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A Mass Spectrometric Analysis of 4-hydroxy-2-(*E*)-nonenal Modification of Cytochrome *c*

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Abstract

Cytochrome *c* is a key mitochondrial respiratory protein that is particularly susceptible to modification during oxidative stress. The nature of this susceptibility is linked to the mitochondrial membrane being rich in esterified linoleic acid, which predisposes this organelle to the formation of lipid peroxidation (LPO) products such as 4-hydroxy-2-(*E*)-nonenal (4-HNE). To better understand the nature of cytochrome *c* modification by 4-HNE we initiated an *in vitro* study utilizing a combination of MALDI-TOF mass spectrometry, LC-ESI-MS/MS, and isotope labeling to monitor 4-HNE modification of cytochrome *c* under various conditions. The overwhelming reaction observed is Michael addition by Lys side-chains in addition to modification of His 33. While the Lys-4-HNE adducts were generally observed to be reversible, the 4-HNE-His 33 was observed to be stable with half of the formed adduct surviving the denaturation and proteolysis protocols used to generate proteolytic peptides for LC-ESI-MS/MS.

Keywords

4-Hydroxy-2-nonenal (4-HNE); lipid oxidation; protein modification; reactive oxygen species

Introduction

4-hydroxy-2-(*E*)-nonenal (4-HNE) is a reactive aldehyde derived from polyunsaturated fatty acids (PUFAs) produced *in vivo* during times of oxidative stress. Since its discovery in 1964^[1], 4-HNE has generally become accepted as a modulator of numerous cellular systems and implicated in a number of degenerative diseases including Alzheimer's disease, atherosclerosis, cataracts, and cancer.^[2-3] The etiology of 4-HNE and disease progression is not well understood, but has generally been ascribed to a model whereby long term chronic diseases induce heightened levels of chronic inflammation, subsequently leading to increased reactive oxygen species (ROS) generation, and consequent formation of lipid peroxidation (LPO) products, such as 4-HNE.^[4-10] Our group has recently argued that disease progression could also be linked to a decreased ability to catabolize lipid peroxidation (LPO) products in a localized manner.^[11-12] Regardless, it has been suggested that oxidative damage to mitochondrial components plays a critical role in the pathology of several of the aforementioned disease states. PUFAs found in mitochondrial membranes are highly susceptible to oxidative damage given their unusually high content.^[13] Peroxidation of these lipids results in unsaturated lipid hydroperoxides that undergo fragmentation giving rise to various aldehydes, including malonaldehyde, 2-alkenals, and 4-hydroxy-2-alkenals such as 4-hydroxy-2-nonenal (4-HNE). Among these reactive aldehydes, 4-HNE has achieved the status of the best recognized and most studied LPO product^[14]. Several studies have proposed that 4-HNE represents an important mediator of free-radical-induced

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mitochondrial dysfunction^[15-36], as 4-HNE has been shown to modify and inactivate several enzymes in the mitochondria.^[15-17, 20, 24-27, 29, 33-35] Although no 4-HNE–cytochrome *c* adducts have been found *in vivo*, cytochrome *c* was reported to be released during 4-HNE-induced cell apoptosis.^[18, 23, 36] Given the subcellular localization of cytochrome *c* to the mitochondria inner membrane, the membrane lipid content, and the inherent redox chemistry of this organelle it is a highly plausible hypothesis that modification of cytochrome *c* by LPO products is a common physiologic event.^[15, 37-38]

