Supporting Information

In Vivo Targeting of Hydrogen Peroxide by Activatable Cell-Penetrating Peptides

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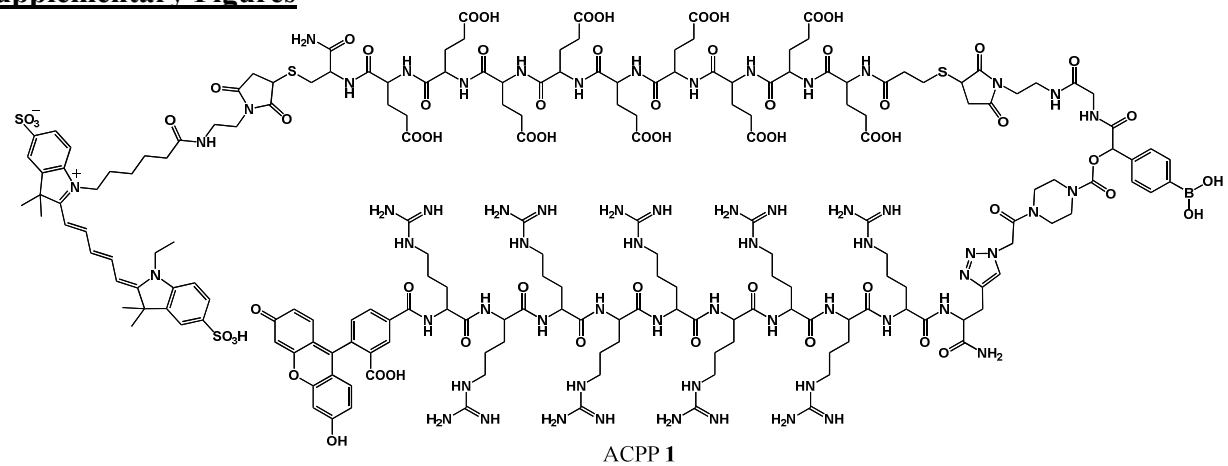
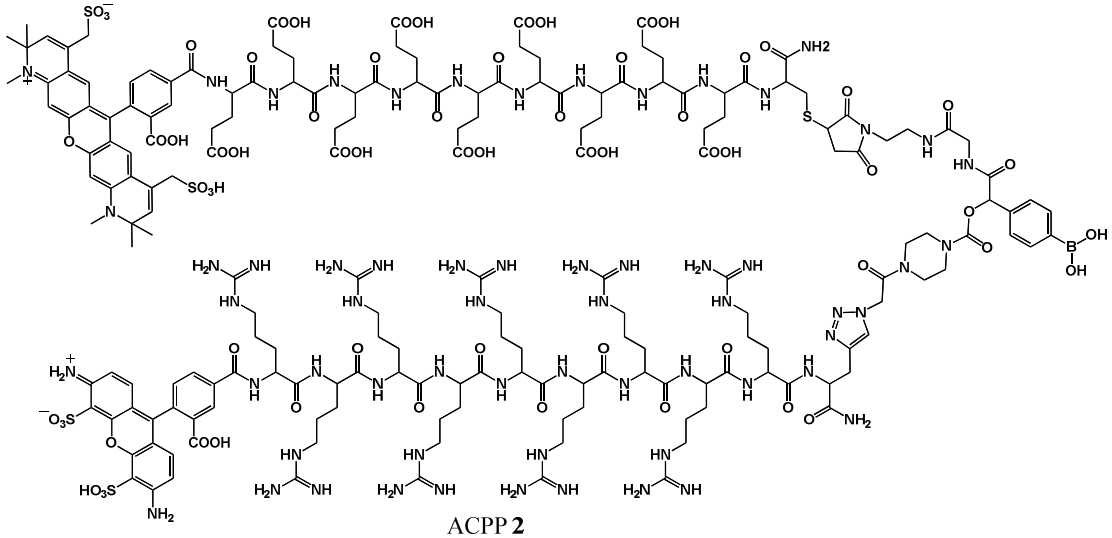
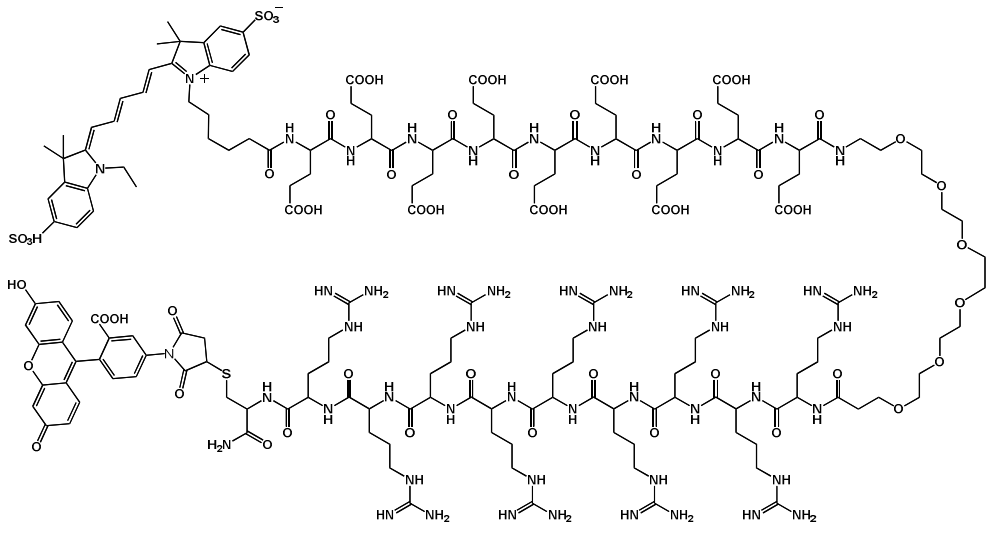
Supplementary Figures



ACPP **1**



ACPP **2**



Uncleavable ACPP **3**

Figure S1. Chemical structures of ACPPs **1** and **2** and uncleavable ACPP **3.**

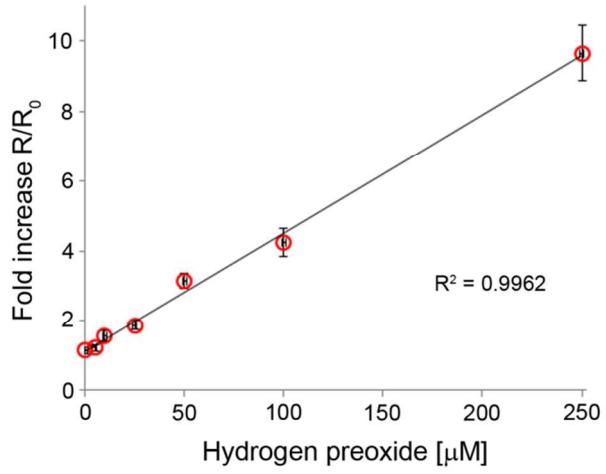


Figure S2. Line graph representation for Figure 2A showing the linear dependency of ACPP **1** FRET change on the H_2O_2 concentration. Experiments were performed in triplicate. Error is ± standard deviation.

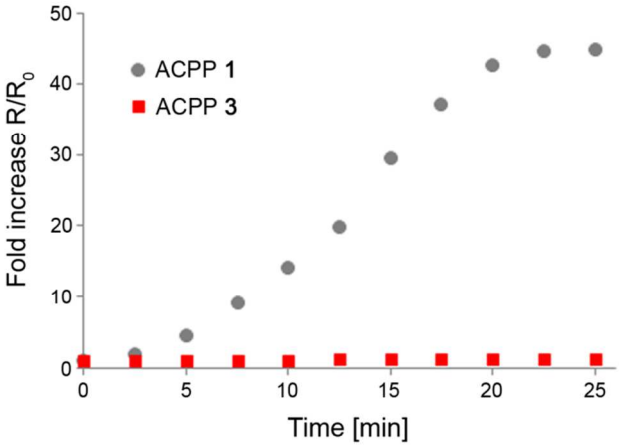


Figure S3. Uncleavable ACPP **3** does not respond to H_2O_2. ACPP **1** or uncleavable ACPP **3** (1 μM each) were incubated with H_2O_2 (2 mM) in PBS pH 7.4 and FRET changes were monitored every 2.5 minutes.

