

Introduction

Angiotensin (Ang) II is the major bioactive peptide involved in the renin/angiotensin system (RAS[1]). In addition to the circulating classical RAS, it has been identified also in various tissues including the heart, brain, kidneys, pancreas, and vessel wall (*1*−*3*). Thus, Ang II can be synthesized in various local tissues as well as in the circulation. Circulating RAS and local tissue RAS especially in the heart and kidney are thought to operate in a complementary fashion (*4*). Ang II is involved in the regulation of cardiovascular homeostasis and the development of various cardiovascular diseases, such as hypertension, atherosclerosis, and heart failure (*5*). Although the mechanisms by which Ang II contributes to each of these diseases remains unclear, oxidative stress is considered a central mechanism due to the involvement of reactive oxygen species (ROS) in numerous signaling pathways of Ang II (*6, 7*). Ang II stimulates ROS generation via the activation of NADPH oxidase mainly through its G protein-coupled Ang II type 1 (AT_1) receptor (*6, 8*). The ROS generated by Ang II then activate downstream ROS-sensitive kinases that are critical to mediating cardiovascular remodeling (*9*). Another major consequence of ROS-derived damage to the cardiovascular system is lipid peroxida-

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[1] Abbreviations: ACE, angiotensin-converting enzyme; Ang, angiotensin; APA, aminopeptidase A; Arg, arginine; Asp, aspartic acid; AT_1 receptor, angiotensin II type 1 receptor; *t*Boc, N^{α}-*tert*-butoxycarbonyl; *t*Bu, *tert*-butyl; CHCA, α-cyano-4-hydroxycinamic acid; Cys, cysteine; ESI, electrospray ionization; GSH, glutathione; His, histidine; Hip-Lys, N^{α}-hippuryl-L-lysine; HNE, 4-hydroxy-2(*E*)-nonenal; Ile, isoleucine; LC, liquid chromatography; Lys, lysine; MALDI-TOF, matrix-assisted laser desorption ionization-time-of-flight; MH^+, protonated molecule; MS, mass spectrometry; MS/MS, tandem mass spectrometry; MS^n, multiple tandem mass spectrometry; ONE, 4-oxo-2(*E*)-nonenal; Phe, phenylalanine; PLP, pyridoxal 5′-phosphate; Pro, proline; PSD, postsource decay; PUFA, polyunsaturated fatty acid; RAS, renin/angiotensin system; ROS, reactive oxygen species; t_R, retention time; Tyr, tyrosine; Val, valine.

10.1021/tx100228q
Published on Web 10/26/2010

tion with the generation of the genotoxic aldehydes such as 4-oxo-2(*E*)-nonenal (ONE) and 4-hydroxy-2(*E*)-nonenal (HNE) (*10*). However, there has been little attention given to the potential for the formation of lipid hydroperoxide-derived modifications to Ang II that can modulate the biological activity of Ang II.

ONE and HNE are among the most abundant, toxic, and reactive lipid-derived aldehydes that are generated from the homolytic decomposition of ω-6 polyunsaturated fatty acid (PUFA) hydroperoxides (*11*). These aldehydes have been shown to be cytotoxic, and some toxic effects have been attributed to their ability to modify DNA bases (*12*−*14*) and the consequent mutations (*15, 16*). In addition, ONE and HNE are involved in the regulation of inflammation, apoptosis, and other cellular signaling through modifications on amino acid residues of critical signaling kinases (*17*). Other protein dysfunctions and altered gene regulation can also arise from the modification and cross-linking of proteins by these aldehydes (*11, 18*). ONE and HNE can diffuse across membranes, allowing them to covalently modify peptides or proteins not only localized in the cell but also relatively far from the site of their formation. The electron-withdrawing functional groups of ONE and HNE make them reactive Michael acceptors toward the sulfhydryl group of Cys, the imidazole nitrogen of His, and the ε-amino group of Lys. Although the order of modification preference by ONE and HNE was found to be similar (Cys $\gg$ His > Lys), ONE was much more reactive than HNE toward thiol and imidazole nucleophiles (*11, 19*). In the case of ONE, the aldehyde carbon (C-1) is another favorable reaction site for the ε-amino group of Lys (*11*), the guanidido group of Arg (*20*), and α-amino group of Asp (*21*) through the formation of Schiff base adducts. On the basis of the results of studies using model amino acids and simple peptides, extensive research has focused on ONE- and HNE-derived modifications to proteins and their functional changes (*11, 19, 22*−*24*).

Ang II is an octapeptide (Asp^1-Arg^2-Val^3-Tyr^4-Ile^5-His^6-Pro^7-Phe^8) generated from the cleavage of Ang I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) at the C-terminus by angiotensin-converting enzyme (ACE). Ang II contains amino acids that can be easily modified under nitrosative and oxidative stress environments. The nitration and nitrosylation of Tyr^4 and the nitrosation of Arg^2 on Ang II have been reported (*25, 26*). An *in vivo* study using the nitrated Ang II showed that nitration of Tyr^4 residue inhibits vasoconstrictive properties of Ang II (*25*). The metal-catalyzed oxidation of two His residues in Ang I (*27*) has been investigated using a copper(II)/ascorbate system. The amino acids, Arg^2, His^6, and the N-terminus of Ang II are also well-known nucleophilic sites that readily react with the α,β-unsaturated aldehydes, ONE and HNE. Furthermore, numerous structure−activity relationship studies have identified Asp^1, Arg^2, and His^6 as well as Tyr^4 and Phe^8 of Ang II to be critical for its biological activity and binding to AT_1 receptor (*28*−*32*). We reasoned that the functional changes of Ang II could result from the modification of these amino acid residues by ONE and/or HNE arising from Ang II-induced ROS damage to the cardiovascular system. Therefore, the goal of this study was to carry out the mass spectrometric characterization of Ang II modifications by ONE and HNE. Physiologically relevant conditions and the concentration ratio of Ang II and aldehydes were employed for the reactions. Liquid chromatography (LC)/UV, LC-electrospray ionization/mass spectrometry (ESI/MS) and matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF)/MS were used to identify the modified peptides. Tandem mass spectrometry (MS/MS) and postsource decay (PSD)-TOF/MS analyses were performed to confirm each peptide modification before and after sodium borohydride reduction. The relative importance of different modifications has been explored by prolonged incubation of each modified peptide. Finally, the formations of ONE- and HNE-modified Ang II were compared with the products of the model reactions using N^{α}-*tert*-butoxycarbonyl (*t*Boc)-amino acids.

Materials and Methods

Materials. ONE and HNE were purchased from Cayman Chemical Co. (Ann Arbor, MI). Human Ang II was obtained from Calbiochem (a brand of EMD, San Diego, CA). Ang I, α-cyano-4-hydroxycinamic acid (CHCA), diethyl ether, sodium hydroxide, trifluoroacetic acid, and formic acid were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Sodium borohydride and glutathione (GSH) were purchased from Sigma-Aldrich Inc. (St. Louis, MO). N^{α}-*tert*-Butoxycarbonyl (*t*Boc)-Arg·0.75H_2O and *t*Boc-His were obtained from Watanabe Chemical Industries, Ltd. (Hiroshima, Japan). Chelex-100 chelating ion-exchange resin (100−200 mesh size) was purchased from Bio-Rad Laboratories (Hercules, CA). LC grade acetonitrile and ethanol were obtained from Kanto Chemical Co. Inc. (Tokyo, Japan). Purified water was purchased from Daiwa-Yakuhin Co. Ltd. (Sendai, Japan) and further filtered through Ultrapure Water System, CPW-100 (Advantec Toyo Kaisha, Ltd. Tokyo, Japan). Oasis HLB cartridge was obtained from Waters Corp. (Milford, MA).

Liquid Chromatography. Chromatographies for LC systems 1−3 were carried out on a Nanospace SI-1 semimicrocolumn LC system (Shiseido Co. Ltd., Tokyo, Japan) equipped with a UV detector. Chromatography for LC system 4 was carried out on a Surveyor MS pump (separation module) equipped with a Surveyor AS autosampler, column oven, and PDA detector (Thermo Fisher Scientific Inc., Waltham, MA). LC systems 1, 3, and 4 employed a Jupiter C18 column (150 × 2.0 mm i.d., 5 μm, 300 Å; Phenomenex, Torrance, CA). LC System 2 employed a SunFire C18 column (150 × 2.1 mm i.d., 3.5 μm; Waters, Milford, MA). For LC system 1, solvent A was water/acetonitrile (98:2, v/v) containing 0.08% trifluoroacetic acid, and solvent B was acetonitrile/water (98:2, v/v) containing 0.08% trifluoroacetic acid. The linear gradient was as follows: 20% B at 0 min, 27.5% B at 4 min, 52.5% B at 30 min, 90% B at 31 min, 90% B at 36 min, 20% B at 37 min with a flow rate of 0.2 mL/min. For LC systems 2 and 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 for LC system 2 was as follows: 20% B at 0 min, 20% B at 2 min, 25% B at 4 min, 35% B at 25 min, 90% B at 26 min, 90% B at 29 min, 20% B at 30 min with a flow rate of 0.2 mL/min. The linear gradient for LC system 3 was as follows: 20% B at 0 min, 20% B at 2 min, 31% B at 17 min, 33.4% B at 20 min, 90% B at 21 min, 90% B at 24 min, and 20% B at 25 min with a flow rate of 0.2 mL/min. The separations using LC systems 1−3 were performed at ambient temperatures. For LC systems 4, solvent A was water containing 1% formic acid, and solvent B was acetonitrile containing 1% formic acid. The linear gradient for LC system 4 was as follows: 0% B at 0 min, 70% B at 18 min, 70% B at 20 min, 0% B at 21 min, and 0% B at 35 min with a flow rate of 0.25 mL/min and a column oven temperature at 25 °C.

