Supporting Information for

LDL has an Enormous Capacity to Bind HNE. Detection and Characterization of Lysyl and Histidyl Adducts Containing Multiple Molecules of HNE

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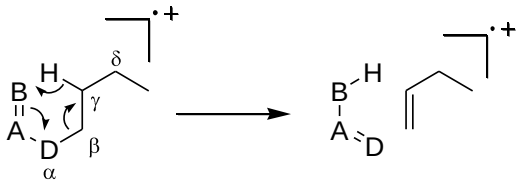
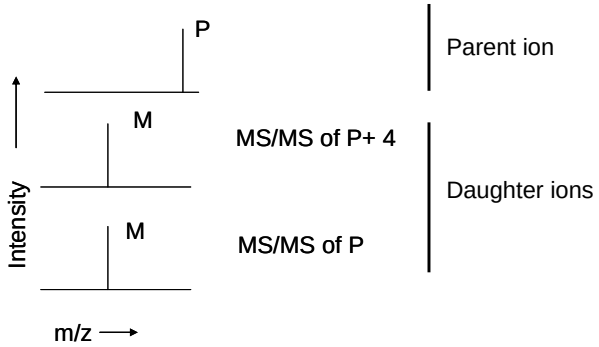
[8,8,9,9-$^2H_4$]-HNE is optimal for detecting multiple adducts. Choosing the right kind of deuterated HNE is important for many practical reasons. Firstly, the deuterium atoms should be in a position that precludes it from loss or any chemical reactivity. The carbons 1-4 are involved in the adduction chemistry and the protons at carbon 5-7 may be involved in the McLafferty type rearrangements (*1*) that could occur in the mass spectrometric ionization process as shown below.



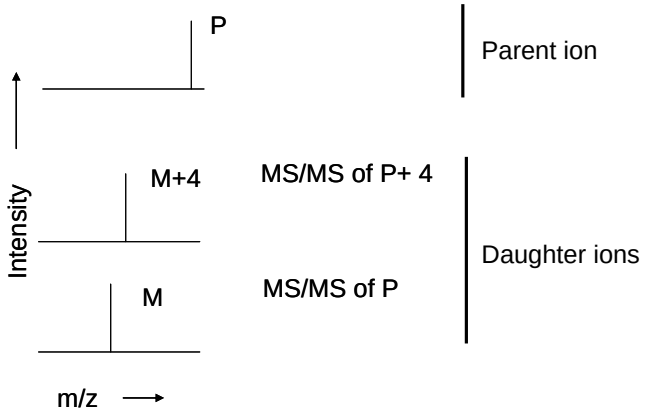
Secondly, the number of deuteria present should be sufficient to readily differentiate adducts of labeled and unlabeled HNE from the isotopic peaks that arise from the various isotopic abundances of some of the elements. Since our study was to be focused on the elements C, H, O, N and S, the highest isotopic peak (> 1%) is at M+1 for carbon and M+2 for sulphur Table 2.2. Thirdly, the number of deuteria incorporated should be easily achieved synthetically. The use of multiples of two is readily achieved by hydrogenation of alkenes. All these considerations indicated that the deuterium atoms should be located at C8 or C9 of the HNE molecule and that there should be > 2 deuterium atoms. We, therefore, chose to incorporate four deuterium atoms at the C8 and C9 carbons by reduction of an alkyne bond. Consequently, we prepared a deuterated alkylating reagent by deuteration of an alkyne triple bond.

Methodology. Incubation of amino acids individually with a mixture of deuterium labeled (d_4) HNE and unlabeled (d_0) HNE in the ratio 1:1 can potentially yield adducts that can be formed from either the d_0 or d_4 HNE. Analysis of these reaction mixtures by mass spectrometry revealed that unique signatures are conferred upon the spectra. Sets of ions are found with mass differences in multiples of 4 Da owing to the presence of various combinations of d_0 and d_4 HNE. Thus, HNE with 4 deuterium atoms will have a molecular weight of 156 + 4 Da. Therefore, an ion with 'n' HNE will have 'n + 1' peaks with successive mass differences of 4 Da. Accordingly, adducts containing more than one molar equivalent of HNE per molar equivalent of N-acetyl-His should appear as triplets (1:2:1) or multiplets of n + 1 ions, where 'n' corresponds to the number of equivalents of HNE present. Based on this premise, the following conclusions can be drawn from the pattern of daughter ions derived from tandem MS/MS each of the molecular ion (P) and their corresponding isotopic isomers (P + 4 and/or P + 8):

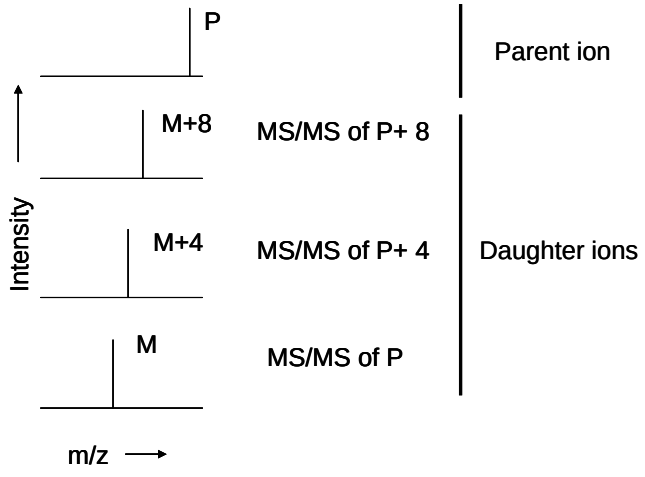
a. If the daughter ion appears as M (from P) with no M+4 but M (from P + 4) also, then the fragment has no C5-C9 alkyl chain of HNE.



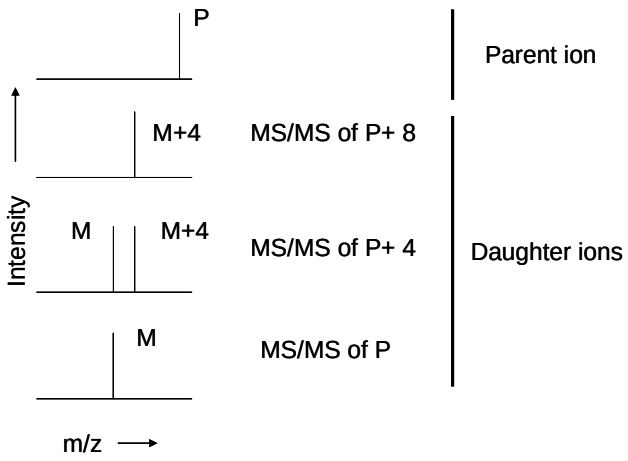
b. If the daughter ion appears as both M (from P) and M+4 (from P + 4), then the daughter ion has one C5-C9 alkyl chain from HNE.



c. If the daughter ion appears as M (from P), M+4 (from P + 4), and M+8 (from P + 8), then the daughter ion has two C5-C9 alkyl chains from HNE.



d. If the daughter ion of a parent ion with two C5-C9 alkyl chains from HNE appears as [M (from P)] , [M & M+4 (from P + 4)] and [M+4 (from P + 4)], then the daughter ion has one C5-C9 alkyl chain of HNE.



N-acetyl histidine adducts:

The Michael adduct that is formed by reaction of HNE and histidine residues is well known(*2*). The fragmentation pattern of this Michael adduct is illustrated in Figure S1 along with the tandem MS spectra's.

