

Electronic Supplementary Information

Selective Lysosomal H₂O₂ – ROS Imaging with a Naphthalimide Probe Forming Hydroxylamine without Overoxidation

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METHODS

Cell Viability Assay (MTT Assay) in SK-Lu-1 Cell Line

Cell viability was evaluated using the colorimetric MTT assay, as originally described by Mosmann (1983), with minor modifications. Briefly, cells were seeded in 96-well plates at a density of 5×10^3 to 1×10^4 cells per well, depending on the cell line, in a final volume of 100 μ L of appropriate culture medium. Cell viability was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay in SK-Lu-1 cells treated with **LysoH₂O₂** across a concentration gradient of 1–5 μ M for 24 hours.

At the end of the treatment period, 10 μ L of MTT solution (5 mg/mL in phosphate-buffered saline, PBS) was added to each well and the plates were incubated for an additional 3–4 hours at 37 °C under a humidified 5% CO₂ atmosphere. During this incubation, metabolically active cells reduced the yellow tetrazolium salt (MTT) to insoluble purple formazan crystals.

Following incubation, the medium was carefully removed and 100 μ L of a solubilization solution (typically dimethyl sulfoxide, DMSO) was added to each well to dissolve the formazan crystals. Absorbance was measured at 570 nm using a microplate reader, with background subtraction at 630–690 nm where applicable. All treatments were performed in triplicate or quadruplicate, and data were expressed as percentage of viable cells relative to untreated controls. Control experiments were performed using LysoTracker™ Deep Red (Invitrogen, L12492) to validate lysosomal targeting and staining specificity. Cell viability assessment by MTT assay confirmed that **LysoH₂O₂** exhibits ~80% cell viability (~20% cytotoxicity) across the working concentration range of 1–5 μ M in SK-Lu-1 cells, establishing its biocompatibility for live-cell fluorescence imaging applications.

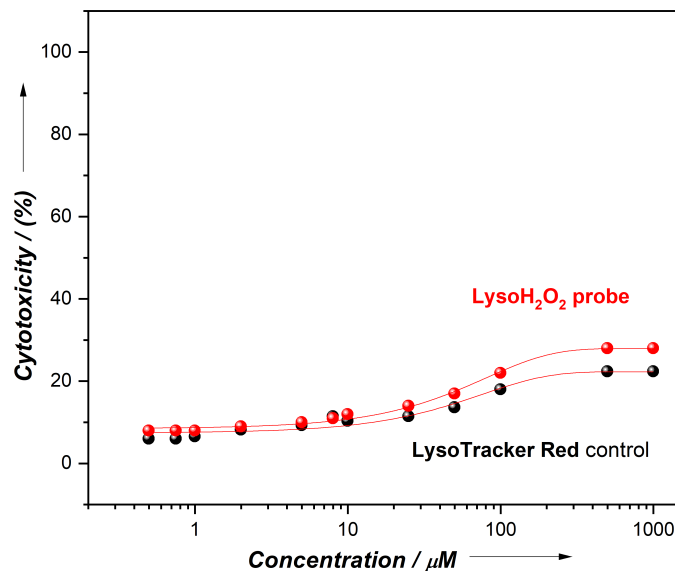


Figure S1. Cell viability assessment by MTT assay confirmed that **LysoH₂O₂** exhibits ~80% cell viability (~20% cytotoxicity) across the working concentration range of 1–5 μM in SK-Lu-1 cells, establishing its biocompatibility for live-cell fluorescence imaging applications.

General Probe Synthesis

Synthesis of 6-(2-morpholinoethyl)-1*H*-benzo[*de*]isoquinoline-1,3(2*H*)-dione (**LysoH₂O₂**). General Methods: All commercial reagents were used as received. NMR spectra were recorded on a 300 MHz spectrometer. Chemical shifts (δ) are reported in ppm relative to residual solvent peaks. High-resolution mass spectrometry (HRMS) was performed using electrospray ionization (ESI).

Procedure: A mixture of 4-bromo-1, 8-naphthalic anhydride, 1 mmol, 0.278 g and sodium azide NaN₃ (0.195 g, 3 mmol) were dissolved in *N,N*-dimethylformamide (DMF) / H₂O (v/v, 10/1, 20 mL) and stirred at 80 °C for 4 h. Then, the solvent was poured into ice water (10 ml), and the precipitated solid was filtered. Then, the solid was dissolved in DMF (20 ml), added with 2ml of aqueous NaHS (0.224 g, 4 mmol) and further stirred at 90 °C for 1h. Next, the reaction solution was added with ice water and adjusted to weak acidity. Subsequently, a solid was filtered and added 10 mL DMF containing 4-(2-aminoethyl)morpholine (1.3 mmol, 0.75 mL) and heated at 120 °C under controlled pressure for 8 hours with vigorous stirring. After cooling to room temperature, the crude material was dissolved in acetone (~25 mL) and purified by flash column chromatography on silica gel (eluent: DCM/MeOH 95:5). The product was isolated as a yellow solid. Yield: 67% (0.225 g). ¹H NMR (300 MHz, CDCl₃) δ: 8.61 (dd, *J* = 26.60 Hz, 1H), 8.42 (dd, *J* = 12.87 Hz, 1H), 8.12 (dd, *J* = 21.45 Hz, 1H), 7.68 (m, *J* = 22.32 Hz, 1H), 6.90 (d, *J* = 13.73 Hz, 1H), 4.97 (s, 2H), 4.34 (t, *J* = 21.45 Hz, 2H), 3.71 (m, *J* = 21.45 Hz, 4H), 2.72 (t, *J* = 26.60 Hz, 2H), 2.63 (s, 4H). ¹³C NMR (75 MHz, CDCl₃) δ 154.2, 147.1, 133.7, 129.3, 127.2, 124.8, 122.1, 119.5, 117.21, 109.4, 107.81, 67.25, 57.16, 55.18, 36.41. HRMS (ESI+) *m/z*: Calculated for C₁₈H₂₀N₃O₃ [M+H]⁺: 326.14. Found: 326.1498.

