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Supporting Information

Flavin-mediated photo-oxidation for the detection of mitochondrial flavins

Dong-Nam Lee,[a] Eunjin Kim,[b] Jae Hong Lee,[c] Jong Seung Kim,*[c] Chulhun Kang,*[b] and Jong-In Hong*[a]

[a] Department of Chemistry, College of Natural Sciences, Seoul National University, Seoul 08826, Republic of Korea, [b] Graduate School of Medical Science, Kyung Hee University, Yongin 446-701, Republic of Korea. [c] Department of Chemistry, Korea University, Seoul 136-701, Republic of Korea.

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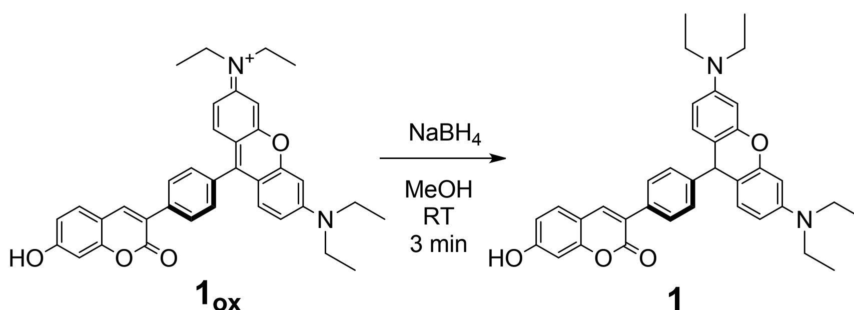
General procedure

All fluorescence and UV−Vis absorption spectra were recorded with Jasco FP 6500, multimode plate reader (Molecular Devices, SpectraMax M2e), and Beckman coulter DU 800 spectrophotometers. All ^{1}H and ^{13}C NMR spectra were collected in $CDCl_3$ or DMSO-d_6 on a Bruker 300 and Varian 400 MHz spectrometer. All chemical shifts are reported in ppm value using the peak of residual proton signals of TMS as an internal reference. DFT calculations were performed by Hartree-Fock/6-311G method using program "Gaussian 09". HRMS data were received directly from the Korea Basic Science Institute. The fluorescence imaging of cells and tissues was performed with a confocal laser scanning microscope (Carl-Zeiss LSM 5 Exciter, Oberko, Germany). All analytes were purchased from Aldrich and used as received. All solvents were analytical reagents from Duksan Pure Chemical Co., Ltd. DMSO for spectral measurements was of HPLC reagent grade, without fluorescent impurity. De-ionized water was used in all studies.

Synthesis of probe 1 (3-(4-(3,6-bis(diethylamino)-9*H*-xanthen-9-yl)phenyl)-7-hydroxy-2*H*-chromen-2-one)

Compound **1_{ox}** (*N*-(6-(diethylamino)-9-(4-(7-hydroxy-2-oxo-2*H*-chromen-3-yl)phenyl)-3*H*-xanthen-3-ylidene)-*N*-ethylethanaminium) was prepared by the literature method[1] and synthesis of **1** is described below.



Scheme S1. Synthesis of probe **1**

To a stirred solution of **1_{ox}** (3 mmol) in MeOH (60 mL, 0.05 M) was added excess amount of $NaBH_4$ (60 mmol, 20 equiv.), and the reaction mixture was stirred at room temperature for 3 min (pink color of the solution was changed to yellow color). The solvent was evaporated under reduced pressure, and crude product was purified by flash column chromatography using 1:50 MeOH:CH_2Cl_2 as eluent to afford the product which was further crystallized from MeOH to give a violet solid in 56% yield. Compound **1** is air-sensitive. So, the synthetic process was completed within 1 hour to minimize the air-oxidation. ^{1}H NMR

(DMSO-d_6, 300 MHz): δ 10.59 (s, 1H), 8.06 (s, 1H), 7.56−7.53 (m, 3H), 7.19 (d, 2H, J = 8.1 Hz), 6.85−6.78 (m, 3H), 6.73 (s, 1H), 6.36−6.33 (m, 4H), 5.08 (s, 1H), 3.29 (m, 8H), 1.09 (t, 12H, J = 6.9 Hz,); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 161.8, 159.9, 154.9, 152.1, 148.5, 147.6, 140.4, 132.6, 130.4, 129.2, 128.5, 123.9, 113.8, 113.2, 111.8, 107.7, 102.8, 98.9, 77.2, 44.5, 42.6, 12.6; HRMS (FAB$^+$, m-NBA) m/z calculated for $C_{36}H_{36}N_2O_4$ 560.2675, observed 560.2670.

Spectroscopic measurements

Stock solutions of **1** (5 mM in THF) was diluted to 5 μM in 3:7 CH_3CN:buffer (10 mM HEPES, pH = 7.4). Time-dependent fluorescence spectra of **1** in the presence of 2 equiv. FMN (auto-oxidation *vs.* FMN-promoted oxidation) were recorded under varied light exposure (UV hand lamp, ambient light, and dark conditions). The oxidation rate constant was measured under pseudo-1st order reaction conditions, assuming that the concentration of flavin remains constant due to short life time of the reduced form. The varying irradiation intensity (λ_{irr} = 450 nm) was feasible by adjusting the irradiation slit width (1×3, 5×3, 10×3 nm) of the fluorometer (Jasco FP 6500). The irradiation wavelength was also controlled by the fluorometer (λ_{irr} = 300, 360, 450, 515, 565 nm), where the slit width of photoirradiation was fixed to 5×3 nm (sensitivity: medium). After photoirradiation, the fluorescence intensity of **1** was measured at 600 nm with excitation at 565 nm (monitoring slit width: 5×3 nm). For selectivity and competition experiments, stock solutions of various oxidants such as NAD^+, Fe(III), 6-biopterin, folic acid, H_2O_2, OCl^-, $OH^{\cdot}$, $O_2^{\cdot -}$, and GSSG were prepared in deionized water and diluted to different concentrations as shown in Table S1.

Table S1. Preparation of stock solutions and working solutions of **1** and various oxidants

Stock and working solutions	Preparation	Stoichio-metry to probe **1**
5 mM of **1** stock solution	2 μmole of **1** in 0.4 mL of THF	
5 μM of **1** working solution	1000× dilution in 3:7 CH_3CN:buffer (HEPES 10 mM, pH = 7.4)	
FMN	2 μL of 10 mM FMN stock solution into 2 mL of 5 μM **1** solution	2 equiv.
NAD^+, Fe(III), H_2O_2, OCl^-	2 μL of 100 mM each stock solution into 2 mL of 5 μM **1** solution	20 equiv.
6-biopterin, folic acid	10 μL of 10 mM each stock solution into 2 mL of 5 μM **1** solution	10 equiv.
$O_2^{\bullet -}$ [a]	0.1 mg of KO_2 as solid into 2 mL of 5 μM **1** solution	141 equiv.
GSSG [a]	1.0 mg of GSSG as solid into 2 mL of 5 μM **1** solution	163 equiv.
$^{\bullet}OH$ [b]	2 μL of 100 mM Fe(II) stock solution into 2 mL of 100 μM H_2O_2 solution, following by addition of 2 μL of 5 mM **1** stock solution	20 equiv.

