Supporting Information for: 4-Hydroxy-7-oxo-5-heptenoic Acid (HOHA) Lactone is a Biologically Active Precursor for the Generation of 2-(ω-Carboxyethyl)pyrrole (CEP) Derivatives of Proteins and Ethanolamine Phospholipids

*Hua Wang, Mikhail Linetsky, Junhong Guo, Jaewoo Choi, Li Hong, Amanda S. Chamberlain, Scott J. Howell, Andrew M. Howes and Robert G. Salomon***

Synthesis of CEP-PC-dipeptide (11):

1-Palmityl-2-(4,7-dioxoheptanoyl)-*sn*-glycero-3-phosphatidylcholine

1-Palmityl-2-(Ac-Gly-Lys-OMe-CEP)-*sn*-glycerophosphatidylcholine

Table S1. The optimized mass spectrometer parameters as well as the MRM transitions and collision energies for each individual analyte.

Figure S1. (A) ESI-MS scan of authentic CEP-dipeptide **9**; (B) ESI-MS/MS scan of m/z 382.5 at collision energy 30 eV and the proposed daughter ion structures.

Figure S2. The LC-MS chromatogram for authentic furan-dipeptide **10a** and CEP-dipeptide **10** as well as their mixture.

Figure S3. ESI-MS spectrum of authentic CEP-EA, **14** and d_4-CEP-EA as well as their daughter ions in the positive ion mode.

Figure S4. (A) ESI-MS scan of HOHA-lactone-PFB oxime in the positive ion mode. (B) ESI-MS/MS scan of m/z 336 at collision energy 25 eV and the proposed daughter ion structures.

Post-translational modification of ARPE-19 cells by HOHA-Lactone.

PAGE and Western blot analysis of proteins in ARPE cell lysates.

Detection CEP in ARPE-19 cells incubated with H_2O_2.

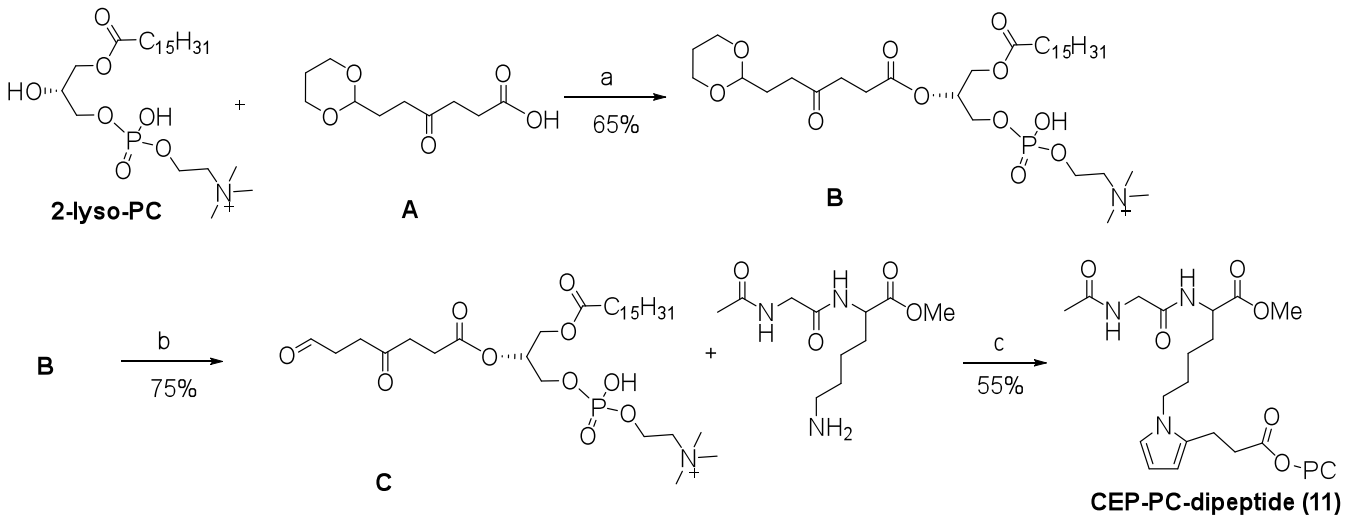
Figure S5. Modification of ARPE-19 cells by HOHA-lactone.

Figure S6. ARPE-19 cells treated with 5 μM H_2O_2 and then mmunostained with mouse monoclonal anti-CEP antibody.

Figure S7. ^{1}H NMR spectrum of 3-(furan-2-yl)propanoyl amide **10a** (400 Mz, CD_3OD)

Figure S8. ^{13}C NMR spectrum of 3-(Furan-2-yl)propanoyl amide **10a** (101 Mz, CD_3OD)

Synthesis of CEP-PC-dipeptide (11) Through Paar-Knorr Pyrrole Synthesis.