During the past few decades, there has been an intensive effort to ascertain the chemical nature of protein modification by 4-HNE. Although the 4-HNE Michael adducts with Cys and His are stable to isolation under model reaction conditions, Michael adducts to Lys ϵ -amino groups are formed reversibly^[39-40], and can be isolated only following reductive trapping with NaBH₄.^[40-41] One hypothesis is that 4-HNE-modified proteins accumulate and serve as useful biomarkers for the occurrence and/or the extent of oxidative stress.^[34, 42-46] Immunochemical methods, because of their sensitivity and specificity, continue to serve as a reliable approach to identifying stable 4-HNE adducts, especially in tissues from *in vivo* sources.^[47-49] However, the last decade has seen increased reliance on mass spectrometric approaches to study the modification of amino acids, peptides, and proteins by 4-HNE.^[42-44, 50-69] The large bulk of these studies focus on long-lived adducts in the absence of reductive stabilization, and have identified mainly 4-HNE-His and 4-HNE-Cys Michael adducts. This is consistent with the known chemical reversibility of 4-HNE-Lys Michael adducts^[39-40], and consequently suggests that the finding of 4-HNE-Lys Michael adducts with reductive stabilization may reflect conditions where 4-HNE is present at steady-state. In this study, we report a carefully developed set of methods to better understand the profile of steady state Lys-4-HNE Michael adducts in cytochrome *c*.

Experimental Procedures

General

Horse heart cytochrome *c* was purchased from Acros. Chymotrypsin was purchased from Sigma. Sequencing grade modified trypsin was received as a kit with buffer from Promega. PD-10 columns (Sephadex G-25) were obtained from GE Healthcare. 4-HNE was prepared as in our previous work.^[40, 70] 4-HNE-*d*₁₁ was prepared as for 4-HNE, except substituting *n*-C₅D₁₁Br. All other reagents were of the highest grade commercially available. 18 M Ω Millipore water was used throughout.

Modification of cytochrome *c* by 4-HNE

Cytochrome *c* (1.0 mg/ml) was incubated with 4-HNE-*d*₀ (2.0 mM) in phosphate buffer (50 mM, pH 7.4) at 37 °C for 6 h. After incubation, aliquots were either quenched with NaBH₄ directly, after passage through a PD-10 column to remove buffer salts and excess 4-HNE, or after being dialyzed against phosphate buffer (50 mM, pH 7.4) for 6 h or 8 h and 24 h. To make the weighing convenient and accurate, NaBH₄ was mixed with NaCl in a 1:9 weight ratio. The amount of NaBH₄ used to quench the reaction was 10 times (molar ratio) of 4-HNE throughout. *Modification of Cytochrome c by 4-HNE-d₁₁*: Incubation of cytochrome *c* (1.0 mg/ml) with 4-HNE-*d*₁₁ (2.0mM) was performed in phosphate buffer (50 mM, pH 7.4) at 37 °C for 6h followed by treatment with NaBH₄ (1.0 mg). Modification of Cytochrome *c* by a 1:1 mixture of 4-HNE-*d*₀ and 4-HNE-*d*₁₁: Cytochrome *c* (1.0 mg/ml) was incubated with 4-HNE-*d*₀ and 4-HNE-*d*₁₁ in a 1:1 ratio (4.0 mM or 0.5 mM) at 37 °C for 6h followed by treatment with NaBH₄ (1.0 mg).

Proteolytic digestion

The solutions of 4-HNE modified cytochrome c were dried on Speed Vac and denatured in guanidine buffer (50 mM Tris, pH 8.0, 6.0 M guanidine hydrochloride) at room temperature for 1h followed by dilution with NH_4HCO_3 (50 mM, pH 7.8) to 1.0 mg/ml. The samples were digested with modified sequencing grade trypsin in a 1:40 trypsin /protein ratio or with chymotrypsin in a 1:10 chymotrypsin/protein ratio for 24h at 37 °C. Digestions were stopped with 1 μL formic acid and stored at -20 °C for LC-MS-MS analysis.