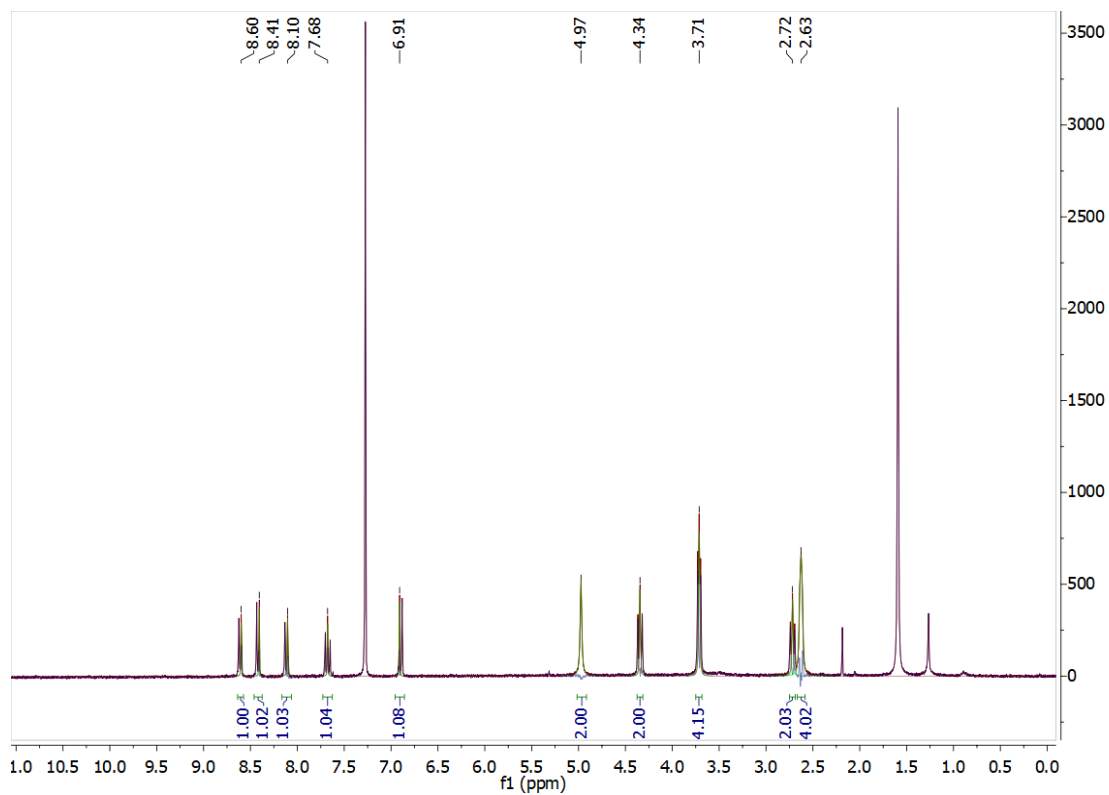


Figure S2. ^1H NMR spectrum (300 MHz, CDCl₃) of LysoH₂O₂.

