# Visible-Light Responsive Supramolecular Enzyme Mimics for Combating Antimicrobial Resistance

Rahul Mahashaya[#], Rishab Pandey[#], Alisha Kamra, Ashish Mazumdar, Subinoy Rana[*] and P. Rajamalli[*]

Affiliation: Materials Research Centre, Indian Institute of Science Bangalore, Karnataka, India

[#] The authors have contributed equally

***Correspondence to:** rajamalli@iisc.ac.in; Phone: +91-80-22932780

**Materials and methods**

## Materials

All commercially available reagents were used without any further purification. Methyl-3,4,5-trihydroxybenzoate was procured from Sigma-Aldrich. Benzohydride and Terephathaldhyde were purchased from SRL. Potassium carbonate and tertiary butyl ammonium iodide were obtained from TCI. Hydrazine monohydrate was acquired from the Spectrochem. 3,3',5,5'-tetramethylbenzidine (TMB) substrate was purchased from SRL. Agar and Luria broth medium (LB) were obtained from HiMedia. Human serum was procured from Sigma-Aldrich. Benzyl chloride and other solvents were purchased from Finar. Milli-Q water was used for all of the studies.

## Methods

**Nuclear Magnetic Resonance (NMR) Spectroscopy.** NMR spectra were recorded on Bruker AV400 NMR spectrometer operating at 400 MHz for $^{1}H$ and at 100 MHz for $^{13}C$.

**HRMS.** Mass spectrometry data was acquired on the MicroMass QTOF Mass Spectrometer.

**UV-Vis and Fluorescence Spectroscopy.** UV-visible spectra were measured at room temperature on PerkinElmer Lambda750 spectrometer and fluorescence emission on a Hitachi F-7000 spectrophotometer using quartz cuvette with pathlength of 1 cm. Enzyme mimetic kinetic assays were performed at 25 °C using a Molecular Devices SpectraMax® M5e microplate reader.

**Field Emission Scanning Electron Microscopy (FESEM).** The Scanning Electron Microscopy imaging was done using the Ultra55 FESEM Karl Zeiss MonoCL instrument with 3 kV and 5 kV emission voltage.

**Fluorescence lifetime**. The emission lifetime measurements were done using a time-correlated single photon counting (TCSPC) spectrometer (Horiba).

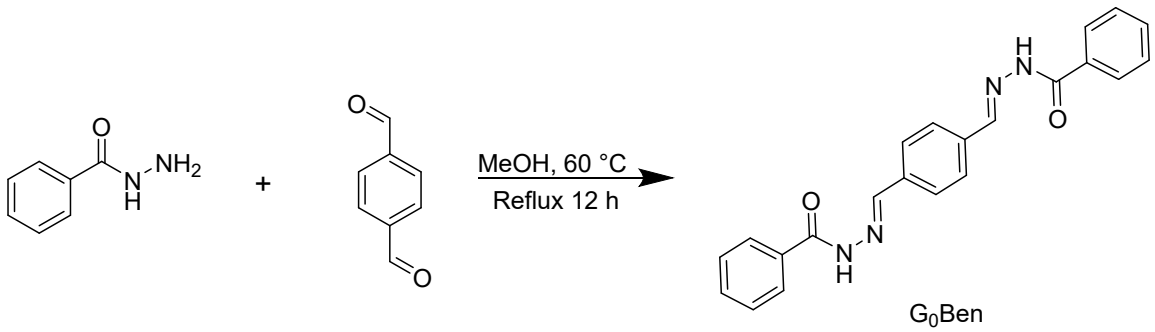
**Thermogravimetric Analysis (TGA).** TGA was carried out on TA Q50 thermogravimetric analyzer.

**Photocurrent measurement** was performed on Keithley SMU 2400 instrument.

**Powder X-ray diffraction (PXRD).** PXRD patterns were attained on Bruker D8 ADVANCE X-ray diffractometer with Cu Kα radiation (λ = 1.54 Å) at 40 kV.

**Solid state UV (DRS).** The spectra were recorded on UV-Vis NIR-PerkinElmer LAMBDA 1050 spectrophotometer.

**Synthesis of $G_0$Ben:**




**Scheme S1.** Synthesis of $G_0$Ben.

5 mmol of benzohydrazide were dissolved in the solvent mixture of methanol and chloroform. To the solution was added terephthaldehyde (2.3 mmol), and the reaction mixture was stirred at 60 ˚C for 12 h. The precipitates obtained were filtered and recrystallized in MeOH to obtain the desired product $G_0$Ben in high purity with 85% yield.[1] The compound synthesized was characterized using $^{1}$H and $^{13}$C NMR spectroscopy.