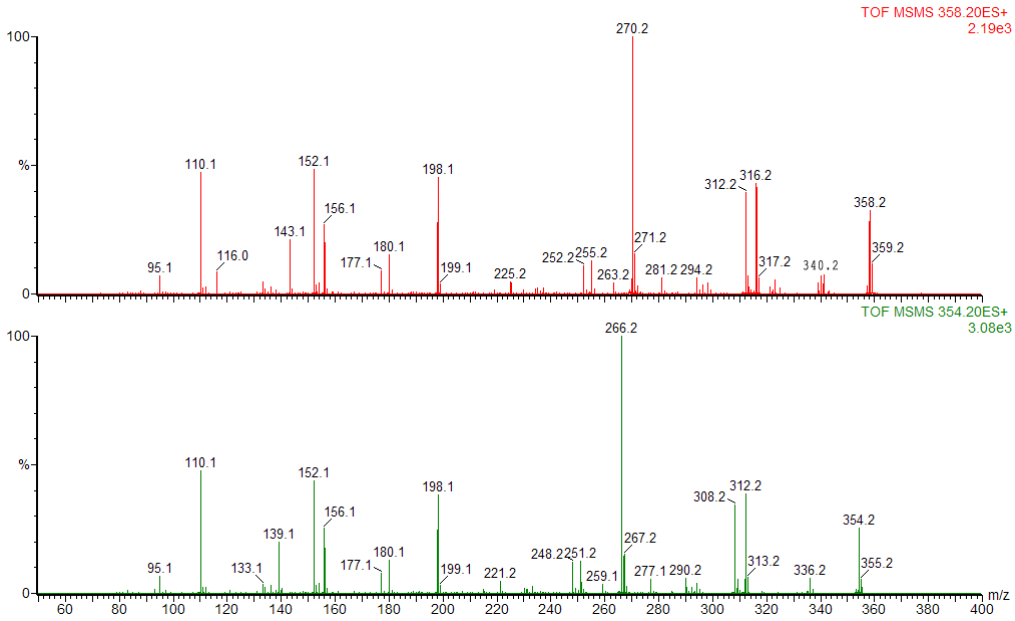
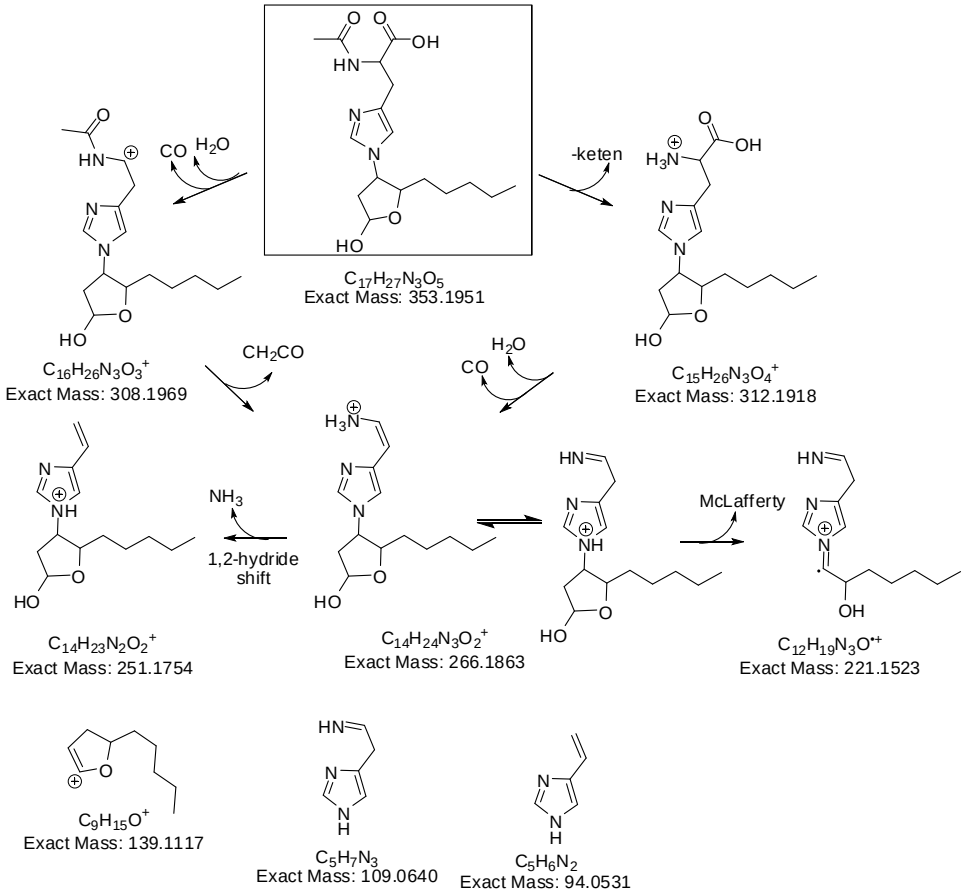


Figure S1: ESI-TOF-MS/MS analysis of Michael adducts parent ions at *m/z* 354 (lower panel) and *m/z* 358 (upper panel) from the infusion of reaction mixture from 2:1 HNE and Adduct with N-acetyl Acetyl-histidine (1:1).

Scheme S1: Fragmentation of HNE-Histidine Michael adduct



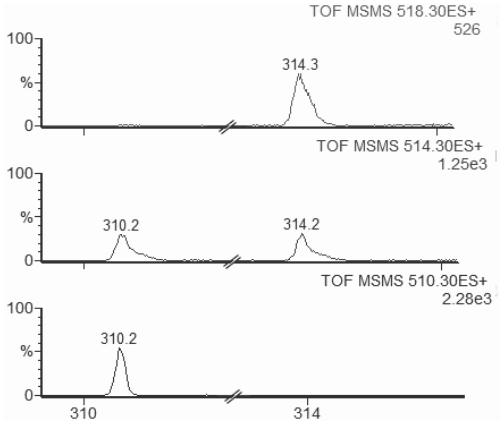


Figure S2. Tandem MS/MS in the mass range *m/z* 309-316 of the parent ions at A) *m/z* 518, B) *m/z* 514 and C) *m/z* 510 from (N-acetyl-His)-HNE_2 (Figure 5C).

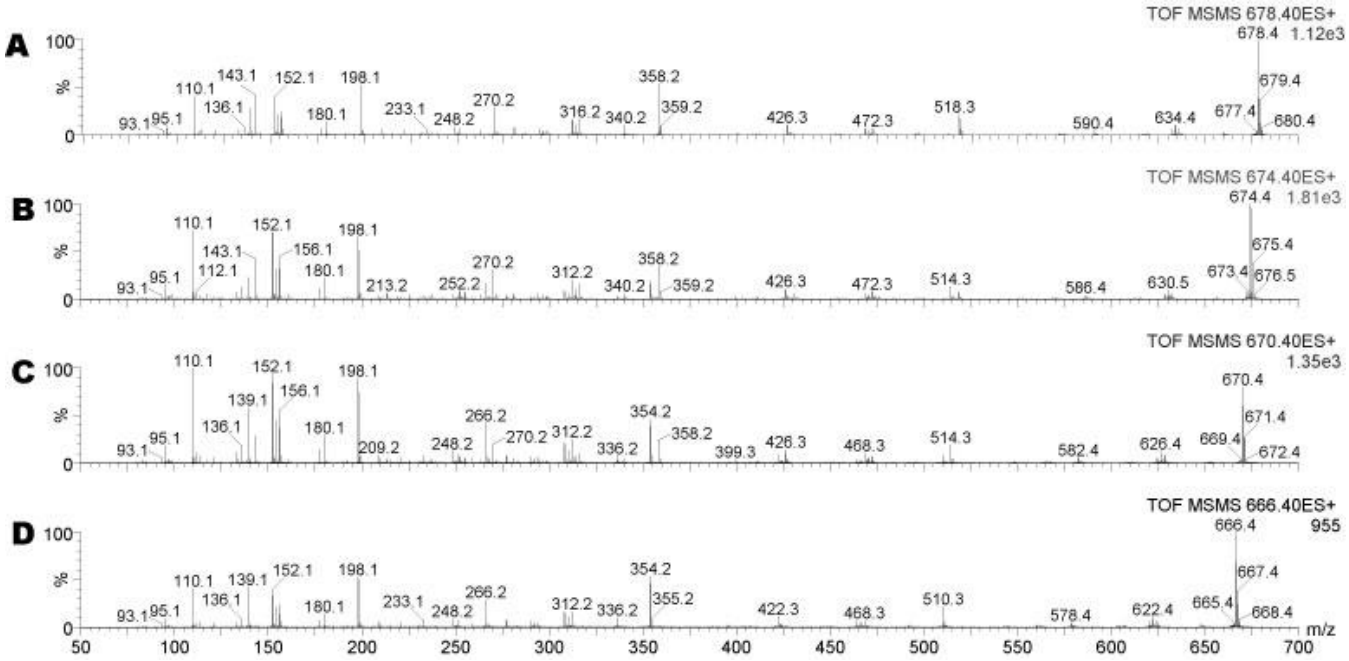
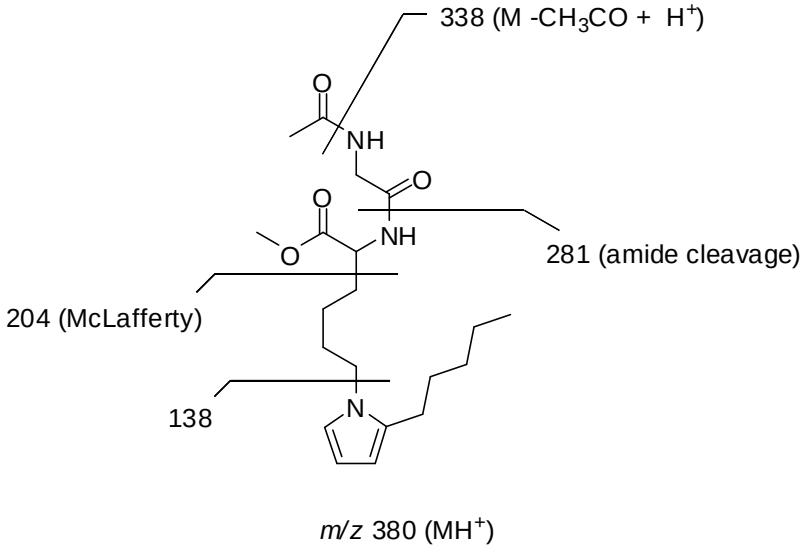


Figure S3. ESI-TOF-MS/MS of molecular ions A) *m/z* 678, B) *m/z* 674 and C) *m/z* 670 D) *m/z* 666 from infusion of the product mixture from the reaction of HNE (d_4:d_0, 1:1) with N-acetyl histidine.