Reagents and Conditions: a.DCC, DMAP, CHCl3. b. AcOH/H_2O (3:1 v/v), 50 °C. c. $CHCl_3$/MeOH (1:2 v/v)

1-Palmityl-2-(4,7-dioxoheptanoyl)-*sn*-glycero-3-phosphatidylcholine (B). In a flame-dried 25 mL round-bottom flask, a mixture of 1-palmityl-*sn*-glycero-3-phosphatidylcholine (2-lyso-PC, 50 mg, 0.1 mmol), 6-[1,3]dioxolan-2-yl-4-oxo-hexanoic acid (**A)** (30 mg, 0.14 mmol), 4-dimethylamino-pyridine (DMAP, 12.2 mg, 0.1 mmol) was thoroughly dehydrated by azeotropic evaporation of molecular sleeve dried toluene (50 mL), was dissolved in 6.0 mL of dry chloroform. Dicyclohexylcarbodiimide (DCC, 103 mg, 0.5 mmol) was added. The reaction mixture was stirred overnight under argon, and the residue was purified by flash chromatography on a silica gel column (chloroform/methanol/H_2O = 16:9:1, R_f = 0.20) to give pure **Compound B** (45 mg, 65%). ^{1}H NMR (400 MHz, CD_3OD) δ 5.26 – 5.13 (m, 1H), 4.55 (t, *J* = 5.0 Hz, 1H), 4.35 (dd, *J* = 11.9, 3.6 Hz, 1H), 4.28 (dd, *J* = 7.3, 4.0 Hz, 4H), 4.22 – 4.13 (m, 1H), 4.07 – 3.92 (m, 4H), 3.75 (td, *J* = 12.4, 2.5 Hz, 2H), 3.69 – 3.60 (m, 2H), 3.22 (s, 9H), 2.83 – 2.66 (m, 2H), 2.63 – 2.45 (m, 4H), 2.33 (q, *J* = 7.6 Hz, 2H), 1.96 (ddd, *J* = 17.5, 12.5, 7.5 Hz, 1H), 1.78 (td, *J* = 7.4, 5.0 Hz, 2H), 1.66 – 1.49 (m, 2H), 1.40 – 1.17 (24H), 0.88 (t, *J* = 6.9 Hz, 3H). ^{13}C NMR (100 MHz, CD_3OD) δ 209.3, 173.8, 173.5, 172.9, 172.6, 101.1, 71.0, 66.7, 66.3, 63.7, 62.5, 62.3, 59.3, 53.5, 53.4, 48.5, 36.6, 36.6, 36.3, 33.8, 33.6, 31.9, 29.6, 29.6, 29.5, 29.3, 29.0.

1-Palmityl-2-(Ac-Gly-Lys-OMe-CEP)-*sn*-glycerophosphatidylcholine (CEP-PC-dipeptide, 11) 40 mg of **Compound B** (58 μmol) was dissolved in 10 mL of AcOH-water (3:1) solution, and the mixture was stirred at 50 °C overnight. TLC analysis showed complete reaction. The solvent was evaporated to dryness and the residue was purified by flash chromatography on a silica gel column

(chloroform/methanol/H_2O = 16:9:1, R_f = 0.15) to give pure DOHA-PC ester **Compound C** (27.5 mg, 75%). ESI-MS of m/z $(M+H)^+$: calcd 638.39; found 638.67.

PC ester compound C (10 mg, 16 μmol) was mixed with Ac-Gly-Lys-OMe dipeptide (5 mg, 20 μmol) in 10 mL of methanol. The mixture was stirred at room temperature overnight under argon whereupon TLC analysis showed complete reaction. The reaction mixture was then concentrated under reduced pressure. Then the residue was purified by preparative TLC (elution solvent: chloroform/methanol/H_2O = 16:9:2, R_f = 0.10) to give pure CEP-PC-dipeptide **11** (7.6 mg, 55%). ^1H NMR (400 MHz, CD_3OD) δ 6.56 (m, 1H), 5.96 – 5.87 (m, 1H), 5.78 (s, 1H), 5.27 – 5.16 (m, 1H), 4.62 (m, 1H), 4.38 (d, *J* = 11.9 Hz, 2H), 4.19 (dd, *J* = 18.2, 11.8 Hz, 4H), 4.00 (m, 2H), 3.84 (dd, *J* = 10.4, 6.3 Hz, 3H), 3.68 (s, 3H), 3.60 (m, H), 3.19 (s, 9H), 2.84 (d, *J* = 6.8 Hz, 2H), 2.69 (d, *J* = 7.8 Hz, 2H), 2.55 (m, 2H), 2.29 (d, *J* = 8.2 Hz, 2H), 2.22 – 2.11 (m, 1H), 1.99 (s, 3H), 1.91 (5H), 1.85 – 1.76 (m, 2H), 1.70 (s, 2H), 1.56 (s, 2H), 1.27 (s, 24H), 0.97 – 0.81 (m, 3H).

	CEP-dipeptide, CEP-lysine	CEP-PC-dipeptide, LysoPC (13:0)	HOHA-lactone-PFB oxime
Ion mode	Positive	Positive	Positive
Capillary(kV)	3.0	3.0	4.0
Cone (V)	40	40	60
Hex 1 (V)	40	40	35
Aperture (V)	0	0	0
Hex 2 (V)	1.0	1.0	1.0
LM 1 Resolution	15	15	15
HM 1 Resolution	15	15	15
Ion Energy 1	0.7	0.7	0.7
LM 2 Resolution	15	15	15
HM 2 Resolution	15	15	15
Ion Energy 2	3.0	3.0	3.0

Analytes	MRM transition ion pair (m/z)	Collision energy (eV)
CEP-dipeptide	382.3 > 206.3	25
	382.3 > 265.3	25
	382.3 > 283.0	30
	382.3 > 322.2	25
CEP-PC-dipeptide	859.6 > 184.0	30
CEP-lysine	269.2 > 84.0	25
LysoPC (13:0)	454.0 > 184.0	30
HOHA-lactone-PFB oxime	336.3 > 139.1	25

Table S1. The optimized mass spectrometer parameters as well as the MRM transitions and collision energies for each individual analyte.

