

Electronic Supplementary Information (ESI)

for

Lipid Radicals Cause Light Induced-Retinal Degeneration

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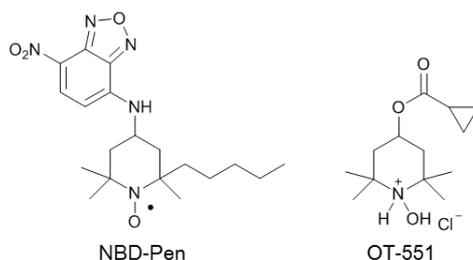
‡These two authors contributed equally to this work.

General Section

Apparatus and chemicals

Materials were purchased from commercial suppliers and used without further purification. All solvents were purified prior to use. TLC analysis was performed using precoated silica plates. ^1H NMR (500 MHz) and ^{13}C NMR (500 MHz) spectra were recorded on a Bruker Advanced Three using $\text{DMSO-}d_6$ as the solvent and tetramethylsilane as an internal standard. HR-MS data were obtained with a Bruker micrOTOF MS spectrometer. Fluorescence images of retinal tissues were obtained with a Zeiss LSM 700 confocal microscope. Bright-field images were obtained with a Keyence BZ-9000 microscope. HPLC data were obtained with a Nexra HPLC System. Fluorescence and absorbance measurements were recorded on an EnSpire Multimode Plate Reader. ESR spectra were monitored using an X-band (9.45 GHz) ESR spectrometer JES-FA100.

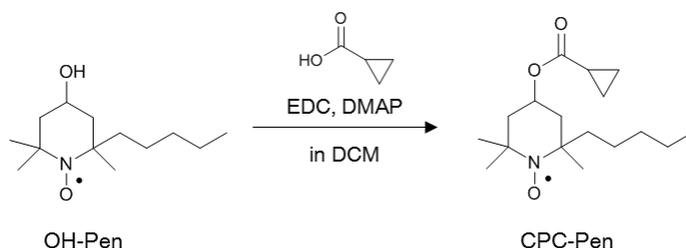
Synthesis of NBD-Pen and OT-551



NBD-Pen and OT-551 were prepared as previously described.^{1,2}

- 1 K. Yamada, F. Mito, Y. Matsuoka, S. Ide, K. Shikimachi, A. Fujiki, D. Kusakabe, Y. Ishida, M. Enoki, A. Tada, M. Ariyoshi, T. Yamasaki, and M. Yamato, *Nat. Chem. Biol.*, 2016, **12**, 608-613.
- 2 US Pat., 7442711, 2008.

Synthesis of CPC-Pen



OH-Pen (2.28 g, 10.0 mmol) was added to a flask containing dry dichloromethane (DCM; 20 mL), cyclopropanecarboxylic acid (1.72 g, 20.0 mmol), *N,N*-dimethyl-4-aminopyridine (DMAP; 1.22 g, 10.0 mmol), and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC; 1.91 g, 10.0 mmol). The mixture was stirred for 17 h, and then water was added to stop the reaction. The products were extracted with CHCl_3 three times. The organic phase was separated, and dried with Na_2SO_4 . After

filtration, the solvent was removed by vacuum distillation. The product was obtained as a yellow solid with a yield of 91% after the residue was purified by column chromatography with dichloromethane as the eluent. ¹H-NMR (500 MHz, DMSO-*d*₆): δ 7.13 (s, 1H), 4.84–4.90 (m, 1H), 1.92–1.95 (m, 1H), 1.79–1.82 (m, 1H), 1.70–1.76 (m, 1H), 1.55–1.59 (m, 1H), 1.47–1.54 (m, 1H), 1.38–1.44 (m, 1H), 1.07–1.32 (m, 16H), 0.793–0.860 (m, 7H); ¹³C-NMR (500 MHz; DMSO-*d*₆), 173.5, 66.6, 60.7, 57.7, 43.5, 39.7, 33.7, 32.5, 31.3, 27.1, 24.6, 22.7, 22.1, 14.0, 12.7, 8.02, 7.99; HRMS (m/z): [M+Na]⁺ calcd. for C₁₇H₃₀NNaO₃, 319.2123; found, 319.2113.