**$^{1}$H NMR of $G_0$Ben:**

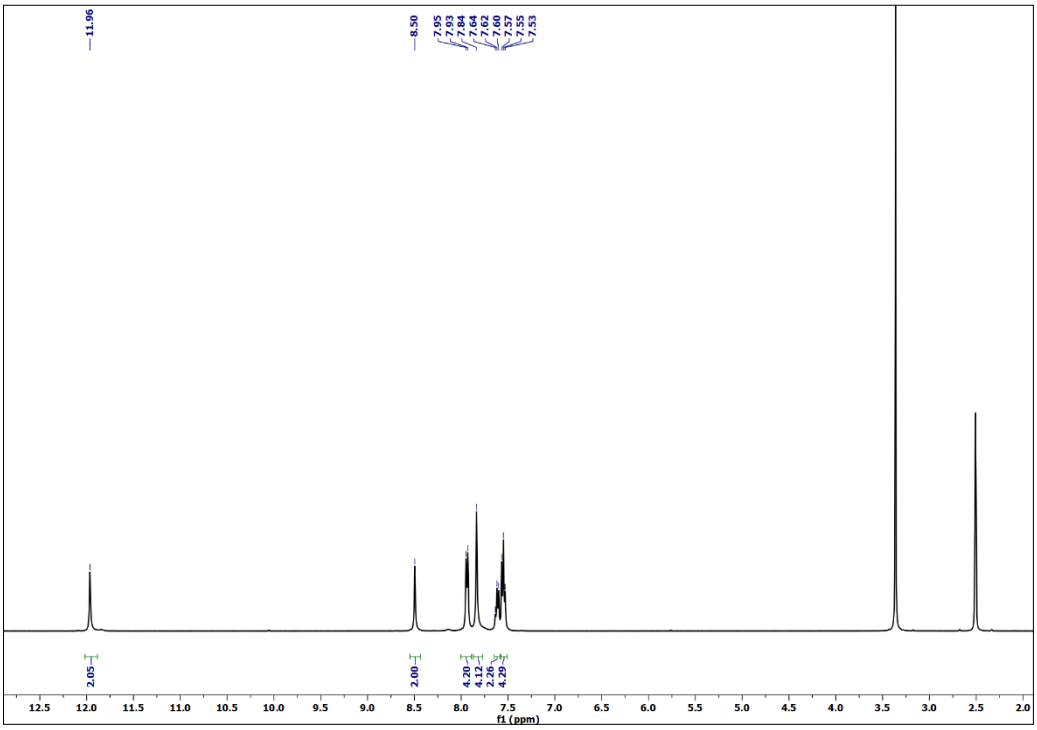



**Figure S1.** $^{1}$H NMR spectrum of $G_0$Ben.

**$^{13}$C NMR of $G_0$Ben:**

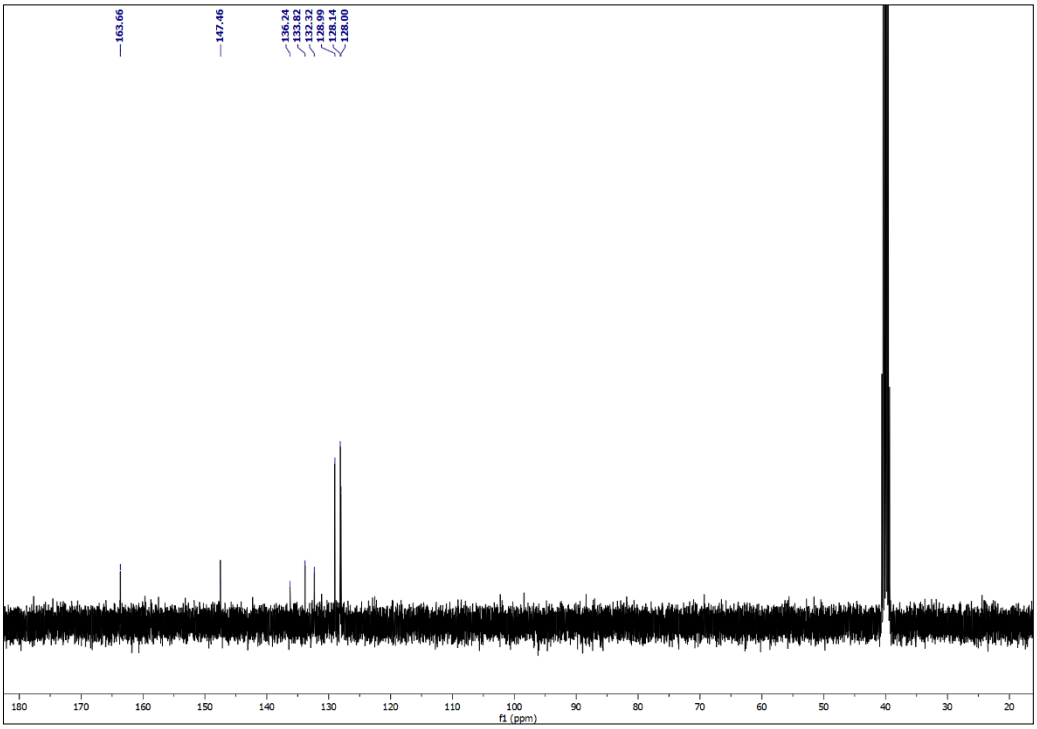
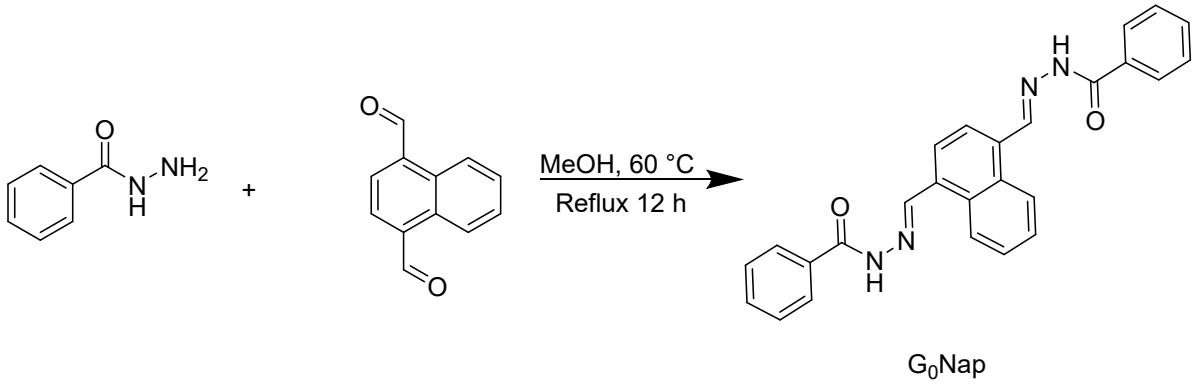



**Figure S2.** $^{13}C$ NMR spectrum of $G_0$Ben.

**Synthesis of $G_0$Nap:**




$G_0$Nap

**Scheme S2.** Synthesis of $G_0$Nap.

4.2 mmol of benzohydrazide was dissolved in the MeOH and $CHCl_3$ mixture. To the solution was added Napththalene-1,4-dicarbaldehyde (2 mmol), and the reaction mixture was stirred at 60 ˚C for 12 h. The precipitates obtained were filtered and recrystallized in MeOH to get the desired product $G_0$Nap in high purity with 89% yield. The compound synthesized was characterized using $^1H$ NMR spectroscopy and mass spectrometry.

