Electronic Supplementary Material (ESI) for Biomaterials Science.

Visualizing biofilm by targeting eDNA with long wavelength probe CDr15

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Experimental

Quantum Yield Measurements.

Quantum yield was calculated by measuring the integrated area of emission spectra for CDr15 utilizing Nile blue (Φ = 0.27) in ethanol as reference. CDr15 and Nile blue were excited at 570 nm, and emission spectra were collected from 590 to 850 nm.

$$\Phi_f^i = \left(\frac{F^i}{F^s}\right)\left(\frac{f_s}{f_i}\right)\left(\frac{n_i^2}{n_i^2}\right)\Phi_f^s \quad (1)$$

Quantum yields were calculated using eq 1, Φ_f^i and Φ_f^s represented the quantum yield of sample and standard, respectively. *F* represented the integrated emission area of the fluorescence spectra, *n* represented the refractive index of solvent, and *f* represented absorption factor ($f = 1\text{-}10^{-A}$, where A represented absorbance) at the excitation wavelength selected for sample and standard. Emission was integrated from 590 to 850 nm, and quantum yield of CDr15 in DMSO was calculated to be 0.009.

Bacterial strains and growth conditions.

P. aeruginosa strains used in this research are PAO1, *ΔwspF*, *pYhiH*, *Δfap, Δpsl, Δpsl-GFP*, *Δpel, Δpel-GFP*, *Δfap, lasIrhII,* and *lasIrhII*-GFP and cultured at a 37˚C incubator in LB media. LB medium was contained 10 g of yeast extract (BD, Bioscience Co.), 5 g of bacto peptone (BD, Bioscience Co.) and 10 g of NaCl per liter of distilled water. Each media plate is with 15 g of Agar per liter of distilled water. All media were used after autoclaving for culture.

Screening for biofilm binding compound using mutant *P. aeruginosa*.

Seven *P. aeruginosa* strains, PAO1, *ΔwspF*, *pYhiH*, *Δpsl-GFP*, *Δpel-GFP*, *Δfap,* and *lasIrhII* strains were cultured at a 37˚C incubator in LB media. 2 pieces of cover glass were incubated in 50 mL conical tube by adding 5 mL LB media. Compounds were treated on the cover glass

where biofilm was formed. The compound showed stronger fluorescence intensity in all strains biofilm except *pYhjH* and *lasIrhII* strains biofilm was chosen as eDNA staining probe of biofilm.

Confirmation of CDr15 selectivity.

Δpel-GFP, Δpsl-GFP, and lasIrhII-GFP strains were inoculated in chamber slide with ABTG media after pre-culture with LB media. After three days, CDr15 compound treated to biofilm and incubated for 30 min.

The validation of CDr15 target.

For target confirmation of CDr15, DNA (chromosomal DNA) and RNA were isolated from PAO1. Mammalian DNA was isolated from HEK293 cells. DNA of bacteria and HEK293 cells was extracted with the DNeasy Tissue Kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. RNA from bacteria was extracted using the RNeasy mini kit (Qiagen, Hilden, Germany). DNA or RNA (1, 2, three μg/μL) was incubated with CDr15 (5 μM) for 30 min and measured by SpectraMax®M2 Spectrophotometer (Molecular Devices).

Compound (1 μM) was treated to PAO1 biofilm growing on the cover slide with or without DNase (5 μg/μL) (Sigma–Aldrich, St. Louis, MO, USA). Image of biofilm was taken after 30 min staining. All biofilm images were acquired under a confocal laser scanning microscope.

Competition assay

CV (crystal violet) and Ho (Hoechst 33342) (Sigma–Aldrich, St. Louis, MO, USA) were used as CDr15 competitor. Bacteria DNA (1 μg/μL) and competitor (CV or Hoechst) were mixed and incubated for 30 min. Then the sample was treated with 5μM CDr15 for 30 min. The fluorescence intensity of the mixed sample was analyzed by SpectraMax®M2 Spectrophotometer (Molecular Devices) instrument, and the obtained data were analyzed using the Microsoft Office Excel.

Picosecond time-resolved fluorescence.

Picosecond time-resolved fluorescence was obtained by time-correlated single photon counting (TCSPC) method. A home-made cavity-dumped optical parametric oscillator (OPO) based on periodically poled stoichiometric lithium tantalite (PPSLT) operating in the near-infrared was used to generate the optical pulse of 60 fs at 1220 nm. The gain medium was pumped by Ti: sapphire femtosecond laser (Tsunami, Spectra-Physics), which provides 1.0 W at 800 nm with a repetition rate of 81.5 MHz. Ti: sapphire laser was pumped by a frequency doubled diode-pumped solid-state laser (Millennia Prime 6, Spectra-Physics). Pump pulses were provided by the second harmonic generation (610 nm) in a 3 mm thick lithium triborate (LBO) crystal. The fluorescence of the sample was collected into a monochromator (SP-300i, Princeton Instruments), and detected by a silicon avalanche photodiode (id100-50, ID Quantique), which provides instrumental response function as 55 ps (FWHM).

Fixation and permeabilization of HEK293 cell.

HEK293 cell (ATCC CRL-1573) were obtained from the American Type Culture Collection (ATCC) and cultured with DMEM containing 10% fetal bovine serum, 100 μg / ml of penicillin, and streptomycin (Thermo Fisher Scientific, Rockford IL, USA). Cells were fixed in 4% paraformaldehyde (Thermo Fisher Scientific, Rockford IL, USA) for 30 min at room temperature and washed three times with PBS. For the permeabilization of the cell, 0.1% Triton x-100 (Sigma–Aldrich, St. Louis, MO, USA) was treated to the fixed cells for 10 min and washed three times with PBS. Cells were stained with Hoechst 33342 (1.62 μM) or SYTOX green (5 μM) (Thermo Fisher Scientific, Rockford IL, USA), for 5 min at room temperature.