MALDI-TOF MS analysis of 4-HNE modified cytochrome c

Prior to MALDI-TOF MS analysis, 10 μL aliquots of modified cytochrome c solution were mixed and acidified by addition of 5 μL of 1% TFA (or 2.5% TFA in the case of NaBH_4 reduction). The mixture was desalted using Millipore C18 ZipTip using the protocol of the manufacturer. MALDI-TOF MS analyses were performed with a Bruker BiFlex III MALDI-TOF equipped with a pulsed N_2 laser (3 ns pulse at 337 nm). Protein spectra were collected in linear mode using sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid) as the matrix with an accelerating potential of 19 kV. Each spectrum was the accumulation of 1000 laser shots. The native MW weight of cytochrome c (12361) was used for calibration.

HPLC/ESI-MS/MS analysis of 4-HNE-modified peptides

LC-MS-MS analysis was performed on a Thermo Finnigan LCQTM Advantage LC/MS/MS system. The Surveyor MS pump was equipped with a Grace Vydac C18 column (5 μm particle size, 80 Å pore size, 4.6 mm ID $\times$ 250 mm) using a H_2O -MeOH gradient. ESI mass spectrometry was performed in positive mode using nitrogen as the sheath and auxiliary gas. The capillary temperature was 300 °C, and the capillary voltage set to 30V. Two scan events were used: (i) m/z 400-2000 full scan MS, (ii) data-dependent full scan MS/MS on the most intense ion from the MS full spectrum. The MS/MS collision energy was set at 35 %. This data-dependent multistage MS fragmentation was used to sequence the peptides and modified peptides. Interpretation of the modified peptides was performed with the help of "PeptideMass" (<http://us.expasy.org/tools/peptide-mass.html>), which can predict the masses of cleaved peptides of cytochrome c and the known modification mass increment to a predicted proteolytic peptide. In some cases, with the aid of the near coelution of peptides modified with the unlabeled or deuterium-labeled aldehydes, the modified peptides with abnormal cleavage could be determined manually and confirmed by tandem mass.

Results and Discussion

MALDI-TOF analysis of 4-HNE-modified cytochrome c as a function of reaction workup

Previous studies of intact model proteins modified by LPO-derived aldehydes have been successful in identifying the average number of aldehydes adducted per protein monomer using MALDI analysis. The "fine structure" of the spectrum can further give evidence of whether simple Michael adductions are taking place (peaks appear cleanly with the mass shift of the MW of the modifier) or more complex chemistry such as competing dehydration reactions, etc. We carried out a comparative study of cytochrome c modified by 2.0mM 4-HNE for 6 h under several different work up procedures. MALDI-TOF analysis was subsequently performed on the crude reaction mixture following the indicated procedures.

Initially, the "quenching" effect of NaBH_4 was demonstrated by comparing the spectra of samples right after reaction, followed by PD-10 clean up or dialysis with or without borohydride reduction. In interpreting Figure 1, it can be seen that up to five molecules of 4-HNE can be bound to each single monomer of cytochrome c in the crude reaction mixture (trace A) or following NaBH_4 stabilization (trace B), though the native unmodified protein still gives a strong signal, and the average modification is just under two bound 4-HNE

molecules. Passing the reaction solution through a PD-10 column alone (trace C) or followed by borohydride reduction (trace D) does not show an appreciable effect. However, reversibility is seen during dialysis of the modified protein for either 8 h (traces E & F) or 24 h (traces G & H). Substantial reversibility is seen when the cytochrome *c* samples are subjected to guanidinium denaturation (traces I & J). It is important to note that at no condition does addition of NaBH₄ result in increased 4-HNE modification of the protein. This suggests that “quenching” of reactions with this reducing agent essentially traps those adducts present in solution rather than creating new modifications as occurs in the equilibrium-driven reductive alkylation of amines using carbonyl compounds and NaBH₃CN.^[71]