**$^1H$ NMR of $G_0$Nap**

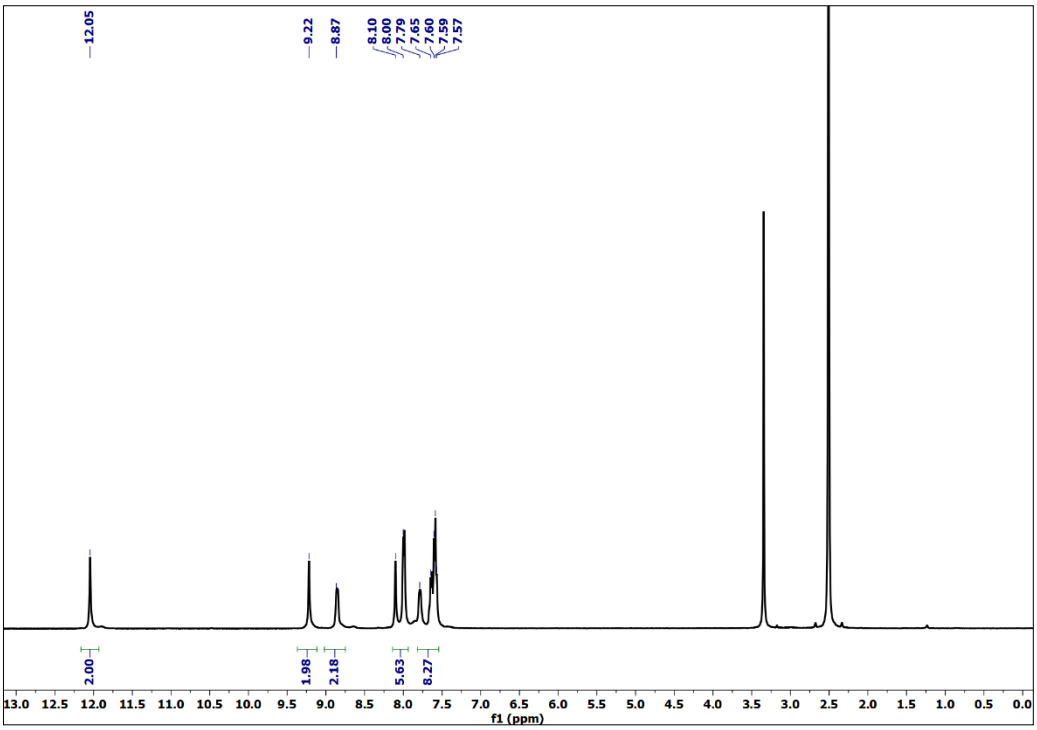



**Figure S3.** $^{1}H$ NMR spectrum of $G_0Nap$.

**HRMS of $G_0Nap$:**

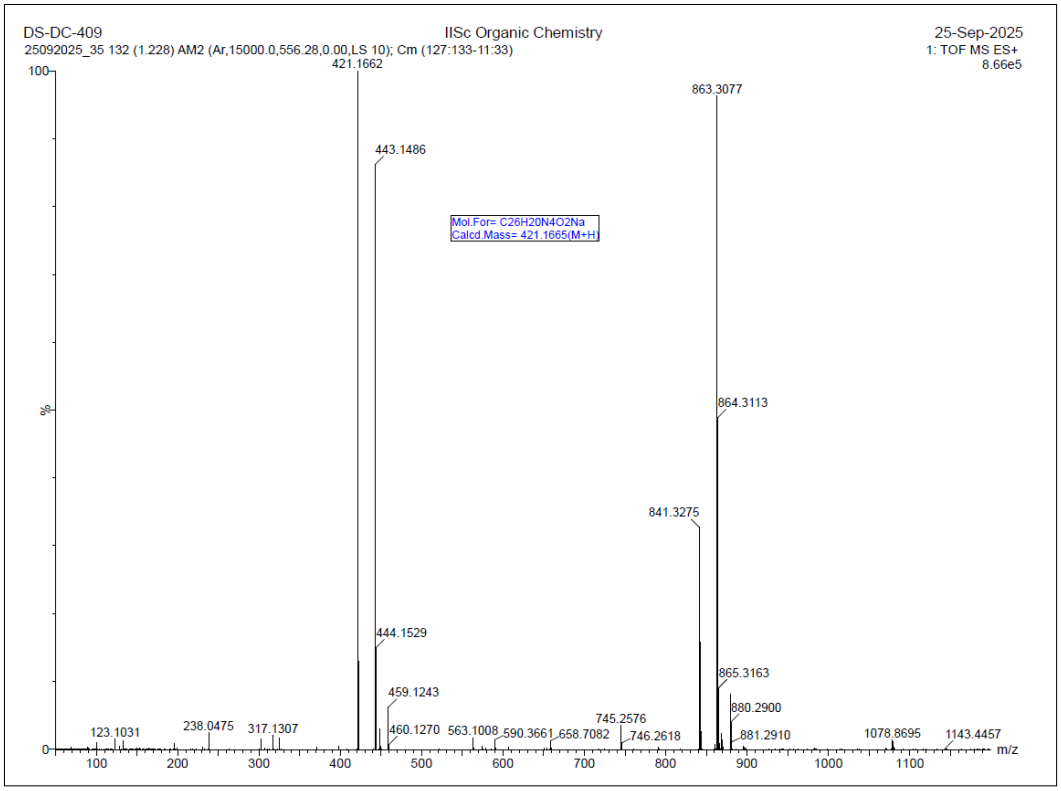
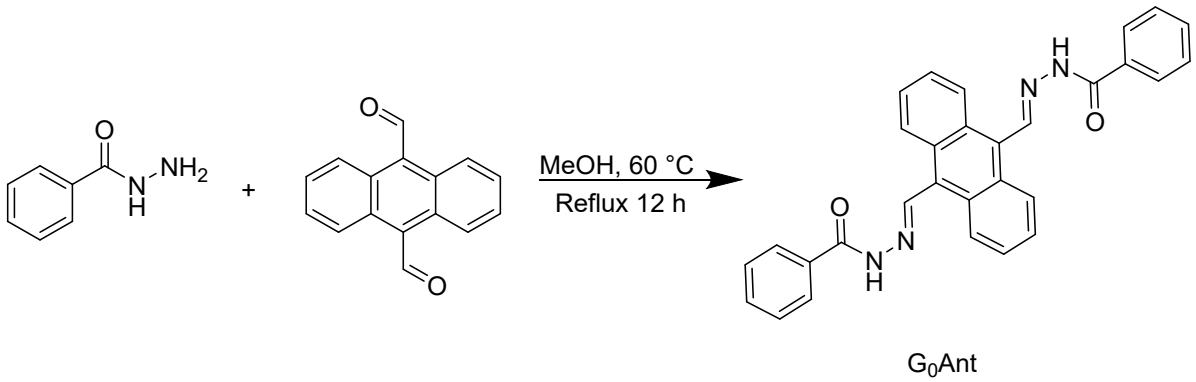



**Figure S4.** HRMS spectrum of $G_0Nap$. Calculated m/z = 421.1665, Found m/z = 421.1662.

**Synthesis of $G_0$Ant:**




**Scheme S3.** Synthesis of $G_0$Ant.

3.80 mmol of benzohydrazide were dissolved in the solvent mixture of MeOH and $CHCl_3$. To the solution was added anthracene-9,10-dicarbaldehyde (1.72 mmol), and the reaction mixture was allowed to stir at 60˚C overnight (~12 h). The precipitates obtained were filtered and recrystallized in MeOH to obtain $G_0$Ant as the desired product in high purity with ~87% yield. The synthesized compound was characterized using $^1$H NMR spectroscopy and mass spectrometry.

**$^1$H NMR of $G_0$Ant**

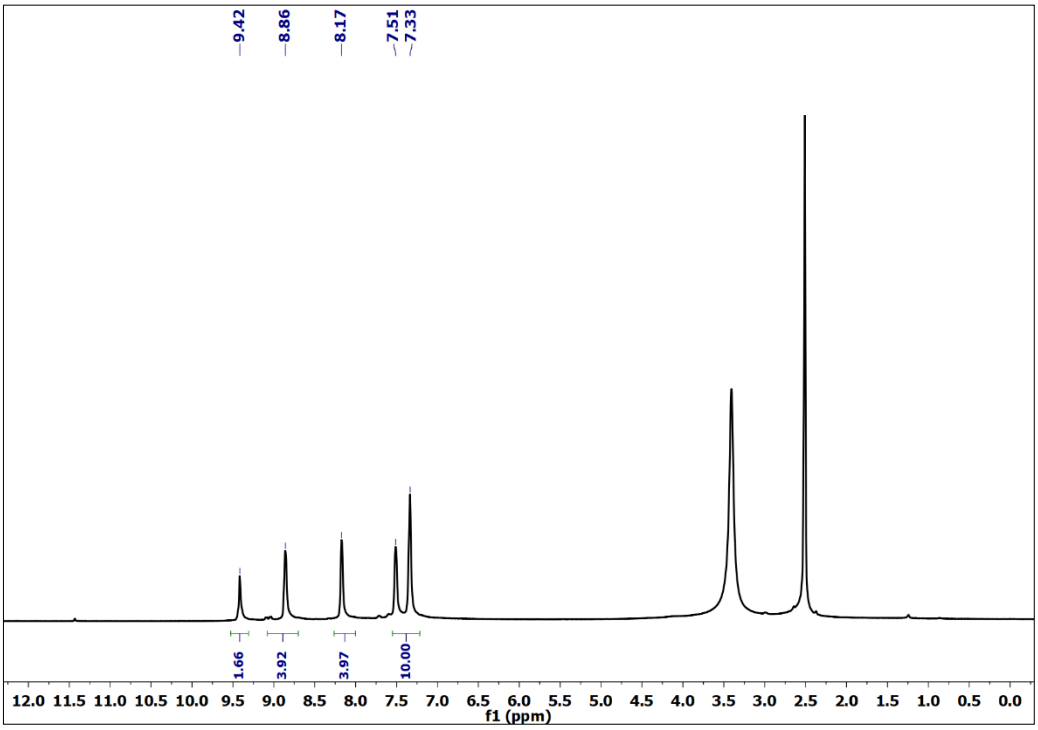



**Figure S5.** $^1$H NMR spectrum of $G_0$Ant.

**HRMS of $G_0$Ant:**

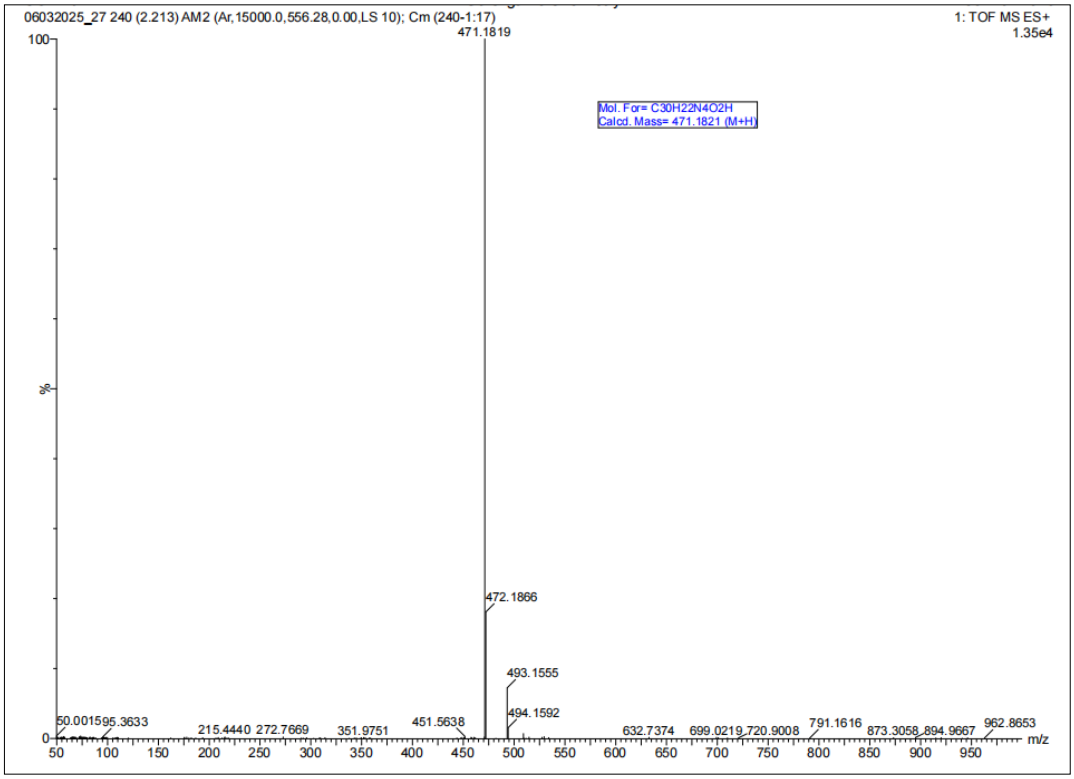



**Figure S6.** HRMS spectrum of $G_0$Ant. Calculated m/z = 471.1821, Found m/z = 471.1819.

## Photophysical Studies

*Absorbance measurements*

The 1 mM stock of $G_0$Ben, $G_0$Nap and $G_0$Ant were prepared in DMF. The absorbance spectra of a 2 mL solution of 20 μM of the three compounds were measured in varying volume fractions of DMF: $H_2O$ (0-90% $H_2O$ v/v) on a PerkinElmer Lambda750 spectrometer using a quartz cuvette with a 1 cm pathlength.

