Supporting Information

Development of a Novel Amplifiable System to Quantify Hydrogen Peroxide in Living Cells

Lingfei Wang[1], Hanfeng Lin[1,2], Bin Yang[1,2], Xiqian Jiang[1], Jianwei Chen[1], Sandipan Roy Chowdhury[1,2], Ninghui Cheng[3], Paul A. Nakata[3], David M. Lonard[4], Meng. C. Wang[5], Jin Wang[1,2,4]*

[1]*Verna and Marrs McLean Department of Biochemistry and Molecular Pharmacology, Baylor College of Medicine, Houston, Texas 77030, United States.*

[2]*Center for NextGen Therapeutics, Baylor College of Medicine, Houston, Texas 77030, United States.*

[3]*Department of Pediatrics, Baylor College of Medicine, Houston, Texas 77030, United States.*

[4]*Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas 77030, United States.*

[5]*Department of Molecular and Human Genetics, Huffington Center on Aging, and Howard Hughes Medical Institute, Baylor College of Medicine, Houston, Texas 77030, United States.*

Corresponding Author:

E-mail: wangj@bcm.edu

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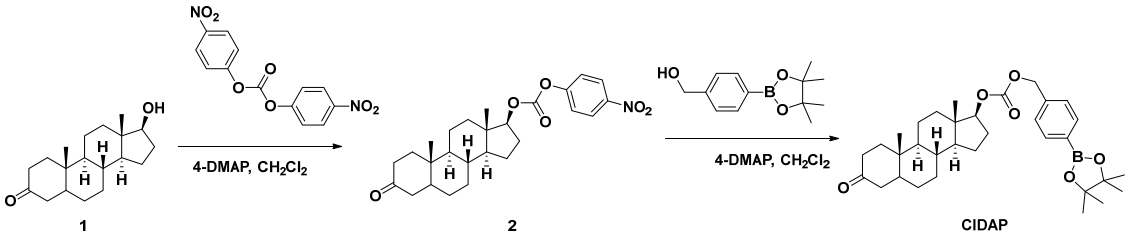
General information

Instruments and materials

All the chemicals were purchased from Sigma-Aldrich, Combi-blocks or Alfa Aesar unless otherwise specified. All reagents were used as purchased without further purification. Anhydrous solvents were purchased from Acros Organics and used as received. ^{1}H NMR and ^{13}C NMR spectra were collected on Bruker Avance III HD 600 MHz spectrometer. Chemical shifts (δ) are reported in ppm and coupling constants (*J*) are in Hertz (Hz). The following abbreviations were used to explain the multiplicities: s = singlet, d = doublet, t = quartet, m = multiplate, br = broad. Purifications using flash chromatography were performed on a Teledyne ISCO CombiFlash Rf 200. Purifications using preparative LC-MS were conducted on an Agilent 1260 Infinity II Preparative LC/MSD system. High-resolution mass spectra were collected on ThermoFisher Orbitrap Exploris 120 Mass Spectrometer. LC-MS analysis were performed on an Agilent 6130 Single Quadrupole liquid chromatograph-mass spectrometer.

Synthesis Procedures

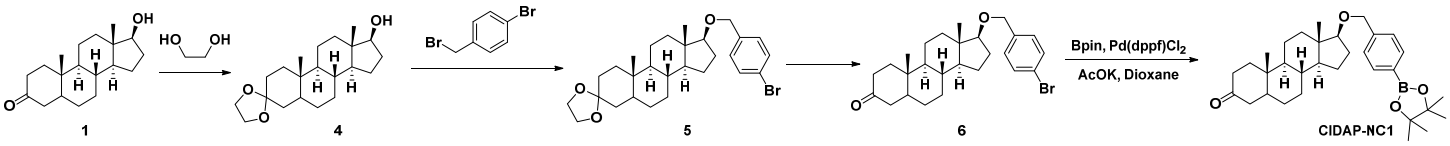
*Synthesis of **CIDAP***



(8R,9S,10S,13S,14S,17S)-10,13-dimethyl-3-oxohexadecahydro-1H-cyclopenta[a]phenanthrene-17-yl(4-nitrophenyl)carbonate (**2**): Under a N_2 atmosphere, a mixture of dihydrotestosterone (100 mg, 0.344 mmol), bis(4-nitrophenyl) carbonate (313 mg, 1.03 mmol) and 4-dimethylaminopyridine (4-DMAP 126 mg, 1.03 mmol) in 0.3 mL of anhydrous dichloromethane (DCM) was stirred for 6 hours. The reaction was diluted with dry DCM after completion and washed with water. The organic layer was collected and concentrated. The mixture was purified on flash chromatography and the product was obtained as a white solid with 75% yield.

(8R,9S,10S,13S,14S,17S)-10,13-dimethyl-3-oxohexadecahydro-1H-cyclopenta[a]phenanthren-17-yl(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl)carbonate (**CIDAP**): Under a N_2 atmosphere, a mixture of **2** (13 mg, 0.028 mmol), 4-(Hydroxymethyl)phenylboronic acid pinacol ester (20 mg, 0.856 mmol) and 4-dimethylaminopyridine (11.1 mg, 0.856 mmol) in 0.3 mL of anhydrous DCM was stirred overnight. The reaction was extracted with DCM after completion. The organic layer was concentrated and purified with preparative LC-MS. The product was obtained as white powder with 50 % yield. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.80 (d, *J* = 7.9 Hz, 2H), 7.38 (d, *J* = 7.9 Hz, 2H), 5.15 (s, 2H), 4.52 (t, *J* = 8.4 Hz, 1H), 2.42 – 2.33 (m, 1H), 2.31 – 2.15 (m, 3H), 2.10 – 2.07 (m, 1H), 2.02 – 1.99 (m, 1H), 1.81 (dt, *J* = 12.6, 3.0 Hz, 1H), 1.70 (d, *J* = 16.0 Hz, 1H), 1.66 – 1.61 (m, 2H), 1.55 – 1.51 (m, 1H), 1.49 – 1.43 (m, 1H), 1.39-1.23 (m, 18H), 1.18 (td, *J* = 12.9, 4.3 Hz, 1H), 1.10-1.01 (m, 4H), 0.94-0.87 (m, 1H), 0.82 (s, 3H), 0.77 (td, *J* = 12.6, 4.2 Hz, 1H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 212.01, 155.33, 138.57, 135.16, 127.47, 86.71, 84.02, 69.37, 53.84, 50.60, 46.78, 44.80, 42.90, 38.65, 38.26, 36.93, 35.87, 35.31, 31.33, 28.90, 27.54, 25.00, 23.53, 21.04, 12.19, 11.60. HRMS (ESI) m/z: calcd. for $C_{33}H_{48}BO_6$ $[M + H]^+$ 551.3544, found 551.3549.

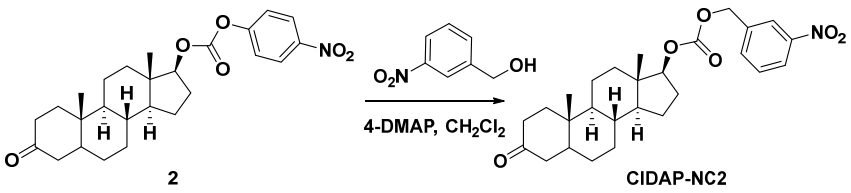
*Synthesis of **CIDAP-NC1***



(8R,9S,10S,13S,14S,17S)-10,13-dimethylhexadecahydrospiro[cyclopenta[a]phenanthrene-3,2'-[1,3]dioxolan]-17-ol (5)[1]: Under a N_2 atmosphere, DHT (290 mg, 1 mmol) was dissolved in anhydrous toluene in a Dean-Stark/water separator. Ethylene glycol (6.7 mL) and p-toluenesolfonic acid (9.5 mg, 0.05 mmol) were added and the mixture was refluxed for 12 h. The reaction was quenched with saturated $NaHCO_3$ and concentrated. The resulting mixture was extracted with EA and concentrated. The concentrated product (**4**) was directly used for the next step without further purification. The mixture of **4** (0.5 mmol, 167 mg) was dissolved in anhydrous THF and NaH (60 %, 5 mmol, 200 mg) was added slowly at room temperature. The mixture was heated to reflux and stirred for 0.5 h, then 1-bromo-4-(bromomethyl)benzene (1.5 mmol, 375 mg) was added. The mixture was kept refluxing for another 1 h and cooled to room temperature. The reaction was diluted with water and extracted with DCM. The organic layer was collected and concentrated under reduced pressure to give the compound 5. The mixture of **5** was directly dissolved in hydrochloric acid (4 M in dioxane) and stirred for 2 h to remove the protecting group on the ketone. The reaction was monitored by TLC and extracted with DCM after completion. The organic layer was concentrated and purified on flash chromatography to obtain compound **6**.

(8R,9S,10S,13S,14S,17S)-10,13-dimethyl-17-((4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl)oxy)hexadecahydro-3H-cyclopenta[a]phenanthren-3-one (**CIDAP-NC1**): Under a N_2 atmosphere, compound **6** (100 mg, 0.218 mmol) from the previous steps was dissolved in anhydrous dioxane and bis(pinacolato)diboron (60.8 mg, 0.239 mmol) was added. 1,1'-Bis(diphenylphosphino)ferrocene-palladium (II) (5.35 mg, 0.006 mmol) was added, followed by the addition of potassium acetate (64.1 mg, 0.653 mmol). The mixture was stirred at 70ºC for 24 h and monitored by TLC. The resulting mixture was extracted with DCM. The organic layer was concentrated and purified on a preparative LC-MS to obtain the final compound as a white solid. ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.77 (d, *J* = 8.0 Hz, 2H), 7.33 (d, *J* = 8.1 Hz, 2H), 4.58 – 4.52 (m, 2H), 3.38 (dd, *J* = 8.9, 7.7 Hz, 1H), 2.41 – 2.35 (m, 1H), 2.31 – 2.24 (m, 2H), 2.09 – 1.91 (m, 4H), 1.69 (dq, *J* = 13.1, 3.6 Hz, 1H), 1.56 – 1.46 (m, 4H), 1.45 – 1.38 (m, 2H), 1.36 – 1.27 (m, 16H), 1.12 (td, *J* = 12.8, 4.2 Hz, 1H), 1.01 (s, 3H), 0.97 – 0.91 (m, 1H), 0.89 – 0.82 (m, 4H), 0.70 (ddd, *J* = 12.4, 10.5, 4.2 Hz, 1H).^{13}C NMR (150 MHz, $CDCl_3$): 212.23, 142.70, 134.89, 126.67, 88.26, 83.87, 71.62, 54.09, 51.13, 46.86, 44.85, 43.25, 38.71, 38.32, 37.93, 35.87, 35.35, 31.40, 28.96, 28.01, 25.17, 25.01, 23.55, 21.25, 12.02, 11.63; HRMS (ESI) m/z: calcd. for $C_{32}H_{48}BO_4$ $[M + H]^+$ 507.3646, found 507.3651.

*Synthesis of **CIDAP-NC2***



(8R,9S,10S,13S,14S,17S)-10,13-dimethyl-3-oxohexadecahydro-1H-cyclopenta[a]phenanthren-17-yl (3-nitrobenzyl) carbonate (**CIDAP-NC2**): Under a N_2 atmosphere, a mixture of **2** (9 mg, 0.020 mmol), 3-nitrobenzyl alcohol (3 mg, 0.02 mmol) and 4-dimethylaminopyridine (4.9 mg, 0.04 mmol) in 0.3 mL of anhydrous DCM was stirred for overnight. The reaction was extracted with DCM after completion. The organic layer was concentrated and purified with preparative TLC plates. The product was obtained as white powder with 40 % yield. ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.27 (s, 1H), 8.21 (d, *J* = 8.4 Hz, 1H), 7.72 (d, *J* = 7.2 Hz, 1H), 7.58 (t, J = 8.4 Hz, 1H), 5.225 (s, 2H), 4.56-4.53 (m, 2H), 2.38-2.34 (m, 1H), 2.32 – 2.17 (m, 3H), 2.11 – 2.07 (m, 1H), 2.03 – 1.99 (m, 1H), 1.83 (dt, *J* = 12.6, 3.0 Hz, 1H), 1.73 (m, 1H), 1.67 – 1.24 (m, 9H), 1.24 (td, *J* = 12.6, 4.2 Hz, 1H), 1.10-1.02 (m, 4H), 0.95-0.91 (m, 1H), 0.84 (s, 3H), 0.78-0.73 (m, 1H). ^{13}C NMR (150 MHz, $CDCl_3$): 133.94, 129.65, 123.40, 122.97, 87.04, 67.81, 53.70, 50.43, 46.64, 44.65, 42.80, 38.52, 38.11, 36.80, 35.73, 35.73, 35.16, 29.71, 28.74, 27.38, 23.38, 20.89, 14.13, 12.07, 11.48; HRMS (ESI) m/z: calcd. for $C_{27}H_{36}NO_6$ $[M + H]^+$ 470.2542, found 470.2542.