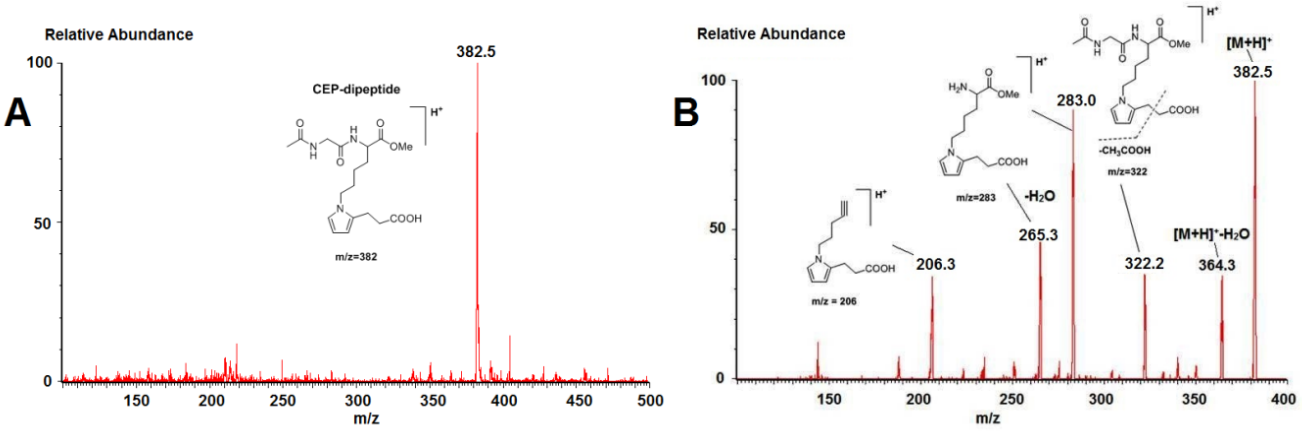


Figure S1. (A) ESI-MS scan of authentic CEP-dipeptide **9**; (B) ESI-MS/MS scan of m/z 382.5 at collision energy 30 eV and the proposed daughter ion structures.

