Supplementary Information for:-

Fragmentation of the quinoxaline *N*-oxide bond to the •OH radical upon one-electron bioreduction

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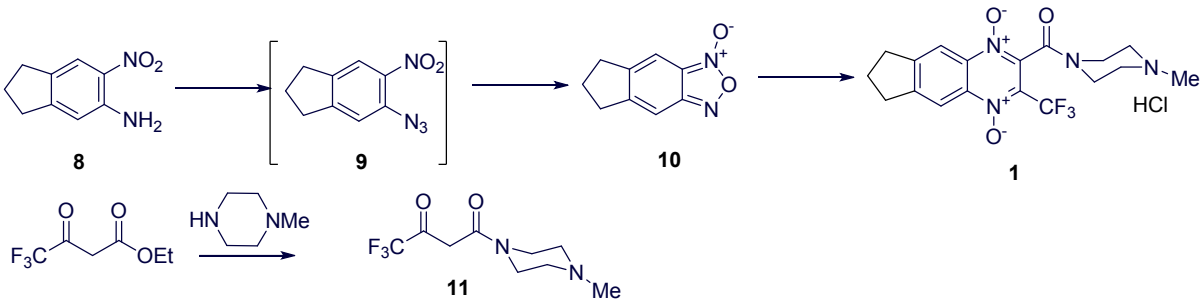
A. Synthesis of quinoxaline 1,4-dioxide compounds, 1 and 2.

Analyses were carried out in the Campbell Microanalytical Laboratory, University of Otago, Dunedin, NZ. All final products were analysed by reverse-phase HPLC, (Altima C18 5 μm column, 150 × 3.2 mm; Alltech Associated, Inc., Deerfield, IL) using an Agilent HP1100 equipped with a diode-array detector. Mobile phases were gradients of 80% acetonitrile/20% H_2O (v/v) in 45 mM ammonium formate at pH 3.5 and 0.5 mL/min. Final compound purity was determined by monitoring at 330 ± 50 nM and was >95%. Melting points were determined on an Electrothermal 2300 Melting Point Apparatus. NMR spectra were obtained on a Bruker Avance 400 spectrometer at 400 MHz for ^1H and 100 MHz for ^{13}C spectra. Spectra were obtained in $CDCl_3$ unless otherwise specified, and were referenced to Me_4Si. Chemical shifts and coupling constants were recorded in units of ppm and Hz, respectively. Low resolution mass spectra were gathered by direct injection of methanolic solutions into a Surveyor MSQ mass spectrometer using an atmospheric pressure chemical ionization (APCI) mode with a corona voltage of 50 V and a source temperature of 400 °C. High resolution mass spectra (HRMS) were measured on a Bruker microTOF-QII Hybrid Quadrupole Time of Flight (TOF-Q) mass spectrometer interfaced

with either an Electrospray Ionization (ESI) or Atmospheric Pressure Chemical Ionization (APCI) probe allowing positive or negative ions detection. Solutions in organic solvents were dried with anhydrous $MgSO_4$. Solvents were evaporated under reduced pressure on a rotary evaporator. Thin-layer chromatography was carried out on aluminium-backed silica gel plates (Merck 60 F_{254}) with visualization of components by UV light (254 nm) or exposure to I_2. Column chromatography was carried out on silica gel (Merck 230–400 mesh). DCM refers to dichloromethane, EtOAc refers to ethyl acetate, EtOH refers to ethanol, MeOH refers to methanol, pet. ether refers to petroleum ether boiling fraction 40-60 °C.

2-(4-Methylpiperazine-1-carbonyl)-3-(trifluoromethyl)-7,8-dihydro-6*H*-cyclopenta[*g*]quinoxaline 1,4-Dioxide (1).