Mass Spectrometry. MALDI-TOF/MS and MALDI-PSD-TOF/MS experiments were carried out on an AXIMA-CFR *Plus* MALDI-TOF mass spectrometer (Shimadzu Biotech, Kyoto, Japan) located at the Department of Instrumental Analysis (Technical Division, School of Engineering, Tohoku University) for ONE-modified Ang II. For HNE-modified Ang II, a Voyager-DE STR MALDI-TOF mass spectrometer (Applied Biosystems Inc., Foster City, CA) located at the Biomedical Research Core (School of Medicine, Tohoku University) was used. All spectra presented herein were acquired in the positive ion mode with an accelerating voltage of 20 kV and in the reflectron mode with an average of 100−150 laser shots. TOF/MS experiments were performed in the

mass range of *m/z* 100−1500. Calibration was performed by using four or five internal calibrants from the following: monoisotopic masses of matrix monomer at *m/z* 190.0504, matrix dimer at *m/z* 379.0930, matrix trimer at *m/z* 568.1400, Ang II at *m/z* 1046.5418, and Ang I at *m/z* 1296.6800. For MALDI-PSD-TOF/MS analyses, PSD fragments were calibrated using monoisotopic masses of fragment ions generated from Ang II (MH^+ at *m/z* 1046.5418, $MH^+ - NH_3$ at *m/z* 1029.5152, y_7 at *m/z* 931.5148, $b_7 + H_2O$ at *m/z* 899.4734, b_6 at *m/z* 784.4100, a_6 at *m/z* 756.4151, b_5 at *m/z* 647.3511, a_5 at *m/z* 619.3562, $b_4 - NH_3$ at *m/z* 517.2405, y_3 at *m/z* 400.1979, $b_3 - NH_3$ at *m/z* 354.1772, and y_2 at *m/z* 263.1390). Aliquots (0.5 μL) of the samples in 50% aqueous acetonitrile were loaded onto MALDI sample plates followed by 0.5 μL of matrix solution (saturated CHCA in 50% aqueous acetonitrile containing 0.1% trifluoroacetic acid and 1−2 μM of internal calibrants) and allowed to dry at room temperature. For LC/MS analyses of the reaction between Ang II and ONE or HNE, a TSQ-7000 triple quadrupole mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA) equipped with an ESI source was used in positive ion mode with LC system 3. The TSQ-7000 operating conditions were as follows: heated capillary temperature, 330 °C; sheath gas (nitrogen) pressure, 90 psi; and auxiliary gas (nitrogen) pressure, 30 (arbitrary units). Full scanning analyses were performed in the range of *m/z* 300−1300. Collision-induced dissociation (CID) experiments coupled with MS/MS experiments employed argon as the collision gas at 2.5 mTorr in the second (rf-only) quadrupole. The collision energy was set at 30−35 eV. For LC/MS analyses of the reaction between *t*Boc-amino acid and ONE or HNE, a LCQ-DECA ion trap mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA) equipped with an ESI source was used in positive ion mode with LC system 4. The LCQ-DECA operating conditions were as follows: heated capillary temperature, 300 °C; ion spray voltage, 5.0 kV; sheath and auxiliary gas (nitrogen) pressures, 90 and 15 (arbitrary units), respectively. Full scanning analyses were performed in the range of *m/z* 100−600. CID experiments coupled with multiple tandem mass spectrometry (MS^n) employed helium as the collision gas. The relative collision energy was set at 35% of the maximum (1 V).

Reaction of Ang II with ONE or HNE. A solution of ONE (92.4 μg, 0.6 μmol) in 20 μL of methylacetate/ethanol (1:1, v/v) or HNE (93.7 μg, 0.6 μmol) in 20 μL of ethanol was added to Ang II (209.2 μg, 0.2 μmol) in the chelex-treated 50 mM sodium phosphate buffer (pH 7.4, 180 μL). The reaction mixture was incubated for 24 h at 37 °C. After incubation, excess ONE or HNE was removed by extraction using hexane or diethyl ether (200 μL × 2), respectively. A portion of the sample (10 μL) was analyzed by LC/UV (235 nm) using LC system 1 for the reaction with ONE and LC system 2 for the reaction with HNE. The reactions with decreased concentrations of Ang II and ONE or HNE (Ang II/aldehyde = 100 μM:300 μM and 10 μM:30 μM) were also conducted with the same incubation conditions in the absence and presence of GSH (10 μM). A portion (10 μL) of the reaction mixture was analyzed by LC-ESI/MS/PDA using LC system 3 for the reaction with ONE and LC system 2 for the reaction with HNE.

Sodium Borohydride Reduction of ONE- and HNE-Modified Ang II. Four ONE-modified Ang II and eleven HNE-modified Ang II peptides were purified using LC systems 1 and 2, respectively. Each solution of ONE- or HNE-modified Ang II in 50 mM sodium phosphate buffer (pH 7.4, 100 μL) was treated with 100 μL of 0.1 M sodium borohydride in 0.1 M sodium hydroxide. The reaction mixtures were incubated for 1 h at 37 °C. After being cooled down to room temperature, the resulting modified peptides were purified and desalted chromatographically on Oasis HLB (1 mL, 10 mg) cartridges. The cartridges were conditioned with acetonitrile (1 mL) followed by 0.1% aqueous trifluoroacetic acid (1 mL). The reaction solutions (200 μL) were loaded on the cartridges and washed with 0.1% aqueous trifluoroacetic acid (200 μL × 3). The peptides were recovered by elution with 60% aqueous acetonitrile containing 0.1% trifluoroacetic acid (200 μL × 2) and evaporated to dryness under nitrogen. The samples were redissolved in 50% aqueous acetonitrile (20 μL). Aliquots (0.5 μL) were analyzed by MALDI-TOF/MS and PSD-TOF/MS as described above or by LC-ESI/MS using LC system 3 for the ONE-modified Ang II and LC system 2 for the HNE-modified Ang II.

Time Course for the Formation of Modified Ang II by ONE or HNE. A solution of ONE (92.4 μg, 0.6 μmol) in 20 μL of methylacetate/ethanol (1:1, v/v) or HNE (93.7 μg, 0.6 μmol) in 20 μL of ethanol was added to Ang II (209.2 μg, 0.2 μmol) in the chelex-treated 50 mM sodium phosphate buffer (pH 7.4, 180 μL). The reaction mixture with ONE or HNE was incubated at 37 °C for 48 h or 14 days, respectively. A portion of the sample (10 μL) was analyzed at various time points by LC/UV (235 nm) without the extraction of the aldehyde using LC system 1 for the ONE reaction mixture and LC system 2 for the HNE reaction mixture.

Prolonged Incubation of Isolated HNE-Modified Ang II. A portion of isolated HNE-modified Ang II was further incubated in the chelex-treated 50 mM sodium phosphate buffer (pH 7.4, 100 μL) for 7 days at 37 °C and analyzed by LC/UV (235 nm) using LC system 2.

Reaction of *t*Boc-Arg with ONE. A solution of ONE (46.3 μg, 0.3 μmol) in 10 μL of methylacetate/ethanol (1:1, v/v) was added to *t*Boc-Arg·$0.75H_2O$ (28.8 μg, 0.1 μmol) in the chelex-treated 50 mM sodium phosphate buffer (pH 7.4, 90 μL). The reaction mixture was incubated for 48 h at 37 °C. A portion of the sample (10 μL) was analyzed at various time points by LC-ESI/MS using LC system 4. After 48 h of incubation, a portion of the reaction mixture (10 μL) was treated with 20 μL of 0.1 M sodium borohydride in 0.1 M sodium hydroxide. The reaction mixtures were incubated for 1 h at 37 °C and analyzed by LC-ESI/MS using LC system 4 without further purification.

Reaction of *t*Boc-His with HNE. A solution of HNE (46.9 μg, 0.3 μmol) in 10 μL of ethanol was added to *t*Boc-His (25.5 μg, 0.1 μmol) in the chelex-treated 50 mM sodium phosphate buffer (pH 7.4, 90 μL). The reaction mixture was incubated for 14 days at 37 °C. A portion of the sample (10 μL) was analyzed at various time points by LC-ESI/MS using LC system 4. After 7 days of incubation, a portion of the reaction mixture (10 μL) was treated with 20 μL of 0.1 M sodium borohydride in 0.1 M sodium hydroxide. The reaction mixtures were incubated for 1 h at 37 °C and analyzed by LC-ESI/MS using LC system 4 without further purification.

Results

Analysis of the Reaction between Ang II and ONE. LC/UV (235 nm) analysis of the products from the reaction between Ang II and ONE at 37 °C for 24 h revealed the presence of four major products together with residual Ang II (Figure 1A). Each product peak was isolated using LC system 1 and analyzed by MALDI-TOF/MS or LC-ESI/MS as described in the Materials and Methods section. The MALDI-TOF/MS spectrum of the most polar modified peptide (O1) eluting at 8.7 min revealed an MH^+ at *m/z* 1001.5 corresponding to a loss of 45 Da from Ang II (MH^+; *m/z* 1046.5). The ONE-modified peptides that eluted at 10.9 min (O2) and 14.1 min (O3) had an identical MH^+ at *m/z* 1200.7, which corresponded to an increase in mass of 154 Da (+ ONE). The last eluting ONE-modified peptide had a retention time of 16.0 min (O4). It showed an MH^+ at *m/z* 1182.6, corresponding to an increase in mass of 136 Da (+ [ONE−H_2O]). The LC-ESI/MS analyses of the reactions with decreased concentrations of Ang II and ONE (Ang II/ONE = 100 μM:300 μM and 10 μM:30 μM) revealed the presence of O1−O4 as major products (data not shown). When 10 μM of Ang II was reacted with ONE in the presence of GSH (10 μM), the total modification yield was reduced to 66.0%.

Analysis of the Reaction between Ang II and HNE. LC/UV (235 nm) analysis of the products from the reaction between Ang II and HNE at 37 °C for 24 h revealed the presence of 11 major products together with residual Ang II (Figure 1B). Each product peak was isolated using LC system 2 and analyzed by

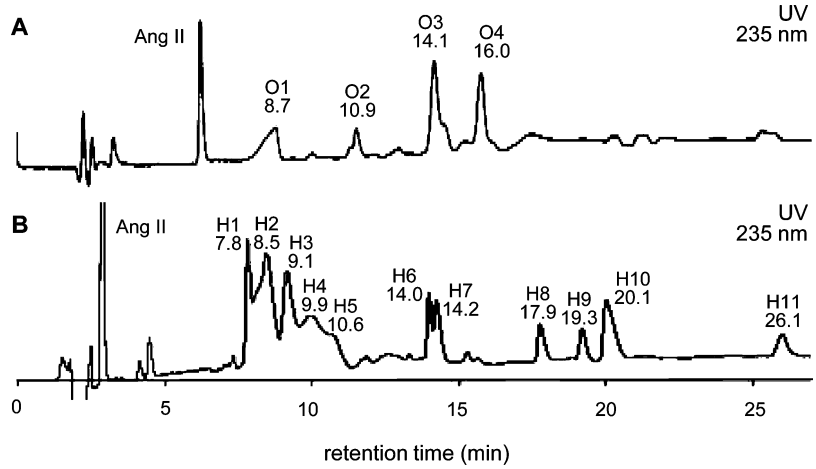


Figure 1. LC/UV (235 nm) analyses of the reactions of Ang II with (A) ONE and (B) HNE at 37 °C for 24 h.

