Supplementary information to

'Oxidative Tryptophan modification by terpene- and squalene-hydroperoxides and a possible link to cross-reactions in diagnostic tests'

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Synthesis of test compounds:

Hydroperoxide of Linalool **2**. A 45:55 mixture of (5*E*)-7-hydroperoxy-3,7-dimethylocta-1,5-diene-3-ol (linalool-7-OOH) and 6-hydroperoxy-3,7-dimethylocta-1,7-diene-3-ol (*cis/trans*-linalool-6-OOH, consisting of 2 diastereomers) was prepared from synthetic linalool by oxidation in a microemulsion made from water, sodium dodecyl sulfate, n-butanol, and dichloromethane, using sodium molybdate /35% aq. and hydrogen peroxide as a source of singlet oxygen.[1] This linalool hydroperoxide reference contained 80.2% of total linalool hydroperoxides (55:45 mixture of 6-/7-hydroperoxides) as determined with a quantitative NMR experiment. Detailed analytical data on this synthetic sample were provided before.[2]

Hydroperoxide of Limonene **1**. A mixture of (4*R*,6*S*)-6-hydroperoxy-1-methyl-4-(prop-1-en-2-yl)cyclohex-1-ene (*trans*-limonene-2-OOH) and (4*R*,6*R*)-6-hydroperoxy-1-methyl-4-(prop-1-en-2-yl)cyclohex-1-ene) (*cis*-limonene-2-OOH) was prepared from (-)-carveol (64:36 *trans*/*cis*) according to the literature [1]. The sample contained 80% limonene-2-OOH based on a 2D NMR experiment (HSQC). Detailed analytical data on this synthetic sample were provided before.[3]

Hydroperoxides of Squalene **3**. The mixture of squalene hydroperoxides was obtained by treating squalene with 35% aq. hydrogen peroxide/sodium molybdate in a microemulsion consisting of water, dichloromethane, sodium dodecyl sulfate and *n*-butanol as described in the literature for the synthesis of linalool hydroperoxides.[1] At 20°C, a mixture of sodium dodecyl sulfate (19.5 g), *n*-butanol (60 ml) and dichloromethane (293 ml) was treated with a solution of sodium molybdate (2.4 g) in water (12.5 ml). The resulting suspension was stirred for 5 min and treated with squalene (5 g). After 5 min stirring, a first portion of 35% hydrogen peroxide in water (1.5 ml) was added and the resulting orange/red solution was stirred for 20 min. Another nine portions of 35% hydrogen peroxide in water (1 ml) were then added every 10 min. (10.5 ml, 10 eq., in total) while a moderate gas release was observed and the reaction temperature rose to 30°C. The reaction mixture was then stirred for 135 min. The solvent was removed in vacuo (46°C, water bath) and dichloromethane (900 ml) was added to the residue (35 g). The resulting suspension was stirred at 20°C for 19 h, filtered, and the filtrate concentrated in vacuo. The residue was dissolved in dichloromethane (200 ml) and washed three times with water (100 ml). The aqueous phases were extracted with dichloromethane (100 ml) and the combined organic phases were dried ($MgSO_4$) and concentrated to give a crude yellow oil (6.8 g).

The analytical data (NMR, TLC) of the crude mixture are in agreement with the literature data of the squalene hydroperoxide mixtures prepared by photooxidation:[4-6]

^{1}H-NMR (400MHz, $CDCl_3$): selected signals δ 9.5-7.5 (br. *s*, OOH), 5.8-5.4 (*m*), 5.2-4.9 (*m*), 4.5-4.2 (*m*), 3.0-2.6 (*m*), 2.3-1.9 (*m*).

TLC (benzene/EtOAc 93:7 v/v and hexane/Et_2O/AcOH 85:15:2 v/v; revelation using 1% N,N,N'N'-tetramethyl-1,4-phenylenediamine in MeOH/H_2O/AcOH 50:50:1 v/v): Rf range of 0.39-0 and 0.19-0, respectively.