Animal experiments

All animal procedures were approved by the Committee on Ethics of Animal Experiments, Graduate School of Pharmaceutical Sciences, Kyushu University, and conducted according to the Guidelines for Animal Experiments of the Graduate School of Pharmaceutical Sciences, Kyushu University. Four-week-old male BALB/c mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). All mice were kept under a 12-h/12-h light/dark cycle (lights on from 07:00 to 19:00) with free access to a standard diet (CLEA Japan, Inc., Tokyo, Japan) and water.

Exposure to light

Mice were kept under dark conditions for 12 h prior to light exposure and allowed food and water *ad libitum*. The pupils were dilated with 0.5% tropicamide eye drops (Santen Pharmaceutical Co., Ltd., Osaka, Japan) at 30 min before light exposure. Unanesthetized mice were exposed to 8000 lux white fluorescent light for 3–10 h in wire-topped cages. All experiments started at 09:00. The temperature was maintained at 26 ± 2.0°C during the exposure to light. After light exposure, all mice were kept under a normal light/dark cycle.

Administration of compounds

CPC-Pen or OT-551 was administered i.p. at a dose of 75 or 100 μmol/kg body weight in PBS containing 10% PEG 300 or an identical volume of solvent 30 min before light exposure.

Preparation of retinal tissue sections

Mice were euthanized by cervical dislocation and each eye was enucleated after exposure to light. The eyes were embedded in Tissue Tek OCT compound (Sakura Finetek Japan Co., Ltd., Tokyo, Japan) and frozen. The tissue blocks were stored at –80°C, and then cut into 8-μm thick sections in a standard manner.

Lipid radical detection by NBD-Pen

At 1, 3, or 5 h after light exposure, NBD-Pen was administered i.p. at a dose of 5.0 μmol/kg body

weight in PBS containing 50% PEG 300. After 30 min, each eye was enucleated and frozen sections were obtained as described above. The sections were mounted in ProLong Gold antifade reagent containing DAPI (Thermo Fisher Scientific, Inc., MA, USA), and fluorescence images were obtained using the LSM 700 confocal laser scanning microscope (Carl Zeiss, Inc., Jena, Germany) equipped with a 40× objective lens. Fluorescence intensity was calculated as the mean value using the image analysis software program Zeiss ZEN2010. The following detection lasers were chosen for fluorescence imaging: DAPI, $\lambda_{\text{ex}} = 405 \text{ nm}$, $\lambda_{\text{em}} = 490 \text{ nm}$; NBD-Pen, $\lambda_{\text{ex}} = 488 \text{ nm}$, $\lambda_{\text{em}} = 505\text{--}600 \text{ nm}$.

Measurement of ONL thickness

Retinal sections (8 μm) were obtained from mice at 7 days after light exposure, and stained with hematoxylin-eosin. Bright-field images of the stained sections were obtained using the BIOREVO BZ-9000 microscope (KEYENCE Corp., Osaka, Japan). The outer nuclear layer (ONL) thickness was measured at 26 points (A–Z; Fig. S3a) with the software program BZ-X Analyzer.

Immunohistochemistry for 4-HNE-modified proteins

Retinal sections (8 μm) were obtained from mice at 1 day after light exposure. 4-HNE immunohistochemical staining was performed using specific antibodies (1:200; kindly provided by K. Uchida, The University of Tokyo, Japan, and T. Shibata, Nagoya University, Japan). Biotinylated goat anti-rabbit IgG (8 $\mu\text{g}/\text{mL}$; Vector Laboratories, CA, USA) was used as the secondary antibody. Bound antibodies were detected using a VECTASTAIN® ABC Kit (Vector Laboratories) and DAB Reagent Set (SeraCare, MA, USA), following the manufacturers' protocols. Bright-field images of the stained sections were obtained using the BIOREVO BZ-9000 microscope. The intensity was measured in P–S sections with the software program BZ-X Analyzer.