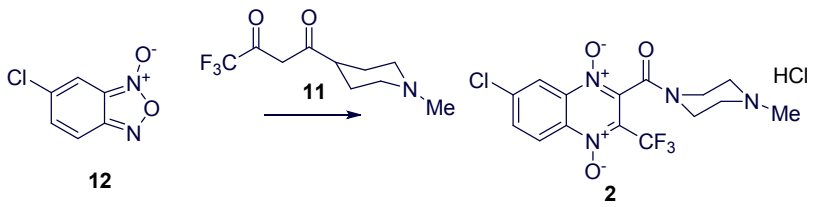


6,7-Dihydro-5H-indeno[5,6-c][1,2,5]oxadiazole 1-Oxide (10). A solution of $NaNO_2$ (0.95 g, 13.7 mmol) in water (10 mL) was added drop wise to a stirred suspension of 6-nitro-2,3-dihydro-1*H*-inden-5-amine[1] (**8**) in conc HCl (17 mL) and water (50 mL) at 5 °C. The mixture was stirred at 5 °C for 30 min. This mixture was added to a stirred solution of NaN_3 (1.21 g, 20.2 mmol) and NaOAc (13.3 g, 162 mmol) in water (130 mL) and the mixture was stirred and allowed to warm to 20 °C over 3 h. The resulting precipitate was filtered, washed with water (20 mL) and air dried to give the crude 5-azido-6-nitro-2,3-dihydro-1*H*-indene (**9**) (1.51 g, 92%) as a yellow amorphous powder: ^{1}H NMR δ 7.79 (s, 1H), 7.16 (s, 1H), 2.03–3.02 (m, 4H), 2.17 (pent, J = 7.5 Hz, 2H). A solution of the azide **9** (1.51 g, 7.39 mmol) in toluene (10 mL) was added drop wise to toluene at reflux temperature and the solution stirred at reflux temperature for 3 h. The solvent was evaporated and the residue purified by chromatography, eluting with 50% EtOAc/pet. ether, to give 1-oxide **10** (1.24 g, 95%) as a yellow powder: mp (EtOAc/pet. ether) 102–104 °C; ^{1}H NMR δ 7.28 (br s, 1H), 7.10 (br s, 1H), 2.89–2.94 (m, 4H), 2.12 (pent, J = 7.4 Hz, 2H); ^{13}C NMR δ 154.1, 152.7, 149.3, 115.2, 110.8, 105,9, 32.6, 26.1 (2). Anal. calc. for $C_9H_8N_2O_2$: C, 61.36; H, 4.58; N, 15.90. Found: C, 61.53; H, 4.62; N, 15.65%.

4,4,4-Trifluoro-1-(4-methylpiperazin-1-yl)butane-1,3-dione (11). A mixture of ethyl 4,4,4-trifluoro-3-oxobutanoate (1.0 g, 5.44 mmol) and 1-methylpiperazine (0.54 g, 5.44 mmol) in dry toluene (5 mL) was stirred in a sealed tube at 110 ºC for 12 hr. The mixture was cooled to 20 °C and partitioned between EtOAc (50 mL) and water (50 mL). The aqueous layer was extracted with EtOAc (50 ml). The combined organic fraction was extracted with aqueous HCl (1 M, 2 × 25 mL). The combined aqueous extracts were cooled and the pH adjusted to pH 9 with aqueous NH_3 solution and then extracted with EtOAc (3 × 75 mL). The combined organic fraction was washed with brine (10 mL), dried and the solvent evaporated. The crude oil was purified by column chromatography, eluting with 5% MeOH/DCM, to give a mixture of keto- and enol amides **11** (811 mg, 63%) as a yellow oil: ^{1}H NMR δ 15.26 (s, 0.5H), 5.81 (s, 0.5H), 4.12 (s, 1H), 3.68–3.72 (m, 2H), 3.51–3.54 (m, 2H), 2.42–2.48 (m, 4H), 2.33 and 2.31 (2 s, 3H); MS *m/z* 239.2 (MH^+, 100%). HRMS calcd for $C_9H_{14}N_2O_2$ (MH^+) *m/z* 239.1002, found 239.1006 (1.5 ppm).