N-acetyl Lysine-glycine methyl ester adducts:

Scheme S2: Possible fragmentation of the molecular ion *m/z* 380 corresponding to an HNE-(N-acetyl-Gly-Lys-OMe) pyrrole adduct.



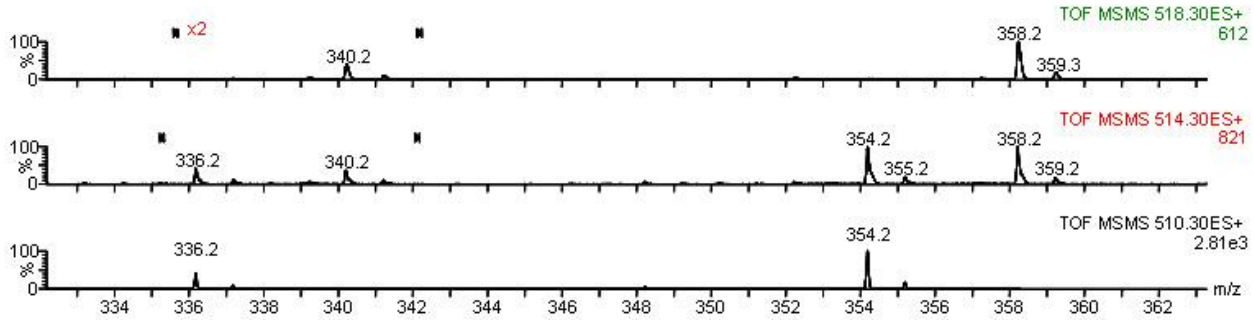


Figure S4: Loss of a molecule of H_2O from the product of McLafferty rearrangement product **12**

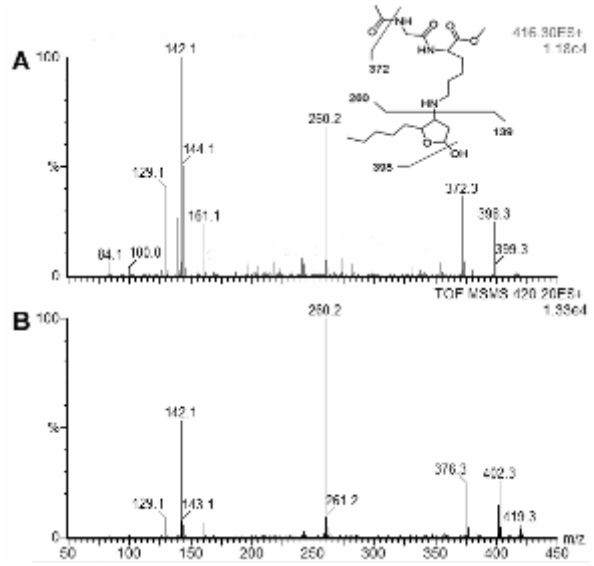


Figure S5: ESI-MS/MS of molecular ions at A) *m/z* 416 and B) *m/z* 420 corresponding to a 1:1 HNE-N-acetyl glycine lysine Michael adduct. Inset – proposed fragmentation of the N-acetyl glycine lysine Michael adduct.

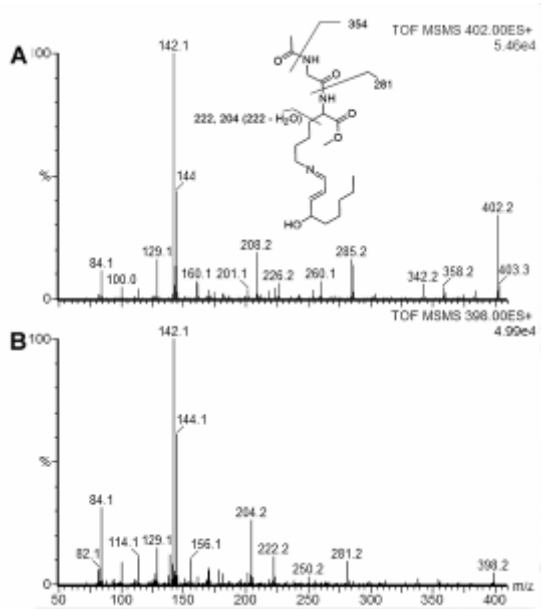


Figure S6: ESI-MS/MS analysis of molecular ions A) *m/z* 402 and B) *m/z* 398 corresponding to an HNE- N-acetyl glycine lysine methyl ester Schiff base adduct. Inset – proposed fragmentation of the Schiff base adduct.

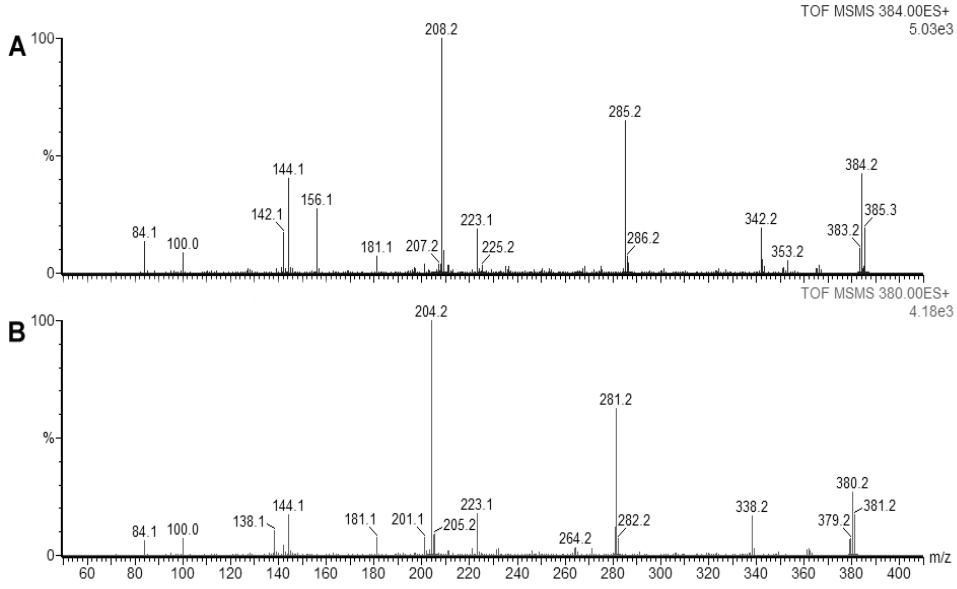
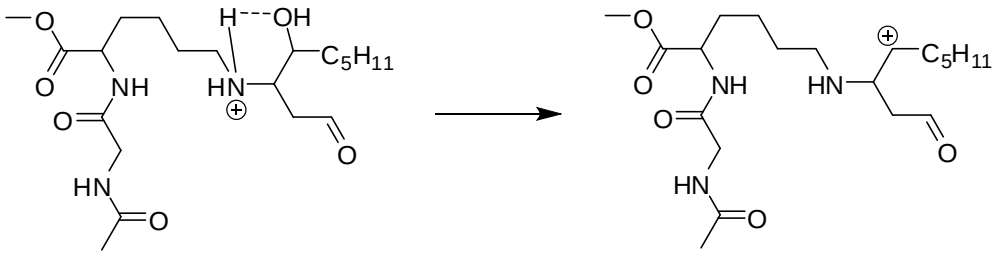


Figure S7: ESI-MS/MS analysis of molecular ions at A) *m/z* 384 and B) *m/z* 380 corresponding to the HNE-(N-acetyl-Gly-Lys-OMe) pyrrole adduct.