TUNEL staining

Retinal sections (8 μm) were obtained from mice at 24 h after light exposure. Terminal-transferase-mediated dUTP nick end-labeling (TUNEL) staining was performed using the Apoptosis in situ Detection Kit Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan), following the manufacturer's protocol.

Measurement of n-octanol/water partition coefficients of nitroxides

CPC-Pen or OT-551 was dissolved at 2 mM in a pre-equilibrated mixture of n-octanol and water (1:1, v/v). After 1 h, the concentrations of nitroxide in the water (C_w) and octanol (C_o) phases were determined by HPLC using a Nexra HPLC System (Shimadzu Co., Kyoto, Japan). LC separations were achieved with a C18 reverse-phase column (InertSustain C18; 3.0 μm , 2.0 \times 150 mm; GL

Sciences Inc., Tokyo, Japan) equilibrated with 40% A (H₂O containing 5.0 mM NH₄OAc) and 60% B (ACN:H₂O, 95:5, containing 5.0 mM NH₄OAc) at 40°C. The reaction mixture (10 µL) was injected onto the HPLC column and eluted at a flow rate of 0.4 mL/min with a linear gradient: (1) 0–30 min, 85%–10% A and 15%–90% B; (2) 30–60 min, 10%–5% A and 90%–95% B. The compounds were monitored using a diode array detector (SPD-M20A; Shimadzu Co.) with absorption at 190 nm. Three repetitions were performed. Log P values were calculated as follows: $\log P = \log (C_o/C_w)$.

ESR measurement for determining the reactivity of CPC-Pen

CPC-Pen (5 µM) was reacted with ROS or lipid radicals in PBS (pH 7.4) and the remaining radical levels were immediately measured using ESR. Hydroxyl radicals were generated by H₂O₂ (0.5 mM) and FeSO₄·7H₂O (5 µM). H₂O₂ and NaClO were used at 0.5 mM. Lipid radicals were generated by arachidonic acid (0.5 mM) and lipoxygenase (25 µg/mL). ESR spectra were monitored using an X-band (9.45 GHz) ESR spectrometer (JES-FA100; JEOL Ltd., Tokyo, Japan) at room temperature, microwave power at 10 mW, field modulation at 100 kHz, modulation width at quarter to half line width for each compound and an external magnetic field range of 5 mT.

Thiobarbituric acid reactive substances (TBARS) assay

Mice were euthanized by cervical dislocation and eyes were enucleated. Mice retinas were homogenized with 1.15% potassium chloride. The homogenate was centrifuged at 3000 rpm for 10 minutes. CPC-Pen or OT-551 (5 µM 0.5% DMSO) and FeSO₄ · 7H₂O (30 µM) was added to supernatant and incubated at 37°C for 1 h. Then, 5.9% sodium dodecyl sulfate, 8.3% acetic acid and 0.33% thiobarbituric acid were added and incubated at 100°C for 1 h. Samples were cooled and n-butanol/ pyridine (15:1) solution was added. After centrifugation at 2000 rpm for 10 minutes, samples were plated to a 96-well microplate and fluorescence was measured ($\lambda_{ex} = 512$ nm, $\lambda_{em} = 553$ nm) using an EnSpire Multimode Plate Reader.

Statistics

All data are presented as the mean \pm s.d. To assess the statistical significance of differences in data, the Dunnett test or Tukey–Kramer test was used. StatView Version 5.0 software (SAS Institute Inc., NC, USA) was used for all statistical analyses. The sample numbers used in each experiment are indicated in the figure legends.

