Electronic Supplementary Information

Simultaneous Photoactivation of a Fluoroquinolone Antibiotic and Nitric Oxide with Fluorescence Reporting

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Chemicals

All chemicals were purchased by Sigma-Aldrich and used as received. Organic solvents were removed under reduced pressure at 30 °C. Synthetic-purity solvents were used. All solvents used for the spectrophotometric studies were spectrophotometric grade. Ultrapure water (MilliQ) was used.

Synthesis of N-Nitroso Norfloxacin (NF-NO)

Norfloxacin (100 mg, 0.31 mmol) was solubilized in a mixture of THF and acetic acid 1:1 (v/v, 4 mL) and the solution was cooled down with an ice bath. After, sodium nitrite (173 mg, 2.5 mmol) was added to the solution and kept under stirring at 0 ºC for one hour and at room temperature overnight. The reaction mixture was diluted in dichloromethane (DCM), and washed with saturated sodium bicarbonate solution (3 × 20 mL), water (3 × 20 mL), dried over Na_2SO_4 and concentrated to dryness, yielding **NF-NO** as a white solid (44 mg, 40.7%). The product was characterized by NMR spectroscopy (^{1}H-NMR, ^{13}C-NMR, gCOSY and DEPT-edited HSQC). ^{1}H NMR (500 MHz, d_6-DMSO) δ 8.95 (s, 1H), 7.97 (d, J = 13.1 Hz, 1H), 7.24 (d, J = 7.1 Hz, 1H), 4.58 (m, 2H), 4.48 – 4.41 (m, 2H), 3.98 – 3.92 (m, 2H), 3.64 – 3.58 (m, 2H), 3.38 (m, 2H), 1.41 (t, J = 7.1 Hz, 3H). ^{13}C-NMR (126 MHz, d_6-DMSO) δ 176.60, 166.84, 153.99, 152.24, 149.12, 137.69, 120.27, 111.91, 111.73, 106.98, 50.40, 49.53, 49.09, 48.56, 39.61, 29.46, 14.87. NMR spectra are shown in Figures S1, S2, S3 and S4. For comparison, we performed ^{1}H-NMR and gCOSY for commercial norfloxacin, shown in Figures S5 and S6. ESI-MS m/z 349.13 $[M+H]^+$. Theoretical MS m/z 348.12.

Instrumentation

^{1}H and ^{13}C NMR spectra were recorded on a Varian UNITY Inova at 500 MHz. Chemical shifts (δ) are given in parts per million (ppm) and the coupling constants (J) are given in Hz. The following abbreviations are used to designate peak multiplicity: s = singlet, bs = broad singlet, d = doublet, dd = doublet of doublets, t = triplet, q = quartet, quint = quintuplet, m = multiplet.

The separation of the analytes was carried out using an UHPLC system (consisting of thermostated autosampler, column compartment, Diode Array Detector, vacuum degasser and a quaternary pump) (Agilent Series 1290, Agilent Technologies, Santa Clara, CA, USA) equipped with a reversed phase C18 analytical column of 50 mm × 2.1 mm and 1.9 µm particle size (Poroshell 120 EC-C18). Column temperature was maintained at 25 °C. The injected sample volume was 2 µL. Mobile phases A and B were water with 0.1% formic acid and acetonitrile with 0.1% formic acid, respectively. The optimized chromatographic method held the initial mobile phase composition (10% B) constant for 1 min, followed by a linear gradient to 90% B in 4 min, this composition was held for 1 min, then returned to initial conditions in 1 min, a 4-min post-run

time was used for each analysis. The flow-rate used was 0.2 mL/min. This UHPLC system was connected to an ultra-high definition quadrupole time-of-flight mass spectrometer Model 6545 Agilent (Agilent Technologies, Santa Clara, CA, USA) equipped with electrospray Jet Stream Technology, operating in positive ion mode, using the following operation parameters: capillary voltage: 3000 V; nebulizer pressure: 35 psi; drying gas: 11 L min^{-1}; gas temperature: 320 °C; sheath gas flow: 11 L/min; sheath gas temperature: 350 °C; nozzle voltage: 1000 V, fragmentor voltage: 175 V; skimmer voltage: 65 V; and octopole RF: 750 V. Accurate mass measurements of primary ions were used to identify **NF** and **NF-NO**. Mass spectra were recorded across 100–1700 m/z range at 2 GHz.