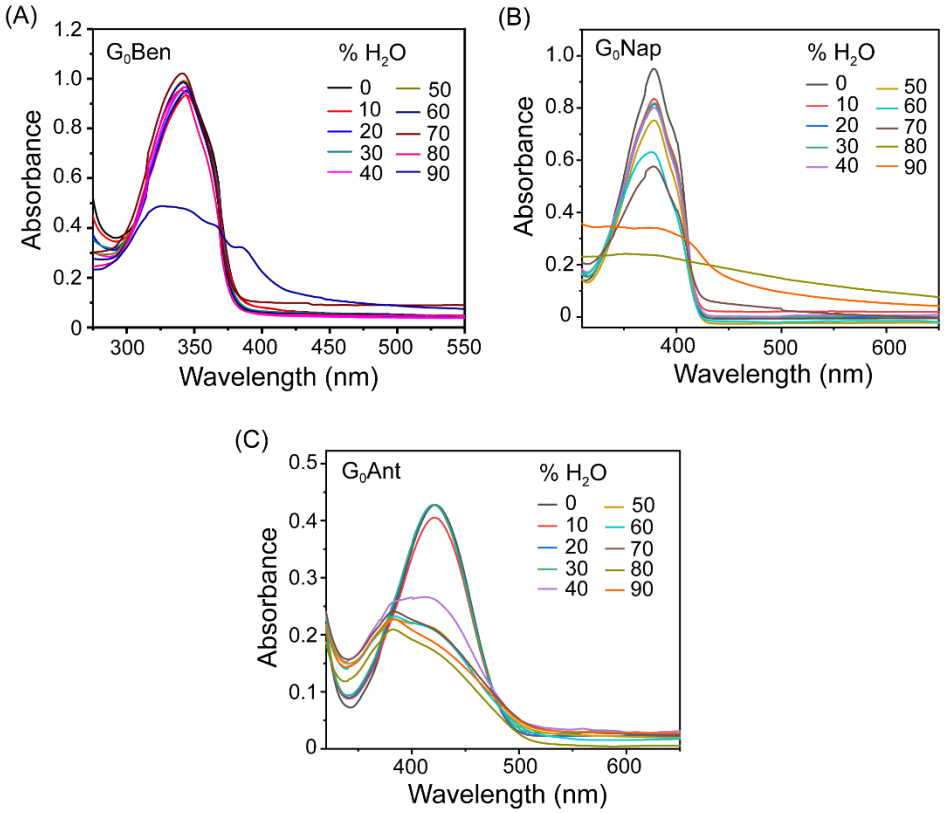



**Figure S7. (A)** UV-Vis absorption spectra of $G_0$Ben with increasing water fraction in DMF-water mixture (0–90% $H_2O$ v/v). **(B)** UV-Vis absorption spectra of $G_0$Nap with increasing water fraction in DMF-water mixture (0–90% $H_2O$ v/v). **(C)** UV-Vis absorption spectra of $G_0$Ant with increasing water fraction in DMF-water mixture (0–90% $H_2O$ v/v).

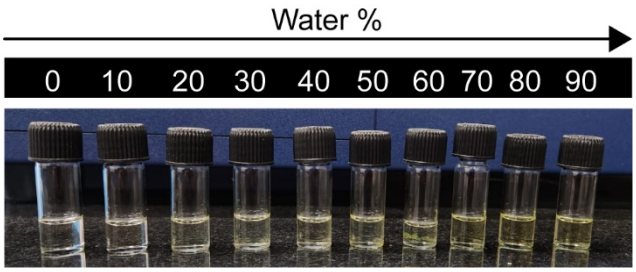



**Figure S8.** Photograph of the solutions of $G_0$Ant at different water fractions under visible light.

*Fluorescence measurements*

The 1 mM stock of $G_0$Ben, $G_0$Nap and $G_0$Ant were prepared in DMF. Fluorescence spectra of the 20 µM, 2 mL of the compounds were measured upon excitation at 350 nm, 375 nm and 430 nm for $G_0$Ben, $G_0$Nap and $G_0$Ant, respectively in varying volume fractions of DMF: $H_2O$ (0-90% $H_2O$ v/v) on a Hitachi F-7000 spectrophotometer.

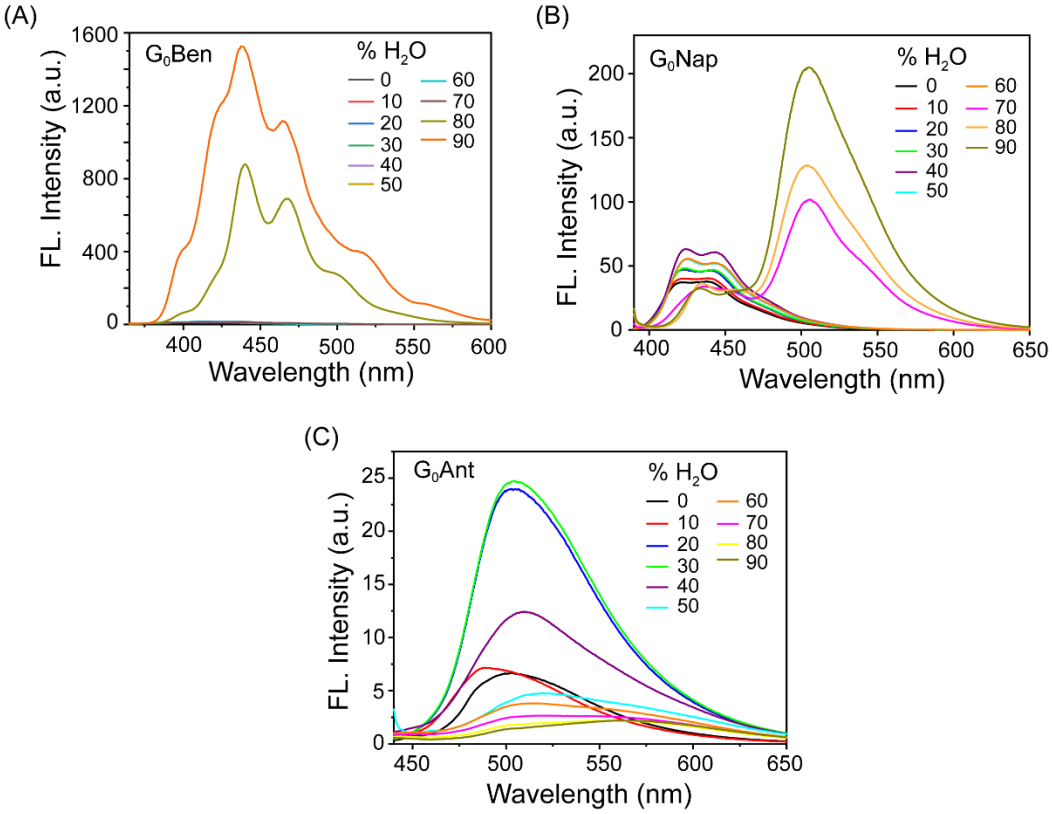



**Figure S9. (A)** FL spectra of $G_0$Ben at varying water fractions in DMF-water mixture (0–90% $H_2O$ v/v). Ex. 350 nm. **(B)** FL spectra of $G_0$Nap at varying water fractions in DMF-water mixture (0–90% $H_2O$ v/v). Ex. 380 nm. **(C)** FL spectra of $G_0$Ant at varying water fractions in DMF-water mixture (0–90% $H_2O$ v/v). Ex. 430 nm.