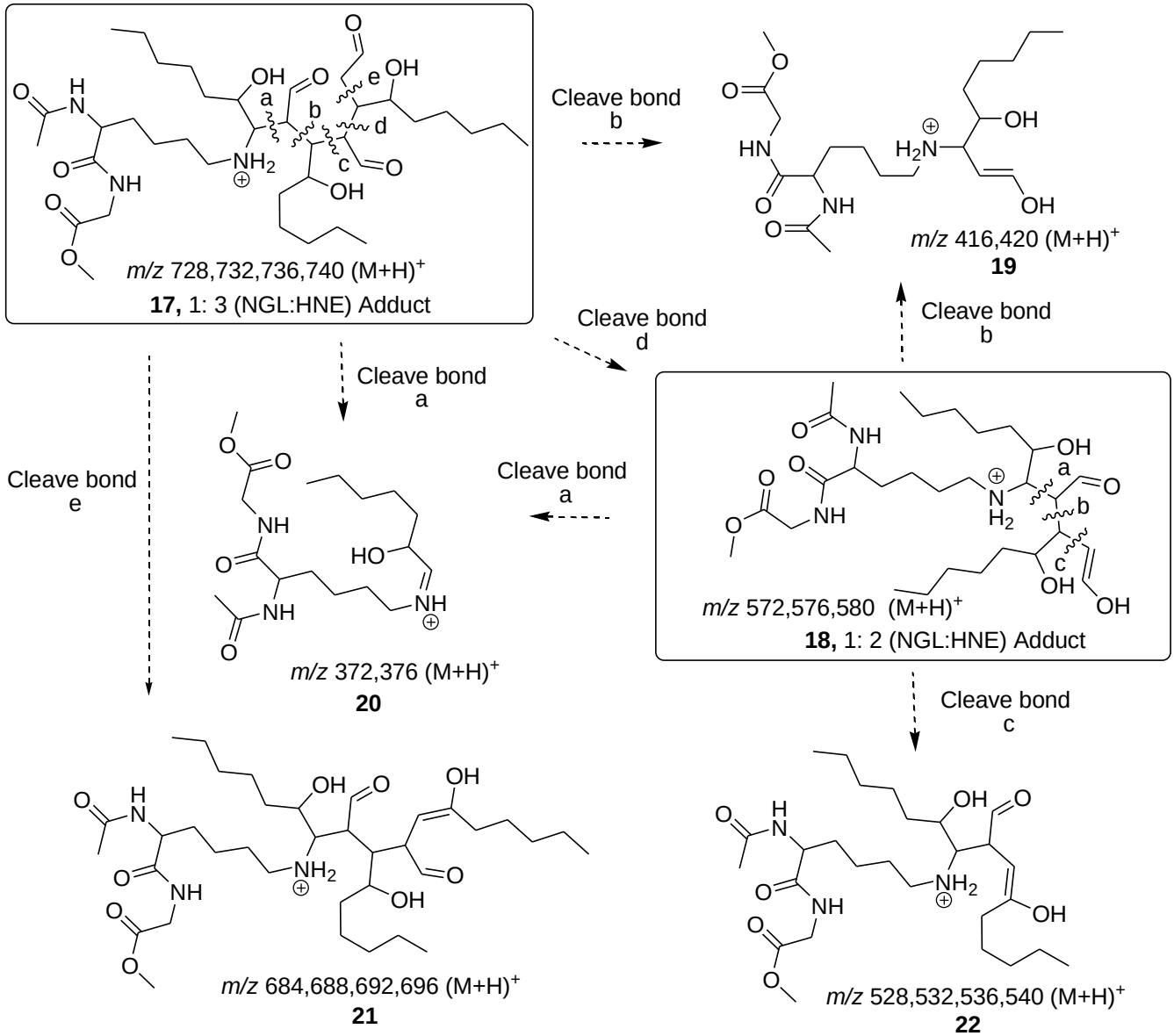
Scheme S3: Suggested mechanism for H_2O loss from Lysyl-HNE Michael adducts.



Chemical Formula: $C_{20}H_{38}N_3O_6^+$
Exact Mass: 416.2755

Chemical Formula: $C_{20}H_{36}N_3O_5^+$
Exact Mass: 398.2649

Scheme S4. Structural characterization of the 2:1 and 3:1 HNE-N-acetyl glycine lysine methyl ester adducts by MS/MS analysis.



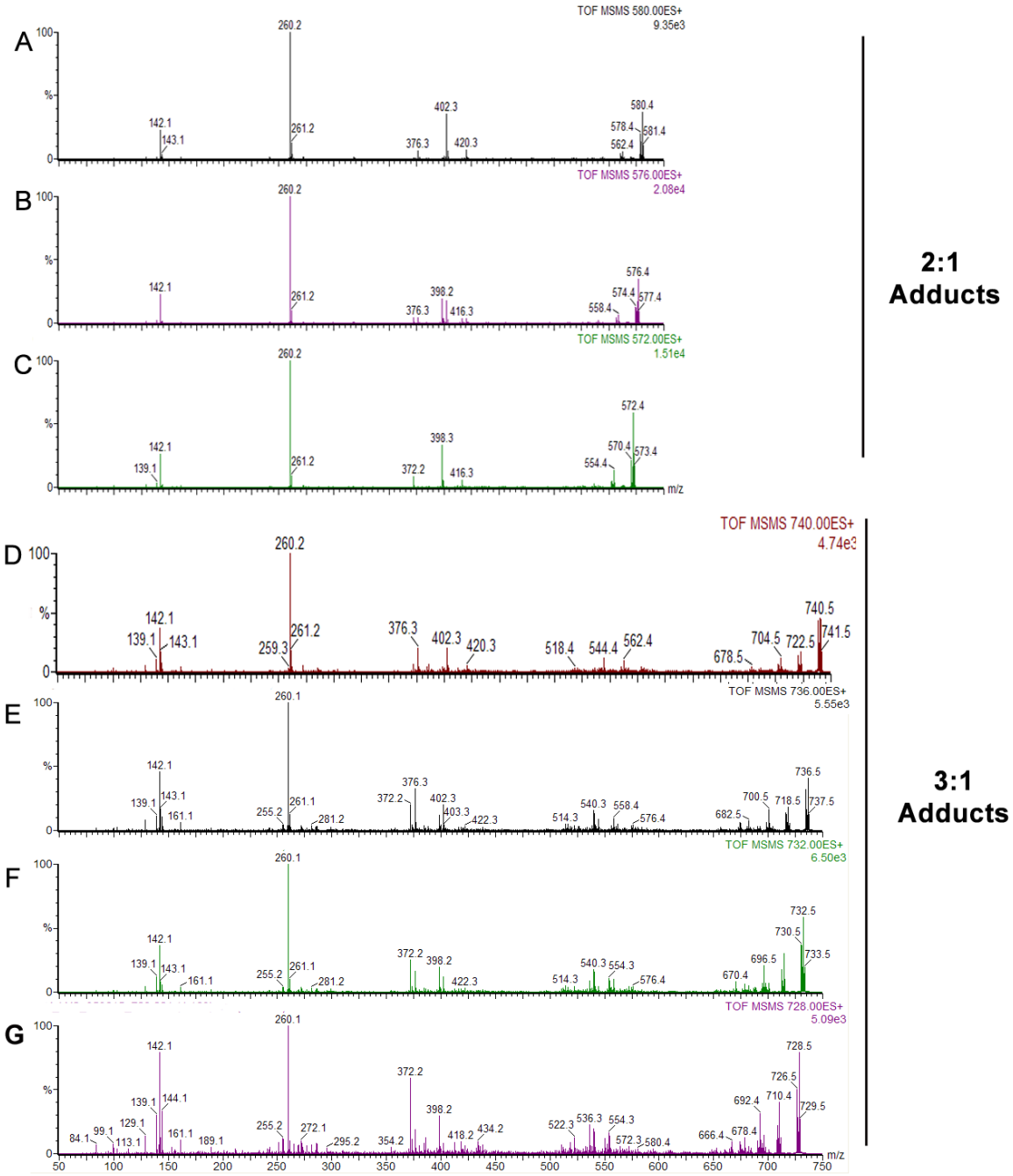


Figure S8: ESI-MS/MS analysis of molecular ions at A) *m/z* 580 B) *m/z* 576and B) *m/z* 572 corresponding to the 2:1 HNE-(N-acetyl-Gly-Lys-OMe) adduct and D) *m/z* 740 E) *m/z* 736, F) *m/z* 732 and G) *m/z* 728 corresponding to the 3:1 HNE-(N-acetyl-Gly-Lys-OMe) adduct.

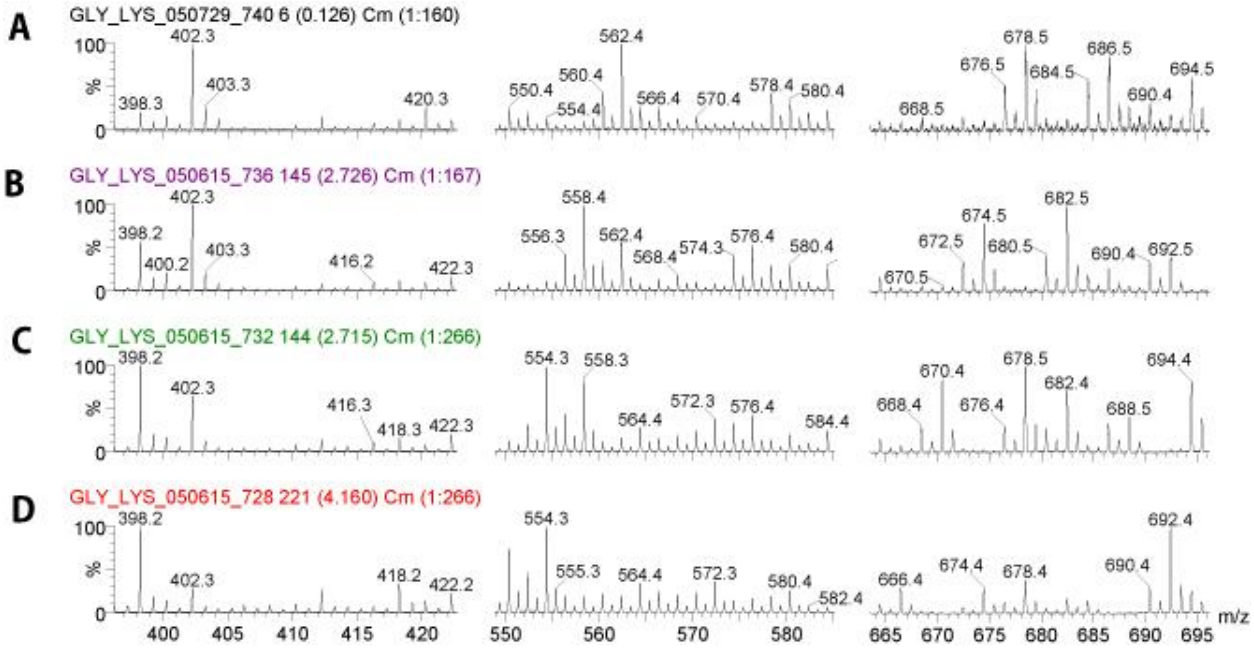


Figure S9: ESI-MS/MS analysis of molecular ions at A) *m/z* 740 B) *m/z* 736 B) *m/z* 732 and *m/z* 728 showing three regions to illustrate the presence of peaks generated due to loss of water from the Mclafferty rearrangement products (Scheme 5) of HNE-(N-acetyl-Gly-Lys-OMe) 3: 1 adduct.