Biofilm staining.

The compound was treated to a different stage of biofilm depending incubation time; 1, 3, 5 days. For imaging of living biofilm, PAO1-GFP strains were inoculated in chamber slide for 1, 3, 5 days with ABTG media at room temperature and supplied fresh media after removing old media, every day. CDr15 (1 μM) was treated to each stage of biofilm with media at chamber slide and incubated for 30 minutes**.** Biofilm images were acquired under a confocal laser scanning microscope.

HHA-FITC (EY Laboratories, Inc. San Mateo, USA) was treated to 3-day-old biofilm with ABTG media at chamber slide and incubated for 30 minutes. After washing with PBS, CDr15 (1 μM) was incubated for 30 min. Biofilm images were acquired under a confocal laser scanning microscope.

For double-compound staining, CDy14 (1 μM) and CDr15 (1 μM) were treated to 3-day-old biofilm and incubated for 30 min. Images were acquired under a confocal laser scanning microscope. The software packed with Nikon generated the 3-D images and optical Z-sections.

Confocal image acquisition of biofilm.

A Nikon A1R laser confocal microscope (Nikon, Tokyo, Japan) equipped with an objective lens (NA 0.95, 40x magnification, PLAPO) and laser including 17.7 mW 488 nm (laser power 1.4), 20 mW 561 nm (laser power 2.0), and 40mW 640 nm (laser power 2.3) were used to capture biofilm stacks. All biofilm samples were sequentially scanned. Emitted fluorescence was recorded within the range 500–550 nm, 572-622 nm, and 662-737 nm in order to visualize GFP, CDy14 and CDr15 fluorescence.

40 × objective and 100 x objective (bacteria membrane staining with FM1-43) were used in imaging experiments. Image capture, two-dimensional (2D) projections of z-stacks and 3D reconstructions were performed using NIS Nikon software.

Three stacks of horizontal plane images (512 × 512 pixels corresponding to 119 × 119 μm) with a z-step of 1 μm were acquired for each biofilm at different areas in the well.

Super-resolution images.

PAO1 (O.D.600 = 0.1) strain was inoculated in 8-well chamber with ABTG media and incubated for 20 hours at the 37˚C condition to get biofilm. CDr15 (1 μM) was treated to PAO1 biofilm for 30 min at 37˚C incubator before analyzing fluorescent signals with super-resolution structure illumination microscopy (SR-SIM). The image of the CDr15 (1 μM) treated PAO1 biofilm was captured using the LSM780 ELYRA PS.1 system superresolution structured illumination system (Carl Zeiss, Germany) with a 100× plan-apochromatic oil immersion objective lens (numerical aperture, 1.46). In general, acquisition time for a single data set was 3–6 s. GFP and CDr15 were excited using a 488 nm and 640 nm optically pumped semiconductor laser line for observation and data collection. The acquired images were further processed with the Zen 2011 software (Carl Zeiss).

Generation of corneal infection model.

PAO1-GFP and *lasIrhII* -GFP were cultured at 37˚C with 100 rpm shaking overnight. Each strain was wash and diluted with PBS. C57BL/6 black mice were anesthetized with ketamine/xylazine by subcutaneous injection. Under anesthesia conditions, light scratches were generated using the mini blade in the corneal region of the eye. Bacteria (*PAO1-GFP* and *lasIrhII -GFP*) in PBS buffer infected in scratched corneal and mice were maintained for 1 day for inducing biofilm in corneal regions. All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of National University of Singapore (NUS) and approved by the Institutional Animal Care and Use Committee for Biological Resource Center at A*STAR, Singapore

Strain	Genotype	Main matrix composition and biofilm formation	Reference
PAO1	Wild type PAO1 strain	proper biofilm formation	(1)
ΔwspF	wspF mutation	Enhancement of biofilm Formation by upregulated c-di-GMP	(2)
pYhjH	*yhjH gene encoding*	Decrease of biofilm Formation by down regulated c-di-GMP	(2)
Δfap	Fap mutation	Forming biofilm excluding amyloids	(3)
Δpsl	*Psl* gene mutation	Forming biofilm excluding Psl polysaccharide	(4)
Δpel	*Pel* gene mutation	Forming biofilm excluding Pel polysaccharide	(4)
rasIrhII	*lasIrhII gene mutation*	Forming biofilm excluding eDNA	(1)

