Supporting Information

Accompanying the manuscript "A fluorescence assay for the detection of hydrogen peroxide and hydroxyl radicals generated by metallonucleases"

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S-1 Experimental section

A. General fluorescence experiment conditions

All fluorescence experiments were carried out at room temperature in MOPS buffer (50 mM, pH 7.4) if not stated otherwise. The concentration of other components (TPA, PBSF, scavenger compounds, ascorbic acid and ROS source) are indicated in the description of the related experiments. The components were added to Eppendorf tubes in the following order: Millipore water, buffer, fluorogenic dye, scavenger, ROS source. The fluorescence spectra were measured using a Cary Eclipse (Varian) spectrofluorimeter in emission scan mode (slit width 5 nm, excitation: λ_{ex} = 320 nm for TPA, λ_{ex} = 485 nm for PBSF) using quartz cells from Hellma Analytics. Ethanol (HPLC grade), DMSO (HPLC Grade), Tris base, MOPS, L-ascorbic acid (≥ 99.9998%), catalase (bovine liver, 2000–5000 U/mg), superoxide dismutase (bovine liver, 5000 U/mg), pyruvate (98%), methional (98%), HCl (37 wt.% in water), hydrogen peroxide (30 wt.% in water, nonstabilized), sodium formate (≥ 99%), mannitol, sodium azide, potassium iodide (≥ 99%, p. a.), sodium iodide (≥ 99%, p. a.), TMP (98%), ^{t}BuOH (≥ 99%), iron(II) sulfate heptahydrate and bleomycin sulfate were purchased from Fisher Scientific, Sigma-Aldrich, VWR, Acros Organics and Riedel-de Haën.

B. Detection of HO$^{\cdot}$ by TPA

Disodium terephthalate (TPA, 99+%, Alfa Aesar) was used as a 5 mM stock solution in water, which was brought to pH 7.4 with 1 M HCl and stored at r.t. Hydroxyl radicals were generated in a Fenton reaction with $FeSO_4$ (10 µM), EDTA (10 µM), H_2O_2 (100 µM) and ascorbate (100 µM). In a total volume of 1000 µL, TPA (500 µM) was incubated with the Fenton reaction mixture for 2.5 h to give the fluorescent HTPA.
No change in reactivity and fluorescence intensity of the TPA stock solution was observed after 3 months of storage at room temperature.

C. Detection of H_2O_2 by PBSF

Pentafluorobenzenesulfonyl fluorescein (PBSF, 98+%, Cayman Chemical Company) was used as a 10 mM stock solution in ethanol and was stored at -20 °C (note: storage in water is not recommended by the vendor). 500 µL of a solution of PBSF (25 µM) and H_2O_2 (0.92 mM) were incubated for 2.5 h to give the fluorescent fluorescein.
No change in reactivity and fluorescence intensity of the PBSF stock solution was observed after 3 months of storage at -20 °C.

It should be noted that the selectivity of PBSF towards hydrogen peroxide is not presented clearly in the original literature, although it is the main conclusion of that work.[1] For reasons of clarity in the context of the present work, the results therein (extracted from Figure 1 and Table 2 of reference 1) are concluded and discussed in the following:

Sample	response – blank	Comment (no response - / positive response +)
blank	0	-
H_2O_2	50	+
HO$^{\cdot}$	< 0	-
^{t}BuOOH	0	-
$O_2^{\cdot-}$	41	+ → H_2O_2 as a side-product from the XO reaction [a]
$O_2^{\cdot-}$ + catalase	< 0	- → H_2O_2 as a side-product from the XO reaction was eliminated [b]
$O_2^{\cdot-}$ + SOD	34	+ → H_2O_2 was formed as a product from the SOD reaction [c]

[a] **Superoxide radical anions** were generated by degradation of hypoxanthine with xanthine oxidase (XO). This oxidative degradation reaction produces superoxide AND hydrogen peroxide.[2] Unfortunately, this fact is not mentioned anywhere in reference 1, but explains well the fluorescence response on superoxide (41) being close to the one of hydrogen peroxide (50). Selectivity over superoxide was additionally shown in cells, where superoxide generation was initiated by paraquat (PQ): PBSF did not respond in contrast to DCFH, which is an "all ROS"-fluorogenic probe. Similarly, the selectivity over **singlet oxygen** was shown: Upon activation with methylene blue (MB), $^1O_2$ was generated, and PBSF again did not respond.

[b] When **catalase** was added, the hydrogen peroxide-related response was eliminated (the value dropped from 41 to <0), showing that superoxide alone does not activate PBSF.

[c] When **superoxide dismutase** was added, the enzymatic reaction caused the generation of hydrogen peroxide leading then again to a value close to the one of hydrogen peroxide (34).

D. Determination of scavenging effects

The above described TPA assay (**B**) was supplemented with one of the following scavenging agents: catalase (2 mg/mL), DMSO (200 mM), KI (10 mM), mannitol (10 mM), methional (10 mM), NaI (10 mM), NaN_3 (10 mM), HCOONa (10 mM), pyruvate (10 mM), SOD (500 U/mL), ^{t}BuOH (200 mM), TMP (10 mM).

The above described PBSF assay (**C**) was supplemented with one of the following scavenging agents: catalase (0.4 mg/mL), DMSO (40 mM), KI (2 mM), mannitol (2 mM), methional (2 mM), NaI (2 mM), NaN_3 (2 mM), HCOONa (2 mM), pyruvate (2 mM), SOD (100 U/mL), ^{t}BuOH (40 mM), TMP (2 mM).

E. Fluorescence assay for metallonucleases

The metallonucleases $[Cu(phen)_2]^{2+}$, $[Cu(oxacyclen)]^{2+}$ and $[Cu(bpa\text{-}ethoxyethan\text{-}1\text{-}ol)]^{2+}$ were prepared as described before.[3–5] $[Cu(phen)_2](NO_3)_2$ (10.9 mg, 20 µmol) was dissolved in 1 mL water at 40 °C and then diluted to obtain a 200 µM stock solution. $[Cu(oxacyclen)(NO_3)]NO_3$ (11.5 mg, 32 µmol) was dissolved in 1 mL water at room temperature and then diluted to obtain a 320 µM stock solution. $[Cu(bpa\text{-}ethoxyethan\text{-}1\text{-}ol)(ClO_4)](ClO_4)$ (11.6 mg, 20 µmol) was dissolved in 1 mL water at room temperature and then diluted to obtain a 200 µM stock solution. Bleomycin sulfate (7.0 mg, 4.6 µmol) was dissolved in 460 µL water at room temperature to obtain a 10 mM stock solution. Iron(II) sulfate heptahydrate (83.4 mg, 300 µmol) was dissolved in 15 mL water and then diluted to obtain a 200 µM stock solution. $[Fe(bleomycin]^{2+}$ was generated *in situ* by mixing equimolar amounts of the iron(II) sulfate and the bleomycin sulfate stock solutions.