NMR structure elucidation of 5*:*

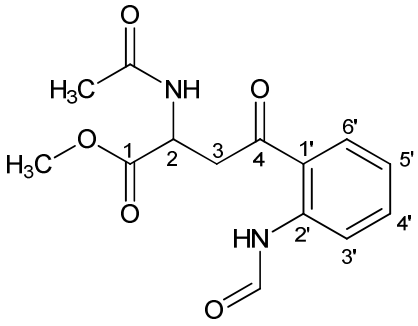
All NMR measurements were performed at room temperature on a Bruker Avance III 600 MHz instrument equipped with a TCI microCryoprobe 1.7 mm. The ^{1}H NMR was referenced by the residual HDO line at 4.79 ppm. In the HSQC and HMBC experiments the ^{13}C dimension was referenced to TSP (Sodium 3-trimethylsilyl-tetradeuterio-propionate) as an external standard at 0.0 ppm.

^{1}H NMR: spectral width = 20.5 ppm, acquisition time = 2.66 s, recycle time = 1.0 s, time domain = 64K points, size of real spectrum = 64 K points, window function = exponential multiplication, line broadening 0.2, number of scans = 16. Gradient selected and multiplicity edited HSQC: spectral width = 8.0 ppm (^{1}H) x 160.0 ppm (^{13}C), acquisition time: 120 ms, recycle time: 2.0 s, time domain = 1152 (^{1}H) x 256 (^{13}C) points, size of real spectrum = 1024 x 1024 points, window function = squared sine-bell (phase shift = 2) in both dimensions, number of scans = 4. Gradient selected HMBC: spectral width = 8.0 ppm (^{1}H) x 200.0 ppm (^{13}C), acquisition time = 357 ms, recycle time = 2.0 s, time domain = 3392 (^{1}H) x 512 (^{13}C) points, size of real spectrum = 2048 (^{1}H) x 1024 (^{13}C) points, window function = sine-bell (phase shift = 4, ^{1}H), squared sine-bell (phase shift = 2, ^{13}C), number of scans = 16. COSY-DQF: spectral width = 8.0 ppm (^{1}H) x 8.0 ppm (^{1}H), acquisition time = 320 ms, recycle time = 2.0 s, time domain = 3072 x 512 points, size of real spectrum = 2048 x 1024 points, window function = squared sine-bell (phase shift = 2) in both dimensions, number of scans = 2. NOESY: spectral width = 8.0 ppm (^{1}H) x 8.0 ppm (^{1}H), acquisition time = 346 ms, recycle time = 2.0 s, time domain = 3328 x 512 points, size of real spectrum = 2048 x 1024 points, window function = squared sine-bell (phase shift = 2) in both dimensions, number of scans = 4, mixing time = 750 ms.

The structure of compound **5** has been determined by careful examination of the following gradient enhanced 2D-NMR experiments: a multiplicity-edited HSQC, a HMBC and a double quantum filtered COSY. The oxidative ring opening of the tryptophan derivative **4** has been evidenced by the presence of a cross-peak correlating to a ketone shift at 201.7 ppm to the aromatic proton H6′ detected in the HMBC spectrum. Additionally, the HSQC (figure S2) reveals a formamide functionality characterized by the typical cross-peak with the coordinates 163.0/8.30 [^{13}C/^{1}H] ppm. The formyl proton is correlated to the aromatic ring by the HMBC spectrum leading to the quaternary sp2-carbon 2′ at 135.5 ppm, confirming its position. All correlations stemming from the HMBC and COSY-DQF spectra are reported in Table S1.

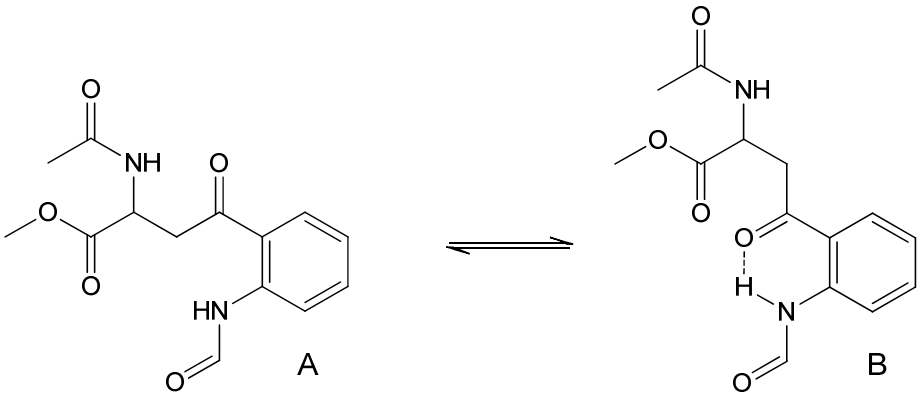
Table S1. Correlations of COSY-DQF and HMBC spectra for the major conformer of compound **5.**

