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Charge Derivatized Amino Acids Facilitate Model Studies on Protein Side-Chain Modifications by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

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Abstract

The α -amino groups of histidine and lysine were derivatized with *p*-carboxylbenzyltriphenylphosphonium to form the pseudo dipeptides, PHis and PLys, which can be sensitively detected by MALDITOF mass spectrometry due to the fixed positive charge of the phosphonium group. Detection limits of PHis and PLys by MALDITOF mass spectrometry were both 30 fmol with a signal-to-noise ratio of 5:1. These pseudo dipeptides were excellent surrogates for His or Lys-containing peptides in model reactions mimicking proteins with reactive electrophiles, prominently those generated by peroxidation of polyunsaturated fatty acids including 4-hydroxy-2(*E*)-nonenal (HNE), 4-oxo-2(*E*)-nonenal (ONE), 2(*E*)-octenal, and 2(*E*)-heptenal. An air saturated solution of linoleic acid ($d_0:d_5 = 1:1$) was incubated in the presence of Fe(II) and ascorbate with these two pseudo dipeptides, and the reaction products were characterized by MALDITOFMS and LCESIMS. By using PHis and PLys, the previously reported ONE-derived His-furan adduct was detected along with evidence for a cyclic α,β -unsaturated ketone. A dimer formed from ONE was found to react with PHis through Michael addition. Alkenals were found to form two novel adducts with PLys. 2(*E*)-Octenoic acid–His Michael adduct and *N*^ε-pentanoyllysine were identified as potential protein side-chain adducts modified by products of linoleic acid peroxidation. In addition, when PHis or PLys and AcHis or BocLys were exposed to the linoleic acid peroxidation, an epoxy-keto-octadecenoic acid mediated His–His cross-link was detected, along with a His–ONE/9,12-dioxo-10-dodecenoic acid–Lys derived pyrrole cross-link was observed.

Keywords

MALDITOF; phosphonium; amino acid; modification; lipid peroxidation; linoleic acid; cross-link

Lipid peroxidation by reactive oxygen species (ROS) generated during oxidative stress is suggested to play a causative role in many aging and neurodegenerative diseases.^{1–5} Free radical mediated lipid peroxidation releases a number of reactive bifunctional aldehydes such as acrolein, 4-hydroxy-2(*E*)-nonenal (HNE) and 4-oxo-2(*E*)-nonenal (ONE), which are capable of covalently modifying proteins.^{6–16} However, most previous studies have incubated amino acids/peptides/proteins with individual purified aldehydes to identify specific protein modifications. We are unaware of any systematic studies on protein side-chain modification by the heterogeneous ensemble of lipid peroxidation products created by

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polyunsaturated fatty acid oxidation. Our purpose is to characterize the potential protein side-chain modifications generated by exposure to lipid peroxidation products using simple model peptides to facilitate characterization of the modified structure and then use these known modifications to characterize modification of proteins from both *in vitro* and *in vivo* reactions.

Mass spectrometry is one of the commonly used tools to study covalent protein modifications.^{7, 17, 18} Since specific protein modifications may be present at low levels following exposure to lipid peroxidation products, a mass spectrometry-sensitive model peptide will enhance the ability to detect and characterize the resulting products. Charge derivatized peptides were extensively studied and showed an enhanced response in mass spectrometry.¹⁹⁻²⁴ Among them triphenylphosphonium (TPP) derivatized peptides displayed an enhanced sensitivity in MALDITOFMS since the quaternary phosphonium ion only requires the desorption step, obviating the need for protonation.²¹

Therefore, we have prepared TPP-derivatized amino acids to serve as “model peptides” in this study. His and Lys residues have been reported to be modified by various lipid peroxidation products.^{6-8, 11, 12, 15, 16} Both present nucleophilic side chains in most proteins which are reactive towards α,β -unsaturated aldehydes, making these amino acids the subjects of this study. Since the α -amino groups of His and Lys can also react with lipid peroxidation products, the TPP group is appended to the α -amine through a peptide bond so that interference by α -amine modification will be eliminated. The ethyltriphenylphosphonium group used by Liao and Allison²¹ has been replaced by a *p*-carboxybenzyltriphenylphosphonium group, which has the twin advantages of a greater molecular weight (MW) which elevates its derivatives above the noise of matrix-cluster peaks in the low *m/z* range of the MALDITOF mass spectrum²⁵ and coupling via a peptide bond to more closely reflect the reactivity of the His and Lys residues present in proteins and peptides.

In this study, these model dipeptides were first incubated with well-known α,β -unsaturated aldehydes such as HNE ($d_0:d_{11} = 1:1$), ONE ($d_0:d_9 = 1:1$) and alkenals. Then they were exposed to the non-enzymatic oxidative mixture of either d_0 - or d_5 -linoleic acid (LA). LA is the most abundant polyunsaturated fatty acid (PUFA) in mammalian tissue.²⁶ Most degradation products of PUFAs are derived from LA.^{27, 28} Therefore, to understand the role of PUFA peroxidation in the etiology of aging and neurodegenerative diseases, the chemical nature of protein side-chain modification by LA peroxidation products is required.

EXPERIMENTAL

General

α -Bromo-*p*-toluic acid, α -cyano-4-hydroxycinnamic acid (CHCA), 2(*E*)-heptenal (heptenal) and hexanoic anhydride were purchased from Acros Organics. *N* $^{\alpha}$ -Acetyl-histidine (AcHis), *N* $^{\alpha}$ -Boc-lysine (BocLys), 2(*E*)-octenal (octenal), 2(*E*)-octenoic acid and triphenylphosphine were purchased from Aldrich. *N* $^{\epsilon}$ -Boc-Lysine was purchased from Sigma. *N*-Hydroxysuccinimide (NHS) and *N,N'*-dicyclohexylcarbodiimide (DCC) were purchased from Fisher Biotech. H-His(1-Trt)-OH was purchased from Bachem. Valeric anhydride was purchased from Pearson. LA was purchased from Fluka. (17,17,18,18,18- $^2\text{H}_5$)-(9*Z*,12*Z*)-Octadecadienoic acid (d_5 -LA) (X. Zhu, L. M. Sayre, unpublished data), (4-(1-carboxy-5-hexanamidopentylcarbamoyl)benzyl)-triphenylphosphonium bromide (*N* $^{\epsilon}$ -Hexanoyl-PLys)²⁹, HNE³⁰ and ONE³¹ were prepared according to our previous studies. (4-(1-Carboxy-5-pentanamidopentylcarbamoyl)-benzyl)triphenylphosphonium bromide (*N* $^{\epsilon}$ -pentanoyl-PLys) was synthesized according to the similar preparation procedure of *N* $^{\epsilon}$ -hexanoyl-PLys from PLys and valeric anhydride. (5,5,6,6,7,7,8,8,9,9,9- $^2\text{H}_{11}$)-4-