The data were processed using Masshunter workstation qualitative analysis version 10.0 software. Accurate mass measurements of each peak from the extracted ion chromatograms were obtained using a calibrant solution. This solution contains the internal reference masses (purine ($C_5H_5N_4$ at m/z 121.0509 and HP-921 [hexakis-(1H,1H,3H-tetrafluoro-pentoxy)phosphazine] ($C_{18}H_{18}F_{24}N_3O_6P_3$) at m/z 922.0098. The instrument provides a mass resolving power of 30,000 ± 500 (m/z 1522).

UV-Vis spectra absorption and fluorescence emission spectra were recorded with a Perkin Lambda 365 spectrophotometer and a Spex Fluorolog-2 (mod. F111) spectrofluorimeter, respectively, under nitrogen or in air-equilibrated solutions, using either quartz cells with a path length of 1 cm. Fluorescence decays were acquired using the same fluorimeter above, equipped with a TCSPC Triple Illuminator. The samples were excited with a pulsed diode excitation source (Nanoled) at λ_{exc} = 370 nm, and the decays were collected at λ_{em} = 415 nm. The system allowed measurement of fluorescence lifetimes with a time resolution of 200 ps. The multiexponential fit of the fluorescence decay was obtained using the following equation:

$$I(t) = \Sigma\alpha_i \exp^{(-t/\tau i)}$$

where I is the fluorescence intensity, α is the relative amplitude and τ is the lifetime.

For the flash photolysis experiments, all of the samples were excited with the third harmonic of Nd–YAG Continuum Surelite II–10 laser (355 nm, 6 ns FWHM), using quartz cells with a path length of 1.0 cm. The excited solutions were analyzed with a Luzchem Research mLFP–111 apparatus with an orthogonal pump/probe configuration. The probe source was a ceramic xenon lamp coupled to quartz fibre-optic cables. The laser pulse and the mLFP–111system were synchronized by a Tektronix TDS 3032 digitizer, operating in pre-trigger mode. The signals from a compact Hamamatsu photomultiplier were initially captured by the digitizer and then transferred to a personal computer, controlled by Luzchem Research software operating in the National Instruments LabView 5.1 environment. The solutions were deoxygenated via bubbling with a vigorous and constant flux of pure nitrogen (previously saturated with solvent). In all of these

experiments, the solutions were renewed after each laser shot (in a flow cell of 1 cm optical path), to prevent sample photodegradation. The sample temperature was 295 ± 2 K. The energy of the laser pulse was measured at each shot with a SPHD25 Scientech pyroelectric meter.

Direct monitoring of NO release for samples in solution was performed by amperometric detection with a World Precision Instrument, ISO-NO meter, equipped with a data acquisition system, and based on direct amperometric detection of NO with short response time (< 5 s) and sensitivity range 1 nM – 20 μM. The analog signal was digitalized with a four-channel recording system and transferred to a PC. The sensor was accurately calibrated by mixing standard solutions of $NaNO_2$ with 0.1 M H_2SO_4 and 0.1 M KI according to the reaction [1S]:

$$4H^+ + 2I^- + 2NO_2^- \rightarrow 2H_2O + 2NO + I_2$$

Irradiation was performed in a thermostated quartz cell (1 cm pathlength, 3 mL capacity) using a continuum laser with λ_{exc} = 405 nm having a bean diameter of 1.5 mm. NO measurements were carried out under stirring with the electrode positioned outside the light path in order to avoid NO signal artefacts due to photoelectric interference on the ISO-NO electrode.

Steady-state photolysis and photouncaging quantum yield.

Photolysis experiments were performed by irradiating the samples in solution in a thermostated quartz cell (1 cm pathlength, 3 mL capacity) under gentle stirring, by using a blue Light Emitting Diode (LED) (λ_{exc} = 415-420 nm) having an irradiance on the samples of *ca*. 60 mW cm^{-2}. The photouncaging quantum yield was determined within the 20% transformation by using the following equation [2S]:

$$\Phi = [\mathbf{NF}] \times V/t \times (1\text{-}10^{-A}) \times I$$

where, [**NF**] is the concentration of phototransformed **NF-NO**, V is the volume of the irradiated sample, t is the irradiation time, A is the absorbance of the sample at the excitation wavelength and I the intensity of the excitation light source. The concentrations of the phototransformed **NF-NO** was determined spectrophotometrically, by taking into account the absorption changes at 230 and $\Delta\varepsilon_{230}$ = 6000 M^{-1} cm^{-1}. I was calculated by potassium ferrioxalate actinometry.