Table S1. Strains used in this study

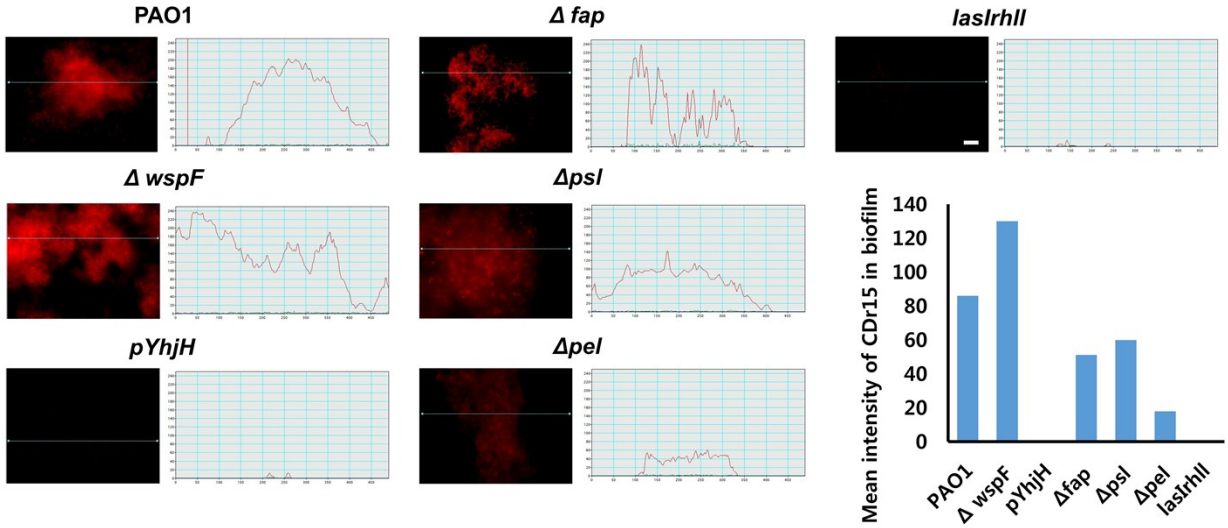


Fig. S1 CDr15 intensity of the different mutant strains. The CDr15 staining images of biofilm with different strains. Scale bars, 10 μm. Profile showed the mean fluorescence intensity of CDr15 along white line in every image by DigitalMicrograph software.

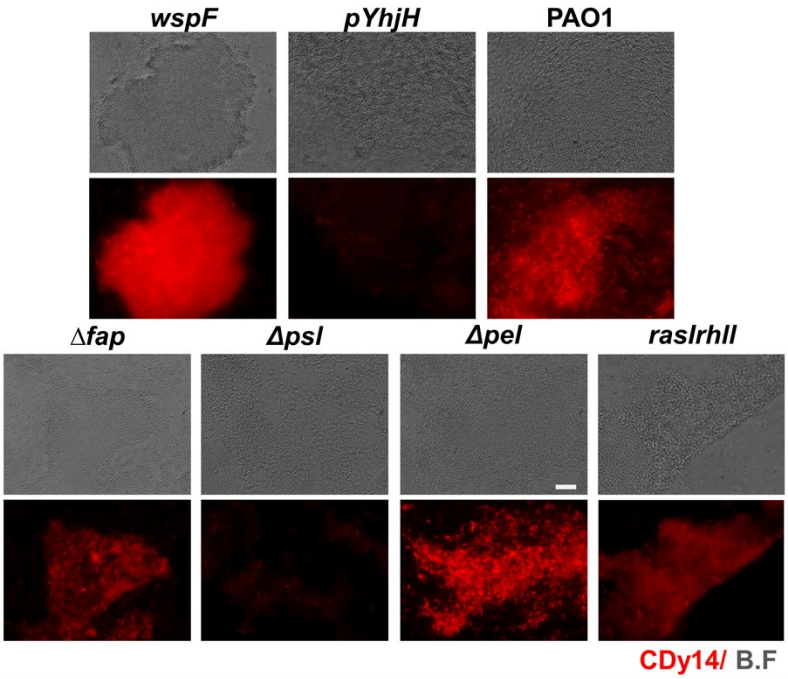


Fig. S2 Biofilm image of CDy14 on the different mutant strains. The biofilm, which was formed on slide glasses, were treated with CDy14 (1 μM) for 60 minutes, respectively. The staining CDy14 on biofilm of the different strains. Scale bars, 10 μm.

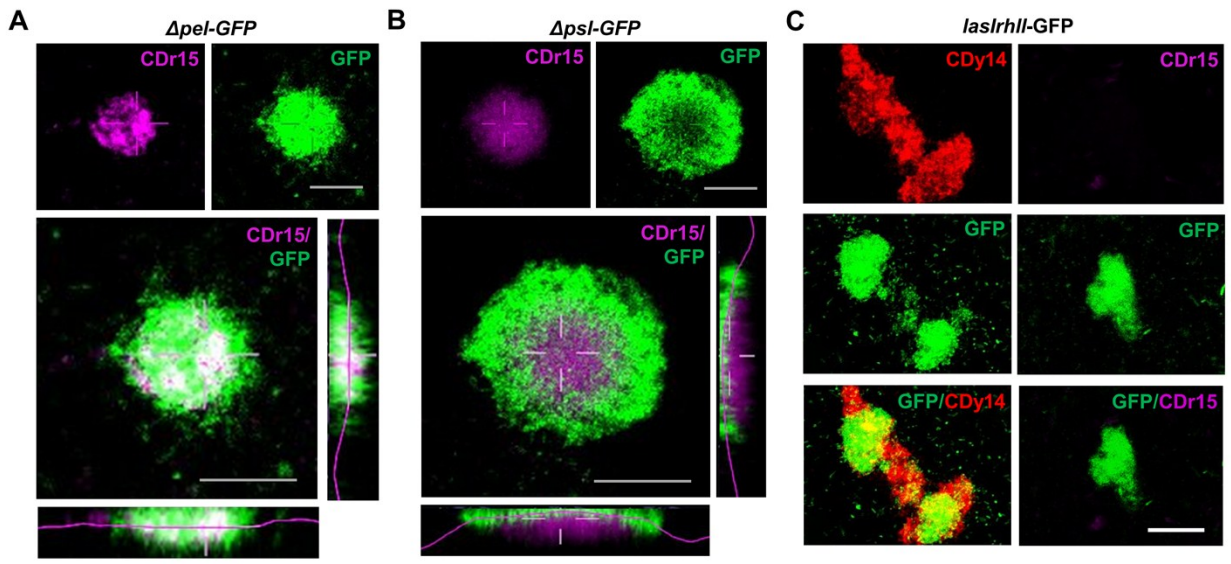


Fig. S3 The selectivity of CDr15 on biofilm of chamber slide. The biofilm images of **(A)** *Δpel*-GFP and **(B)** *Δpsl*-GFP. Horizontal sectioned images which were the matrix close to the surface (square) and two vertical sectioned images of the matrix (rectangle) were taken by confocal microscopy. **(C)** No staining of CDr15 on biofilm of *lasIrhlI*-GFP; eDNA-deficient *P. aeruginosa*. The left column showed CDy14 stain, and the right column showed CDr15 stain pattern. Scale bar, 20 μm.