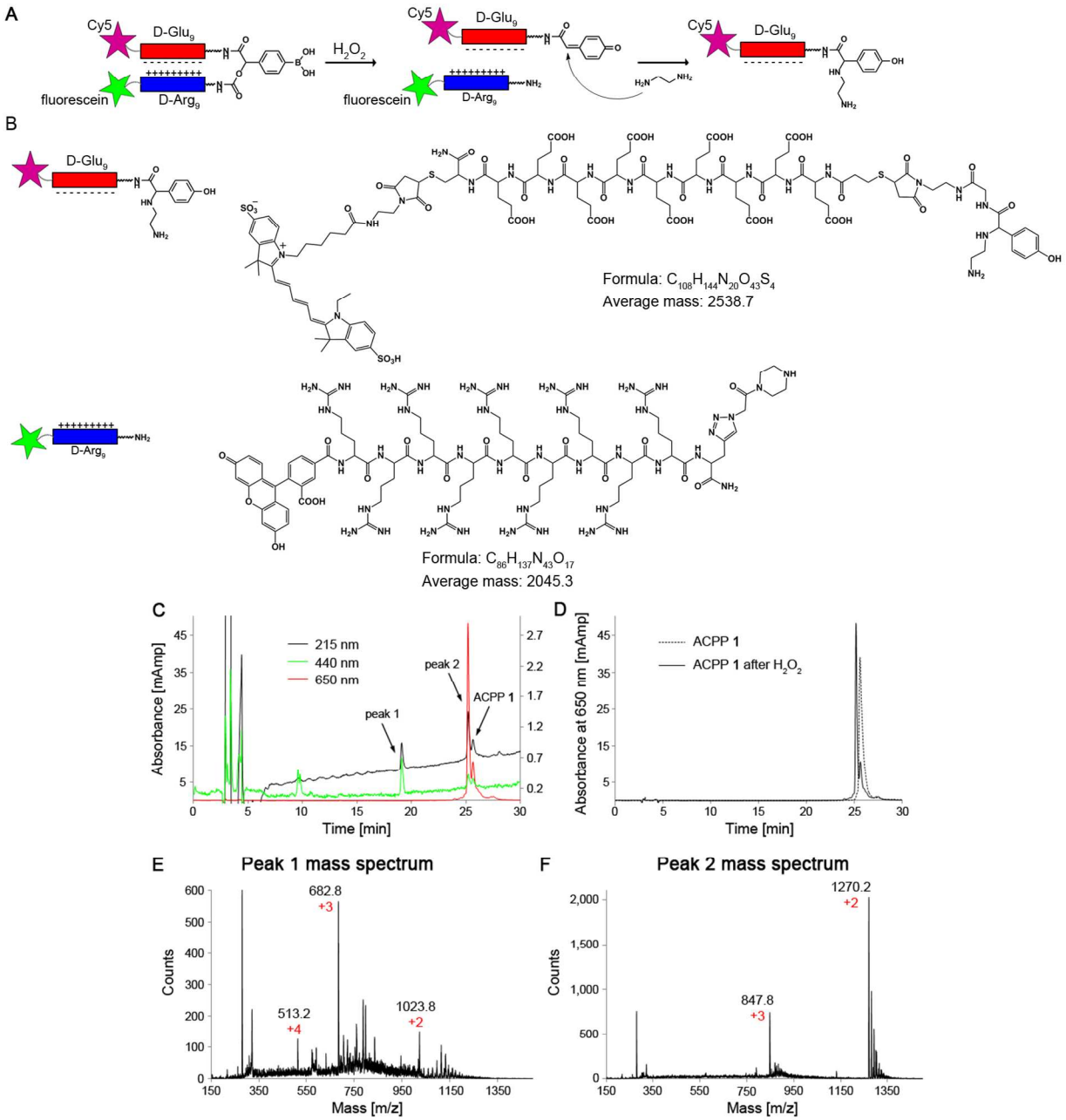


Figure S4. Analysis of the reaction products of ACPP **1** with H_2O_2 by HPLC-MS. A) Mechanism of the reaction of ACPP **1** with H_2O_2 in the presence of ethylenediamine. The reaction was performed in ethylenediamine buffer in order to capture the nascent unstable quinone methide and to improve the ionization of the Cy5-e_9 fragment. B) Structures of expected reaction products. (C) Overlaid HPLC chromatograms (215, 440 and 650 nm) of ACPP **1** (1 μM) after 30 minutes reaction with H_2O_2 (1 mM) in ethylenediamine buffer (50 mM, pH 7.05). Some unreacted ACPP **1** can still be observed (~15%), which is evident from the overlay of ACPP **1** chromatogram before and after the reaction with H_2O_2 (D). The two newly formed peaks (peaks 1 and 2) were identified as the fluorescein-r_9 and Cy5-e_9-ethylenediamine fragments of ACPP **1** (structures are shown in B) by mass spectra analysis (E and F, respectively). C) Fluorescein-r_9 fragment: ESI-MS (positive mode) calculated average mass ($C_{86}H_{137}N_{43}O_{17}$) 2045.3, found $[M+2H]^{2+}$ = 1023.8, $[M+3H]^{3+}$ = 682.8,

$[M+4H]^{4+}$ = 513.2. D) Cy5-e_9-ethylenediamine fragment: ESI-MS (positive mode) calculated average mass ($C_{108}H_{144}N_{20}O_{43}S_4$) 2538.7, found $[M+2H]^{2+}$ = 1270.2, $[M+3H]^{3+}$ = 847.8.

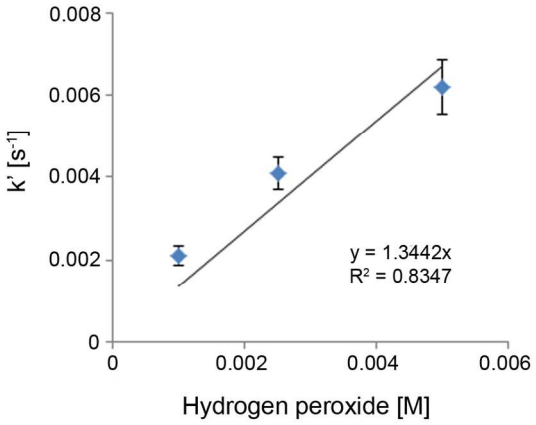


Figure S5. Measurement of the second-order rate constant for the reaction of boronic acid in ACPP **1** with H_2O_2 under pseudo first-order approximation. Values of k' were from measuring the rate of reaction of ACPP **1** (1 μM) with H_2O_2 (1, 2.5 and 5 mM). Measurements were performed in triplicate. Error is ± standard deviation.

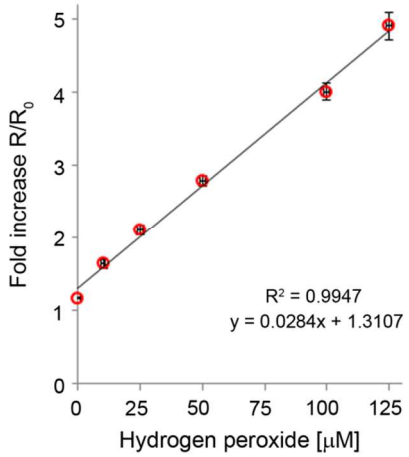


Figure S6. Line graph representation for Figure 3A, showing the linear dependency of ACPP **1** FRET change on H_2O_2 concentration. ACPP **1** (1 μM) was incubated with HL-60 cells (5 x 10^4 cells in 100 μL growth media) and hydrogen peroxide was added at the indicated concentrations. The change in 524/672 nm fluorescence emission ratio after 60 minutes is depicted. Experiments were performed in triplicate. Error is ± standard deviation.

