Supporting Information

A Redox-based Superoxide Generation System using Quinone/Quinone Reductase

Shailesh Kumar Singh[a] and Syed Masood Husain[a]*

I. General remarks

All the chemical reagents were obtained from Sigma-Aldrich and Himedia Laboratories, India. HPLC grade solvents were obtained from Spectrochem Pvt. Ltd., India. UV-Vis spectroscopy and activity measurements were performed on Agilent Technologies Cary 300 UV-Vis. spectrophotometer. Reactions were monitored using Agilent Technologies 1260 Infinity HPLC system equipped with EZChrom Elite v3.3.2SP2 software. Fluorescence spectrophotometric assays were performed on Synergy H1 microplate reader equipped with Gen5 v2.07.17 software (Biotek Instruments, Inc.) Substrate synthesis reactions were monitored by thin-layer chromatography (TLC, 0.25 mm E. Merck silica gel plates, $60F_{254}$) and visualized by using UV light. Column chromatography was performed on silica gel 60-120/230-400 mesh obtained from S. D. Fine Chemical Co., India. ^{1}H-NMR spectra were recorded on BRUKER 400 Ultra Shield instruments using deuterated solvents. Chemical shifts (δ) of the ^{1}H NMR are reported in parts per million (ppm) with a solvent resonance as an internal standard. Proton coupling constants (*J*) are reported as absolute values in Hertz (Hz). For ^{1}H NMR: chloroform 7.26, acetone-d_6 2.05, dimethylsulfoxide-d_6 2.50.

II. Expression and purification of enzymes

The strains *E. coli* DH5α and BL21(DE3) (Sigma-Aldrich) were used for cloning and expression, respectively. Synthesized NfsB gene from *E. coli* K-12 cloned into the pET19b vector using restriction sites *5'*-NdeI, *3'*-BamHI yielding NfsB_his, N-terminal histidine-tagged was procured from Eurofins Genomics India Pvt Ltd. Nucleotide and protein sequences for NfsB are given in Fig. S1 and Fig. S2, respectively. Glucose dehydrogenase (GDH) plasmid was generously provided by Prof. Werner Hummel (University of Bielefeld, Germany). The NfsB_pET19b recombinant plasmid was transformed into competent *E. coli* BL21 cells by applying a heat shock at 42 °C for 45 seconds. The transformed cells were grown overnight on SOB-agar medium containing 100 µg/mL ampicillin. One clone was picked and dispersed in 5 mL of LB-Lennox media (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.0) containing ampicillin (100 $\mu g \cdot mL^{-1}$), followed by incubation overnight (37 °C, 160 rpm). The overnight cultures were diluted to 500 mL of medium each (ampicillin 100 µg/mL) and incubated at 37 °C, 160 rpm. IPTG (0.2 mM) was added after reaching the mid-log phase (OD_{600nm} = 0.6) followed by incubation for 4 hours at 37 °C, 160 rpm. Cells were harvested (by centrifugation at 9600 rpm, 4 °C for 15 minutes) and resuspended in KPi buffer (50 mM, pH = 7.5; 5 mL per 1 L culture medium). The cells were disrupted by sonication (6 times 10 s, Vibra-Cell Processors, model no. VCX500, Sonics), followed by centrifugation (30 minutes, 12000 g, 4 °C). For purification of NfsB_his, Ni-NTA affinity gel was equilibrated with KPi buffer (50 mM, pH = 7.5) containing 25 mM imidazole. Crude enzyme preparation was added to the gel column (2 mL enzyme preparation per 1 mL affinity gel) and incubated on ice for 30 minutes. Non-specifically bound proteins were washed off with KPi buffer (50 mM, pH = 7.5) containing 25 mM, 50 mM and 100 mM imidazole. Desired enzymes were eluted with KPi buffer containing 500 mM imidazole. The volume of the elution fractions was reduced by ultrafiltration (Vivaspin® 15R centrifugal filter Units, 10 kDa nominal molecular weight limit, Sartorius) and desalted by size-exclusion chromatography (Econo-Pac 10DG desalting gel column, Bio-Rad). Purified NfsB_his was stored in KPi buffer (50 mM, pH = 7.5) containing 20% (v/v) glycerol at –20 °C. The purity of the protein was confirmed by SDS-PAGE, performed in 15% resolving gel (Fig. S1). The concentration of the protein was determined by measuring the UV absorption at 280 nm (NanoVue, GE Healthcare; extinction coefficient 22460 $M^{-1} \cdot cm^{-1}$, molecular weight 26.672 kDa).

```
ATGGATATCATTTCTGTCGCCTTAAAGCGTCATTCCACTAAGGCATTTGATGCCAGCAAAAAACTTACCCCGGAACAGGCCGAGCAGAT
CAAAACGCTACTGCAATACAGCCCATCCAGCACCAACTCCCAGCCGTGGCATTTTATTGTTGCCAGCACGGAAGAAGGTAAAGCGCGT
GTTGCCAAATCCGCTGCCGGTAATTACGTGTTCAACGAGCGTAAAATGCTTGATGCCTCGCACGTCGTGGTGTTCTGTGCAAAAACCGC
GATGGACGATGTCTGGCTGAAGCTGGTTGTTGACCAGGAAGATGCCGATGGCCGCTTTGCCACGCCGGAAGCGAAAGCCGCGAACGA
TAAAGGTCGCAAGTTCTTCGCTGATATGCACCGTAAAGATCTGCATGATGATGCAGAGTGGATGGCAAAACAGGTTTATCTCAACGTCG
GTAACTTCCTGCTCGGCGTGGCGGCTCTGGGTCTGGACGCGGTACCCATCGAAGGTTTTGACGCCGCCATCCTCGATGCAGAATTTGG
TCTGAAAGAGAAAGGCTACACCAGTCTGGTGGTTGTTCCGGTAGGTCATCACAGCGTTGAAGATTTTAACGCTACGCTGCCGAAATCTC
GTCTGCCGCAAAACATCACCTTAACCGAAGTGTAA
```

Fig. S1. DNA sequence of NfsB.

```
MGHHHHHHHHHHSSGHIDDDDKHMDIISVALKRHSTKAFDASKKLTPEQAEQIKTLLQYSPSSTNSQPWHFIVASTEEGKARVAKSAAGNYVF
NERKMLDASHVVVFCAKTAMDDVWLKLVVDQEDADGRFATPEAKAANDKGRKFFADMHRKDLHDDAEWMAKQVYLNVGNFLLGVAALGLD
AVPIEGFDAAILDAEFGLKEKGYTSLVVVPVGHHSVEDFNATLPKSRLPQNITLTEV
```

Fig. S2. Protein sequence of NfsB_his.

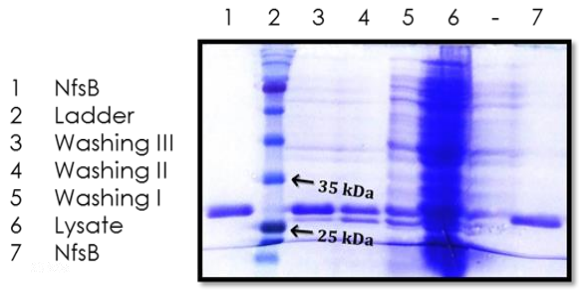


Fig. S3. SDS-PAGE for NfsB_his in 15% acrylamide resolving gel.