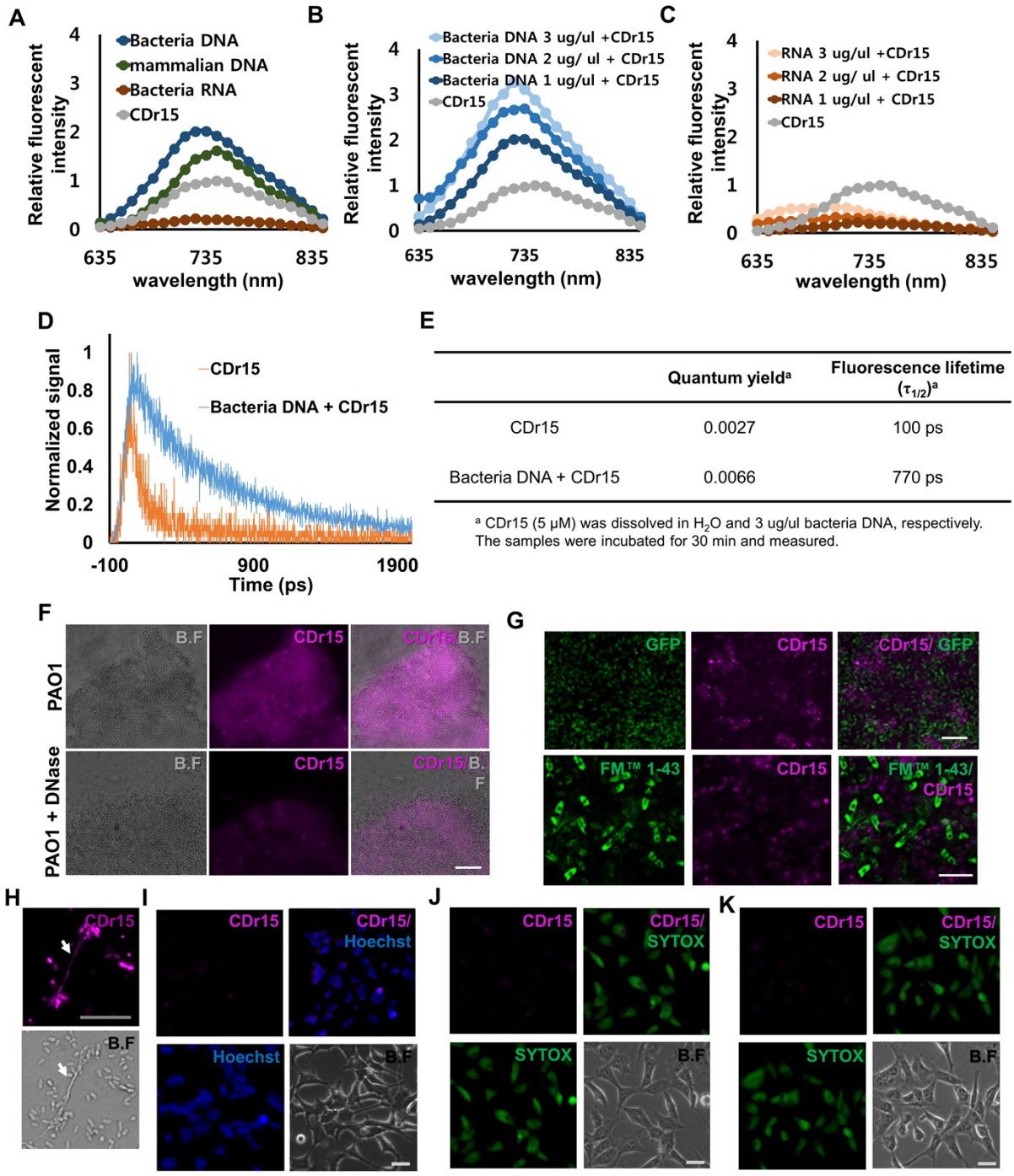


Fig. S4. The target validation of CDr15. (A) Measurement of relative fluorescence intensity for binding response between nucleic acids; mammalian DNA (1 μg/μL), bacterial DNA (1 μg/μL, chromosomal DNA) and RNA (1 μg/μL). The fluorescence intensity was measured by

spectrophotometer. The affinity of CDr15 and the different amount of **(B)** bacterial DNA (chromosomal DNA) and **(C)** bacterial RNA (1, 2, 3 μg/μL) was tested by spectrophotometer. **(D)** Normalized fluorescence lifetime signal of CDr15 and its binding to DNA. **(E)** Quantum yield and fluorescence lifetime change of CDr15 upon DNA binding. CDr15 (5 μM) was dissolved in H_2O and 3 ug/ul bacteria DNA, respectively. **(F)** 1 μM CDr15 was treated for 30 min on DNase (5 μg/μL) treated (lower) and non-treated biofilm of PAO1 (upper). Images were taken by the fluorescence microscopy. Scale bars, 10 μm. **(G)** Upper: CDr15 (1 μM) stained biofilm of PA01-GFP strain. The image was taken by the SR-SIM. Scale bars, 10 μm. Lower: CDr15 (1 μM) and FM 1-43 (1.65 μM, bacteria membrane staining dye) stained biofilm. The image was taken by confocal. Scale bars, 10 μm. **(H)** Presence of eDNA in bacteria twitching motility. No staining mammalian cell (HEK293) **(I)** in live, **(J)** fixed cell and **(K)** fixed & permeabilized cell.