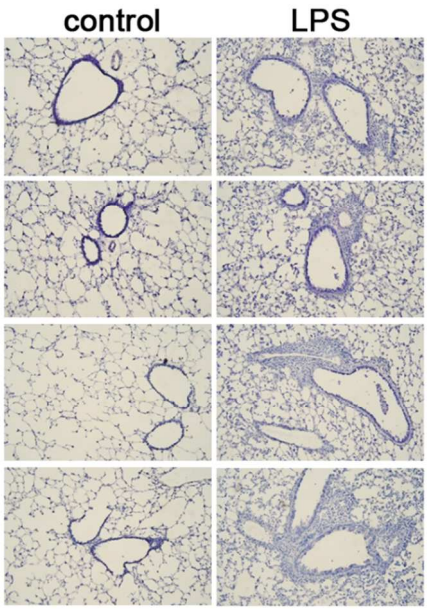


Figure S7. Hematoxylin and eosin (H&E) staining of control and LPS mice shows recruitment of inflammatory cells to the airways of mice treated with LPS.

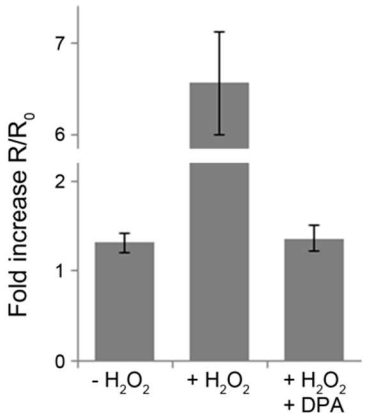


Figure S8. Penicillamine inhibits ACPP **1** reaction with H_2O_2. Changes in FRET ratio after 60 minutes of ACPP **1** (1 μM) in PBS pH 7.4 (-H_2O_2), with 100 μM H_2O_2 (+H_2O_2) or with 100 μM H_2O_2 and 1 mM D-penicillamine (+H_2O_2+DPA). Experiments were performed in triplicate. Error is ± standard deviation.

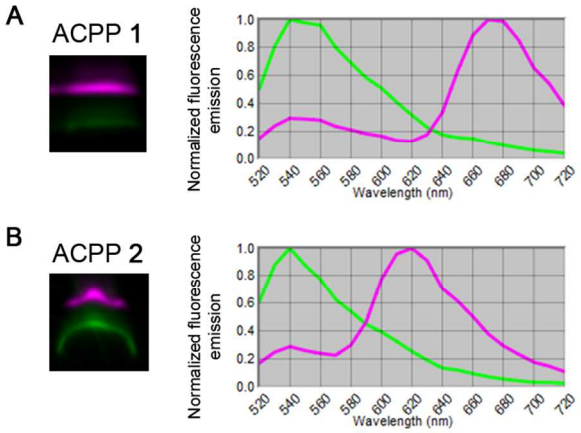


Figure S9. Fluorescence emission spectra analysis of SDS-PAGE of lung extracts from LPS-challenged mice treated with H_2O_2-ACPPs. On the left are the SDS-PAGE of lung extracts from LPS-challenged mice treated with the corresponding ACPP (A) **1** or (B) **2**. On the right are the fluorescence emission spectra (λ_{ex} = 466/44 nm) of each band on the gel, separately normalized to each spectrum's maximum. The higher molecular bands showed fluorescence emission spectra that closely resemble the corresponding intact ACPP. The lower molecular bands showed fluorescence emission spectra that closely resemble the corresponding cleaved ACPP. The bands were therefore pseudocolored to reflect the ACPP state: intact (purple) or cleaved (green).

Methods

General synthetic and analytical methods

Fmoc-protected D-amino acids were purchased from EMD Millipore. 4-bromomandelic acid was from Matrix Scientific. Cy5 maleimide (PA25031) was from GE Health Care. 5-carboxyfluorescein succinimidyl ester, Alexa Fluor 488 succinimidyl ester and Alexa Fluor 594 succinimidyl ester were from Invitrogen. PROLI NONOate was purchased from Cayman Chemical. Catalase from bovine liver was from Sigma (C9322). *N*-succinimidyl 3-(2-pyridylthio)propionate was from Molecular Biosciences Inc. All other chemicals and reagents were purchased from either Combi-Blocks Inc. or Sigma-Aldrich and used as received unless otherwise noted. Anhydrous solvents and reagents (DCM, THF, 1,4-dioxane, DMSO) were obtained as SureSeal bottles from Sigma-Aldrich. Thin-layer chromatography and flash chromatography were performed using EMD pre-coated silica gel 60 F-254 plates and silica gel 60 (230-400 mesh). UV absorbance and fluorescence spectra were recorded on a Cary 3E (Varian) and Fluorolog 2 (Spex) fluorimeter, respectively. Peptides were synthesized using Protein Technologies Inc. Prelude system through standard solid phase Fmoc syntheses on Rink-amide resin preloaded with D-Glu or D-Arg (AnaSpec). Analytical and preparative HPLCs were performed on Agilent HPLCs, with Luna C18(2) columns (Phenomenex) using water (solvent A) and acetonitrile (solvent B) with 0.05% TFA as an additive. Low resolution ESI mass spectrometry was performed on an Agilent LC/MSD Trap XCT coupled to an Agilent HPLC. High resolution mass spectra were acquired on a ThermoFisher Orbitrap XL hybrid mass spectrometer coupled to an Agilent HPLC. ^{1}H- and ^{13}C-NMR spectra were collected in d_6-DMSO or $CDCl_3$ (Cambridge Isotope Laboratories, Cambridge, MA) at 25 ºC using a Varian Unity Inova spectrometer at 500 MHz at the Department of Chemistry and Biochemistry NMR Facility at the University of California, San Diego. All chemical shifts are reported in the standard δ notation of parts per million using the peak of residual proton signals of d_6-DMSO or $CDCl_3$ as an internal reference. All ACPPs (**1**-**3**) were prepared as 100 μM stock solutions in dd water and diluted to working concentrations as described.

Abbreviations: DMF: dimethylformamide, THF: tetrahydrofuran, Et_3N: triethylamine, HATU: 2-(7-aza-1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, DCM: dichloromethane, DMSO: dimethyl sulfoxide, TFA: trifluoroacetic acid, DIPEA: N,N-diisopropylethylamine, dppf: 1,1'-bis(diphenylphosphino)ferrocene, EDC: 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide, DMAP: 4-dimethylaminopyridine, NMM: N-methylmorpholine, TBTA: Tris-(benzyltriazolylmethyl)amine, FBS: fetal bovine serum, PMA: phorbol myristate acetate, SDS: sodium dodecyl sulfate, dd: double distilled, RT: room temperature.

Preparative HPLC conditions. Preparative separations were performed on an Agilent HPLC system (1200 series) with Luna 10 μm PREP C18(2) column (250.0 X 21.2 mm, 100 Å), using a water-acetonitrile solvent system (solvent A = water, solvent B = acetonitrile, both with 0.05% TFA as an additive) at flow rate of 15 mL/min.

Sensitivity test. ACPP **1** (1 μM) was incubated with H_2O_2 (0-250 μM) in 100 μL PBS 50 mM pH 7.4 and fluorescence emission at 524 and 672 nm (λ_{ex} = 488 nm) was monitored every 2.5 minutes for 60 minutes. Experiments were performed in triplicate. Error is ± standard deviation.