### Aggregation of $G_0$Ant in DMSO-water mixture

*Absorbance measurements*: The 1 mM stock of $G_0$Ant were prepared in DMSO. The absorbance spectra of a 2 mL solution of 20 µM of the compound was measured in varying volume fractions of DMSO: $H_2O$ (0-90% $H_2O$ v/v) on a PerkinElmer Lambda750 spectrometer using a quartz cuvette with a 1 cm pathlength.

*Fluorescence measurements*: The 1 mM stock of $G_0$Ant was prepared in DMSO. Fluorescence spectra of the 20 µM, 2 mL of the compounds in DMSO: $H_2O$ (90% $H_2O$ v/v) were measured upon excitation at 430 nm on a Hitachi F-7000 spectrophotometer.

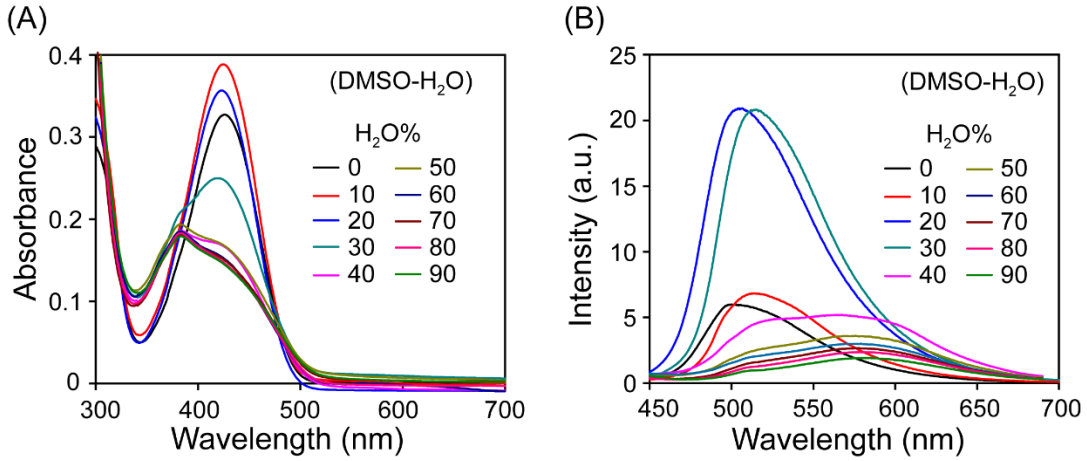



**Figure S10. (A)** UV-Vis absorption spectra of $G_0$Ant with increasing water fraction in DMSO-water mixture (0–90% $H_2O$ v/v). **(B)** FL spectra of $G_0$Ant at varying water fractions in DMSO-water mixture (0–90% $H_2O$ v/v). Ex. 430 nm.

### Fabrication of the assembly

From 1 mM stock of the compounds ($G_0$Ben, $G_0$Nap and $G_0$Ant) in DMF, 20 μM was taken in the DMF-water mixture (90% $H_2O$ v/v) and incubated for 10 min.

### Critical aggregation concentration (CAC)

CAC of the $G_0$Ant was determined by increasing its concentration in a solution of 90% $H_2O$ (v/v) DMF-water mixture. The solutions were excited at 430nm, and the corresponding emission was recorded. The fluorescence started increasing with increasing concentrations of the suprazymes. CAC was determined via the intersection point of the liner parts of the titration curves, which gave the CAC value of ~4.5 μM.

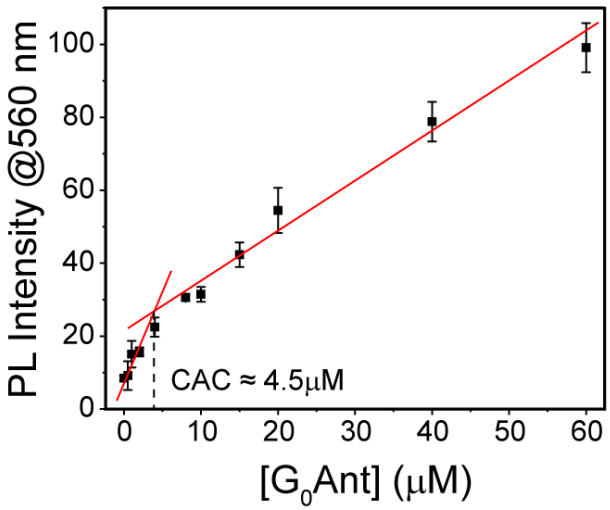



**Figure S11.** CAC of $G_0$Ant in the 90% *f*$H_2O$ (v/v). (Ex. 430 nm and Em. 560 nm).

**Fluorescence Lifetime**

Fluorescence lifetime was determined using a time-correlated single photon counting (TCSPC) spectrometer (Horiba). 20 μM of $G_0$Ben, $G_0$Nap and $G_0$Ant were taken in a DMF-water mixture (90% $H_2O$ v/v), and the fluorescence decay curve was recorded upon excitation with a laser of 415 nm. All the fluorescence decays were fitted using a tri-exponential function, with the $R^2$ value close to 1. The average lifetime was calculated using the equation below:

$$\tau(avg) = \frac{A_1(\tau_1)^2 + A_2(\tau_2)^2 + A_3(\tau_3)^2}{A_1\tau_1 + A_2\tau_2 + A_3\tau_3}$$

τ corresponds to the different lifetimes that are fitted to the data. A corresponds to the amplitudes or pre-exponential factors associated with each of the three exponential terms.

**Field Emission Scanning Electron Microscopy**

Samples for SEM imaging of $G_0$Ben, $G_0$Nap and $G_0$Ant were prepared using 20 μM each in a DMF-water mixture (90% $H_2O$ v/v) and incubated for 15 min, followed by drop casting 10 μL of the sample on silicon wafers. The samples were dried in air and then in vacuum. The samples were sputter coated with ~4 nm layer of gold before imaging under SEM. The SEM imaging was done using Ultra55 FESEM Karl Zeiss MonoCL instrument with 5 kV emission voltage and appropriate magnification.

**DFT Calculations**

A time-dependent density functional theory (TD-DFT) was performed for $G_0$Ben, $G_0$Nap and $G_0$Ant using the Gaussian 09 program package in the delta-cluster of SERC facility @IISc. The structures were used as the input geometries for time-dependent density functional theory (TD-DFT) calculations. GaussView 5.0 was used to analyze the molecular orbitals for the three molecules.

**NMR Titration**

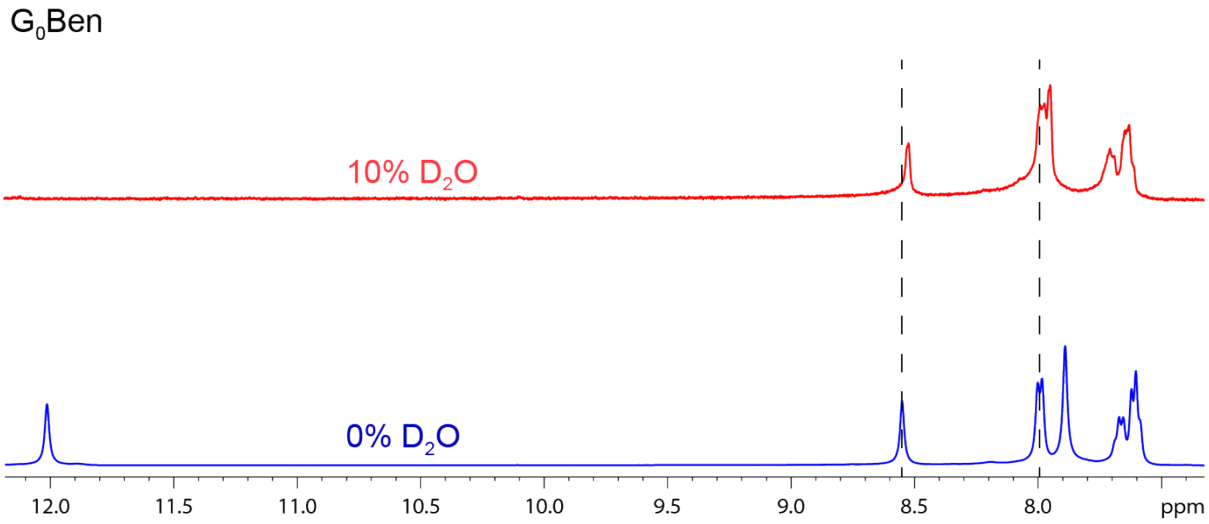



**Figure S12A.** $^{1}H$ NMR spectrum of $G_0$Ben in the monomeric form (0% $D_2O$) and the aggregated form (10% $D_2O$) in DMSO-$d_6$.