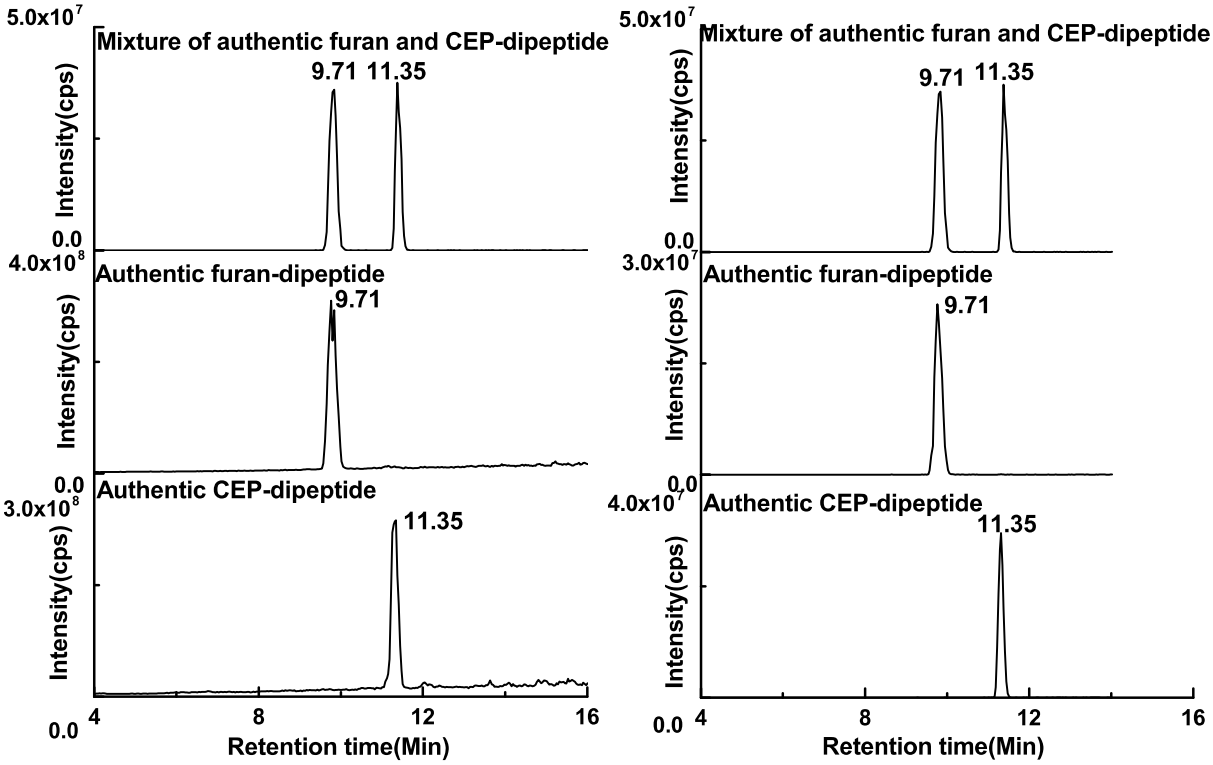


Figure S2. The LC-MS chromatogram for authentic furan-dipeptide (**10a**) and CEP-dipeptide (**10**) and their mixture. (A) Total ion chromatogram (TIC) for furan-dipeptide and CEP-dipeptide as well as their mixture. (B) Selected ion chromatogram (SIC) at m/z = 382.3 (M+1) from TIC chromatogram A. ESI mass spectrometry was performed on a Thermo Finnigan LCQ Deca instrument in the positive ion mode using nitrogen as the sheath and auxiliary gas. The Surveyor LC system was equipped with a Luna C18 (2) column (2.0 mm i.d. × 150 mm length, 5 µm, Phenomenex). The heated capillary temperature was 300 °C, the source voltage was 4.5 kV, and the capillary voltage was 31.00 V. The data were processed with Qual browser in Xcalibur software.

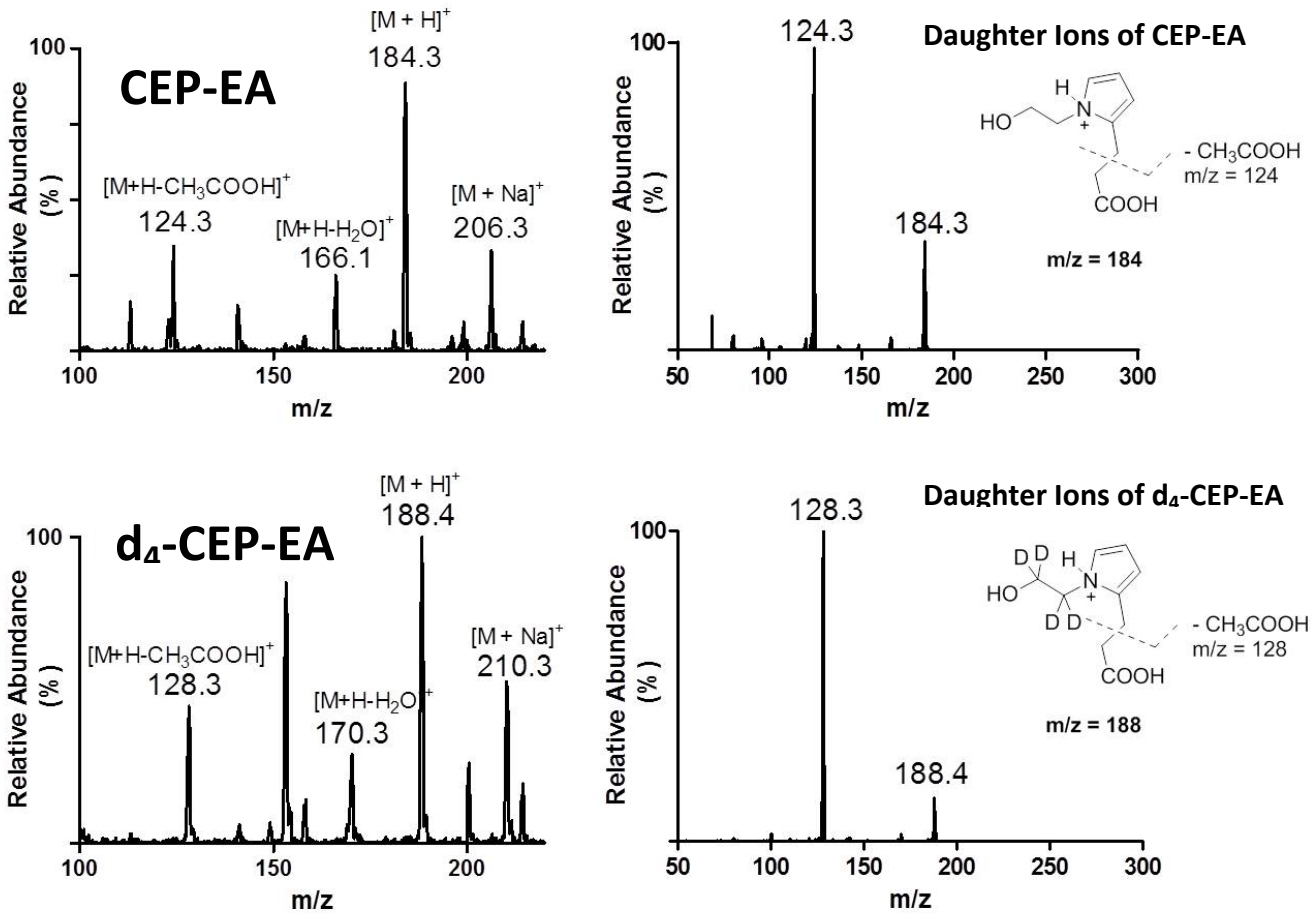


Figure S3. ESI-MS spectrum of authentic CEP-EA, **14** and d_4-CEP-EA as well as their daughter ions in the positive ion mode.