#C position	δC, assignment	δH, mult. (J in Hz)	COSY-DQF	HMBC
1	173.2, $C_{quat.}$			
2	48.4, CH	4.89, m	H-3	C-1, C-3, C-4
3	41.3, CH_2	3.61-3.73, m	H-2	C-1, C-2, C-4
4	201.7, $C_{quat.}$			
1′	125.5, $C_{quat.}$			
2′	135.5, $C_{quat.}$			
3′	123.1, CH	8.07, d, (8.8)	H-4′	C-1′, C-5′
4′	134.5, CH	7.62, t, (8.1)	H-3′, H-5′	C-2′, C-6′
5′	125.0, CH	7.32, t, (8.1)	H-4′, H-6′	C-1′, C-3′
6′	130.5, CH	7.93, d (8.6)	H-5′	C-4, C-2′, C-4′
CH_3O	52.9, CH_3	3.71, s		C-1
$\underline{CH_3}CO$	21.5, CH_3	1.96, s		$CH_3\underline{C}O$
$CH_3\underline{C}O$	173.8, $C_{quat.}$			
CHO	163.0, CH	8.30, s		C-2′



The ^{1}H NMR reveals a second set of signals partially overlapped with those of the main component, present as a minor proportion of 1.0 to 2.6. These signals belong to a conformer of **5** according to the observations made in the NOESY experiment depicted in figure S3. Beside the expected cross-peaks related to Nuclear Overhauser Effects (NOE) with an opposite phase to the diagonal signals, multisite chemical exchange cross-peaks are also visible as in-phase signals. This NOESY experiment is described as an EXSY (**EX**change **S**pectroscop**Y**) experiment[7] if the exchange signals are targeted. Each of these represents a positional exchange between the two categories of conformers. The affected protons are the formyl proton of the formamide moiety, as well as the two ortho-aromatic protons H3' and H6'. This suggests a conformational equilibrium between random conformers A and a hydrogen bonded conformer B. An alternative conformational restriction engaging a hydrogen bond from the acetamidic NH to the same ketone was discarded in regard to the position of the involved protons in the

chemical exchange. Furthermore, the aromatic ortho-disubstitution brings the formamide NH in close proximity to the ketone thus making it prone to hydrogen bonding.



Methyl 2-acetamido-4-(2-formamidophenyl)-4-oxobutanoate (**5**, main conformer) ^{1}H NMR (D_2O, 600 MHz) δ 1.96 (3H, s, CH_3CO), 3.61-3.73 (2H, m, H3), 3.71 (3H, s, CH_3O), 4.89 (1H, m, H2), 7.32 (1H, t, *J*=8.1 Hz, H5′), 7.62 (1H, t, *J*=8.1 Hz, H4′), 7.93 (1H, d, *J*=8.6 Hz, H6′), 8.07 (1H, d, *J*=8.8 Hz, H3′), 8.30 (1H, s, CHO), 8.39 (2H, s, N<u>H</u>CHO and N<u>H</u>$COCH_3$). ^{13}C NMR (D_2O, 150 MHz, chemical shifts extracted from HSQC & HMBC experiments) δ 21.5 ($\underline{CH_3}CO$), 41.3 (C3), 48.4 (C2), 52.9 (CH_3O), 123.0 (C3′), 125.0 (C5′), 125.5 (C1′), 130.5 (C6′), 134.5 (C4′), 135.5 (C2′), 163.0 (CHO), 173.2 (C1), 173.8 ($\underline{C}OCH_3$), 201.7 (C4).