Nitrite assay

NO_2^- were first evaluated through the well-known, highly sensitive (detection limit on the order of the picomoles) fluorometric bioassay of Misko *et al.* [3S] based on the ring closure of the nonfluorescent 2,3- diaminonaphthalene (DAN) with nitrite, the main oxidation product of NO, to form the highly fluorescent product 2,3-diaminonaphthotriazole (DANT). Aliquots of 2 mL of solutions of **NF-NO** were irradiated with the blue LED for 30 minutes or kept in the dark. Afterward, it was added to 200 μL of DAN solution (DAN 0.30 M in 0.62 M HCl) and stirred for

20 min at room temperature. NaOH 3 M (300 µL) was then added to the previous solution and stirred for 20 min at room temperature. The resultant solution was put into the fluorescent cuvette and the fluorescence emission and excitation spectra were recorded using these parameters: for emission spectra λ_{exc} = 360 nm, slit 1, integration time 0.1s; for excitation spectra λ_{em} = 405 nm, slit 1, integration time 0.1s.

Biological assay

The ATCC *Staphylococcus aureus* ATCC-25923 strain was cultivated in BHI broth incubated at 37ºC. The strain was centrifuged at 6000 rpm for 10 minutes, the supernatant was discarded and PBS was added. The solution was homogenized in a vortex and continued to standardization at 10^8 in a spectrophotometer. For the Minimum inhibitory concentration (MIC) a serial dilution from the NF and NF-NO were performed in Mueller Hinton medium (MH) from the concentration of 50 µg mL^{-1} up to 0.2 µg mL^{-1}. After carrying out the serial dilution, 5 µL of the standardized *Staphylococcus aureus* solution was added to each well; two plates were prepared, with the above dilutions for both compounds, and one was kept protected from light, and the other was irradiated. Irradiation was carried out at 10 using a LED table (420 nm, 43 mW/cm^2). After that, the plates were incubated at 37ºC for 16 hours, and the reading was then carried out, observing the turbidity of the wells, indicating bacterial growth.

Confocal images

To capture images using Confocal scanning microscopy, a 24-hour biofilm with the ATCC strain of *S. aureus* was assembled on a glass coverslip. A concentration of 25 µg/mL was used to obtain the signal. After 10 minutes irradiation (420 nm, 43 mW/cm^2), the images were obtained by confocal fluorescence microscopy with λexc: 405nm and capture above 410 nm.

References

[1S] P. N. Coneski and M. H. Schoenfisch, *Chem. Soc. Rev.*, 2012, **41**, 3753–3758

[2S] M. Montalti, A. Credi, L. Prodi and M. T. Gandolfi, *Handbook of Photochemistry*, 3rd ed., CRC Press, Boca Raton, 2006.

[3S] T. P. Misko, R. J. Schilling, D. Salvemini, W. M. Moore and M. G. Currie, *Anal. Biochem.* 1993, **214**, 11-16.