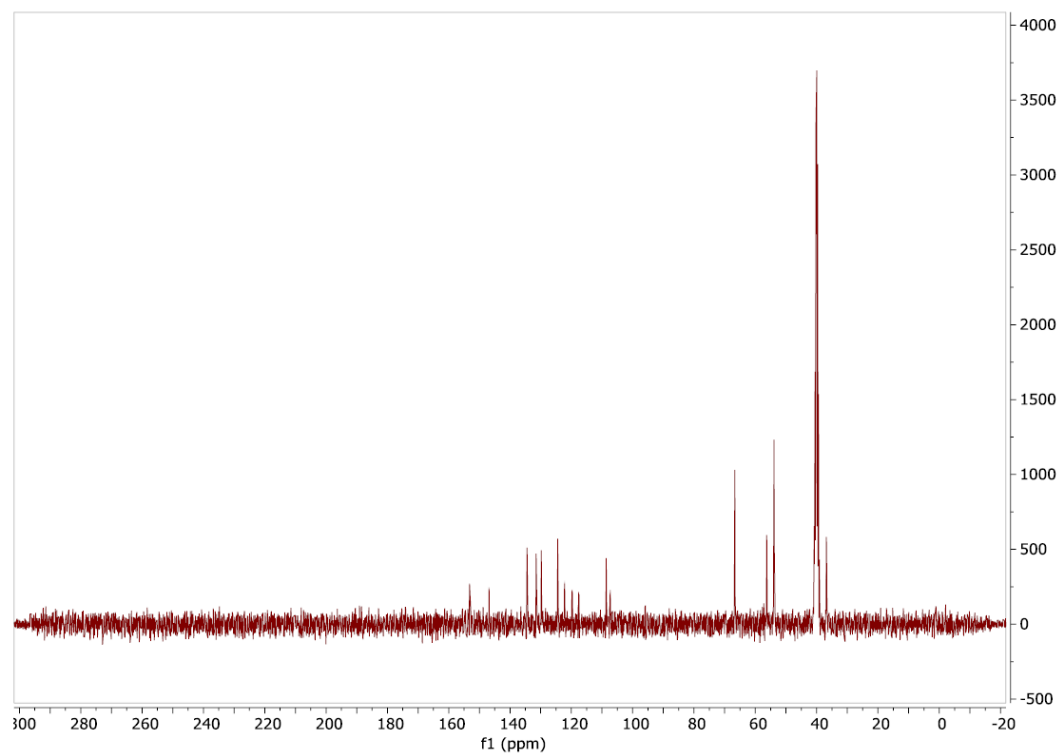


Figure S3. ^{13}C NMR spectrum (75 MHz, CDCl₃) of LysoH₂O₂.

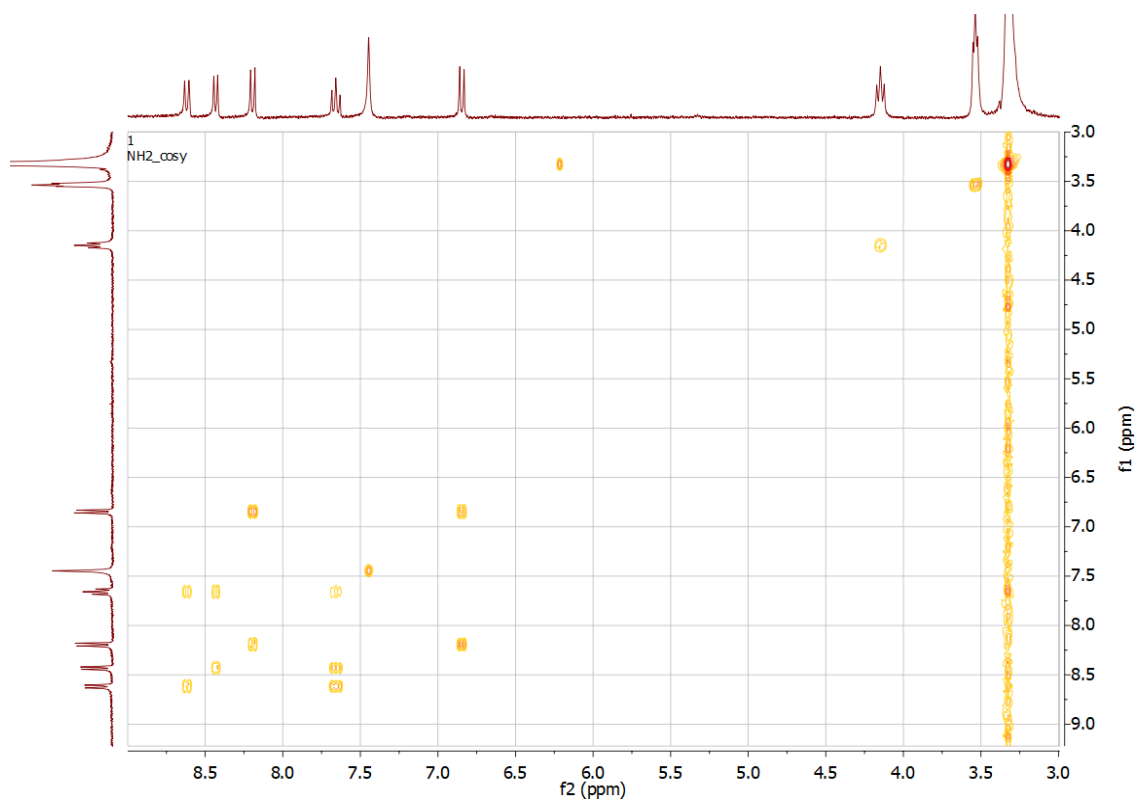


Figure S4. ^1H - ^1H COSY NMR spectrum (300 MHz, CDCl_3) of **LysoH₂O₂**.