The fluorescence assay experiments were carried out as described in **B** and **C**, but the ROS source was substituted by the metallonucleases $[Cu(phen)_2]^{2+}$, $[Cu(oxacyclen)]^{2+}$, $[Cu(bpa\text{-}ethoxyethan\text{-}1\text{-}ol)]^{2+}$ or $[Fe(bleomycin]^{2+}$ (40 µM). Ascorbic acid (250 µM) was added to reduce copper(II) to copper(I) and iron(III) to iron(II),[6] respectively. For the detection of hydroxyl radicals generated by $[Fe(bleomycin]^{2+}$ an additional experiment was carried out with the same conditions that were described above, but with supplementation of hydrogen peroxide (0.92 mM) based on the Fe(II)-catalyzed Fenton reaction.

F. DNA cleavage (standard ROS quenching assay)

The DNA cleavage activity of $[Cu(phen)_2]^{2+}$ towards pBR322 plasmid DNA (Roth) was monitored by agarose gel electrophoresis in duplicates. DNA (0.025 µg/mL) in MOPS buffer (50 mM, pH 7.4) was mixed with $[Cu(phen)_2]^{2+}$ in the presence and absence of ascorbic acid (0.25 mM) and of ROS scavengers [DMSO (200 mM), ^{t}BuOH (200 mM), NaN_3 (10 mM), pyruvate (10 mM), SOD (313 U/mL)]. Millipore water was added up to a total reaction volume of 8 µL. Samples were incubated for 30 min. 1.5 µL of loading buffer (37 µM bromophenol blue, 12.7 mM saccharose) were added to the samples and loaded onto an 1% agarose gel made from SeaKem LE agarose (Lonza) in 0.5X TBE buffer (Fisher Scientific) containing ethidium bromide (0.2 µg/mL, Fisher Scientific). Electrophoresis was carried out at 40 V for 2 h. DNA bands were visualized and photographed in a Bio-Rad GelDoc™ EZ Gel Documentation System. The different DNA forms were quantified by using the Image Lab™ Software. Intensities were put into relation to the DNA reference and for supercoiled DNA (form I) a correction factor of 1.22 was used.[7]

G. LC-MS study

An HPLC system (Agilent Technologies 1260 Infinity II) was coupled to a mass spectrometer (Agilent Technologies 6230 TOF MS). Absorption was measured at 485 nm [λ_{max}(PBSF)] as well as 510 nm, 460 nm, 300 nm and 200 nm. A C18 column (250 mm, 5 µm particle size, 100 Å pore size, LiChrospher® 100 RP-18, Merck) was used. The mobile phase consisted of (A) water and (B) acetonitrile containing each 0.1% TFA (v/v), using linear gradients of 5% B (v/v) in 10 min, followed by 5–95% B in 30 min, followed by 95% B in 10 min. The sample was prepared according to procedure **E** (for incubation conditions *cf.* **A** and **C**), but 20x higher concentrated to warrant the detection by the LC-MS system. MOPS buffer was used 10x lower concentrated to avoid enrichment on the column, however, the concentration (5 mM) was still high enough to ensure a constant pH value.

S-2 Sensitivity assay

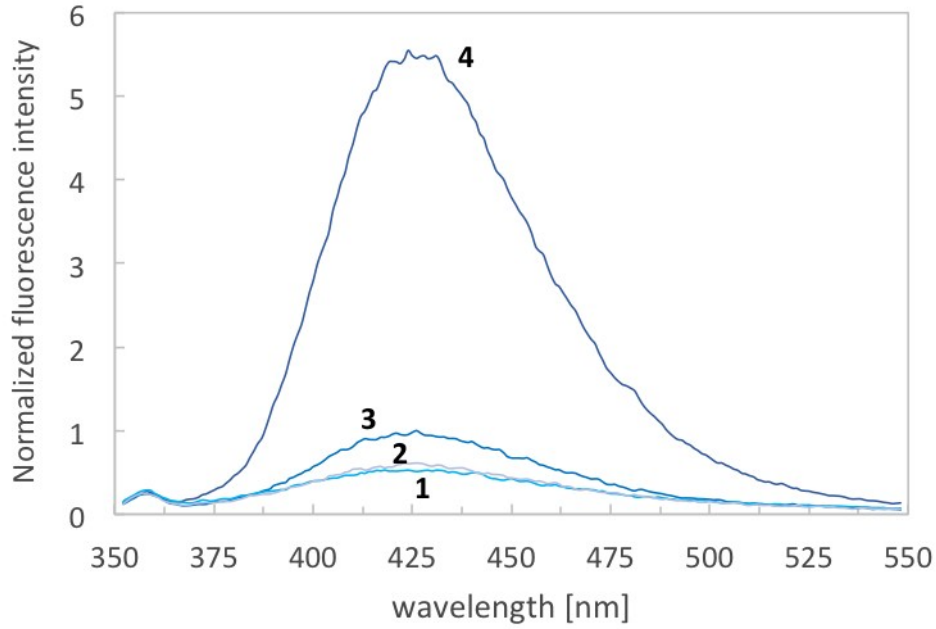


Figure S-2.1 Normalized emission spectra of TPA (0.5 mM) in the presence of $FeSO_4$ (10 µM), H_2O_2, EDTA (10 µM) and ascorbic acid (0.1 mM) in MOPS buffer (50 mM, pH 7.4) at r.t. after 2.5 h. 1: only TPA; 2: 0.01 mM H_2O_2; 3: 0.1 mM H_2O_2; 4: 1 mM H_2O_2.