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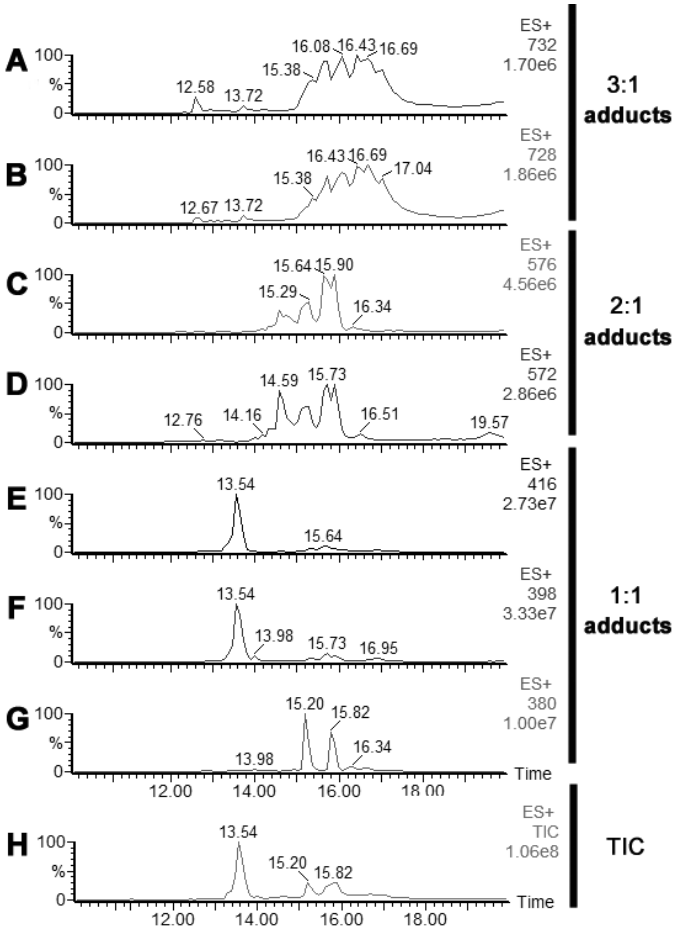


Figure S10: LC-ESI/SIM/MS of the products from reaction of HNE (d_4:d_0, 1:1) with N-acetyl-Gly-Lys-OMe monitored through 8 channels are shown here. LC-SIM chromatograms for molecular ions at A) *m/z* 732 (M+4 of 3:1 adduct), B) *m/z* 728 (M of 3:1 adduct). C) *m/z* 576 (M+4 of 2:1 adduct), D) *m/z* 572 (M of 2:1 adduct); E) *m/z* 416 (M of 1:1 Michael adduct); F) *m/z* 398 (M of 1:1 Schiff base adduct); G) *m/z* 380 (M of 1:1 pyrrole adduct); and H) total ion chromatogram (TIC).

N-acetyl cysteine adducts:

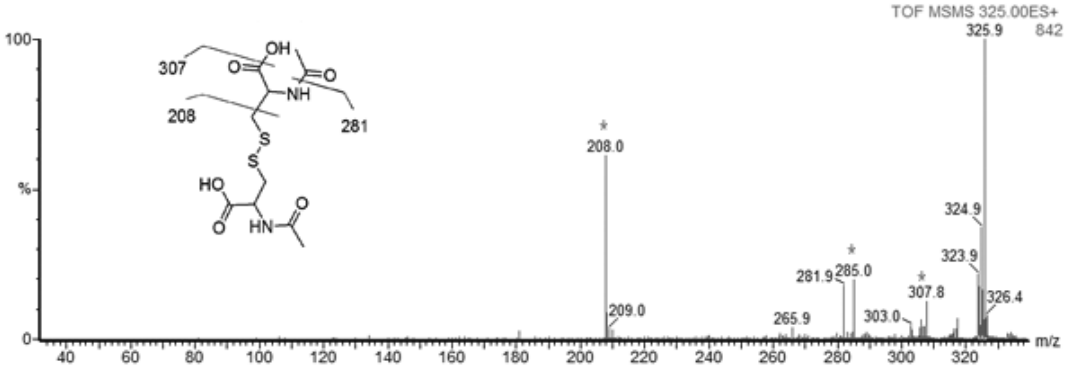


Figure S11: ESI-MS/MS analysis for N-acetyl-cysteine dimer at *m/z* 325. Suggested mode of fragmentations for N-acetyl-cysteine dimer *m/z* 324.

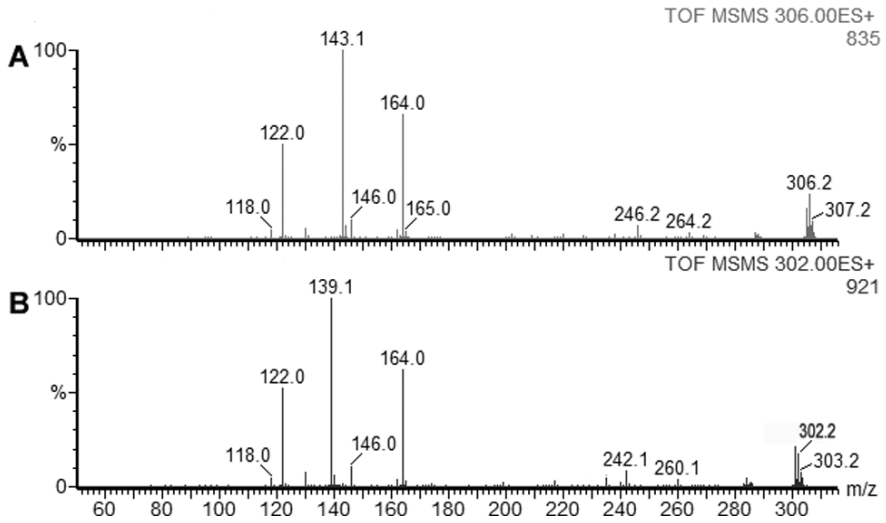


Figure S12: The ESI-TOF-MS/MS analysis of the dehydrated (N-acetyl-cysteine)–HNE Michael adduct.

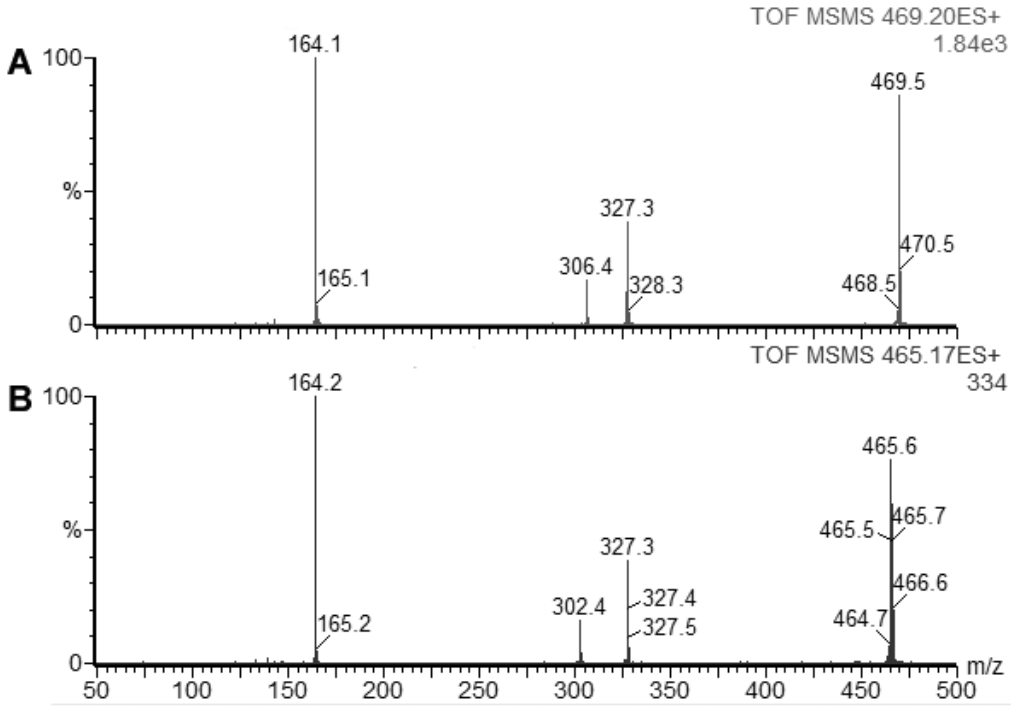


Figure S13: ESI-MS/MS analysis for molecular ion at A) *m/z* 469 and B) *m/z* 465 corresponding to LC-ESI analysis of the product mixture from the reaction of HNE-(with N-acetyl-cysteine) (1:2) adduct.