[a] The reagent was added as solid into 5 μM **1** solution.

[b] $^{\bullet}OH$ was prepared by the method of Fenton's reagent.

Theoretical calculations

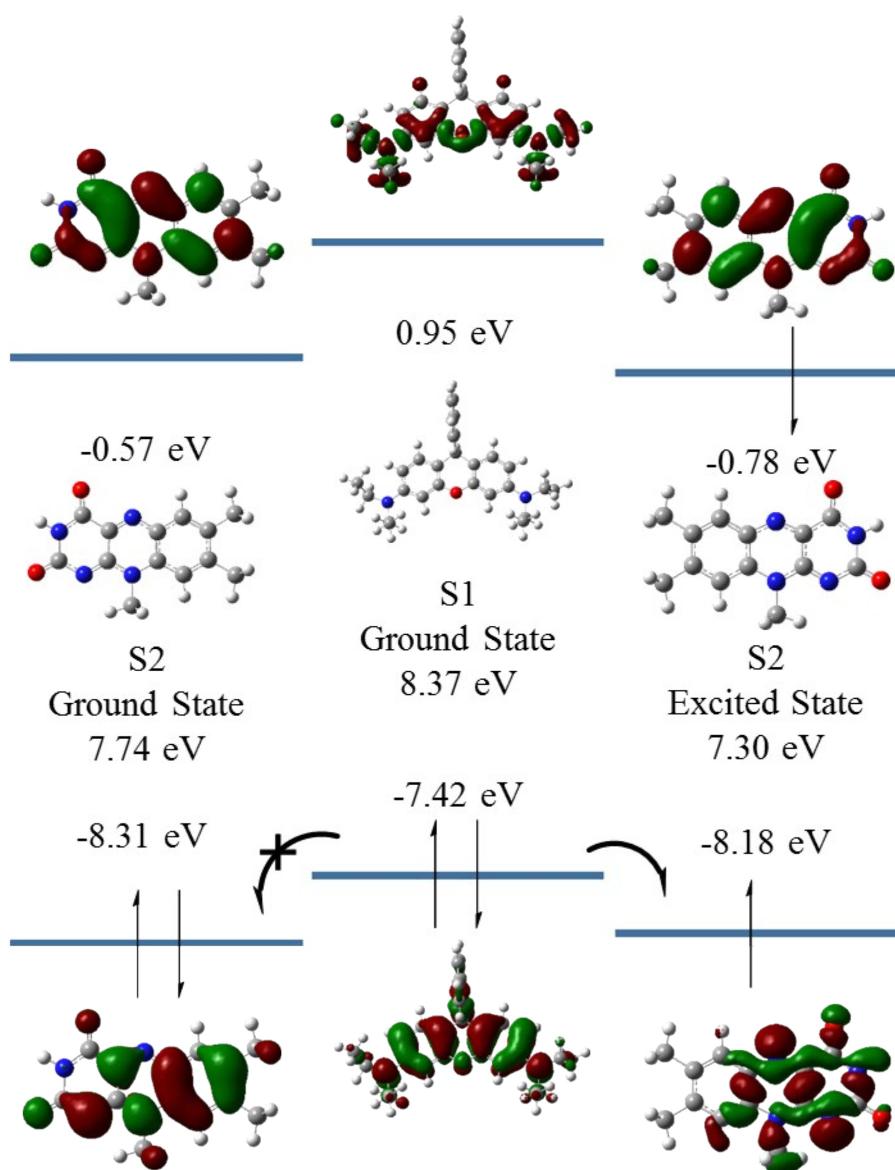


Figure S1. DFT calculations for geometry optimizations of probe **1** and the isoalloxazine ring. All molecular orbital calculations were performed with MINDO3 method.[2] The energy level of singly occupied molecular orbital (SOMO) was obtained by the TD-SCF method.

UV−Vis. and Fluorescence spectroscopy

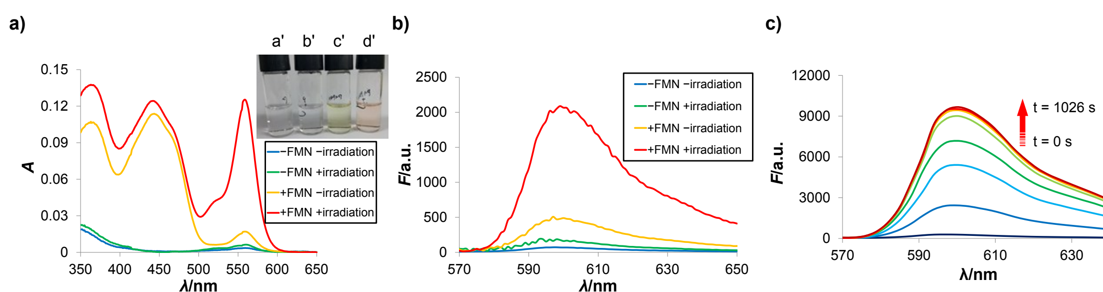


Figure S2. UV−Vis. (a) and fluorescence (b) spectra of **1** (5 μM) in the absence (blue and green line) and presence (orange and red line) of 2.0 equiv. FMN in 3:7 CH_3CN:buffer (HEPES 10 mM, pH = 7.4). The spectrum of FMN-driven photo-oxidation was recorded after irradiation with UV hand lamp (365 nm, 6 W) for 3 min. (c) The time-dependent fluorescence spectra in the presence of 2.0 equiv. FMN upon photoirradiation (365 nm, 6 W). The inset in panel (a) shows the color change of **1** under the conditions of (a') −FMN −irradiation, (b') −FMN +irradiation, (c') +FMN −irradiation, and (d') +FMN +irradiation.