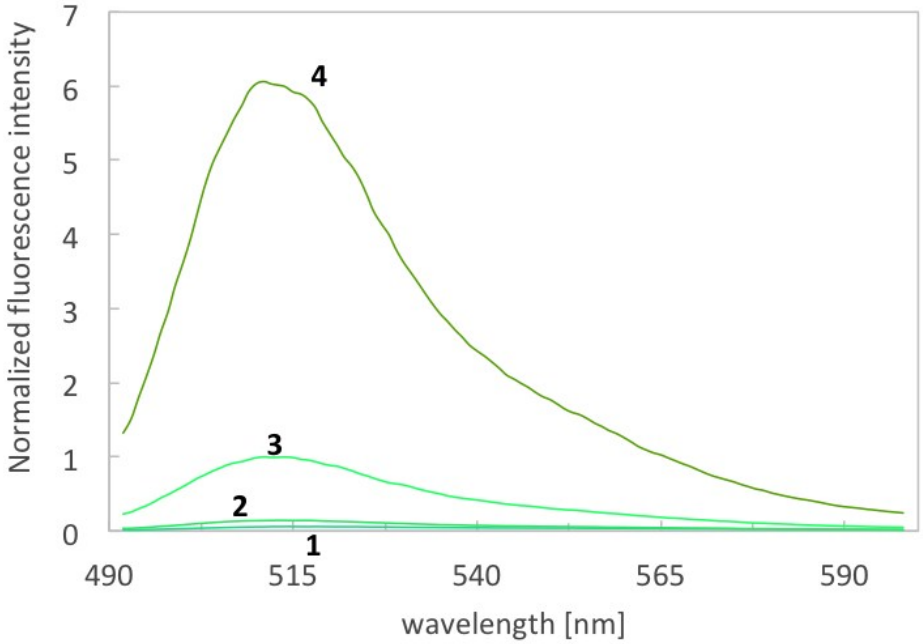


Figure S-2.2 Normalized emission spectra of PBSF (25 µM) in the presence of H_2O_2 in MOPS buffer (50 mM, pH 7.4, 41.2 mM EtOH) at r.t. after 2.5 h. 1: only PBSF; 2: 0.092 mM H_2O_2; 3: 0.92 mM H_2O_2; 4: 9.2 mM H_2O_2.

S-3 TPA applied on metallonucleases

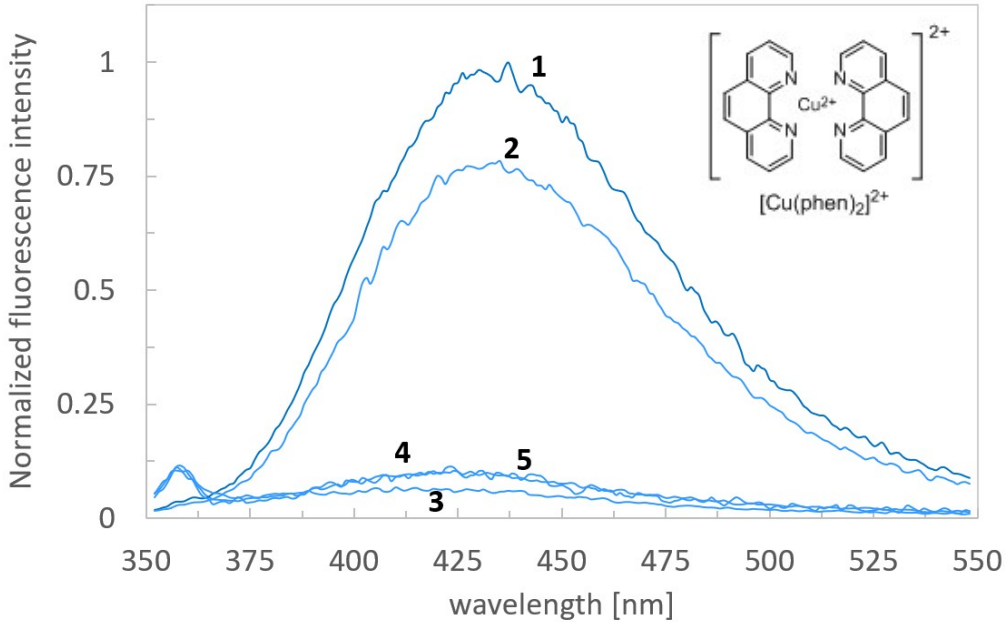


Figure S-3.1 Normalized emission spectra of TPA (0.5 mM) and $[Cu(phen)_2]^{2+}$ (40 µM) in MOPS buffer (50 mM, pH 7.4) at r.t. after 2.5 h. 1: TPA + $[Cu(phen)_2]^{2+}$ + ascorbic acid (0.25 mM); 2: TPA + $[Cu(phen)_2]^{2+}$ + ascorbic acid (0.25 mM) + DMSO (200 mM); 3: TPA + $[Cu(phen)_2]^{2+}$; 4: TPA; 5: TPA + ascorbic acid (0.25 mM).