2-(4-Methylpiperazine-1-carbonyl)-3-(trifluoromethyl)-7,8-dihydro-6*H*-cyclopenta[*g*]quinoxaline 1,4-Dioxide (1). A mixture of 1-oxide **10** (390 mg, 2.22 mmol), ketoamide **11** (528 mg, 2.22 mmol), $CaCl_2$ (493 mg, 4.44 mmol) and K_2CO_3 (20 mg) in EtOH (20 mL) was stirred at 50 °C for 16 h in the dark. The reaction was cooled to 20 °C and the solvent evaporated. The residue was purified by column chromatography, eluting with a gradient (0–5%) of MeOH/DCM, to give quinoxaline dioxide **1** (412 mg, 47%) as a yellow powder: mp (MeOH/H_2O) 206–208 °C; ^{1}H NMR δ 8.41 (s, 1H), 8.39 (s, 1H), 3.80–3.91 (m, 2H), 3.39 (ddd, *J* = 13.1, 7.5, 3.3 Hz, 1H), 3.29 (ddd, *J* = 13.1, 6.4, 3.3 Hz, 1H), 3.17 (br t, *J* = 7.4 Hz, 4H), 2.63 (br dt, *J* = 11.1, 5.0 Hz, 1H), 2.56 (ddd, *J* = 11.4, 6.3, 3.3 Hz, 1H), 2.45 (ddd, *J* = 11.2, 6.4, 4.0 Hz, 1H), 2.31–2.38 (m, 4H), 2.27 (pent, *J* = 7.4 Hz, 2H); ^{13}C NMR δ 157.0, 153.5, 152.5, 137.9, 137.8, 135.4, 129.1 (q, *J* = 30.3 Hz), 119.3 (q, *J* = 275.1 Hz), 115.2, 115.1, 54.1, 54.0, 46.1 (2), 42.0, 33.3, 33.2, 25.8; MS 397.3 (MH^+, 100%). Anal. calcd for $C_{18}H_{19}F_3N_4O_3 \cdot ¼H_2O$: C, 53.93; H, 4.90; N, 13.98. Found: C, 53.96; H, 4.60; N, 13.80%. HPLC purity: 95.3%.

7-Chloro-2-(4-methylpiperazine-1-carbonyl)-3-(trifluoromethyl)quinoxaline 1,4-Dioxide (2).



A mixture of 5-chlorobenzo[c][1,2,5]oxadiazole 1-oxide (258 mg, 1.15 mmol), ketoamide **11** (360 mg, 1.15 mmol), $CaCl_2$ (255 mg, 2.3 mmol) and K_2CO_3 (20 mg) in dry EtOH (10 mL) was stirred at 45 °C for 2 days in the dark. The solvent was evaporated and the residue was purified by column chromatography, eluting with a gradient (0–5%) of MeOH/DCM, to give quinoxaline dioxide **2** (157 mg, 35%) as a yellow foam: ^{1}H NMR δ 8.59 (d, *J* = 2.2 Hz, 1H), 8.57 (d, *J* = 9.2 Hz, 1H), 7.87 (dd, *J* = 9.2, 2.2 Hz, 1H), 3.79–3.89 (m, 2H), 3.38 (ddd, *J* = 13.1, 7.2, 3.4 Hz, 1H), 3.30 (ddd, *J* = 13.1, 6.5, 3.4 Hz, 1H), 2.62 (dt, *J* = 11.7, 5.0 Hz, 1H), 2.55 (ddd, *J* = 11.4, 6.5, 3.4 Hz, 1H), 2.48 (dt, *J* = 11.0, 5.2 Hz, 1H), 2.32–2.40 (m, 4H); ^{13}C NMR δ 156.4, 141.6, 138.8, 137.1, 134.1, 130.2 (d, *J* = 35 Hz), 122.5, 120.3, 119.1 (d, *J* = 275 Hz), 54.6, 54.0, 53.6, 46.1, 42.1; MS 391.3 (MH^+, 100%), 393.3 (MH^+, 30%). Anal. calcd for $C_{15}H_{14}ClF_3N_4O_3$: C, 46.11: H, 3.61; N, 14.34. Found: C, 46.46; H, 3.65; N, 14.15%. HPLC purity: 98.5%.