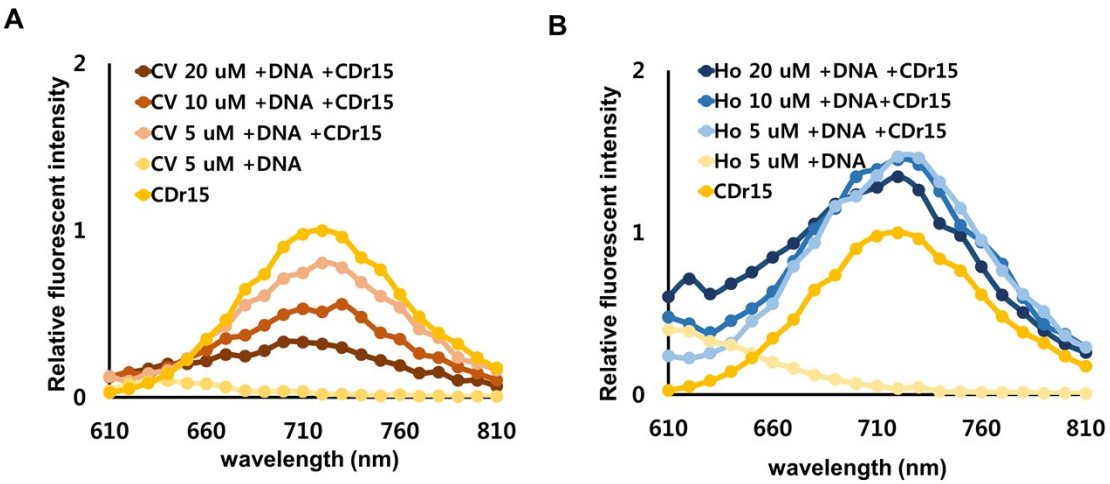


Fig. S5. CDr15 competition assay. CDr15 (5μM) fluorescence intensity was measured after CV or Ho treatment with DNA (1 μg/μl) by spectrophotometer. CV (crystal violet, DNA intercalator) and Ho (Hoechst, minor groove binder) were used as competitors. **(A)** CDr15 fluorescence intensity decreased when the concentration of CV increased. **(B)** No significant change of CDr15 fluorescence intensity when the concentration of Ho increased.

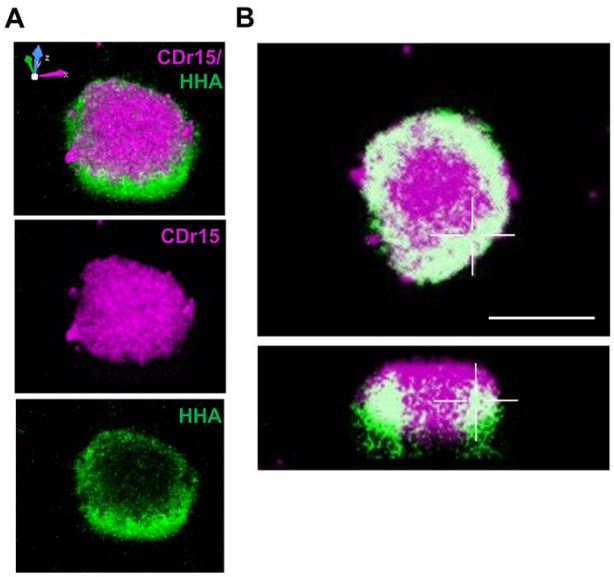


Fig. S6. Localization of CDr15 on PAO1 biofilm with HHA-FITC. (A) The localization of CDr15 on biofilm with HHA-FITC antibody stain on PAO1 biofilm. Confocal 3D images were taken with size 141.82 μm x 141.82 μm x 45 μm (x,y and z); calibration xy: 0.28 μm, z: 1 μm. **(B)** The biofilm images and horizontal sectioned images, which were the matrix close to the surface (square) and vertical sectioned image of the matrix (rectangle) were taken by confocal microscopy. Scale bar: 20 μm.