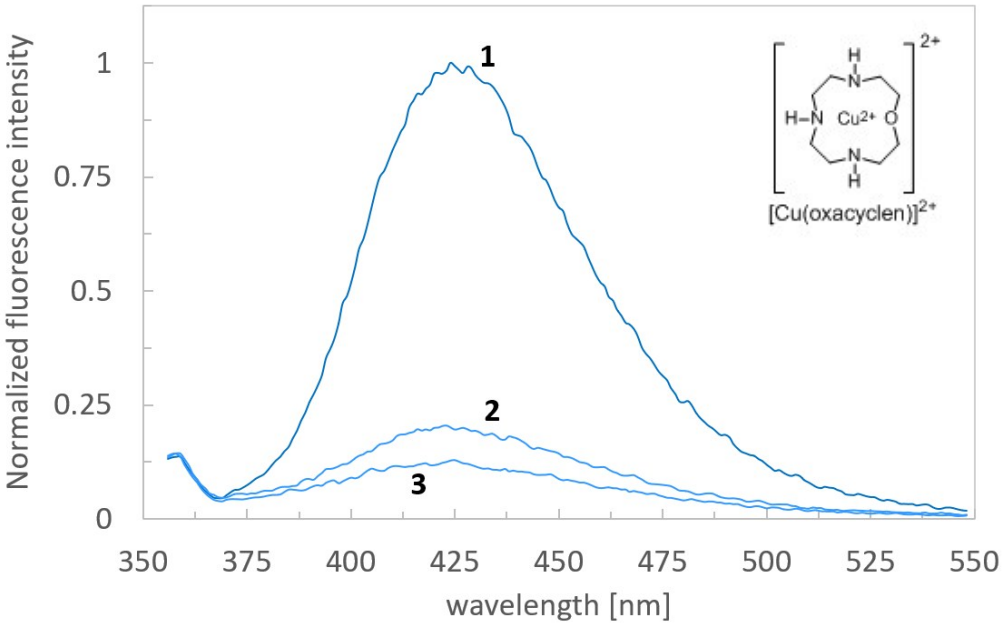


Figure S-3.2 Normalized emission spectra of TPA (0.5 mM) and $[Cu(oxacyclen)]^{2+}$ (40 µM) in MOPS buffer (50 mM, pH 7.4) at r.t. after 2.5 h. 1: TPA + $[Cu(oxacyclen)]^{2+}$ + ascorbic acid (0.25 mM); 2: TPA + $[Cu(oxacyclen)]^{2+}$ + ascorbic acid (0.25 mM) + DMSO (200 mM); 3: TPA + $[Cu(oxacyclen)]^{2+}$.

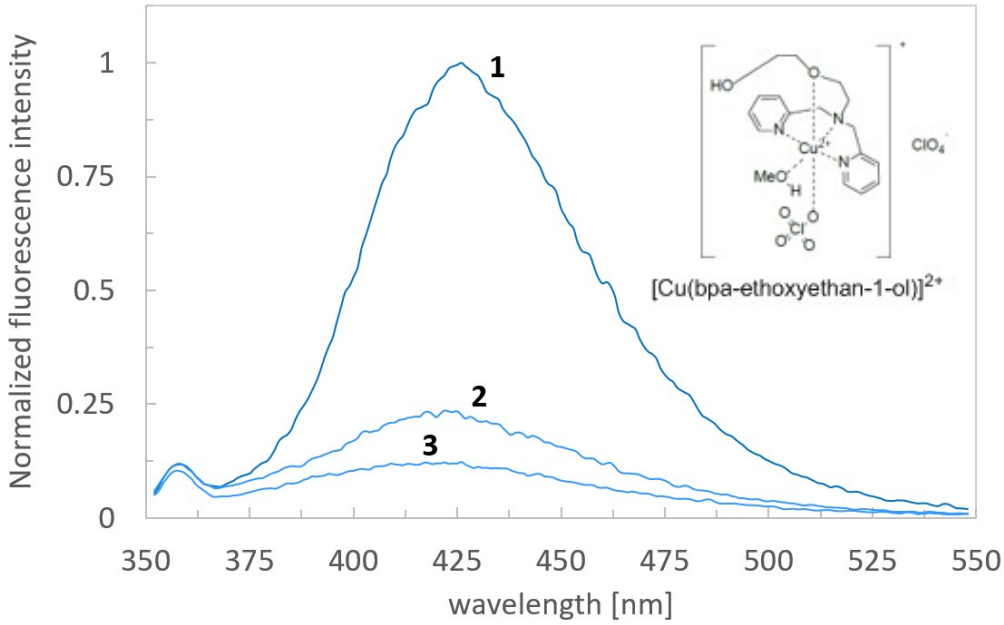


Figure S-3.3 Normalized emission spectra of TPA (0.5 mM) and $[Cu(bpa\text{-}ethoxyethan\text{-}1\text{-}ol)]^{2+}$ (40 µM) in MOPS buffer (50 mM, pH 7.4) at r.t. after 2.5 h. 1: TPA + $[Cu(bpa\text{-}ethoxyethan\text{-}1\text{-}ol)]^{2+}$ + ascorbic acid (0.25 mM); 2: TPA + $[Cu(bpa\text{-}ethoxyethan\text{-}1\text{-}ol)]^{2+}$ + ascorbic acid (0.25 mM) + DMSO (200 mM); 3: TPA + $[Cu(bpa\text{-}ethoxyethan\text{-}1\text{-}ol)]^{2+}$.

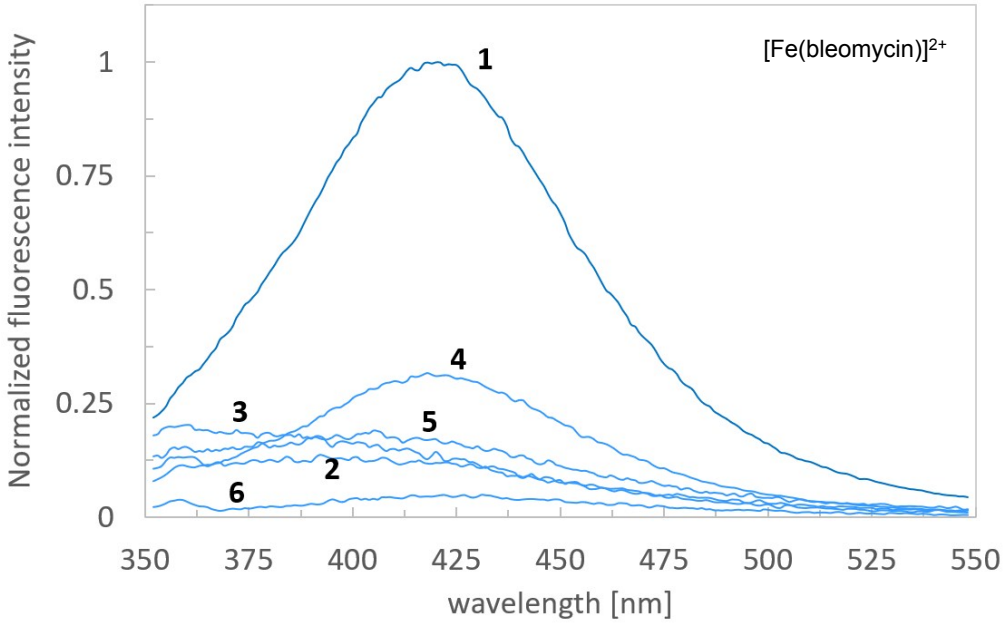


Figure S-3.4 Normalized emission spectra of TPA (0.5 mM) and $[Fe(bleomycin)]^{2+}$ [bleomycin sulfate (40 µM) and $FeSO_4 \cdot 7\ H_2O$ (40 µM)] in MOPS buffer (50 mM, pH 7.4) at r.t. after 2.5 h. 1: TPA + $[Fe(bleomycin)]^{2+}$ + ascorbic acid (0.25 mM) + H_2O_2 (0.92 mM); 2: TPA + $[Fe(bleomycin)]^{2+}$ + ascorbic acid (0.25 mM) + H_2O_2 (0.92 mM) + DMSO (200 mM); 3: TPA + $[Fe(bleomycin)]^{2+}$; 4: TPA + $FeSO_4 \cdot 7\ H_2O$ (40 µM) + ascorbic acid (0.25 mM) + H_2O_2 (0.92 mM); 5: TPA + $[Fe(bleomycin)]^{2+}$ + ascorbic acid (0.25 mM); 6: TPA + H_2O_2 (0.92 mM).