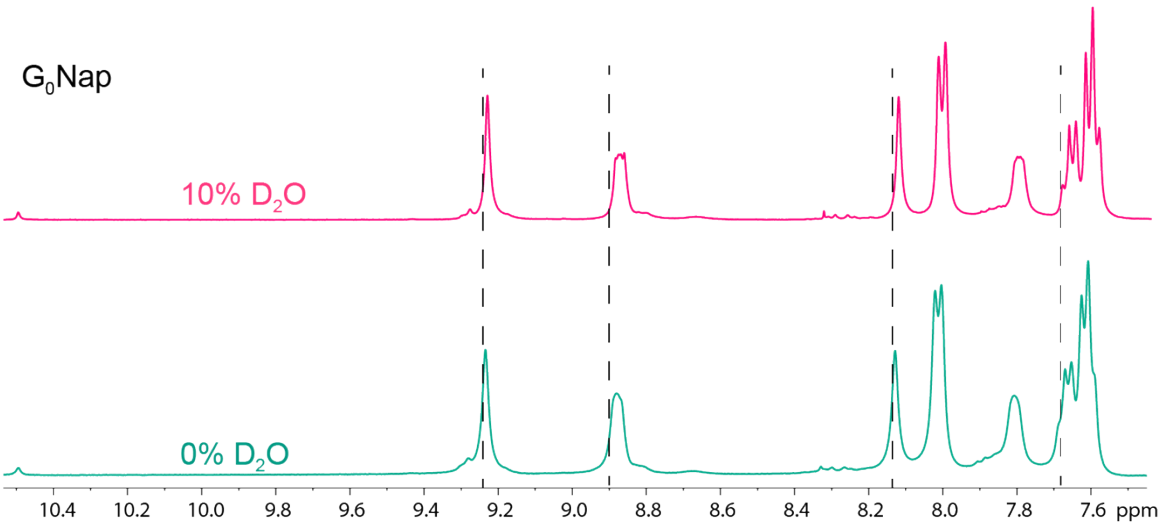



**Figure S12B.** $^{1}H$ NMR spectrum of $G_0$Nap in the monomeric form (0% $D_2O$) and the aggregated form (10% $D_2O$) in DMSO-$d_6$.

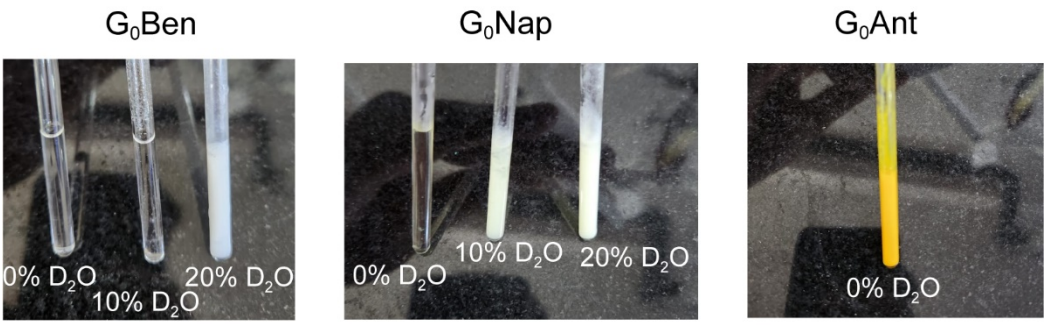



**Figure S13.** Photograph of the solutions of $G_0$Ben, $G_0$Nap and $G_0$Ant in different percentage of $D_2O$ in DMSO-$d_6$-$D_2O$ mixtures for NMR.

**X-Ray Diffraction**

The powder X-ray diffraction pattern was recorded on the assembly of $G_0$Ant on a glass slide (1.5X1.5 cm). The film sample of the $G_0$Ant assembly was prepared by dropcasting and drying the assembly on the slide. XRD pattern was recorded from 5° to 80° at a rate of 0.02° $s^{-1}$.

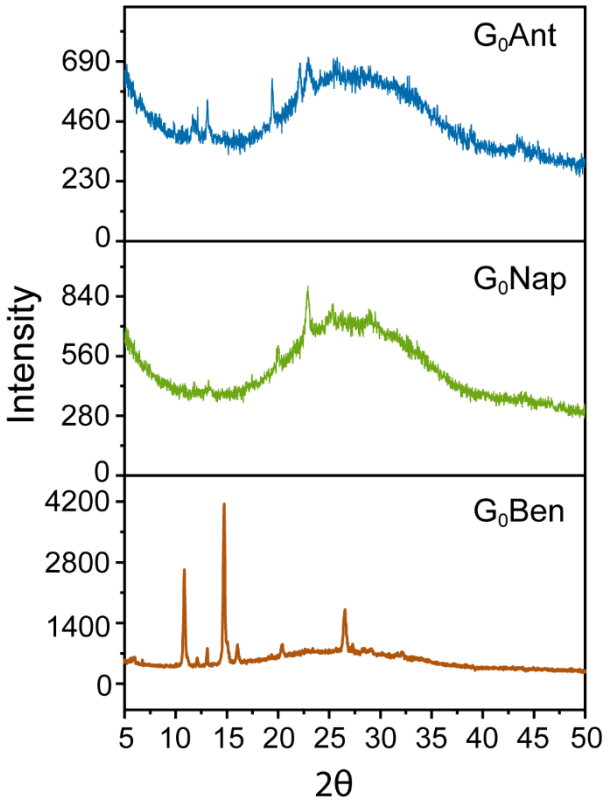



**Figure S14.** Powder XRD patterns of $G_0$Ben, $G_0$Nap and $G_0$Ant in the assembled form.

**Solid state UV-Vis spectroscopy**

To investigate the band gap energies of the inherent $G_0$Ben, $G_0$Nap and $G_0$Ant molecules, solid-state UV DRS spectra was recorded for their powdered samples on UV-Vis NIR-PerkinElmer LAMBDA 1050 spectrophotometer. The resulting data was used to plot tauc plot.

**Photocurrent**

The photocurrent was measured for 20 µM of assembly of $G_0$Ant in a DMF-water mixture (90% $H_2O$ v/v) dropcasted onto a silicon wafer and dried. The current response under the alternate exposure to white light was recorded upon applying a bias of 4V on Keithley SMU 2400 instrument.

**Enzyme mimicking assay**

The activity was monitored in 100 mM acetate buffer pH 4.0 by oxidation of TMB. Catalytic reactions were performed with $G_0$Ant suprazymes (20 µM) in 90% $H_2O$ (v/v) and 0.5 mM TMB in 200 µL of solution. The reaction solution was irradiated for 10 min with white light (20 $mWcm^{-2}$) at room

temperature, and the absorbance maximum was monitored at 652 nm for TMB, using a SpectraMax® M5e microplate reader.

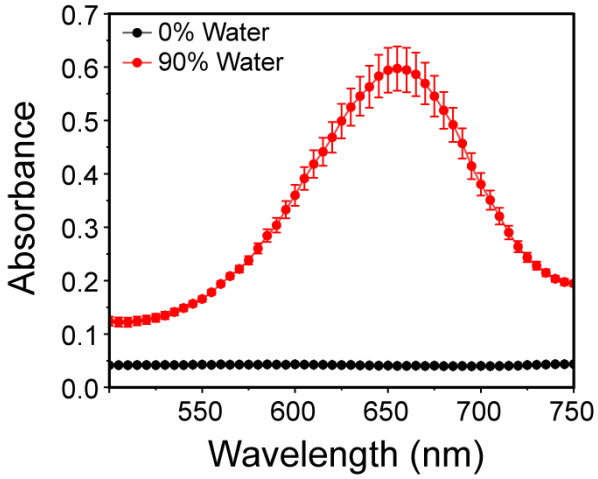



**Figure S15.** Oxidase-like photocatalytic activity of $G_0$Ant (20 µM) in 0% $H_2O$ and 90% $H_2O$ with TMB (0.5 mM) and white light irradiation in acetate buffer (100 mM, pH 4.0) for 10 min.