Hydroxy-2(*E*)-nonenal (d_{11} -HNE) and (6,6,7,7,8,8,9,9,9- $^2\text{H}_9$)-4-oxo-2(*E*)-nonenal (d_9 -ONE) were prepared from pentylbromide- d_{11} and butylbromide- d_9 , respectively (unpublished data). ZipTips were purchased from Millipore. All NMR spectra were recorded by Varian Inova 600 or Inova 400 NMR spectrometer.

MALDITOFMS

The matrix solution was 20 mg/mL CHCA in 70% aqueous acetonitrile and 0.1% trifluoroacetic acid (TFA). Sample preparation: 8 μL of the incubation mixture was mixed with 4 μL of 0.1% TFA aqueous solution, and then extracted with a ZipTip which was previously wet by 50% aqueous acetonitrile and equilibrated with 0.1% TFA. The ZipTip was washed with 0.1% TFA several times, and the sample was eluted with 1 μL of CHCA onto the MALDI stainless steel target. The detection limit of PHis and PLys was determined according to the published method.³² Sample preparation for the determination of detection limit: aqueous solutions of various concentrations of PHis or PLys were mixed with CHCA (v/v = 1/1) in a small vial and 1 μL of the resulting mixture was directly spotted on the MALDI stainless steel target. The MALDITOF mass spectra were acquired with a Bruker BiFlex III MALDITOF mass spectrometer equipped with a pulsed nitrogen laser (3 ns pulse at 337 nm) after the samples were dried at 25 °C. All spectra were collected in the positive ion reflectron mode with an average of 250 laser shots. All data were processed with the programs XMass (Bruker Daltonics) and *m* over *z* (Proteometrics, LLC).

LCESIMSMS

Reversed-phase HPLC was performed with a Surveyor LC system equipped with a 5 μm 4.6 $\times$ 250 mm Agilent ZORBAX SB-C18 column with a gradient elution program at a flow rate of 400 $\mu\text{L}/\text{min}$. Eluent A was a mixture of 95% H_2O , 5% MeOH and 0.1% formic acid. Eluent B was a mixture of 95% MeOH, 5% H_2O and 0.1% formic acid. The gradient program was from 70% B for 5 min, 70% B to 100% B over 40 min, 100% B to 70% B over 5 min, 70% B for 5 min. ESI-MS was performed with a Thermo Finnigan LCQ Advantage instrument in the positive ion mode using nitrogen as the sheath and auxiliary gas. The capillary temperature was 300 °C, the capillary voltage was 14.00 V, and the source voltage was 4.50 kV. Typically two scan events were used: (1) *m/z* 400–1000 full scan MS; (2) data dependent scan MS/MS on the most intense ion from event 1 or from the predefined parent mass list. The spectra were recorded using dynamic exclusion of previously analyzed ions for 0.5 min with two repeats and a repeat duration of 0.5 min. The MS/MS normalized collision energy was set to 35%.

Incubation of PHis or PLys and α,β -unsaturated aldehydes with or without NaBH_4 quenching

A solution of PHis or PLys (50 mM, 8 μL) added to pH 7.4 HEPES buffer (100 mM, 172 μL) was mixed with a 20 mM solution of a single α,β -unsaturated aldehyde in EtOH (HNE ($d_0:d_{11}$ = 1:1), ONE ($d_0:d_9$ = 1:1), octenal or heptenal; 20 μL). The mixtures were incubated at 37 °C for 3 d. Half of the reaction mixture (100 μL) was treated with NaBH_4 (2 M in 0.2 M NaOH solution, 10 μL) at 25 °C overnight.

Incubation of PHis, PLys, AcHis or BocLys and d_0 - or d_5 -LA in the presence of Fe(II) and L-ascorbic acid (Asc) with or without NaBH_4 quenching

A solution of PHis, PLys, AcHis or BocLys (50 mM, 24 μL) added to air saturated pH 7.4 HEPES buffer (100 mM, 504 μL) was incubated with a solution of d_0 -LA or d_5 -LA in EtOH (200 mM, 15 μL), $\text{FeSO}_4\cdot(\text{NH}_4)_2\text{SO}_4$ (50 mM, 6 μL), Asc (100 mM, 6 μL) and EtOH (45 μL) at 37 °C. After 3 d, d_0 -LA and d_5 -LA-containing solutions were combined. Half of the

reaction mixture (300 μ L) was treated with NaBH₄ (5 M in 0.2 M NaOH solution, 30 μ L) at 25 °C overnight.

Incubation of PHis or PLys and *d*₀- or *d*₅-LA in the presence of Fe(II), Asc and AcHis or BocLys with or without NaBH₄ quenching

A solution of PHis or PLys (50 mM, 24 μ L) added to pH 7.4 HEPES buffer (100 mM, 480 μ L) was incubated with a solution of *d*₀-LA or *d*₅-LA in EtOH (200 mM, 15 μ L), FeSO₄·(NH₄)₂SO₄ (50 mM, 6 μ L), Asc (100 mM, 6 μ L), EtOH (45 μ L) and AcHis or BocLys (50 mM, 24 μ L) at 37 °C. After 3 d, *d*₀-LA and *d*₅-LA-containing solutions were combined. 300 μ L of the combined aliquot was treated with NaBH₄ (5 M in 0.2 M NaOH solution, 30 μ L) at 25 °C overnight.