Hay, M.P., Hicks, K.O., Pchalek, K., Lee, H.H, Blaser, A., Pruijn, F.B., Anderson, R.F., Shinde, S., Wilson, W.R., Denny, W.A. *J. Med. Chem.* **2008**, *51*, 6853–6865.

B. Pulse radiolysis data

(i) Formation of the radical anions of 1 and 2.

The one-electron reduction of **1** and **2** to their radical anions ($A^{\cdot-}$) was carried out by reaction with the $CO_2^{\cdot-}$ radical species, which is exclusively formed from the primary radicals of N_2O-saturated irradiated water with a yield of 0.68 μmol J^{-1}.[1]

$H_2O \text{ ^^^^}\rightarrow e^-_{aq} + {\cdot}OH + H^{\cdot} + H_2O_2 + H_2 + H_3O^+$
$e^-_{aq} + N_2O \rightarrow {\cdot}OH + OH^- + N_2$
${\cdot}OH / H^{\cdot} + HCOO^- \rightarrow CO_2^{\cdot-} + H_2O(H_2)$
$CO_2^{\cdot-} + A \rightarrow CO_2^{\cdot-} + A^{\cdot-}$

(ii) Measurement of one-electron reduction potentials, *E*(1) vs. NHE.

One electron reductions of compounds **1** and **2** (A) and benzylviologen (BV^{2+}) redox indicator, were carried out by scavenging the e^-_{aq} species produced on the pulse radiolysis (4 MeV electrons) of aqueous solutions containing *tert*-butanol (0.2 M) to scavenge the $^{\bullet}OH$ radical and H-atom. The establishment of redox equilibria for 3 mixtures BV^{2+} (0.05-0.10 mM) and A (0.1-0.3 mM) was observed at 600 nm where the one-electron reduced benzylviologen ($BV^{+\bullet}$) absorb and the radicals anions of **1** and **2** absorb minimally.

H_2O ^^^^→ e^-_{aq} + $^\bullet OH$ + $^\bullet H$ + H_2O_2 + H_2 + H_3O^+

$^\bullet OH$ / $^\bullet H$ + $(CH_3)_3COH$ → $^\bullet CH_2(CH_3)_2COH$ + $H_2O(H_2)$

e^-_{aq} + A / BV^{2+} → $A^{\bullet-}$ / $BV^{+\bullet}$

$BV^{+\bullet}$ + A $\overset{K}{\rightleftharpoons}$ BV^{2+} + $A^{\bullet-}$

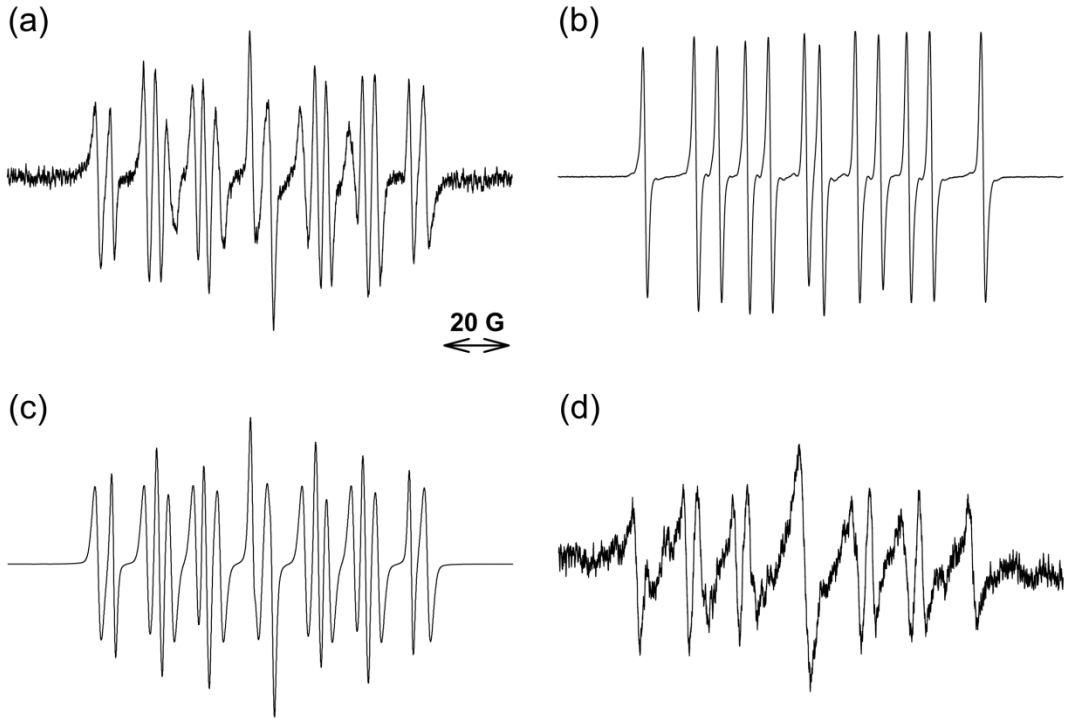
The average equilibrium constants, *K*, for **1** and **2** vs. BV^{2+} ($E(1)$ = –375 ± 10 mV) [2] of 0.183 ± 0.009 and 19.4 ± 3.8 were used to calculate ΔE values –44 ± 1 mV and 76 ± 5 mV using the Nernst equation. Correction for ionic strength effects (–6 mV) yielded $E(1)$ values for **1** and **2** of –430 ± 10 mV and –306 ± 10 mV respectively.