Supplementary Figures

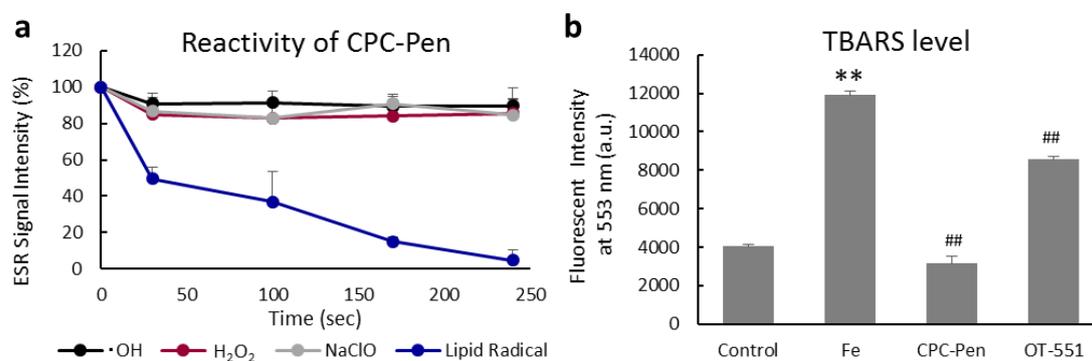


Fig. S1 *In vitro* studies of CPC-Pen and OT-551 reactivity. (a) The reactivity of CPC-Pen for ROS or lipid radicals was determined by ESR. $\cdot\text{OH}$; hydroxyl radicals, H_2O_2 ; hydrogen peroxide, NaClO ; sodium hypochlorite. (b) TBARS assay was performed to determine the LPO inhibiting activity of CPC-Pen and OT-551. Iron (II) sulfate (Fe) $30\ \mu\text{M}$ was added to retinal homogenates to induce LPO. The reaction time was 1 h. Data are shown as the mean \pm s.d. of three experiments. ** $p < 0.01$ versus Control, ## $p < 0.01$ versus Fe.