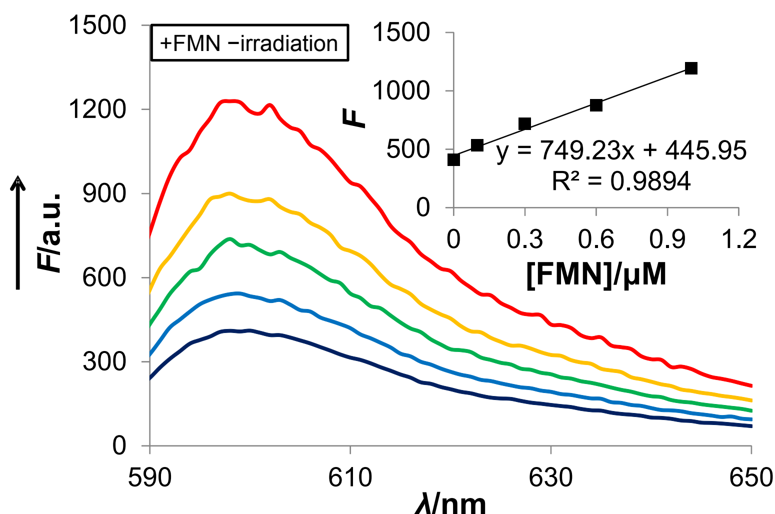
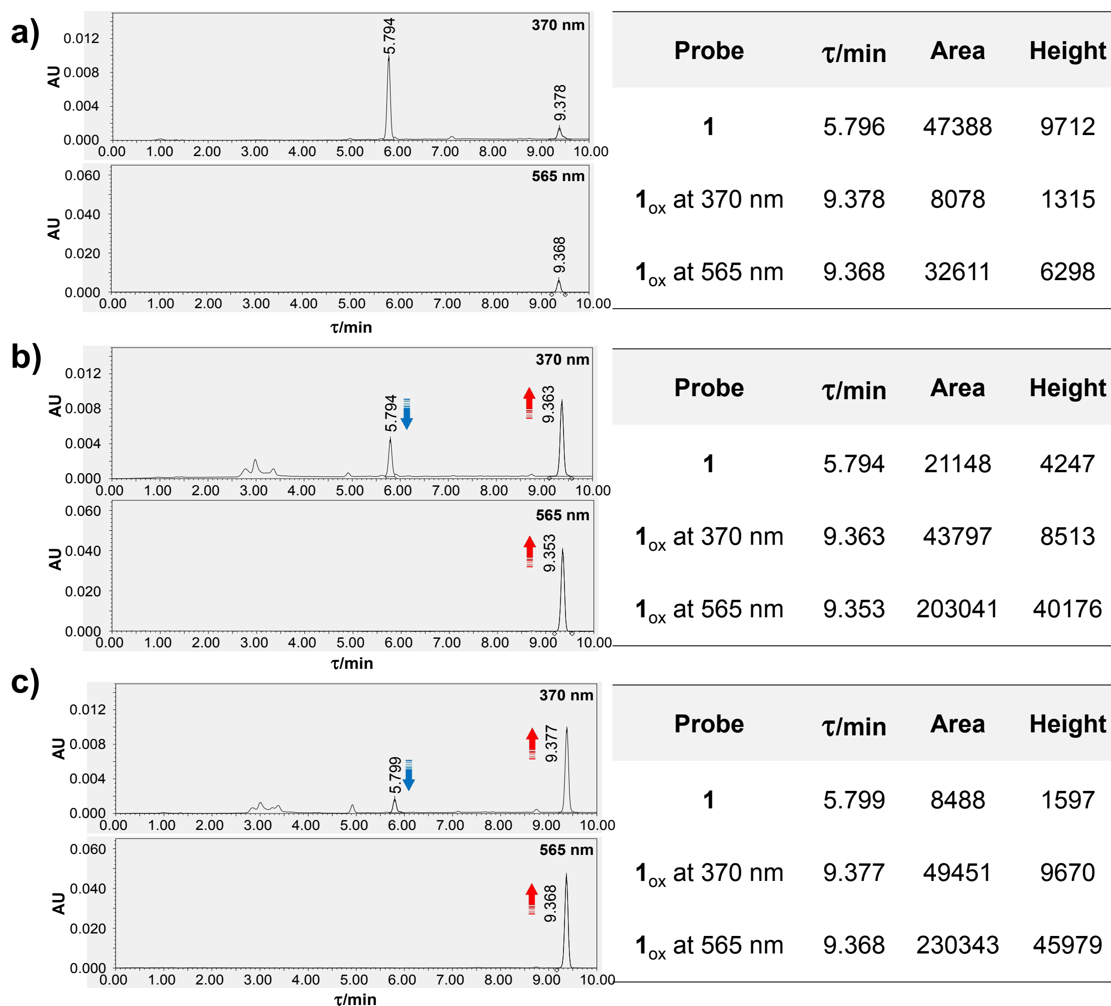


Figure S3. Fluorescence spectra of **1** (5 μM) in presence of varied concentration of FMN (0−1.0 μM) in 3:7 CH_3CN:buffer (HEPES 10 mM, pH = 7.4) under ambient light exposure. The fluorescence was measured after excitation at 565 nm after aging for 30 m. Inset shows linear plot of fluorescence intensity at 600 nm.

HPLC analysis



a)

Probe	τ/min	Area	Height
1	5.796	47388	9712
$\mathbf{1}_{ox}$ at 370 nm	9.378	8078	1315
$\mathbf{1}_{ox}$ at 565 nm	9.368	32611	6298

b)

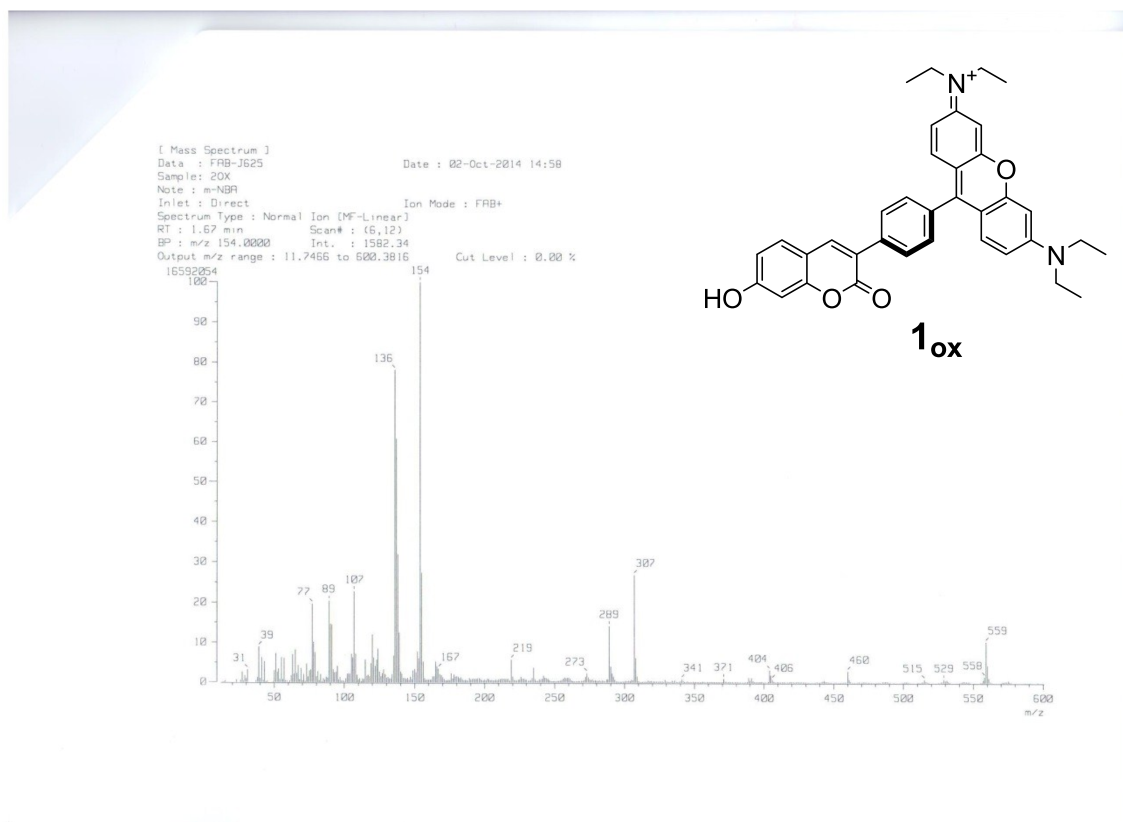
Probe	τ/min	Area	Height
1	5.794	21148	4247
$\mathbf{1}_{ox}$ at 370 nm	9.363	43797	8513
$\mathbf{1}_{ox}$ at 565 nm	9.353	203041	40176