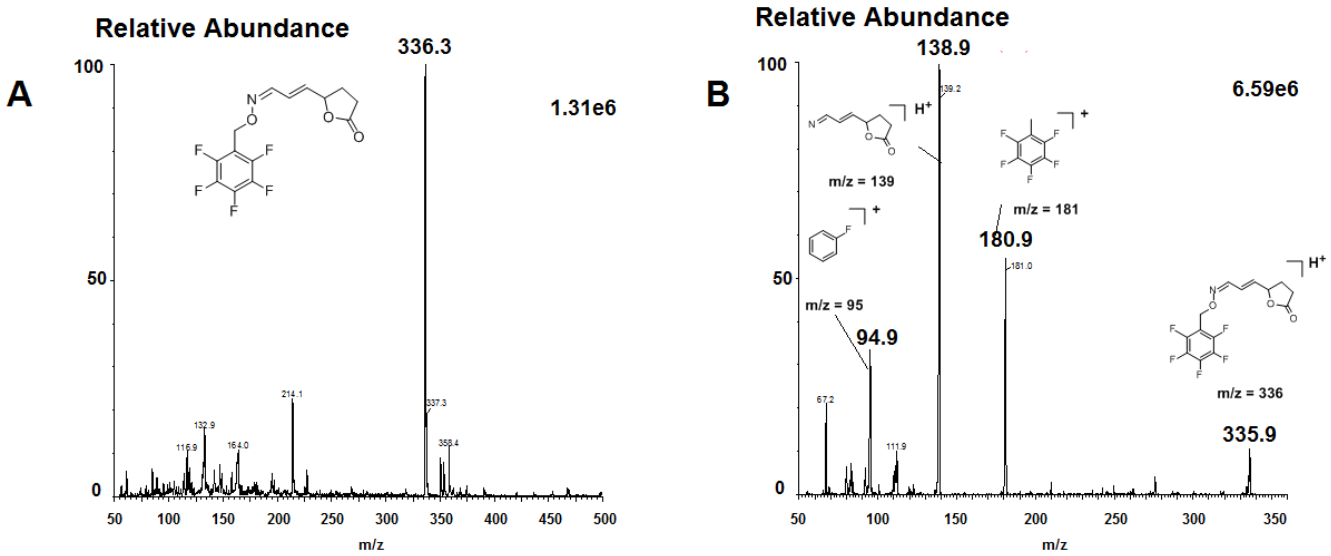


Figure S4. (A) ESI-MS scan of HOHA-lactone-PFB oxime in the positive ion mode. (B) ESI-MS/MS scan of m/z 336 at collision energy 25 eV and the proposed daughter ion structures.

Post-translational modification of ARPE-19 cells by HOHA-Lactone. ARPE-19 cells were grown on 100-mm dishes in humidified CO_2 incubator at 37 °C and 5% CO_2 in Ham's F12 medium and Dulbecco's modified Eagle's medium (DMEM) (50:50 ratio), containing L-glutamine and 10% heat-inactivated FBS. Cells were trypsinized and passaged every 2-3 days. Cells (30,000 cells/ per well) were plated on a 8-chamber well (Lab-Tek II Chamber Slide System, Nunc, Rochester, NY) in the DMEM/F12 complete medium (with 10%FBS). The following day, the cells were starved in the DMEM/F12 basal medium for 4-5 hours, washed three times with a basal medium and the wells were aspirated. Solutions of either basal medium or HOHA-Lactone (10 μM) in basal medium were added to the respective wells. The ARPE19 cells were incubated overnight at 37 °C and 5% CO_2. After incubation, the cells were aspirated, fixed and permeabilized with acetone (-20 °C). The sliders were blocked with 1:100 diluted normal goat serum. The cells were probed with mouse anti CEP-monoclonal antibody (1:100) followed by incubation with goat anti rabbit FITC antibody (1:200; Invitrogen, Carlsbad, CA). The sliders were mounted in VectaShield containing DAPI mounting medium (Vector Laboratories, Burlingame, CA). The cell images were taken at 10x magnification using a Leica DMI 6000 B inverted microscope.

PAGE and Western blot analysis of proteins in ARPE cell lysates. For visualization of CEP presence in ARPE-19 cell lysates from dose-dependent studies by Western blot, ARPE-19 cells ($1x10^6$ cells per 100 mm diameter plate) were treated for 2h with 0, 5,10, 20, 30, 40 50 and 100 μM HOHA-lactone in HBSS in 5% CO_2/95% air at 37 °C. Cells were aspirated afterwards, supplemented with basal medium and incubated overnight in in 5% CO_2/95% air at 37 °C. Next day, the cells were thoroughly washed with warm basal medium, scraped by a rubber policeman. Cells were transferred to 15-ml conical tubes and centrifuged at 480*g* at 4 °C for 10 min. The medium was carefully aspirated and the cells were washed three times with ice-cold PBS followed by centrifugation at 480 *g* at 4 °C for 10 min. Cells were disrupted upon incubation with a RIPA lysis buffer, 50 mM HEPES (pH 7.4), 0.5% sodium deoxycholate, 0.1% sodium dodecylsulphate, 1 mM phenylmethanesulfonyl fluoride (PMSF), and 1x HALT protease inhibitor cocktail for 15 min at 4 °C. Cell lysate was further placed on ice and sonicated at 40% power (5 cycles of 5 sec on and 5 sec off). The cell lysate was spun at 14,000 g at 4 °C for 20 min and the supernatant was collected and snap-frozen in liquid nitrogen till further studies. Protein determination was performed using a BCA kit Pierce Biotechnology (Rockford, IL) following directions suggested by the manufacturer. ARPE19 cell RIPA extracts (10 μg protein/lane) were separated by SDS-PAGE (4-20% gels; Life Sciences) and then electrotransferred onto a nitrocellulose membrane