Synthesis of (4-carboxybenzyl)triphenylphosphonium bromide (1)

Triphenylphosphine (4.20 g, 16.0 mmol) was added to a solution of α -bromo-*p*-toluic acid (1.72 g, 8.0 mmol) in 60 mL of anhydrous acetonitrile and stirred at 25 °C for 40 h. The reaction mixture was concentrated under reduced pressure, and the residue was washed with ethyl ether until all excess triphenylphosphine was removed. This afforded the titled compound as a white solid (3.80 g, 100%). m.p. 270–271 °C; ¹H NMR (400 MHz, CD₃OD) δ 7.91 (m, 3H), δ 7.85 (d, 2H, *J* = 8.0 Hz), δ 7.65–7.78 (12H), δ 7.11 (d, 2H, *J* = 8.0 Hz), δ 5.03 (d, 2H, *J* = 15.6 Hz); ¹³C NMR (100 MHz, CD₃OD) δ 167.62, 135.43 (d, *J* = 3.0 Hz), 134.22 (d, *J* = 9.9 Hz), 133.01 (d, *J* = 8.3 Hz), 131.18 (d, *J* = 5.3 Hz), 130.29 (d, *J* = 13 Hz), 130.01 (d, *J* = 3.0 Hz), 117.71 (d, *J* = 86.2 Hz), 29.47 (d, *J* = 48.0 Hz); HRMS (FAB) calcd for C₂₆H₂₂O₂P⁺ (M⁺) 397.1352, found, 397.1352.

Synthesis of (4-(1-carboxy-2-(1-trityl-1*H*-imidazol-4-yl)ethylcarbamoyl)benzyl)triphenylphosphonium bromide (2)

1 (478.8 mg, 1.00 mmol), *N*-hydroxysuccinimide (121.6 mg, 1.05 mmol) and DCC (217.7 mg, 1.05 mmol) were dissolved in 10 mL of anhydrous DMF at 0 °C and stirred at 25 °C for 5 h. After the active ester was formed, a solution of H-His(1-Trt)-OH (397.8 mg, 1 mmol) in 40 mL mixture of DMF and pH 9.0 Bicine buffer (0.5 M) (v/v = 1/1) was added at 0 °C, and stirred overnight at 25 °C. The reaction mixture was filtered to remove the dicyclohexylurea, and the filtrate was extracted with 600 mL of CH₂Cl₂. The CH₂Cl₂ solution was concentrated by rotary evaporation, and the residue was purified with flash column chromatography, eluting with CH₂Cl₂/MeOH (v/v = 1/2). This afforded the titled compound as a light yellow solid (440 mg, 51.4%). ¹H NMR (400 MHz, CDCl₃) δ 7.40–7.64 (16H), δ 7.02–7.12 (11H), δ 6.92–7.00 (7H), δ 6.62 (d, 2H, *J* = 6.8 Hz), δ 5.04 (m, 2H), δ 4.50 (dd, 1H, *J*₁ = 5.2 Hz, *J*₂ = 11.2 Hz), δ 3.10–3.26 (2H); ¹³C NMR (100 MHz, CDCl₃) δ 174.61, 165.09, 142.83, 139.06, 137.47, 135.79 (d, *J* = 3.3 Hz), 135.19, 134.38 (d, *J* = 9.9 Hz), 134.05 (d, *J* = 10.0 Hz), 131.02 (d, *J* = 3.3 Hz), 130.38 (d, *J* = 12.2 Hz), 129.97, 128.03, 127.87, 127.53, 119.75, 117.69 (d, *J* = 84.6 Hz), 55.42, 53.76, 32.09, 30.12 (d, *J* = 47.3 Hz); HRMS (FAB) calcd for C₅₁H₄₃N₃O₃P⁺ (M⁺) 776.3037, found, 776.3026.

Synthesis of (4-(1-carboxy-2-(1*H*-imidazol-4-yl)ethylcarbamoyl)benzyl)triphenylphosphonium bromide hydrobromide (Pseudo histidine dipeptide or PHis)

A solution of **2** (380 mg, 0.44 mmol) in 50 mL of HBr (48%) was stirred at 45 °C. After 3 h, it was diluted with 150 mL water, and the mixture was extracted with ethyl ether three times (3×50 mL) and CH₂Cl₂ three times (3×50 mL) sequentially. The aqueous phase was concentrated under reduced pressure. Then a little water was added, and it was concentrated again. This cycle was applied several times to remove HBr. Finally a yellow solid was obtained (270 mg, 87.5%). ¹H NMR (400 MHz, D₂O) δ 8.34 (s, 1H), δ 7.46 (m, 3H), δ

7.20–7.31 (12H), δ 7.16 (d, 2H, J = 8.0 Hz), δ 7.02 (s, 1H), δ 6.72 (dd, 2H, J_1 = 2.0 Hz, J_2 = 8.0 Hz), δ 4.59 (dd, 1H, J_1 = 5.6 Hz, J_2 = 9.2 Hz), δ 4.53 (d, 2H, J = 15.2 Hz), δ 3.15 (dd, 1H, J_1 = 5.2 Hz, J_2 = 15.2 Hz), δ 3.01 (dd, 1H, J_1 = 9.2 Hz, J_2 = 15.2 Hz); ^{13}C NMR (150 MHz, D_2O) δ 173.26, 169.45, 135.19 (d, J = 2.4 Hz), 133.94 (d, J = 9.8 Hz), 133.36, 132.63 (d, J = 4.2 Hz), 131.99 (d, J = 8.6 Hz), 131.25 (d, J = 4.8 Hz), 129.92 (d, J = 12.8 Hz), 128.84, 127.62 (d, J = 3.2 Hz), 117.07, 116.78 (d, J = 86.1 Hz), 52.14, 29.54 (d, J = 48.5 Hz), 26.04; HRMS (FAB) calcd for $\text{C}_{32}\text{H}_{29}\text{N}_3\text{O}_3\text{P}^+$ (M^+) 534.1941, found, 534.1938.