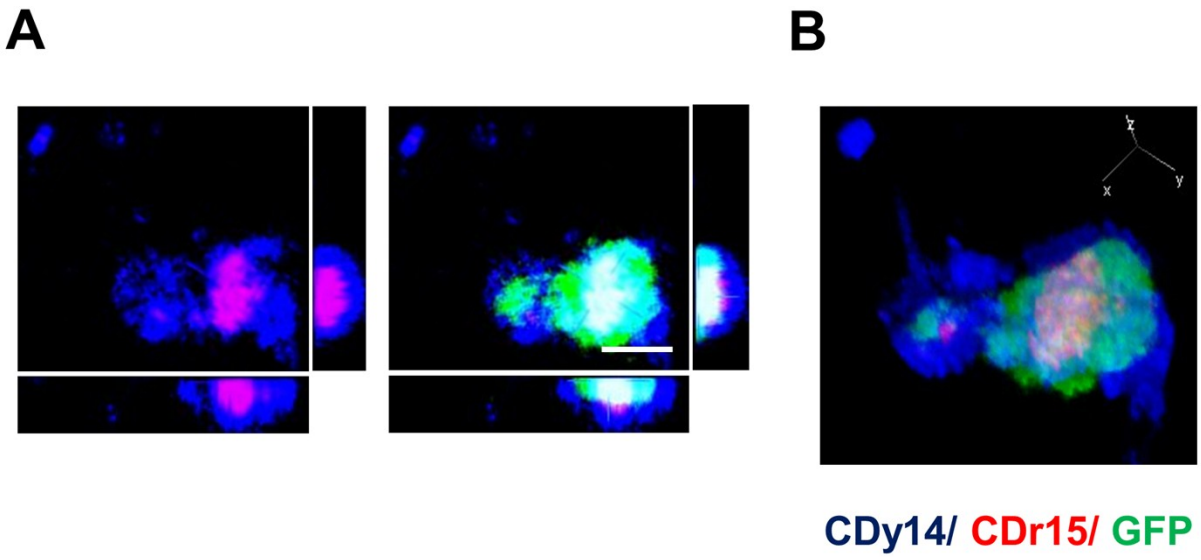
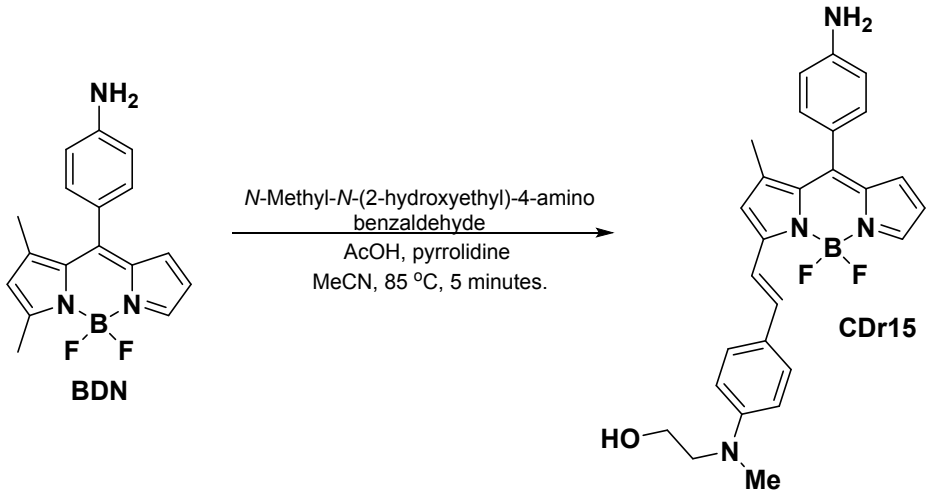


Fig. S7. Localization of CDy14 and CDr15 on biofilm. (A) The horizontal sectioned images were taken with biofilm on the chamber slide; the matrix close to the middle (square) and two vertical sectioned images of the matrix (rectangle). The schematic figure showed the different localization of CDy14 and CDr15 in microcolony of biofilm. Scale bar: 20 μm. **(B)** The 3D images were taken by the confocal microscopy with size 119 μm x 119 μm x 9 μm (x, y, and z); calibration xy: 0.23 μm, z: 0.5 μm. The image was reconstructed by Image J.

General synthetic procedure and characterization of compound CDr15.

General information: All the chemicals and solvents were purchased from Sigma Aldrich, Alfa Aesar, Fluka, MERCK or Acros and used without further purification. Normal phase purifications were carried out using Merck Silica Gel 60 (particle size: 0.040-0.063 mm, 230-400 mesh). Analytical characterization was performed on an HPLC-MS (Agilent-1200 series) with a DAD detector and a single quadrupole mass spectrometer (6130 series) with an ESI probe. An analytical method, unless indicated, the gradient solvent system was water: acetonitrile (ACN) (95:5 to 5:95) with 0.1% HCO_2H in the run time of 10 min; C18 (2) Luna column (4.6 × 50 mm, 2.5 µm particle size). ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker Avance 300 and 600 MHz NMR spectrometer, and chemical shifts were expressed in parts per million (ppm). All the photo-physical studies of CDr15 were performed in SpectraMax®M2 Spectrophotometer (Molecular Devices) instrument, and the obtained data were analyzed using the Microsoft Office Excel and Origin 8.5.



Supplementary Scheme 1: Synthetic scheme of CDr15

General Procedure for the Synthesis of compound CDr15: To a solution of compound (BDN)[5] (1x equiv) in anhydrous acetonitrile was added with a corresponding aldehyde (4x equiv), followed by acetic acid (6x equiv) and pyridine (6x equiv), then heated at 85 °C for 5 min. After solvent evaporation, the crude residue was purified by flash column chromatography to afford the condensed product as a solid.

Characterization of compound CDr15.

CDr15. ^{1}H NMR (600 MHz, Acetone-d_6) δ 7.57 (dt, *J* = 13.3, 10.4 Hz, 5H), 7.21 – 7.15 (m, 2H), 7.01 (s, 1H), 6.86 (d, *J* = 7.7 Hz, 4H), 6.47 (d, *J* = 3.6 Hz, 1H), 6.40 (dd, *J* = 3.8, 2.2 Hz, 1H), 5.15 (s, 2H), 3.91 (m, 1H), 3.80 (m, *J* = 4.8 Hz, 2H), 3.62 (t, *J* = 5.9 Hz, 2H), 3.13 (d, *J* = 1.2 Hz, 3H), 1.79 (s, 3H); ^{13}C NMR (151 MHz, Acetone-d_6) δ 206.37, 159.86, 152.28, 152.08, 150.98, 146.54, 142.08, 141.98, 135.78, 131.50, 130.83, 130.61, 124.71, 124.54, 122.88, 120.35, 120.27, 115.55, 114.60, 113.86, 112.86, 60.01, 55.20, 39.38, 16.03; ESI-MS *m/z* : calc for $C_{27}H_{27}BF_2N_4O$ (M+H) 473.2, found: 473.2; Extinction coefficient (ε): 52760 $M^{-1}cm^{-1}$ (Solvent: DMSO, Wavelength (λ): 618 nm).