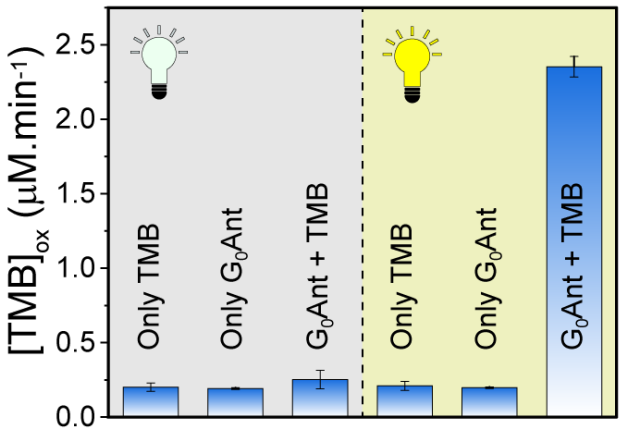



**Figure S16.** Rate of TMB (0.5 mM) oxidation in the presence of $G_0$Ant (20 µM) after white light irradiation in acetate buffer (100 mM, pH 4.0) for 10 min.

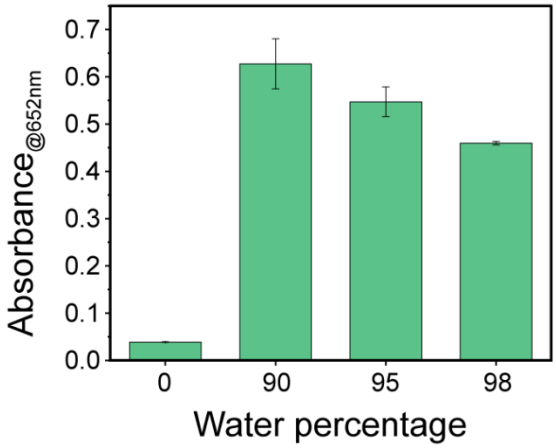



**Figure S17.** Oxidase-like photocatalytic activity of $G_0$Ant (20 μM) in different $H_2O$ fractions in DMF-water mixture.

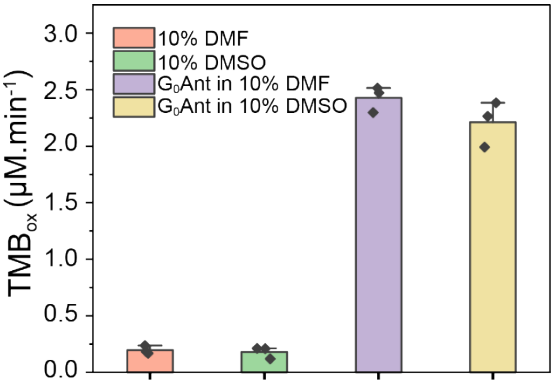



**Figure S18.** Rate of TMB (0.5 mM) oxidation in the presence of $G_0$Ant in 10% DMF and 10% DMSO after white light irradiation in acetate buffer (100 mM, pH 4.0) for 10 min.

**Oxidase activity at varying $G_0$Ant concentrations**

The activity was monitored in 100 mM acetate buffer pH 4.0 by oxidation of TMB. Catalytic reaction was performed with increasing concentrations of $G_0$Ant suprazymes in 90% H2O (v/v) and 0.5 mM TMB in 200 μL of solution. Absorbance spectra were recorded after irradiating with light for 10 min.

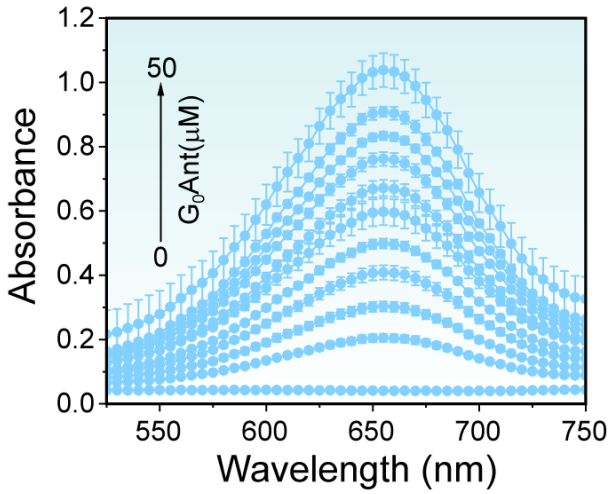



**Figure S19.** Absorbance spectra of TMB oxidation with increasing concentrations of the $G_0$Ant assembly upon irradiating with light for 10 min.

**Enzyme mimicking Kinetics**

The catalytic oxidation of TMB was used to study the oxidase-mimicking activity of $G_0$Ant suprazymes. The activity was monitored in 100 mm acetate buffer pH 4.6. Catalytic reactions were performed with $G_0$Ant suprazymes (20 μM) in 90% $f H_2O$ (v/v) and 0.5 mM substrates in 200 μL of the solution. The reaction solution was irradiated for 10 min with white light (20 $mWcm^{-2}$) at room temperature, and the absorbance maximum was monitored at 652 nm for TMB, using a SpectraMax® M5e microplate reader.

The kinetics studies were carried out using TMB as the substrate from 0-0.5 mM. The absorbance @652 nm was measured as a function of irradiation time. The acquired data was fitted using the Michaelis-Menten equation (1), and the kinetic parameters were determined.

(1)

$$\frac{1}{V_0} = \frac{K_M}{V\max\,[S]} + \frac{1}{V_{max}}$$

where $K_M$ denotes the Michaelis-Menten constant, $V_{max}$ represents the maximum velocity, $V_0$ is the initial rate, and S represents the substrate concentrations.

**Detection of ROS**

The ROS generation was measured by using UV–vis absorbance spectroscopy. The activity assay was performed in the presence of various scavengers to determine the type of ROS generated. The scavenger was incubated with the suprazyme prior to the light exposure for measuring the oxidase activity. TMB oxidation in the presence of $G_0$Ant under white light irradiation. Reaction conditions: 0.5 mM TMB, 20 μM $G_0$Ant, 20 mM IPA, 0.5 mM L-His, 1.0 mM $NaN_3$, 5 mM KI, and EDTA 10mM. Light irradiation time: 10 min.

The estimation of the superoxide radical was confirmed by the reaction with DHR and TMBD substrates. DHR assay was performed, to 200 µL solution of 20 µM $G_0$Ant in DMF-water mixture (90% $H_2O$ v/v), 10 µM of DHR was added and irradiated with white light for a duration of 10 min, and the Fluorescence spectra were recorded on a microplate reader. For TMPD assay, to 200 µL solution of 20 µM $G_0$Ant in Water−DMF mixture (90% $H_2O$ v/v), 0.5 mM TMPD was added and irradiated with white light for a duration of 10 min, and the absorbance spectra were recorded on a microplate reader.

**Recyclability of $G_0$Ant suprazyme after multiple**

50 µM of the $G_0$Ant assembly was used for the oxidation of TMB followed by centrifugation and then redispersed in 90% H2O: 10% DMF solution. Similarly, repeated for 5 cycles.

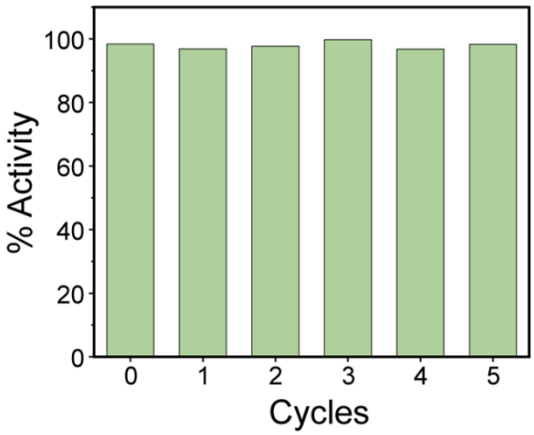



**Figure S20.** The percentage catalytic activity of $G_0$Ant after multiple successive cycles of TMB oxidation, following recovery by centrifugation.

**Antimicrobial Activity**

The antimicrobial activity of $G_0$Ant (DMF-water mixture (95% $H_2O$ v/v)) was studied on MRSA as Gram-positive bacteria and *E. coli* as Gram-negative bacteria. The freeze-dried bacterial species were revived by transferring into nutrient agar plates. To prepare the primary culture, a few bacteria colonies from the agar plate were taken and cultured in LB medium (2 g/ 100 mL) overnight for 12 h. The secondary culture was prepared by subculturing 100 µL of the primary culture in 10 mL of fresh LB media until it reached the mid-log phase ($A_{600\ nm}$= 0.25).