1. Q. G. Mulazzani, M. Venturi, M. Z. Hoffman and M. A. J. Rodgers, *J. Phys. Chem.*, 1986, **90**, 5347-5352.
2. J. W. Van Leeuwen, C. Van Dijk and C. Veeger, *Eur. J. Biochem.*, 1983, **135**, 601-607.

C. EPR Methodology and EPR control experiments

All chemicals were obtained from Sigma except the spin trap, 5-diethoxyphosphoryl-5-methyl-pyrroline 1-oxide (DEPMPO), which was obtained from Alexis International. All solutions were freshly prepared in Milli-Q water. Diethylene triamine pentaacetic acid (DETAPAC) (100 μM) was added to obliterate any adventitious ions in solution and also to negate formation of any ·OH radical arising from Fenton-type chemistry. Aqueous solutions of **1** & **2** (in phosphate buffer, pH 7.0), NADPH, were degassed separately with N_2 gas. Superoxide dismutase from bovine erythrocytes (SOD), Catalase, glucose-6-phosphate (G-6-P), glucose-6-phosphate dehydrogenase (G-6-P-D), sPOR P_{450} and the spin trap, were added into the sample vial under N_2 atmosphere. All additions were carried out on ice with NADPH being added last to initiate the enzyme incubation. An EPR flat quartz cell, designed for the variable temperature cavity, was used for all measurements. Solutions for EPR measurements were transferred under N_2 from the sample vial to the EPR cell, which had been flushed previously with N_2, and inserted into the EPR cavity. Utmost care was taken to keep the system deaerated at all times. The temperature of the cavity was raised to 310 K to start the enzyme incubation. EPR spectra were recorded at 20 mW power over a range of 100 G at a modulation width of 0.5 G, scan time of 2 minute, time constant of 0.03 second and were averaged (100 scans) to improve S/N ratio.

Fig. S1. EPR spectra obtained in absence of **1** and **2** in aqueous solutions containing sPOR protein (6 ng.ml^{-1}), DETAPAC (100 μM), SOD (300 units.ml^{-1}), catalase (1500 units.ml^{-1}), glucose-6-phosphate-dehydrogenase (13 units.ml^{-1}), glucose-6-phosphate (10 mM), and NADPH (1 mM) at pH 7, 310 K in presence of DEPMPO (25 mM).