In vitro biochemical assays

Calculation of second-order rate constant by LC-MS

The responses of **CIDAP** with different concentrations of H_2O_2 were measured by monitoring the response of produced DHT on LC-MS. The 100 mM H_2O_2 stock solution was prepared by adding 11 μL of 30 % H_2O_2 to 989 μL water. The final concentration of CIDAP in the reactions was prepared as 250 μM in PBS buffer (pH 7.4), and the H_2O_2 concentrations were set to 5 mM, 10 mM, and 15 mM. The reactions were performed at 37ºC with shaking at 1000 rpm. Aliquots of the reaction mixture were taken for LC-MS analysis at various time points (from 0 min to 150 min). Due to limited UV absorbance of DHT, the data was collected by integrating the area of DHT response in the mass spectrum (Figure S2A).

As the H_2O_2 concentration is significantly higher than CIDAP concentration, this reaction is considered as a pseudo-first-order reaction. Therefore, a rate constant, k_{obs}, was generated for each curve using GraphPad Prism Software. The second-order rate constant (K, $M^{-1}min^{-1}$) can be calculated by plotting k_{obs} versus the corresponding H_2O_2 concentration, using the following equation: $k_{obs} = K[M]$, where M is the concentration of H_2O_2. The slope of this linear plot is the second-order rate constant of this reaction (Figure S2B).

Selectivity of CIDAP

H_2O_2 was prepared as described before. Other ROS were generated according to the methods reported by Chang group.[2,3] ROS solutions (5 mM) were added to the probe solution (250 μM), respectively, and kept shaking (1000 rpm) at 37ºC for 2 h, unless otherwise specified. The resulting mixture was taken for LC-MS analysis (Figure S3A). The conversion rate of CIDAP to DHT with each ROS was calculated using the response of 250 μM DHT in LC-MS as full conversion (100 %).

Additional tests were performed to confirm the reactivity of CIDAP with 50 μM of peroxynitrite ($ONOO^-$) and hypochlorite (OCl^-), respectively. CIDAP and DHT were prepared as described before. The reactions were conducted in 20 mM HEPES buffer at pH 7.4 and incubated at 37 ºC. The conversion rate of CIDAP to DHT at each condition can be calculated using the response of 250 μM DHT in LC-MS as full conversion (100 %). 10 μL of the reaction mixture was taken for LC-MS analysis after 2 hours of incubation (Figure S3B). The side reaction between CIDAP and peroxynitrite was shown in Figure S3B.

Standard curve of CIDAP in LC-MS

CIDAP was prepared in 100% EtOH at concentrations of 20 nM, 200 nM, 2 μM, and 20 μM. 10 μL of the CIDAP standard solution was subsequently injected into an Agilent 1290 Infinity II LC System coupled with an Agilent 6495 Triple Quadrupole mass spectrometer. The quantities of both CIDAP and DHT were monitored via multiple reaction monitoring (MRM), with CIDAP transmission set at m/z 551.3 -> 217.1 and a fragmentation energy of 40V. The integrated area of CIDAP responses was plotted against the aforementioned concentrations of CIDAP. The equation was generated using the linear regression program provided by GraphPad Prism Software.

Cell-based assays

Cell culture

All cell lines (unless otherwise specified) were cultured in Dulbecco's Modified Eagle's Medium (Corning™) supplemented with 10 % Fetal Bovine Serum and 1 % penicillin-streptomycin (Gibco), which will be referred as complete medium in the following section. Cells were cultured with a controlled atmosphere with CO_2 level at 5 % and temperature at 37ºC. All cell lines were purchased from the American Type Culture Collection (ATCC). HeLa GRX3-KD cell line was kindly provided by Cheng Group at Baylor College of Medicine.[4] HEK293T cells, HeLa cells, HeLa GRX3-KD cells and MDA231 cells were maintained in DMEM complete medium as described before. HepG2 cells were maintained in Eagle's Minimum Essential Medium (EMEM, Corning™) supplemented with 10 % Fetal Bovine Serum and 1 % penicillin-streptomycin. ASPC-1 cells were maintained in RPMI-1640 medium supplemented with 10 % Fetal Bovine Serum and 1 % penicillin-streptomycin.

General co-transfection protocol

Cells were plated in flat-bottom clear 6-well plates (Corning™) at a density of 0.2 x 10^6 cells/mL with 3 mL complete growth medium and incubated for 24 h (37ºC, 5 % CO_2) before co-transfection. After the incubation, the complete medium was removed from the cells and replaced with 3 mL Opti-MEM™ I reduced serum medium (Gibco™, no phenol red). The plasmids (pGL3-SV40-LgBit-hAR and pGL3-SV40-hAR-SmBit) were kindly provided by Michelini group.[5] For each well, the cells were transiently co-transfected with 1 μg pGL3-SV40-LgBit-hAR and 1 μg pGL3-SV40-hAR-SmBit, mixed with 5 μL P3000 reagent and 5 μL Lipofectamine™ 3000 reagent from Lipofectamine™ 3000 transfection kit (Invitrogen) following the standard protocol. After 48 h post-transfection, the cells were detached with Trypsin 1X, and centrifuged for 5 min at 200 × g. The cell pellet was resuspended in phenol-red free Opti-MEM™ I reduced serum medium with 4 % FBS. The cells were seeded in a 96-well white microplate with 2 × 10^4 cells/well in 100 μL medium and incubated for 4-6 h to attach before the treatment of the probe.

Estrogen-mediated luciferase assay

HeLa cell line was cultured using DMEM complete medium as described before. The cells were plated in the flat-bottom clear 6-well plates at a density of 0.2 × 10^6 cells/mL with 3 mL growth medium and incubated for 24 h (37ºC, 5 % CO_2) before co-transfection. After the incubation, the complete medium was removed from the cells and replaced with 3 mL Opti-MEM™ I reduced serum medium. For each well, the cells were transiently co-transfected with 50 ng of pCR3.1-hERα and 1.25 μg pGRE-E1b-LUC, (kindly provided by Lonard group), using Lipofectamine™ 2000 transfection kit following the standard protocol.[6] After 24 h post-transfection, the cells were detached with Trypsin, and centrifuged for 5 min at 200 x g. The cell pellet was resuspended in phenol-red free Opti-MEM™ I reduced serum medium with 4 % FBS. The cells were seeded in 96-well white microplates with 2 × 10^4 cells/well in 100 μL medium. The cells were treated with estradiol (E2) from 0 to 1 nM. The cells were washed with PBS after 24 h of incubation and followed by addition of luciferase substrate (Promega). The luminescence readouts were obtained by the plate reader (Figure S1A).

Androgen-mediated luciferase assay

HeLa and HEK293T cells were cultured using DMEM complete medium as described before, respectively. The cells were plated in the flat-bottom white 96-well plate at a density of 0.2 × 10^6 cells/mL with 100 μL growth medium at each well and incubated for 24 h (37ºC, 5 % CO_2) before co-transfection. After the incubation, the complete medium was removed from the cells and replaced with 100 μL Opti-MEM™ I reduced serum medium. For each well, the cells were transiently co-transfected with 1 ng of pCR3.1-AR and 125 ng pGRE-E1b-LUC (kindly provided by Lonard group), using Lipofectamine™ 3000 transfection kit following the standard protocol. After 24 h post-transfection, the cells were treated with DHT from 0 to 1 nM. The cells were washed with PBS after 24 h of incubation and followed by addition of luciferase substrate (Promega). The luminescence readouts were obtained on the plate reader (Figure S1B).

nLuc control assay

nLuc proteins were diluted to 10 ng/well with 100 μL of phenol-red free OptiMEM medium and incubated with 5 μL of CIDAP or ethanol (final concentration range from 0 to 33 nM with at least triplicates for each concentration. The mixture was then treated with 1X Nano-luc substrate. The luminescence signal was measured immediately with plate reader (Figure S2C).

General protocol for H_2O_2 quantification

The HEK293T cell line was cultured using DMEM complete medium as described before. The cells were plated in a flat-bottom clear 6-well plates at a density of 0.2 × 10^6 cells/mL with 3 mL complete growth medium and incubated for 24 h (37ºC, 5 % CO_2) before co-transfection. After the incubation, the complete medium was removed from the cells and replaced with 3 mL Opti-MEM™ I reduced serum medium. For each well, the cells were transiently co-transfected with 1 μg pGL3-SV40-LgBit-hAR and 1 μg pGL3-SV40-hAR-SmBit, using Lipofectamine™ 3000 transfection kit following the standard protocol. After 48 h post-transfection, the cells were detached with Trypsin, and centrifuged for 5 min at 200 x g. The cell pellet was resuspended in phenol-red free Opti-MEM™ I reduced serum medium with 4 % FBS. The cells were seeded in 96-well white microplates with 2 x 10^4 cells/well in 100 μL medium and divided into two groups for DHT and CIDAP. The cells were kept in the incubator for an additional 4 h to adapt. The stock solutions for both DHT and CIDAP were prepared at 10 μM in ethanol. The cells were treated with 5 μL of DHT or CIDAP in ethanol solutions added to 100 μL medium

(final concentration range from 0 to 11 nM) with at least triplicates for each concentration. After 2 h of, the medium was removed and replaced with 1X Nano-luc substrate (Promega) in phenol-red free OptiMEM reduced serum medium (100 µL/well). The luminescence signal was measured immediately with the plate reader.

The dose-response curve of each compound was generated with GraphPad Prism Software. The response was normalized with background signal as 0 (the lowest signal for this system) and the averaged highest signal in DHT group as 1 (the highest potential this system possesses). The half maximal effective concentration (EC_{50}) for both compounds ($EC_{50,DHT}$ and $EC_{50,CIDAP}$) were calculated, using this equation: Y=Bottom + X*(Top-Bottom)/(EC_{50} + X), where X is the concentration of DHT or CIDAP, Y is the normalized response. All the normalization and calculations were performed with GraphPad Prism Software.

As the reaction of CIDAP with endogenous H_2O_2 is considered pseudo-first-order reaction, the concentration of endogenous H_2O_2 can be obtained with the following equation:

$$\ln[P]_t = -k[H_2O_2]t + \ln[P]_0 \quad \leftrightarrow \quad [P]_t/[P]_0 = e^{-k[H_2O_2]t}$$

Where $[P]_t$ is the concentration of CIDAP at time t (t = 120 min), $[P]_0$ is the initial concentration of CIDAP, $[H_2O_2]$ is the concentration of cellular H_2O_2 and K is the second-order rate constant measured by LC-MS as described before. To obtain the ratio of $[P]_t$ to $[P]_0$, the following equation is used:

$$[P]_t/[P]_0 = 1 - (EC_{50,DHT})/(EC_{50,CIDAP})$$

Where $EC_{50,DHT}$ and $EC_{50,CIDAP}$ are generated as described before. In this system, $EC_{50,CIDAP}$ is the initial concentration of CIDAP ($[P]_0$), while $EC_{50,DHT}$ can be considered as the actual concentration of DHT converted from CIDAP in the system after reaction with H_2O_2. Therefore, the difference between $EC_{50,DHT}$ and $EC_{50,CIDAP}$ equals to the remaining amount of CIDAP in the system ($[P]_t$). With all the information acquired, the concentration of H_2O_2 can be calculated by the following equation:

$$[H_2O_2]=\ln(1 - EC_{50,DHT}/EC_{50,CIDAP}))/(-kt)$$

This equation was integrated into the global fitting program of GraphPad Prism Software for dose-response curves. The EC_{50} ratio was established based on the EC_{50} of both curves generated from the CIDAP and DHT datasets. The software's modified program directly computed and generated both the concentration of peroxide and the standard error of the mean (SEM). For more details, please refer to the prism file with the name of "H2O2_Calculation_Method". To validate the robustness and accuracy of the method, simulated sigmoidal curves for both DHT and CIDAP were generated in Excel, with EC50 values of 0.5 nM and 5 nM, respectively. Each data point included a 10 % error margin. Peroxide concentration was calculated directly from the equation, given the known EC50 values, resulting in a concentration of 4.47 µM. Using the newly established Prism analysis method, the peroxide concentration was determined to be 4.54 ± 0.6 µM. This value is highly consistent with the concentration calculated from the equation, and the error margin produced by the program aligns well with the original 10 % error setup for the simulated curves.

Investigation on hydrolysis rate

Transfected HEK293T cells were prepared following the co-transfection protocol described before. The cells were seeded on tissue-treated 96-well white plates and divided into 3 groups for DHT, CIDAP, CIDAP-NC1 and CIDAP-NC2. The cells in each group were treated with 5 µL of DHT or CIDAP in ethanol solutions at 0 and 1.1 nM. After 2 h incubation, the medium was removed and replaced with 1X Nano-Luc substrate in OptiMEM reduced serum medium (4 % FBS, phenol-red free). The results were collected on the plate reader and visualized in GraphPad Prism.