For the plate count assay, the secondary cultured bacteria solution was centrifuged at 5000 rpm to discard the old media, and the bacteria were washed twice with PBS (10 mm, pH ≈7.4) through centrifugation. Ultimately, the bacterial pellet was redispersed in PBS to prepare the bacterial inoculum. Bacterial solution (100 µL) in PBS was spread on the freshly prepared agar plate. For agar plates, sterile polystyrene Petri dishes were filled with 10 mL of solution (containing 9.75 g of agar in 250 mL of Milli-Q water and autoclaved at 120 °C) per plate. The hot agar solution was poured into Petri dishes. Once the agar was dried, the plates were used for the antibacterial study. The $G_0$Ant suprazyme treated bacteria in dark and white light (10 min) were spread on the agar plates. The plates were then kept at 37 °C overnight. After ≈12 h, the images of the plates were taken.

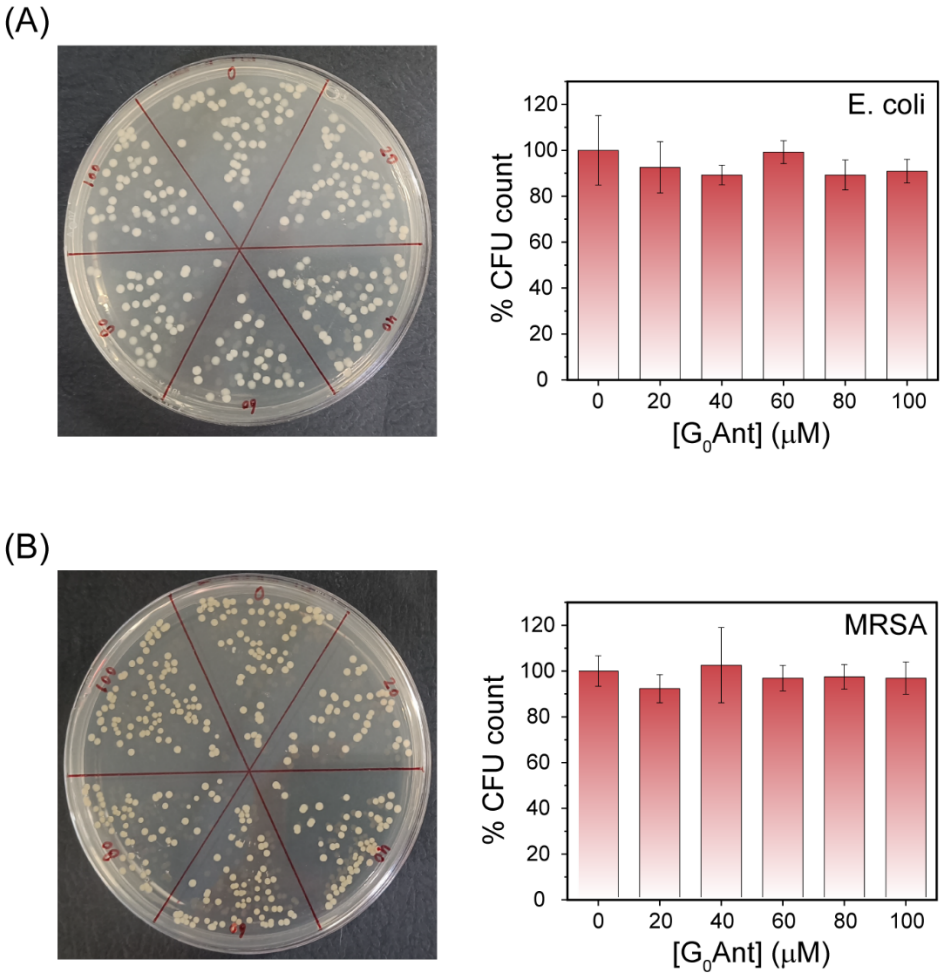



**Figure S21.** (A) The bactericidal activity of bacteria after treated with different concentrations of $G_0$Ant (0, 20, 40, 60, 80 and 100 μM) against E. Coli without light by using colony forming unit (CFU) method. (B) The bactericidal activity of bacteria after treated with different concentrations of $G_0$Ant (0, 20, 40, 60, 80 and 100 μM) against MRSA without light by using CFU method.

**SEM of bacteria samples**

The SEM analysis was conducted for the visualization of the bacterial death mediated by $G_0$Ant. The untreated *E. coli* and *S. aureus* and the $G_0$Ant treated samples of bacteria were irradiated with white light for 15 min., followed by fixation with 3 % glutaraldehyde to preserve cell wall morphology. The bacterial samples suspended in EtOH-water were dropped on cleaned silicon wafers and air-dried in a dirt-free atmosphere. The samples were then gold sputtered with ~5 nm layer before imaging.

**Cytotoxicity**

The cytotoxicity of $G_0$Ant assembly was tested on HEK-293 cell line using the standard protocol. First, HEK-293 cells were seeded at ≈15 000 cells/100μL cell media in a 96-well plate to reach the desired confluency. Post 24 h incubation, the cells were washed with DPBS and incubated for with the different concentrations of the $G_0$Ant suprazyme in triplicates for reliable readings. The plate was then incubated

for 24 h at 37 °C with 5% $CO_2$. After 24 h, the cells were washed once with DPBS and incubated with media containing (3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) MTT solution (2 mg $mL^{-1}$ in 5% EtOH, 100 µL per well). After 3 h incubation with MTT, the solution was removed, and 200 µL of biological grade DMSO was added to each well to dissolve the resulting formazan crystals. Further, after 30 min, absorbance was measured at 570 nm using a Molecular Devices SpectraMax® M5e microplate reader, and cellular viability was determined relative to the control group.

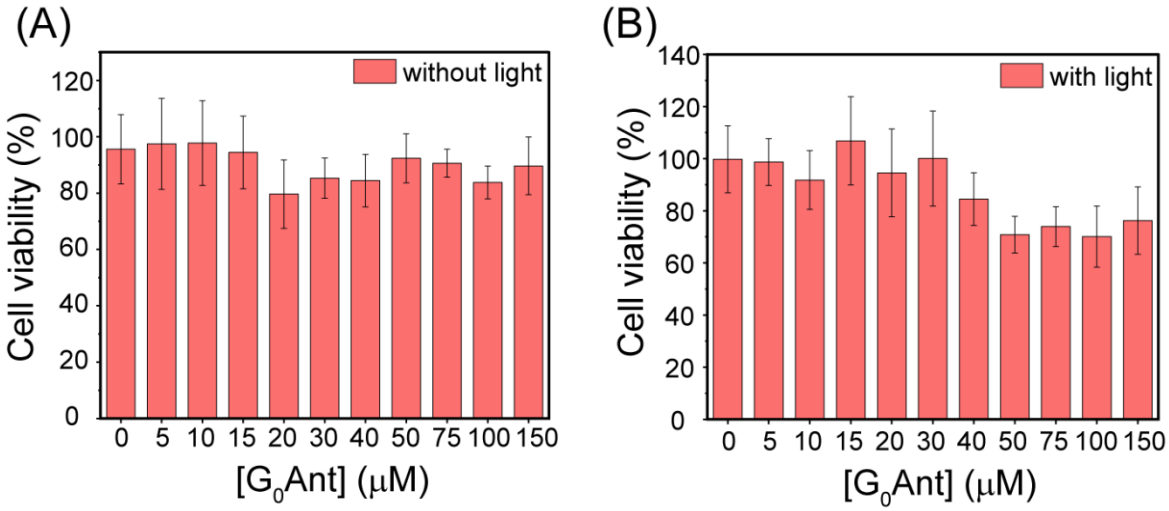



**Figure S22.** MTT cell viability assays of $G_0$Ant at different concentrations against HEK-293 cells in (A) absence and (B) presence of light.

## Reference:

1. Kamra, A.; Mahashaya, R.; Rajamalli, P.; Rana, S., A Tailorable Metal-Free Supramolecular Oxidase Equivalent with Photoswitchable Functions. *Small* **2025,** *21* (31), 2411416.