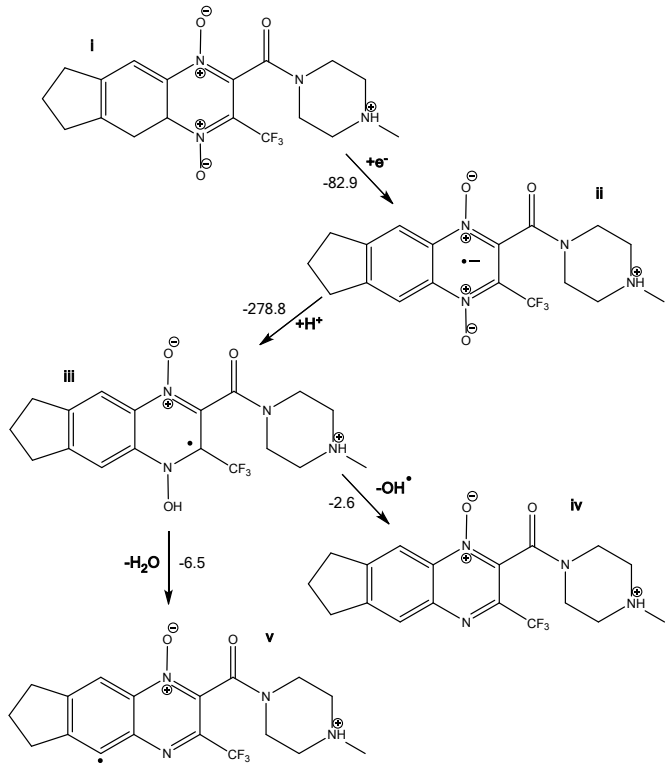
Supplementary Fig.2: EPR spectra obtained upon reduction of **2** (14.2 mM (a), 15.6 mM (b); 16.1 mM (d)) by sPOR protein (6 ng.ml^{-1}) in solutions at 310 K containing DETAPAC (100 μM), SOD (300 units.ml^{-1}), catalase (1500 units.ml^{-1}), glucose-6-phosphate-dehydrogenase (13 units.ml^{-1}), glucose-6-phosphate (10 mM), and NADPH (1 mM) at pH 7 plus; (a) DEPMPO (25 mM); (b) DEPMPO (25 mM) and DMSO (2 M); (c) simulated spectrum (NIH WinSim software) of DEPMPO-C species (0.69) and DEPMPO-OH (0.31), r = 0.966; (d) DEPMPO (25 mM) and glucose-6-phosphate (180 mM).

D. DFT information

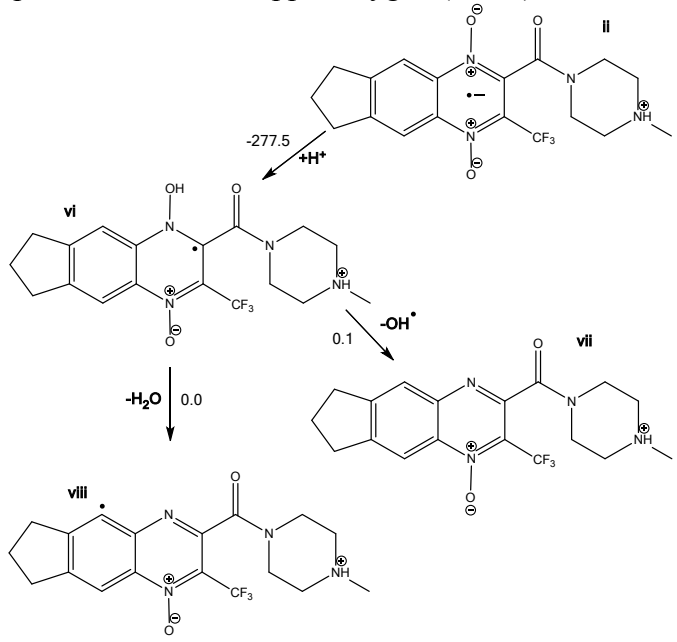
Methodology

The geometry optimisation and energy calculations were performed with Gaussian 09 software suite using unrestricted DFT [1]. The non-local B3LYP functional hybrid method was employed [2-4] and the standard 6-31+G(d, p) [5, 6] basis set was used for the geometry optimisation and frequency analysis. The zero-point vibrational energies (ZPE) were scaled according to Wong (0.9804) [7]. In all cases, the normal modes revealed no imaginary frequencies indicating that they represent minima on the potential energy surface. The subsequent energy calculations were performed with the larger 6-311+G(2df, p) basis set. All of the structural optimisation and energy calculations were carried out with a water simulation based on the polarised continuum model (IEFPCM) [8]. The piperazine moiety is protonated on the distal nitrogen. The electron affinities and proton affinities were calculated as described in Forseman and Frisch [9]. In general, the reaction steps were calculated by subtracting the ZPE corrected energies of the reactants from the products. The results are given in Schemes S1a/b, S2a/b and Table S1.