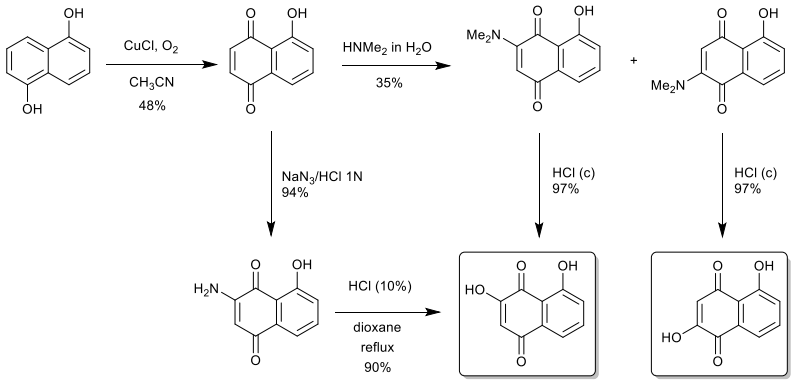
For GDH containing plasmid, transformation and expression were performed following the same protocol as for NfsB_his. The harvested cells were resuspended in lysis buffer (50 mM HEPES, 10% Glycerol, pH = 7.5; 5 mL per harvested cells of 1 L medium). The cells were disrupted by sonication (6 times for 10 s each, Vibra-Cell Processors, model no. VCX500, Sonics), followed by centrifugation (30 minutes, 12000 g, 4 °C). Glycerol (20% v/v) was added, and the crude enzyme preparation (GDH lysate) was frozen at –20 °C.

Activity measurements

NfsB_his: 200 µL of a 1 mM solution of either NADPH and 10 µL of a 2 mM lawsone solution were added to 780 µL of KPi buffer (50 mM, pH = 7.5). The reaction was started by addition of 10 µL of a solution of purified NfsB_his and gentle mixing. The decline of the NAD(P)H concentration was monitored photometrically over time (λ = 340 nm; 25 °C; path length: 1 cm). The concentration of the enzyme solution was chosen to not exceed an initial absorbance decay of 0.4 min^{-1} for both cofactors.[1]

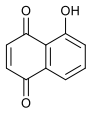
GDH: GDH was assayed as described elsewhere.[2]

III. Substrate synthesis



Scheme S1. Preparation of 5-hydroxynaphthalene-1,4-dione (**juglone, 8**); 2,5-dihydroxynaphthalene-1,4-dione (**2-hydroxy-juglone, 10**) and 2,8-dihydroxynaphthalene-1,4-dione (**3-hydroxy-juglone, 11**).[3,4]

5-hydroxynaphthalene-1,4-dione (8)



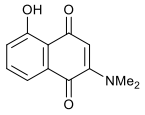
$C_{10}H_6O_3$: 174.2 g.mol^{-1}

A suspension of powdered CuCl (2 g, 0.20 mol) in acetonitrile (170 mL) was placed in a 250 mL three-neck flask with a gas inlet tube and a strong current of air was bubbled through it. The reaction flask was covered with aluminium foil protect from light. To this vigorously stirred solution, 1,5-dihydroxynaphthalene (5 g, 0.31 mol) was added portion wise over 30 minutes at room temperature. The resultant mixture was stirred overnight (16 hours). The mixture was filtered, washed with acetonitrile, and the solvent removed under reduced pressure. Finally, the crude product was purified in a Soxhlet extractor with *n*-hexane as solvent to afford juglone (2.626 g, 48%) as orange-red solid.[3]

R_f = 0.32 ($CHCl_3$/MeOH 90/10).

^{1}H NMR ($CDCl_3$): δ (ppm) = 6.95 (s, 2H, H-2 and H-3), 7.29 (dd, ^3J = 7.4 Hz, ^4J = 2.2 Hz, 1H, CH_{ar}), 7.58 – 7.69 (m, 2H, CH_{ar}), 11.91 (s, 1H, OH-5).

2-(dimethylamino)-5-hydroxynaphthalene-1,4-dione



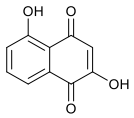
$C_{12}H_{11}NO_3$: 217.2 g.mol^{-1}

A suspension of juglone (2 g, 0.1 mol) in water (100 mL) was stirred vigorously and to this aqueous dimethylamine solution (3 mL, 40% w/v) was added. A yellow solution turned red immediately and then dark brown. After 2 hours, the suspended solid was filtered, washed and dried. This afforded a red crystalline solid (870 mg, 35%). The compound is used directly for the next reaction without any further purification[4].

R_f = 0.32 ($CHCl_3$/MeOH 90/10).

^{1}H NMR (CDC*l*$_3$): δ (ppm) = 3.24 (s, 6H, $N(CH_3)_2$), 5.71 (s, 1H, H-3), 7.19 (dd, ^3J = 7.9 Hz, ^4J = 1.6 Hz, 1H, CH_{ar}), 7.43 – 7.50 (m, 2H, CH_{ar}), 12.95 (s, 1H, OH-5).

2,5-dihydroxynaphthalene-1,4-dione (10)



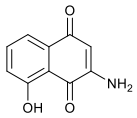
$C_{10}H_6O_4$: 190.1 g.mol^{-1}

To the solution of 2-dimethylaminojuglone (500 mg, 2.30 mmol) in dioxane (100 mL), conc. HCl (0.5 mL) was added. The mixture was refluxed for 2 hours and then cool down to room temperature. The solvent was removed under reduced pressure and the residue was dissolved in ethyl acetate (20 mL). The organic layer is washed with H_2O (x2) and then with brine. The organic layer was dried over $MgSO_4$, filtered and the solvent was removed under reduced pressure to afford brown solid (394 mg, 90%).[4]

R_f = 0.32 ($CHCl_3$/MeoH 90/10).

^{1}H NMR (CDC*l*$_3$): δ (ppm) = 6.31 (s, 1H, H-3), 7.33 (dd, ^3J = 8.4 Hz, ^4J = 1.2 Hz, 1H, H-7), 7.45 (bs, 1H, OH-2), 7.55 – 7.64 (m, 1H, H-8), 7.69 (dd, ^3J = 7.4 Hz, ^4J = 1.2 Hz, 1H, H-6), 12.32 (s, 1H, OH-5).

3-amino-8-hydroxy-naphthalene-1,4-dione

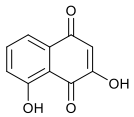


$C_{10}H_7NO_3$: 189.17 g.mol^{-1}

To a stirred solution of juglone (100 mg, 0.57 mmol) in 4.8 mL of methanol under an argon atmosphere was added a solution of sodium azide (220 mg, 3.38 mmol) in 1.6 mL of water and acidified to pH 4 (with 1 N HCl). The reaction was heated to at 30-35 °C for 22 hours, and then the mixture was cooled, and extracted with ethyl acetate. The organic layer was washed successively with water and brine, dried over anhydrous Na_2SO_4, and concentrated. The residue was purified by column chromatography on silica gel (ethyl acetate/hexane) to give the titled compound (101 mg, 94%) as red crystal; 1H NMR data were consistent with that reported in the literature.[4]

^{1}H NMR (acetone-*d*$_6$): δ (ppm) = 5.93 (s, 1H, H-3), 6.55 (s, 2H, NH_2), 7.17 (d, ^3J = 7.4 Hz,1H, H-6), 7.51 (d, ^3J = 7.6 Hz, 1H, H-7), 7.72 (t, ^3J = 7.3 Hz, 1H, H-6), 11.60 (s, 1H, OH-8)

3,8-dihydroxy- naphthalene-1,4-dione (11)

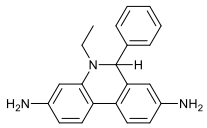


$C_{10}H_6O_4$: 190.15 g.mol^{-1}

A suspension of compound 3-Amino-8-hydroxy-naphthalene-1,4-dione (2.4 mmol) in conc. HCl (165 mL) was heated under reflux for 24 hours. The mixture was poured into cold water and extracted with CH_2Cl_2. The organic layer was dried over anhydrous Na_2SO_4, filtered, and the solvent evaporated. The residue was purified by column chromatography on silica gel (ethyl acetate/hexane, 2:8) to give the titled compound (442 mg, 97%) as orange crystal.[4]