Fig. S2

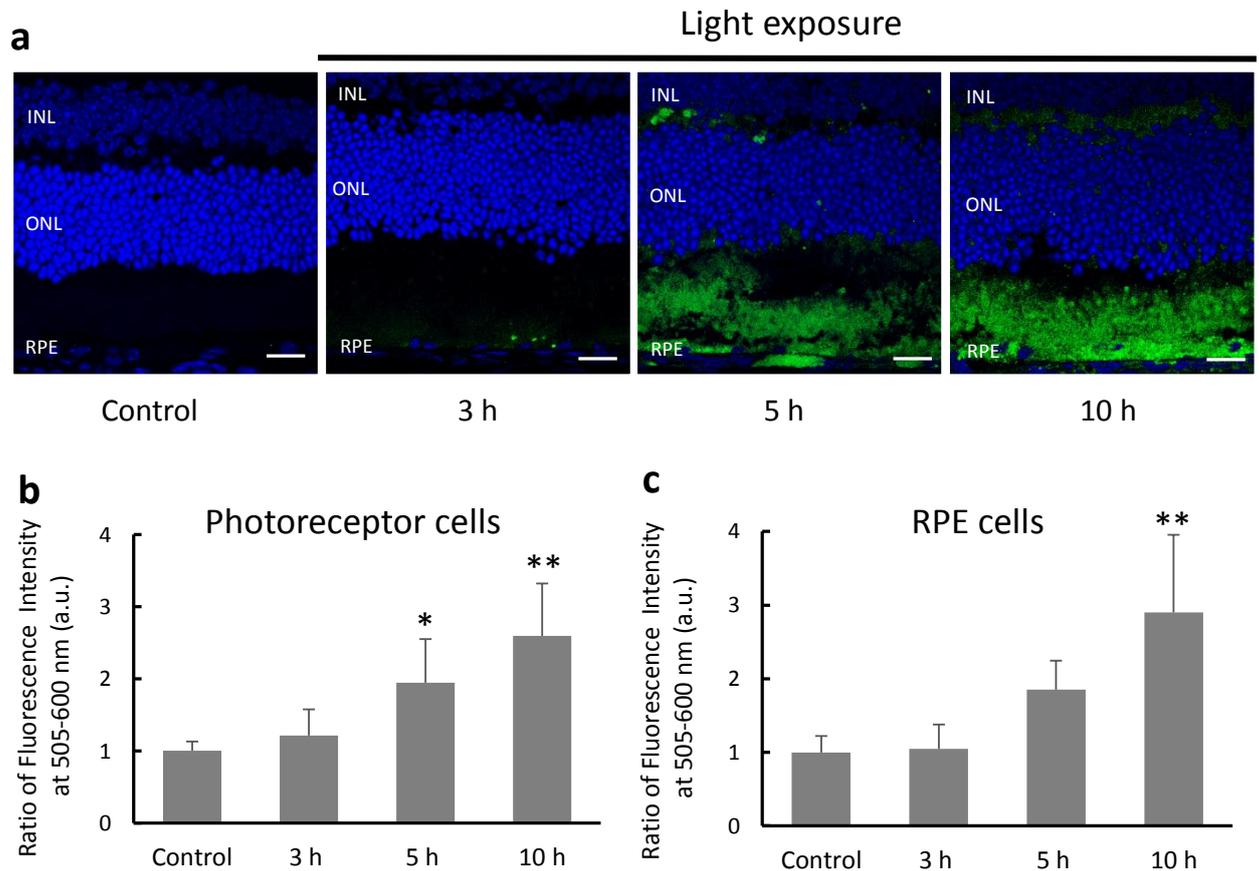


Fig. S2 Fluorescence images of the retina in NBD-Pen-administered mice. Mice were kept under 8000 lux light for 3, 5, or 10 h. After 3 h of exposure, NBD-Pen (5.0 $\mu\text{mol/kg}$ body weight in PBS containing 50% PEG 300) was administered i.p. After 30 min, the eyes were enucleated. Frozen retinal sections (8 μm) were mounted with DAPI and their fluorescence was measured by confocal microscopy (NBD-Pen: λ_{ex} = 488 nm, λ_{em} = 505–600 nm; DAPI: λ_{ex} = 405 nm, λ_{em} = 490 nm; scale bars = 25 μm). (a) Fluorescence images of the retina. (b, c) Fluorescence intensity was calculated in photoreceptor cells (b) and RPE cells (c). Data are shown as the mean \pm s.d. of three experiments. * p < 0.05, ** p < 0.01 versus Control.