Synthesis of (4-(5-(tert-butoxycarbonylamino)-1-carboxypentylcarbamoyl)benzyl)triphenylphosphonium bromide (3)

1 (1.90 g, 4.0 mmol), *N*-hydroxysuccinimide (0.48 g, 4.2 mmol) and DCC (0.87 g, 4.2 mmol) were dissolved in 24 mL of anhydrous DMF at 0 °C and stirred at 25 °C for 4 h. A solution of *N*^ε-Boc-Lysine (0.98 g, 4.0 mmol) in 140 mL mixture of DMF and pH 9.0 bicine buffer (0.5 M) (v/v = 1/1) was added at 0 °C, and stirred overnight at 25 °C. The reaction mixture was filtered, and the filtrate was extracted with CH_2Cl_2 . The CH_2Cl_2 solution was concentrated under reduced pressure, and the resulting residue was purified by flash column chromatography with ethyl acetate-MeOH (v/v = 4/1). This afforded the titled compound as a yellow solid. ^1H NMR (400 MHz, CD_3OD) δ 7.90 (broad, 3H), δ 7.60–7.82 (14H), δ 7.12 (broad, 2H), δ 5.07 (d, 2H, J = 12 Hz), δ 4.51 (broad, 1H), δ 3.02 (broad, 2H), δ 1.93 (broad, 1H), δ 1.84 (broad, 1H), δ 1.40–1.60 (4H), δ 1.39 (s, 9H); ^{13}C NMR (100 MHz, CDCl_3) δ 175.33, 166.60, 156.37, 135.33, 134.43 (d, J = 9.9 Hz), 134.10 (d, J = 3.3 Hz), 131.48, 130.70 (d, J = 8.2 Hz), 130.41 (d, J = 12.2 Hz), 127.83, 117.42 (d, J = 85.4 Hz), 53.95, 31.79, 30.50, 29.84 (d, J = 45.8 Hz), 28.60, 23.12; HRMS (FAB) calcd for $\text{C}_{37}\text{H}_{42}\text{N}_2\text{O}_5\text{P}^+$ (M^+) 625.2826, found, 625.2820.

Synthesis of (4-(5-amino-1-carboxypentylcarbamoyl)benzyl)triphenylphosphonium bromide hydrobromide (Pseudo lysine dipeptide or PLys)

3 (400 mg, 0.57 mmol) was stirred in 100 mL of aqueous 3 N HBr, heated with a hot gun. During 30 min of heating, the solid gradually dissolved. The solution was stirred at 25 °C for another 1.5 h, then extracted with CH_2Cl_2 five times (5×100 mL). The aqueous phase was concentrated under reduced pressure. A little water was added, and the aqueous solution was concentrated again. This cycle was applied several times to remove HBr. This afforded the titled compound as a yellow solid (390 mg, 100%). ^1H NMR (400 MHz, D_2O) δ 7.52 (m, 3H), δ 7.23–7.35 (14H), δ 6.75 (dd, 2H, J_1 = 2.0 Hz, J_2 = 8.0 Hz), δ 4.52 (d, 2H, J = 15.2 Hz), δ 4.19 (dd, 1H, J_1 = 5.6 Hz, J_2 = 9.2 Hz), δ 2.70 (t, 2H, J = 7.4 Hz), δ 1.69 (m, 1H), δ 1.59 (m, 1H), 1.41 (m, 2H), δ 1.19 (m, 2H); ^{13}C NMR (150 MHz, D_2O) δ 175.21, 169.40, 135.07, 133.74 (d, J = 10.4 Hz), 132.69, 131.54 (d, J = 7.8 Hz), 131.06, 129.83 (d, J = 12.8 Hz), 127.60, 116.61 (d, J = 86.1 Hz), 52.98, 39.01, 29.67, 29.40 (d, J = 49.2 Hz), 26.05, 22.16; HRMS (FAB) calcd for $\text{C}_{32}\text{H}_{34}\text{N}_2\text{O}_3\text{P}^+$ (M^+) 525.2302, found, 525.2297.

RESULTS AND DISCUSSION

Reactions of PHis and PLys with α,β -unsaturated aldehydes

The pseudo dipeptides, PHis and PLys, were synthesized according to Figure 1. Their detection limits were each 30 fmol with a signal-to-noise ratio of 5:1. Their MALDITOF mass spectra (Figure 2) show that there is minimal background above m/z 500. In contrast, there are many matrix-related noise signals lower than m/z 500. If PHis or PLys is modified, the product signals will be at greater m/z in a region unobscured by background or matrix peaks so that even minor peaks will be readily observed.

α,β -Unsaturated aldehydes have been widely reported to modify both protein and DNA. Among them, HNE is one of most abundant and reactive products of lipid peroxidation.

Reactions of HNE with His or Lys are known to form His Michael, Lys Michael and Lys Schiff base adducts.^{7, 10-12, 16} HNE ($d_0:d_{11} = 1:1$) was used in this study because the modified products would appear as doublets ($m/m+11$) and be readily identified. After incubation of HNE ($d_0:d_{11} = 1:1$) and PHis for 3 d with or without subsequent NaBH₄ reduction, the mixtures were subjected to MALDITOFMS. Without reduction the major peaks are the d_0 -HNE or d_{11} -HNE-PHis Michael adducts with mass shifts of 156 and 167 Da, respectively, which appear as a doublet at m/z 690.32/701.43 (data not shown). After incubation with NaBH₄, the carbonyl group of the HNE-PHis Michael adduct is reduced, resulting in an additional mass shift of 2 Da, producing the doublet m/z 692.26/703.36 (data not shown). The spectra primarily contain peaks corresponding to HNE-PHis Michael adducts and potentially two additional minor adducts (<5% relative intensity), which indicates that the modification of PHis reproduces the already identified HNE-derived modifications. Similarly, the reaction of HNE and PLys produces the HNE-PLys Michael adduct as the primary product (data not shown).