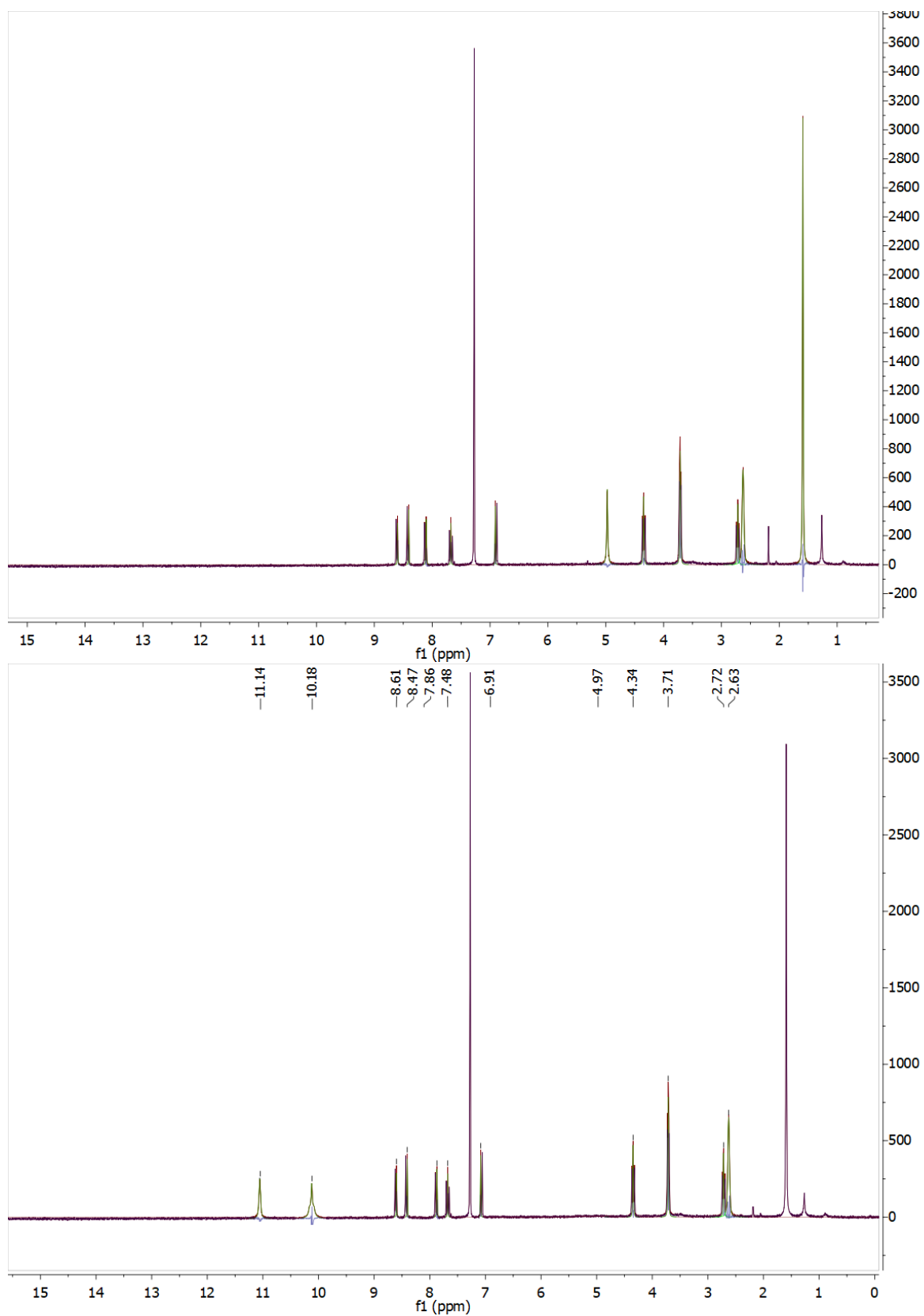


Figure S5. $^1\text{H-NMR}$ spectra (300 MHz, CDCl_3) of LysoH_2O_2 (above) and after adding 1 equiv H_2O_2 14 % (below, δ : 10.15 (s, 1H, -OH signal) and 11.05 (s, 1H, -NH- signal).