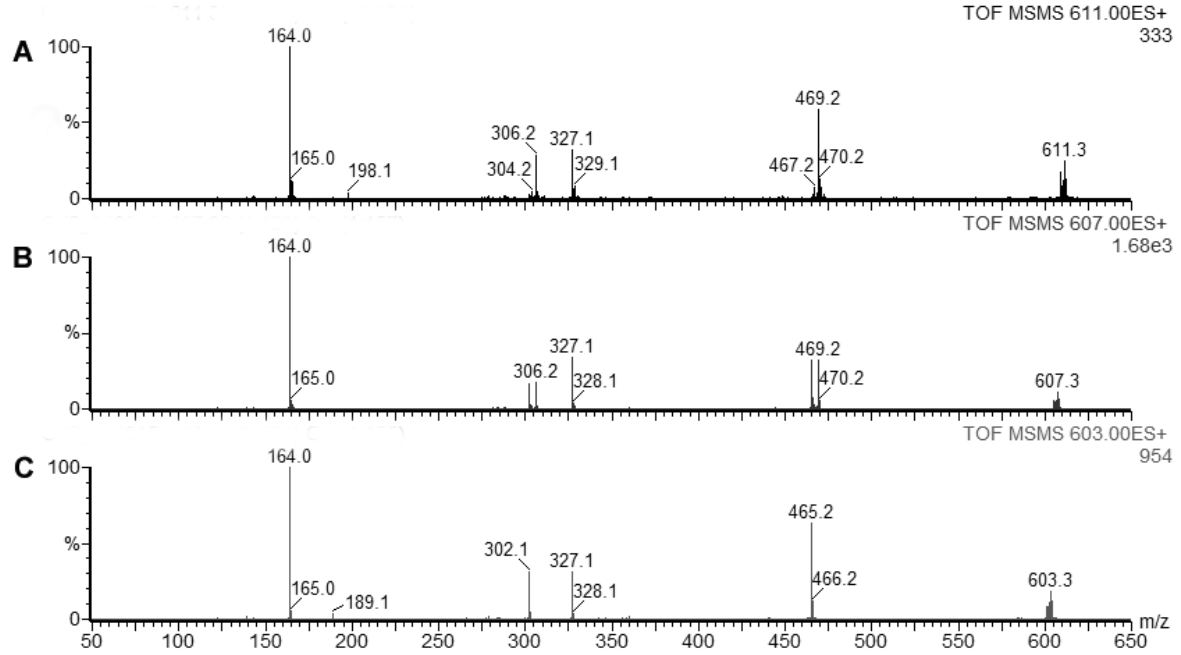
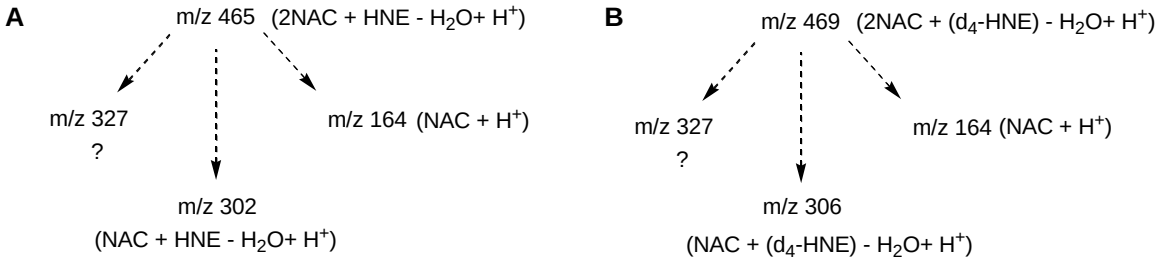
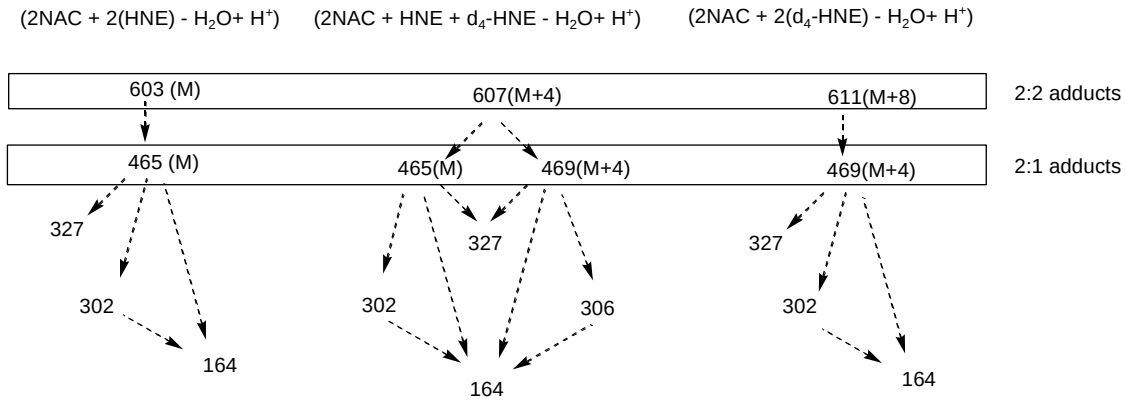


Figure S14: ESI-MS/MS analysis of the ions with A) *m/z* 611, B) *m/z* 607 and C) *m/z* 603, which correspond to 2:2 (N-acetyl-cysteine)-HNE adducts.

Scheme S5: Daughter ions formed from the tandem MS/MS of 2:1 (N-acetyl-Cys): HNE adduct A) d_0-HNE-2(N-acetyl-Cys) adduct B) d_4-HNE-2(N-acetyl-Cys) adduct, (NAC = N-acetyl-cysteine).



Scheme S6: Daughter ions formed from the tandem MS/MS of 2:2 (N-acetyl-cysteine: HNE) adduct *m/z* 603, 607 and 611. (NAC = N-acetyl-cysteine)



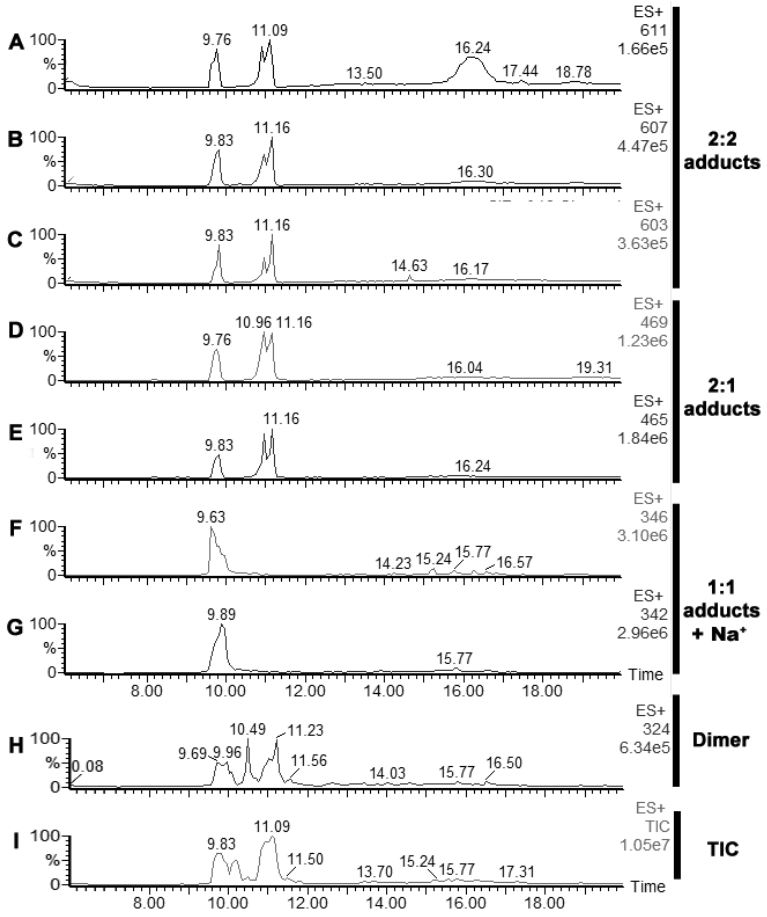


Figure S15. LC-ESI/SIM/MS analysis of the molecular ions observed in (N-acetyl-cysteine)-HNE reaction mixture through 8 channels. (Chromatograms shown for A) *m/z* 611, B) *m/z* 607, C) *m/z* 603, D) *m/z* 469, E) *m/z* 465, F) *m/z* 346, G) *m/z* 342 H) *m/z* 324 and I) TIC.