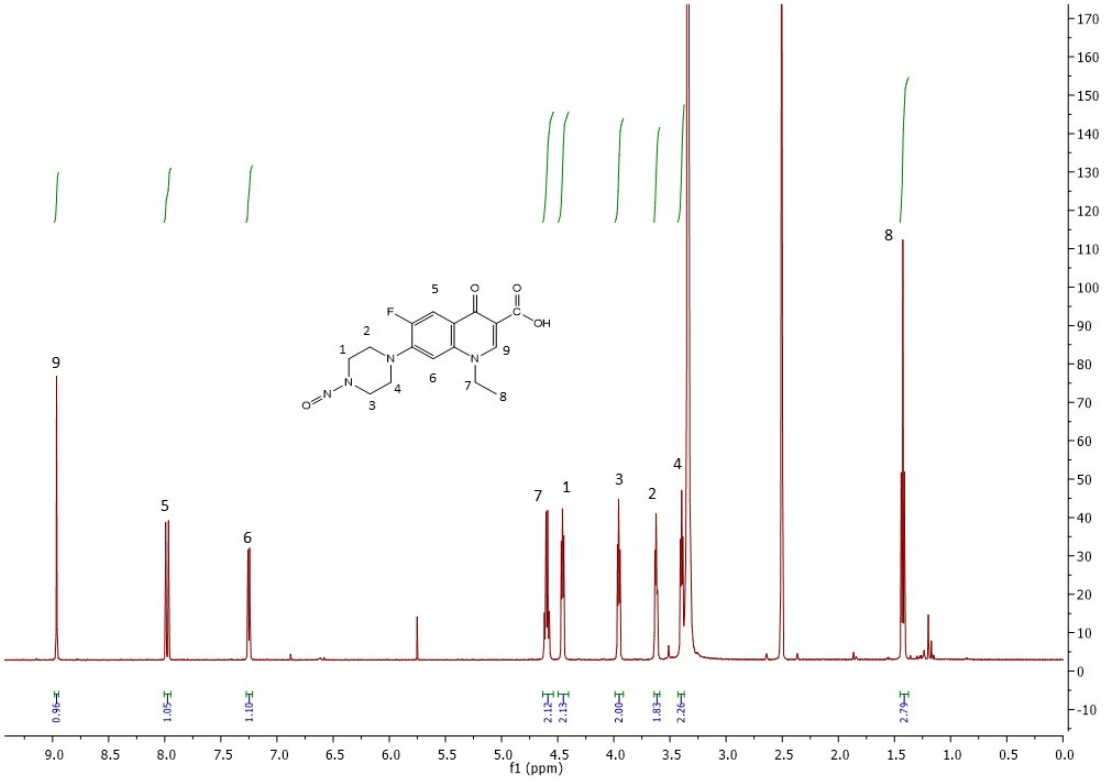

Figure S1. ^{1}H-NMR spectrum of **NF-NO** in d_6-DMSO.

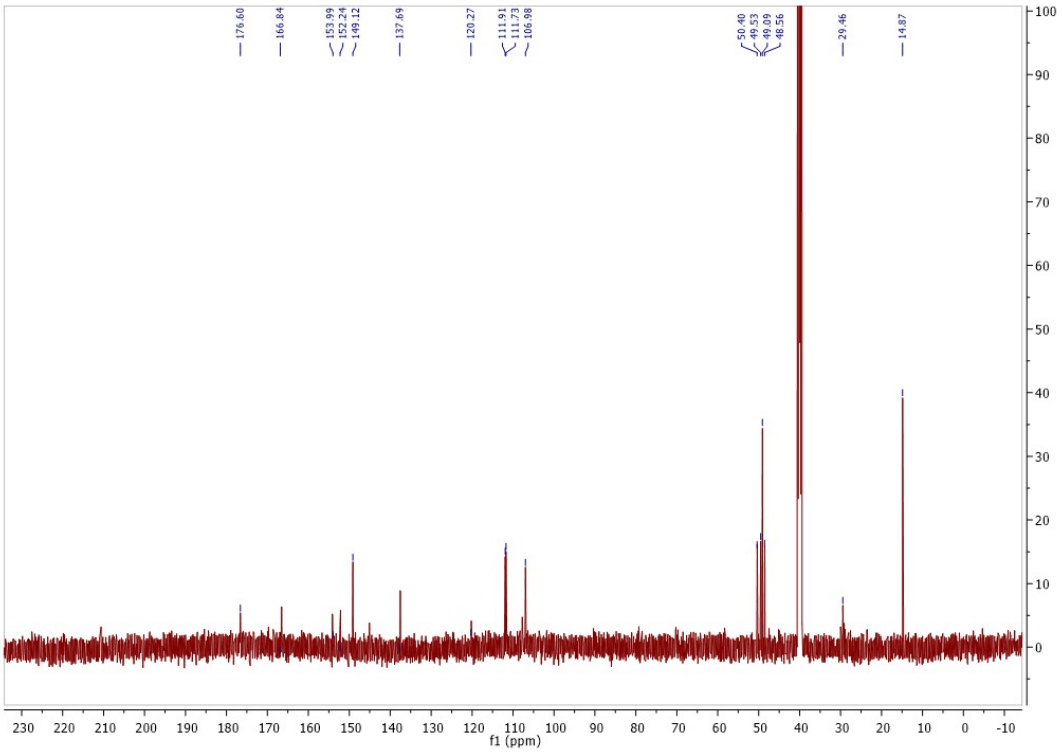

Figure S2. ^{13}C-NMR spectrum of **NF-NO** in d_6-DMSO.