^{1}H NMR (acetone-d_6): δ (ppm) = 6.22 (s, 1H, H-3), 7.27 (dd, ^3J = 8.5 Hz, ^4J = 1.1 Hz, 1 H, H-5), 7.56 (dd, ^3J = 7.4 Hz, ^4J = 1.0 Hz, 1 H, H-7), 7.77 (dd, ^3J = 8.4 Hz, ^3J = 7.5 Hz, 1 H, H-6), 11.41 (s, 1 H, OH-8).

hydroethidine (HE, 3)

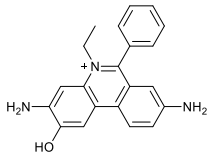


$C_{21}H_{21}N_3$: 315.42 g.mol^{-1}

200 mg (0.50 mmol) of ethidium bromide is dissolved in 6 mL absolute methanol, 88 mg $NaBH_4$ is added at 0 °C. The reaction is carried out under argon, in the absence of light and at 0 °C for 24 hours. The solvent is evaporated under reduced pressure, and 6 ml of a 3% NaOH solution is added. The solution is extracted 6 times with 20 mL ether. The ether fractions are pooled and dried over anhydrous Na_2SO_4. Ether is evaporated under reduced pressure, and a light brown crystallized residue is obtained which is recrystallized from ether.[5]

^{1}H NMR (dmso-d_6): δ (ppm) = 1.07 (t, ^3J = 7.3 Hz, 3H, C$\underline{H}_3$), 3.10 (q, ^3J = 7.1 Hz, 1H, C$\underline{H}_2$), 3.38 (q, ^3J = 7.0 Hz, 1H, CH_2), 4.86 (d, ^2J = 18.3 Hz, 4H, NH_2), 5.30 (s, 1H, H-5), 5.95 (d, ^4J = 2.2 Hz, 1H, H-2 and H-4), 6.31 (d, ^4J = 2.3 Hz, 1H, H-7), 6.44 (dd, ^3J = 8.4, ^4J = 2.4 Hz, 1H, H-9), 7.07 – 7.23 (m, 5H, Φ-5), 7.25 (d, ^3J = 8.7 Hz, 1H, H-1), 7.28 (d, ^3J = 8.5 Hz, 1H, H-10).

2-hydroxyethidium (2-OH-E$^+$, 5)



$C_{21}H_{20}N_3O^+$: 330.41 g.mol^{-1}

A solution of Fremy's salt (19.6 mg, 0.073 mmol) dissolved in a phosphate buffer (50 mL, pH 7.4, 0.1 M) was slowly added to a solution of hydroethidine (10 mg, 0.032 mmol) in 500 ml of phosphate buffer (0.1 M, pH 7.4) while stirring in the dark. The reaction mixture was continuously stirred for 30 minutes. The reaction mixture was then filtered and extracted with a mixture of chloroform and methanol (ratio 2:1, 100 ml x 5). The product was purified using a silica gel column (prewashed with chloroform) with methanol as an eluent and the second band was collected. After removing the solvent in vacuo, a red-orange coloured product was obtained.[6] The product was further purified using C_{18} reverse phase cartridge (Waters, *sep-pak*) and analyzed by NMR.[7]

^{1}H NMR (dmso-d_6): δ (ppm) = 1.40 (t, ^3J = 7.2 Hz, 3H, CH_3), 4.46 (q, ^3J = 7.1 Hz, 2H, CH_2), 6.25 (d, ^4J = 2.4 Hz, 1H, H-7), 7.44 (s, 1H, H-4), 7.49 (dd, ^3J = 9.1 Hz, ^4J = 2.4 Hz, H-9), 7.65 – 7.69 (m, 2H, Φ-2), 7.72 – 7.77 (m, 3H, Φ-3), 7.93 (s, 1H, H-1), 8.32 (d, ^3J = 9.2 Hz, 1H, H-10).

IV. Optimization of superoxide generation system

Quantification of superoxide by cytochrome c reduction assay

Amount of superoxide generated by Q/QR (quinone/quinone reductase) system was estimated using SOD (superoxide dismutase)-inhibitable cytochrome c reduction assay.[8] 20 µL of a 6.9 mM ferricytochrome c solution, 10 µL of a 20 mM lawsone solution (in 2-propanol) and 10 µL of NADPH solution were added to 950 µL potassium phosphate buffer (50 mM, pH = 7.8). The reaction was started by addition of 10 µL of NfsB-his solution of 0.2 U/mL and gentle mixing. The increase in ferrocytochrome c concentration was monitored photometrically over time (λ = 550 nm; 25 °C; path length: 1 cm). The same reaction was performed in the presence of SOD, i.e., 10 µL of SOD (200 U) was added to the buffer before adding other components. Another reaction without lawsone served as a control. Subtracting the absorbance at 550 nm due to reaction without SOD by reaction with SOD gives the specific absorbance of ferricytochrome c reduction by superoxide (Fig. S4).

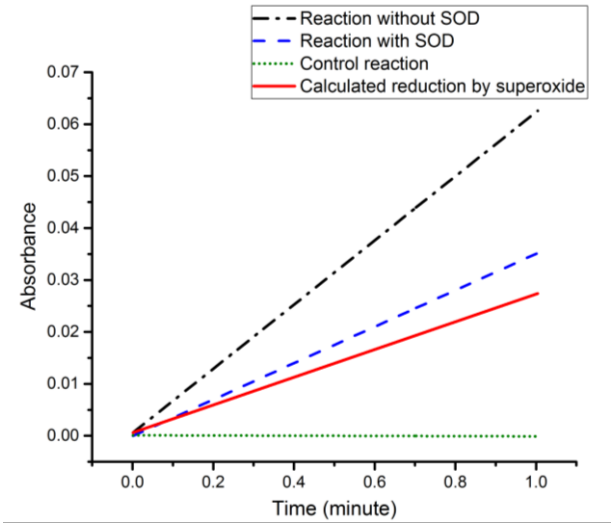
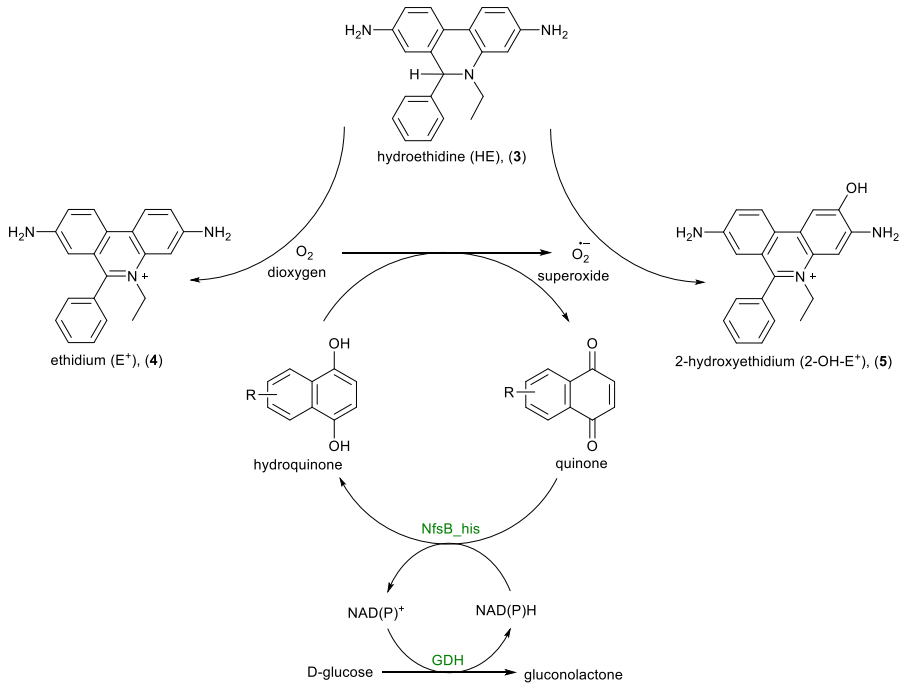


Fig. S4. SOD-inhibitable cytochrome c reduction assay for lawsone/NfsB system. Dash-dotted-black and dashed-blue lines represent the cytochrome c reduction by lawsone/NfsB system in absence and presence of SOD respectively. Solid-red line represents superoxide specific cytochrome c reduction while dotted-green line represents the control reaction in the absence of lawsone.