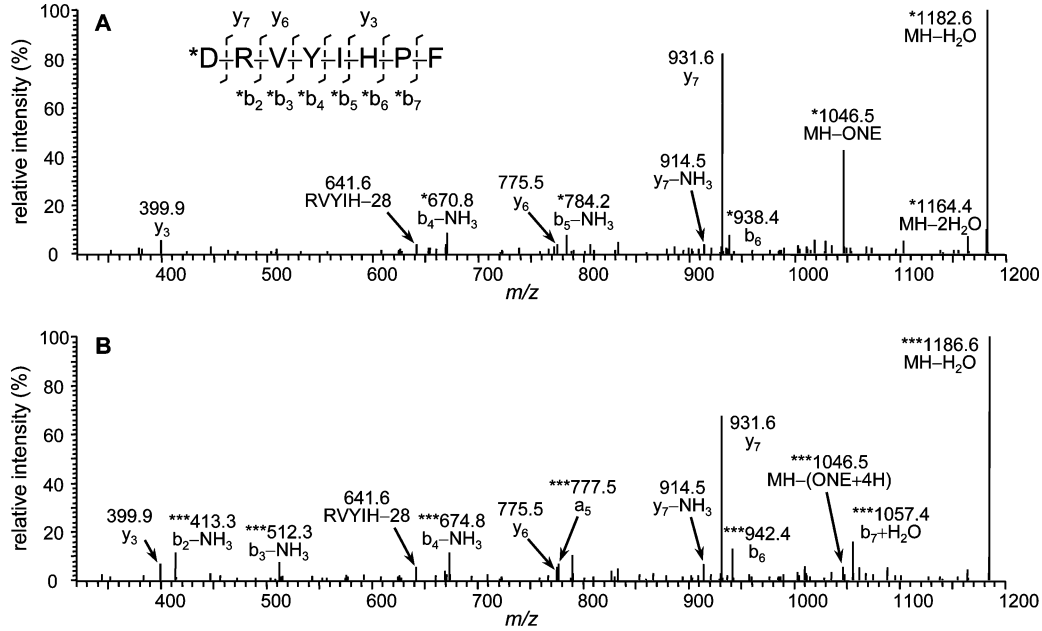
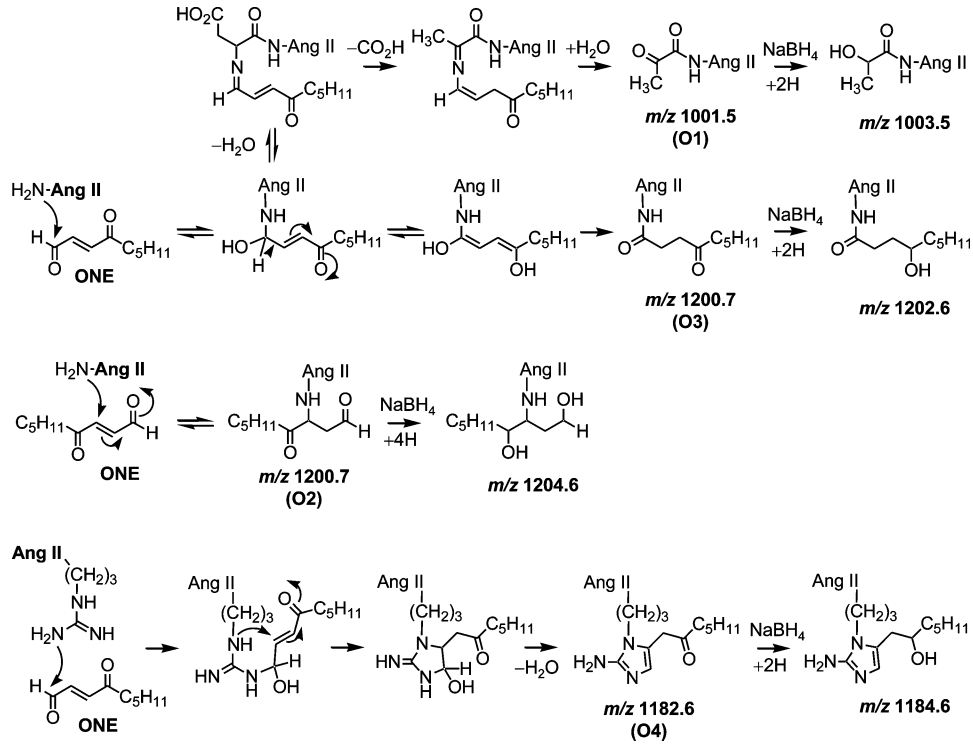


Figure 2. LC-ESI/MS/MS analyses of ONE-modified Ang II ([N-ONE]-Ang II, O2) (A) before (MH^+; *m/z* 1200.7) and (B) after (MH^+; *m/z* 1204.6) reduction with $NaBH_4$. * represents a modified ion (+ 154 Da). *** represents a modified ion (+ 154 Da + 4H). The *m/z* value of each peak indicates the monoisotopic mass observed.

MALDI-TOF/MS or LC-ESI/MS. The MALDI-TOF/MS spectra of the HNE-modified peptides eluting between 7.8 and 10.6 min (H1−H5) revealed an identical MH^+ at *m/z* 1202.5 corresponding to an increase in mass of 156 Da (+ HNE) from Ang II. The HNE-modified peptides that eluted at 14.0, 14.2, 17.9, 19.3, and 20.1 min (H6−H10) had an identical MH^+ at *m/z* 1184.3, which corresponded to an increase in mass of 138 Da (+ [HNE−H_2O]). The last eluting HNE-modified peptide had a retention time of 26.1 min (H11). It showed an MH^+ at *m/z* 1358.7, corresponding to an increase in mass of 312 Da (+ 2HNE). The LC-ESI/MS analyses of the reactions with decreased concentrations of Ang II and HNE (Ang II/HNE = 100 μM:300 μM and 10 μM:30 μM) revealed the presence of H1−H10 as major products. However, H11 was not detected from the both reactions (data not shown). When 10 μM of Ang II was reacted with HNE in the presence of GSH (10 μM), the total modification yield was reduced to 63.2%.

MALDI-PSD-TOF/MS or LC-ESI/MS/MS Analyses of ONE-Modified Ang II before and after Sodium Borohydride Reduction. The most polar ONE-modified peptide (O1, 8.7 min) with an MH^+ at *m/z* 1001.5 has been characterized as pyruvamide-Ang II by MALDI-PSD-TOF/MS analysis and the comparison with a pyridoxal 5′-phosphate (PLP)-derived authentic standard (*21*). The MS/MS analysis of the ONE-modified peptide O2 (10.9 min) at *m/z* 1200.7 revealed that a modification occurred at the N-terminus of Ang II (Figure 2A). Thus, all the b-ions that were detected (b_4 to b_7) appeared with an increase of 154 Da (+ ONE) when they were compared with the corresponding b-ions of Ang II. However, the y-ions (y_3, y_6, and y_7) and an internal fragment ion (RVYIH−28) remained unmodified. The peak at *m/z* 1046.5, corresponding to the MH^+ of the parent peptide (Ang II), was generated from neutral loss of the ONE molecule (retro-Michael addition), which indicates the addition of ONE via a Michael addition mechanism (*33*). The ONE modified peptide O2 was then subjected to sodium borohydride reduction. After the reaction, the resulting product was further purified and desalted by solid-phase extraction as described in the Materials and Methods section. LC-ESI/MS spectrum of the reduced O2 revealed an MH^+ at *m/z* 1204.6 that corresponded to the addition of four hydrogen atoms. MS/MS analysis of *m/z* 1204.6 resulted in the generation of all modified a- and b-ions (a_5 and b_2 to b_7) and all intact y-ions (y_3, y_6, and y_7), which is consistent with the modification of the N-terminus of Ang II (Figure 2B). All of the modified a-

Scheme 1. Proposed Mechanism for the Formation of ONE-Modified Ang II and Their $NaBH_4$ Reduction Products[a]



[a] The *m/z* values represent MH^+ of the monoisotopic ion.

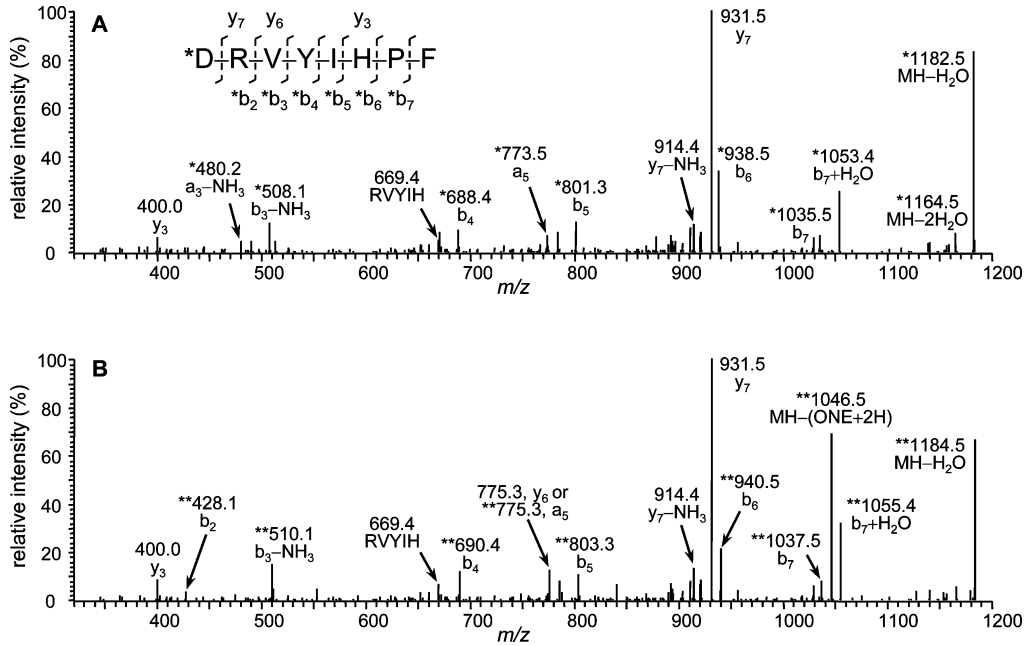


Figure 3. LC-ESI/MS/MS analyses of the ONE-modified Ang II ([N-ONE]-Ang II, O3) (A) before (MH^+; *m/z* 1200.7) and (B) after (MH^+; *m/z* 1202.6) reduction with $NaBH_4$. * represents a modified ion (+ 154 Da). ** represents a modified ion (+ 154 Da + 2H). The *m/z* value of each peak indicates the monoisotopic mass observed.

and b-ions showed an increase in mass units of 4 Da when compared with those in the MS/MS spectrum of O2 (Figure 2A). This confirmed that four hydrogen atoms were added to two carbonyl groups of ONE after the Michael addition of N-terminal α-amino group of Ang II. The stabilization of the retro reaction (decreased formation of parent peptide ion at *m/z* 1046.5) after hydride reduction (Figure 2B) also indicates the formation of ONE Michael addition product. On the basis of these results, the ONE-modified peptide O2 was characterized as a Michael addition product of [N-ONE]-Ang II (Scheme 1).