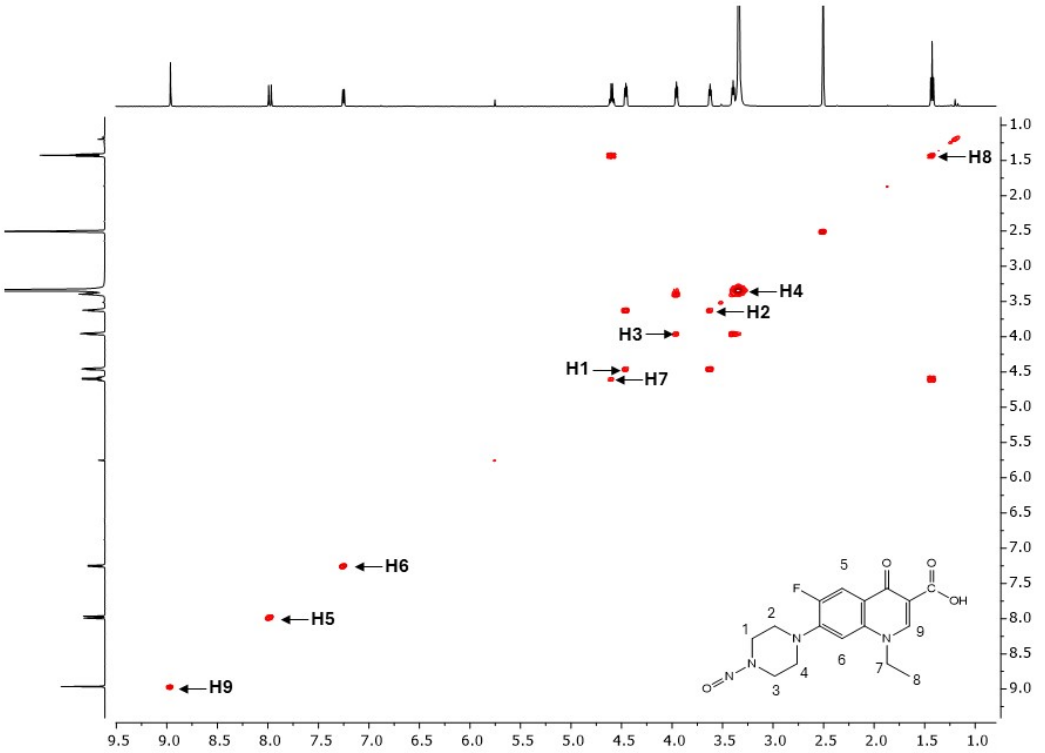

Figure S3. gCOSY of **NF-NO** in d_6-DMSO.