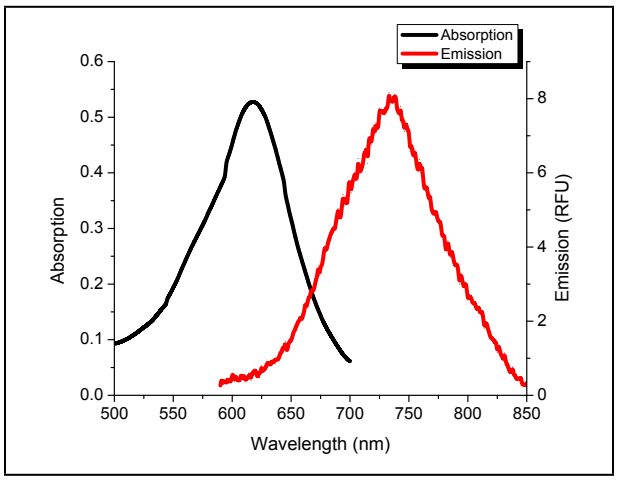
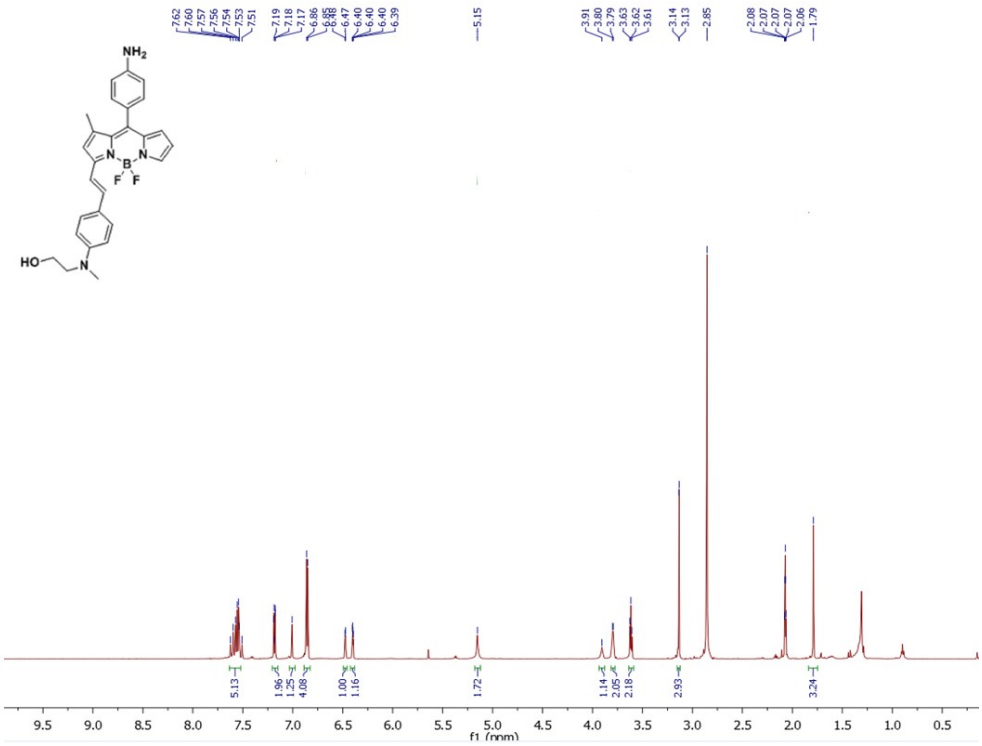


Fig. S8 Absorption and emission spectra of CDr15. Absorption and emission spectra of **CDr15** were measured at the concentration of 10 μM in DMSO. Excitation wavelength (λ_{ex}) = 570 nm.

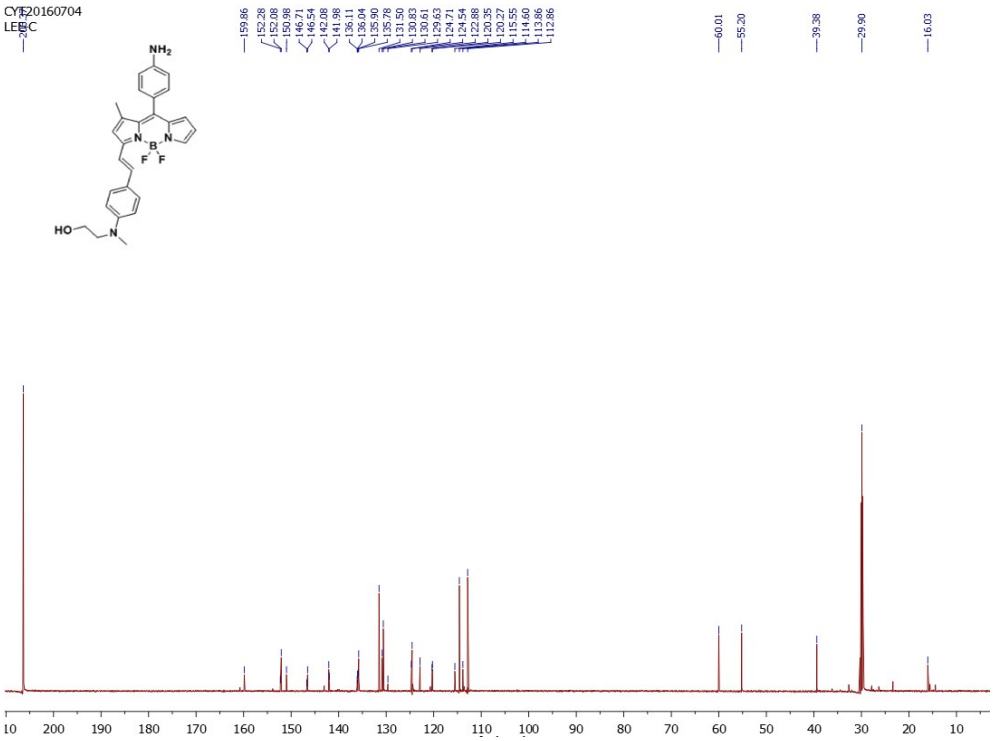
Compound	M^+ (cal)	M^+ 1(exp)	Abs (nm)	Em (nm)	QY	Purity (%)
CDr15	472.22	473.2	618	733	0.009	99

Table S2. Spectroscopic properties and purity table for CDr15. Absorbance maximum (λ_{abs}), fluorescent emission maximum (λ_{em}), and quantum yield (QY) in DMSO. Concentration is 10 μM. Excitation wavelength (λ_{ex}) = 570 nm.