ONE was reported to actively modify cysteine, histidine and lysine residues.^{7, 11, 14-16, 31, 33} However, model studies of the reactions of ONE with amino acids to track the stable products surviving long-time incubations that would be more typical of *in vivo* conditions have not been fully characterized. ONE ($d_0:d_9 = 1:1$) and PHis were incubated for 3 d. Before borohydride reduction three doublets ($m/m+9$) and a triplet ($m/m+9/m+18$) appeared in the MALDITOF mass spectrum (Figure 3A), which indicated that four major products were generated. The mass shifts of the doublets m/z 670.30/679.36, 688.33/697.39 and 706.35/715.39 are 136/145 Da, 154/163 Da and 172/181 Da, respectively. These are consistent with the results of Blair and co-workers.³³ Based largely on the observed mass shifts, they proposed that these masses correspond to a furan adduct, a Michael adduct or isomeric cyclic adduct, and a hydrated Michael adduct, respectively. However, after borohydride reduction we found that the doublet m/z 670.30/679.36 ($M+136/145$) was augmented by 2 Da to m/z 672.23/681.29 ($M+138/147$) (Figure 3B). Considering the fact that the furan would not be reduced by NaBH₄, the product must be something else which should contain a borohydride reducible functional group, such as a ketone, aldehyde or Schiff base. A possible structure of this unknown product might be the cyclic α,β -unsaturated ketone **6** (Figure 4A). It was reported that the 4-ketoaldehyde was readily converted to the 2-cyclopentenone derivative through base catalyzed cyclization.³⁴⁻³⁶ Therefore, product **6** is proposed to result from the dehydration of the product **5** which is cyclized from the ONE-His Michael adduct **4**. The fact that the doublet m/z 688.33/697.39 ($M+154/163$) has a 4 Da mass shift after reduction indicates that it is either the ONE-His Michael adduct **4**, as Blair's group reported, or its cyclic product **5**. The doublet m/z 706.35/715.39 ($M+172/181$) corresponds to the hydrated Michael adduct **7a** and/or its isomeric cyclic adduct **7b** (Figure 4B) according to the same report.³³ Our results corroborate this hypothesis. After borohydride reduction **7a** and **7b** would be converted into the reduced Michael adduct **8** (Figure 4B). We show in Figure 3B that the m/z 706.35/715.39 doublet was completely converted to a different adduct, consistent with its dehydration and reduction into **8**.

It is very interesting that a triplet peak m/z 842.49/851.56/860.62 (1/2/1) is observed after incubation of PHis and ONE ($d_0:d_9 = 1:1$) (Figure 3A), demonstrating that this product contains a binomial distribution of two d_0 - or d_9 -ONE molecules. The mass increment is consistent with the His Michael adduct of an ONE condensation dimer (found by our group which will be described elsewhere) which contains an α,β -unsaturated ketone. After reduction, the triplet exhibits a 6 Da mass shift (Figure 3B), consistent with the structure of an ONE dimer-His Michael adduct which has three reducible carbonyls.

After the incubation of PLys and ONE ($d_0:d_9 = 1:1$) for 3 d, two doublets m/z 661.18/670.24 and 679.20/688.26 with almost identical intensities, which correspond to the ONE-Lys pyrrolinone adduct11· 15 and 4-ketoamide adduct15, respectively, were detected (Figure 3C). It is very interesting that after reduction the reduced 4-ketoamide adduct is observed while the peak corresponding to the pyrrolinone adduct is entirely displaced (Figure 3D). We propose that the pyrrolinone adduct is first hydrated to the 4-ketoamide isomer and then trapped by NaBH_4 reduction (Figure 4C).

Alkenals are known to form Michael adducts with His37· 38 and various pyridinium adducts with Lys when high concentrations of alkenals are used.^{39· 40} We used a relatively low concentration of alkenals in this study as their biological concentration is low and our model peptides are highly sensitive to the MALDITOFMS. After incubation of octenal with PHis for 3 d, there is only an abundant singlet with a 126 Da mass shift observed in the MALDI spectrum (data not shown), which matches the PHis Michael adduct of octenal. With reduction, the singlet has a 2 Da mass shift as expected. Interestingly, for the PLys modification by alkenals, two novel adducts are observed. Two major peaks, $M+122$ and $M+140$, are detected in the incubation mixture of PLys and octenal for 3 d by MALDITOFMS (Figure 5A). The MW of octenal is 126 and MW of 2(*E*)-nonenal (nonenal) is 140. These two unanticipated peaks could correspond to a nonenal-PLys Schiff base and a nonenal-Michael adduct, respectively, derived from contaminating nonenal. This hypothesis is untenable, because even if the commercial octenal contains contaminating nonenal, the octenal present should form the homologous Schiff base and Michael adduct with PLys. However, the octenal-PLys Schiff base and Michael adduct are not detected at all. In order to further prove that these products were derived from the major enal, PLys was incubated with heptenal (MW 112) and a similar result was obtained (Figure 5B). After the reduction of the 3 day incubation mixture of PLys and octenal, the $M+122$ peak disappeared and only an abundant $M+142$ peak was observed (data not shown), which corresponds to a 2 Da mass shift of $M+140$. While the unexpected additional mass of 14 Da could be derived by oxidation and subsequent loss of 18 attributed to dehydration, the structures of these two products are still unknown. Further study to elucidate their structures is in progress.

Reaction of PHis and PLys with oxidative products of linoleic acid ($d_0:d_5 = 1:1$)

Degradation of LA by metal-catalyzed oxidation generates a large number of electrophilic aldehydes and ketones,^{27· 41· 42} most of which are able to covalently modify protein nucleophiles. Metal-catalyzed oxidation of LA leads to two classes of oxidative products. One class is the oxidative cleavage product (OCP) which contains either the LA ω -terminus (ω OCP) such as HNE and ONE, or the carboxy terminus (cOCP) such as 9-hydroxy-12-oxo-10(*E*)-dodecenoic acid (HODA) and 9-12-dioxo-10(*E*)-dodecenoic acid (DODE) from the cleavage of the LA carbon chain. Since d_5 -LA has a d_5 -labeled ω -terminus with an isotopic natural abundance carboxy terminus, the modified pseudo dipeptide incorporating an ω OCP should appear as a doublet in the spectrum while a cOCP modification appears as a singlet. The cOCP is always 72 Da greater than its symmetry-related ω OCP analog. Because the ω OCP and cOCP should be generated with approximately equal probability,⁴¹ any doublet ($m/m+5$) derived from the ω OCP should be accompanied by a singlet ($m+72$) derived from the related cOCP.

The second class is comprised of oxidative addition products (OAPs) which incorporate oxygen into LA without cleavage of the carbon chain, such as epoxyketoctadecenoic acids (EKODEs). As the OAPs contain both the ω - and carboxy termini of LA, the modified pseudo dipeptide incorporating an OAP only appears as a doublet ($m/m+5$) without any accompanying peak ($m+72$). This difference generated by isotopic labeling can be used to differentiate modification of peptides by OCPs from OAPs.