Method to quantify the relative amounts of adducts formed. The LC-ESI/SIM chromatograms for each of the adduct was integrated using the Masslynx® software ver 3.5. The total peak area of the chromatogram for each of the adduct (A) was determined and using the formulae below the values of relative amounts in percentage was calculated. The error bars reflect the results of two independent experiments.

$$\text{Relative percentage of an adduct A(m)} = \frac{A(m)}{\{ A(1) + A(2) + \ldots + A(n) \}} \times 100$$

n – the total number of adducts

A(n) – Total area of the chromatogram for the extracted ion chromatogram of adduct n.

NMR Spectra of Unknown Compounds:

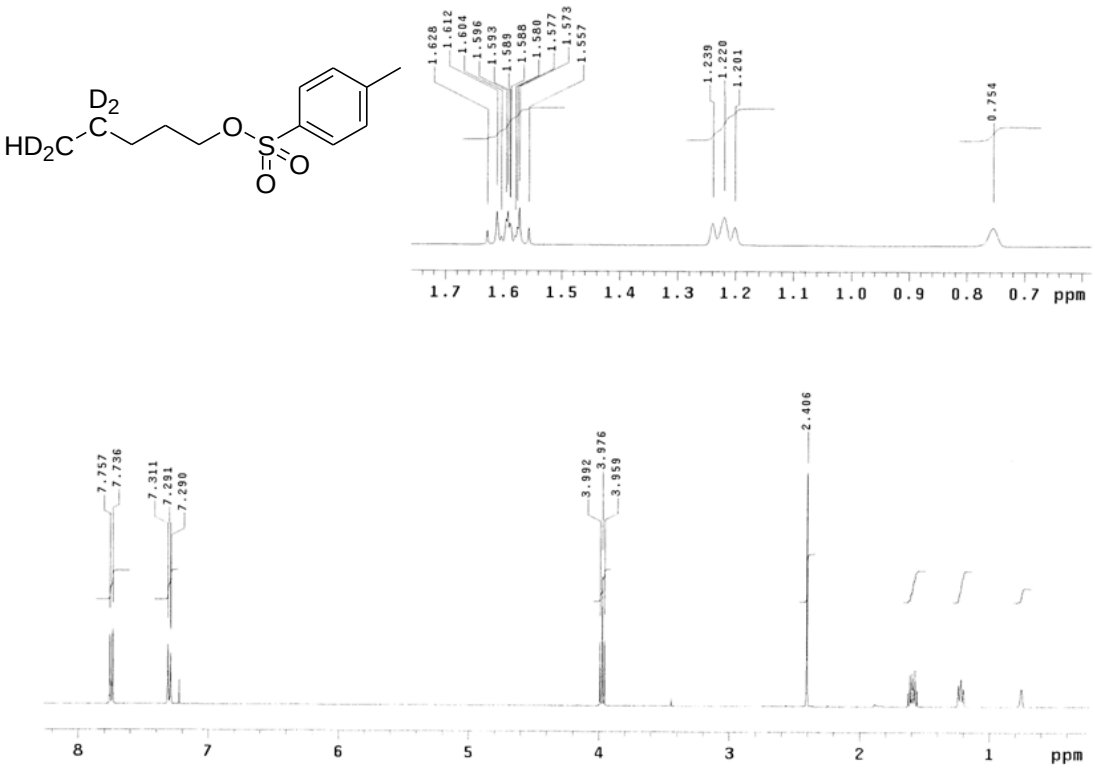


Figure S16: ^{1}H NMR of **3**

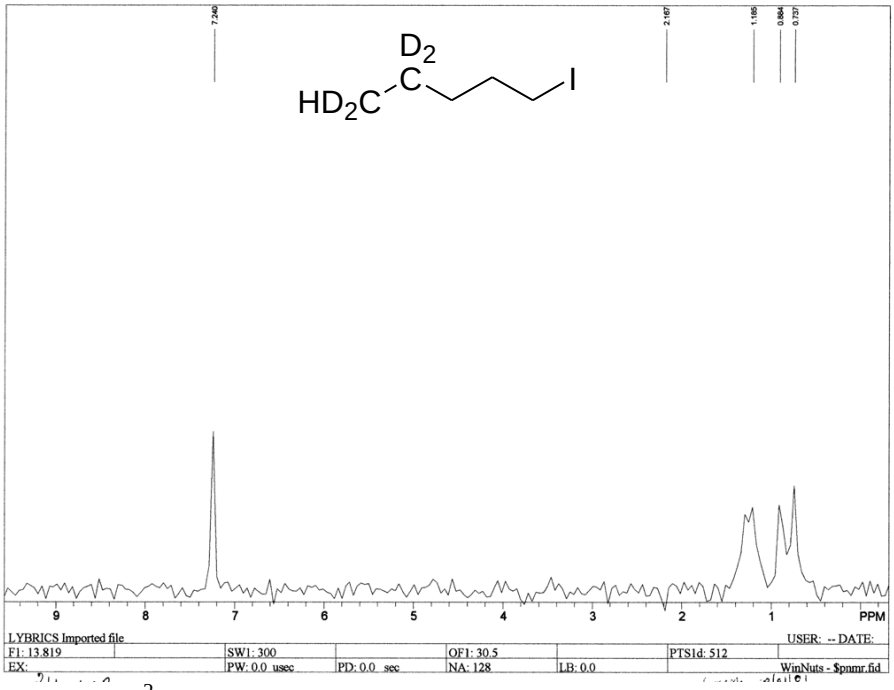
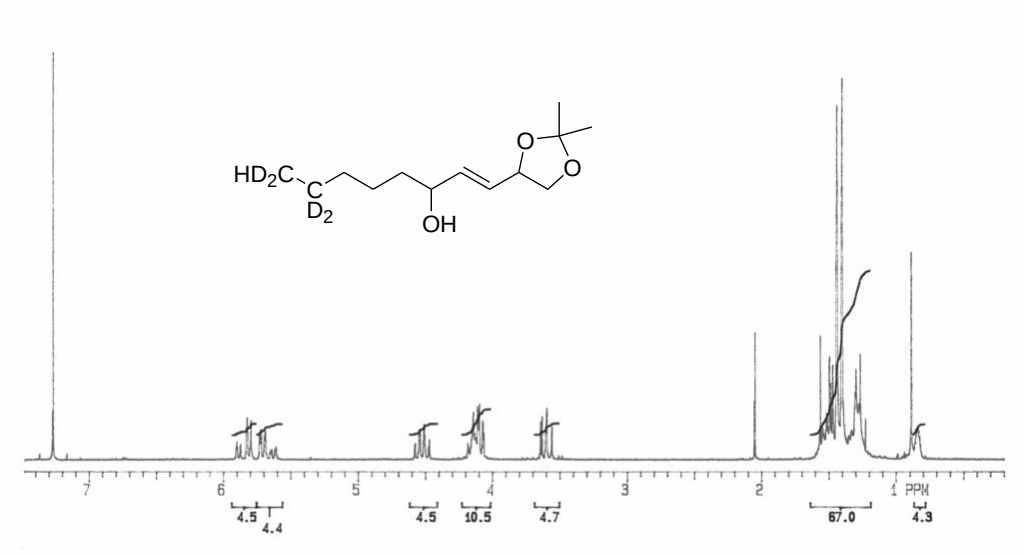


Figure S17: ^{2}H NMR of **4**

a)



a)

b)

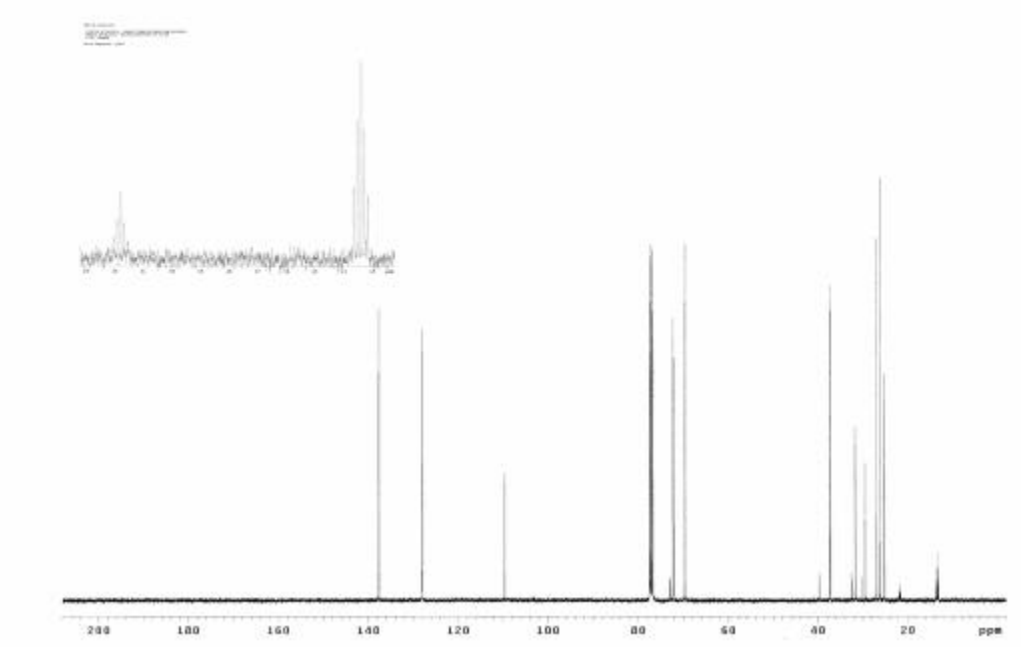
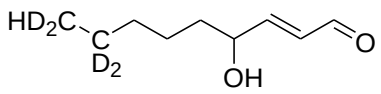