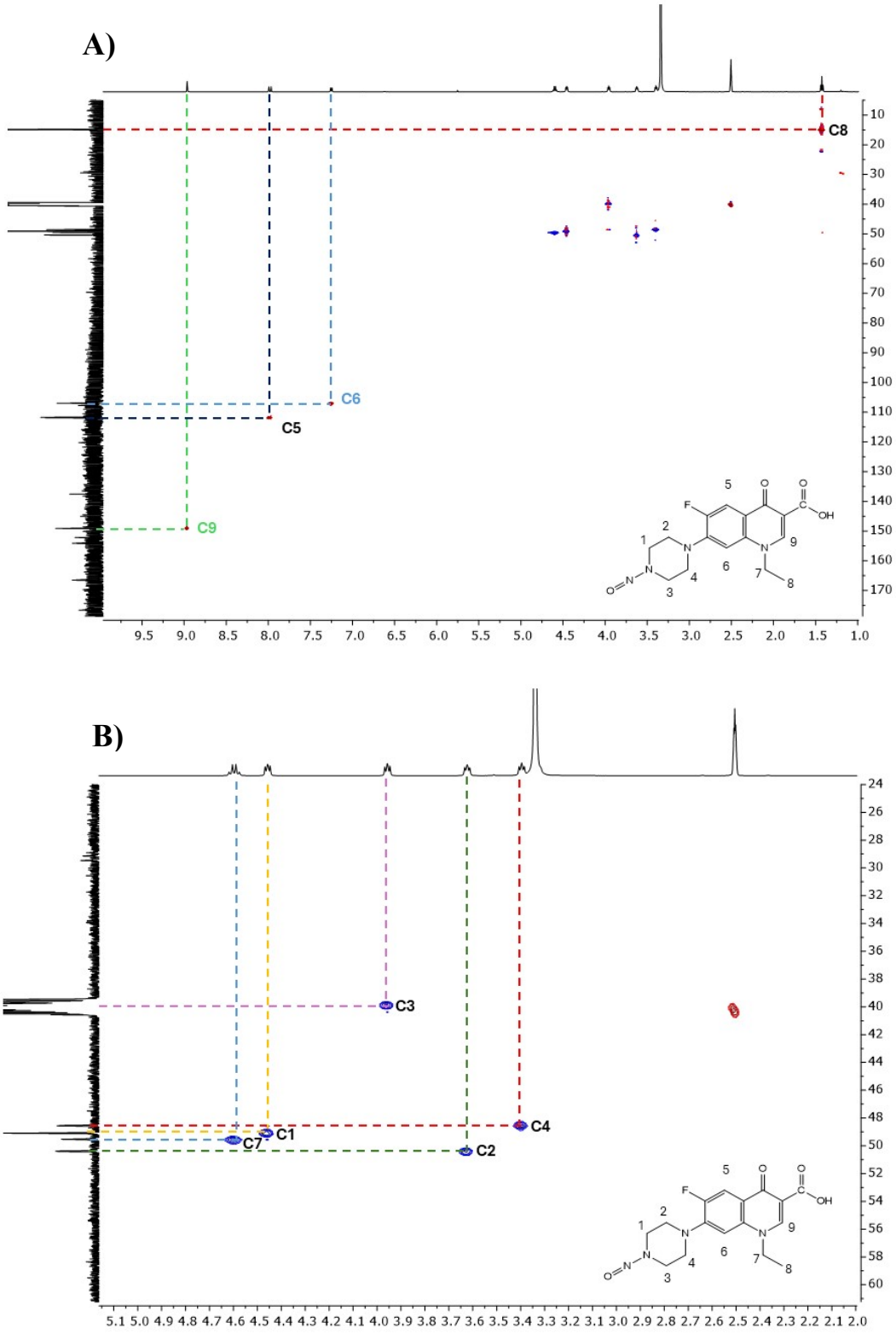

Figure S4. (A) HSQC of **NF-NO** in d_6-DMSO and (B) zoom of the region between 2.0 ppm and 5.1 ppm.