Optimization on DHT/CIDAP incubation time

Transfected HeLa cells were prepared following the co-transfection protocol described before. The cells were seeded on tissue-treated 96-well white plates and divided into 2 groups for DHT and CIDAP. The cells in each group were treated with 5 µL of DHT or CIDAP in ethanol solutions (0.01, 0.1, 1 and 10 nM). The incubation was stopped at 0, 0.5, 1, 2, 4, and 8 h for data collection. The results were obtained on the plate reader and plotted in GraphPad Prism (Figure S3A).

Quantification of exogenous H_2O_2 in HEK293T cells

Transfected HEK293T cells were prepared following the co-transfection protocol described before. The cells were seeded on tissue-treated 96-well white plates and divided into 2 groups for DHT and CIDAP. The cells in each group were treated with 5 μL of DHT or CIDAP in ethanol solutions (final concentration range from 0 to 11 nM). After 1 h incubation with the compounds, the medium was removed and replaced with 50 μM H_2O_2 in OptiMEM reduced serum medium (4 % FBS, phenol-red free). The cells were incubated with H_2O_2 for another hour and measured with the plate reader.

Titration of H_2O_2 in HEK293T cells

Transfected HEK293T cells were prepared following the co-transfection protocol described before. The cells were seeded on tissue-treated 96-well white plates and divided into 2 groups for DHT and CIDAP. The cells in each group were treated with 5 μL of DHT or CIDAP in ethanol solutions final concentration range from 0 to 11 nM). After 1 h incubation with the compounds, the medium was removed and replaced with 0 μM, 5 μM, 10 μM, 20 μM, and 50 μM H_2O_2 in OptiMEM reduced serum medium (4 % FBS, phenol-red free). The cells were incubated with H_2O_2 for another hour and the data was collected on the plate reader (Figure S3B).

Quantification of menadione induced H_2O_2 in HeLa cells

Transfected HeLa cells were prepared following the co-transfection protocol described before. The cells were seeded on tissue-treated 96-well white plates and divided into 2 groups for DHT and CIDAP. The cells were treated with 10 μM menadione and 25 μM Z-VAD-FMK for 1 h, followed by the treatment with 5 μL of DHT or CIDAP in ethanol solutions (final concentration range from 0 to 11 nM). After 2 h incubation with the compounds, the data was collected on the plate reader.

Quantification of H_2O_2 level in GRX3-KD HeLa cells

Transfected wild-type HeLa cells GRX3-KD HeLa cells were prepared following the co-transfection protocol described before. The cells were seeded on tissue-treated 96-well white plates and divided into 2 groups for DHT and CIDAP. The cells were treated with 5 μL of DHT or CIDAP in ethanol solutions (final concentration range from 0 to 11 nM). After 2 h incubation with the compounds, the data was collected on the plate reader.

Application of CIDAP in various cell lines

MDA231, ASPC1, and HepG2 cells were transfected following the general transfection protocol described before. The cells were seeded tissue-treated 96-well white plates and divided into 2 groups for DHT and CIDAP. The cells were treated with 5 μL of DHT or CIDAP in ethanol solutions (final concentration range from 0 to 11 nM. After 2 h of incubation with DHT/CIDAP, the medium was removed and replaced with 1X Nano-luc substrate (Promega) in phenol-red free OptiMEM reduced serum medium (100 μL/well). The luminescence signal was measured immediately on the plate reader. The individual dose-response curves for each cell line were shown in Figure S4. The H_2O_2 level for each cell line was calculated based on the EC_{50} as described before.

Investigation on the permeability of DHT

Transfected HEK293T cells were prepared following the co-transfection protocol described before. The cells were seeded on tissue-treated 96-well white plates and divided into 2 groups for DHT and CIDAP. The cells in each group were treated with DHT at 1, 5 and 10 nM. 0.05 % digitonin or water (negative control) was added to the cells simultaneously. The incubation was stopped and measured at 0, 5, 10, 20, 30, 60, 120 min time points. The results were collected on the plate reader and visualized in GraphPad Prism, as shown in Figure S9. The findings indicate that there was minimal disparity observed between the digitonin and DHT groups, suggesting that DHT exhibited a rapid transmembrane permeability.

The mitochondria permeability of CIDAP

Female C57BL6 mice were subjected to overnight fasting before being sacrificed to obtain liver samples for mitochondria extraction. The isolation protocol for mitochondria followed the guidelines provided in this literature.[7] Mitochondria were extracted from the livers of three mice, resulting in a combined wet weight of 187 mg. Subsequently, 60 mg of mitochondria

(equivalent to a volume of approximately 30 µl) was suspended in 300 µl of sucrose buffer (0.25 M, 10 mM Tris, with pH adjusted to 7.4 using MOPS). This suspension was then incubated at room temperature for 2 hours in the presence of 1% ethanol, 10 µM CIDAP in 1% ethanol, or 100 µM CIDAP in 1% ethanol. Following the incubation period, the mitochondria were washed twice with sucrose buffer, followed by centrifugation at 17,000 x g for 10 minutes. After the final wash, 100 µl of ice-cold 100% acetonitrile was added to the mitochondria pellets to extract small molecules, followed by three freeze-and-thaw cycles. Proteins and mitochondrial debris were removed by centrifugation at 17,000 x g for 3 minutes at 4°C. 10 µl of the supernatant was injected into an Agilent 1290 Infinity II LC System coupled with an Agilent 6495 Triple Quadrupole mass spectrometer. The quantities of CIDAP and DHT were monitored using multiple reaction monitoring (MRM) with CIDAP transmission at m/z 551.3 -> 217.1 at a fragmentation energy of 40V, and DHT transmission at m/z 291.2 -> 255.2 at a fragmentation energy of 19V.

DFT calculations

All DFT calculations were performed using the Gaussian 16 Version D.01 suite of programs at Texas Advanced Computing Center (TACC). The geometries listed below were optimized at the M06-2X/6-31G(d) level of theory with SRCF solvation method in water. The charge distribution of C in the carbonate for each compound can be found in Table S1.

Matlab Simbiology simulation

The model simulation was performed using Matlab Simbiology (Release 2022b, MathWorks, Natick, MA, USA). The flowcharts of DHT and CIDAP models were demonstrated in Figure S5-6. Information of compartments and kinetic parameters for each model were listed in Table S2-3. The simulated data was generated by varying the ranges of values for cell crossing rates, initial concentrations of DHT/CIDAP, H_2O_2 concentrations, and time points.

Supplementary figures, tables, NMR and DFT geometries

Supplementary figures

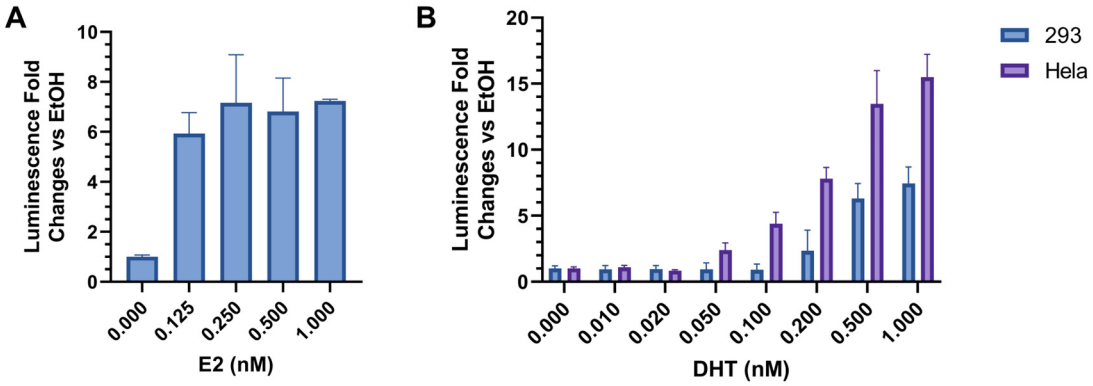


Figure S1. (A) The bioluminescence response of luciferase in HeLa cells after 24 h of incubation with estradiol (E2), driven by estrogen response elements (ERE). Data are mean ± SEM of experimental replicates (n = 2). Ethanol was used as the solvent and the control. (B) The bioluminescence response of luciferase in HEK293T and HeLa cells after 24 h of incubation with DHT, driven by androgen response elements (ARE). Data are mean ± SEM of experimental replicates (n = 6). Ethanol was used as the solvent and the control.

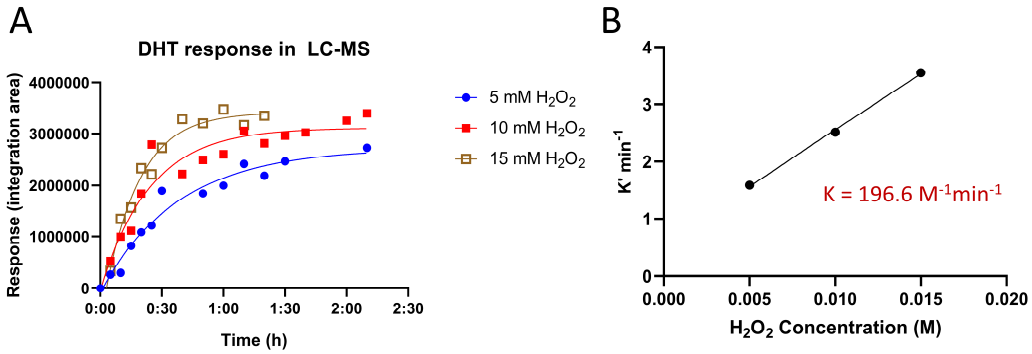


Figure S2. The rate constant of CIDAP with peroxide was determined by monitoring the generation of DHT in the mixture. (A) Each data point was collected by integrating the area of DHT response in mass spectrometry. The method was described under in vitro assays. Blank samples were run in between each data point to avoid the DHT carry-over. The data was plotted in GraphPad Prism and k_{obs} for each curve was generated. (B) The k_{obs} was plotted against the corresponding H_2O_2 concentration. The rate constant (K) was the gradient of this fitted linear regression.

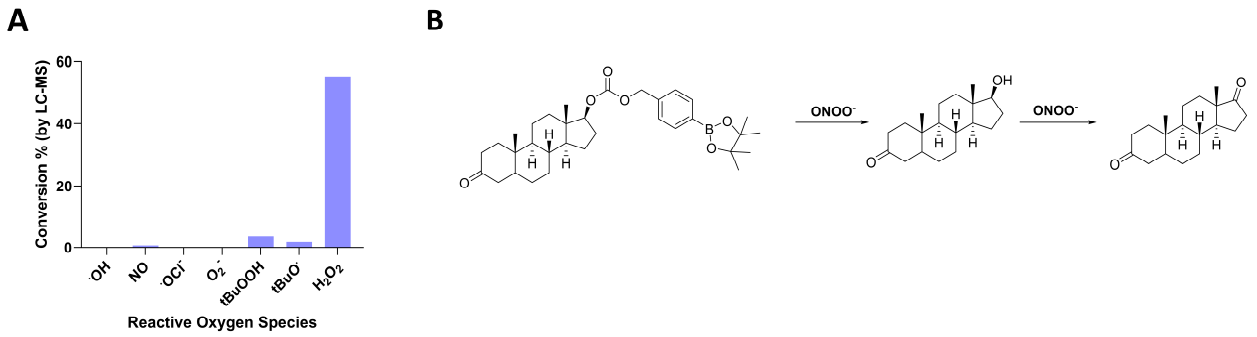


Figure S3. (A) The selectivity of CIDAP among various reactive oxygen species (ROS). 250 μM CIDAP was incubated with 5 mM of each ROS at 37°C for 2 h, unless otherwise specified. The production of DHT was measured in LC-MS and the conversion rate was calculated based on the response of 250 μM DHT. The results confirmed that CIDAP exhibits a high selectivity of peroxide over other ROS. (B) 250 μM of CIDAP was incubated with 50 μM of peroxynitrite in 20 mM HEPES (pH 7.4). It was found that peroxynitrite reacts with CIDAP rapidly and generates DHT. DHT can be further oxidized by peroxynitrite. The final oxidized product was confirmed by LC-MS results.