Figure S18: a) ^{1}H NMR b) ^{13}C NMR of **6**



7

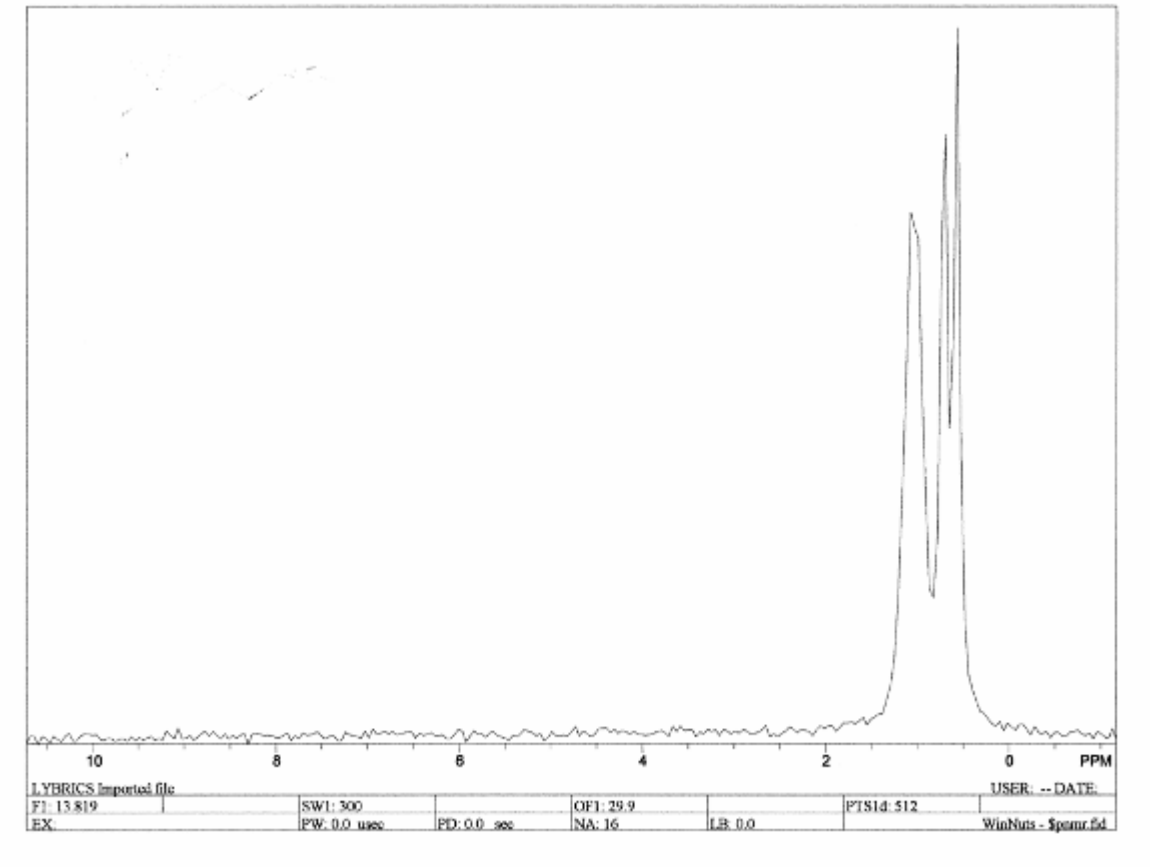


Figure S19: ^{2}H NMR of **7**

a)

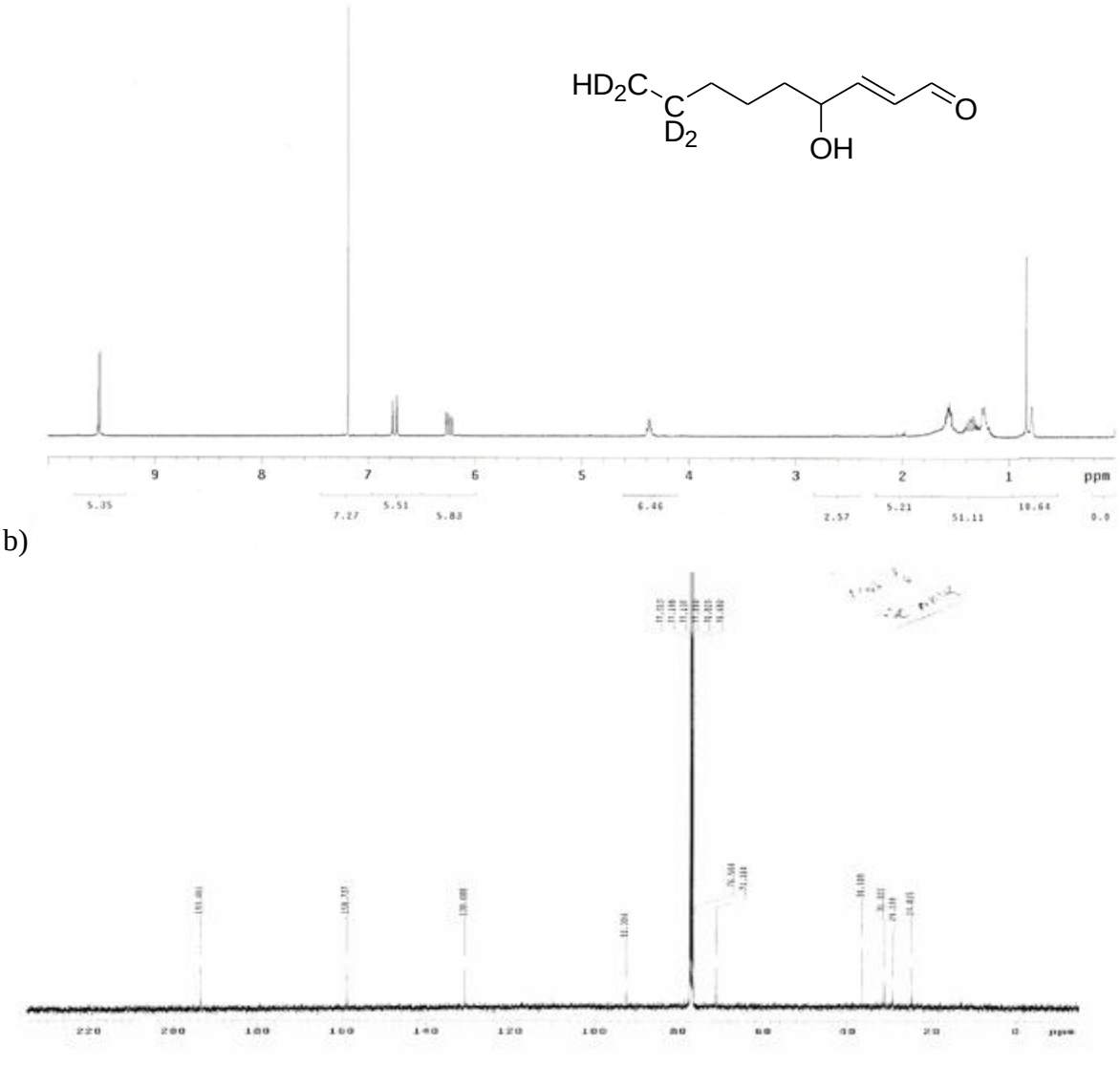


Figure S20: a) ^{1}H NMR and b) ^{13}C NMR of **7**

References:

(1) Kingston, D. (1974) Intramolecular hydrogen transfer in mass spectra. II. The McLafferty Rearrangement and related reactions. *Chemical Reviews 74*, 215.

(2) SDBS. http://www.aist.go.jp/RIODB/SDBS/ (National Institute of Advanced Industrial Science and Technology,). *2005*.

(3) Sayre, L. M., Arora, P. K., Iyer, R. S. and Salomon, R. G. (1993) Pyrrole formation from 4-hydroxynonenal and primary amines. *Chem Res Toxicol 6*, 19-22.

(4) Xu, G. and Sayre, L. M. (1998) Structural characterization of a 4-hydroxy-2-alkenal-derived fluorophore that contributes to lipoperoxidation-dependent protein cross-linking in aging and degenerative disease. *Chem Res Toxicol 11*, 247-251.

(5) Nadkarni, D. V. and Sayre, L. M. (1995) Structural definition of early lysine and histidine adduction chemistry of 4-hydroxynonenal. *Chem Res Toxicol 8*, 284-291.