(0.45μ Bio-Rad, Hercules, CA). The blot was blocked with 5% bovine serum albumin (BSA) in Tris buffered saline containing 0.1% Tween-20 (TBST) for an hour at room temperature. The blots were subsequently probed with the monoclonal anti-CEP antibody overnight at 4 °C in 5% BSA-TBST buffer overnight at 4 °C. After washing with TBST, the membrane was incubated with the goat-antimouse secondary antibody-HRP conjugate at room temperature for 1 h. The membrane was washed again with TBST and immunoblot was developed with the SuperSignal West Pico Chemiluminescent Substrate from Pierce Biotechnology (Rockford, IL) according to the manufacturer's instructions. Representative Western blot from three independent experiments is shown.

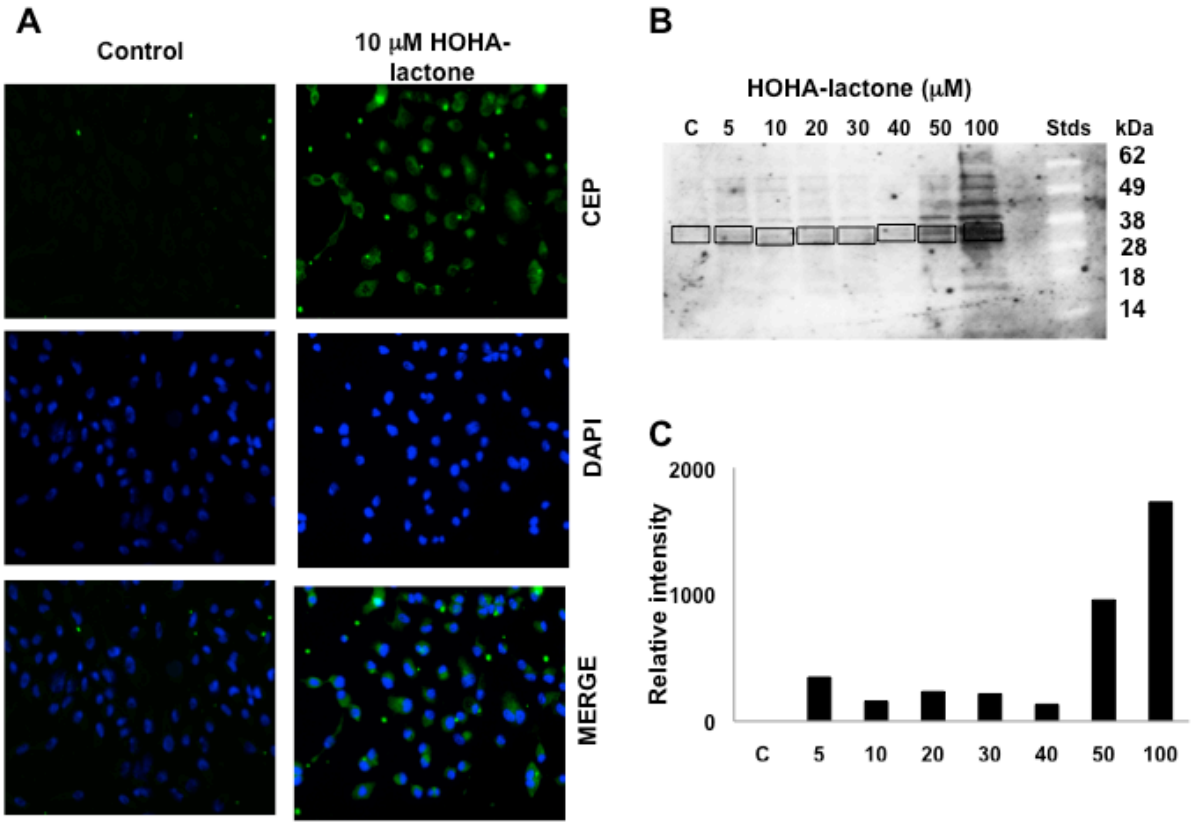


Figure S5. Modification of ARPE-19 cells by HOHA-lactone. (A) CEP immunostaining of ARPE-19 cells (30,000 cells/ per well; 8-chamber well Lab-Tek II Chamber Slide System) treated with 10 μM HOHA-lactone for 2 h followed by incubation in basal medium overnight in 5% CO_2/95% air at 37 °C; (B) Detection of CEP in cell lysates harvested from ARPE-19 cells treated with increasing concentrations of HOHA-lactone under conditions similar to the ones described in Panel A; (C) Relative optical density of the CEP-positive bands (see rectangles in Panel B) determined by using MetaMorph image-processing software. Panels B and C show a Western blot representative of three independent experiments, which showed similar results.