MALDI-TOF and LC-ESI-MS/MS analysis of 4-HNE-modified Cytochrome *c*

The data reported in Figure 1 conveys a sense of the average level of monomer modification, but provides no information on the nature of amino acids modified or on the distribution of modification. To determine the maximal possible information on amino acid residues of cytochrome *c* modified by 4-HNE, we exposed the protein to a 1:1 mixture (4.0 mM) of 4-HNE-*d*₀ and 4-HNE-*d*₁₁ for 6 h followed by a NaBH₄ quench, and subsequent workup (with prior denaturation) for either tryptic or chymotryptic digestion. While the concentration of 4-HNE used is high, the goal of the studies is to gauge the spectrum of possible 4-HNE adducts and their relative stability once formed. Additional data is provided in supporting information of a 0.5 mM incubation to better gauge the relative reactivities of individual lysine residues at lower 4-HNE concentrations (see Supporting Information). The MALDI-TOF spectra of the two proteolytic digests (not shown) readily permitted visualization of modified vs. unmodified peptides on the basis that the former appear as *m/m* + 11 doublets. Tentative assignment of these modifications could be made using the native tryptic or chymotryptic map, but we instead resorted to a complete LC-ESI-MS/MS analysis for confirmation of the modification site through analysis of *b* and *y* ions in the MS/MS spectra of both the tryptic (Table 1) and chymotryptic (Table 2) digests, as in our previous study on apomyoglobin.^[65] In contrast to what was seen for apomyoglobin, the data for this 6 h incubation show exclusively reduced Michael adducts for most of the nineteen Lys residues and for primarily one of the three His residues (H33) in the protein monomer. The His serving as an axial ligand to the heme iron (H18) is not modified, and the third Histidine residue (H26) is modified infrequently. The prevalence for modification of H33 has been previously reported^[63], and this appears to be a “hot-spot” for 4-HNE modification of cytochrome *c*.

Interestingly, using selected ion chromatograms to distinguish the 4-HNE-*d*₀ vs. 4-HNE-*d*₁₁ adducts from the full scan ion chromatogram, we found that the peaks of reduced 4-HNE-*d*₀ modified peptide routinely eluted just slightly later than the peaks of reduced 4-HNE-*d*₁₁ modified peptide on HPLC. This phenomenon has been previously described and is attributable to the shorter C-D bond length (as compared to C-H) and the consequent reduction in polarizability of that bond.^[72-74]

In the chymotrypsin digest (Table 2), five peptides were found to be modified by HNE that were not predicted by the standard chymotryptic map (<http://us.expasy.org/tools/peptide-mass.html>). The ability to detect HNE-modified peptides by the successional appearance of peak *M*+1 and peak *M*+1+11 was particularly helpful to distinguish these abnormal cleavage products. One example of the tandem mass spectra assignment for an abnormal chymotryptic cleavage peptide containing the key modified H33 residue is shown in Figure 2.

The LC-ESI-MS/MS results showing a nearly random distribution of reduced HNE-Lys Michael adducts over the surface of cytochrome *c*, suggesting that there is little selectivity in

the nature of HNE-Lys adduction using 4 mM HNE. This is not unexpected based on the structure of cytochrome *c* and the structure-function role of the Lys residues.^[37-38] At the same time, since the MALDI spectra in Figure 1 (using 2 mM HNE) suggested an average of 2 HNE-molecules per monomer, it appears that other than the fairly selective Histidine modification (H33), the Lysine modification reflects a nearly stochastic distribution at some overall (fairly low) degree of modification. At lower concentrations of 4-HNE, there does appear to be some level of selectivity. For example, in comparing the peptides formed from incubations of 4.0 mM and 0.5 mM 4-HNE (compare Table 1 to S1 and Table 2 to S2) peptides with adducted residues K25, K87, K88, and K99 are no longer detected in the 0.5 mM incubation. Further, Adduction of K39, K55, and K79 are only detected in the chymotryptic digest. In titrating the concentrations of 4-HNE, we found that no lysine adducts form below a threshold concentration of 80 μ M, which interestingly correlates to the concentration at which 4-HNE exceeds a 1:1 molar ratio of cytochrome *c*. Our interpretation of this result is that upon saturation of H33 residues, the excess 4-HNE will begin adducting Lys residues.