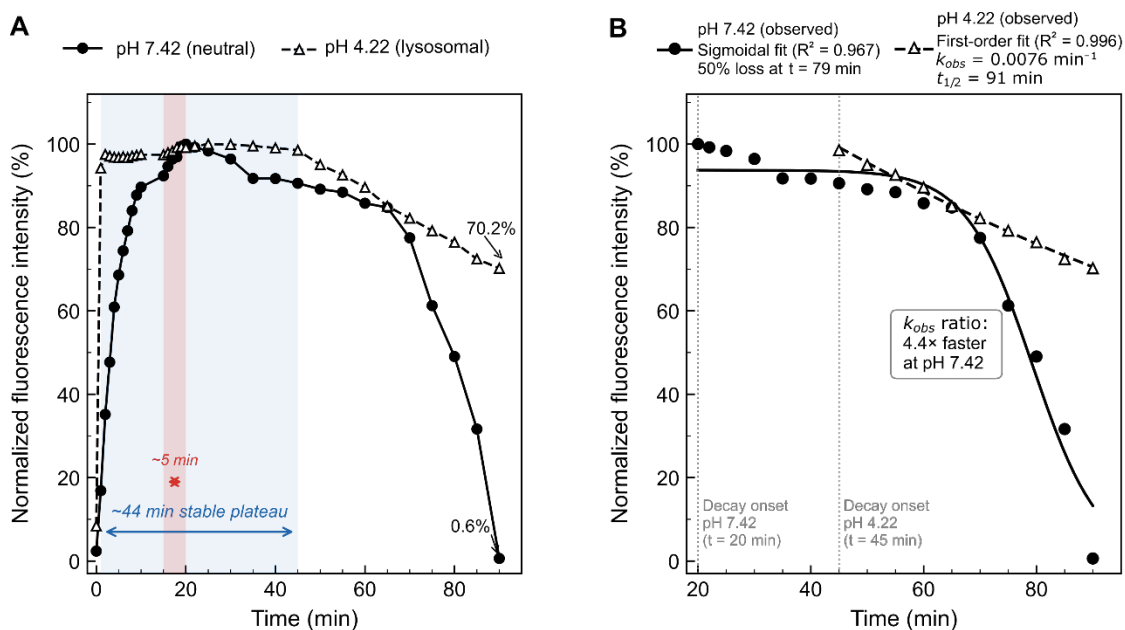


Figure S6. Extended fluorescence time-course profiles ($\lambda_{exc} = 430$ nm, $\lambda_{em} = 550$ nm) of **LysoH₂O₂** (40 μ M) following activation by 2 equiv. H₂O₂ at pH 4.22 (5 mM HTAB micellar medium, open triangles, dashed line) and pH 7.42 (aqueous medium, filled circles, solid line), monitored over 90 minutes. (A) Full normalized profiles showing the rapid fluorescence turn-on upon H₂O₂ addition, the stable plateau phase at pH 4.22 (~44 min), and the contrasting immediate decay onset at pH 7.42. Shaded regions indicate the plateau windows at each pH. Retention values at $t = 90$ min are indicated. (B) Decay phase analysis with kinetic fits: sigmoidal decay fit for pH 7.42 (50% signal loss at $t = 79$ min, $R^2 = 0.967$) and pseudo-first-order decay fit for pH 4.22 ($k_{obs} = 0.0076 \text{ min}^{-1}$, $t_{1/2} = 91$ min from decay onset at $t = 45$ min, $R^2 = 0.996$). Vertical dotted lines indicate decay onset times. The 4.4-fold difference in decay rate constants between pH 7.42 and pH 4.22 quantifies the efficiency of the lysosomal acid-protection mechanism. Data are normalized to the maximum fluorescence intensity observed at each pH condition.

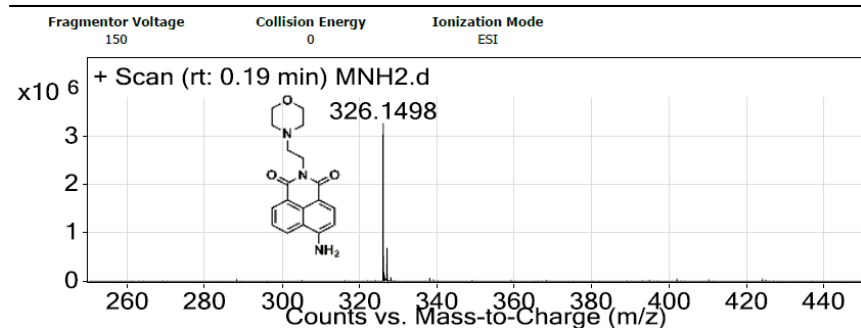


Reporte de Análisis Cualitativo LANCIC IQ



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Acq Method	Ine directa pos (ACN).m	Acquired Time	2/10/2025 1:28:09 PM
IRM Calibration Status	Success	DA Method	Everardo ESI MS.m
Comment			
Sample Group		Info.	
Stream Name	LC 1	Acquisition SW	6200 series TOF/6500 series
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User Spectra



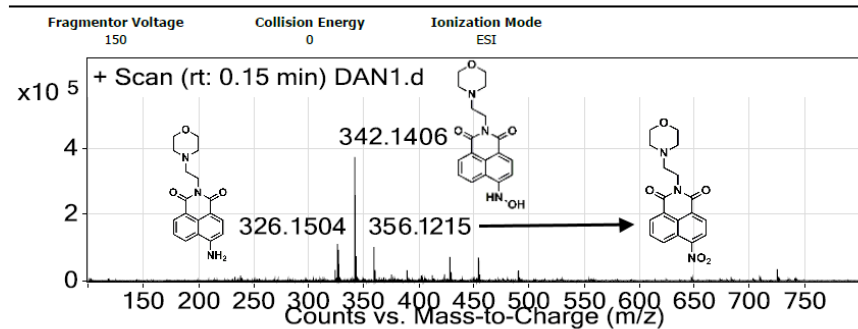
Peak List

m/z	z	Abund
326.1498	1	3295260.56
326.2962		207846.56
327.1529	1	710666.12

Figure S7. HRMS (ESI-TOF⁺) spectra of **LysoH₂O₂** (m/z: Calculated for C₁₈H₂₀N₃O₃ [M+H]⁺: 326.14, found: 326.1498).

Data Filename	MNHOH.d	Sample Name	MNHOH
Sample Type	Sample	Position	P1-F2
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IRM Calibration Status	Success	DA Method	Everardo ESI MS.m
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Sample Group		Info.	
Stream Name	LC 1	Acquisition SW	6200 series TOF/6500 series
		Version	Q-TOF B.06.01 (B6172 SP1)