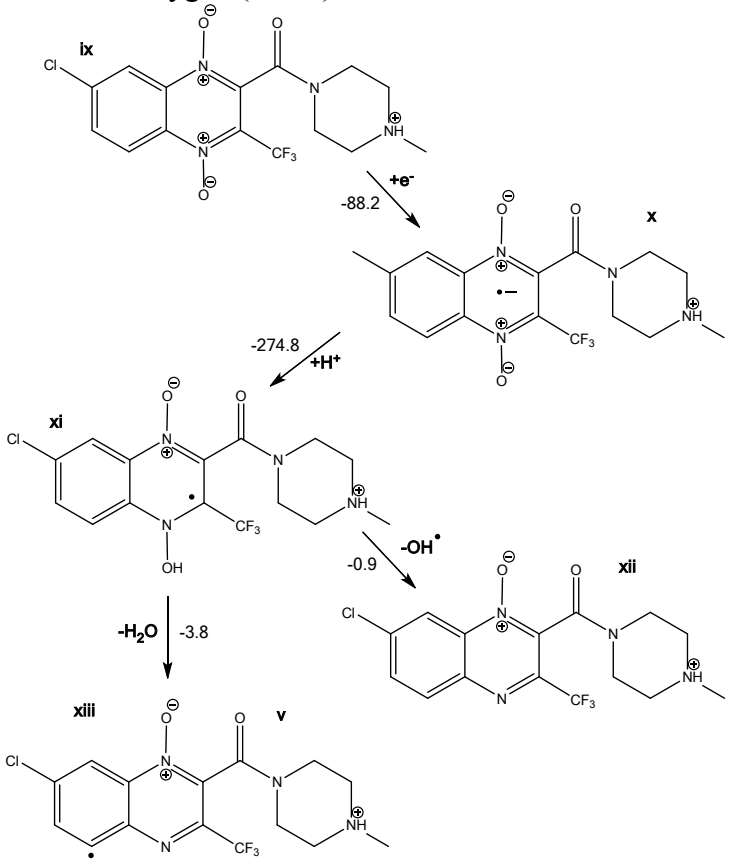
Scheme S1a: The thermochemical cascade of derivative **1** initiated by one-electron reduction with consequent protonation on the lower oxygen (N4-O). All the values are in kcal mol^{-1}.



Scheme S1b The thermochemical cascade of derivative **1** initiated by one-electron reduction with consequent protonation on the upper oxygen (N1-O). All the values are in kcal mol^{-1}.



Scheme S2a The thermochemical cascade of derivative **2** initiated by one-electron reduction with consequent protonation on the lower oxygen (N4-O). All the values are in kcal mol^{-1}.



Scheme S2b The thermochemical cascade of derivative **2** initiated by one-electron reduction with consequent protonation on the upper oxygen (N1-O). All the values are in kcal mol^{-1}.