c)

Probe	τ/min	Area	Height
1	5.799	8488	1597
$\mathbf{1}_{ox}$ at 370 nm	9.377	49451	9670
$\mathbf{1}_{ox}$ at 565 nm	9.368	230343	45979

Figure S4. Time-dependent HPLC/UV−Vis. spectra (top line: 370 nm, bottom line: 565 nm) of **1** in 3:7 CH_3CN:buffer (HEPES 10 mM, pH 7.4) with corresponding values in the absence (a) and presence (b, c) of 2.0 equiv. FMN (b: after irradiation for 3 min, c: after irradiation for 7 min).

HPLC/HRMS spectrum of 1_{ox}



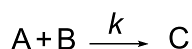
[Elemental Composition]
Data : FAB-J649 Date : 07-Oct-2014 14:24
Sample: 2OX
Note : m-NBA
Inlet : Direct Ion Mode : FAB+
RT : 0.10 min Scan#: (1,9)
Elements : C 150/0, H 150/0, N 10/0, O 10/0
Mass Tolerance : 1000ppm, 3mmu if m/z < 3, 5mmu if m/z > 5
Unsaturation (U.S.) : 0.0 - 100.0

Observed m/z	Int%	Err [ppm / mmu]	U.S.	Composition
559.2604	100.0	-1.0 / -0.6	25.5	C 37 H 31 N 6
		-3.4 / -1.9	25.0	C 39 H 33 N 3 O
		-5.8 / -3.3	24.5	C 41 H 35 O 2
		+6.2 / +3.4	21.5	C 32 H 31 N 8 O 2
		+3.7 / +2.1	21.0	C 34 H 33 N 5 O 3
		+1.3 / +0.8	20.5	C 36 H 35 N 2 O 4
		+8.5 / +4.8	16.5	C 31 H 35 N 4 O 6
		+6.1 / +3.4	16.0	C 33 H 37 N O 7
		-4.4 / -2.4	12.5	C 25 H 35 N 8 O 7
		-6.8 / -3.8	12.0	C 27 H 37 N 5 O 8
		+2.8 / +1.6	8.5	C 20 H 35 N 10 O 9
		+0.4 / +0.2	8.0	C 22 H 37 N 7 O 10

Figure S5. HPLC/HRMS spectrum of $\mathbf{1}_{ox}$ and its corresponding data.

Determination of Reaction Rate Constants (*k*)

For reaction,



$$-\frac{d[A]}{dt} = k[A][B]$$

$$-\frac{d[A]}{dt} = k_{obs}[A] \qquad \because \text{ [B] = constant concentration}$$

$$-\int_{[A]_0}^{[A]_t} \frac{d[A]}{[A]} = \int_0^t k_{obs}\, dt$$

$$\ln\frac{[A]_0}{[A]_t} = k_{obs} t$$

For unimolecular reaction, $[A]_t + [C]_t = [C]_{max}$

$$k_{obs} t = \ln\frac{[A]_t + [C]_t}{[A]_t} = \ln\left(1 + \frac{[C]_t}{[C]_{max} - [C]_t}\right) \approx \ln\left(1 + \frac{C_t}{C_{max} - C_t}\right) \qquad (1)$$

In our case,

$[A]_t$ = concentration of **1** unreacted after time elapse of ‘t’ sec

[B] = concentration of FMN

$[C]_t$ = concentration of the $\mathbf{1}_{ox}$ after time elapse of ‘t’ sec

$[C]_{max}$ = maximum concentration of $\mathbf{1}_{ox}$

A_t, C_t, and C_{max} are fluorescence intensity at 600 nm of $[A]_t$, $[C]_t$, and $[C]_{max}$, respectively.

Using equation (1), we calculated the initial rate constants under different light conditions as tabulated in **Table S2**.

Table S2. Initial oxidation rate constants[a] of **1**[b] in the absence (auto oxidation) and presence of FMN[c] under various light conditions

Light condition	Auto oxidation	FMN addition
UV irradiation [d]	$5.60 \pm 0.38 \times 10^{-1}$	5.08 ± 0.62
Ambient light	$3.34 \pm 0.37 \times 10^{-2}$	$1.83 \pm 0.22 \times 10^{-1}$
Dark	$6.88 \pm 0.47 \times 10^{-4}$	$1.14 \pm 0.02 \times 10^{-3}$

[a] k_{obs}/s^{-1} × 10^{-3}

[b] 5 μM in 3:7 CH_3CN:buffer (HEPES 10 mM, pH = 7.4)

[c] 2 equiv. FMN was added. We assumed that there was no photo-decomposition of FMN during the measurement.