The rate of superoxide generation equals the rate of ferricytochrome c reduction which is calculated using Beer-Lambert's law.[9] Molar extinction coefficient for ferricytochrome c reduction at 550 nm is 2.1×10^4 $M^{-1}cm^{-1}$ which can be obtained by subtracting the molar extinction coefficient of ferricytochrome c from that of ferrocytochrome c reduction. The calculated rate of superoxide generation from lawsone/NfsB system was found to be 1.26 µM/min for the given conditions.

Detection of superoxide

Experiments, as shown in Scheme S2, were performed under oxygen, protected from light at room temperature. All the reactions were performed in 50 mM potassium phosphate buffer (pH 7.8) with 100 µM diethylenetriaminepentaacetic acid (DTPA), and dimethyl sulfoxide (DMSO) was used as a co-solvent for HE and quinones. Detection of superoxide was confirmed by comparing with a reference sample of 2-OH-E^+ using NMR spectroscopy (Fig. S5). The reaction mixtures were purified using C_{18} reverse phase cartridge (Waters, *sep-pak*) in water/methanol solvent system following a known procedure.[7] The fractions containing methanol were evaporated and dried under vacuum. The NMR spectra were recorded in DMSO-d_6.



Scheme S2. Generation of superoxide by quinone and quinone reductase based (Q/QR) system under oxygen and its detection by hydroethidine (HE) which reacts with superoxide forming 2-hydroxyethidium (2-OH-E$^+$) as a marker product for superoxide. D-Glucose and glucose dehydrogenase (GDH) system was used for regeneration of NADPH ensuring its supply to NfsB_his. The system continues to produce superoxide until D-glucose is consumed or the environment became anaerobic.

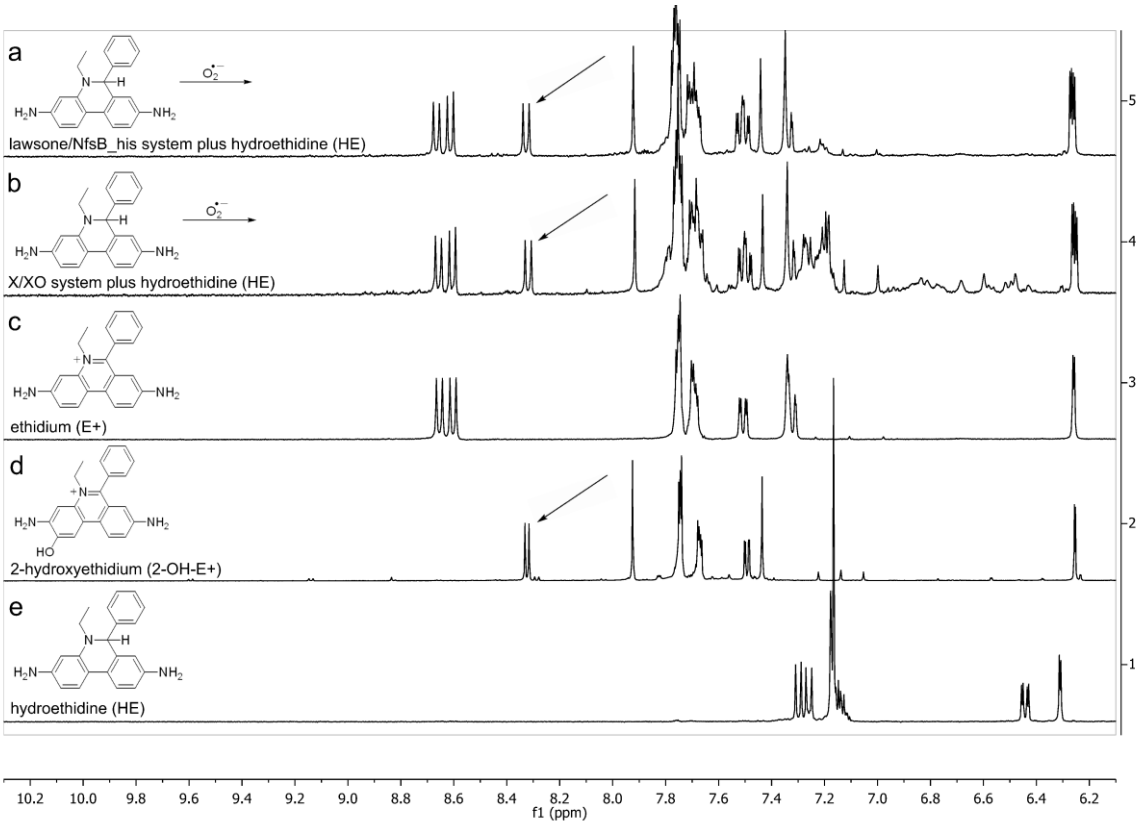


Fig. S5. Comparison of ^{1}H-NMR spectra (recorded in DMSO-d_6) of HE oxidation by X/XO and Q/QR system. The black arrows highlight a characteristic peak of 2-hydroxyethidium at 8.32 ppm.

Quantification and optimization of Q/QR system

For further optimization, we employed HPLC for quantitative comparison of superoxide generation under varying conditions. Following method was developed to analyze 2-hydroxyethidium and ethidium in the samples —

Flow rate: 0.5 mL/min; Typical injection volume: 5 µL; Gradient: 37-47% Acetonitrile (with 0.1 % TFA) in water (with 0.1 % TFA) over 0-23 minutes; DAD/Reference: 470 nm (bandwidth = 4)/600 nm (bandwidth = 100), 370 nm (bandwidth = 4)/600 nm (bandwidth = 100); Column: Kromasil 100-5-C18 4.6 x 250 mm.

For quantification of 2-OH-E^+ and E^+ in the reaction mixture, external standard quantitation procedure was used in which both calibration and unknown samples were analyzed under the same conditions. The results (peak area measured using the data system) from the unknown sample are then related to those of a calibration sample using the calibration curve (Fig. S6). Response factor at 470 nm was calculated for both 2-OH-E^+ and E^+ taking standard amount using formulae:

$$Response\ Factor = \frac{Peak\ Area}{Standard\ Amount} = Analyte$$

$$Amount\ of\ Analyte = \frac{Peak\ Area}{Response\ Factor} = Sample\ Amount$$

The presence of unreacted hydroethidine (HE) was checked by analyzing the peak at 370 nm.