Detection CEP in ARPE-19 cells incubated with H_2O_2. Cells (30,000 cells/ per well) were plated on a 8-chamber well (Lab-Tek II Chamber Slide System, Nunc, Rochester, NY) in the DMEM/F12 complete medium (with 10%FBS) and incubated overnight. The following day, the cells were starved in the DMEM/F12 basal medium for 4-5 hours, washed three times with HBSS and the wells were aspirated. Solutions of either HBSS or H_2O_2 (5 μM) in HBSS were added to the respective wells and the cells were incubated at 37 °C and 5% CO_2 for two hours. The cells were aspirated and washed three times with DMEM/F12 basal medium followed by overnight incubation at 37 °C and 5% CO_2. Cell fixation in cold acetone and immunostaining was performed essentially as described for HOHA-Lactone treated ARPE-19 cells (see above).

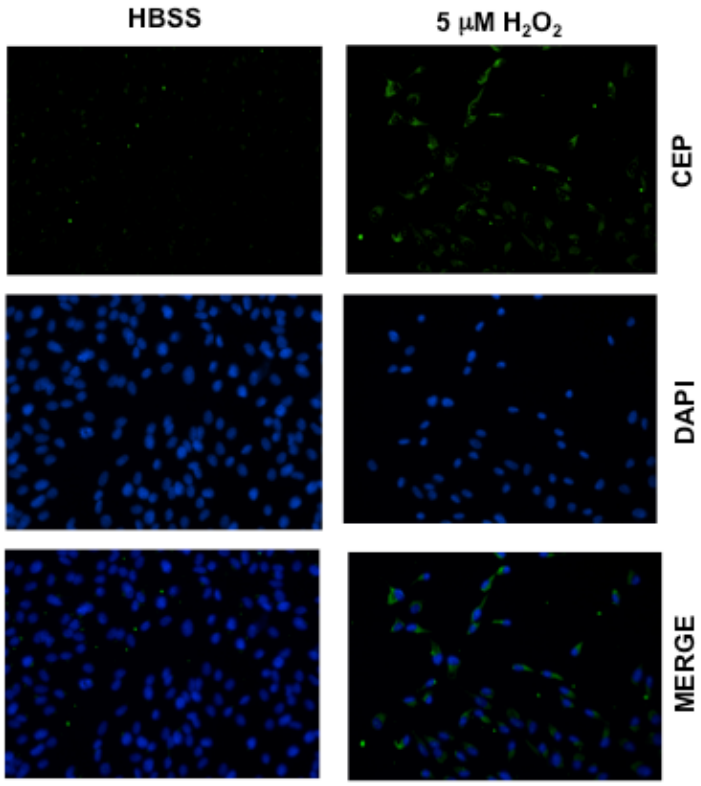


Figure S6. ARPE-19 cells treated with 5 μM H_2O_2 and then immunostained with mouse monoclonal anti-CEP antibody (see Material and Methods for details). The figure shows a representative of two independent experiments, which showed very similar results.