The MS/MS spectrum of *m/z* 1200.7, MH^+ of the ONE-modified peptide O3 (14.1 min), was identical to that of O2 except for the absence of the peak for the parent peptide at *m/z* 1046.5 (Figure 3A). All of the a- and b-ions detected (a_3 to a_5, b_3 to b_7) were observed with an increase of 154 Da (+ ONE) from the corresponding b-ions of Ang II. However, the y-ions (y_3 and y_7) and an internal fragment ion (RVYIH) were intact. Thus, the modification occurred at the N-terminus of Ang II. The ESI/MS spectrum of O3 after a reaction with sodium borohydride exhibited an MH^+ at *m/z* 1202.6 that corresponded

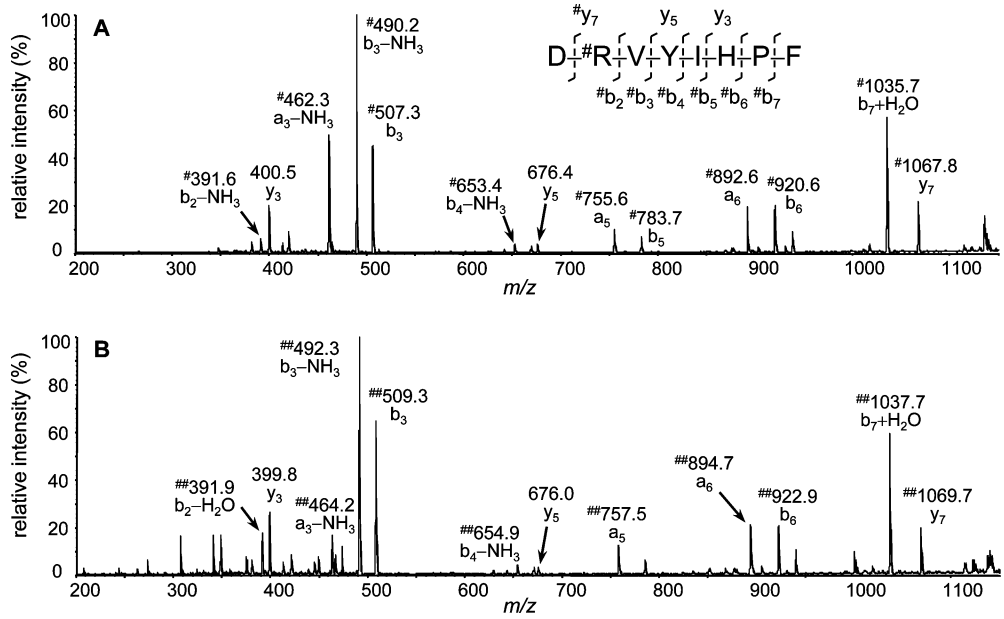


Figure 4. MALDI-PSD-TOF/MS analyses of the ONE-modified Ang II ([Arg^2(ONE−H_2O)]-Ang II, O4) (A) before (MH^+; *m/z* 1182.6) and (B) after (MH^+; *m/z* 1184.6) reduction with $NaBH_4$. # represents a modified ion (+ 136 Da). ## represents a modified ion (+ 136 Da + 2H). The *m/z* value of each peak indicates the monoisotopic mass observed.

to the addition of two hydrogen atoms. The MS/MS analysis of *m/z* 1202.6, which resulted in the generation of all modified a- and b-ions (a_5 and b_2 to b_7) and all intact y-ions (y_3, y_6 and y_7), is consistent with the modification at the N-terminus of Ang II (Figure 3B). All modified a- and b-ions showed an increase in mass units of 2 Da when compared with those in the MS/MS spectrum of O3 (Figure 3A). This confirmed that two hydrogen atoms were added to the N-terminus. The detection of an intense native Ang II peak at *m/z* 1046.5 after reduction (Figure 3B) indicates facile loss of reduced ONE through intramolecular lactonization of 4-hydroxyamide, which has been reported as a characteristic MS/MS property of 4-ketoamide adducts (*34*). On the basis of these results, the ONE-modified peptide O3 was characterized as a 4-ketoamide form of [N-ONE]-Ang II (Scheme 1).

The MALDI-PSD-TOF/MS analysis of *m/z* 1182.6, an MH^+ of ONE-modified peptide O4 (16.0 min), revealed that a modification occurred at Arg^2 of Ang II (Figure 4A). All of the a- and b-ions detected (a_3 to a_6 and b_2 to b_7) were modified with a mass increase of 136 Da (+ [ONE−H_2O]). Most of the y-ions that were detected (y_3 to y_5) contained the intact amino terminus. However, an intense y_7 ion was observed with an increase mass of 136 Da when it was compared to the corresponding y_7 ion of Ang II. Accordingly, the ONE adduct represents the stable adduct containing a substituted imidazole ring formed by the reaction of ONE with the guanidido group present in Arg^2 as previously described by Oe et al. (*20*). The MALDI-TOF/MS spectrum of the reduced O4 revealed an MH^+ at *m/z* 1184.6 that corresponded to the addition of two hydrogen atoms. PSD-TOF/MS analysis of *m/z* 1184.6 resulted in the generation of all modified a- and b-ions (a_3 to a_6 and b_2 to b_7), as well as a modified y_7 ion and intact y-ions (y_3 to y_5), which is consistent with modification of Arg^2 (Figure 4B). All of the modified a-, b-, and y-ions showed an increase in mass units of 2 Da when compared with those in the PSD-TOF/MS spectrum of O4 (Figure 4A). This confirmed that two hydrogen atoms were added to the heptanone moiety on the imidazole ring of the ONE-modified Arg^2. From these results, the ONE-modified peptide O4 was characterized as [Arg^2(ONE−H_2O)]-Ang II (Scheme 1).

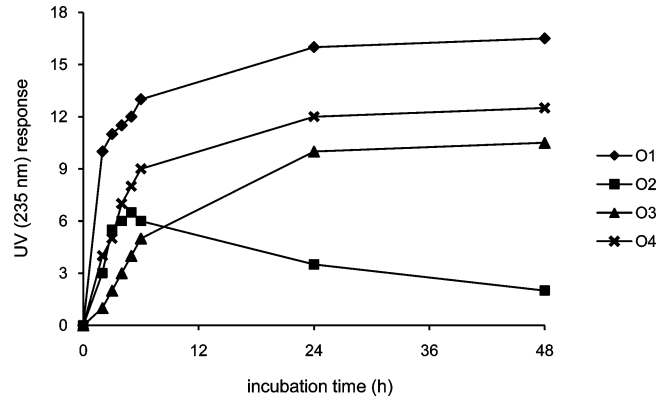


Figure 5. Time course for the formations of ONE-modified Ang II (O1−O4) at 37 °C for 48 h.

Time Course for the Formation of ONE-Modified Ang II. The reaction between Ang II and ONE in phosphate buffer (pH 7.4) at 37 °C was monitored by LC/UV (235 nm) for 48 h (Figure 5). At early reaction times, the pyruvamide-Ang II (O1) was the most abundant product, followed by [Arg^2(ONE−H_2O)]-Ang II (O4), the Michael addition product of [N-ONE]-Ang II (O2), and the 4-keto amide form of [N-ONE]-Ang II (O3). The formations of O1, O3, and O4 increased during the incubation and reached their maximum levels after 48 h. However, the formation of O2 was maximal after 5 h of the reaction and then decreased gradually as the incubation progressed. It was the least abundant product after 10 h of incubation. O1 was the most abundant product throughout the incubation.

LC-ESI/MS and MS^n Analyses of the Reaction between *t*Boc-Arg and ONE. LC-ESI/MS analysis of the products from the reaction between *t*Boc-Arg and ONE at 37 °C for 48 h revealed the presence of one major product with MH^+ at *m/z* 411.2 (retention time (t_R) = 15.1 min, + 136 Da; *t*Boc-Arg-ONE B) (Figure 6A). Minor product with MH^+ at *m/z* 429.2 (t_R = 14.6 min, + 154 Da; *t*Boc-Arg-ONE A) and residual *t*Boc-Arg (MH^+, *m/z* 275.1, t_R = 9.7 min) were also observed. At the initial stage of the reaction (0.5−4 h), the main product was *t*Boc-Arg-ONE A. There was then a gradual decrease in

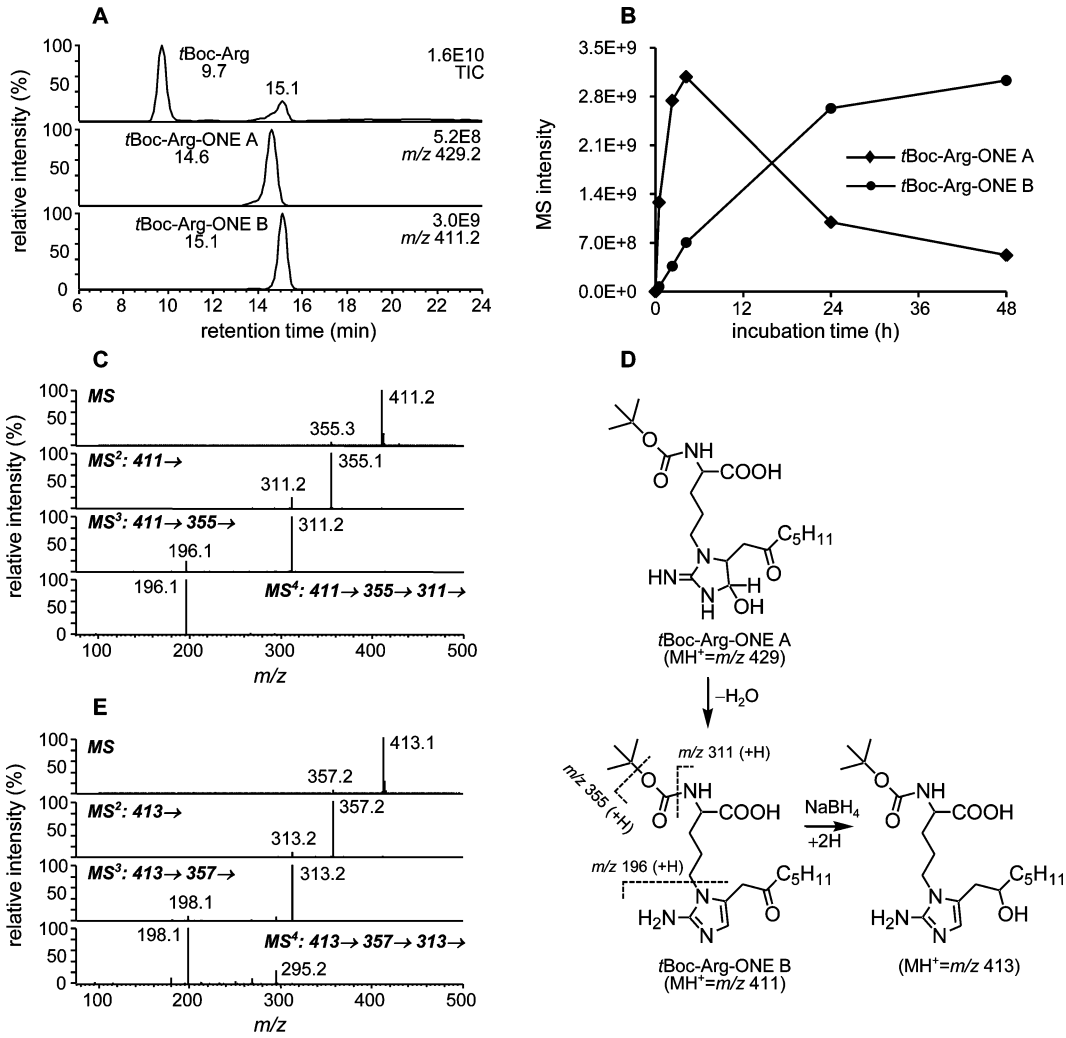


Figure 6. (A) LC-ESI/MS analysis of the reaction between *t*Boc-Arg and ONE at 37 °C for 48 h. (B) Time course for the formations of *t*Boc-Arg-ONE A and B at 37 °C for 48 h. (C) MS^n analyses of *t*Boc-Arg-ONE B. (D) Proposed structures of *t*Boc-Arg-ONE A and B, and reduction product of *t*Boc-Arg-ONE B with $NaBH_4$. (E) MS^n analyses of *t*Boc-Arg-ONE B after reduction with $NaBH_4$.

the formation of *t*Boc-Arg-ONE A as the incubation progressed with a concomitant increase in the formation of *t*Boc-Arg-ONE B, which reached its maximum level after 48 h (Figure 6B). The mass spectrum of *t*Boc-Arg-ONE B (Figure 6C) showed an intense MH^+ at *m/z* 411.2 with a minor fragment ion at *m/z* 355.3 (MH^+ − *tert*-butyl (*t*Bu) + H) (Figure 6D). MS^2 analysis of MH^+ resulted in the formation of *m/z* 355.3 as a major product ion with a lower abundance ion at *m/z* 311.2 (MH^+ − *t*Boc + H). MS^3 analysis of *m/z* 355.1 revealed two product ions at *m/z* 311.2 and *m/z* 196.1 (protonated form of 2-amino-4-(heptane-2′-one)imidazole) (Figure 6D). This ion was an exclusive product ion in the MS^4 analysis of *m/z* 311.2. LC-ESI/MS analysis of the reaction mixture after treatment with sodium borohydride showed an exclusive conversion of *t*Boc-Arg-ONE B to its reduced form with MH^+ at *m/z* 413.2, corresponding to the addition of two hydrogen atoms. In the MS^n analyses of *m/z* 413.2 (Figure 6E), a similar series of product ions was observed as compared with *t*Boc-Arg-ONE B (Figure 6C) except for the shift of two mass units. The intense product ion at *m/z* 198.1 in the MS^4 analysis confirmed the addition of two hydrogen atoms to the heptanone moiety at the substituted imidazole ring.