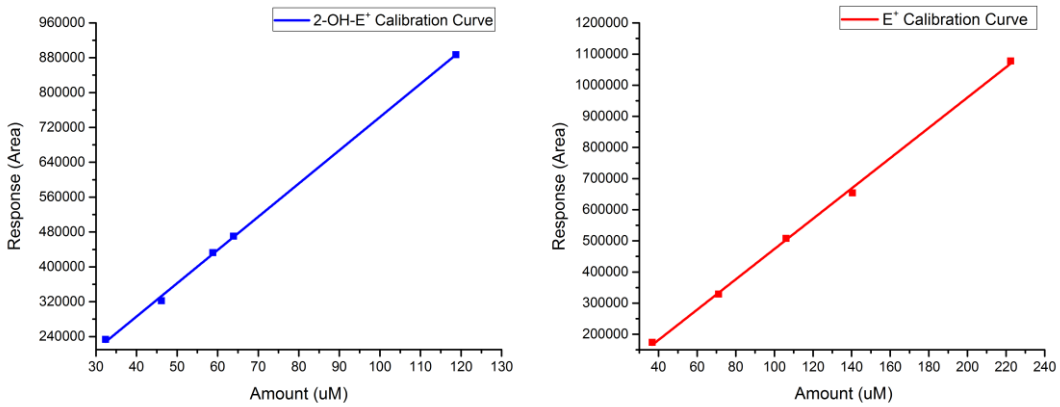


Fig. S6. Calibration curves for 2-hydroxyethidium (2-OH-E^+) and ethidium (E^+) at 470 nm.

5 µL of the sample from the reaction mixture was injected, and response areas corresponding to 2-OH-E^+ and E^+ were obtained using the data system. The concentration of 2-OH-E^+ and E^+ in the sample were calculated by respective response factor using the given formula. The percentage amount of 2-OH-E^+ was obtained using the formula:

$$\%[2\text{–}hydroxyethidium] = \frac{[2\text{–}hydroxyethidium]\ X\ 100}{[Ethidium] + [2\text{–}hydroxyethidium]}$$

The results were expressed as the ratio of [2-OH-E^+] to [E^+].

All the reactions were protected from light and performed at room temperature in 50 mM potassium phosphate buffer containing 100 µM DTPA under oxygen for 24 hours. Dimethyl sulfoxide (DMSO) was used as a co-solvent for dissolving HE and quinones in the reaction. The results from HPLC were expressed as the ratio which represents the relative percentage of 2-hydroxyethidium and ethidium in the reaction mixture calculated from the total concentration of 2-OH-E^+ and E^+ in the final reaction mixture. The data from each experiment represent the relative abundance of superoxide and other oxidants because HE is prone to oxidation under aerobic environment forming 2-OH-E^+ or E^+ on reaction with superoxide or other oxidants respectively. First, control experiments were run to estimate the basal level of superoxide which involved the incubation of HE in buffer under the same reaction conditions (Table S1, entry 1). The basal level of superoxide was diminished when deoxygenated buffer was used (Table S1, entry 3) or SOD was added (Table S1, entry

2) to the reaction. Also, the reactions were performed in HPLC grade water with (Table S1, entry 5 & 6) and without DTPA (Table S1, entry 4) which indicated the role of metal ions in the basal level of superoxide in the buffer. Another control experiment includes the incubation of lawsone with HE and NADPH regeneration system ($NADP^+$/glucose/GDH) in the absence of NfsB under the same conditions (Table S1, entry 7) which do not contribute to superoxide level as the ratio remains same as found at the basal level. Xanthine/Xanthine Oxidase (X/XO) system which is commonly used superoxide generation system served as positive control under the same conditions (Table S1, entry 8 & 9). All the control experiments are listed in Table S1.

Table S1. Control experiments during optimization of Q/QR system.[a]

Entry	Substrate[b]	Enzyme[c]	Reaction medium[d]	[2-OH-E^+]/[E^+]
1	-	-	buffer	32/68
2	-	SOD (20U)	buffer	06/94
3	-	-	deoxygenated buffer	07/93
4	-	-	HPLC grade water (HW)	18/82
5	-	-	HW + 100 µM DTPA	09/91
6	-	-	HW + 200 µM DTPA	05/95
7	lawsone (0.2)	-	buffer	33/67[e]
8	xanthine (5)	XO (0.25U)	buffer	85/15
9	xanthine (5)	XO (2.5U)	buffer	85/15[f]

[a] Each reaction (performed in dark at room temperature for 24 hours) contains HE (1 mg, 3.17 µmol, 1 equivalent) in a total volume of 6 mL. [b] equivalent amount. [c] unit activity. [d] 50 mM KPi, 100 µM DTPA, pH 7.8 is used as buffer. [e] The reaction consists of $NADP^+$, glucose and GDH (glucose dehydrogenase) as NADPH regeneration system. [f] The reaction is scaled up (5X) taking 5 milligrams of HE as the starting material.

Lawsone was used as the substrate for initial optimzation of the system. Further, various quinones were screened for superoxide generation under the optimized conditions. Out of them, menadione was found to be the most efficient in generating SO in the system. Therefore, we planned further optimization with menadione (Table S2).

Table S2. Optimization of Q/QR system with menadione as quinone.[a]

Entry	Menadione	NfsB_his (units)	pH	[2-OH-E^+]/[E^+]
1	0.2	0.25	7.8	63/37[b]
2	0.2	0.25	7.8	72/28
3	0.1	0.25	7.8	60/40
4	0.2	0.25	7.8	72/28
5	0.4	0.25	7.8	61/39
6	0.2	0.125	7.8	68/32
7	0.2	0.25	7.8	72/28
8	0.2	0.5	7.8	72/28
9	0.2	1	7.8	61/39
10	0.2	0.25	6.0	40/60
11	0.2	0.25	7.0	57/43
12	0.2	0.25	7.4	63/37
13	0.2	0.25	7.8	72/28
14	0.2	0.25	8.0	68/32

[a]] Each reaction (performed in dark at room temperature for 24 hours) contains: 50 mM KPi, 100 µM DTPA buffer (6 mL), HE (1 mg, 3.17 µmol, 1 equivalent), glucose (31.7 µmol) and GDH (5 U). Amount of menadione and glucose are shown in equivalence to HE. An equal amount of $NADP^+$ and lawsone are used. [b] Instead of $NADP^+$, NAD^+ is used.

At first, both $NADP^+$ and NAD^+ were tested in the system. The results (Table S2, entry 1 and 2) indicated the preference for $NADP^+$, similar to lawsone. Change in the amount of menadione also indicated the same trend as that of lawsone (Table S2, entry 3, 4 & 5). With the change in units of enzyme used in the system, best results were obtained with 0.25 U and 0.50 U (Table S2, entry 7 and 8). Use of 0.125 U and 1 U of NfsB_his, result in lowering of SO generated in the

system (Table S2, entry 6 and 9). Therefore, we continued with 0.25 U for further optimization of the Q/QR system. Further, we observed that minor changes in pH ranging from 6 to 8 resulted in a change in the ratio of 2-OH-E^+/E^+ (Table S2, entry 10-14). The maximum efficiency of the system, to generate SO, was found to be at pH 7.8, which could be due to the stability of SO radical anion.

Kinetic measurements using fluorescence spectrophotometry

HE was incubated with menadione/NfsB, as well as X/XO system under optimized conditions (refer section ***Quantification and optimization of Q/QR system***). The reaction mixtures were excited at 350, 396 nm and emission were recorded at 395, 579 nm for HE consumption and 2-OH-E^+ formation, respectively.[7,10] The raw data obtained from the kinetic measurements were analyzed and fitted using exponential *ExpDec1* function in OriginPro. The extracted graphs are shown in Fig. S7. Spectral scanning was also performed by exciting at 396 nm and recording emission over 406 nm to 700 nm. The raw data obtained from spectral scanning were analyzed and fitted using polynomial *poly* function in OriginPro. The extracted graphs are shown in Fig. S8.