[d] 365 nm irradiation (6 W) using hand held UV lamp (Spectroline, ENF-260C)

Slit width control

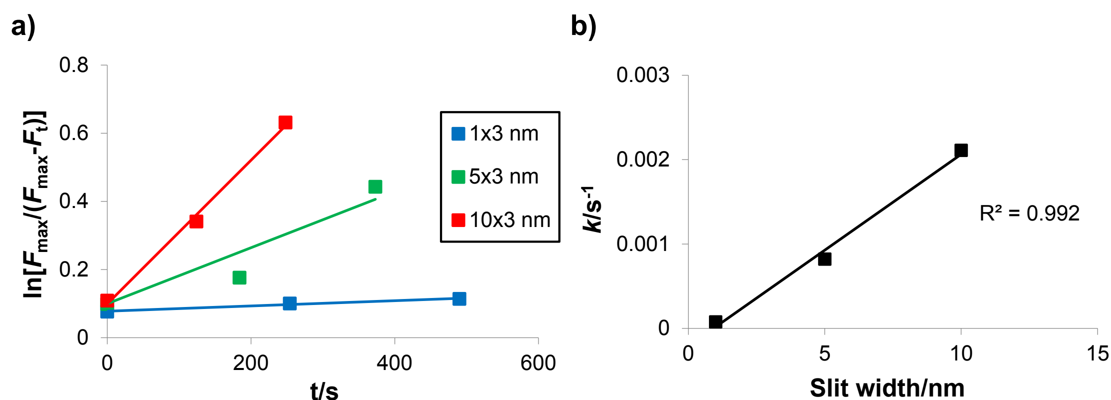


Figure S6. (a) The plot showing the time-dependent fluorescence intensity of **1** (5 μM) in the presence of 2.0 equiv. FMN in 3:7 CH_3CN:buffer (HEPES 10 mM, pH = 7.4) under varying light intensity by adjusting the slit width of a fluorometer (Jasco FP 6500). The photoirradiation wavelength was 450 nm, absorption maxima of FMN. (b) Plot showing the linear relationship between rate constants and slit widths. After photoirradiation, the fluorescence intensity was measured at 600 nm with excitation at 565 nm. The measured rate constants are tabulated in Table S3.

Table S3. Rate constants[a] for oxidation of **1**[b] in the presence of FMN[c] at different slit-widths

Slit width [d]/ nm	k [e]
1×3	$7.68 \times 10^{-2} \pm 1.62$
5×3	$8.21 \times 10^{-1} \pm 0.48$
10×3	2.11 ± 1.50

[a] $k_{obs}/s^{-1} \times 10^{-3}$

[b] 5 μM in 3:7 CH_3CN:buffer (HEPES 10 mM, pH = 7.4)

[c] 2 equiv., We assumed that there was no photo-decomposition of FMN during the measurement.

[d] light exposure at 450 nm (photoirradiation)

[e] fluorescence intensity of **1** was monitored at 600 nm with excitation at 565 nm (monitoring slit width: 5×3 nm)

Photoirradiation wavelength control

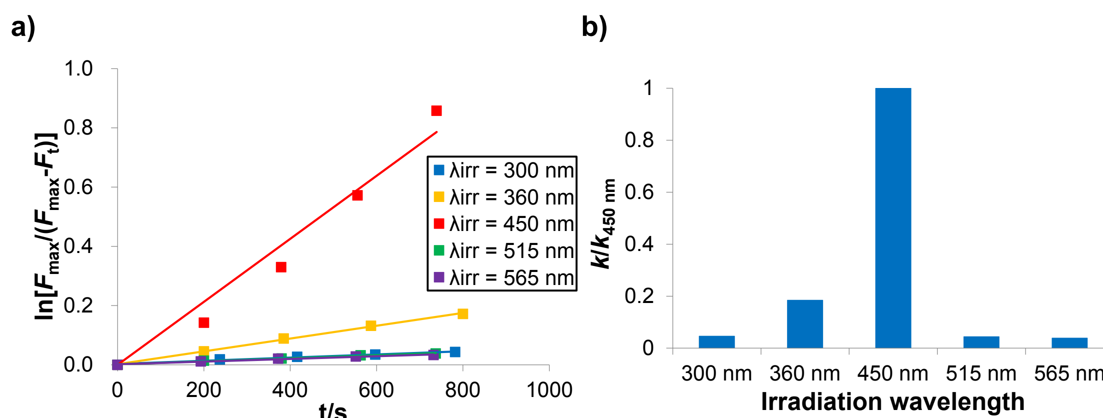


Figure S7. (a) The plot showing the time-dependent fluorescence intensity change of **1** (5 μM) in the presence of 2.0 equiv. FMN in 3:7 CH_3CN:buffer (HEPES 10 mM, pH = 7.4) upon photoirradiation at different wavelengths (λ_{irr} = 300, 360, 450, 515, 565 nm). (b) Bar graph shows ratios ($k/k_{450\ nm}$) of the rate constants upon irradiation at various wavelengths (k, λ_{irr} = 300, 360, 450, 515, 565 nm) to the rate constant upon irradiation at 450 nm ($k_{450\ nm}$). The slit width of photoirradiation was 5×3 nm (sensitivity: medium). After photoirradiation, the fluorescence intensity of **1** was measured at 600 nm with excitation at 565 nm. The obtained rate constants are tabulated in Table S4.

Table S4. Rate constants[a] for flavin-promoted photo-oxidation of **1**[b] in the presence of FMN[c] under photoirradiation at different wavelengths[d]

Irradiation wavelength	$k_{obs}/s^{-1} \times 10^{-3}$
300 nm	5.39×10^{-2}
360 nm	2.16×10^{-1}
450 nm	1.17
515 nm	5.15×10^{-2}
565 nm	4.56×10^{-2}

[a] fluorescence intensity of **1** was monitored at 600 nm with excitation at 565 nm (slit width: 5×3 nm, sensitivity: medium)
[b] 5 μM in 3:7 CH_3CN:buffer (10 mM HEPES, pH = 7.4)
[c] 2 equiv., We assumed that there was no photo-decomposition of FMN during the measurement.
[d] Photoirradiation slit width was 5×3 nm (sensitivity: medium)