S-4 PBSF applied on metallonucleases

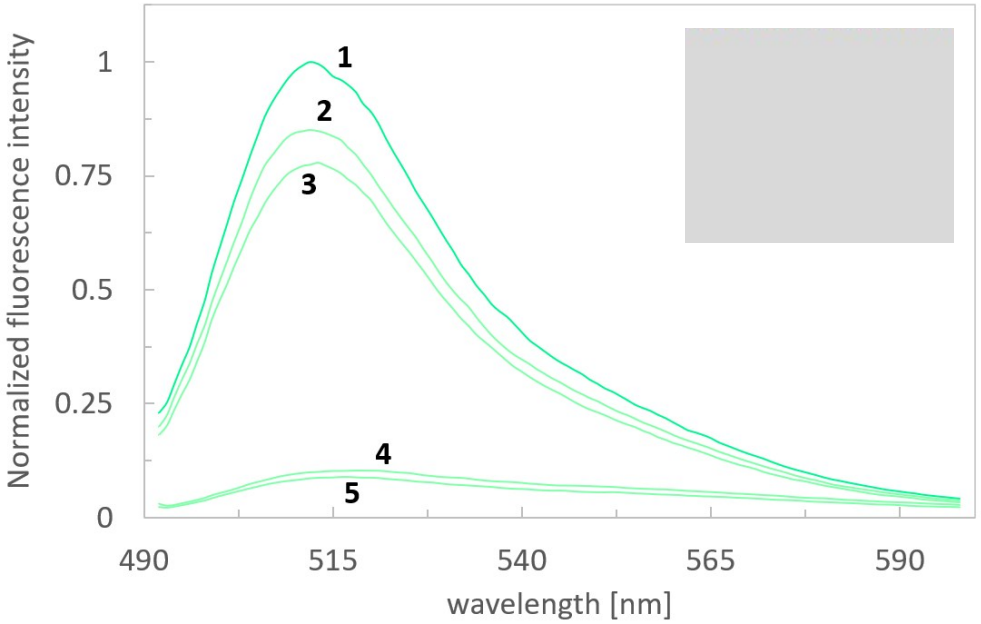


Figure S-4.1 Normalized emission spectra of PBSF (25 µM) and $[Cu(phen)_2]^{2+}$ (40 µM) in MOPS buffer (50 mM, pH 7.4, 41.2 mM EtOH) at r.t. after 2.5 h. 1: PBSF + $[Cu(phen)_2]^{2+}$ + ascorbic acid (0.25 mM); 2: PBSF + $[Cu(phen)_2]^{2+}$ + ascorbic acid (0.25 mM) + pyruvate (2 mM); 3: PBSF + $[Cu(phen)_2]^{2+}$; 4: PBSF; 5: PBSF + ascorbic acid (0.25 mM).

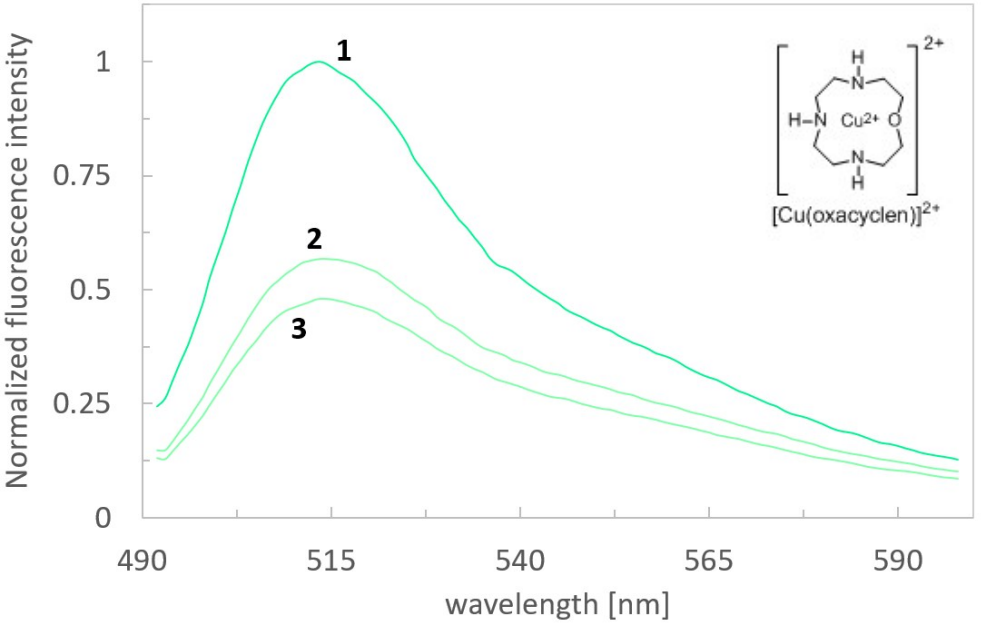


Figure S-4.2 Normalized emission spectra of PBSF (25 µM) and $[Cu(oxacyclen)]^{2+}$ (40 µM) in MOPS buffer (50 mM, pH 7.4, 41.2 mM EtOH) at r.t. after 2.5 h. 1: PBSF + $[Cu(oxacyclen)]^{2+}$ + ascorbic acid (0.25 mM); 2: PBSF + $[Cu(oxacyclen)]^{2+}$ + ascorbic acid (0.25 mM) + pyruvate (2 mM); 3: PBSF + $[Cu(oxacyclen)]^{2+}$.