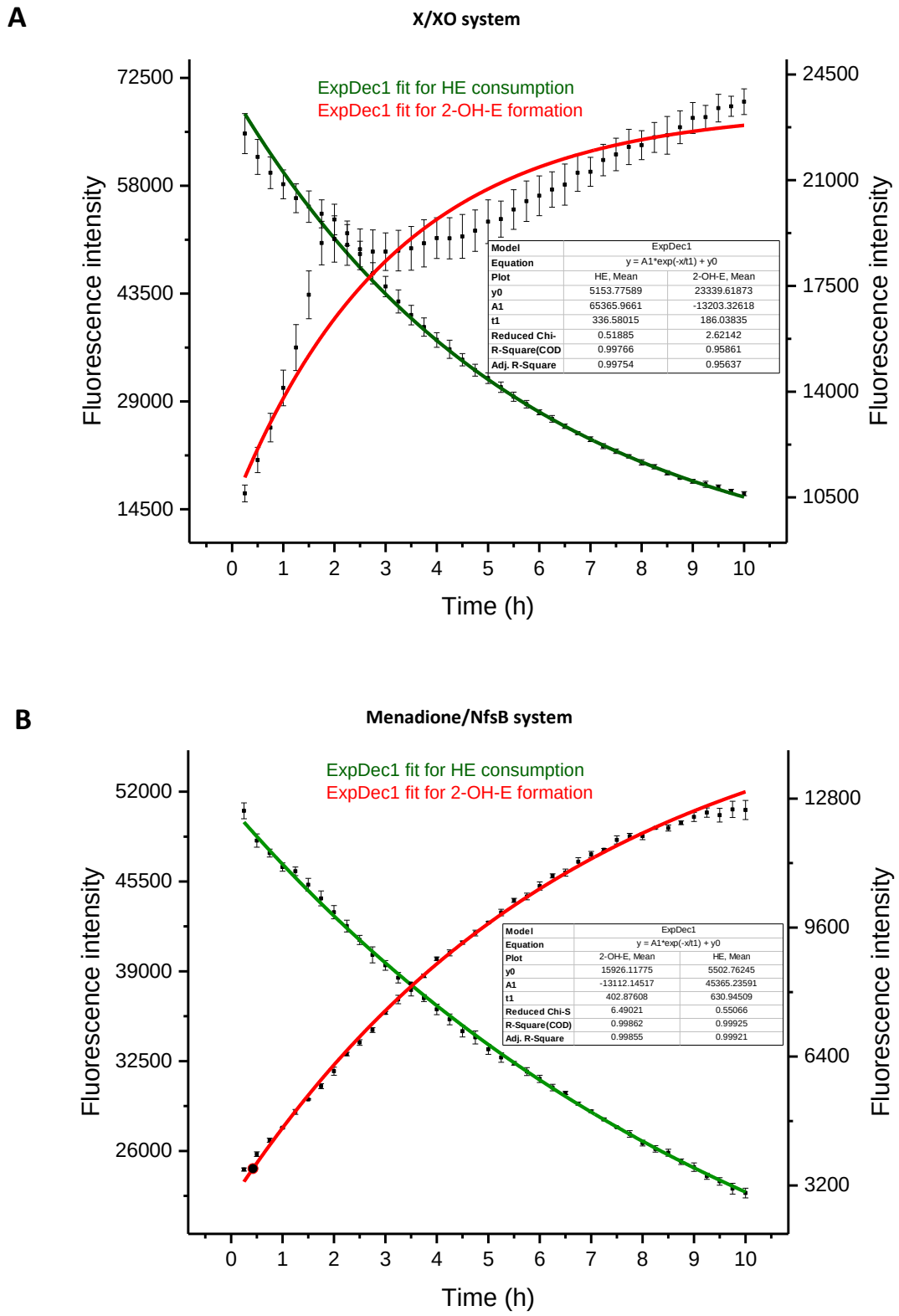


Fig. S7. Kinetics of HE oxidation by superoxide in **(A)** X/XO system, and **(A)** Menadione/NfsB system.

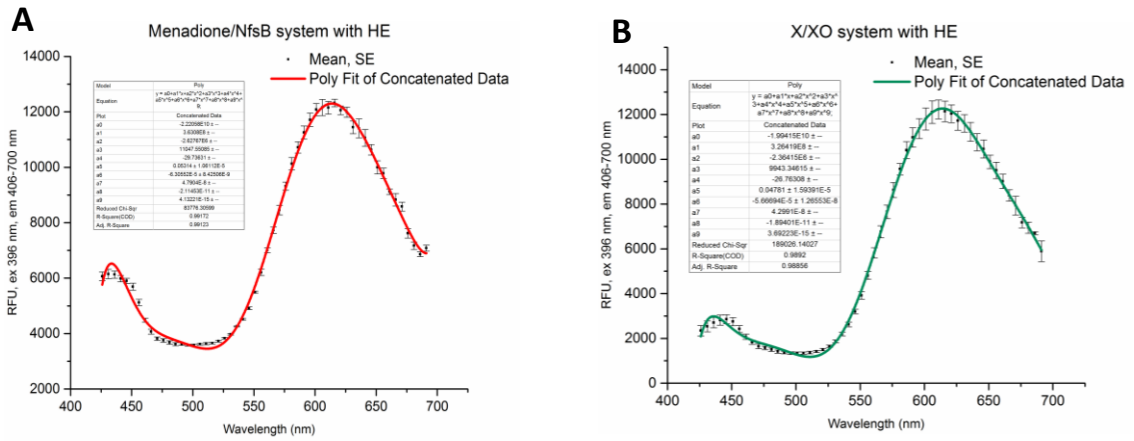


Fig. S8. Spectral scanning of HE oxidation by superoxide in **(A)** Menadione/NfsB system, and **(B)** X/XO system.

V. NMR Spectra

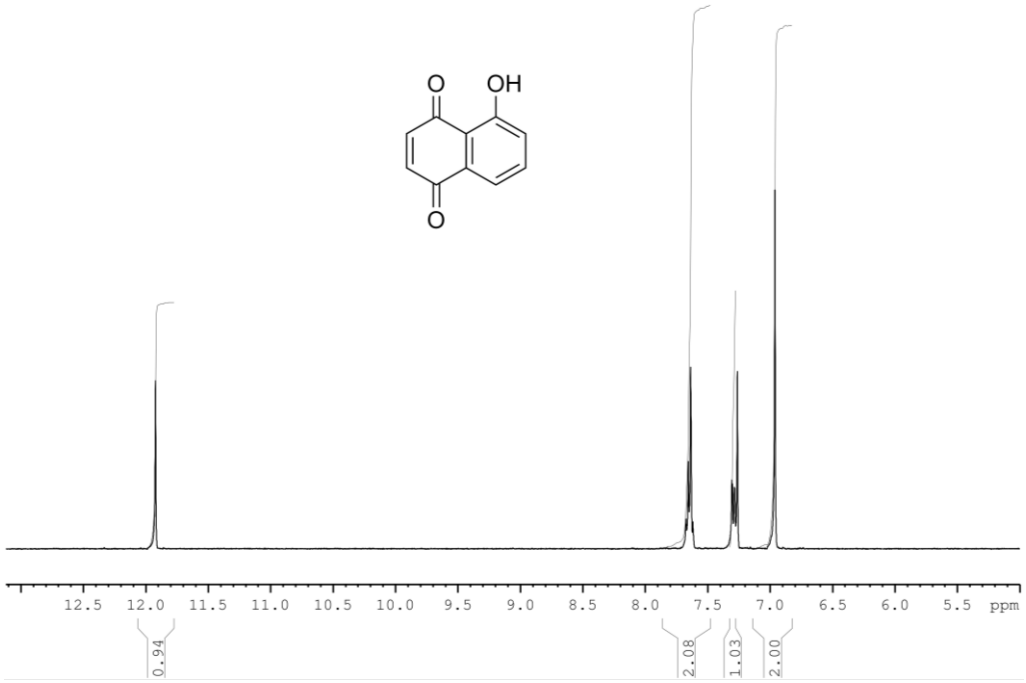


Fig. S9. ^{1}H-NMR of juglone in $CDCl_3$.