MALDI-PSD-TOF/MS or LC-ESI/MS/MS Analyses of HNE-Modified Ang II before and after Sodium Borohydride Reduction. The MALDI-PSD-TOF/MS analysis of *m/z* 1202.5, an MH^+ of HNE-modified peptides eluting at 7.8, 8.5, 9.1, 9.9, and 10.6 min, revealed identical PSD spectra for H1−H5, which agreed that the modifications occurred at His^6 of Ang II (Figure 7A). Thus, ions of a_3 to a_5 and b_2 to b_5 contained the intact amino terminus. Among the observed a- and b-ions, a_6, a_7, and b_6 and b_7 were found to be modified, with a mass increase of 156 Da (+ HNE). However, most of the y-ions appeared as modified ions (y_3 to y_7) with an increase mass of 156 Da (+ HNE). Only the y_2-ion remained unmodified. An intense peak observed at *m/z* 266.5 corresponded to an immonium ion of HNE-modified His. The HNE modified peptides H1−H5 were then subjected separately to sodium borohydride reduction. After the reaction, the resulting products were further purified and desalted as described in the Materials and Methods section. MALDI-TOF/MS analyses of the products from the reduction of H1−H5 revealed that an identical major product was formed with an MH^+ at *m/z* 1204.7, corresponding to the addition of two hydrogen atoms. MALDI-PSD-TOF/MS analysis of *m/z* 1204.7 (Figure 7B) resulted in the generation of the unmodified ions of a_3 to a_5, b_2 to b_5, and y_2 and modified ions of a_6, a_7, b_6, b_7, y_3 to y_7 and His, which is consistent with a modification to His^6. All of the modified ions showed an increase in mass units of 2 Da when compared with those in the PSD spectra of H1−H5 (Figure 7A). This confirmed that two hydrogen atoms were added to the aldehyde carbonyl of HNE at His^6. The intense peak at *m/z* 1046.5 (the MH^+ of Ang II, Figure 7A) was markedly decreased after sodium borohydride

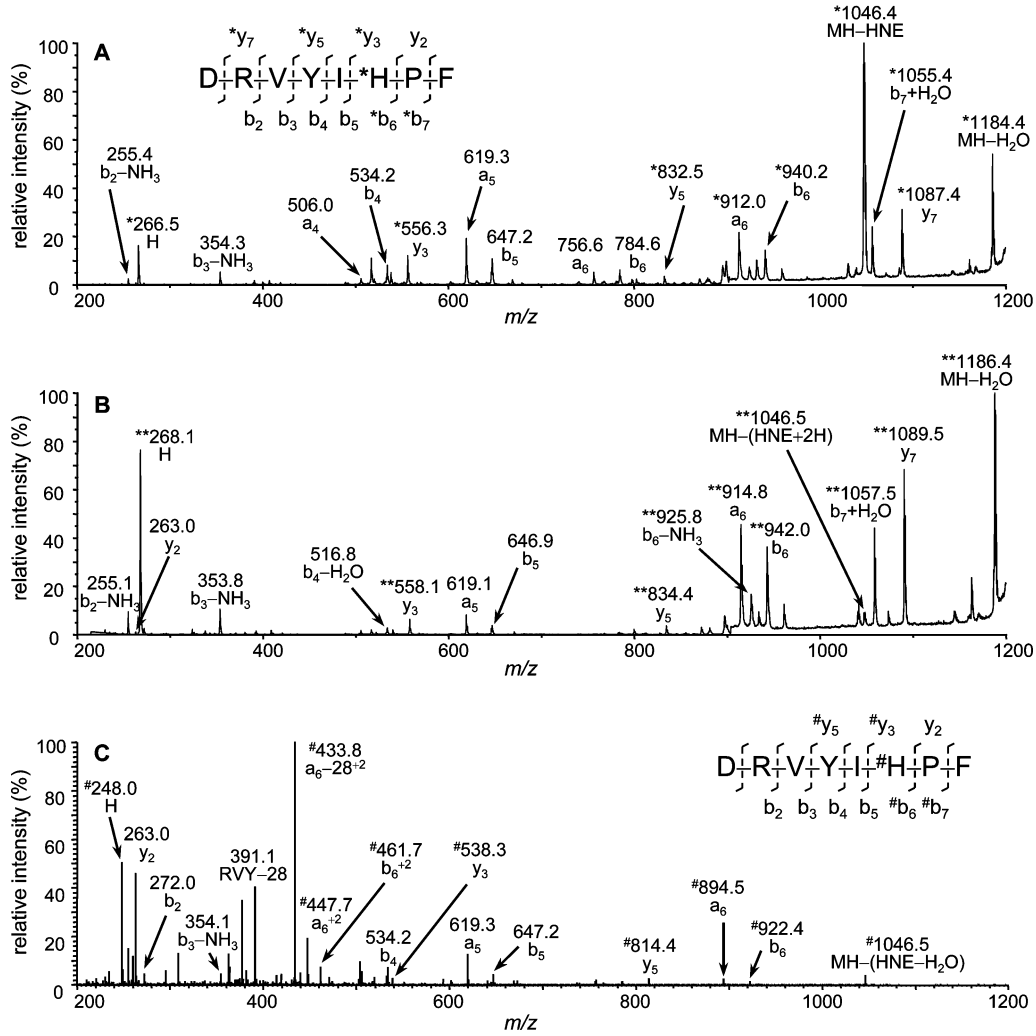


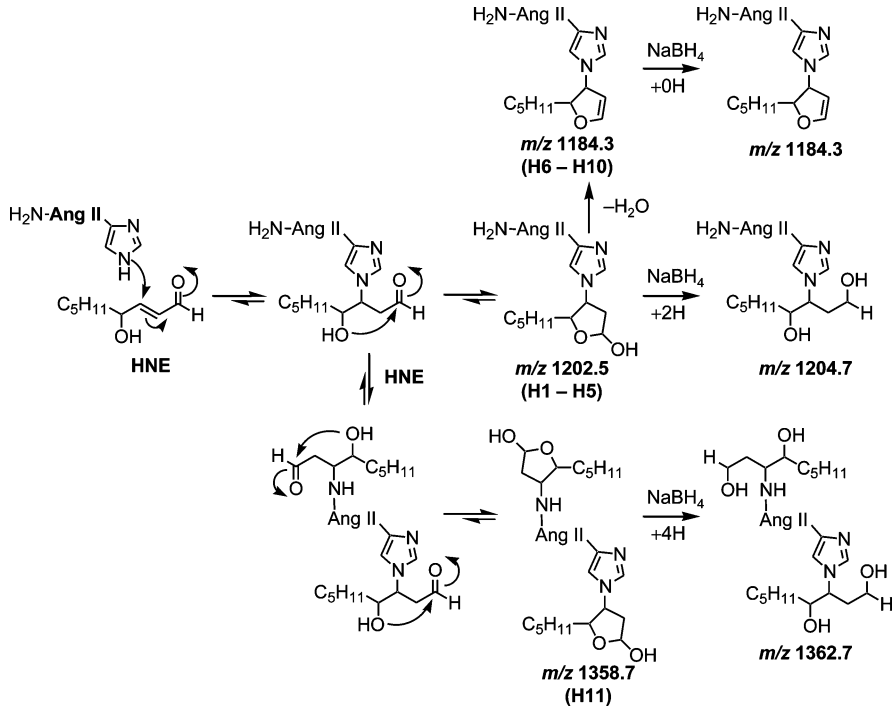
Figure 7. MALDI-PSD-TOF/MS analyses of the HNE-modified Ang II ([His^6(HNE)]-Ang II, H1 as the representative of H1−H5) (A) before (MH^+; *m/z* 1202.5) and (B) after (MH^+; *m/z* 1204.7) reduction with $NaBH_4$. * represents a modified ion (+ 156 Da). ** represents a modified ion (+ 156 Da + 2H). (C) LC-ESI/MS/MS analysis of the HNE-modified Ang II ([His^6(HNE−H_2O)]Ang II, H6 as the representative of H6−H10) before and after (MH^+; *m/z* 1184.6) reduction with $NaBH_4$. # represents a modified ion (+ 138 Da). The *m/z* value of each peak indicates the monoisotopic mass observed.

reduction (Figure 7B), which indicates that the addition of HNE occurred via a Michael addition mechanism (*33*). On the basis of these results, the most polar HNE-modified peptides H1−H5 were characterized as isomers of Michael addition products of [His^6(HNE)]-Ang II (Scheme 2).

The LC-ESI/MS/MS analysis of *m/z* 1184.3, an MH^+ of HNE-modified peptides eluting at 14.0 (H6), 14.2 (H7), 17.9 (H8), 19.3 (H9), and 20.1 min (H10), revealed identical PSD spectra for H6−H10 that showed modifications occurred also at His^6 of Ang II (Figure 7C). Thus, the ions of a_3 to a_5, b_2 to b_5, and y_2 were detected intact. The observed modified ions were a_6, a_7, b_6, b_7, and y_3 to y_5 with an increase mass of 138 Da (+ [HNE−H_2O]). An intense peak for the immonium ion of modified His (His + [HNE−H_2O]) was also observed at *m/z* 248.0. MALDI-TOF/MS analyses of the products from the reduction of H6−H10 revealed one major product with an MH^+ at *m/z* 1184.3, which is identical to the parent H6−H10. This confirmed that no hydrogen was added by the reaction with sodium borohydride. The MS/MS spectra of the products were also identical to those obtained for H6−H10 (Figure 7C). It has been reported that the dihydrofuran, a dehydration product of the cyclic hemiacetal form of the HNE Michael addition products, is inert to sodium borohydride reduction (*33*). On the basis of these results, the HNE-modified peptides H6−H10 were characterized as isomers of dehydrated Michael addition products of [His^6(HNE−H_2O)]-Ang II (Scheme 2).