Second-order rate constant measurement. Second-order rate constant measurement was performed under pseudo first-order conditions. ACPP **1** (1 μM) was incubated with a large excess of H_2O_2 (1, 2.5 and 5 mM) in PBS 50 mM pH 7.4 and fluorescence emission at 524 and 672 nm (λ_{ex} = 488 nm) was monitored every 1.5 minutes until no change in the 524/672 emission ratio was observed (~20 minutes). The measured ratio values were converted into ACPP **1** concentration and plotted as ln([ACPP **1**)] vs. time [sec]. The obtained k' (k' = k[H_2O_2]) values were then plotted vs. [H_2O_2] to extract the second-order rate constant. Error is ± standard deviation.

Selectivity test. ACPP **1** (1 μM) was incubated with various ROS (100 μM) in 100 μL PBS 50 mM pH 7.4 and the fluorescence emission at 524 and 672 nm (λ_{ex} = 488 nm) was monitored every 5 minutes for 60 minutes. ROS were generated as follows: hydrogen peroxide (H_2O_2), tert-butyl hydroperoxide (TBHP) and hypochlorite (^{-}OCl) were introduced from 10 mM stock solutions in double distilled degassed water prepared from commercially available 32%, 70% and 5% aqueous solutions, respectively. Superoxide ($O_2^{\cdot -}$) was delivered from 10 mM stock of potassium superoxide (KO_2) in DMSO. Nitric oxide (NO•) was generated from PROLI NONOate. The catalase concentration, when present, was 0.5 mg/mL.

ACPPs photostability. The photostability of ACPPs **1** and **2** was assessed by comparing their photobleaching rates to the respective non-conjugated dyes (Cy5 and 5(6)-carboxyfluorescein, and Alexa594 and Alexa488, respectively) in PBS (50 mM pH 7.4). Dyes were irradiated with light (480/30 nm for fluorescein and Alexa488, 568/40 nm for Alexa594 and 615/40 nm for Cy5) using an Oriel solar-simulator equipped with a 1500 watt xenon arc lamp for the durations indicated. The absorbance of the molecules was measured at each time point.

Cell culture methods. HL-60 cells were purchased from ATCC (CCL-240). Cells were cultured in phenol-red free RPMI-1640 medium (Cellgro), supplemented with 10% FBS, penicillin (100 units/mL), streptomycin (100 μg/mL) and glutamine (2 mM). Cells were cultured in a humidified atmosphere containing 5% CO_2 at 37 °C and maintained at a density bellow 1 x 10^6/mL.

Cellular assays. HL-60 cells in their complete growth medium (5×10^4 cells in 100 μL medium) were transferred to black 96-well plates with clear bottoms. ACPP **1** (1 μM from stock solution of 100 μM in dd water) and H_2O_2 (0-125 μM, from appropriate stock solutions in dd water so 1 μL stock was added) were added to the cells. Fluorescence emission at 524 and 672 nm (λ_{ex} = 488 nm) was monitored for 60 minutes using a Tecan Infinite M100 Pro plate reader. For the stimulation experiment, under similar conditions, cells were treated with either dd water (2 μL), catalase (final concentration 0.5 mg/mL), PMA (final concentration 0.5 μM) or PMA+catalase (0.5 μM and 0.5 mg/mL, respectively). The fluorescence emission at 524 and 672 upon excitation at 488 nm was monitored for 60 minutes. All experiments were performed in triplicate.

Induction of *in vivo* lung inflammation. Six- to eight-week-old female C57BL/6 (Tyrc BrdCrHsd, albino, Harlan Sprague Dawley) mice were administered 10 μg lipopolysaccharide (LPS) from *Escherichia coli* 055:B5 (Sigma-Aldrich) in 35 μL of phosphate buffered saline intranasally (i.n.) on four consecutive days; they were sacrificed twenty-four hours after the final i.n. challenge with LPS. Age- and sex-matched control mice were also tested. The University of California, San Diego Institutional Animal Care and Use Committee approved of all animal experimental protocols for the experiments included in this paper.

Animal imaging. The ACPPs (10 nmol **1** and **3** or 5 nmol **2** in 100 μL dd water) were administered i.n. while mice were under anesthesia with isoflurane. To knockdown ROS activity, some mice were tested with pre-administration of D-penicillamine (1 μmol, i.n., 5 min before ACPP administration). Six hours after ACPP administration, mice were euthanized by isoflurane overdose followed by cervical dislocation. Then, the skin and ribcages were removed, after which the lungs were inflated with a mix of Tissue-Tek optimized cooling temperature (OCT) compound (Sakura) and PBS (3:2) and isolated for imaging with a whole-body mouse imager (Maestro, CRi). For the ACPP **1**, lungs were imaged with the following settings: (1) direct fluorescein: ex 466/44 nm, em 520/10; (2) direct Cy5: ex620/20, em 670/10; and (3) ratiometric: λ_{ex} = 466/44, λ_{em} = 520-720 in 10 nm steps (numerator 520 – 600; denominator 670 – 720). For ACPP **2**, lungs were imaged with (1) direct 488: λ_{ex} = 466/44, λ_{em} = 520/10, (2) direct 594: λ_{ex} = 590/23, λ_{em} = 620/10, and (3) ratiometric: λ_{ex} = 466/44, λ_{em} = 520-720 in 10 nm steps (numerator 520 – 580; denominator 620 – 720). Ratiometric images were produced with custom software by dividing the numerator (cleaved ACPP) by the denominator (uncleaved ACPP) and creating a pseudocolor for the ratio value varying from blue (lowest ratio) to red (highest ratio). ROIs were delineated and analyzed for the average lung ratio using ImageJ (National Institutes of Health).

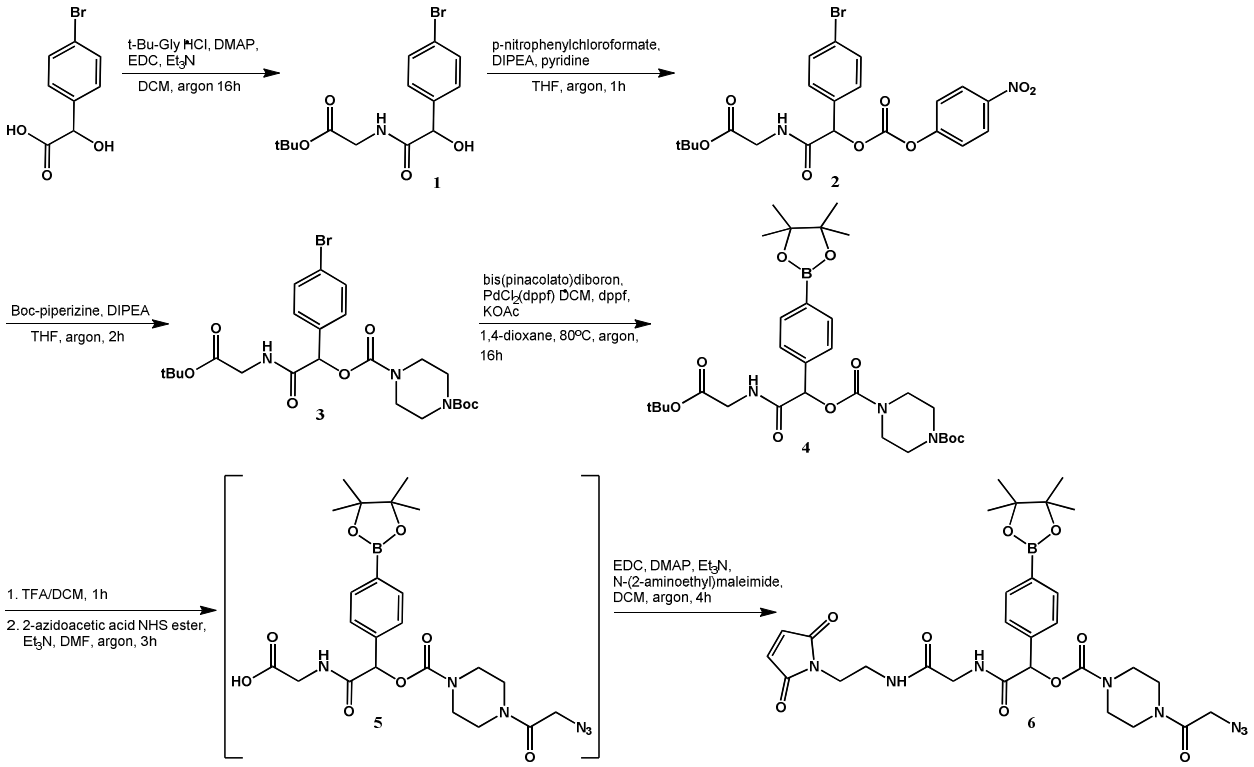
H&E staining. Lung sections (5 μm) were cut and stained with hematoxylin and eosin to verify lung inflammation in the LPS model.