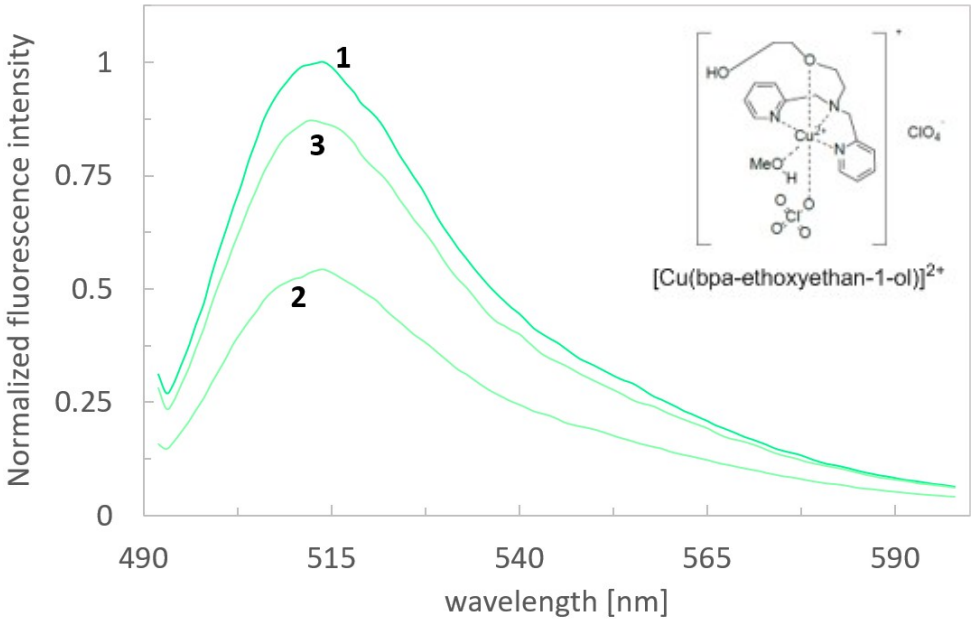


Figure S-4.3 Normalized emission spectra of PBSF (25 µM) and $[Cu(bpa\text{-}ethoxyethan\text{-}1\text{-}ol)]^{2+}$ (40 µM) in MOPS buffer (50 mM, pH 7.4, 41.2 mM EtOH) at r.t. after 2.5 h. 1: PBSF + $[Cu(bpa\text{-}ethoxyethan\text{-}1\text{-}ol)]^{2+}$ + ascorbic acid (0.25 mM); 2: PBSF + $[Cu(bpa\text{-}ethoxyethan\text{-}1\text{-}ol)]^{2+}$ + ascorbic acid (0.25 mM) + pyruvate (2 mM); 3: PBSF + $[Cu(bpa\text{-}ethoxyethan\text{-}1\text{-}ol)]^{2+}$.

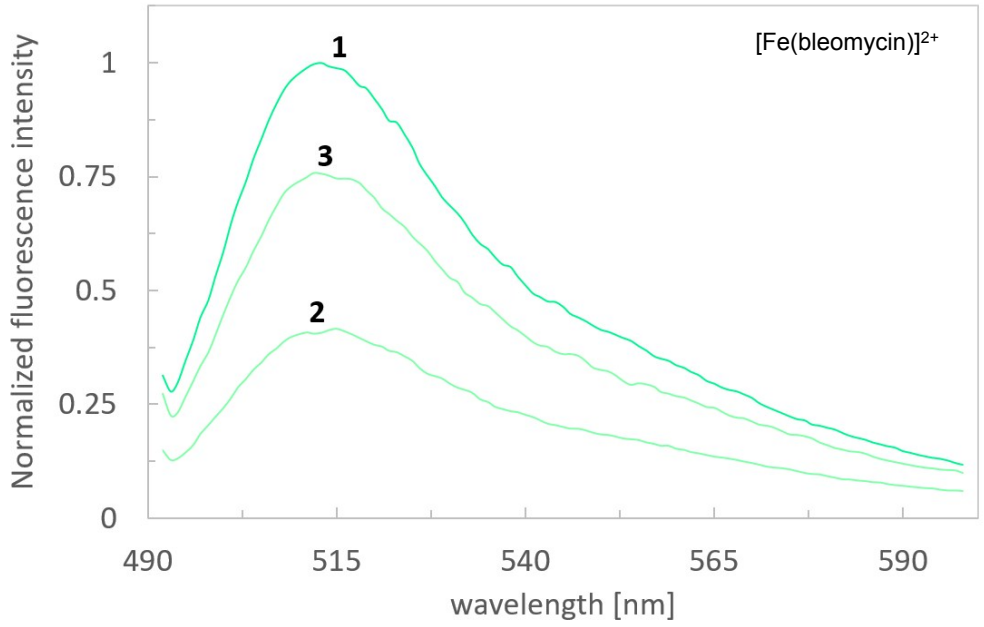
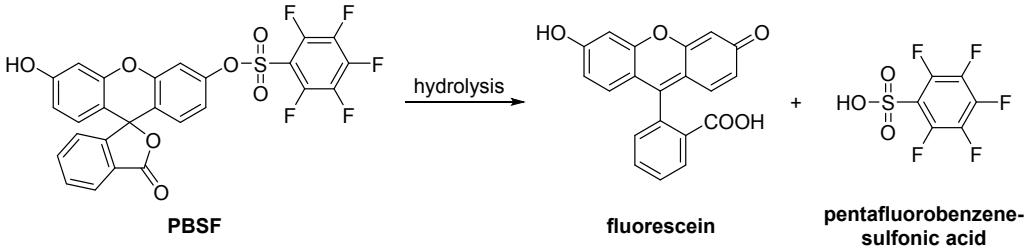


Figure S-4.4 Normalized emission spectra of PBSF (25 µM) and $[Fe(bleomycin)]^{2+}$ [bleomycin sulfate (40 µM) and $FeSO_4 \cdot 7\,H_2O$ (40 µM)] in MOPS buffer (50 mM, pH 7.4, 41.2 mM EtOH) at r.t. after 2.5 h. 1: PBSF + $[Fe(bleomycin)]^{2+}$ + ascorbic acid (0.25 mM); 2: PBSF + $[Fe(bleomycin)]^{2+}$ + ascorbic acid (0.25 mM) + pyruvate (2 mM); 3: PBSF + $[Fe(bleomycin)]^{2+}$.

S-5 LC-MS study on the hydrolysis of PBSF by $[Cu(phen)_2]^{2+}$



PBSF **fluorescein** **pentafluorobenzene-sulfonic acid**

Table S-5 Molecular formula (M), retention time (t_R), $[M + H]^+$ or $[M + Na]^+$ and area of PBSF and its hydrolysis products according to the extracted chromatograms and corresponding mass spectra in Figure S-5. LC-MS conditions are described in S-1G.