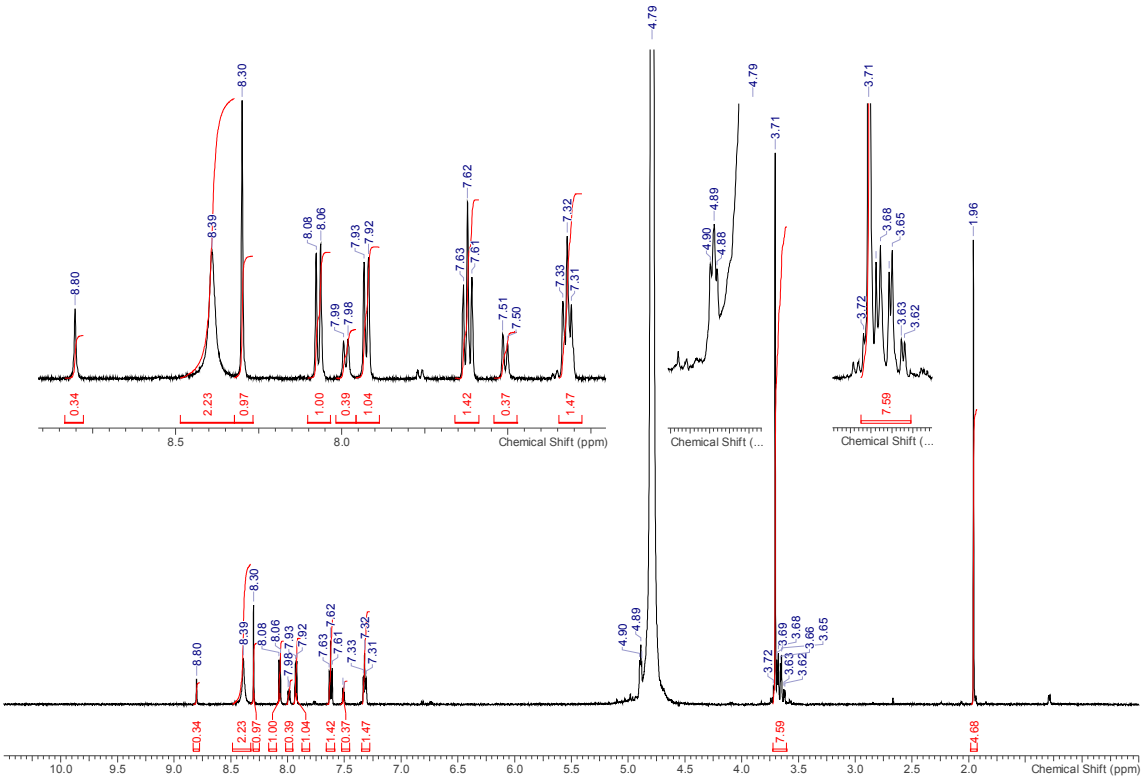


Figure S1. ^{1}H NMR (D_2O) spectrum of compound **5**, including embedded expansions.