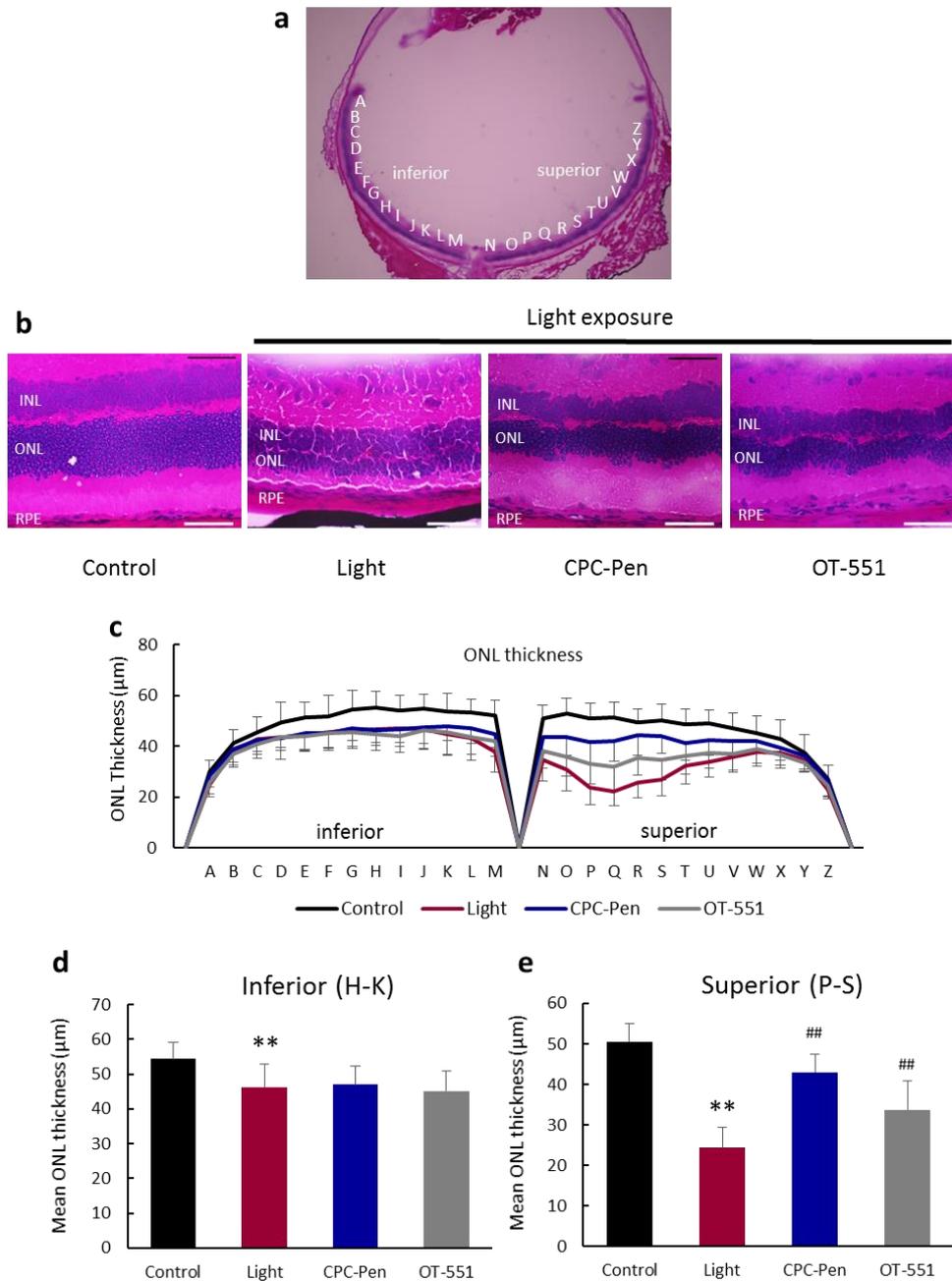
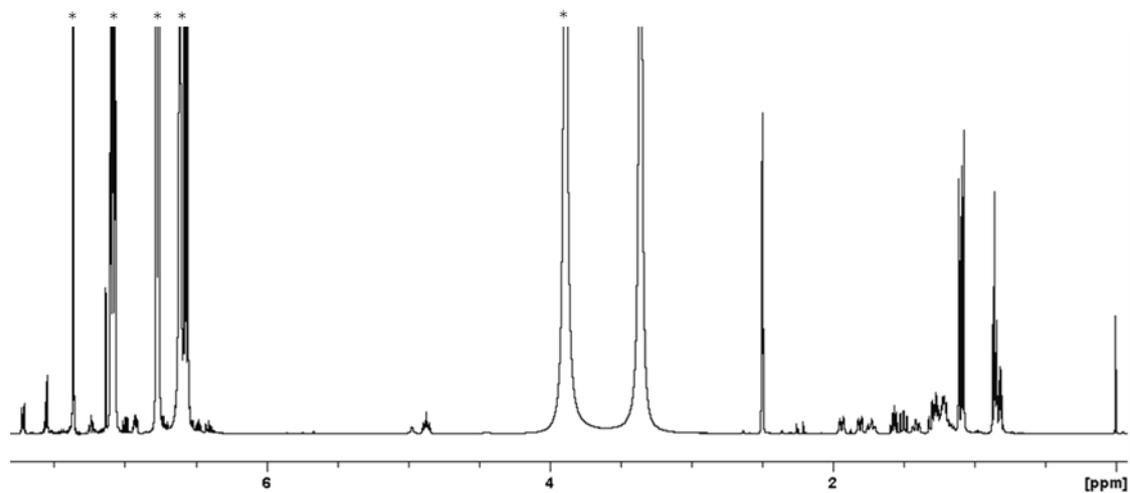


Fig. S3 Effect of CPC-Pen or OT-551 on retinal damage after light exposure. CPC-Pen or OT-551 (75 $\mu\text{mol/kg}$ body weight in PBS containing 10% PEG 300) was administered i.p. at 30 min before light exposure. Mice were kept under 8000 lux light for 10 h, and then under a normal light/dark cycle for 6 days. On day 7, the eyes were enucleated. (a) Frozen sections (8 μm) were stained with hematoxylin-eosin and evaluated. (b) Images of stained P-S sections (scale bars = 50 μm). (c, d) ONL thicknesses in the whole retina. The mean thicknesses in the inferior hemisphere (H-K) (c) and superior hemisphere (P-S) (d) are shown. Data are shown as the mean \pm s.d. of six experiments. ** $p < 0.01$ versus Control, ## $p < 0.01$ versus Light.