***In vitro* H_2O_2 scavenging by D-penicillamine.** ACPP **1** (1 μM) was incubated with D-penicillamine (1 mM) and H_2O_2 (100 μM) in 100 μL PBS 50 mM pH 7.4. Fluorescence emission at 524 and 672 nm (λ_{ex} = 488 nm) was monitored every 2.5 minutes for 60 minutes.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Lungs from mice that had been pre-injected with the appropriate ACPP were harvested and diluted 9x in a 1:1 mixture of tricine SDS sample buffer (Life Technologies) and PBS. The lung samples in buffer were then sonicated and boiled at 100°C for two minutes. The samples were diluted an additional 1:2 in tricine SDS sample buffer, run on a 10 – 20% Novex tricine gel (Life Technologies), and imaged with the Maestro (CRi) at the same settings as described in animal imaging for direct and ratiometric imaging.

Statistical methods. Data were expressed as the mean ± standard deviation for all experiments. Statistical significance was determined using paired or unpaired two-tailed *t*-test, as appropriate.

Synthetic procedures and characterization



Scheme S1. Synthetic scheme for the H_2O_2-senstitive linker **6**.

Compound 1. 4-Bromo-mandelic acid (racemic mixture, 723 mg, 3.13 mmol, 1 eq) was dissolved in dry DCM (15 mL) under argon atmosphere. Then, *tert*-butyl glycine hydrochloride (1077 mg, 6.32 mmol, 2.05 eq), DMAP (38 mg, 0.31 mmol, 0.1 eq), Et_3N (890 µL, 6.32 mmol, 2.05 eq) and EDC (972 mg, 6.26 mmol, 2 eq) were sequentially added. The reaction was stirred at room temperature for 16 hours. The reaction solution was diluted with DCM (50 mL) and washed with sat. NH_4Cl, brine and water. The organic phase was separated, dried over $MgSO_4$, filtered and the solvent was removed under reduced pressure. The crude product was purified on silica gel column (EtOAc:Hex 1:3 to 1:1). After removal of solvents under reduced pressure, 677 mg (1.96 mmol, yield 62%) of **1** as white foam were obtained.

^{1}H-NMR ($CDCl_3$, 500 MHz): δ = 7.51 (2H, d, *J* = 7.8 Hz), 7.33 (2H, d, *J* = 8.8 Hz), 6.69 (1H, broad singlet), 5.08 (1H, s), 3.92 (2H, t, *J* = 5.3 Hz), 1.45 (9H, s). ^{13}C-NMR ($CDCl_3$, 125 MHz): δ = 171.8, 168.8, 138.2, 132.2, 128.7, 123.0, 83.0, 73.7, 42.1, 28.2. ESI-MS (positive mode) calculated ($C_{14}H_{18}BrNO_4$) 343.0, found *m*/*z* $[M+Na]^+$ 366.1 and 368.1 (Br 79/81 respectively).

Compound 2. Compound **1** (677 mg, 1.96 mmol, 1 eq) was dissolved in dry THF (15 mL) under argon atmosphere and cooled to 0°C. Then, 4-nitrophenol chloroformate (1,184 mg, 5.88 mmol, 3 eq) was added, followed by DIPEA (1.36 mL, 7.84 mmol, 4 eq) and pyridine (32 µL, 0.4 mmol, 0.1 eq). The reaction was allowed to warm to room temperature and stirred for additional 1 hour. The reaction solution was diluted with EtOAc (50 mL) and washed with sat. NH_4Cl, brine and water.

The crude product was purified on silica gel column (EtOAc:Hex 15:85 to 3:7). After removal of solvents under reduced pressure, 827 mg (1.62 mmol, yield 83%) of **2** as white foam were obtained.

^{1}H-NMR ($CDCl_3$, 500 MHz): δ = 8.29 (2H, d, *J* = 8.8 Hz), 7.56 (2H, d, *J* = 8.8 Hz), 7.41 (2H, d, *J* = 8.3 Hz), 7.39 (2H, d, *J* = 9.3 Hz), 6.81 (1H, t, *J* = 5.3 Hz), 6.02 (1H, s), 4.00 (2H, d, *J* = 5.4 Hz), 1.48 (9H, s). ^{13}C-NMR ($CDCl_3$, 125 MHz): δ = 168.6, 166.9, 155.2, 151.1, 145.8, 133.2, 132.5, 129.5, 125.6, 124.3, 121.9, 83.2, 79.0, 42.1, 28.2. ESI-MS (positive mode) calculated ($C_{21}H_{21}BrN_2O_8$) 508.0, found *m*/*z* $[M+Na]^+$ 531.3 and 533.5 (Br 79/81 respectively).

Compound 3. Compound **2** (774 mg, 1.52 mmol, 1 eq) mmol was dissolved in dry THF under argon atmosphere. DIPEA (291 μL, 1.67 mmol, 1.1 eq) and 1-Boc-piperizine (312 mg, 1.67 mmol, 1.1 eq) were added. The reaction was stirred at room temperature for 2 hours. The reaction solution was diluted with EtOAc (50 mL) and washed with sat. NH_4Cl, brine and water. The organic phase was separated, dried over $MgSO_4$, filtered and the solvent was removed under reduced pressure. The crude product was purified on silica gel column (EtOAc:Hex 1:3 to 1:1). After removal of solvents under reduced pressure, 522 mg (0.94 mmol, yield 62%) of **3** as white solid were obtained.

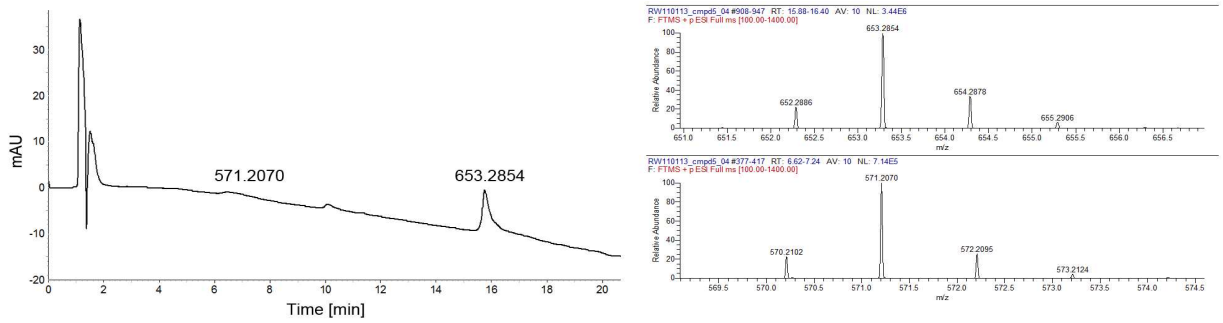
^{1}H-NMR ($CDCl_3$, 500 MHz): δ = 7.50 (2H, d, *J* = 9.3), 7.33 (2H, d, *J* = 9.3), 6.64 (1H, broad singlet), 6.03 (1H, s), 3.93 (2H, dd, J_1 = 4.4 Hz, J_2 = 1.5 Hz), 3.69-3.33 (8H, m), 1.46 (18H, s). ^{13}C-NMR ($CDCl_3$, 125 MHz): δ = 168.8, 168.3, 154.7, 153.5, 134.8, 132.1, 129.3, 123.5, 83.0, 80.6, 76.0 44.3, 44.1, 42.1, 28.6, 28.2. ESI-MS (positive mode) calculated ($C_{24}H_{34}BrN_3O_7$) 555.2, found *m*/*z* $[M+Na]^+$ 578.2 and 580.2 (Br 79/81 respectively).