The next question was whether the various conditions described in Figure 1, which removed most of the adducted 4-HNE, left a modified form where the low level of 4-HNE modification represented a more selective degree of 4-HNE modification. LC-ESI-MS analysis of cytochrome *c* modified by 2.0 mM 4-HNE followed by reduction showed the same modified peptides as seen for 4.0 mM 4-HNE (Tables 1 and 2). Both of these concentrations represent supersaturation conditions with respect to the molar equivalents of lysine residues, but regardless led us to the conclusion that none of the Lys Michael adducts were selective, or would represent “hot-spots” for Lys modification. The only differences between the two conditions were the 4-HNE-Lys Michael adducts in GKHK, KKTER, KTGQAPGFTYTDANK, and KYIPGTMIFAGIK in the trypsin digest (Data not shown) and AGIKKKTEREDL, KEETLM, and the doubly-modified KTGPNLHGLF in the chymotryptic digest (Table 3).

As is seen in Table 3, all the samples that were then gel filtered (Column D) or dialyzed against phosphate buffer for 8 h (Column F) and 24 h (Column H), with NaBH₄ reduction prior to denaturation and proteolysis still showed the same set of Lysine and Histidine modifications in the chymotryptic digests, but with decreasing intensity as a function of length and extent of gel filtration or dialysis respectively. For example, in the first entry (peptide K*GITW) the ion intensity is diminished from 0.98 to 0.91 after either gel filtration, or 8 hours of dialysis. After 24 hours of dialysis the intensity is decreased to 0.76, suggesting that even extensive dialysis does not selectively remove reversibly-formed 4-HNE-Lys Michael adducts, or that these treatments do not remove a small pool of non-covalently-bound 4-HNE that can still equilibrate onto Lys residues. On the other hand, in the absence of reduction, taking the modified sample through denaturation and proteolysis (Column J) resulted in the loss of almost all 4-HNE-Lys Michael adducts and about half of the 4-HNE-H33 Michael adduct. This is consistent with the finding that the modified sample exposed to denaturing buffer (pH 8.0) and then a mock proteolysis in pH 7.8 NH₄HCO₃ for 24 h at 37 °C (Figure 1, trace J) had the most drastic effect on reducing the level of 4-HNE modification as assessed by MALDI-TOF analysis of the undigested protein. The intensity of most of 4-HNE (*d*₀) modified peptides at this stage was so small that if without the help of peaks of 4-HNE (*d*₁₁) modified peptides, it would have been extraordinarily difficult to identify the 4-HNE (*d*₀) modified peptides. The loss of 4-HNE-Lys adducts in the latter case must reflect their greater reversibility, relative to gel filtration or dialysis, under conditions of the elevated pH, raised temperature (37 °C vs r.t.) and/or the dilution of equilibrating 4-HNE-Lys Michael adducts residues by competing ammonium ions.

Conclusions

Amongst the many reports wherein mass spectrometry has been used to assess the nature of protein modification by 4-HNE, most of the adducts that have been characterized to form in simple physiologic model studies have been observed. In the case of the most readily formed and widely-known 4-HNE Michael adduct, the facts (i) that the predominance of studies has reported 4-HNE Michael adducts on Cys and His residues, (ii) that the 4-HNE-Lys Michael adduct is reversibly formed [39-40], and (iii) that most of the reports of 4-HNE-Lys Michael adducts reflect mass spectral analysis only after reductive quenching with NaBH₄, raises the question as to whether these adducts are physiologically relevant and likely to be seen *in vivo*. In this regard, it is important to recognize that “physiologically relevant” is a relative term that can vary based on the question being asked. One may be interested to know the nature of long-lived 4-HNE adducts expected to be found in tissues *in vivo* long after the 4-HNE modification event has occurred. In such case, it appears that in the absence of a stabilizing derivatization, 4-HNE-Lys adducts would be expected only in unusual situations where the microenvironment of the adduct connotes an unusual degree of irreversibility. On the other hand, one may be interested to know the nature of modifications likely to be present at equilibrium under conditions of active oxidative stress, wherein some steady-state concentration of the aldehyde modifier maintains an equilibrium-dependent modification. To learn about the latter question, it would be more appropriate to analyze protein modification after reversibly-formed adducts are stabilized using NaBH₄. In the latter regard, it is important to note from the current study that NaBH₄ appears to be useful for trapping adducts that are indeed present at the time of the quench, in contrast to the case where NaBH₃CN can be used to drive the equilibrium formation of protein Schiff base adducts.^[71] Thus, the finding of reduced Schiff bases using NaBH₃CN cannot be cited as evidence for the existence of the Schiff base, and instead probably reflects simply the fact that free 4-HNE was present in the system.