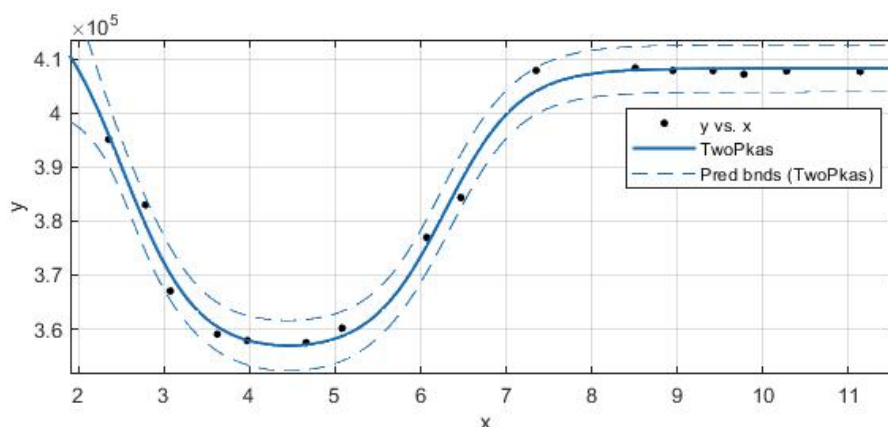
User Spectra



Peak List

m/z	z	Abund
326.1504	1	111884.59
327.153	1	93804.62
342.1406	1	385306.57
356.1215	1	92353.7
428.1815	1	70188.59
454.1722	1	70014.18
725.2416	1	35472.96

Figure S8. HRMS (ESI-TOF+) spectra of **LysoH₂O₂** after adding 1 equiv H₂O₂ 14 % (m/z: Calculated for **hydroxylamine** derivative C₁₈H₂₀N₃O₄ [M+H]⁺: 342.14, found: 342.1406). Also, the **nitro-oxidized** derivative was seen, m/z: C₁₈H₁₈N₃O₅ [M+H]⁺: 356.12, found: 356.1215).



Coefficients with extrapolation (with 95% confidence bounds):

$$P1 = 4.083e+05 \text{ (} 4.063e+05, 4.102e+05 \text{)}$$

$$P2 = 3.554e+05 \text{ (} 3.522e+05, 3.586e+05 \text{)}$$

$$P3 = \text{pKa's} = \text{(} 2.509, 6.285 \text{)}$$

$$P4 = 4.241e+05 \text{ (} 3.962e+05, 4.519e+05 \text{)}$$

Goodness of fit:

$$\text{SSE: } 5.641e+07$$

$$\text{R-square: } 0.9918$$

$$\text{Adjusted R-square: } 0.9888$$

$$\text{RMSE: } 2264$$

Figure S9. Acid-base profiles and pK_a analysis for **LysoH₂O₂**

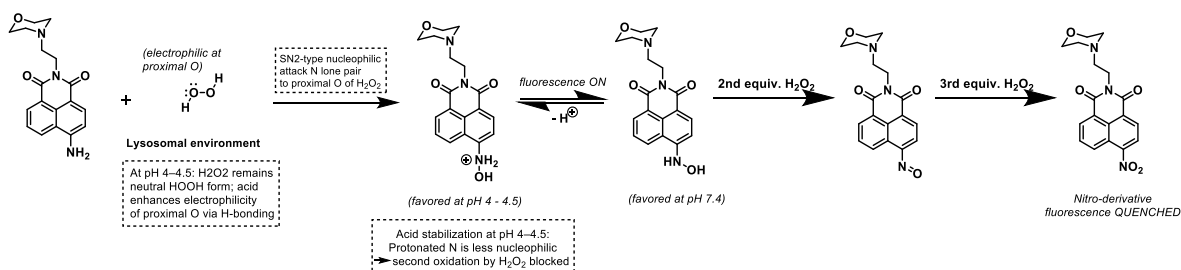


Figure S10. H_2O_2 oxidation mechanism of **LysoH₂O₂**. In the absence of enzymatic catalysis, the reaction of **LysoH₂O₂** with H_2O_2 in the acidic lysosomal environment (pH 4–4.5) proceeds via direct electrophilic oxygen transfer from H_2O_2 to the aromatic amine nitrogen, following a nucleophilic $\text{S}_{\text{N}}2$ -type mechanism at the peroxide oxygen, with water as the leaving group.[E. J. Behrman and J. O. Edwards, in *Progress in Physical Organic Chemistry*, ed. A. Streitwieser and R. W. Taft, Wiley, New York, 1967, **4**, 93–155.] The protonated hydroxylamine intermediate $[\text{Ar}-\text{NH}_2-\text{OH}]^+$ is stabilized at pH 4–4.5 through protonation of the nitrogen, which diminishes its nucleophilicity and prevents a second oxidative event. At physiological pH (7.42), the free base form $\text{Ar}-\text{NH}-\text{OH}$ is a more reactive nucleophile, facilitating overoxidation to the nitroso and subsequently nitro derivatives with complete fluorescence quenching.[B. G. Gowenlock and G. B. Richter-Addo, *Chem. Rev.*, 2004, **104**, 3315–3340.] This pH-gated selectivity constitutes the core operating principle of the lysosomal protection mechanism in **LysoH₂O₂**.

Table S1. Photophysical parameters of **LysoH₂O₂** and its hydroxylamine product in 5 mM HTAB micellar medium at pH 4.22.

Parameter	LysoH ₂ O ₂ (probe)	Hydroxylamine product
λ_{obs} (nm)	480	480
λ_{em} (nm)	480–500	550
ϵ (M ⁻¹ cm ⁻¹)	6,550	6,890
Φf^2	0.008	0.21
Turn-on factor (Φ/Φ_0)	—	26.3
Medium	5 mM HTAB, pH 4.22	5 mM HTAB, pH 4.22

¹ $\lambda_{obs} = \lambda_{em} = 480$ nm for both the probe and the hydroxylamine product.