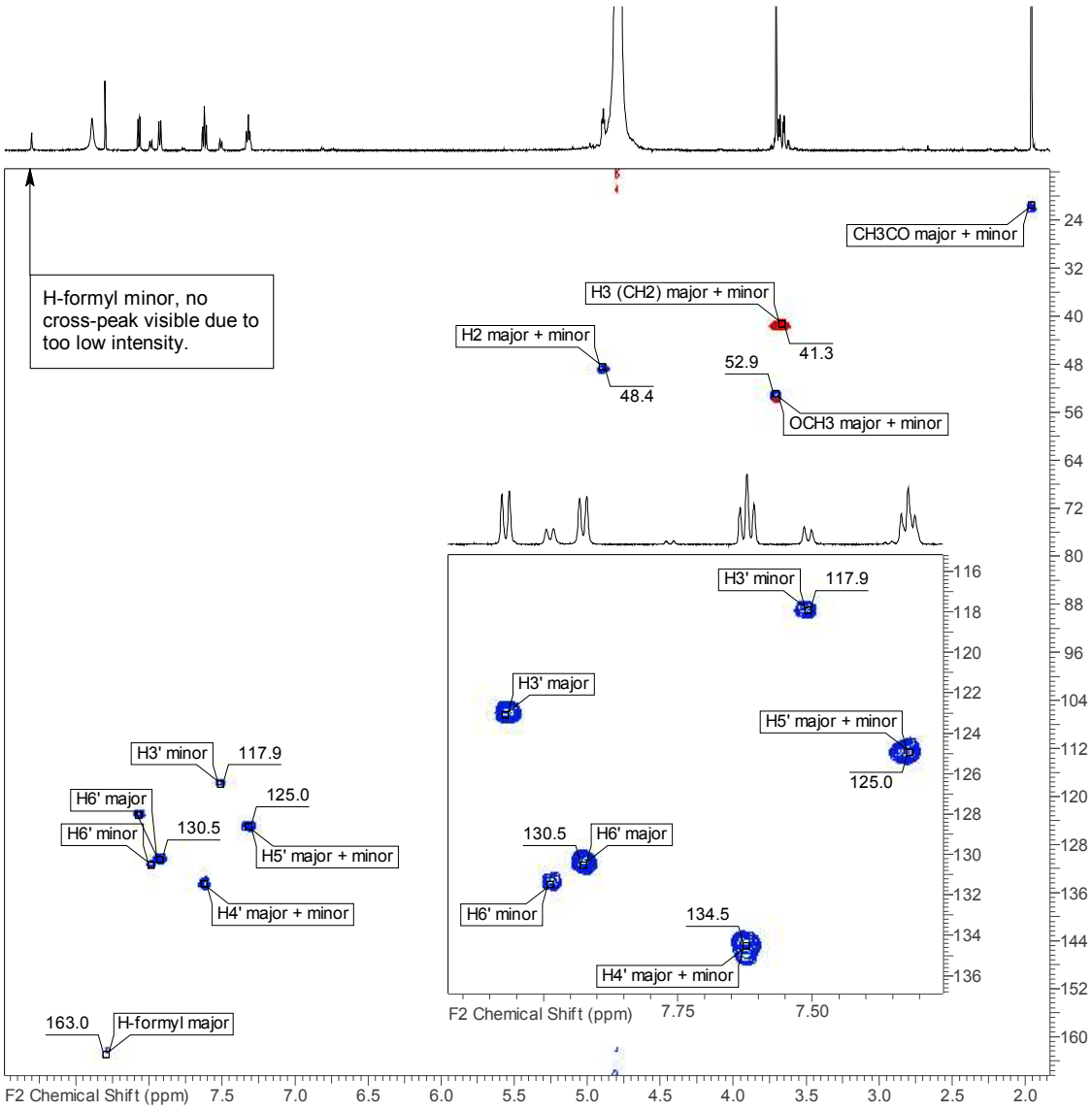


Figure S2. Multiplicity-edited HSQC spectrum (D_2O) of compound **5**, including an embedded expansion of the aromatic protons.