The results of the current study extend our understanding of the nature of protein modification that is likely to underlie the biological activities of 4-HNE, the key aldehyde generated from lipid oxidation during oxidative stress.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

4-HNE	4-hydroxy-2-(<i>E</i>)-nonenal
LPO	Lipid peroxidation
ROS	Reactive oxygen species
PUFA	Polyunsaturated Fatty Acids

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Figure 1.

MALDI-TOF mass spectra of 4-HNE- d_0 modified cytochrome *c*. Cytochrome *c* (1.0 mg/ml) was incubated with 4-HNE- d_0 (2 mM) in phosphate buffer (50mM, pH 7.4) at 37 °C for 6 h. The incubation solution was quenched with NaBH₄ directly, passed through a PD-10 column, or dialyzed against phosphate buffer (50 mM, pH 7.4) for 8 h or 24 h and then quenched with NaBH₄. Aliquots after PD10 separation without reduction were concentrated on a Speed Vac Concentrator (Savant) and incubated with denaturing buffer and/or NH₄HCO₃ “proteolysis buffer” for 20 h at 37 °C. The mass spectra were recorded after (A) incubation, (B) incubation → NaBH₄, (C) incubation → PD-10, (D) incubation → PD-10 → NaBH₄, (E) incubation → dialysis for 8 h, (F) incubation → dialysis for 8 h → NaBH₄, (G) incubation → dialysis for 24 h, (H) incubation → dialysis for 24 h → NaBH₄, (I) incubation → PD-10 → denaturation (pH 8.0), (J) incubation → PD-10 → denaturation → proteolysis “buffer” (pH 7.8),

Figure 2.

Tandem mass spectra of an abnormal chymotryptic peptide TGNLHGLF (residue numbers 28-36) with one molecule of 4-HNE adducted to H33. By comparing the two spectra, the ions marked on the spectra are all 4-HNE modified ions and are assigned as b, y ions. As y4, y5, y7, y8, y9 ions are modified ions, the modified residue should be among HGLF. Only H33 among these four residues is possible to be modified by 4-HNE, and the possibility is confirmed by modified b6, b7, b8 ions.

Table 1

Borohydride-reduced 4-HNE-modified cytochrome *c* peptides detected by HPLC/ESI-MS/MS in the tryptic digest

Peptide sequence	Position	Mass-to-charge ratio (<i>m/z</i>) of 4-HNE- modified peptide		Type of modification	Modified residue
		Theoretical	Detected		
GGKHK	23-27	684.31	684.42	Michael	K25
KKTER	87-91	819.40	819.45	Michael	K87 or k88 ^b
AcGDVEKGKK	1-8	1060.49	1060.45	Michael	K5
KIFVQK	8-13	920.49	920.55	Michael	K8
KYIPGTK	73-79	964.48	964.50	Michael	K73
NKGITWK	54-60	1004.48	1004.54	Michael	K55
KTGQAPGFTYTDANK	39-53	1756.78	879.13 ^a	Michael	K39
KKTEREDLIAYLKK	87-100	1893.01	947.45 ^a	Michael	K87orK88 ^b
MIFAGIKK	80-87	1065.54	1065.58	Michael	K86
KKTEREDLIAYLK	87-99	1764.92	883.17 ^a	Michael	K88
TGPNLHGLFGRK	28-39	1454.72	728.15 ^a	Michael	H33
KTEREDLIAYLKK	88-100	1764.92	883.36 ^a	Michael	K99
YIPGTMIFAGIKK	74-87	1724.91	863.34 ^a	Michael	K79
KYIPGTMIFAGIK	73-86	1724.91	857.81 ^a	Michael	K79
TGPNLHGLFGR	28-38	1326.62	1326.77	Michael	H33
YIPGTMIFAGIK	74-86	1596.81	799.29 ^a	Michael	K79
EDLIAYLKK	92-100	1250.63	1250.60	Michael	K99