² Fluorescence quantum yields (Φf) determined by the relative method using fluorescein in 0.1 M NaOH ($\Phi_{ref} = 0.95$) as the reference standard. All measurements performed at room temperature (25 °C).

Experimental procedure: fluorescence quantum yield determination

Fluorescence quantum yields (Φf) were determined using the relative method of Würth *et al.*,¹ with fluorescein in 0.1 M NaOH ($\Phi_{ref} = 0.95$) as the reference standard. This method requires recording the UV-Vis absorption and fluorescence emission spectra of both the reference and the sample solutions at multiple dilutions under identical instrumental conditions.

Sample preparation. Stock solutions of **LysoH₂O₂** and its hydroxylamine product were prepared in 5 mM HTAB micellar medium at pH 4.22 (acetate buffer). The hydroxylamine product was generated in situ by treatment of **LysoH₂O₂** (40 μ M) with 5 equivalents of H₂O₂ at pH 4.22, followed by confirmation of complete activation by fluorescence spectroscopy prior to quantum yield measurements. Working solutions were prepared at five different concentrations spanning a range of absorbance values between 0.01 and 0.10 at the excitation wavelength ($\lambda_{exc} = 480$ nm) to minimize inner filter effects.

Spectroscopic measurements. UV-Vis absorption spectra were recorded on a Thermo Scientific Evolution diode array spectrophotometer. Fluorescence emission spectra were recorded on an Edinburgh Instruments FS500 spectrofluorometer (or Cary Eclipse fluorimeter) using an excitation wavelength of $\lambda_{exc} = 480$ nm, with matched slit widths for both excitation and emission monochromators. All measurements were performed at room temperature (25 °C) under identical instrumental settings. The fluorescence spectra were corrected for instrument response.

Calculation. The integrated fluorescence intensity was plotted against the corresponding absorbance value at λ_{exc} for each solution. The quantum yield of the sample (Φf) was calculated according to the following equation:

$$\Phi f = \Phi_{ref} \times (m_{sample} / m_{ref}) \times (n_{sample}^2 / n_{ref}^2)$$

where Φ_{ref} is the quantum yield of the reference standard (fluorescein, $\Phi_{\text{ref}} = 0.95$ in 0.1 M NaOH); m_{sample} and m_{ref} are the slopes of the integrated fluorescence intensity versus absorbance plots for the sample and reference, respectively; and n_{sample} and n_{ref} are the refractive indices of the solvents used for the sample and reference solutions, respectively ($n = 1.333$ for aqueous media at 25 °C; refractive index correction factor = 1 when both sample and reference are measured in aqueous solution).

Precautions. All absorbance values were kept below 0.10 to avoid inner filter effects. Samples were freshly prepared prior to each measurement and protected from ambient light to prevent photobleaching. Each measurement was performed in triplicate and the reported Φ_f values represent the mean of three independent determinations.

Reference: ¹ Würth, C., Grabolle, M., Pauli, J. et al. Relative and absolute determination of fluorescence quantum yields of transparent samples. *Nat Protoc* 8, 1535–1550 (2013). <https://doi.org/10.1038/nprot.2013.087>