Compound 4. Compound **3** (300 mg, 0.54 mmol, 1 eq) was dissolved in 1,4-dioxane (7 mL) under argon atmosphere. Then, bis(pinacolato)diboron (548 mg, 2.16 mmol, 4 eq), $Pd(dppf)Cl_2 \cdot DCM$ (88 mg, 0.108 mmol, 0.2 eq), dppf (30 mg, 0.054 mmol, 0.1 eq) and potassium acetate (318 mg, 3.24 mmol, 6 eq) were added. The reaction was heated to 80^{o}C and stirred for 16 hours. After cooling to room temperature, the reaction was diluted with EtOAc (50 mL) and washed with sat. NH_4Cl, brine and water. The organic phase was separated, dried over $MgSO_4$, filtered and the solvent was removed under reduced pressure. The crude product was purified on silica gel column (EtOAc:Hex 1:3 to 7:3). After removal of solvents under reduced pressure, 199 mg (0.33 mmol, yield 61%) of **4** as brownish solid were obtained.

^{1}H-NMR ($CDCl_3$, 500 MHz): δ = 7.81 (2H, d, *J* = 8.3 Hz), 7.45 (2H, d, *J* = 8.7 Hz), 6.57 (1H, broad singlet), 6.07 (1H, s), 3.92 (2H, d, (*J* = 5.4 Hz), 3.68-3.32 (8H, m), 1.46 (9H, s), 1.45 (9H, s), 1.25 (12H, s). ^{13}C-NMR ($CDCl_3$, 125 MHz): δ = 168.8, 168.6, 154.7, 153.7, 138.5, 135.4, 126.8, 84.1, 82.9, 80.5, 76.6, 44.3, 43.0, 42.1, 28.5, 28.2, 24.8. No signal was observed for carbon attached to boronate. ESI-MS (positive mode) calculated ($C_{30}H_{46}BN_3O_9$) 603.3, found *m*/*z* $[M+H]^+$ 604.4.

Compound 6. Compound **4** (100 mg, 0.16 mmol) was dissolved in 2 mL dry DCM/TFA 9:1 and stirred for 1 hour under argon atmosphere. The solvents were removed under reduced pressure, the residue triturated with Et_2O and the white solid obtained was dried under reduced pressure. The crude product was dissolved in dry DMF (4 mL), then 2-azidoacetic acid NHS ester (34.5 mg, 0.175

mmol, 1.1 eq) and Et_3N (89 μL, 0.64 mmol, 4 eq) were added. The reaction was stirred at room temperature under argon atmosphere for 2 hours. When compound **4** was completely consumed and formation of compound **5** was observed (monitored by HPLC-MS), N-(2-aminoethyl)maleimide trifluoroacetate (81 mg, 0.32 mmol, 2 eq), DMAP (2 mg, 0.016 mmol, 0.1 eq), Et_3N (45 μL, 0.32 mmol, 2 eq) and EDC (61 mg, 0.32 mmol, 2 eq) were sequentially added. The reaction was stirred at room temperature under argon atmosphere for additional 4 hours (monitored by HPLC-MS). The reaction was diluted with EtOAc (50 mL) and washed with sat. NH_4Cl, brine and water. The organic phase was separated, dried over $MgSO_4$, filtered and the solvent was removed under reduced pressure. The product was purified using preparative HPLC (10-100% solvent B in 20 minutes, retention time 14.9 minutes) to yield 5 mg of compound **6** (7.7 μmol, 5% yield) as a white solid. Compound **6** was characterized using HPLC coupled to high-resolution mass spectrometer: calculated ($C_{29}H_{38}BN_8O_9^+$) 653.2849, found 653.2854 (difference of calculated vs. found is < 1 ppm) (Chromatogram S1).

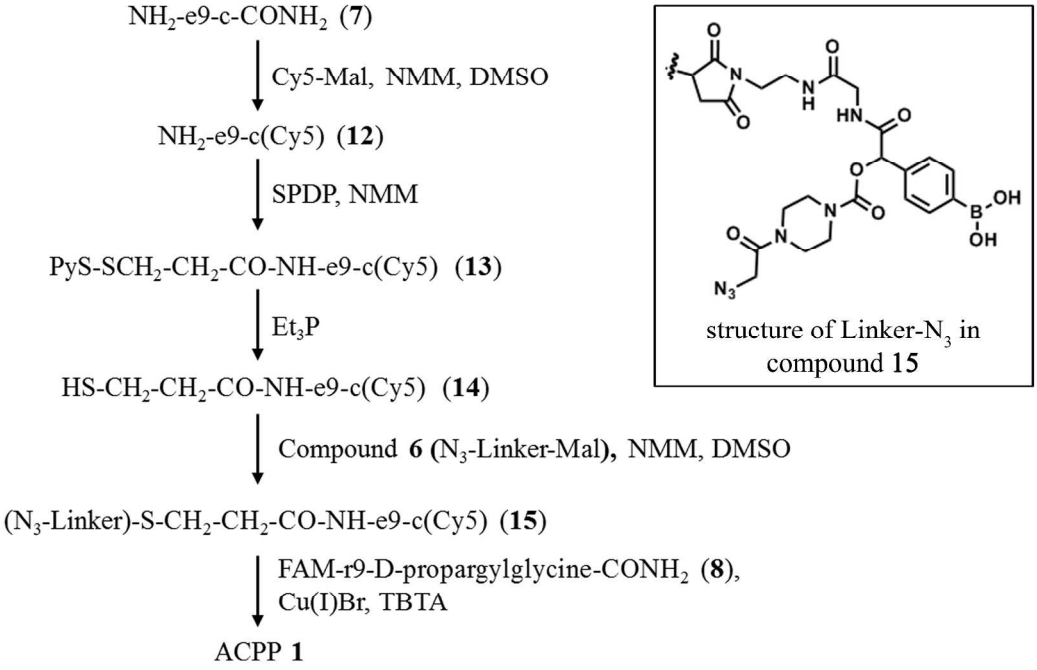


Chromatogram S1. HPLC-HRMS characterization of compound **6**. Left: HPLC chromatogram of compound **6**. Right: high-resolution mass spectra (HRMS) analysis of chromatogram on left showing compound **6** as main product (RT 15.9-16.4 min) and some hydrolysis of the boronate to boronic acid (RT 6.6-7.2 min).

Solid phase peptide synthesis:

NH_2-e$_9$-c-$CONH_2$ (**7**), FAM-r$_9$-D-propargylglycine-$CONH_2$ (**8**), NH_2-e$_9$-c(S-S*t*Bu)-$CONH_2$ (**9**), NH_2-r$_9$-D-propargylglycine-$CONH_2$ (**10**) and NH_2-e$_9$-peg6-r$_9$c-$CONH_2$ (**11**) were synthesized using standard solid phase Fmoc synthesis. Lower case letters in peptide sequence refer to D-amino acids and peg6 denotes $–NH(CH_2\text{-}CH_2\text{-}O)_6\text{-}CH_2\text{-}CH_2\text{-}CO$-. After the completion of solid phase synthesis peptides were cleaved of the resins by treating with cleavage cocktail containing 2% thioanisole, 4% TIPS, 2% water and 92% TFA for 4 hours followed by filtration to remove resin. Peptides were precipitated by addition of ice cold Hex:EtOAc (1:1) to the filtrates and then purified using preparative or semi-preparative HPLC.