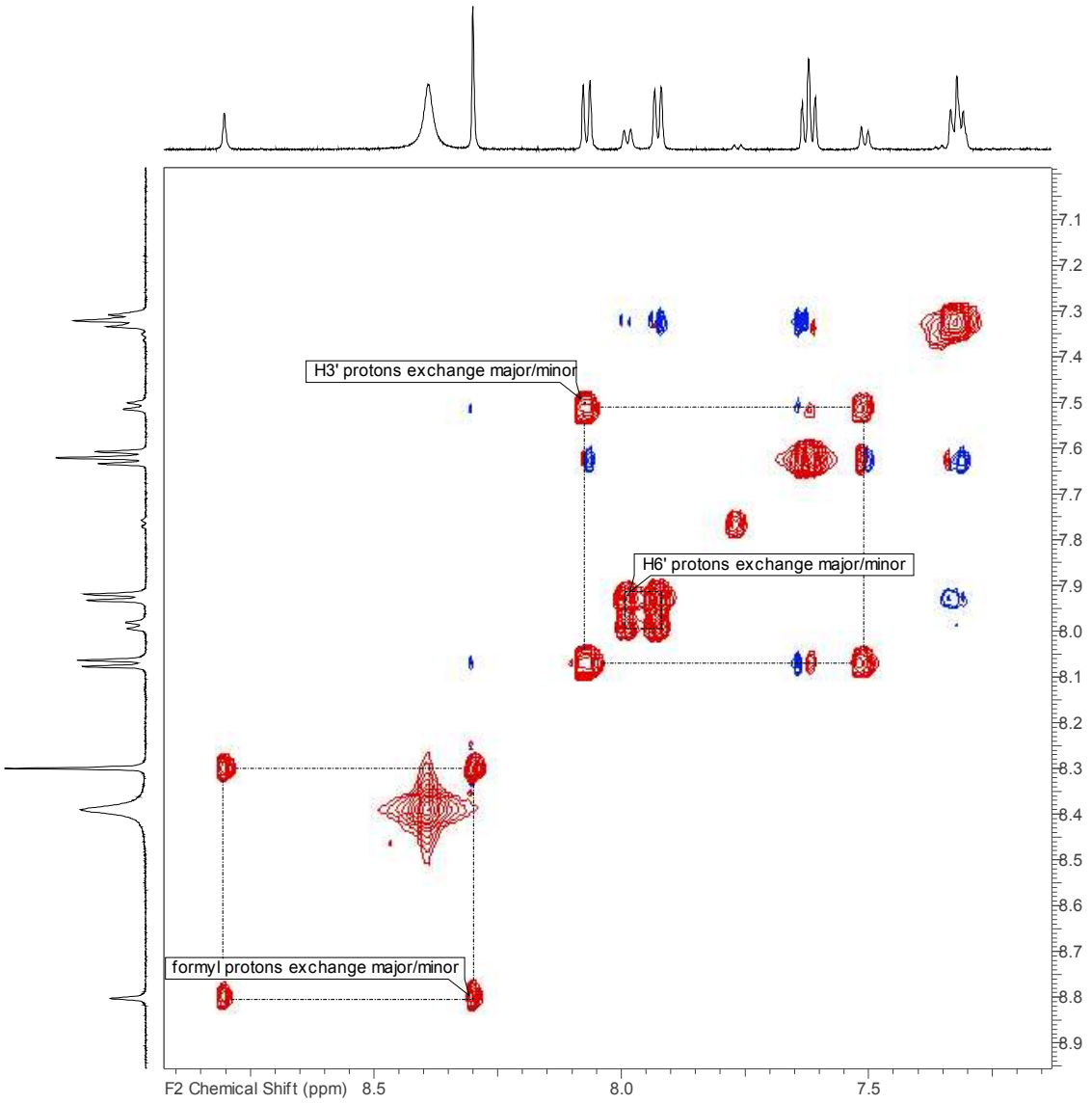


Figure S3. Expansion of the NOESY (EXSY) spectrum (D_2O) of compound **5** in the aromatic region. The labeled cross-peaks indicate chemical exchange between conformers A and B.

LC-MS and LC-MS/MS analysis

The same sample analyzed by NMR was compared to putative **5** formed in presence of **4,** heme, and the different hydroperoxides. As shown in Figure S4, the analyte **5** with theoretical mass $[M+H]^+$ = 293.1132 and experimentally found $[M+H]^+$ = 293.1123/293.1130 respectively was found in the different samples (reference sample and experimental sample with Linalool-OOH and heme). The corresponding peaks from the extracted exact mass of **5** revealed the same retention time and identical MS/MS spectra which proves that they are the same analytes.