The MALDI-PSD-TOF/MS analysis of *m/z* 1358.7, an MH^+ of HNE-modified peptide (H11) eluting at 26.1 min, revealed that one of modifications occurred at His^6 of Ang II (Figure 8A). Modified ions of y_3, y_6, y_7, and His with an increase mass of 156 Da (+ HNE) were observed. The peaks at *m/z* 1202.3 and at *m/z* 1046.5 generated from neutral losses of one and two HNE molecules, respectively, indicate both HNE additions occurred via a Michael addition mechanism. However, another site of modification (+ HNE) could not be identified from the spectrum shown in Figure 8A. MALDI-TOF/MS analysis of the product from the reduction of H11 revealed that one major product was formed with an MH^+ at *m/z* 1362.7, corresponding to the addition of four hydrogen atoms. MALDI-PSD-TOF/MS analysis of *m/z* 1362.7 resulted in the generation of informative ions for structural identification (Figure 8B). Most of ions detected were modified. Ions of a_3 to a_5 and b_3 to b_5 were modified with a mass increase of 158 Da (+ HNE + 2H) and ions of a_6, a_7, b_6, and b_7 were modified with an increase mass of 316 Da (+ 2HNE + 4H), which is consistent with a modification to His^6 and another modification to one of five

Scheme 2. Proposed Mechanism for the Formation of HNE-Modified Ang II and Their $NaBH_4$ Reduction Products[a]



[a] The *m/z* values represent MH^+ of the monoisotopic ion.

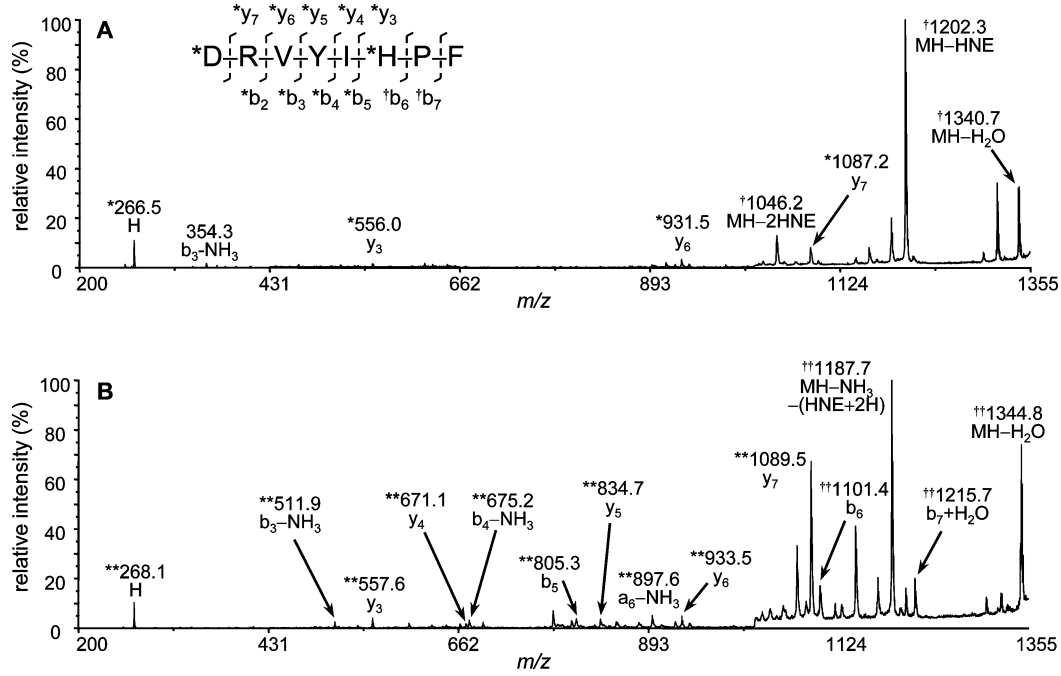


Figure 8. MALDI-PSD-TOF/MS analyses of the HNE-modified Ang II ([N-HNE, His^6(HNE)]-Ang II, H11) (A) before (MH^+; *m/z* 1358.7) and (B) after (MH^+; *m/z* 1362.7) reduction with $NaBH_4$. * represents a modified ion (+ 156 Da). † represents a modified ion (+ 312 Da). ** represents a modified ion (+ 156 Da + 2H). †† represents a modified ion (+ 312 Da + 4H). The *m/z* value of each peak indicates the monoisotopic mass observed.

amino acids from the N-terminus. The ions of y_3 to y_7 modified with an increased mass of 158 Da confirmed a modification occurred at the N-terminus. On the basis of these results, the least polar HNE-modified peptide H11 was characterized as a Michael addition product of [N-HNE, His^6(HNE)]-Ang II (Scheme 2).

Time Course for the Formation of HNE-Modified Ang II. The reaction between Ang II and HNE in phosphate buffer (pH 7.4) at 37 °C was monitored by LC/UV (235 nm) for 14 days. After 40 min of incubation, an LC/UV chromatogram revealed the presence of additional products (H12−H15, Figure 9A) together with isomers of Michael addition products, [His^6(HNE)]-Ang II (H1−H5). Each peak of H12−H15 was isolated using LC system 2. However, they were not stable enough to be rechromatographed by LC/UV. Therefore, the reaction mixture was analyzed by LC-ESI/MS after the incubation for 40 min. The ESI/MS spectra of the HNE-modified peptides (H12−H15) revealed an identical MH^+ at *m/z* 1184.3 corresponding to an increase in mass of 138 Da (+ [HNE−H_2O]) from Ang II. The LC-ESI/MS/MS analysis

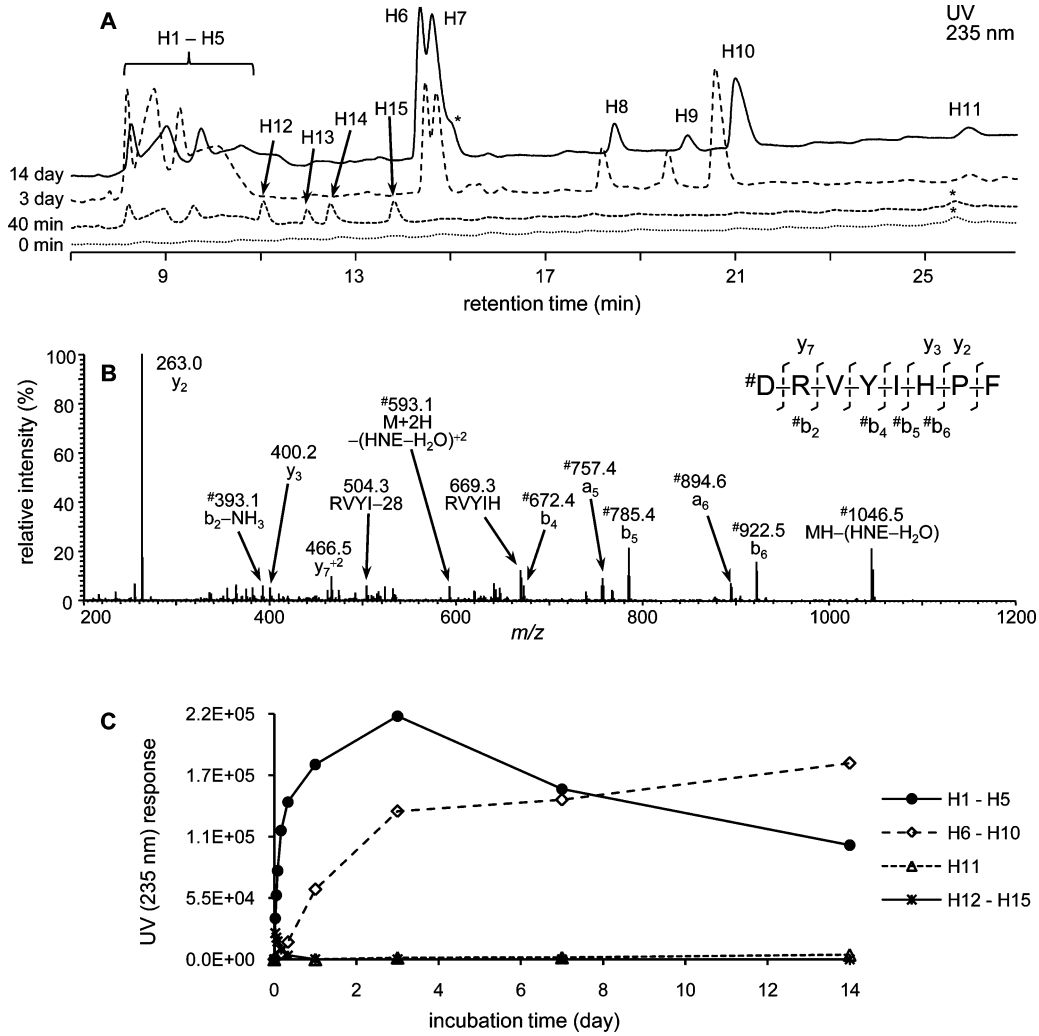


Figure 9. (A) LC/UV (235 nm) analyses of the reaction between Ang II and HNE at 37 °C for 14 days. * represents background interference from the LC/UV system. (B) LC-ESI/MS/MS analysis of the HNE-modified Ang II ([N-(HNE−H_2O)]-Ang II, H12 as the representative of H12−H15) for MH^+ at *m/z* 1184.6. # represents a modified ion (+ 138 Da). The *m/z* value of each peak indicates the monoisotopic mass observed. (C) Time course for the formation of HNE-modified Ang II (H1−H5, H6−H10, H11, and H12−H15) at 37 °C for 14 days.

of *m/z* 1184.3 revealed that modifications occurred at the N-terminus of Ang II (Figure 9B). Thus, all of the b-ions that were detected (b_2 to b_6) appeared with a mass increase of 138 Da in comparison with the corresponding b-ions of Ang II. However, the y-ions (y_2, y_3, and y_7) and internal fragment ions (RVYI−28, RVYIH) remained unmodified. The peaks at *m/z* 1046.5 and 593.1, corresponding to the MH^+ and $(M + 2H)^{+2}$ of the parent peptide, respectively, were generated via neutral loss of the HNE molecule. The reaction mixture was then subjected to sodium borohydride reduction, and each peak was isolated using LC system 2. MALDI-TOF/MS analysis of reduced H12−H15 revealed an identical MH^+ at *m/* 1186.4 that corresponded to the addition of two hydrogen atoms. The PSD/MS spectra of *m/z* 1186.4 were consistent with modification of the N-terminus and confirmed that two hydrogen atoms were added to the N-terminus (data not shown). On the basis of these results, the HNE-modified peptides H12−H15 were characterized as Schiff base adducts of [N-(HNE−H_2O)]-Ang II. As expected from their instability during the isolation process, the formations of H12−H15 rapidly decreased after reaching their maximum levels at 40 min of incubation and could not be detected after 24 h (Figure 9C). At early incubation times, [His^6(HNE)]-Ang II (H1−H5) were the major products followed by [His^6(HNE−H_2O)]-Ang II (H6−H10) (Figure 9C). Maximum levels of H1−H5 were observed after 3 days of incubation. There was then a gradual decrease in the formations of H1−H5. In contrast, the formations of H6−H10 increased steadily reaching maximum levels after 14 days. H6−H10 became the most abundant products after 7 days of incubation. The formation of H11 also increased over time, although it was the least abundant product (Figure 9C).