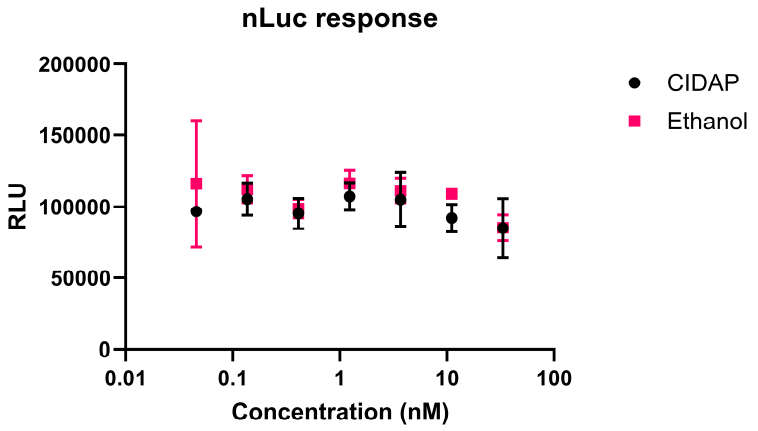


Figure S4. The nLuc response was assessed following incubation with varying concentrations of CIDAP or ethanol, ranging from 0 to 33 nM. The nLuc signal exhibited consistency when treated with ethanol or elevated concentrations of CIDAP. This observation suggests that CIDAP does not act as a coenzyme for nLuc.

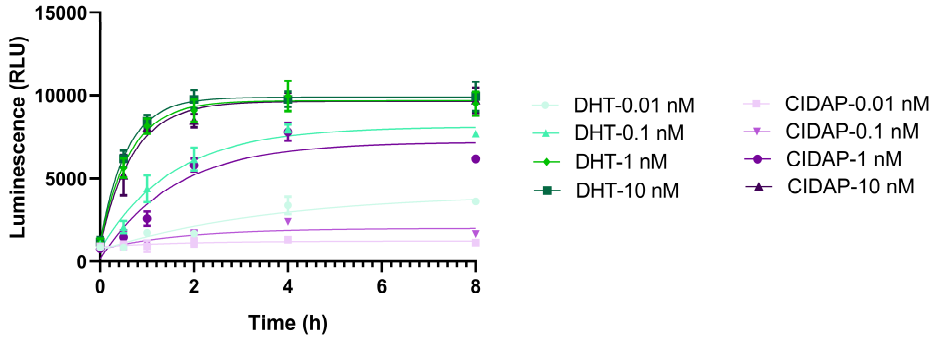


Figure S5. The HEK293T cells were treated with DHT or CIDAP at 4 different concentrations from 0.01 nM to 10 nM. The bioluminescence readouts were collected at various time points as described in the method. Data are mean ± SEM of experimental replicates (n = 3). The goal is to identify the time point that enables maximum signal from each group, completion of DHT induced

dimerization, and partial CIDAP reaction with H_2O_2. The optimal incubation time was determined as 2 h, based on the comparison among DHT, CIDAP, and background signal at each time point.

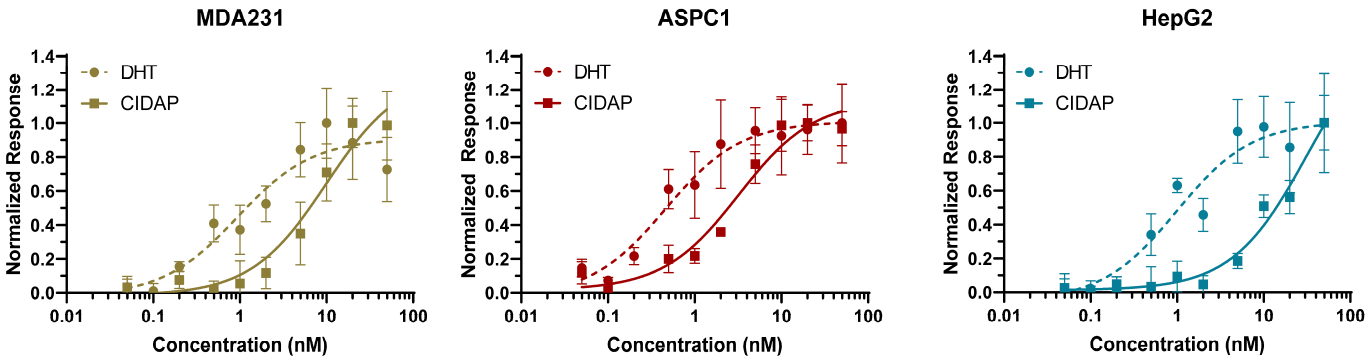


Figure S6. The original bioluminescence dose-response curves for each cell line. Data are mean ± SEM of experimental replicates (n = 4).

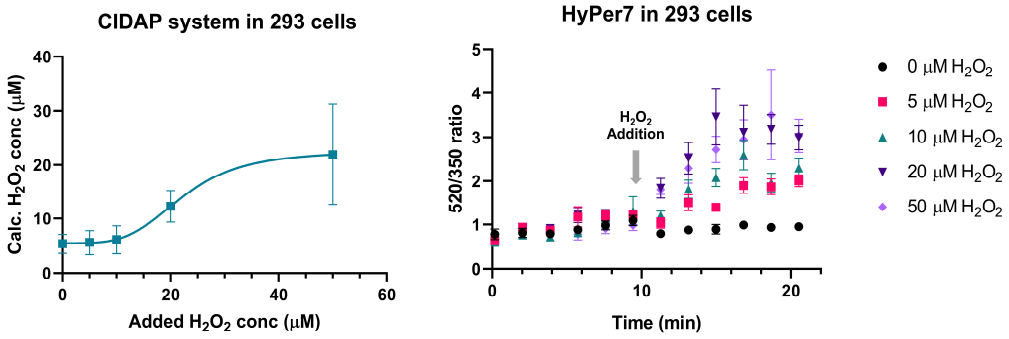


Figure S7. The comparison between CIDAP system and Hyper system. Data are mean ± SEM of experimental replicates (n = 4). Both systems were treated with exogenous peroxide concentrations ranging from 0 to 50 μM. Results indicate that noticeable differences emerged within the CIDAP system when the peroxide level surpassed 10 μM. In contrast, distinct variations were observed in the HyPer7 system even before the peroxide concentration reached 10 μM. Furthermore, it was noted that HyPer7 became saturated for peroxide levels exceeding 10 μM.

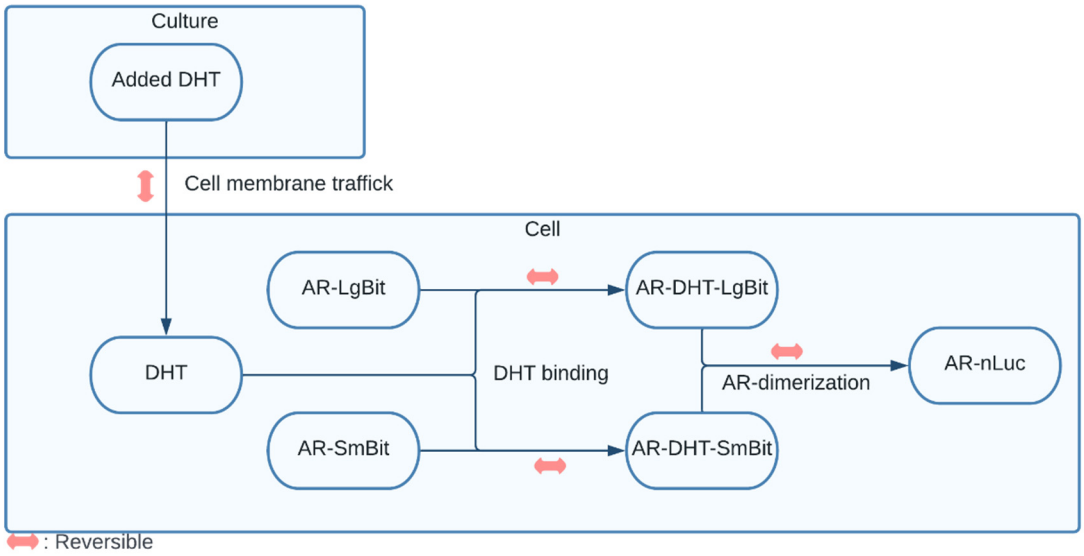


Figure S8. The simulated model of DHT treatment from administration to AR-dimerization for Matlab SimBiology Simulation. DHT is first added into the culture and then diffused into the cells. The intracellular DHT binds to AR-LgBit and AR-Smbit with the same affinity, respectively. The binding of DHT triggers the dimerization of AR-LgBit and AR-Smbit. The dimerization leads to the rapid reconstitution of nLuc. The cell membrane crossing, DHT binding, and AR dimerization steps are considered reversible.

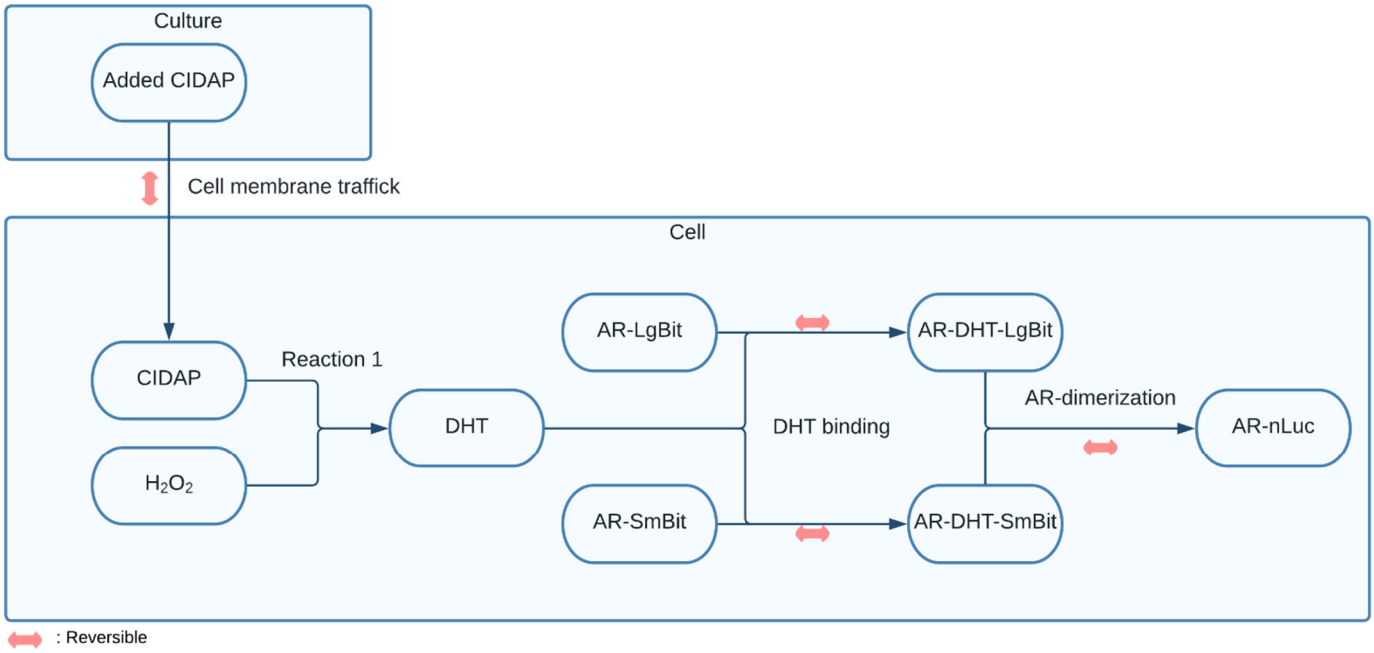


Figure S9. The simulated model of CIDAP treatment from administration to AR-dimerization for Matlab SimBiology Simulation. CIDAP is first added into the culture and then diffused into the cells. The intracellular CIDAP reacts with endogenous H_2O_2 to generate DHT. The DHT binds to AR-LgBit and AR-Smbit with the same affinity, respectively. The binding of DHT triggers the dimerization of AR-LgBit and AR-Smbit. The dimerization leads to the rapid reconstitution of nLuc. The cell membrane crossing, DHT binding, and AR dimerization steps are considered reversible.