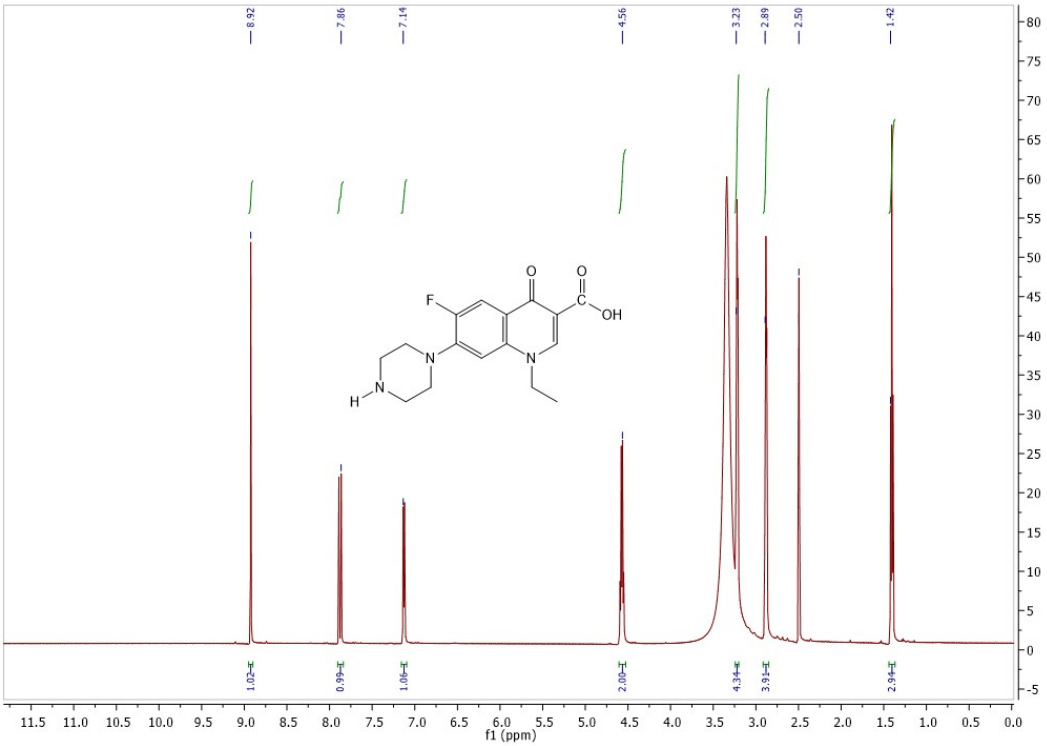

Figure S5. [1]H-NMR spectrum of commercial **NF** in d_6-DMSO.

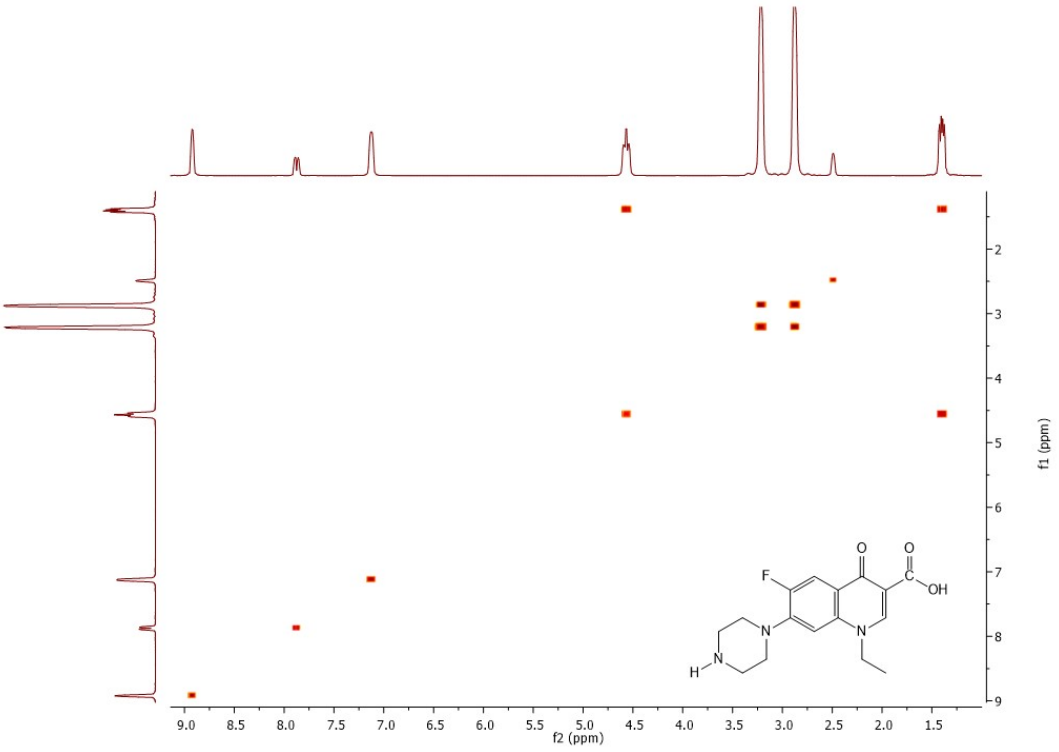

Figure S6. gCOSY of **NF** in d_6-DMSO.