Prolonged Incubation of Isolated HNE-Modified Ang II. To test the possibility that H1−H5 might be the precursors of H6−H10 and H11, each of the isolated H1−H5 was incubated in phosphate buffer (pH 7.4) for 7 days and analyzed by LC/UV (235 nm) using LC system 2 (Figure 10). The identities of incubation products were confirmed by MALDI-TOF/MS and PSD/MS analyses after isolation and by coinjection with isolated H6−H11. The prolonged incubations of individual H1, H2, H3, H4, and H5 resulted in the formation of H7 and H11 (Figure 10a); H6, H10, and H11 (Figure 10b); H8−H11 (Figure 10c); H10 and H11 (Figure 10d); and H10 and H11 (Figure 10e), respectively.

LC-ESI/MS and MS^n Analyses of the Reaction between *t*Boc-His and HNE. LC-ESI/MS analysis of the products from the reaction between *t*Boc-His and HNE at 37 °C for 7 days revealed the presence of one major product with MH^+ at *m/z* 412.2 (t_R = 13.7 min, + 156 Da; *t*Boc-His-HNE) (Figure 11A). Residual *t*Boc-His (MH^+, *m/z* 256.1, t_R = 8.9 min) was also

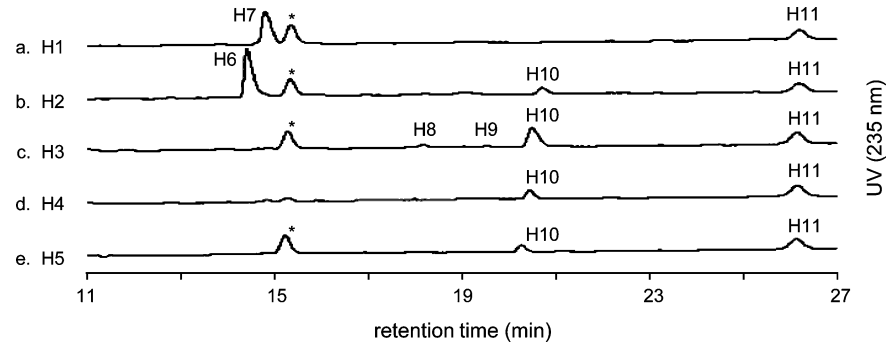


Figure 10. LC/UV (235 nm) analyses for the transformation of H1−H5 (a−e) to H6−H10 after the incubation at 37 °C for 7 days. * represents the background interference from the LC/UV system.

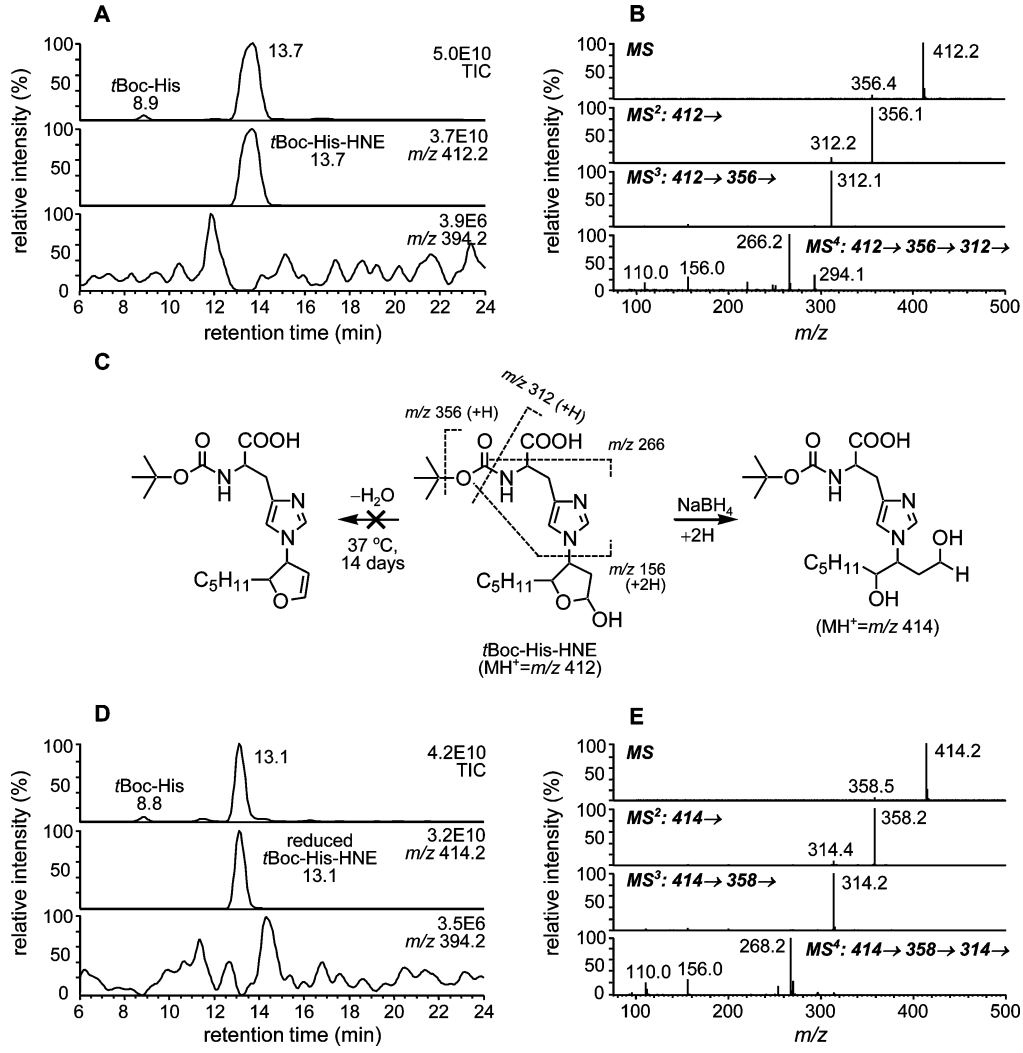


Figure 11. (A) LC-ESI/MS analysis of the reaction between *t*Boc-His and HNE at 37 °C for 7 days. (B) MS^n analyses of *t*Boc-His-HNE. (C) Proposed structure of *t*Boc-His-HNE and reduction product of *t*Boc-His-HNE with $NaBH_4$. (D) LC-ESI/MS analysis of the reaction between *t*Boc-His and HNE at 37 °C for 7 days after reduction with $NaBH_4$. (E) MS^n analyses of *t*Boc-His-HNE after reduction with $NaBH_4$.

observed. The mass spectrum of *t*Boc-His-HNE (Figure 11B) showed an intense MH^+ at *m/z* 412.2 with a minor fragment ion at *m/z* 356.4 ($MH^+ - t\text{Bu} + H$) (Figure 11C). MS^2 analysis of MH^+ resulted in the formation of *m/z* 356.1 as a major product ion with a lower abundance ion at *m/z* 312.2 ($MH^+ - t\text{Boc} + H$). MS^3 analysis of *m/z* 356.1 revealed a product ion at *m/z* 312.1. Finally, the MS^4 spectrum of *m/z* 312.1 showed an intense product ion at *m/z* 266.2 ($MH^+ - t\text{Boc} - \text{COOH}$) with minor ions at *m/z* 294.1 ($MH^+ - t\text{Boc} - H_2O + H$), *m/z* 156.0 ($MH^+ - t\text{Boc} - \text{COOH} - \text{HNE} + 2H$), and *m/z* 110.0 (an immonium ion of HNE). However, the formation of a dehydrated Michael addition product (*t*Boc-His-HNE$-H_2O$) was not detected by LC-ESI/MS during 14 days of incubation (Figure 11C). LC-ESI/MS analysis of the reaction mixture after treatment with sodium borohydride showed an exclusive conversion of *t*Boc-His-HNE to its reduced form with MH^+ at *m/z* 414.2, corresponding to the addition of two hydrogen atoms (Figure 11D). The MS^n analyses of *m/z* 414.2 (Figure 11E) revealed a series of product ions similar to those observed for *t*Boc-His-HNE (Figure 11B). The intense product ion at *m/z* 268.2 in the

MS^4 analysis confirmed the addition of two hydrogen atoms to the hemiacetal form of the HNE moiety.

Discussion

ONE and HNE can be found at the range of nanomolar to lower micromolar in various biological systems, and their levels are increased significantly under oxidative conditions (*19, 35*). The plasma concentration of Ang II in normal human subjects is reported to be in the picomolar to nanomolar range (*36*−*38*). Elevated levels of Ang II in 3- to 5-fold have been detected under various conditions with endothelial activation (*37, 38*). It has been demonstrated that the Ang II concentrations in interstitial fluid (in the nM range) are much higher than the plasma concentrations (*39*). Ang II is also synthesized in various local tissues such as the heart, brain, and kidneys. However, its tissue levels have not been reported. In accordance with this information, we have used 3-fold molar excess of aldehydes for the reaction with Ang II. As for the Ang II, one millimolar concentration was employed for the better characterization of modifications to Ang II. The reactions were also conducted with 100 μM and 10 μM of Ang II in order to confirm the modification profile with more physiologically relevant conditions. The LC-ESI/MS analyses of these reactions revealed the presence of the same major products (O1−O4, H1−H10) as detected in the millimolar scale reaction except for H11. The levels of modified Ang II formed in the reaction of 10 μM Ang II with aldehyde were close to the detection limit of our current LC-ESI/MS conditions. The reactions of Ang II (10 μM) with ONE or HNE in the presence of GSH (10 μM, an average level in plasma) were shown to yield about 65% of modified-Ang II when they were compared with the reactions in the absence of GSH. These results suggest that Ang II could compete for reactive aldehydes with an equimolar amount of GSH.

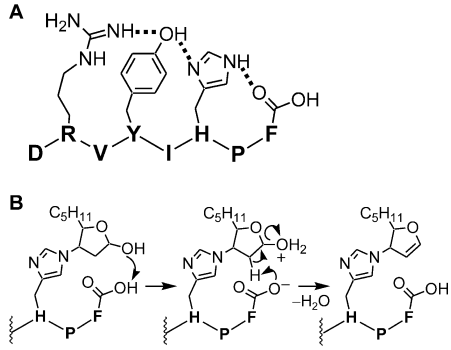
Zhu and Sayre (*34*) have investigated the reaction between N^{α}-hippuryl-L-Lys (Hip-Lys) and ONE after quenching with sodium borohydride at various time points for 50 h. The identities of three major products (Michael addition product, Schiff base, and 4-ketoamide) were confirmed. The time course experiments showed that the Schiff base was the most abundant product at early reaction times, followed by the Michael addition product and the 4-ketoamide. The formations of the Schiff base and the Michael addition product were then rapidly decreased to be undetectable by 10 h when the 4-ketoamide appeared as the major product. Its formation increased steadily to reach a maximum after 50 h, which confirmed the 4-ketoamide as the most stable Lys adduct formed by ONE. Ang II does not contain a Lys residue. However, its N-terminus was shown to be one of the major sites for the reaction with ONE. Three products O1, O2, and O3 were identified as the ONE-derived N-terminal-modified Ang II. Their modifications were characterized by MS, MS/MS, and PSD/MS before and after the sodium borohydride reaction of each isolated compound. The most polar ONE-modified Ang II (O1) has been characterized as a pyruvamide-Ang II (*21*). The initial reaction of ONE with the N-terminal α-amino group of Ang II results in the formation of a Schiff base. Unlike the Lys-ONE Schiff base, it contains an adjacent α-proton, which facilitates tautomerization and decarboxylation of N-terminal Asp (*21*). The subsequent hydrolysis of the resulting imine yields a stable pyruvamide-Ang II (O1) (Scheme 1). As shown in Figure 5, the pyruvamide-Ang II (O1) formed rapidly from the early reaction time, and it was the most abundant product throughout the incubation. The Michael addition product of [N-ONE]-Ang II (O2) was not stable (Figure 5), as expected from the results of previous studies (*34, 40*). The 4-ketoamide of [N-ONE]-Ang II (O3) was the least abundant product in the beginning of the reaction. Its formation increased gradually to reach a maximum level after 48 h (Figure 5), which indicates stability similar to that of Hip-Lys-ONE 4-ketoamide (*34*).