Selectivity test

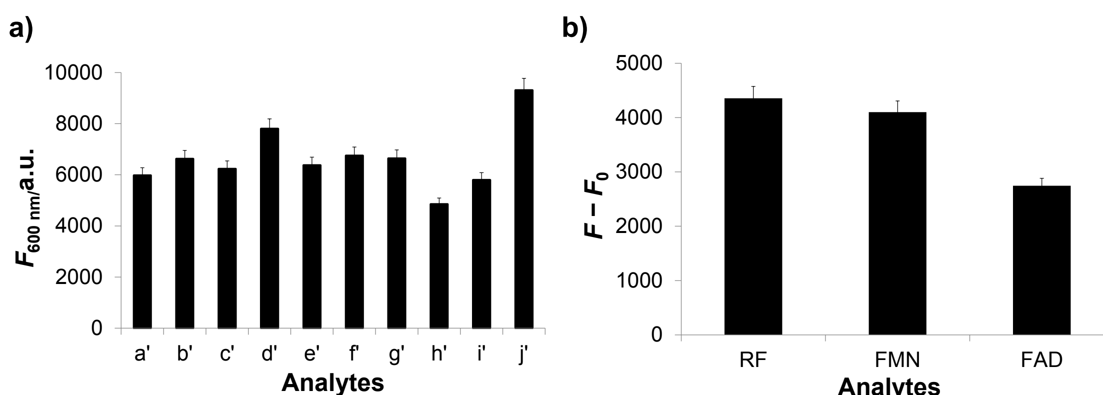


Figure S8. a) The bar diagram showing the fluorescence response of **1** (5 μM) to FMN (2.0 equiv.) in the presence of different oxidants: (a') control (b') NAD^+ (20 equiv.) (c') Fe(III) (20 equiv.), (d') 6-biopterin (10 equiv.), (e') folic acid (10 equiv.), (f') H_2O_2 (20 equiv.), (g') OCl^- (20 equiv.), (h') $^{\cdot}OH$ (20 equiv.), (i') $O_2^{\cdot-}$ (141 equiv.), (j') GSSG (163 equiv.). After the addition of FMN, various mixtures were irradiated with UV hand lamp for 3 min at RT. Data were acquired in 3:7 CH_3CN:buffer (HEPES 10 mM, pH 7.4). b) Fluorescence intensity of **1** (5 μM) resulted from flavin-mediated photo-oxidation (RF; riboflavin, FMN, and FAD). The excitation wavelength was 565 nm.

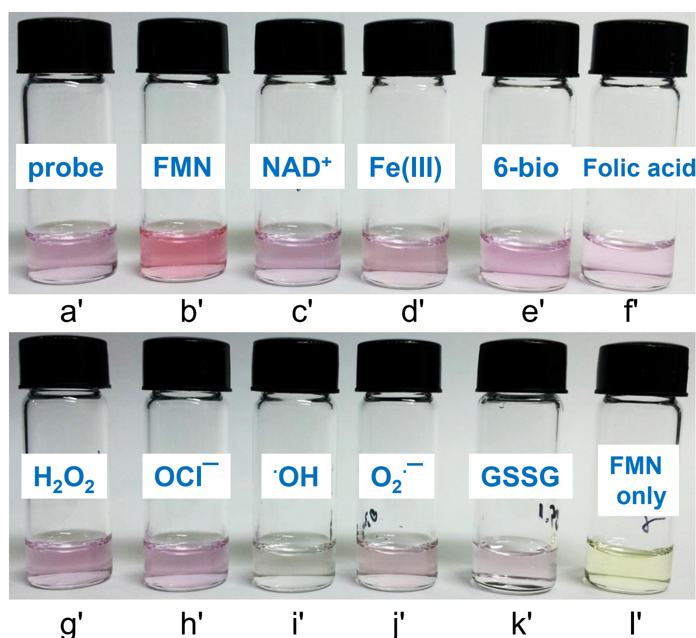


Figure S9. Change in color intensity of **1** (5 μM) in the presence of different oxidants in 3:7 CH_3CN:buffer (HEPES 10 mM, pH 7.4); (a') **1** only (b') FMN (2 equiv.), (c') NAD^+ (20 equiv.), (d') Fe(III) (20 equiv.), (e') 6-biopterin (10 equiv.), (f') folic acid (10 equiv.), (g') H_2O_2 (20 equiv.), (h') OCl^- (20 equiv.), (i') $^{\cdot}OH$ (20 equiv.), (j') $O_2^{\cdot-}$ (141 equiv.), (k') GSSG (163 equiv.), (l') FMN only (10 μM). After the addition of oxidants, the mixtures were irradiated with UV hand lamp for 3 min at RT.

The pH screening

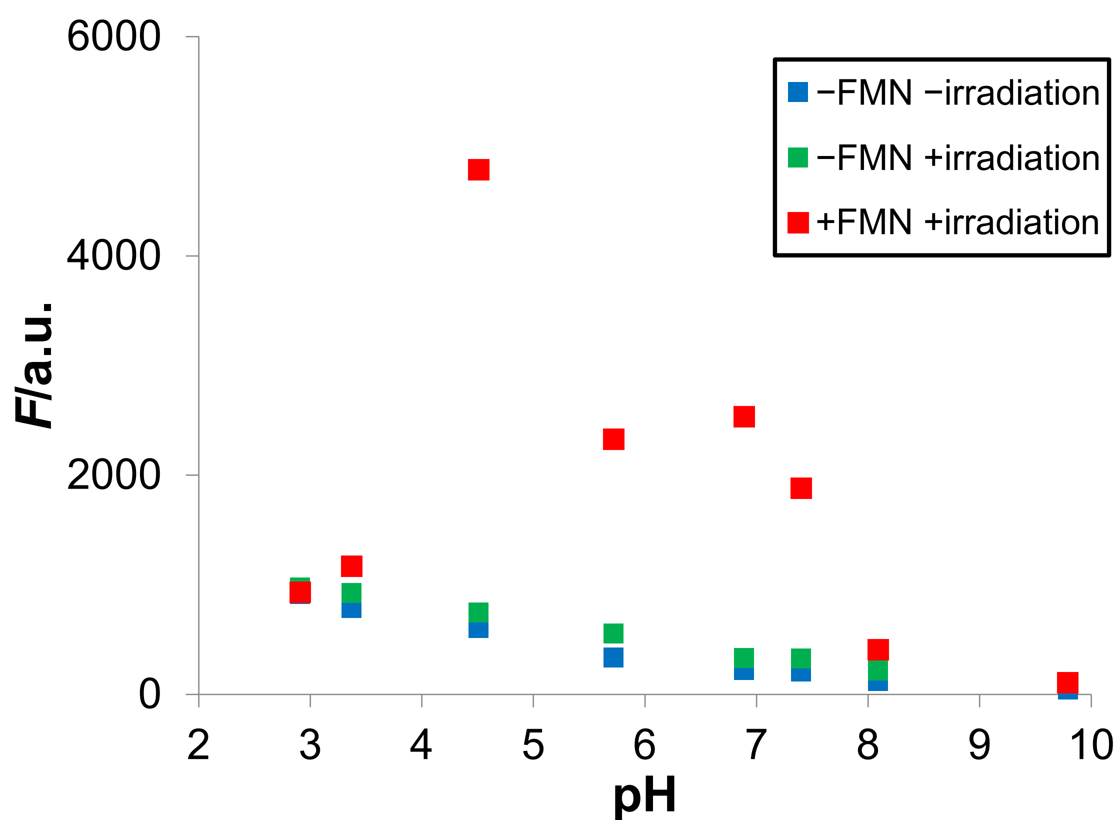


Figure S10. The pH profiles showing changes in the fluorescence intensity of **1** (5 μM) in the absence (blue and green square) and presence of FMN (2.0 equiv., red square) in 3:7 CH_3CN:buffer (HEPES 10 mM, pH 7.4) after the UV hand lamp irradiation for 3 min (green and red square). Three sets of the pH screening were performed in a pH range of 2.91−9.79.