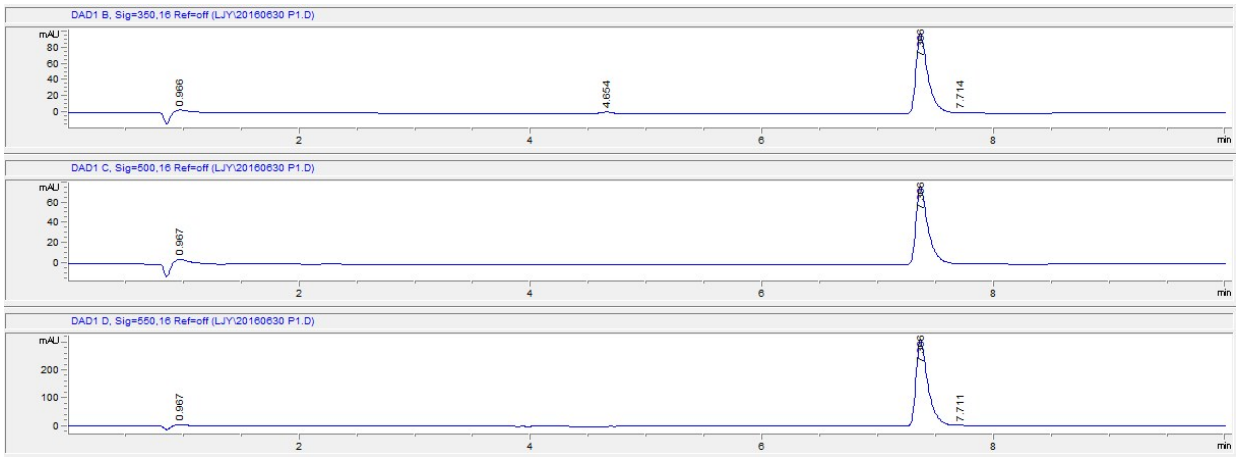
^{1}H NMR (600 MHz, Acetone-d_6) spectrum of CDr15



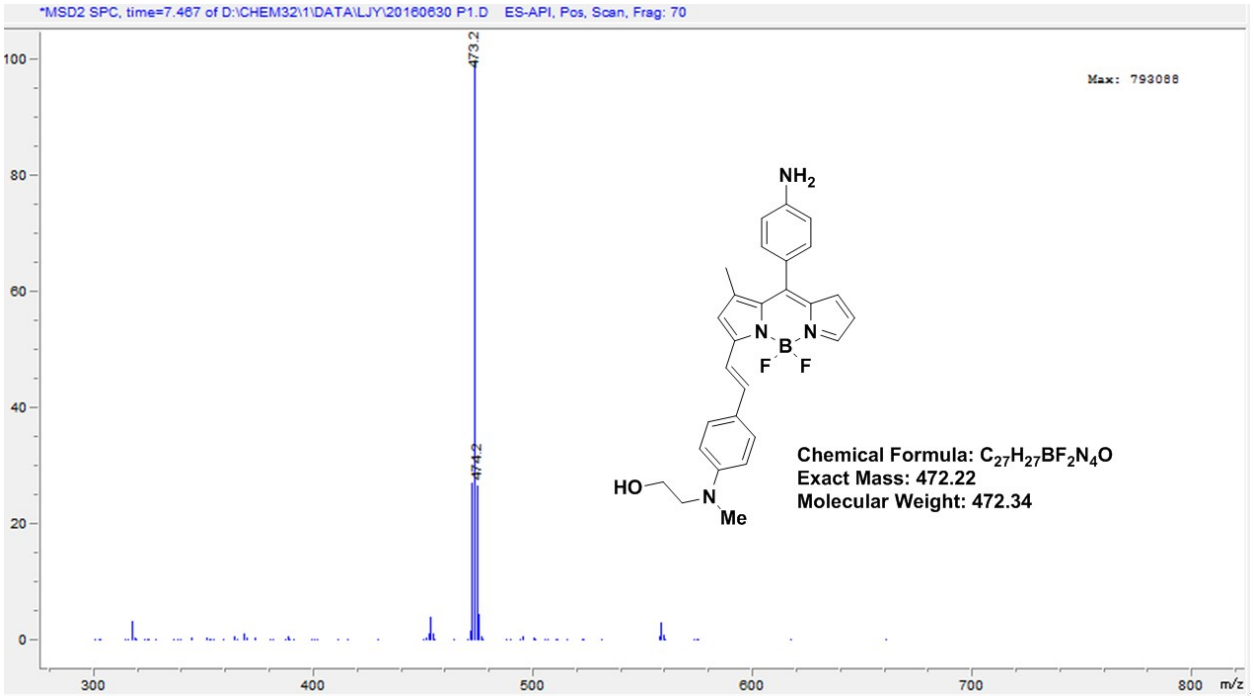
^{13}C NMR (151 MHz, Acetone -d_6) spectrum of CDr15



HPLC Spectrum of CDr15



MS spectrum of CDr15



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