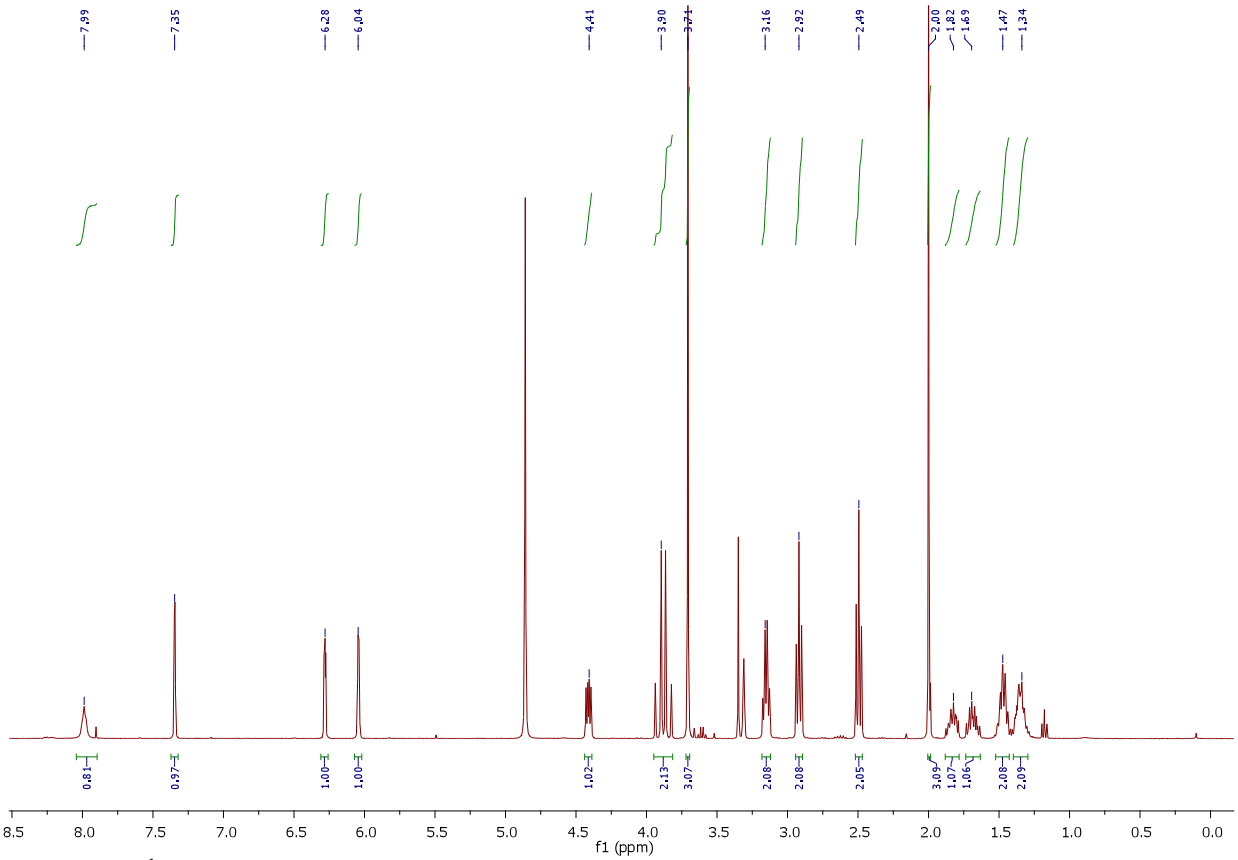


Figure S7. ^{1}H NMR spectrum of 3-(furan-2-yl)propanoyl amide **10a** (400 Mz, CD_3OD)

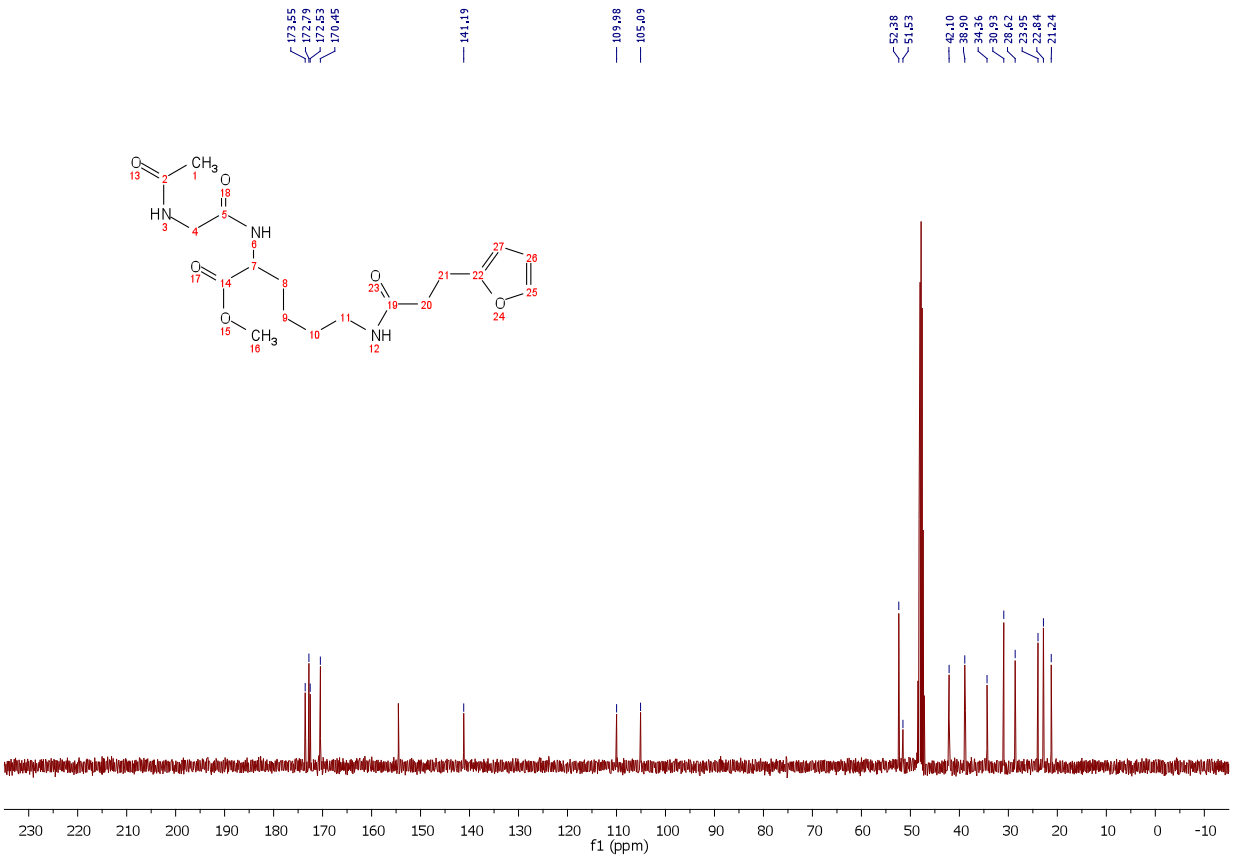


Figure S8. ^{13}C NMR spectrum of 3-(Furan-2-yl)propanoyl amide **10a** (101 Mz, CD_3OD)