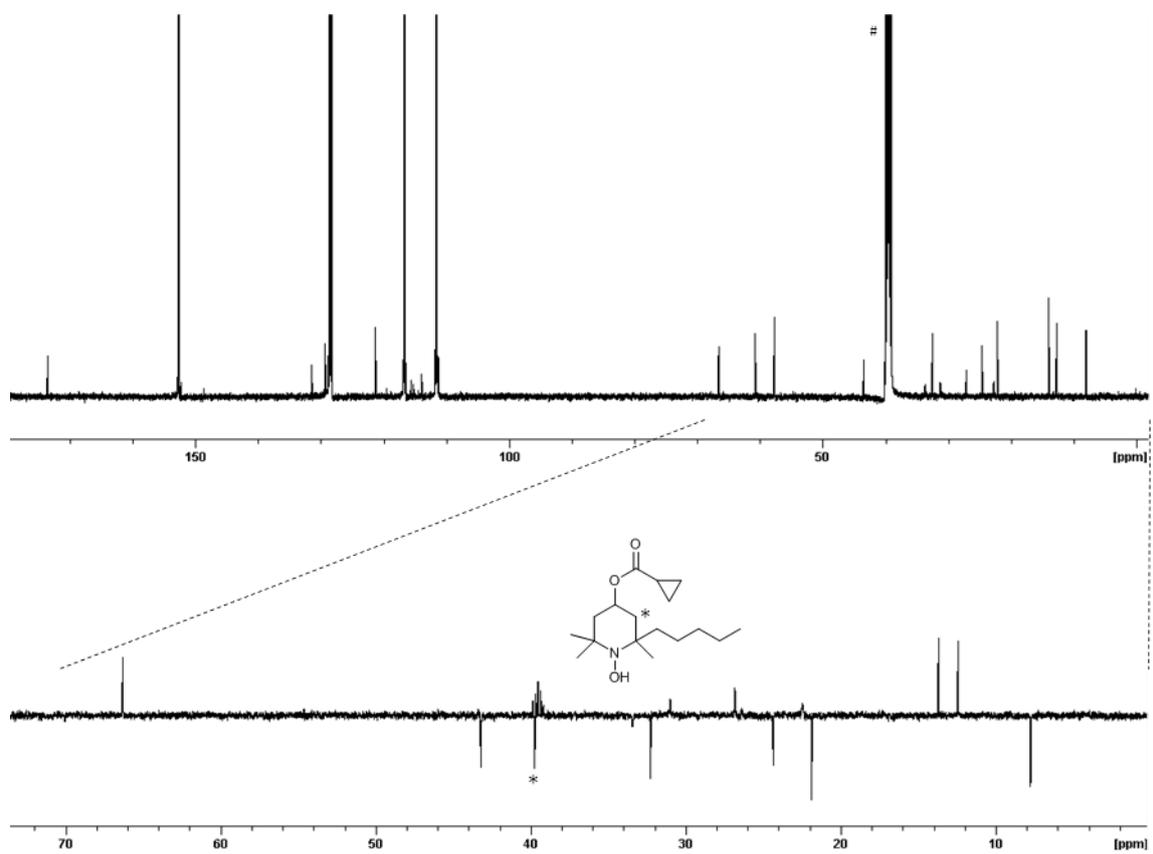
¹H-NMR spectrum of CPC-Pen



CPC-Pen was chemically reduced with phenylhydrazine.

*peaks from phenylhydrazine

¹³C-NMR spectra of CPC-Pen



#peak from DMSO-d6