Figure 6A is the MALDI spectrum of the incubation mixture of PHis and LA ($d_0:d_5 = 1:1$) under non-enzymatic oxidative conditions for 3 d. There are 11 new doublets ($m/m+5$) and 8 new singlets observed in Figure 6A in contrast to the control (Figure 2A). All 8 singlets in Figure 6A correspond to $m/m+5$ doublets (labeled 1–8) at m/z $m+72$, indicating that these singlets represent cOCP modifications that correspond to ω OCP modifications in doublet peaks 1–8. In contrast, there are no accompanying singlets to the doublets 9–11, which suggests that doublets 9–11 are the products of PHis and OAPs. The identities of all of these doublets ($m/m+5$) and singlets ($m+72$) were investigated by LCESIMS/MS in consideration of the peak intensity, the retention time and the MS/MS spectra. The peak intensities of m and $m+5$ in a doublet are almost equal, but that of the corresponding $m+72$ (if available) is greater. The retention time of m is almost identical to $m+5$ with the latter always eluting slightly earlier than the former as it is common for ^2H -labeling.⁴³ The tandem mass spectra of m , $m+5$ and $m+72$ (if available) have similar fragmentation patterns. After borohydride reduction, doublets 1–3 and 5–11 were reduced resulting in an additional 2 Da mass increment, but doublet 4 was not reducible (data not shown). The m/z of the corresponding singlets representing the cOCP derivative changed accordingly.

The structures of the PHis adducts in Figure 6A are assigned or proposed in Table 1 according to the mass shift and products of LA peroxidation reported by Spiteller and co-workers.^{27, 41, 42} Products 1 and 2 match the PHis Michael adducts of heptenal and octenal, respectively. In order to confirm our hypothesis, both incubation mixtures of PHis, LA, Fe(II) and Asc and PHis and heptenal or octenal were applied to LCESIMS/MS. It showed that both the retention times and tandem mass spectra of heptenal and octenal–PHis Michael adducts were the same as products 1 and 2 in the PHis–LA reaction mixture. In the same way, we confirmed that product 4 was the octenoic acid–PHis Michael adduct and 5 was the HNE–PHis Michael adduct. The octenoic acid–PHis Michael adduct is a novel modification of His by lipoxidation end products. The mechanism of the formation of this Michael adduct is complicated. It can be formed directly from the incubation of 2 mM octenoic acid and PHis (data not shown), but its intensity is much less than that we observed from the incubation of 5 mM LA and PHis in the presence of Fe(II) and Asc. It may also come from the catalytic oxidation of the octenal–PHis Michael adduct since it was detected in the reaction mixture of octenal, PHis, Fe(II) and Asc, but it was not found from the reaction of octenal and PHis.

EKODE is one of the abundant non-enzymatic oxidative products of LA, which can form Michael adducts with an imidazole.⁴⁴ Product 9 has a mass shift of 310 Da before reduction and 312 Da after reduction. This result is consistent with its assignment as the EKODE–PHis Michael adduct. The epoxy group of the EKODE–PHis Michael adduct, may be hydrated to form a dihydroxyketooctadecenoic acid–PHis Michael adduct, and would correspond to the mass increment of doublet 11. This modification has been recently found in modified apomyoglobin by our group (D. Lin, L. M. Sayre, unpublished). The structure of product 10 is not clear, but it has a borohydride reducible group since 2 Da is added after reduction. Figure 6A indicates that the EKODE–His Michael adduct has the highest intensity, followed by the HNE/HODA–His Michael adduct.

Figure 6B shows the MALDI spectrum of the incubation mixture of PLys and LA ($d_0:d_5 = 1:1$) for 3 d. There are 10 new doublets ($m/m+5$) and 10 new singlets ($m+72$) observed in Figure 6B in contrast to the control (Figure 2B). The 10 singlets modified by cOCP are also connected to equivalent doublets 1–10 modified by the corresponding ω OCP. These 30 modified species were all confirmed by LCESIMS/MS. After borohydride reduction, doublets 1 and 2 are not reducible. Doublets 3, 5 and 7 disappear at and near their original location. Doublets 4, 6 and 8–10 exhibit mass shifts of 2 Da. The corresponding $m+72$ singlets change in the same way.

The structures of the PLys adducts in Figure 6B are assigned or proposed in Table 2. Doublets 1 and 2 were unaffected by reduction, so they do not have any borohydride reducible functional groups, reminiscent of the formation of *N*^ε-hexanoyllysine.⁴⁵ *N*^ε-Hexanoyl-lysine would not be reduced by NaBH₄. Moreover, the mass shift of 98 Da matches the formation of *N*^ε-hexanoyl-PLys. LCESIMSMS analysis indicated that both the retention time and tandem mass spectrum of product 2 were the same as those of authentic *N*^ε-hexanoyl-PLys, confirming the structure of 2. In the same way, product 1 is identified as the *N*^ε-pentanoyl-PLys, but *N*^ε-pentanoyl-PLys has a lower intensity than *N*^ε-hexanoyl-PLys. The mechanism of *N*^ε-acylation was clarified recently by Uchida and our group.²⁹ Doublets 3 and 6 match the two major products of PLys and heptenal. LCESIMSMS analysis of the retention time and tandem mass spectra proved that they were identical to the products obtained from reaction with heptenal. Doublets 5 and 8 behave similarly to products 3 and 6, and were confirmed to be the products of octenal by reaction with authentic material. The two major doublets 7 and 9 are identical to the spectrum of the reaction mixture of PLys and ONE, and they are verified to be the pyrrolinone and 4-ketoamide adducts formed from ONE, respectively. In addition, a small EKODE–Lys Michael adduct signal has also been observed, but is not shown in Figure 6B.

Assuming there is no fractionation during sample preparation and common ionization efficiencies, the relative intensities suggest that ONE/DODE–Lys pyrrolinone and 4-ketoamide adducts are the major Lys modifications by the LA peroxidation products. As these modifications are derived from ONE and DODE, it suggests that these may be prominent *in vivo* protein modifications following radical initiated lipoxidation.