Detection of bound flavins inside glutathione reductase

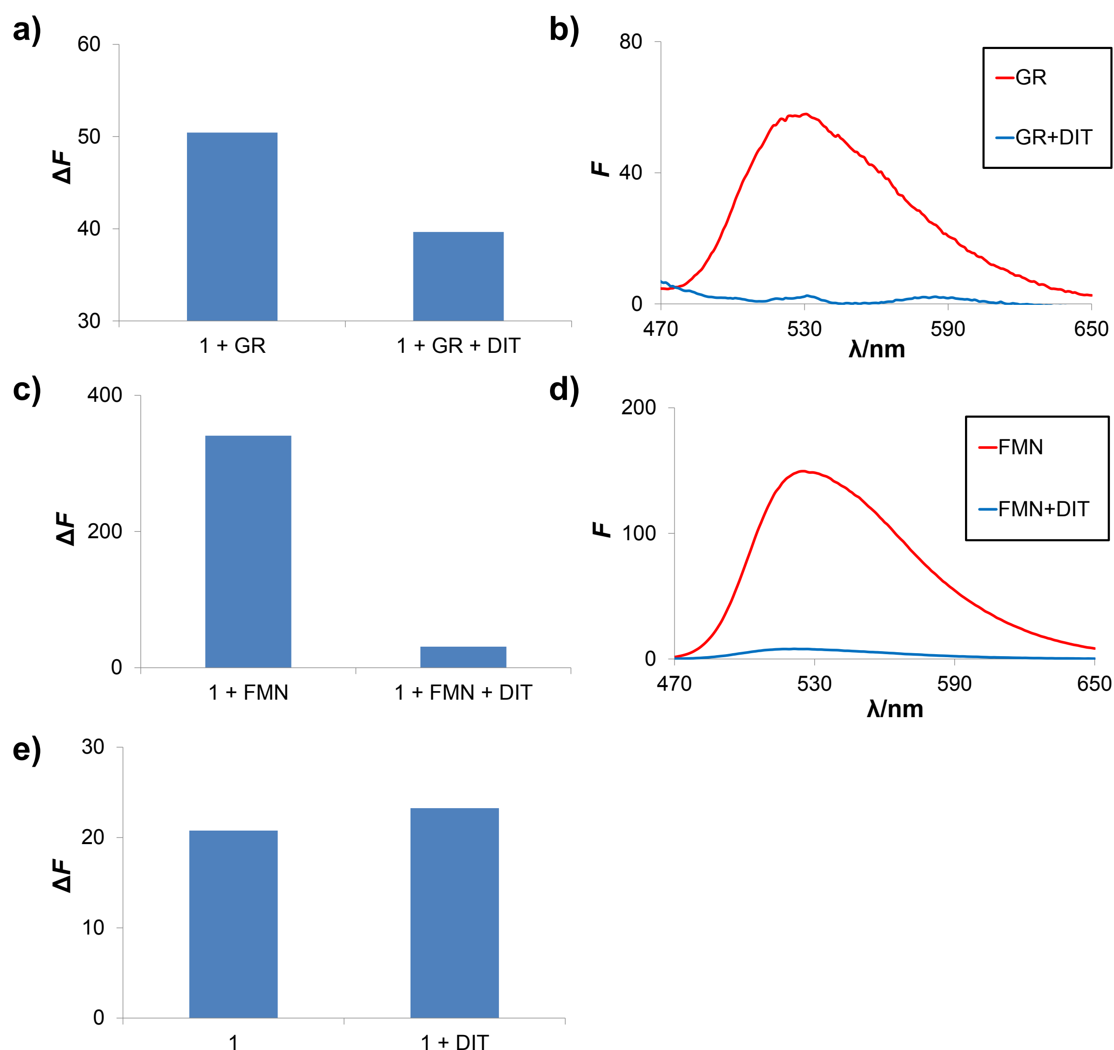


Figure S11. The bar diagram shows that the fluorescence intensity decreases readily when probe **1** (5 μM) was pre-treated with excess sodium dithionite (DIT), prior to its photo-reaction with (a) glutathione reductase (GR, 10 units) and (c) FMN. (b, d) Fluorescence quenching of GR and FMN upon treating with DIT (λ_{ex} = 450 nm). (e) Bar diagram showing negligible emission intensity change of **1** upon treatment with DIT. All spectra were measured 3:7 CH_3CN:buffer (HEPES 10 mM, pH 7.4) after the UV hand lamp irradiation for 3 min after the UV hand lamp irradiation for 3 min. In panels (a), (c) and (e), the excitation wavelength was 565 nm, showing emission changes at 600 nm.