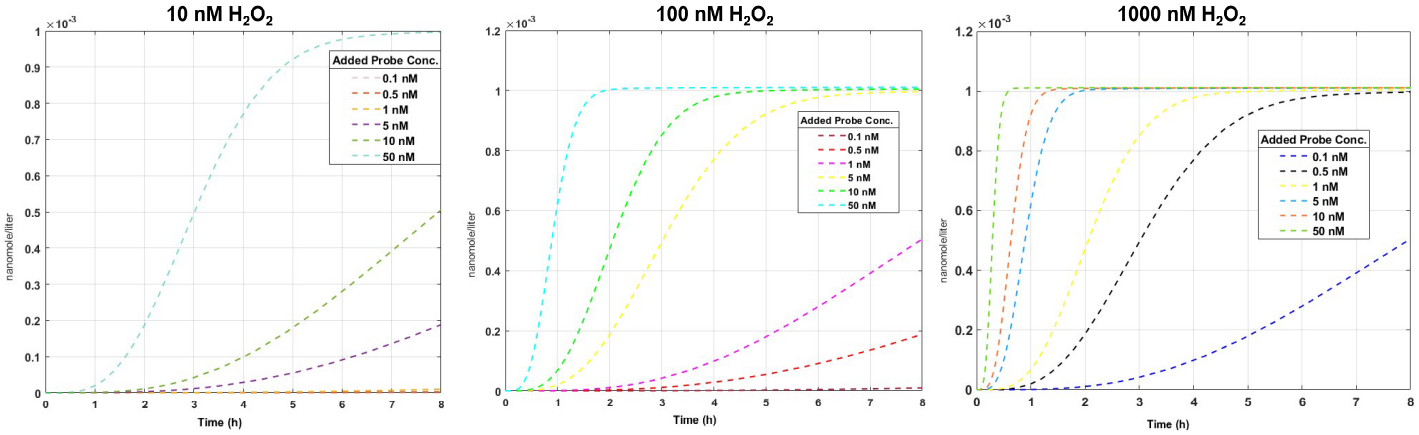


Figure S10. Simulated CIDAP responses were generated from Matlab Simbiology with the settings described in Figure S6. The H_2O_2 level was set at 10 nM, 100 nM, and 1000 nM, respectively. The y axis is the concentration of nLuc output in the system. The time frame was set as 8 h. The results suggested that the CIDAP signal would require longer time to reach saturation if the cellular H_2O_2 concentration is lower.

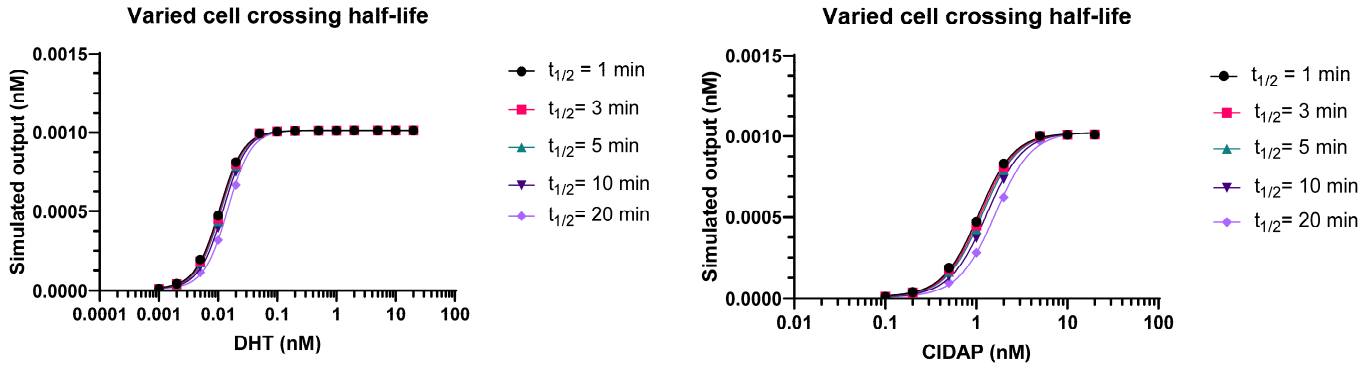


Figure S11: Simulated DHT and CIDAP responses were generated from Matlab Simbiology models with various membrane crossing half-lives. The simulation data was collected at 2 h time point and plotted in GraphPad Prism. The results suggested that the overall luminescent output induced by DHT and CIDAP will likely not be affected by the speed of membrane crossing.

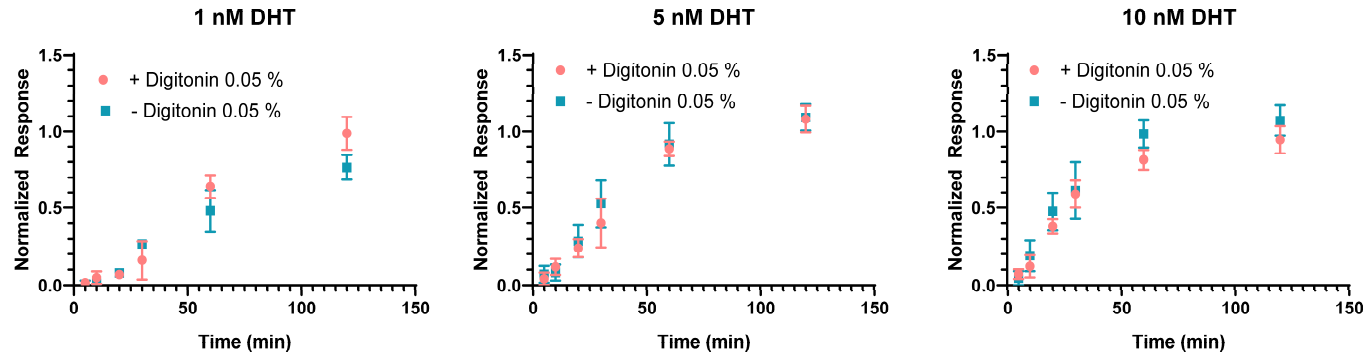


Figure S12. HeLa cells were treated with 0.05 % digitonin (or water as negative control) with DHT simultaneously. The data was collected at different time points from 5 min to 120 min. Data are mean ± SEM of experimental replicates (n = 4). The results were consistent with the simulation results in Figure S11. The responses generated by DHT aligned well among the conditions with and without the presence of digitonin.

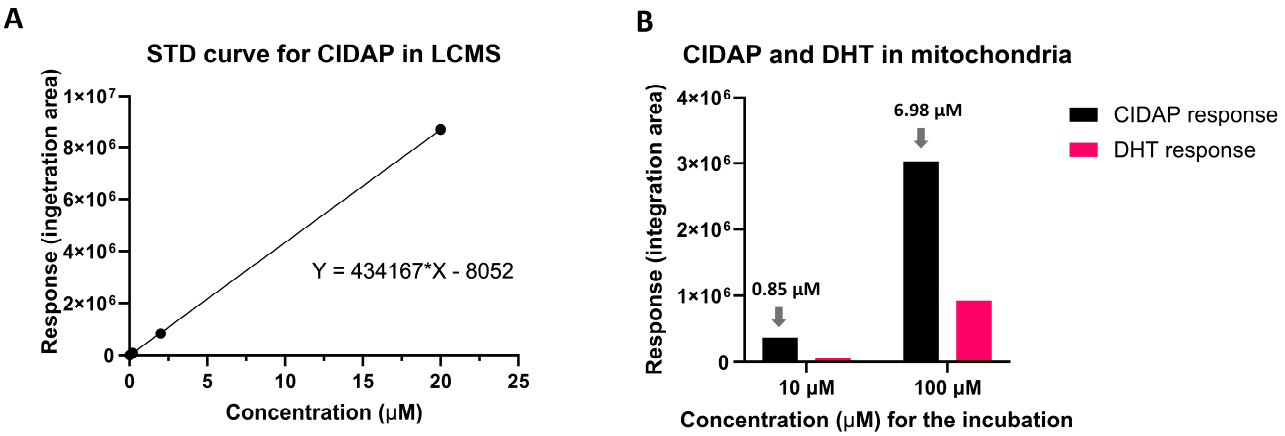
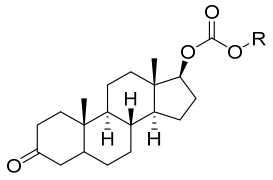
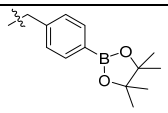
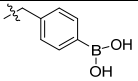
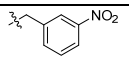
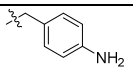


Figure S13. (A) The standard curve of CIDAP response in LC-MS. (B) The response of CIDAP and DHT in mouse liver mitochondria was observed following a 2-hour incubation period with CIDAP. The values depicted in the figure represent the CIDAP amount, which were calculated using the linear equation derived from the standard curve in (A). The results indicate that approximately 10% of CIDAP was detected within the mitochondria after the 2-hour incubation period. Additionally, DHT was also observed as the product of CIDAP and mitochondrial peroxide. This observation further validated the subcellular distribution of CIDAP upon cellular entry.

Supplementary tables

Table S1: Charge distribution (Mulliken) of C in the carbonate from DFT results [a]



R	Charge distribution
	0.344
	0.357
	0.25
	-0.048
	-0.042

a. *We opted for the nitro group because, based on DFT calculations, the charge distribution of the carbon on the carbonate of CIDAP-NC2 closely resembles that of the boronate ester of CIDAP. This similarity suggests that the nitro group would induce a comparable effect in terms of potential hydrolysis. Since charge density correlates with the rate of hydrolysis, we selected the nitro group to simulate a similar charge density as observed in phenylboronate esters. On the other hand, a simple phenyl ester would be less prone to hydrolysis and a less stringent negative control for CIDAP.*

Table S2: Settings of Matlab SimBiology simulation

Compartments*

Name	Value	Units	Constant	Note	Source
Culture	1	L	TRUE	Medium volume in cell culture	
Cell	1	mL	TRUE	Cell volume	

Species

Name	Value	Units	Constant	Note	Source
Added CIDAP	1	nM	TRUE	Initial concentration of CIDAP added to cell Culture	
CIDAP	0	nM	FALSE	The intracellular CIDAP concentration	
DHT	0	nM	FALSE	The intracellular DHT concentration	
H_2O_2	1000	nM	TRUE	The intracellular H_2O_2 concentration	
AR-DHT-LgBit	0	nM	FALSE	The intracellular concentration of DHT and AR-LgBit complex	
AR-LgBit	3	nM	FALSE	The intracellular AR-LgBit concentration	Estimated from experimental results.
AR-nLuc	0	nM	FALSE	The intracellular AR-nLuc concentration	
signal	0	nM	FALSE	The signal produced after nLuc reconstitution	
AR-SmBit	3	nM	FALSE	The intracellular AR-SmBit concentration	Estimated from experimental results.
AR-DHT-SmBit	0	nM	FALSE	The intracellular concentration of DHT and AR-SmBit complex	

Parameters

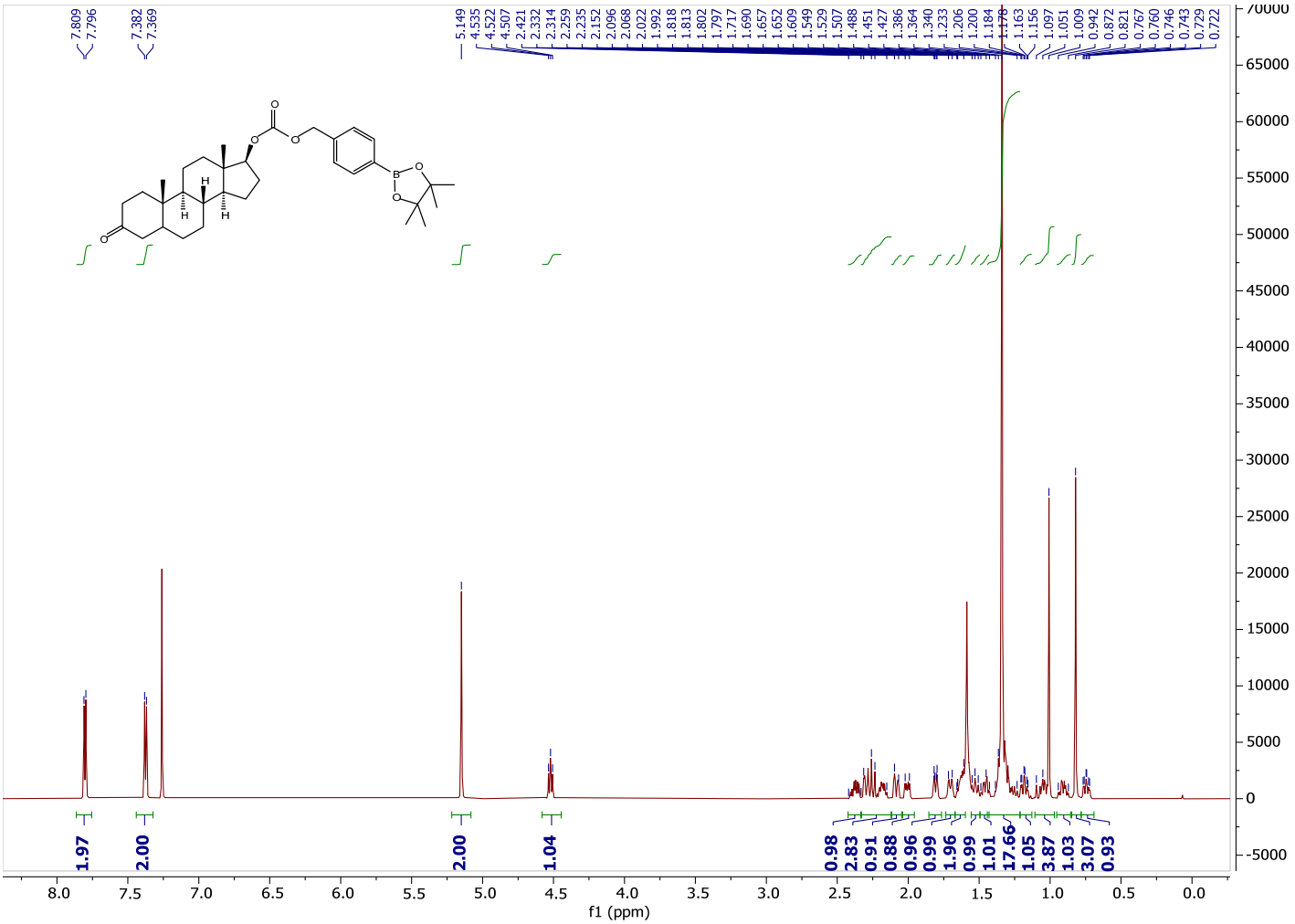
Name	Value	Units	Constant	Notes	Source
kf_r	1.966e-07	$nM^{-1}min^{-1}$	TRUE	The rate constant for Reaction 1	Measured by LC-MS
kf_bind	6e7	$M^{-1}h^{-1}$	TRUE	k_{on} of DHT binding to AR	Ref [8]
k_per	0.6931	1/min	TRUE	Cell membrane crossing rate when $t_{1/2}$ = 1 min	
kon_dimer	8100	$M^{-1}s^{-1}$	TRUE	k_{on} of AR dimerization	Ref [9]
kr_bind	2.888e-4	1/min	TRUE	k_{off} of DHT binding to AR	Ref [8]
koff_dimer	0.072	1/sec	TRUE	k_{off} of AR dimerization	Ref [9]

*Note: The volume of each compartment was scaled up proportionally based on the volume and ratio of 20000 cells/100 µL in a regular 96-well plate.