Characterization of protein side-chain cross-linking by using PHis and PLys

Proteins can be cross-linked by many aldehydes such as ONE, HNE and acrolein.^{6, 11, 31, 46–52} If PHis or PLys and AcHis or BocLys are incubated with LA (*d*₀:*d*₅ = 1:1), Fe(II) and Asc, any cross-linked product between pseudo dipeptides and AcHis or BocLys by the LA peroxidation product will be readily detected by MALDITOFMS. In order to exclude the non-cross-linked products, spectra of the incubation mixture of individual His or Lys surrogate, LA (*d*₀:*d*₅ = 1:1), Fe(II) and Asc are used as the controls. There are three possible cross-links between Lys and His, i.e. Lys–Lys, His–His and His–Lys cross-links. When PLys and BocLys are exposed to the oxidative mixture of LA to explore Lys–Lys cross-links, no new peaks are observed compared to the controls, which indicates that there is no detectable Lys–Lys cross-link formation by the products of LA peroxidation. However, when PHis and AcHis are exposed to the oxidative mixture of LA to probe for His–His cross-links, a new doublet (*m/z* 1041.7/1046.7) is observed compared to the controls (Figure 7). This doublet must incorporate both PHis and AcHis since it is not detected when either PHis or AcHis is incubated with LA individually. The doublet has a mass shift of 310/315 Da to the total MW of PHis and AcHis which matches the MW of an EKODE modification. Moreover, it is not accompanied by a 72 Da higher mass peak, indicating this peak belongs to an OAP modification. EKODE is an OAP, so the cross-link may come from the reaction of PHis and AcHis together with an EKODE molecule. His can mainly attack the carbon-carbon double bond of EKODE through Michael addition.⁴⁴ The epoxide ring may be also attacked by another His to form a ring opened product. **9** and **10** are proposed structures of the His–His cross-link by EKODE I. This doublet is very weak in contrast to the EKODE–PHis Michael adduct (data not shown), which suggests that the ring opening process is very slow. After reduction, this doublet has a mass increase of 2 Da which further corroborates the proposed cross-linked structure. However, the AcHis–EKODE–AcHis cross-link product, expected to appear as a doublet *m/z* 705.38/710.42, is not observed in both spectra corresponding to Figure 7A and 7C (*m/z* range not shown), indicating that the AcHis–EKODE–AcHis cross-link product is less sensitive to MALDITOFMS than the PHis–

EKODE–AcHis cross-link product, presumably because the former doesn't contain a fixed positive charge. The PHis–EKODE–PHis cross-link product, a doubly charged molecule and expected to appear as a doublet m/z 689.30/691.82, is not observed in both spectra corresponding to Figure 7B and 7C either (m/z range not shown), potentially because the MALDI process produces mostly singly charged ions.^{53, 54}

To detect His–Lys cross-links, either PHis and BocLys or PLys and AcHis can be incubated together. When PHis and BocLys are exposed to the oxidative mixture of LA, a new doublet (m/z 898.55/903.44) absent in the controls is also observed (Figure 8A). Furthermore, it has an accompanying 72 Da greater peak (m/z 970.49). Therefore, this should be due to OCP cross-linking. The mass shift of the doublet is 118/123 Da to the total MW of PHis and BocLys, which is consistent with a His–ONE–Lys pyrrole cross-link.^{6, 11, 31} Results from LCESIMSMS proved that the doublet was the ONE cross-linked PHis and BocLys as both retention time and tandem mass spectrum of m/z 898.55 were the same as those from the ONE-incubated PHis and BocLys. Therefore, the peak m/z 970.49 should be the PHis–BocLys cross-linked product by DODE. In addition, all three peaks were not reducible with NaBH_4 treatment as predicted by the pyrrole structure. When PLys and AcHis are exposed to the LA oxidation mixture, a new doublet plus a singlet (m/z 840.63/845.52/912.54) are also observed as anticipated (Figure 8B). They are the d_0 -ONE, d_5 -ONE and DODE cross-linked PLys and AcHis, respectively.

SUMMARY

The above studies show that PHis and PLys are excellent His and Lys surrogates for the model studies using MALDITOFMS with significant advantages. They have a detection limit of 30 fmol with a signal-to-noise ratio of 5:1. Therefore, the derivatives from PHis and PLys have high sensitivity by MALDITOF mass spectrometry. The sensitivity can obviate the routine use of ZipTips, i.e. the incubation mixture and CHCA solution is mixed with 1:1 ratio (v/v) and directly spotted onto the MALDI stainless steel target, almost all products above are still detected (data not shown). In addition, the singly charged Na^+ or K^+ adducts are totally suppressed by the phosphonium ion, which simplifies the interpretation of the mass spectrum. Moreover, all of the products are distributed in a very “clean” range in which every peak represents a modified pseudo dipeptide.

By incubation of PHis or PLys with HNE ($d_0:d_{11} = 1:1$), ONE ($d_0:d_9 = 1:1$) and octenal for 3 d, every stable product previously reported was detected. Two novel adducts of alkenals and PLys were also observed. Although their structures are still unknown, further work to investigate them is warranted. When these two dipeptides were incubated with LA ($d_0:d_5 = 1:1$) in the presence of Fe(II) and Asc, in addition to previously identified products, new adducts such as octenoic acid–PHis Michael adduct and N^{E} -pentanoyl-PLys were detected. These two pseudo dipeptides can also be used to study potential protein side-chain cross-linking. By exposing PHis or PLys and AcHis or BocLys to the LA peroxidation, a His–His cross-link by EKODE was implicated, and a His–ONE/DODE–Lys pyrrole cross-link was observed.

In addition, the use of $d_0:d_x$ mixture of α,β -unsaturated aldehydes and LA along with determining mass increments resulting from borohydride reduction significantly aided the identification of the structures of His and Lys side-chain modifications.