It has been reported that Arg is the least reactive amino acid among the amino acid nucleophiles that can react with ONE or HNE (*11, 41*). The isolation of adducts generated from the reaction of Arg models with ONE or HNE was often found to be difficult. Accordingly, little information has been available on the physiological significance of ONE- and HNE-derived modifications to Arg. Recently, Doorn and Petersen (*41*) have demonstrated the specific reactivity of Arg toward ONE using model peptides, although there are studies showing the formation of HNE-Arg adduct on apolipoprotein B and cytochrome *c* (*42, 43*). Our previous study on the reaction of ONE with *t*Boc-Arg has revealed the formation of a stable adduct containing a substituted imidazole ring through the initial reaction of the guanidido group of Arg at the C-1 aldehyde followed by an intramolecular Michael addition and dehydration (*20*). The tetra peptide LRDE was also shown to react with ONE primarily at Arg to form a stable adduct under physiological conditions (pH 7.0, 37 °C, 36 h) (*20*). We have confirmed in the present study that the Arg-modified [Arg^2(ONE−H_2O)]-Ang II (O4) was formed as one of the major products from the reaction between Ang II and ONE. This product is stable enough to be isolated, which will allow us to test its biological activity on the RAS system.

The model reaction with *t*Boc-Arg and ONE was performed in the same experimental conditions (reactant concentration, reaction buffer, temperature, and time) used for the Ang II reaction. The reaction was monitored by LC-ESI/MS, and the products were characterized by MS^n analyses. *t*Boc-Arg-ONE B, a structure analogous to [Arg^2(ONE−H_2O)]-Ang II (O4), was found as a major product after 48 h of incubation (Figure 6A). It was shown to be converted from *t*Boc-Arg-ONE A by dehydration (Figure 6B), which agreed with the results of a previous study (*20*). In the reaction between Ang II and ONE, [Arg^2(ONE)]-Ang II, an expected precursor of O4, was also detected by LC-ESI/MS analysis (data not shown). MS^n analyses of *t*Boc-Arg-ONE B after the sodium borohydride treatment confirmed the addition of two hydrogen atoms to the heptanone moiety at the substituted imidazole ring (Figure 6D,E). Therefore, the results of this model reaction further support the identity of [Arg^2(ONE-H_2O)]-Ang II (O4) formed in the Ang II reaction.

Uchida and Stadtman (*44*) have demonstrated that the His residue is an important target for the modification by HNE in addition to the sulfhydryl group and primary amino group. They have also proposed that the modification of the His residue by HNE involves a Michael addition of the imidazole group to the double bond of HNE, followed by the reaction of the aldehyde group with the C-4 hydroxyl group of HNE to form a cyclic hemiacetal. The cyclic hemiacetal structure of HNE-His imidazole (N^{π}) Michael addition products were then confirmed by Nadkarni and Sayre (*45*), and their configurational isomers were further characterized by Hashimoto et al. (*46*). The HNE-His Michael addition product formed in the protein/peptide is composed of multiple (at least eight) stereoisomers due to the presence of three chiral centers at C-2, C-4, and C-5 in the tetrahydrofuran moiety, as reported previously (*45, 46*), and the specific conformation of the protein/peptide (*47*). Therefore, the major products formed from the reaction between Ang II and HNE, [His^6(HNE)]-Ang II (H1−H5), represent the isomers of Michael addition product existing as a cyclic

Scheme 3. (A) Schematic Drawing of the Proposed Hydrogen Bonding Interactions (···) among Arg^2, Try^4, His^6, and the C-Terminus of Ang II (*48*−*50*) and (B) Proposed Mechanism for the Dehydration of Michael Addition Product Formed at His^6 of Ang II with HNE



hemiacetal (Scheme 2). It is conceivable that the peaks H1−H5 might contain multiple isomers that were not resolved by the current LC conditions. The formation of a dehydrated Michael addition product was not reported from the reaction of HNE with N^{α}-acetylhistamine or N^{α}-acetyl-L-His at 37 °C for 16 to 24 h (*44*−*46*). It is actually questionable that the dehydration can occur from the tetrahydrofuran moiety since there is no such a driving force. However, the detection of dehydrated HNE-His Michael addition product as a minor product was reported from the incubation of apomyoglobin with HNE at 37 °C for 1 h (*33*). In the reaction of Ang II with HNE at 37 °C for 24 h, we have also observed that the dehydrated His-modified Ang II, [His^6(HNE−H_2O)]-Ang II (H6−H10), was formed as the major products (Figure 1B). When the reaction was continued for 14 days, their formations (especially, H6 and H7) gradually increased (Figure 9A,C). In contrast, there was a concomitant decrease in the levels of H1−H5, suggesting that H1−H5 could be the precursors of H6−H10. To check this possibility, the individual H1−H5 was incubated for 7 days. The Michael addition products H1−H5 were indeed dehydrated to form H6−H10 (Figure 10). The identity of the dehydrated Michael addition product was further confirmed by the reaction with sodium borohydride that resulted in no addition of the hydrogen atom. In addition, the HNE released by a retro-Michael reaction underwent a Michael-type addition to the N-terminus of [His^6(HNE)]-Ang II to generate [N-HNE, His^6(HNE)]-Ang II, H11 (Figure 10).

However, the LC-ESI/MS analysis of the model reaction with *t*Boc-His and HNE revealed an exclusive formation of *t*Boc-His-HNE, a structure analogous to [His^6(HNE)]-Ang II (H1−H5) (Figure 11A). It was not dehydrated to form a [His^6(HNE-H_2O)]-Ang II (H6−H10)-like compound even after 14 days of incubation (Figure 11C). These results indicate that a dehydration of the Michael addition product in Ang II reaction could be induced by the special conformation of Ang II. Indeed, recent conformational studies on Ang II have proposed a bioactive conformation of Ang II as a Tyr^4-Ile^5-His^6 bend, a major His^6-Pro^7 trans conformer, and a cluster of the side chain aromatic rings of the triad key amino acids Tyr^4, His^6, and Phe^8 (*48*−*50*). This proposed conformation is stabilized by guanidinium (Arg^2)-phenolate (Tyr^4) electrostatic and hydrogen bonding interactions as well as hydrogen bonds between hydroxylate (Tyr^4) and imidazole (His^6) and carboxylate (Phe^8) (*48*) (Scheme 3A). This means that the hydroxyl group on the hemiacetal ring formed by the reaction of His^6 with HNE can be easily protonated by the C-terminal carboxyl group, which facilitates its dehydration reaction (Scheme 3B).

As for the reaction of HNE with primary amines, the Michael addition product can be formed reversibly (*11, 45*) in the early stage of the reaction. Also, the formations of stable 2-pentylpyrroles through the Schiff base intermediate have been observed in the HNE-treated proteins as well as model primary amines (*33, 51, 52*). In our experiment, the HNE-derived modifications on N-terminal Ang II were detected as minor products. The formation of Schiff base adducts [N-(HNE−H_2O)]-Ang II (H12−H15) was observed after 40 min of incubation, followed by the decline to undetectable levels after 24 h. However, the 2-pentylpyrroles were not detected by current LC/UV or LC/MS conditions even after 14 days of prolonged incubation. The Michael addition of N-terminal Ang II to HNE was found only as a bis(HNE-modified) product of [N-HNE, His^6(HNE)]-Ang II (H11) (Scheme 2).

In summary, we have identified pyruvamide-Ang II (O1), the 4-ketoamide form of [N-ONE]-Ang II (O3), the Arg-modified [Arg^2(ONE−H_2O)]-Ang II (O4), and the dehydrated Michael addition products of [His^6(HNE−H_2O)]-Ang II (H6−H10) as the major stable adducts from the reaction of Ang II with lipid hydroperoxide-derived ONE or HNE. The model reactions of *t*Boc-Arg with ONE and *t*Boc-His with HNE were also carried out to confirm the unexpected formation of O4 and H6−H10 as major products. *t*Boc-Arg was shown to readily react with ONE to form *t*Boc-Arg-ONE B as Arg^2 of Ang II reacted with ONE. The exclusive formation of the Michael addition product *t*Boc-His-HNE suggested the specific Ang II conformation-induced dehydration of the Michael addition product. Therefore, our results revealed a possible discrepancy in the adduction chemistry of ONE and HNE for model amino acids and endogenous bioactive peptides, which could be governed by the specific amino acid sequence and conformation of the peptides. In the RAS system, the majority of the physiological effects of Ang II on cardiovascular, endocrine, and neuronal systems have been attributed to its interaction via the AT_1 receptor (*3, 6, 8*). Numerous studies conducted to establish the intrinsic roles of each amino acid residue of Ang II have suggested that the N-terminal Asp^1, Arg^2, and His^6 play key roles in the interaction with the AT_1 receptor (*28*−*32*). These studies suggest that the ONE- or HNE-derived modifications to Ang II identified in this study could modulate the biological activity of Ang II by disrupting the interaction with the AT_1 receptor. Ang II is further metabolized to Ang III (Arg-Val-Tyr-Ile-His-Pro-Phe) by aminopeptidase A (APA)-mediated cleavage of the N-terminal Asp residue. Ang II can also be converted to Ang A (Ala-Arg-Val-Tyr-Ile-His-Pro-Phe) through the action of an unknown decarboxlyase in human mononuclear leukocytes on Asp^1. Ang III and Ang A were recently proposed as important regulators of blood pressure (*53*−*55*). The ONE-derived N-terminal modified Ang II (O1 and O3) could inhibit the enzyme activity of APA or the unknown decarboxylase and affect the regulation of cardiovascular function. Therefore, ongoing studies in our laboratory are examining such biological effects of ONE- and HNE-modified Ang II on the RAS system.

Acknowledgment. We gratefully acknowledge the support of a Suzuken Memorial Foundation Grant and KAKENHI (Grant-in-Aid for Scientific Research C: 10004651). We thank the Department of Instrumental Analysis (Technical Division, School of Engineering) and the Biomedical Research Core (School of Medicine) at Tohoku University for the use of MALDI-TOF/MS. We also thank Dr. Ian A. Blair, University of Pennsylvania, for his kind donation of LCQ-DECA with HPLC systems.

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