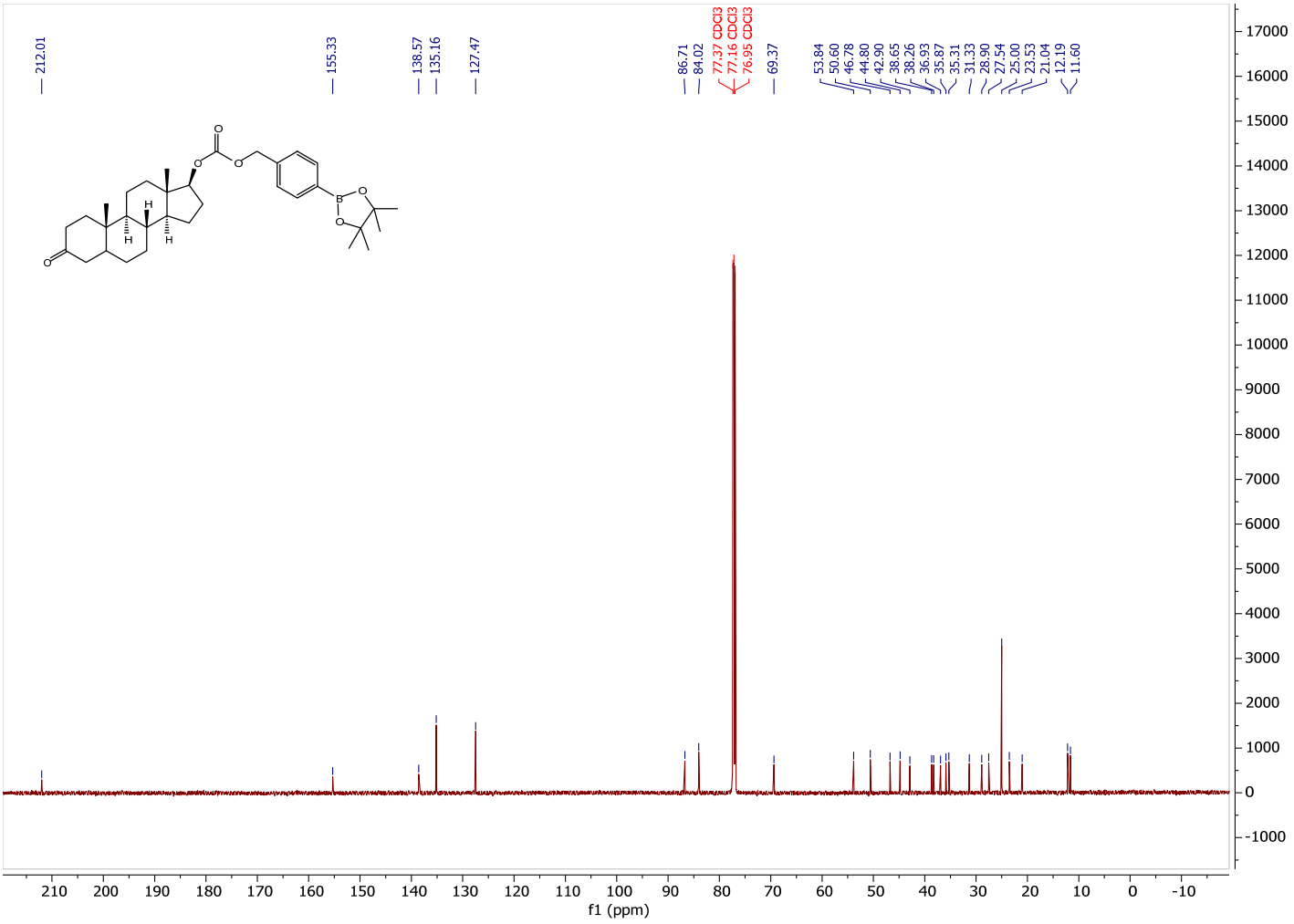
Table S3: Reaction description and kinetic laws for the simulation

Name	Reaction	Kinetic Law	Reaction Rate	Reversible
Cell membrane traffick	Culture.Added DHT <-> Cell.DHT	MassAction	k_per*Culture.Added DHT - k_per*Cell.DHT	TRUE
	Culture.Added CIDAP <-> Cell.CIDAP	MassAction	k_per*Culture.Added CIDAP - k_per*Cell.CIDAP	TRUE
Reaction 1	Cell.CIDAP + Cell.H2O2 -> Cell.DHT	MassAction	kf_r*Cell.CIDAP*Cell.H2O2	FALSE
DHT binding (AR-LgBit)	Cell.DHT + Cell.AR-LgBit <-> Cell.AR-DHT-LgBit	MassAction	kf_bind*Cell.DHT*Cell.AR-LgBit - kr_bind*Cell.AR-DHT-LgBit	TRUE
DHT binding (AR-SmBit)	Cell.DHT + Cell.AR-SmBit <-> Cell.AR-DHT-SmBit	MassAction	kf_bind*Cell.DHT*Cell.AR-SmBit - kr_bind*Cell.AR-DHT-SmBit	TRUE
AR dimerization	Cell.AR-DHT-SmBit + Cell.AR-DHT-LgBit <-> Cell.AR-nLuc	MassAction	kon_dimer*Cell.AR-DHT-SmBit*Cell.AR-DHT-LgBit - koff_dimer*Cell.AR-nLuc	TRUE