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Abbreviations

AcHis	<i>N</i> α-acetyl-histidine
BocLys	<i>N</i> α-Boc-lysine
CHCA	α-cyano-4-hydroxycinnamic acid
DCC	<i>N,N'</i> -dicyclohexylcarbodiimide
EKODE	epoxyketoctadecenoic acid
<i>N</i>^ε-Hexanoyl-PLys	(4-(1-carboxy-5-hexanamidopentylcarbamoyl)benzyl)triphenylphosphonium bromide
HNE	4-hydroxy-2(<i>E</i>)-nonenal
<i>d</i>₁₁-HNE	(5,5,6,6,7,7,8,8,9,9,9- ² H ₁₁)-4-hydroxy-2(<i>E</i>)-nonenal
HODA	9-hydroxy-12-oxo-10(<i>E</i>)-dodecenoic acid
DODE	9,12-dioxo-10(<i>E</i>)-dodecenoic acid
LA	linoleic acid
<i>d</i>₅-LA	(17,17,18,18,18- ² H ₅)-(9 <i>Z</i> ,12 <i>Z</i>)-octadecadienoic acid
MW	molecular weight
NHS	<i>N</i> -hydroxysuccinimide
OAP	oxidative addition product
OCP	oxidative cleavage product
ωOCP	oxidative cleavage product containing ω-terminus
cOCP	oxidative cleavage product containing carboxy terminus
Octenal	2(<i>E</i>)-octenal
ONE	4-oxo-2(<i>E</i>)-nonenal
<i>d</i>₉-ONE	(6,6,7,7,8,8,9,9,9- ² H ₉)-4-oxo-2(<i>E</i>)-nonenal
<i>N</i>^ε-Pentanoyl-PLys	(4-(1-carboxy-5-pentanamidopentylcarbamoyl)benzyl)triphenylphosphonium bromide
PHis	(4-(1-carboxy-2-(1 <i>H</i> -imidazol-4-yl)ethylcarbamoyl)benzyl)triphenylphosphonium bromide hydrobromide
PLys	(4-(5-amino-1-carboxypentylcarbamoyl)benzyl)triphenylphosphonium bromide hydrobromide
PUFA	polyunsaturated fatty acid
TFA	trifluoroacetic acid
TPP	triphenylphosphonium

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Figure 1.
Preparation of PHis and PLys.

Figure 2. MALDI-TOF mass spectra of (A) PHis and (B) PLys. The *p*-carboxybenzyltriphenylphosphonium group increases the MW of the amino acid above the region dominated by matrix/solvent ion clusters. Additionally, the phosphonium ion prevents the formation of singly charged Na^+ or K^+ adducts, which simplifies the interpretation of the mass spectrum.

Figure 3.

MALDI-TOF mass spectra of the reaction mixture of PHis and ONE ($d_0:d_9 = 1:1$) for 3 days prior to NaBH_4 reduction (A) and after NaBH_4 reduction (B) as well as the reaction mixture of PLys and ONE ($d_0:d_9 = 1:1$) for 3 days prior to NaBH_4 reduction (C) and after NaBH_4 reduction (D).

Figure 4.

Proposed mechanisms of formation of α,β -unsaturated ketone adduct (A), reduction of hydrated ONE-His Michael adduct by NaBH_4 (B) and hydration of ONE-Lys pyrrolinone adduct followed by NaBH_4 reduction (C).

Figure 5. MALDI-TOF mass spectra of reaction mixture of PLys and octenal (A) or heptenal (B) at various times.

Figure 6.

MALDI-TOF mass spectra of the following reaction mixtures for 3 days with Fe(II) and Asc: (A). PHis, LA ($d_0:d_5=1:1$), Doublets 1–8 ($m/m+5$) modified by ω OCP, connected by termination cross bar, are connected to their corresponding singlets ($m+72$) modified by cOCP. Doublets 9–11 ($m/m+5$) are modified by OAP without accompanying singlets ($m+72$). (B). PLys, LA ($d_0:d_5 = 1:1$). Doublets 1–10 ($m/m+5$) modified by ω OCP are connected to their corresponding singlets ($m+72$) modified by cOCP.

Figure 7. MALDI-TOF mass spectra of different reaction mixtures (37°C, 3 days) containing LA ($d_0:d_5=1:1$), Fe(II), Asc and various supplements as follows: AcHis (A), PHis (B), PHis and AcHis (C).

Figure 8.

MALDI-TOF mass spectra of different reaction mixtures (37°C, 3 days) containing LA ($d_0:d_5 = 1:1$), Fe(II), Asc and various supplements as follows: (A) BocLys (a), PHis (b), PHis and BocLys (c); (B) AcHis (a), PLys (b), PLys and AcHis (c).

Table 1

Modified PHis adducts by the LA peroxidation products in Figure 6A

No. *	ωOCP modification	Mass shift before reduction (Da)	Mass shift after reduction (Da)	cOCP modification	Mass shift before reduction (Da)	Mass shift after reduction (Da)
1		112	114		184	186
2		126	128		198	200
3^		140	142		212	214
4		142	142		214	214
5		156	158		228	230
6^		168	170		240	242
7^		170	172		242	244

No. *	ωOCP modification	Mass shift before reduction (Da)	Mass shift after reduction (Da)	cOCP modification	Mass shift before reduction (Da)	Mass shift after reduction (Da)
8 [^]		186	188		258	260
	OAP modification				Mass shift before reduction (Da)	
9 [#]				310	312	
10 ^{\$}	unknown			326	328	
11 [#]			etc.	328	330	

* Only shows the products modified by the oxidative products of *dO*-LA

[^] Proposed structure

[#] Only shows one EKODE or DHKODE-His Michael adduct out of six

^{\$} The structure was unknown

Table 2

Modified PLys adducts by the LA peroxidation products in Figure 6B

No. *	ω OCP modification	Mass shift before reduction (Da)	Mass shift after reduction (Da)	cOCP modification	Mass shift before reduction (Da)	Mass shift after reduction (Da)
1		84	84		156	156
2		98	98		170	170
3 [§]	Heptenal product	108	N/A	10-oxo-8(E)-decanoic acid product	180	N/A
4 ^{§^}	Heptenal product	112	114	9-oxo-7(E)-nonenoic acid product	184	186
5 [§]	Octenal product	122	N/A	11-oxo-9(E)-undecenoic acid product	194	N/A
6 [§]	Heptenal product	126	128	10-oxo-8(E)-decanoic acid product	198	200
7		136	156		208	228
8 [§]	Octenal product	140	142	11-oxo-9(E)-undecenoic acid product	212	214
9		154	156		226	228
10 [^]		170	172		242	244

* Only shows the products modified by the oxidative products of dO-LA

[^] Proposed product

[§] The structure was unknown