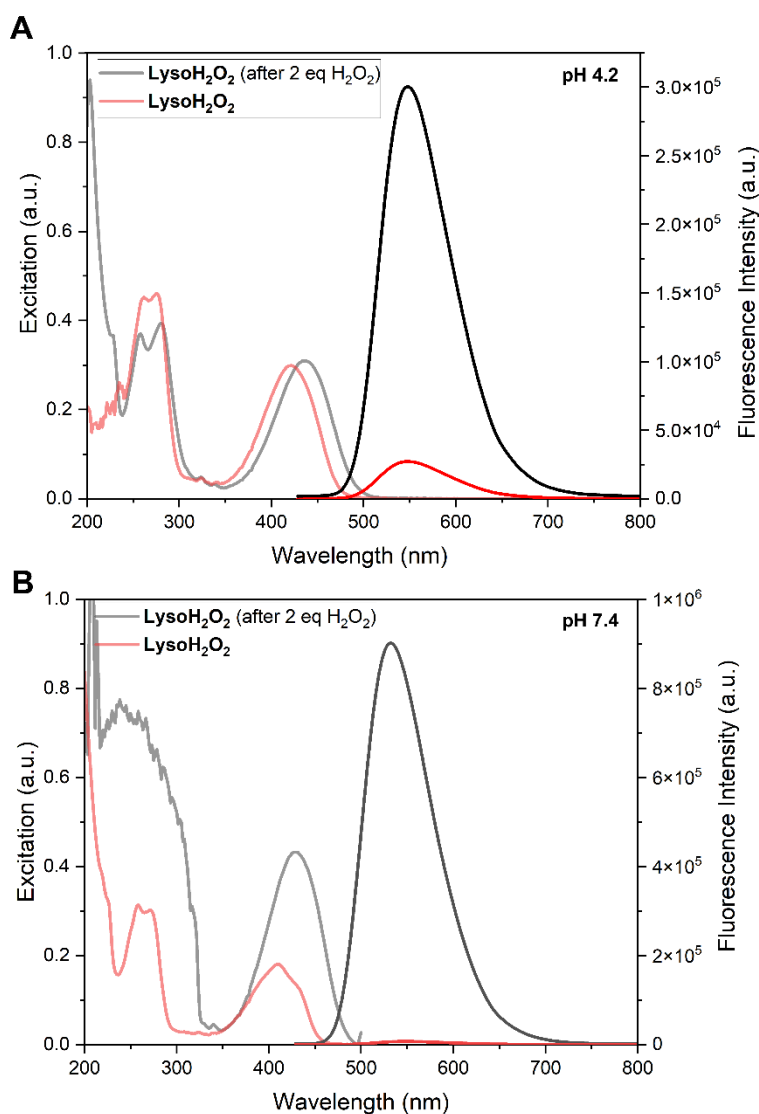


Figure S11. Fluorescence excitation ($\lambda_{\text{em}} = 550$ nm) and emission profiles ($\lambda_{\text{exc}} = 440$ nm) of 40 μM LysoH₂O₂ probe before (red lines) and after (black lines) exposure to 2 equiv. H₂O₂ at pH 4.22 (A) and pH 7.42 (B).

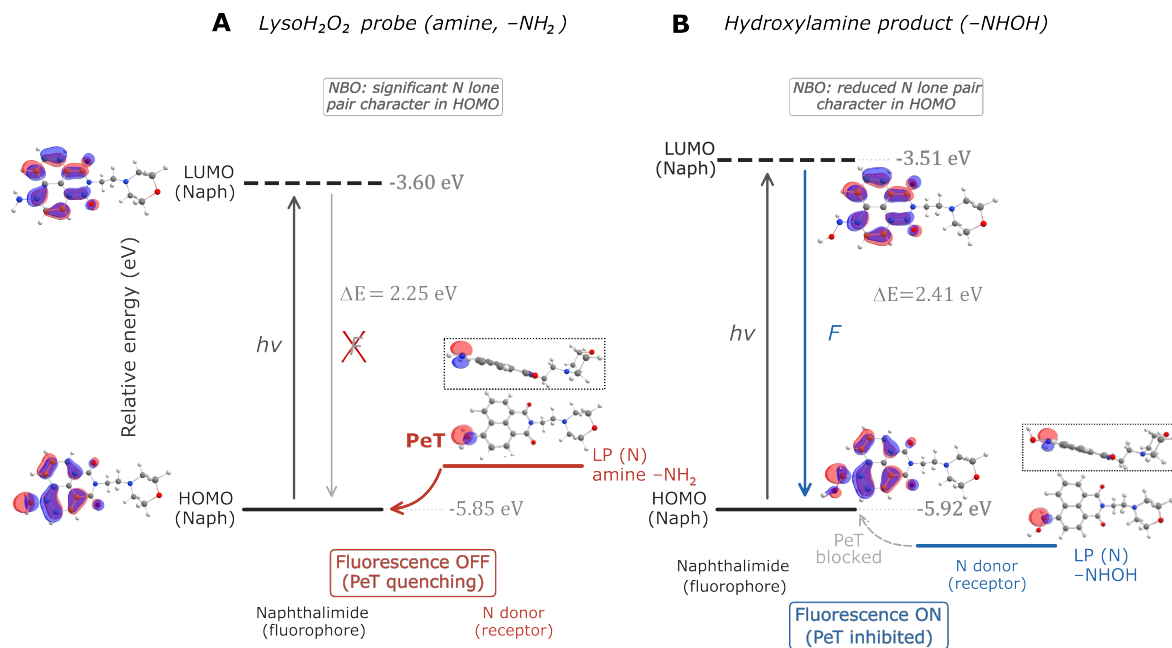


Figure S12. Frontier orbital energy alignment diagram comparing **LysoH₂O₂** (left) and its hydroxylamine product (right), computed at the PBE/6-31G(d,p) level of theory, with NBO orbital representations. Energy levels are given relative to vacuum. In the intact probe, the naphthalimide HOMO and LUMO are located at -5.85 eV and -3.60 eV, respectively ($\Delta E = 2.25$ eV). The nitrogen lone pair (LP) of the amine -NH₂ group lies above the naphthalimide HOMO, enabling photoinduced electron transfer (PeT) from the donor to the photoexcited fluorophore, which quenches fluorescence (OFF state). Upon H₂O₂-mediated oxidation to the hydroxylamine product, the naphthalimide HOMO and LUMO shift to -5.92 eV and -3.51 eV, respectively ($\Delta E = 2.41$ eV, $\Delta\Delta E = +0.16$ eV). The LP of the -NHOH group is lowered below the naphthalimide HOMO, blocking PeT and restoring fluorescence emission (ON state). NBO analysis confirms significant nitrogen lone pair character in the HOMO of the probe (inset, left) and a marked reduction thereof in the hydroxylamine product (inset, right), consistent with PeT inhibition upon oxidation. LP energies are qualitative, derived from isolated fragment NBO calculations of the free amine and hydroxylamine groups. Due to direct conjugation of the amine nitrogen into the naphthalimide π -system, frontier molecular orbitals are delocalized across both donor and acceptor units; the diagram is therefore presented as a qualitative mechanistic representation consistent with established models for 4-aminonaphthalimide-based fluorescent sensors.