Tissue imaging

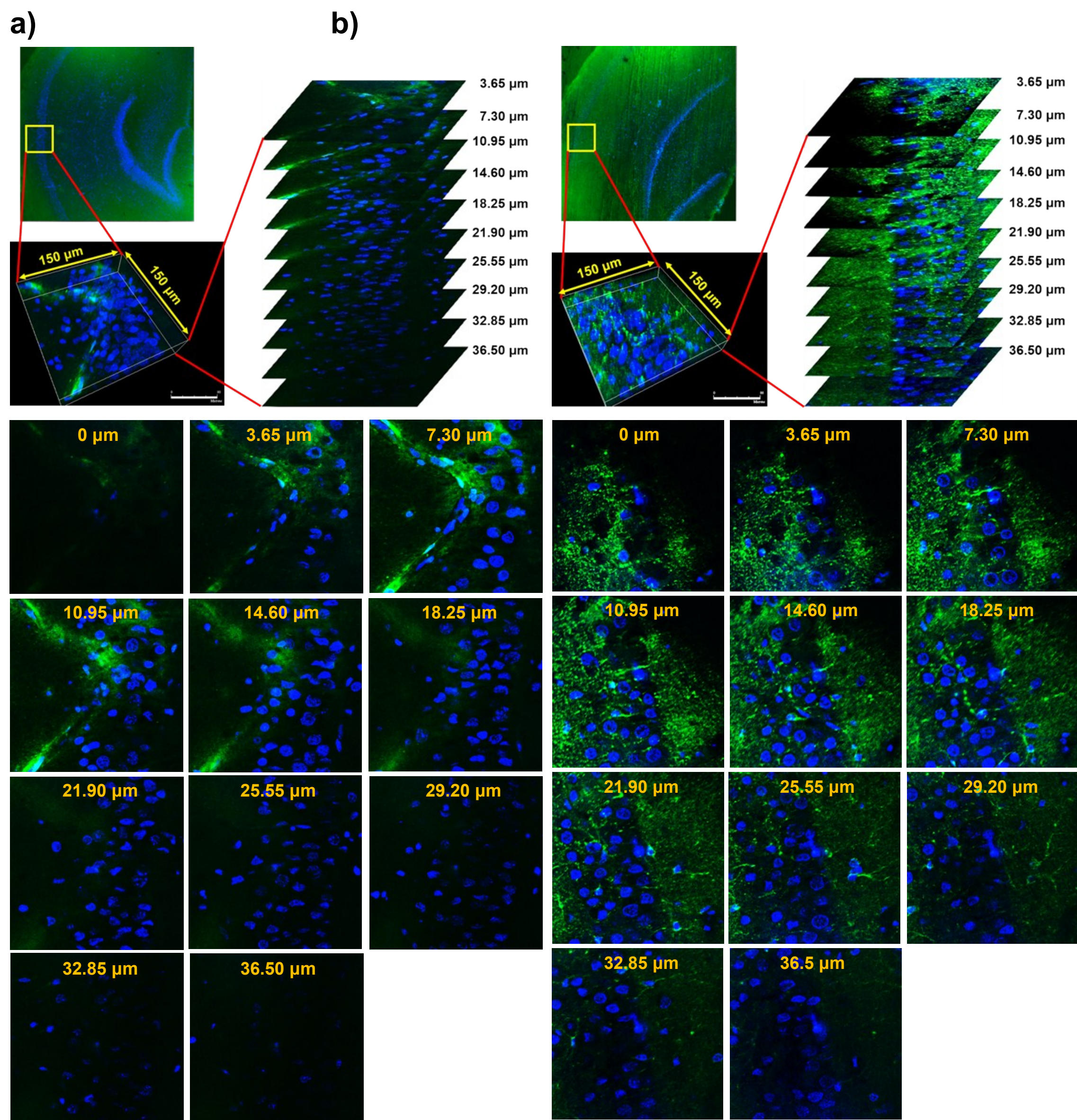


Figure S12. 3-Dimensional confocal images of rat hippocampal tissue stained with **1** (20 μM) at depth of 3.65−36.50 μm before (a) and after (b) treatment with multi-inhibitors (ROT 2.5 μM + MAL 0.5 mM). The images were taken after irradiation with UV hand lamp for 10 min. The excitation wavelength was 543 nm. Scale bar: 80 μm. The green and blue colours are contributed by probe **1** and Hoechst staining, respectively, under UV irradiation.

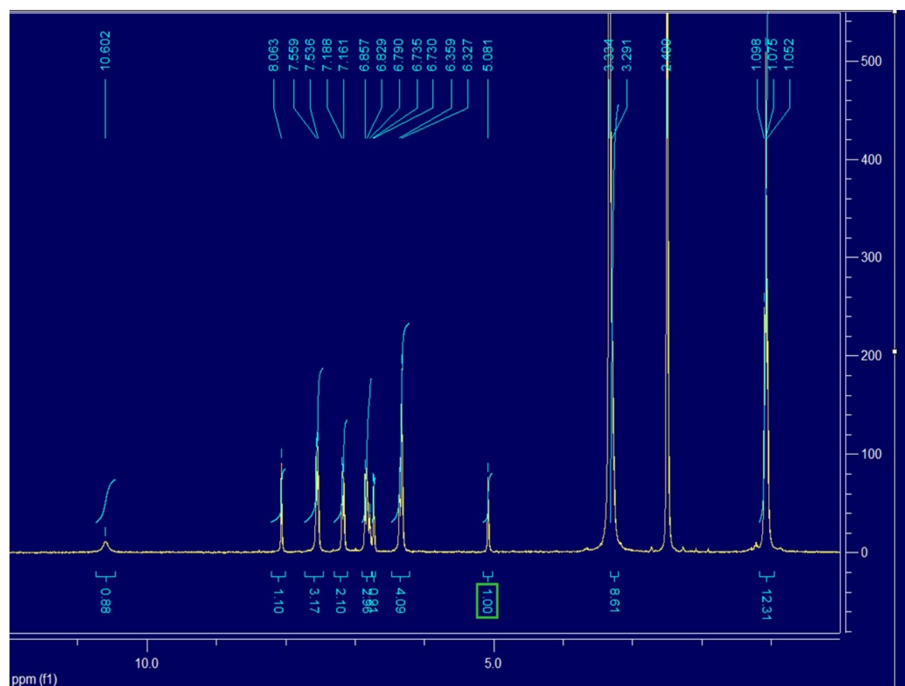


Figure S13. ^{1}H-NMR spectrum of **1**

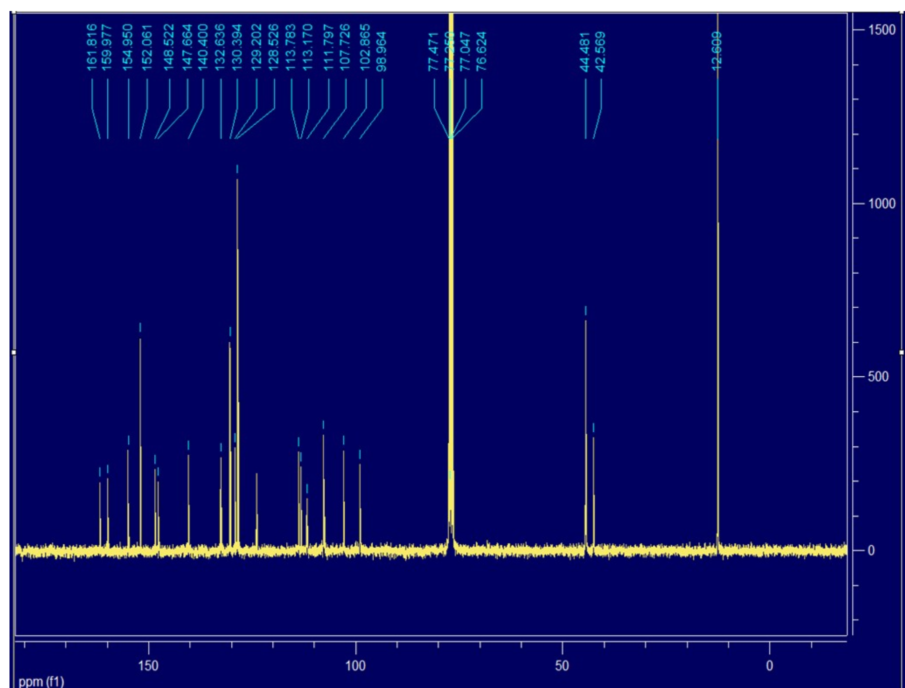


Figure S14. ^{13}C-NMR spectrum of **1**

References

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