Compound	**Molecular formula**	**t_R (min)**	**m/z calcd.**	**m/z found**	**Area**
Fluorescein	$C_{20}H_{12}O_5$	25.5	333.0763 for $[M + H]^+$	333.0784	10552976
Pentafluorobenzene-sulfonic acid	$C_6HF_5O_3S$	22.4	270.9464 for $[M + Na]^+$	271.0058	12186046
PBSF	$C_{26}H_{11}F_5O_7S$	32.7	563.0224 for $[M + H]^+$	563.0255	105028803

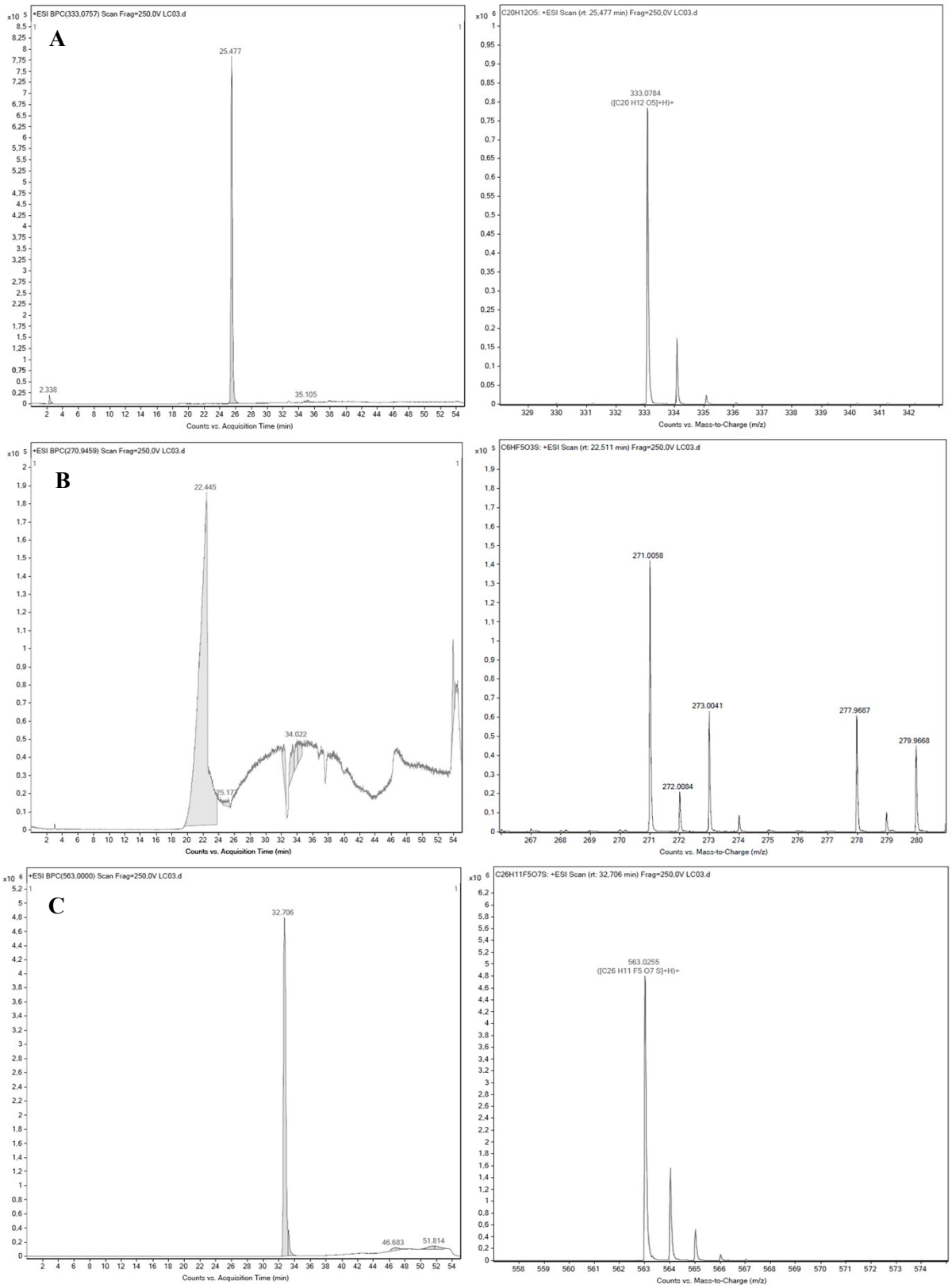
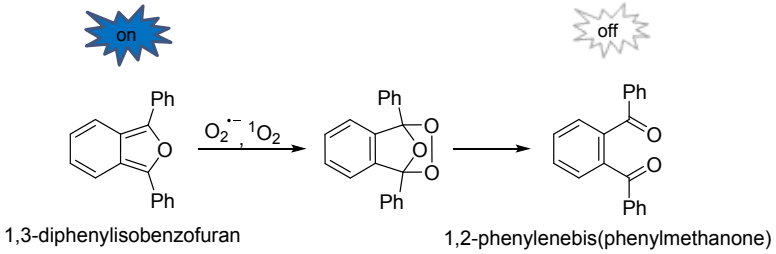


Figure S-5 Extracted chromatograms and corresponding mass spectra of the LC-MS study on the hydrolysis reaction of PBSF (500 μM) by $[Cu(phen)_2]^{2+}$ (800 μM) in MOPS buffer (5 mM, pH 7.4, 41.2 mM EtOH) at r.t. after 2.5 h for **A** fluorescein, **B** pentafluorobenzenesulfonic acid and **C** PBSF.

S-6 DPBF for singlet oxygen and superoxide radical anion detection

The fluorescent dye 1,3-diphenylisobenzofuran (DPBF) is known to be suitable for singlet oxygen and superoxide radical anion detection by forming the non-fluorescent 1,2-phenylenebis(phenylmethanone).[8–11]



1,3-diphenylisobenzofuran 1,2-phenylenebis(phenylmethanone)

The following experiments with DPBF did not establish singlet oxygen and superoxide anion reproducibly. Furthermore, DPBF was only soluble in ethanol or DMSO, whereas an aqueous solution is required for the detection of singlet oxygen and superoxide radical anion generation by metallonucleases in order to ensure conditions similar to those in a physiological environment.