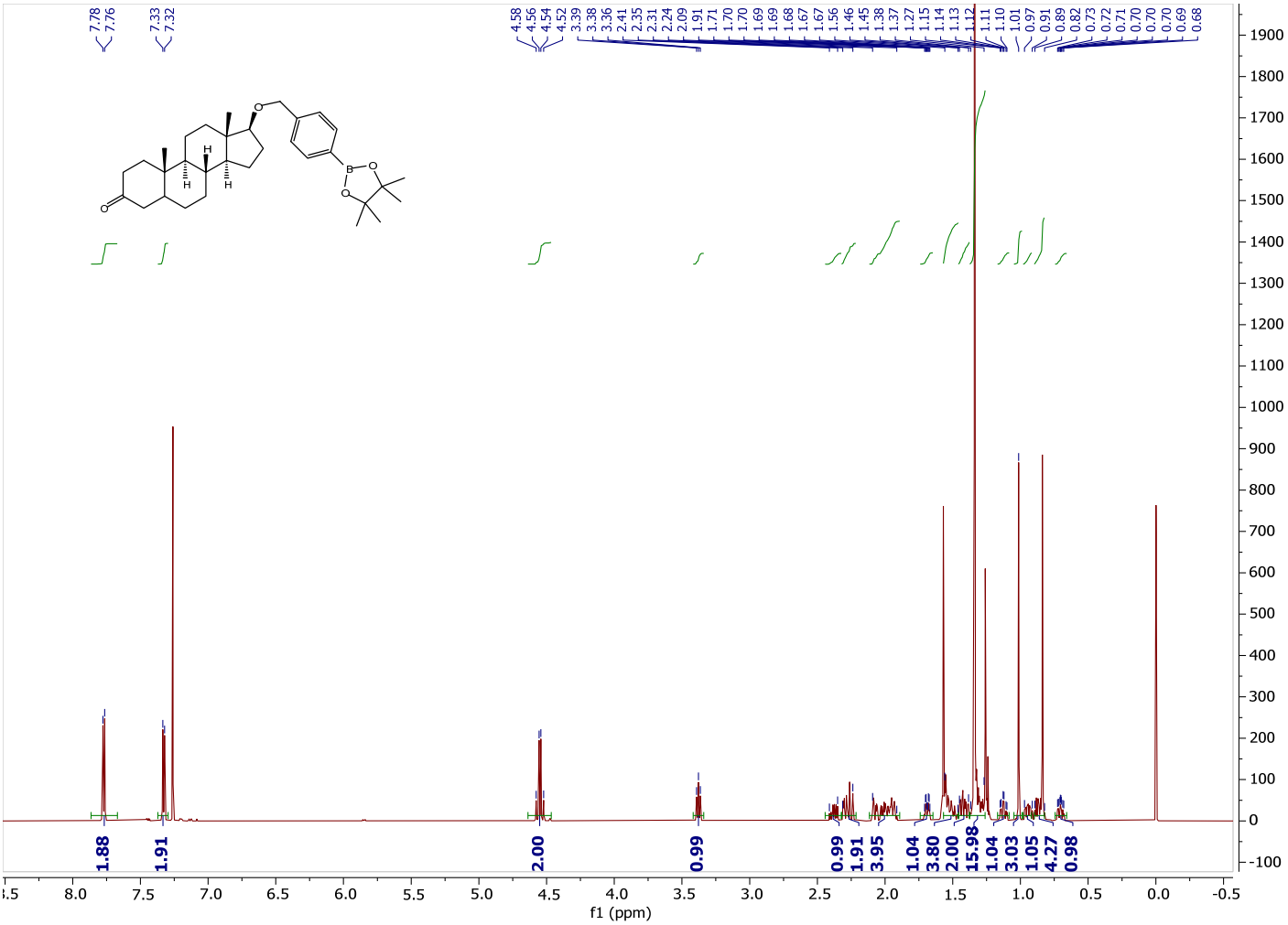
NMR spectra



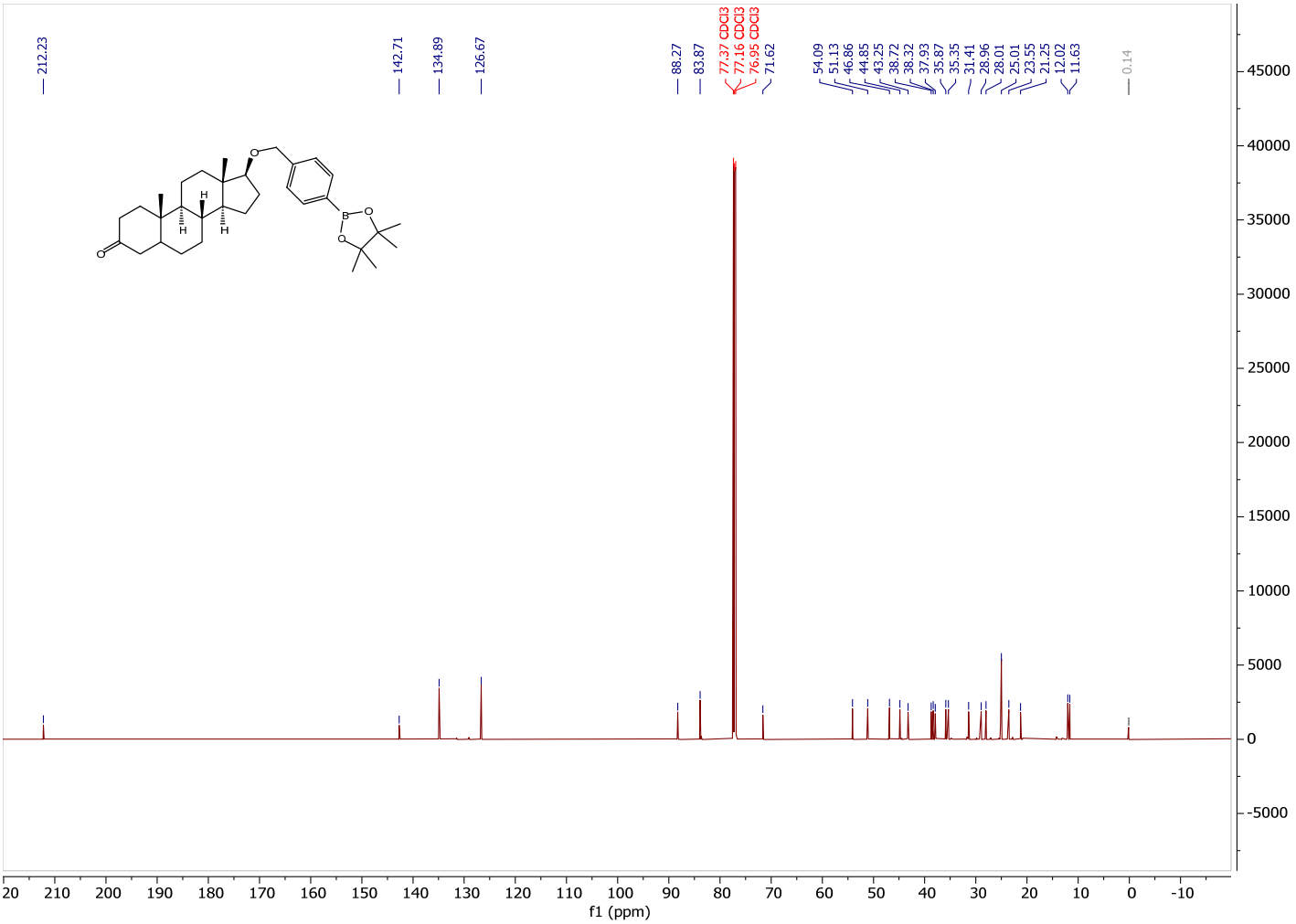
^{1}H NMR of CIDAP



^{13}C NMR of CIDAP



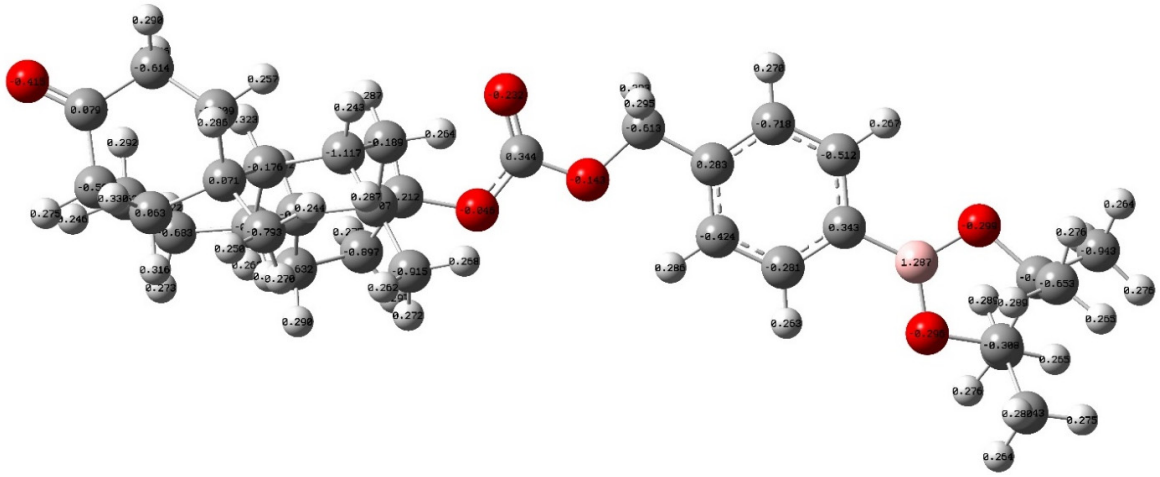
^{1}H NMR of CIDAP-NC1



^{13}C NMR of CIDAP-NC1

DFT geometry

CIDAP



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C	-8.42076	1.92091	-0.99756
C	-7.27052	2.37511	-0.09602
O	-10.69400	1.20295	-0.66698
C	-9.23023	1.00642	1.23868
C	-7.86345	0.29511	1.28739
C	-7.91261	-1.01057	0.48434
C	-6.62906	-1.82541	0.67695
C	-5.35881	-0.93865	0.72736
C	-4.20610	-1.64898	0.02112
C	-3.76161	-3.03193	0.53351
C	-2.35297	-3.22376	-0.09959
C	-2.01422	-1.86951	-0.75445
O	-0.57187	-1.61784	-0.58593
C	-2.89460	-0.83446	-0.03380
C	-2.30647	-0.55333	1.35971
C	-3.17092	0.46584	-0.79404
C	-4.29521	1.25020	-0.09466
C	-5.60428	0.44430	0.06691
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C	9.57450	-0.18571	1.30908
O	8.08334	-0.23613	1.26522
C	11.15930	0.85598	-0.46688
C	9.79739	2.36478	1.00973
C	10.01028	0.03860	2.74372
C	10.06204	-1.53025	0.78192
H	-8.79748	2.74125	-1.61076
H	-8.09035	1.14452	-1.69533
H	-6.46701	2.78590	-0.70726
H	-7.62696	3.19956	0.52846
H	-10.02203	0.38012	1.64819
H	-9.19426	1.92894	1.82864
H	-7.66998	0.02301	2.33034
H	-8.78065	-1.59564	0.79861
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H	-5.98415	0.24329	-0.94660
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H	-5.49937	2.78268	1.82911
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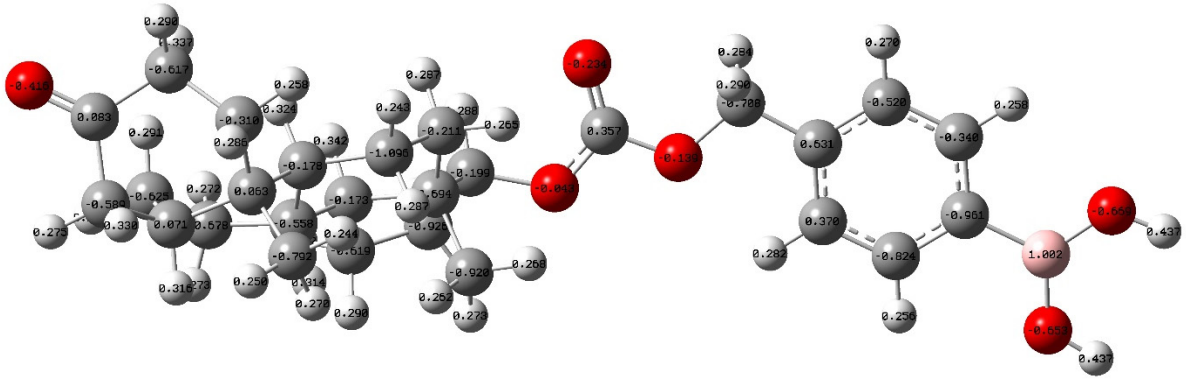
H	2.17239	-0.33630	-3.01221
H	1.76283	1.09934	-2.06532
H	4.30350	1.33631	-3.07554
H	6.61742	1.44476	-2.22071
H	5.48625	-0.97018	1.13892
H	3.16580	-1.07431	0.28187
H	11.28451	1.72532	-1.11139
H	12.01784	0.79961	0.20468

H	11.13915	-0.03642	-1.08761
H	10.64151	2.51696	1.68230
H	9.81907	3.13782	0.24270
H	8.87300	2.46686	1.57890
H	9.75402	-0.83534	3.34150
H	11.09140	0.18119	2.78971
H	9.52202	0.90924	3.17468
H	11.14966	-1.59079	0.82004
H	9.64665	-2.32245	1.40340
H	9.73822	-1.69087	-0.24680

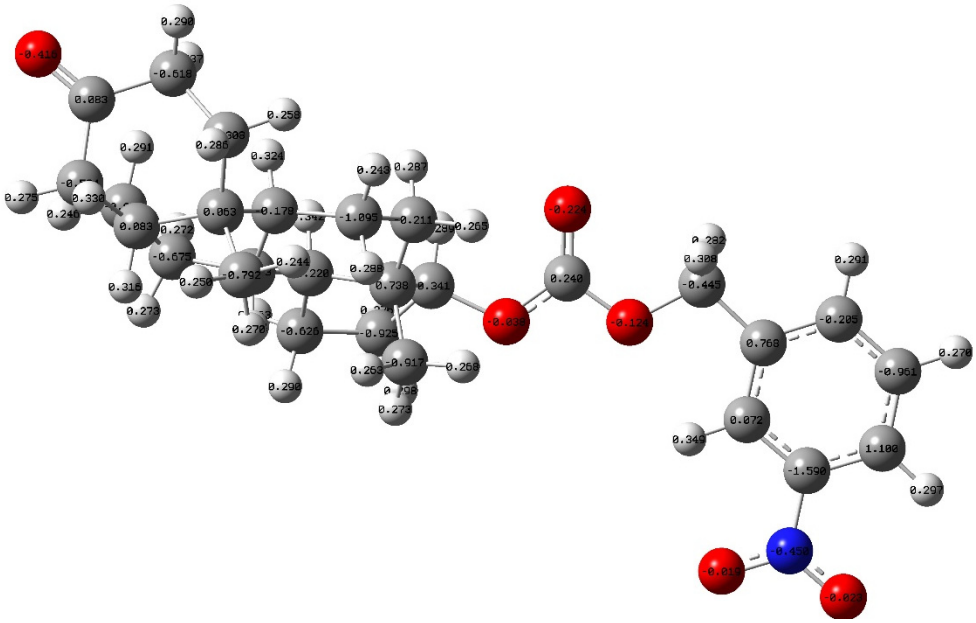


E(RM062X) = -1526.89900438

C	7.74643	-1.23006	-0.30408
C	6.58476	-1.94944	-0.94147
C	5.5054	-2.27753	0.09272
O	8.84311	-1.11968	-0.87684
C	7.48057	-0.64171	1.05383
C	6.09122	0.02579	1.06507
C	6.04251	1.17279	0.04805
C	4.73947	1.96951	0.17575
C	3.51321	1.06831	0.46889
C	2.2881	1.6125	-0.26224
C	1.82564	3.05003	0.03865
C	0.37176	3.09136	-0.51366
C	0.03911	1.63484	-0.89666
O	-1.37572	1.383	-0.57115
C	1.01017	0.7638	-0.08079
C	0.53192	0.7076	1.38028
C	1.28822	-0.63872	-0.62988
C	2.48836	-1.25954	0.10723
C	3.77167	-0.4001	0.03875
C	4.95145	-1.03494	0.84874
C	4.48834	-1.50903	2.23942
C	-2.04153	0.45329	-1.27241
O	-1.59725	-0.21158	-2.20889

O	-3.30092	0.36292	-0.79679
C	-4.17839	-0.61373	-1.43804
C	-5.52767	-0.54905	-0.7767
C	-6.5304	-1.41355	-1.23465
C	-7.79442	-1.39147	-0.65435
C	-8.09259	-0.5054	0.39495
C	-7.07934	0.3542	0.84115
C	-5.80777	0.3361	0.26584
B	-9.50774	-0.47863	1.04244
O	-9.73961	0.42249	2.05688
O	-10.4551	-1.35692	0.56702
H	6.95941	-2.85084	-1.42939
H	6.18542	-1.31417	-1.73879
H	4.6851	-2.8083	-0.39055
H	5.92785	-2.97428	0.82267
H	8.26804	0.06913	1.30167
H	7.51476	-1.45015	1.79263
H	5.94547	0.46908	2.05577
H	6.90231	1.82953	0.20311
H	6.13603	0.77967	-0.96787
H	4.83751	2.71375	0.96998
H	4.56698	2.52416	-0.75095
H	3.31779	1.08265	1.54714
H	2.52992	1.56951	-1.33613
H	2.46511	3.79527	-0.43199
H	1.84154	3.23467	1.1148
H	-0.3369	3.433	0.24004
H	0.27728	3.74893	-1.37578
H	0.16691	1.4468	-1.96295
H	0.23623	1.69157	1.7447
H	-0.33523	0.05005	1.46055
H	1.30696	0.3259	2.04244
H	0.41204	-1.28252	-0.51755
H	1.5021	-0.56603	-1.70157
H	2.68608	-2.24999	-0.30728
H	2.20793	-1.41589	1.14975
H	4.07934	-0.36614	-1.01775
H	3.91728	-0.73483	2.75655
H	3.87345	-2.40732	2.17218
H	5.36033	-1.75043	2.85233
H	-4.24497	-0.37674	-2.50004
H	-3.73106	-1.60254	-1.3341
H	-6.31765	-2.10171	-2.04339
H	-8.5627	-2.06315	-1.01297
H	-7.29081	1.04354	1.64772
H	-5.03689	1.00284	0.62079
H	-10.5935	0.49977	2.50363