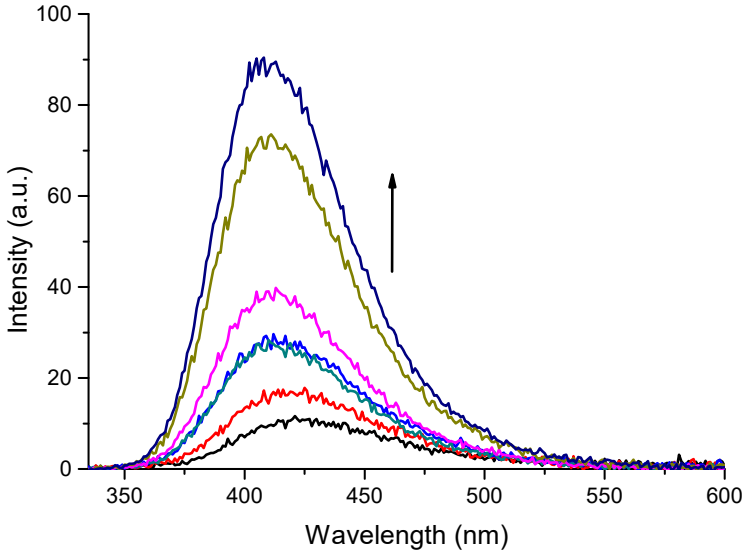

Figure S7. Evolution of the fluorescence emission spectra observed upon exposure of an aqueous solution of **NF-NO** (40 µM) in the presence of TyrOH (800 µM) at λ_{exc} = 420 nm (ca. 20 mW cm^{-2}) for time intervals from 0 to 81 min. T = 25 °C.

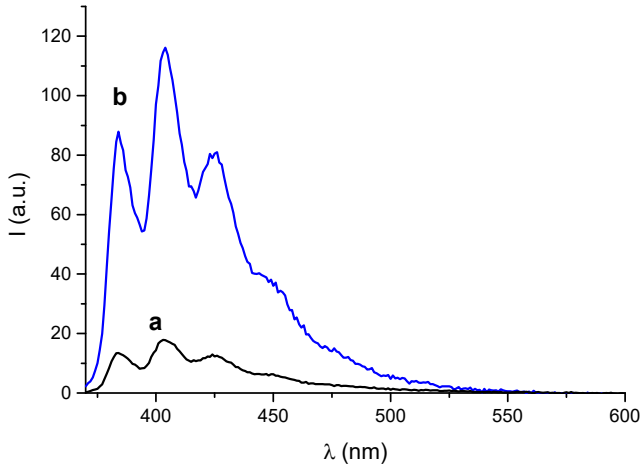

Figure S8. Fluorescence emission spectra obtained after fluorimetric assay of aqueous solutions of **NF-NO** (40 µM) before (a) and after (b) 30 min irradiation with blue light at 420 nm. λ_{exc} = 360 nm, T = 25 °C.

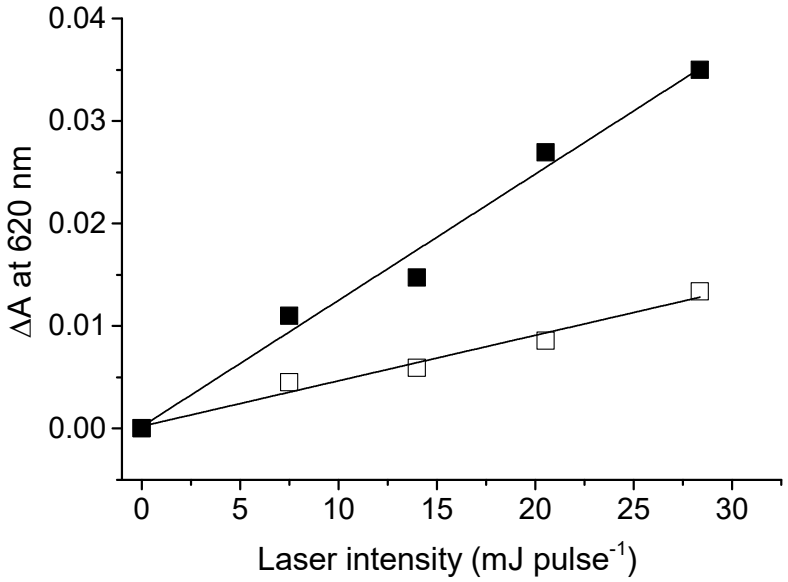

Figure S9. Laser intensity dependence of the absorbance changes, ΔA, taken 0.1 μs after 355 nm laser pulse excitation of N_2-saturated PBS solution (10 mM, pH 7.4) of **NF** (■) and **NF-NO** (□) (both 40 μM) (each point represents signal average of 10 traces).