^a The peptide is doubly charged and the mass is (*m*+1)/2.

^b The MS/MS spectrum can only tell that one of the first two residues at the N-terminal is modified by 4-HNE.

Table 2

Borohydride-reduced 4-HNE-modified cytochrome *c* peptides detected by HPLC-ESI-MS/MS in the chymotryptic digest

Peptide sequence	Position	Mass-to-charge ratio (<i>m/z</i>) of 4-HNE- modified peptide		Type of modification	Modified residue
		Theoretical	Detected		
KGITW ^c	55-59	762.35	762.48	Michael	K55
AcGDVEKGKKIF	1-10	1320.65	1320.68	Michael	K5
AcGDVEKGKKIF	1-10	1320.65	1320.68	Michael	K7
AcGDVEKGKKIF	1-10	1320.65	1320.68	Michael	K8
KTGPNLHGLF ^c	27-36	1241.59	1241.66	Michael	H33
TGPNLHGLF ^c	28-36	1113.50	1113.50	Michael	H33
KTGPNLH ^c	27-33	924.42	924.46	Michael	K27
AGIKKKTEREDL	83-94	1545.79	773.55 ^a	Michael	K87
GRKTGQAPGF	37-46	1176.54	1176.6	Michael	K39
TDANKNKGITW	49-59	1405.64	703.73 ^a	Michael	K55
KEETLM	60-65	908.37	908.45	Michael	K60
LENPKKY	68-74	1049.49	1049.60	Michael	K72
LENPKKY	68-74	1049.49	1049.60	Michael	K73
IPGTKM	75-80	804.36	804.46	Michael	K79
KTGPNLHGLF ^c	27-36	1399.59	700.61 ^a	Michael	K27,H33 ^b

^aThe peptide is doubly charged and the mass is (*m*+1)/2.

^bThe peptide is modified by two 4-HNE molecules on two residues.

^cThe peptide is not predicted by "PeptideMass".

Table 3

Stability Analysis of Individual 4-HNE-Michael adducts.

Ratio		B	D	F	H	J
Modified peptide						
K*GITW ^c	K55	0.98	0.91	0.91	0.76	0.06
AcGDVEK*GKKIF	K5	1.10	0.93	0.93	0.70	0.11
AcGDVEKGKK*IF	K8	1.06	1.07	0.84	0.46	--
AcGDVEKGK*KIF	K7	1.01	1.02	0.69	0.20	0.08
KTGPNLH*GLF ^c	H33	1.00	0.98	0.79	0.47	0.56
TGPNLH*GLF ^c	H33	1.19	1.11	1.04	0.60	0.38
K*TGPNLH ^c	K27	0.98	1.05	0.74	0.20	0.28
GRK*TGQAPGF	K39	0.90	0.93	0.81	0.54	0.12
TDANKNK*GITW	K55	0.95	0.97	0.97	0.86	--
LENPKK*Y	K73	0.97	1.06	0.76	0.40	0.06
LENPK*KY	K72	0.89	0.93	0.67	0.34	0.05
IPGTK*M	K79	0.76	0.98	0.68	0.48	0.11

See Figure 1 for conditions corresponding to column labels.