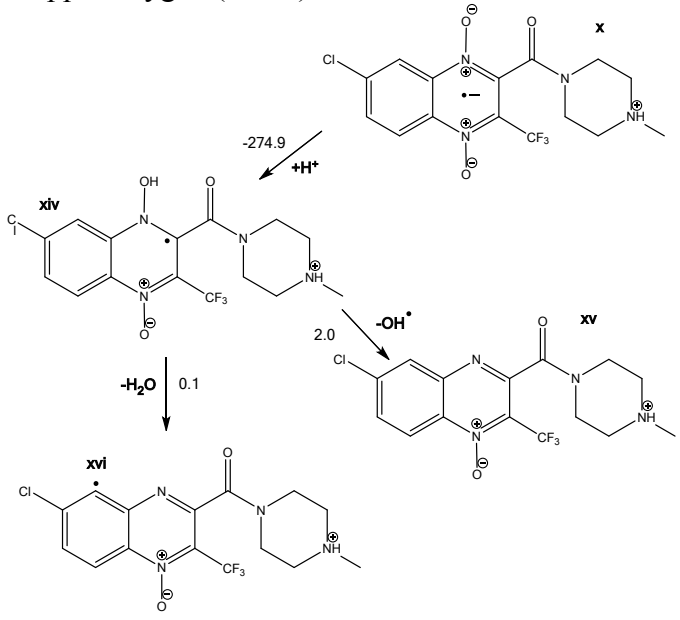


Table S1. The single point and zero point corrected vibrational energies (ZPE) of the optimised structures in hartrees (a.u.). The molecular structures are shown in Schemes S1a/b and S2a/b.

Structure	Energy (a.u.)	ZPE (a.u.)
i	-1442.40004	0.37321
ii	-1442.52816	0.36925
iii	-1442.98446	0.38255
iv	-1367.21430	0.36896
v	-1366.52177	0.35647
vi	-1442.98320	0.38214
vii	-1367.20933	0.36888
viii	-1366.51689	0.35642
ix	-1785.24881	0.30130
x	-1785.38535	0.29726
xi	-1785.83677	0.31070
xii	-1710.06417	0.29747
xiii	-1709.36989	0.28475
xiv	-1785.83690	0.31077
xv	-1710.05957	0.29732
xvi	-1709.36376	0.28481
H_2O	-76.46783	0.02078
OH	-75.76900	0.00826

References

[1] **(also Reference 15 in text)** M. J. Frisch, G. W. Trucks, H. B. Schlegel, G. E. Scuseria, M. A. Robb, J. R. Cheeseman, G. Scalmani, V. Barone, B. Mennucci, G. A. Petersson, H. Nakatsuji, M. Caricato, X. Li, H. P. Hratchian, A. F. Izmaylov, J. Bloino, G. Zheng, J. L. Sonnenberg, M. Hada, M. Ehara, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, T. Vreven, J. J. A. Montgomery, J. E. Peralta, F. Ogliaro, M. Bearpark, J. J. Heyd, E. Brothers, K. N. Kudin, V. N. Staroverov, R. Kobayashi, J. Normand, K. Raghavachari, A. Rendell, J. C. Burant, S. S. Iyengar, J. Tomasi, M. Cossi, N. Rega, J. M. Millam, M. Klene, J. E. Knox, J. B. Cross, V. Bakken, C. Adamo, J. Jaramillo, R. Gomperts, R. E. Stratmann, O. Yazyev, A. J. Austin, R. Cammi, C. Pomelli, J. W. Ochterski, R. L. Martin, K. Morokuma, V. G. Zakrzewski, G. A. Voth, P. Salvador, J. J. Dannenberg, S. Dapprich, A. D. Daniels, O. Farkas, J. B. Foresman, J. V. Ortiz, J. Cioslowski, D. J. Fox, Gaussian 09, Revision D.02., Gaussian, Inc., Wallingford CT, **2009**.

[2] A. D. Becke, *Phys.Rev.A.* **1988**, *38*, 3098-3100.

[3] A. D. Becke, *J.Chem.Phys.* **1993**, *98*, 5648-5652.

[4] C. Lee, W. Yang, R. G. Parr, *Phys.Rev.B.* **1988**, *37*, 785-789.

[5] P. C. Hariharan, J. A. Pople, *Theoret.Chim.Acta* **1973**, *28*, 213-222.

[6] M. J. Frisch, J. A. Pople, J. S. Binkley, *J.Chem.Phys.* **1984**, *80*, 3265-3269.

[7] M. W. Wong, *Chem.Phys.Lett.* **1996**, *256*, 391-399.

[8] J. Tomasi, B. Mennucci, R. Cammi, *Chem. Rev.* **2005**, *105*, 2999-3093.

[9] J. B. Foresman, Æ. Frisch, *Exploring Chemistry with Electronic Structure Methods*, Gaussian Inc., **1996**. Pp. 142 -143.