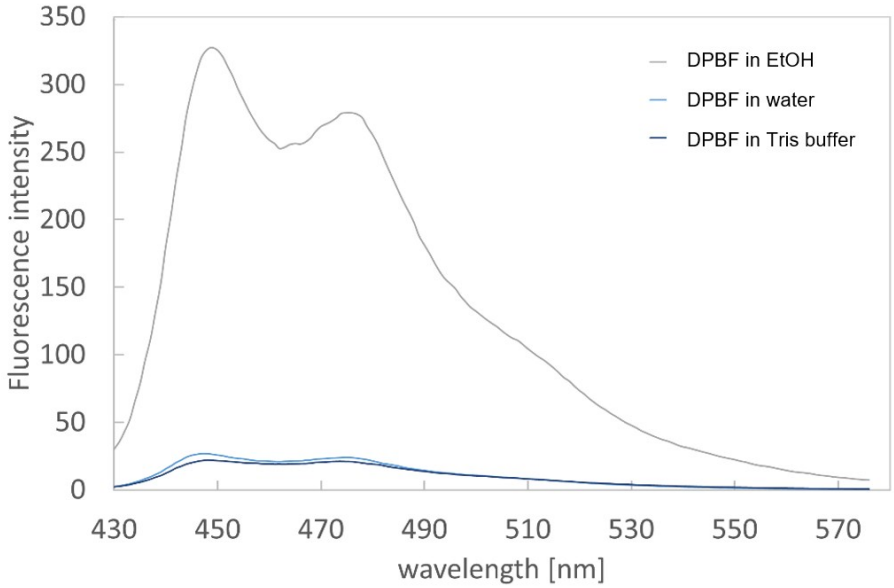


Figure S-6.1 Emission spectra of DPBF (0.5 µM) in ethanol, in water and in Tris buffer (30 mM, pH 7.4), λ_{ex} = 410 nm, λ_{em} = 430–580 nm, direct measurement of spectra without incubation. The stock solution of DPBF was 0.1 mM in ethanol.

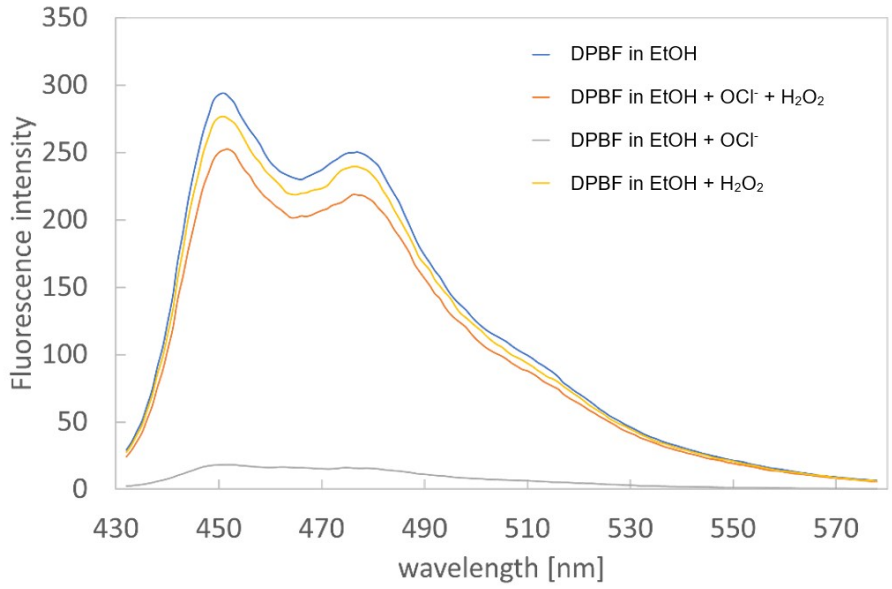


Figure S-6.2 Emission spectra of DPBF (0.5 μM) in ethanol supplemented with the singlet oxygen source NaOCl (106 μM) and hydrogen peroxide (2 mM), λ_{ex} = 410 nm, λ_{em} = 430–580 nm, 3 h incubation time. The stock solution of DPBF was 0.1 mM in ethanol.

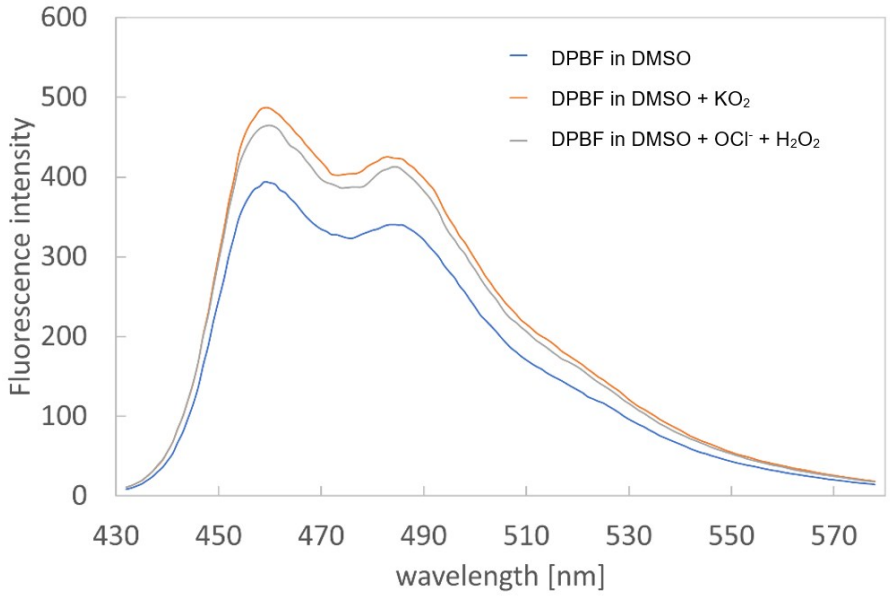


Figure S-6.3 Emission spectra of DPBF (8 μM) in DMSO supplemented with the superoxide radical anion source KO_2 (0.1 mM) or the singlet oxygen source NaOCl (106 μM) and hydrogen peroxide (2 mM), λ_{ex} = 410 nm, λ_{em} = 430–580 nm, 2 h incubation time. The stock solution of DPBF was 1.6 mM in DMSO.

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