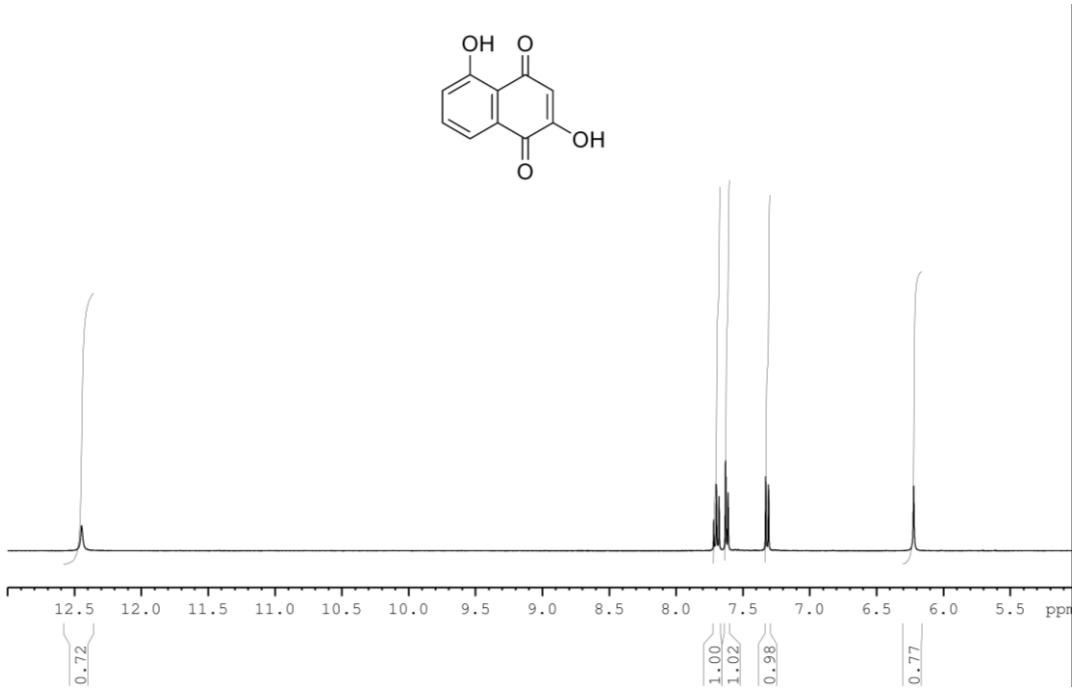


Fig. S10. ^{1}H-NMR of 2-hydroxy-juglone in $CDCl_3$.

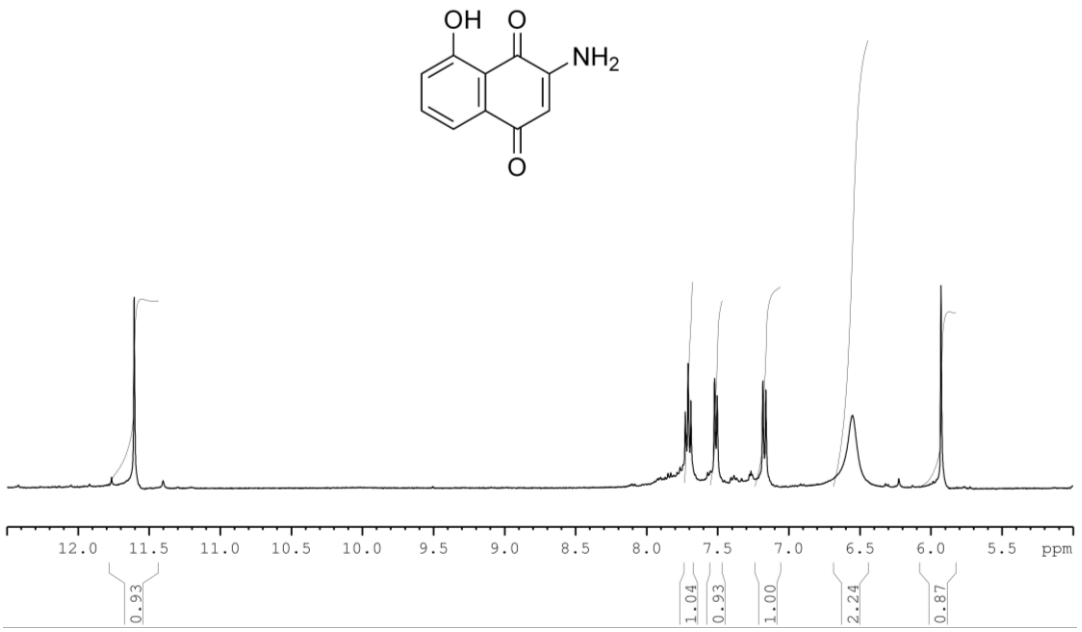


Fig. S11. ^{1}H-NMR of 3-amino-8-hydroxy-naphthalene-1,4-dione in acetone-d_6.

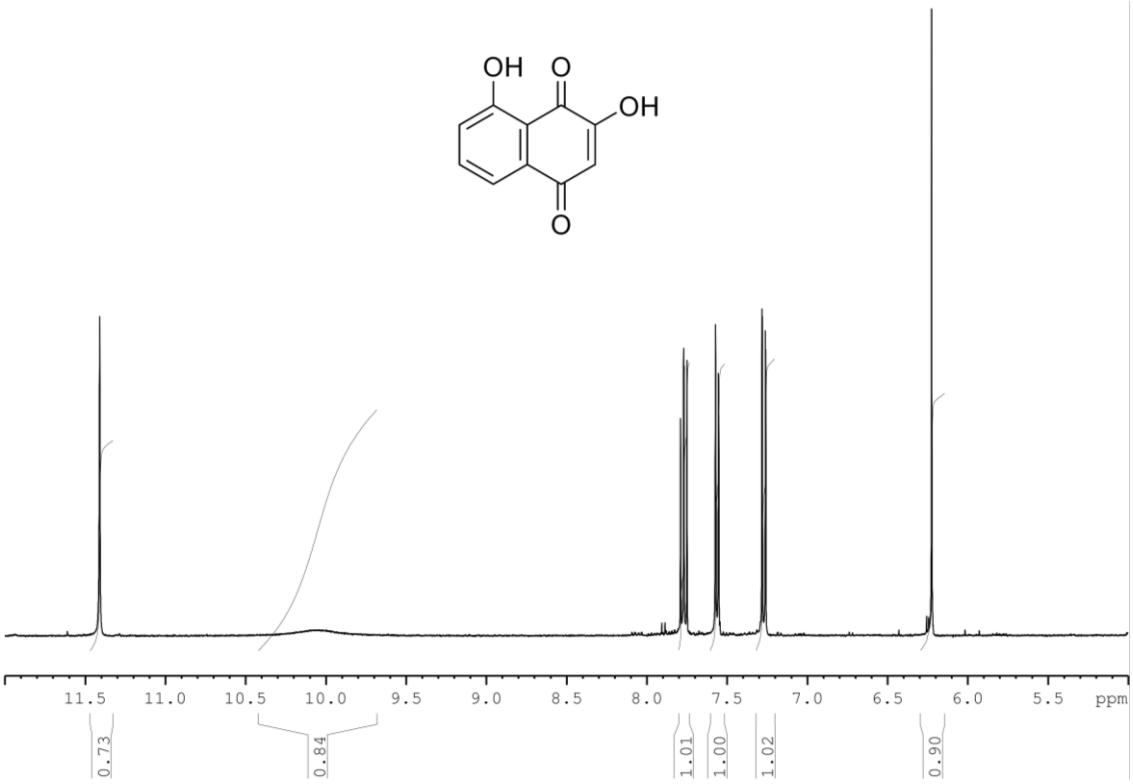


Fig. S12. ^{1}H-NMR of 3-hydroxy-Juglone in acetone-d_6.