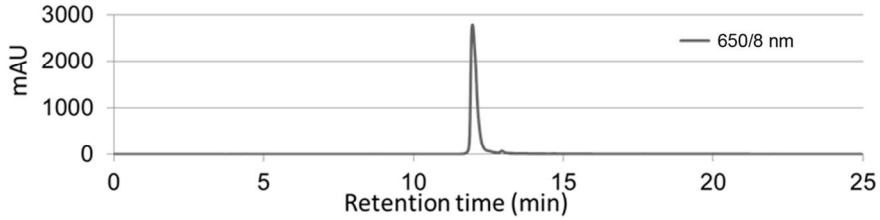
Synthesis of ACPP 1:



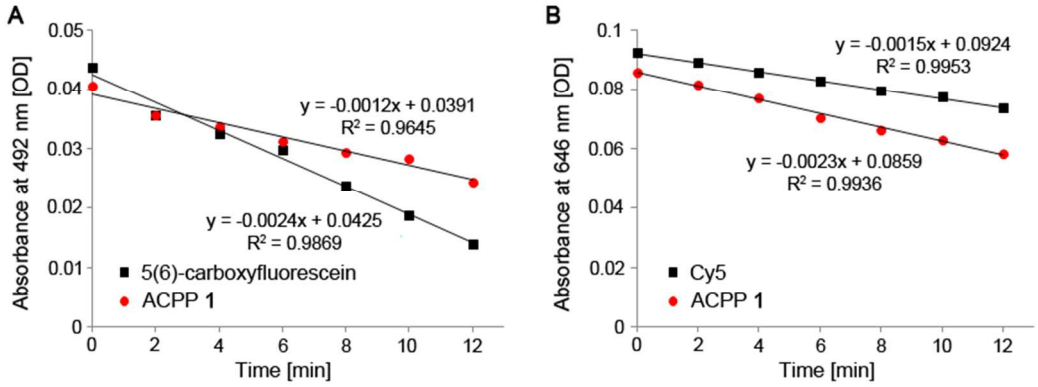
Scheme S2. Synthetic scheme of ACPP **1**.

According to scheme S2: Compound **7** (25 mg) was dissolved in anhydrous DMSO under N_2 atmosphere. Cy5-Mal (12 mg), and NMM (0.6 µl) were added and incubated for 2 hours. After completion of the reaction (as monitored by HPLC-MS) *N*-succinimidyl 3-(2-pyridylthio)propionate (12 mg) and NMM (1 µL) were added and allowed to react overnight. Then, the reaction product **13** was purified using preparative HPLC (7-50% solvent B in 27 minutes) and lyophilized. To lyophilized compound **13** was added triethylphosphine (30 µL) and the solution was stirred for 4 hours. Compound **14** was then precipitated by addition of EtOAc and purified using preparative HPLC (7-60% solvent B in 27 minutes). Compound **14** (3 mg) was dissolved in anhydrous DMSO in a small dry reaction vessel. NMM (0.5 µL) and compound **6** (1.1 mg) were added and the solution was stirred for 1 hour. When compound **14** was completely consumed and formation of compound **15** was observed (as determined by HPLC-MS), compound **8** (2.5 mg), TBTA (1 mg),

and Cu(I)Br (0.5 mg) were subsequently added. The reaction was stirred for additional 12 hours and the product ACPP **1** was purified using semi-preparative HPLC (15-45% solvent B in 20 minutes at 4 mL/min flow rate) (Chromatogram S2). ESI-MS (positive mode) calculated average mass ($C_{193}H_{274}BN_{61}O_{63}S_4$) 4595.7, found $[M]^{3+}$ = 1533.0, $[M]^{4+}$ = 1150.0, M^{4+} of ($M-H_2O$) = 1145.5, $[M]^{4+}$ of ($M-2H_2O$) = 1140.8. ACPP **1** was characterized using HPLC coupled to a high-resolution mass spectrometer: calculated exact mass ($C_{193}H_{274}BN_{61}O_{63}S_4$) 4592.9088, found 4592.9088.

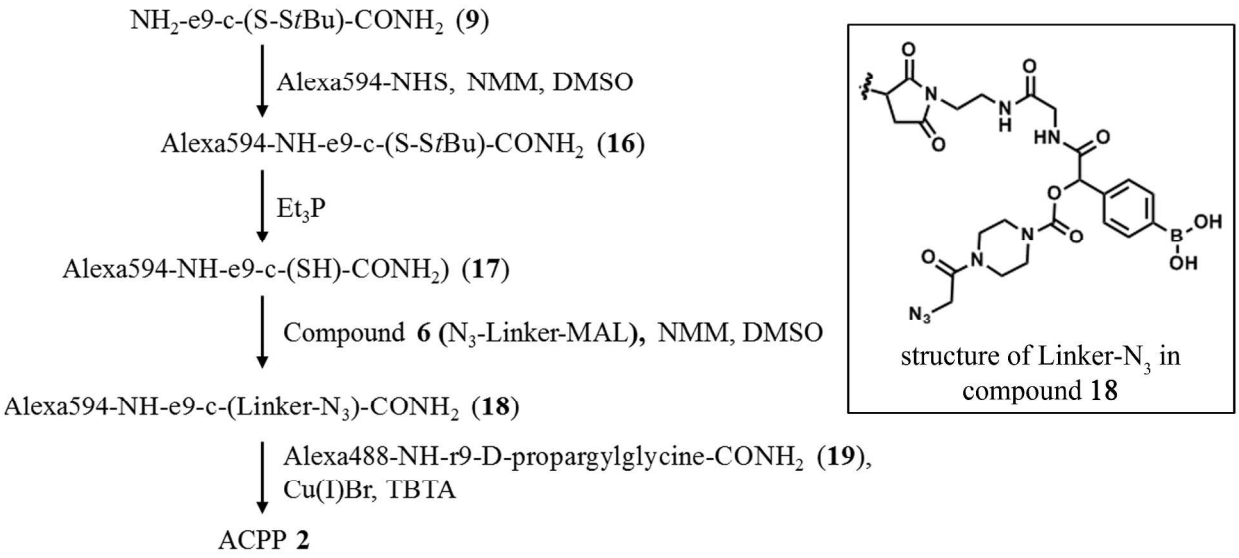


Chromatogram S2. HPLC trace of ACPP **1** obtained by collecting the absorbance at 650/8 nm using analytical HPLC-MS.



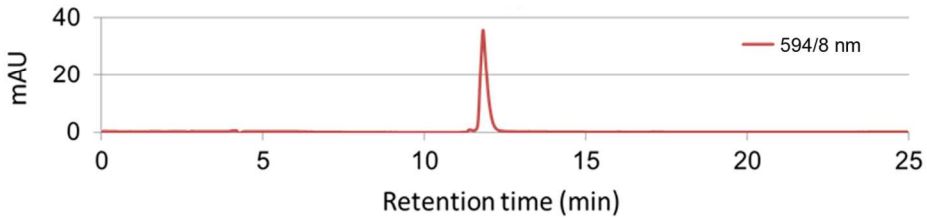
Graph S1. Photostability of ACPP **1**. The bleaching rates of fluorescein and Cy5 in ACPP **1** were compared to their non-conjugated forms. The absorbance of each dye at the appropriate maxima was measured following light irradiation for the indicated time periods.