H	-11.3541	-1.39862	0.9204



E(RM062X) = -1555.33605076

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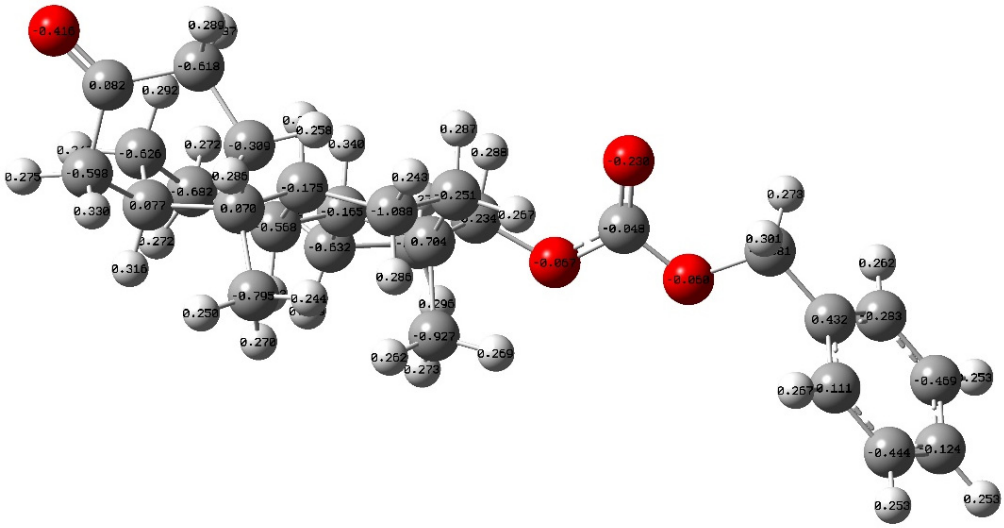
C	5.40285	-1.89267	1.0124
O	8.67968	-1.08496	-0.41241
C	7.26663	0.11109	1.13195
C	5.8403	0.63375	0.87007
C	5.73658	1.2153	-0.54536
C	4.38745	1.91033	-0.75961
C	3.21238	1.15687	-0.08619
C	1.96776	1.25106	-0.96591
C	1.41575	2.64441	-1.32012
C	-0.03039	2.35251	-1.81425
C	-0.26947	0.85884	-1.51479
O	-1.67195	0.69086	-1.09134
C	0.73993	0.4933	-0.41307
C	0.24593	1.05558	0.93088
C	1.10796	-0.98874	-0.29579
C	2.3327	-1.15077	0.62216
C	3.56334	-0.33341	0.1665
C	4.76757	-0.47903	1.15623
C	4.31524	-0.32235	2.62043
C	-4.34632	-1.63403	-0.98122
C	-5.71654	-1.33642	-0.43906
C	-6.72918	-2.29014	-0.60431
C	-8.0102	-2.06458	-0.10518
C	-8.30292	-0.88047	0.56593
C	-7.28348	0.05252	0.7166

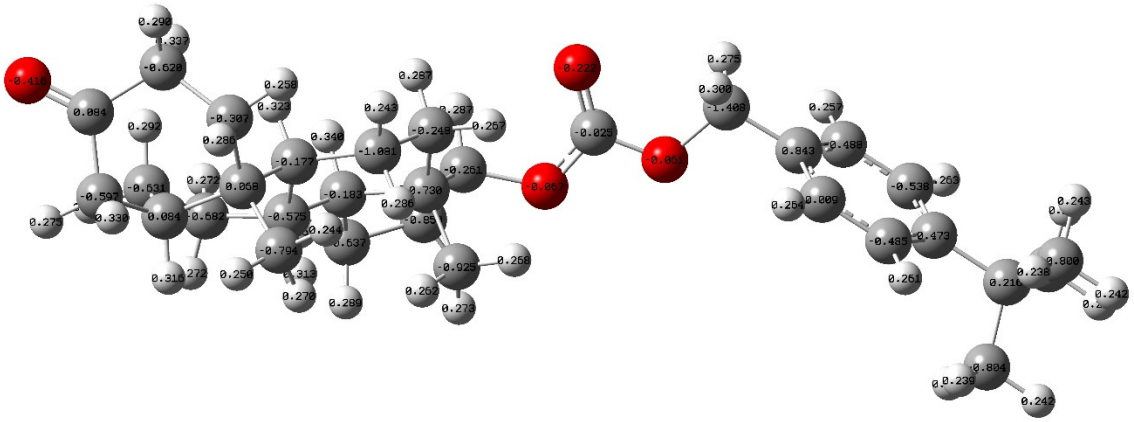
C	-5.99429	-0.14882	0.22936
C	-2.27124	-0.48739	-1.30816
O	-3.53396	-0.43272	-0.82848
O	-1.7787	-1.47008	-1.86139
N	-7.57325	1.29609	1.41312
O	-6.65603	2.14331	1.54816
O	-8.73292	1.48352	1.8579
H	6.90686	-2.98848	-0.12611
H	6.04872	-1.78933	-1.06527
H	4.62126	-2.62801	0.82146
H	5.85573	-2.17409	1.96755
H	8.00777	0.90323	1.03147
H	7.33931	-0.28788	2.14985
H	5.65664	1.4565	1.56906
H	6.55419	1.92265	-0.70582
H	5.86563	0.42358	-1.28827
H	4.43142	2.93041	-0.37001
H	4.19496	1.99511	-1.83287
H	3.00025	1.62977	0.87937
H	2.22732	0.75561	-1.91494
H	2.01563	3.14435	-2.07904
H	1.40565	3.28332	-0.43464
H	-0.76846	2.94709	-1.27697
H	-0.15226	2.55817	-2.87602
H	-0.11725	0.22992	-2.39228
H	-0.11188	2.08045	0.83071
H	-0.58232	0.45039	1.30291
H	1.03296	1.0494	1.6828
H	0.27039	-1.56907	0.09973
H	1.33131	-1.3806	-1.29385
H	2.59491	-2.20891	0.68085
H	2.04748	-0.85097	1.63139
H	3.88284	-0.74746	-0.80224
H	3.6939	0.56586	2.75434
H	3.7545	-1.19315	2.9622
H	5.19211	-0.22016	3.26463
H	-3.87271	-2.44963	-0.43446
H	-4.38843	-1.90275	-2.03607
H	-6.5134	-3.21245	-1.12726
H	-8.78128	-2.80752	-0.24002
H	-9.28741	-0.68318	0.95858
H	-5.23185	0.60049	0.36369



E(RM062X) = -1350.92397674

C	-6.76565	0.98675	-0.73429
C	-5.58859	1.63097	-1.42142
C	-4.57492	2.15584	-0.40192
O	-7.8274	0.75221	-1.33484
C	-6.55956	0.63147	0.71206
C	-5.15683	0.02101	0.90114
C	-5.02393	-1.27303	0.08899
C	-3.71108	-1.99511	0.41179
C	-2.52471	-1.01834	0.61228
C	-1.24867	-1.633	0.04113
C	-0.76411	-2.98478	0.59787
C	0.71846	-3.06448	0.13218
C	1.03012	-1.68116	-0.47375
O	2.4197	-1.32838	-0.13401
C	-0.00449	-0.72254	0.14013
C	0.3946	-0.41122	1.5929
C	-0.2892	0.56069	-0.64603
C	-1.54068	1.25301	-0.07804
C	-2.79614	0.35137	-0.06512
C	-4.03278	1.06978	0.57216
C	-3.65665	1.7762	1.88838
C	3.08853	-0.49639	-0.94863
O	2.66313	-0.00714	-1.9961
O	4.32366	-0.28337	-0.44678
C	5.20203	0.61461	-1.22769
C	6.46771	0.76445	-0.442
C	7.59327	-0.00156	-0.75679
C	8.76223	0.12096	-0.00414
C	8.80942	1.00985	1.07027
C	7.68698	1.77755	1.39039
C	6.52159	1.65511	0.63583

H	-5.95732	2.42963	-2.06725
H	-5.13178	0.88874	-2.08417
H	-3.74289	2.62846	-0.92381
H	-5.0531	2.94672	0.18341
H	-7.34157	-0.05657	1.03142
H	-6.65446	1.54565	1.30853
H	-5.05513	-0.25106	1.95702
H	-5.87483	-1.92405	0.30517
H	-5.07005	-1.05276	-0.98091
H	-3.83464	-2.60129	1.31268
H	-3.47516	-2.68876	-0.40018
H	-2.38674	-0.85034	1.68636
H	-1.43426	-1.77528	-1.03537
H	-1.35677	-3.81938	0.22628
H	-0.83311	-2.99131	1.68767
H	1.39419	-3.24934	0.96661
H	0.87813	-3.85298	-0.60089
H	0.94915	-1.67713	-1.56098
H	0.70133	-1.31056	2.12723
H	1.23571	0.28389	1.60437
H	-0.42538	0.04212	2.14706
H	0.56386	1.24242	-0.60185
H	-0.44549	0.30594	-1.69975
H	-1.74171	2.1554	-0.65875
H	-1.31863	1.58653	0.93656
H	-3.04699	0.136	-1.11516
H	-3.09756	1.11356	2.55261
H	-3.05926	2.67044	1.70666
H	-4.56588	2.08639	2.40935
H	5.37268	0.15914	-2.20083
H	4.68068	1.56115	-1.35691
H	7.55454	-0.69058	-1.59049
H	9.62978	-0.47246	-0.25567
H	9.71469	1.10682	1.65286
H	7.72242	2.46917	2.22018
H	5.65122	2.25113	0.87885



E(RM062X) = -1406.26618268

C	7.12151	1.01901	0.68584
C	5.94791	1.63169	1.40687
C	4.91549	2.17563	0.41658
O	8.193	0.77633	1.26551
C	6.89932	0.70504	-0.76762
C	5.50023	0.08582	-0.95589
C	5.39147	-1.23311	-0.18096
C	4.08139	-1.9587	-0.50726
C	2.88217	-0.98897	-0.66056
C	1.62154	-1.63323	-0.0878
C	1.14052	-2.97259	-0.67725
C	-0.33443	-3.0799	-0.19275
C	-0.64738	-1.71962	0.46285
O	-2.04511	-1.36743	0.16221
C	0.36729	-0.733	-0.13934
C	-0.05886	-0.38309	-1.57562
C	0.65205	0.52951	0.67941
C	1.88667	1.25119	0.11113
C	3.15064	0.36323	0.05279
C	4.37042	1.11252	-0.58095
C	3.96793	1.85279	-1.87044
C	-2.71233	-0.58459	1.02841
O	-2.27237	-0.14616	2.09335
O	-3.95531	-0.36052	0.55799
C	-4.84075	0.48122	1.40689
C	-6.14246	0.61464	0.6928
C	-7.16596	-0.32108	0.88337
C	-8.3674	-0.22449	0.19283
C	-8.58145	0.82466	-0.722
C	-7.55391	1.76826	-0.91456
C	-6.35784	1.65657	-0.21676
N	-9.77282	0.92759	-1.40848
H	6.31777	2.41508	2.07051

H	5.50728	0.86615	2.05391
H	4.08631	2.62486	0.96306
H	5.378	2.98764	-0.15219
H	7.68387	0.03522	-1.11799
H	6.97649	1.63767	-1.33748
H	5.38686	-0.15609	-2.0179
H	6.24558	-1.86866	-0.42833
H	5.45054	-1.04464	0.89441
H	4.19817	-2.53552	-1.42811
H	3.86427	-2.67944	0.28612
H	2.72535	-0.79092	-1.7269
H	1.82615	-1.80586	0.98077
H	1.74607	-3.81223	-0.33925
H	1.19347	-2.94628	-1.76765
H	-1.02088	-3.24262	-1.02306
H	-0.47728	-3.89343	0.51606
H	-0.544	-1.74996	1.54767
H	-0.36524	-1.26944	-2.1314
H	-0.90714	0.3029	-1.5524
H	0.74721	0.095	-2.12944
H	-0.20872	1.20311	0.66941
H	0.82914	0.24591	1.72241
H	2.08805	2.13903	0.71371
H	1.64476	1.61053	-0.89
H	3.41935	0.11981	1.09219
H	3.40633	1.20363	-2.54577
H	3.36368	2.73458	-1.65394
H	4.86621	2.1881	-2.39484
H	-4.94184	-0.01925	2.36791
H	-4.34327	1.43883	1.54747
H	-7.02069	-1.13452	1.58344
H	-9.14892	-0.95461	0.35564
H	-7.70386	2.58396	-1.60922
H	-5.57953	2.39219	-0.37786
H	-9.93413	1.67232	-2.06245
H	-10.51	0.2596	-1.27151

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