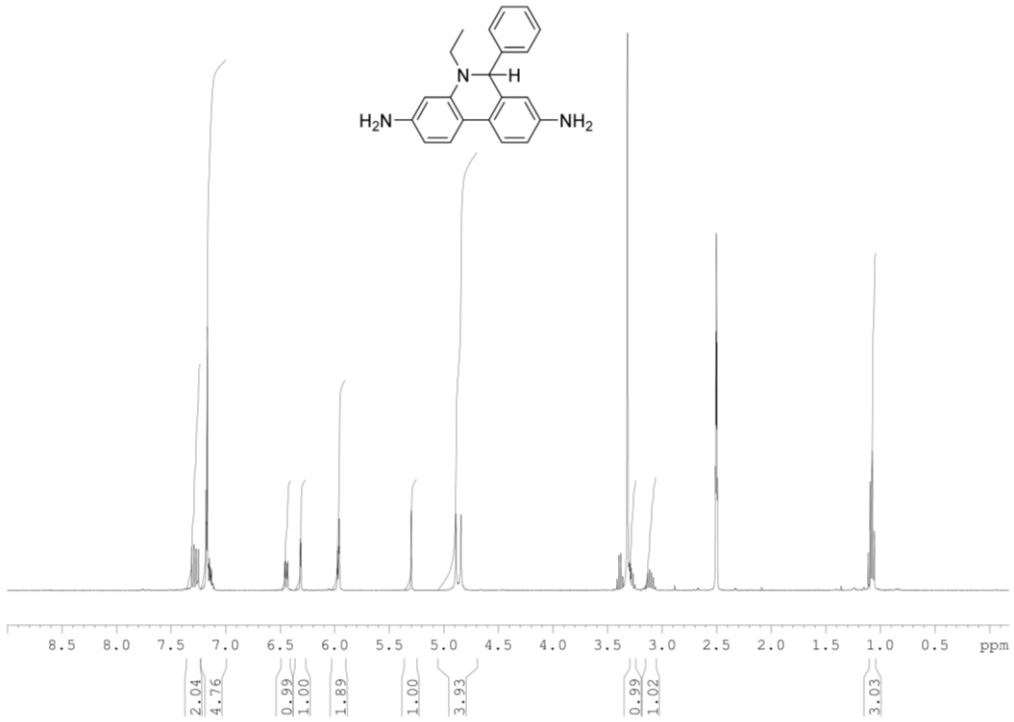


Fig. S13. ^{1}H-NMR of hydroethidine (HE) in DMSO-d_6.

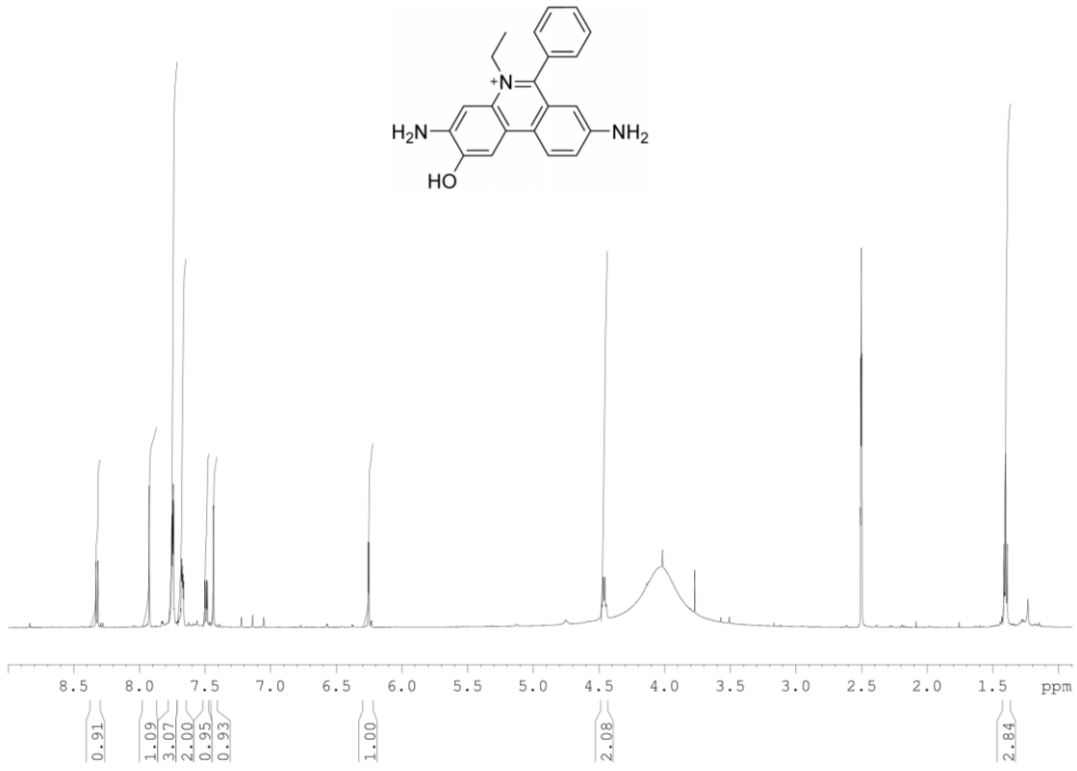


Fig. S14. ^{1}H-NMR of 2-hydroxyethidium (2-OH-E^{+}) in DMSO-d_6.

VI. References

[1] J. Haas, M. A. Schätzle, S. M. Husain, J. Schulz-Fincke, M. Jung, W. Hummel, M. Müller, S. Lüdeke, *Chem. Commun.* **2016**, *52*, 5198–5201.

[2] M. A. Schätzle, S. Flemming, S. M. Husain, M. Richter, S. Günther, M. Müller, *Angew. Chemie Int. Ed.* **2012**, *51*, 2643–2646.

[3] L. F. Tietze, C. Güntner, K. M. Gericke, I. Schuberth, G. Bunkoczi, *European J. Org. Chem.* **2005**, 2459–2467.

[4] E. L. Bonifazi, C. Ríos-Luci, L. G. León, G. Burton, J. M. Padrón, R. I. Misico, *Bioorg. Med. Chem.* **2010**, *18*, 2621–2630.

[5] J. Loccufier, E. Schacht, *Tetrahedron* **1989**, *45*, 3385–3396.

[6] J. Zielonka, H. Zhao, Y. Xu, B. Kalyanaraman, *Free Radic. Biol. Med.* **2005**, *39*, 853–863.

[7] J. Zielonka, J. Vasquez-Vivar, B. Kalyanaraman, *Nat. Protoc.* **2008**, *3*, 8–21.

[8] K. R. Messner, J. A. Imlay, *Methods Enzymol.* **2002**, *349*, 354–361.

[9] H. Zhu, *React. Oxyg. Species* **2016**, *1*, 65–80.

[10] K. M. Robinson, M. S. Janes, M. Pehar, J. S. Monette, M. F. Ross, T. M. Hagen, M. P. Murphy, J. S. Beckman, *Proc. Natl. Acad. Sci.* **2006**, *103*, 15038–15043.