Synthesis of ACPP 2:

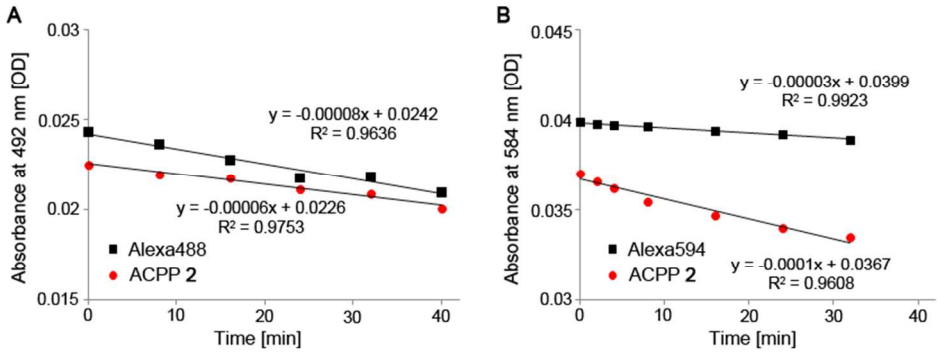


Scheme S3. Synthetic scheme of ACPP **2**.

<u>According to scheme S3</u>: Compound **9** (8.5 mg) was dissolved in anhydrous DMSO under N_2 atmosphere. AlexaFluor594-NHS ester (2 mg) and NMM (1.5 µL) were added and allowed to react overnight. Then, compound **16** was purified by using semi-preparative HPLC. Compound **16** was then dissolved in DMSO in a dry reaction vessel, triethylphosphine (20 µL) was added and allowed to react for 2 hours to get compound **17** that was precipitated by addition of EtOAc and purified using semi-preparative HPLC. To a dry reaction vessel, compound **17**, Compound **6**, NMM, and DMSO were added and allowed to react for 1 hour. When compound **17** was completely consumed and formation of compound **18** was observed (as determined by HPLC-MS), compound **19**, TBTA (1.5 mg), Cu(I)Br (0.5 mg) were added and reacted overnight. The product ACPP **2** was purified using semi-preparative HPLC (Chromatogram S3). ESI-MS (positive mode) calculated ($C_{186}H_{258}BN_{61}O_{67}S_5$) average mass 4591.5, found $[M]^{3+}$ of M-2H_2O = 1519.5, $[M]^{4+}$ of M-2H_2O = 1139.9. ACPP **2** was also characterized using HPLC coupled to a high-resolution mass spectrometer: calculated exact mass ($C_{186}H_{258}BN_{61}O_{67}S_5$) 4588.7353, found 4588.7441 (difference of calculated vs. found is < 2 ppm).



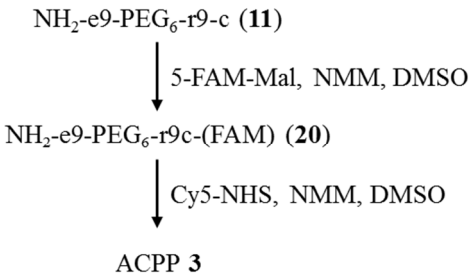
Chromatogram S3. HPLC trace of ACPP **2** obtained by collecting the absorbance at 594/8 nm using analytical HPLC-MS.



Graph S2. Photostability of ACPP **2**. The bleaching rates of Alexa488 and Alexa594 in ACPP **2** were compared to their non-conjugated forms. The absorbance of each dye at the appropriate maxima was measured following light irradiation for the indicated time periods.

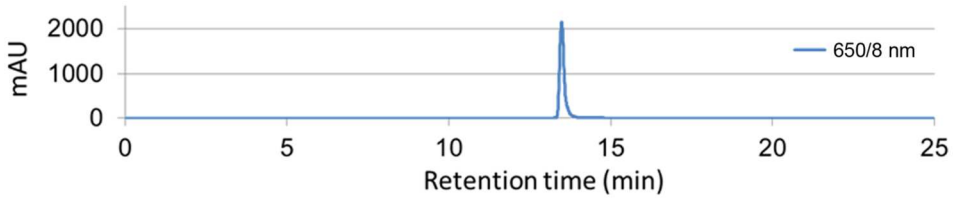
Compound 19 (Alexa488-NH-r9-D-propargylglycine-CONH$_2$). Compound **10** (7.5 mg) was dissolved in anhydrous DMSO under N_2 atmosphere. Then, AlexaFluor488-NHS ester (2 mg, mixed isomers) and NMM (1.5 µL) were added and allowed to react overnight. After completion of the reaction the product was purified using semi-preparative HPLC (0-35% solvent B in 20 minutes then 70% solvent B for additional 5 minutes at the flow rate of 3.5 mL/min).

Synthesis of uncleavable ACPP 3:



Scheme S4. Synthetic scheme of uncleavable ACPP **3**.

According to scheme S4: Compound **11** (6.7 mg) was dissolved in anhydrous DMSO under N_2 atmosphere. Fluorescein 5-maleimide (5-FAM Mal) (2 mg) and NMM (0.4 µL) were added and stirred for 1 hour. When compound **11** was completely consumed and formation of compound **20** was observed (as determined by HPLC-MS), Cy5-NHS ester (0.8 mg) and NMM (0.3 µL) were added and allowed to react overnight. Then, the product ACPP **3** was purified using semi-preparative HPLC (15-55% solvent B in 25 minutes at the flow rate of 3.5 mL/min) (Chromatogram S4). ESI-MS (positive mode) calculated ($C_{175}H_{260}N_{50}O_{58}S_3$) average mass 4089.4, calculated m/z= 4088.8, found *m/z* $[M]^{3+}$ 1363.8, $[M]^{4+}$ 1023.4. ACPP **3** was also characterized using HPLC coupled to a high-resolution mass spectrometer: calculated exact mass ($C_{175}H_{260}N_{50}O_{58}S_3$) 4086.8047, found 4086.8080 (difference of calculated vs. found is < 1 ppm).



Chromatogram S4. HPLC trace of ACPP **3** obtained by collecting the absorbance at 650/8 nm using analytical HPLC-MS.

Mass spectra analysis of intermediate compounds

Compound	Observed Mass (m/z)	Calculated average mass
NH_2-e_9-c-$CONH_2$ (**7**)	1281.8 (641.9 as M^{2+})	1282.2
FAM-r_9-D-propargylglycine-$CONH_2$ (**8**)	1876.2 (626.4 as M^{3+})	1876.10
NH_2-e_9-c(S-S*t*Bu)-$CONH_2$(**9**)	1370 (686.0 as M^{2+})	1370.4
NH_2-r_9-D-propargylglycine-$CONH_2$(**10**)	1517.4 (759.7 as M^{2+})	1517.8
NH_2-e_9-peg6-r_9c-$CONH_2$(**11**)	3023.1 (1008.7 as M^{3+})	3023.3
NH_2-e_9-c(Cy5) (**12**)	1281.6 (641.9 as M^{2+})	1282.2
PyS-SCH2-CH2-CO-NH-e_9-c(Cy5) (**13**)	2257.8 (1129.9 as M^{2+})	2258.4
SH-CH_2-CH_2-CO-NH-e_9-c(Cy5) (**14**)	2148.8 (1075.4 as M^{2+})	2149.3
(N_3-Linker)-S-CH_2-CH_2-CO-NH-e_9-c(Cy5) (**15**)	2719.4 (1342.7 as (M^{2+} of M-$2H_2O$) peak)	2719.6
Alexa594-NH-e_9-c-(S-S*t*Bu)-$CONH_2$ (**16**)	2074.8 (1038.4 as M^{2+})	2075.1
Alexa594-NH-e_9-c-(SH)-$CONH_2$) (**17**)	1986.8 (994.4 as M^{2+})	1987.0
Alexa594-NH-e_9-c-(-Linker-N_3)-$CONH_2$ (**18**)	2557.2 (1261.6 as M^{2+} of M-$2H_2O$ peak)	2557.3
Alexa488-NH-r_9-D-propargylglycine-$CONH_2$ (**19**)	2034.2 (1018.1 as M^{2+})	2034.3
NH_2-e_9-peg_6-r_9c-(FAM) (**20**)	3450.3 (1151.1 as M^{3+})	3450.6

Table S1. Mass spectra analysis of the intermediate compounds *en route* to ACPPs **1-3** as obtained from analytical HPLC-MS.