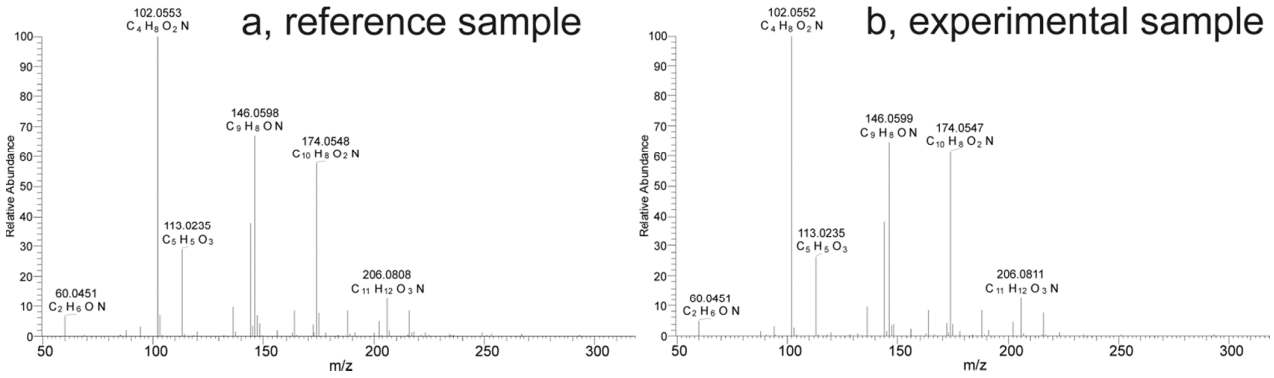


Figure S4. MS/MS spectrum of **5**. (a) reference sample prepared by photooxidation wit Bengal Rose and analyzed in detail by NMR, (b) experimental sample with peak of **5** formed in presence of linalool hydroperoxide and heme. The precursor ion has an experimental mass $[M+H]^+$ = 293.1123 (a) and 293.1130 (b) which is within 5ppm mass deviation (theoretical mass $[M+H]^+$ = 293.1132).

The second most abundant reaction product with $[M+H]^+$ = 277.1388 could not be isolated in sufficient purity for NMR analysis. Figure S5 shows the MS/MS spectrum of this reaction product. Based on its molecular mass we hypothesize that it corresponds to alcohol peak 9 in Gracanin *et al.*[8] This hypothetical structure was thus subjected to analysis by the Mass frontier software (Version 7.0, HighChem Ltd., Slovakia), which predicted fragments corresponding to observed fragments (as indicated in Figure S5). Thus high resolution MS/MS data are consistent with that proposal, but we do not have NMR verification. However, we were in this work mostly interested whether different hydroperoxides trigger formation of *N*-formyl-kynurenine derivatives which had been proposed as possible reasons for cross-sensitization.

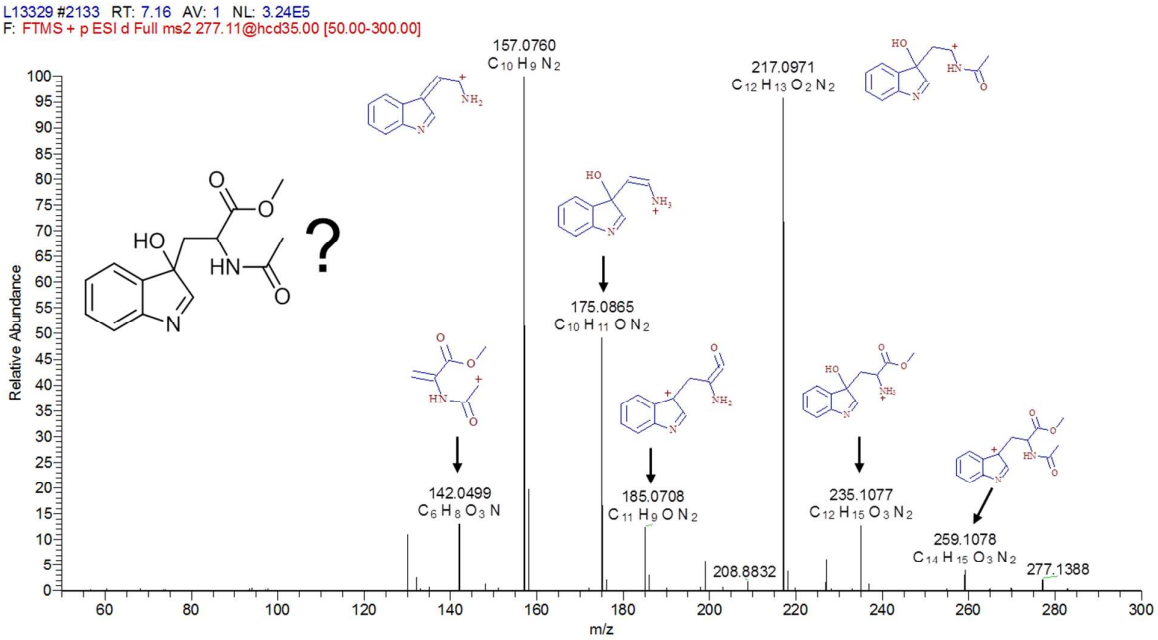


Figure S5. MS/MS spectrum of the second most abundant product formed in incubations of heme, **4** and either **1**,**2** or **3**. The precursor ion has an experimental mass $[M+H]^+ = 277.1388$ (theoretical mass $[M+H]^+ = 277.1183$). Shown is the putative structure in agreement with the literature (peak 9 in [8]) and the fragments predicted by the Mass frontier software aligned to the observed masses.

References:

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