Electronic Supplementary Material (ESI) for ChemComm.

Supporting information

Detailed Experimental Procedures

An Activity-based probe targeting the streptococcal virulence factor C5a peptidase

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1) Strains, reagents, and basic procedures

1.1 Bacterial cultures

Streptococcus pyogenes (ATCC 19615) and *Streptococcus agalactiae* (ATCC 13813) were purchased from American Type Culture Collection (ATCC) and cultured in Brain Heart Infusion media at 37 °C. *Streptococcus pneumoniae* was obtained directly from NIST as a glycerol stock and cultured in tryptic soy broth (TSB) supplemented with 5% sheep's blood at 37 °C. *E. coli* Nissle 1917 was originally obtained by sampling Mutafluor Probiotic and cultured in LB-Lennox at 37 °C. *E. coli* NEB 5-alpha F'IQ containing the RFP plasmid pGW55 with KanR/Ptac:mKate2 was obtained from Joshua Elmore and cultured in LB containing 5 µg/mL kanamycin at 37 °C. [1]

1.2 Expression of *scpA* in Escherichia coli.

A plasmid vector capable of inducible expression of the *scpA* gene was synthesized by Twist Bioscience (https://www.twistbioscience.com). The plasmid has a p15a medium copy-number origin of replication and carries kanamycin resistance. The *scpA* gene is downstream of both a P_{BAD} arabinose-inducible promoter and a T7 promoter. The plasmid was transformed into *Escherichia coli* strain RE1000, a derivative of MG1655 which constitutively expresses the arabinose importer and lacks the *ara* operon for arabinose metabolism.[2] Cells were grown in 1x M9 salts (doi:10.1101/pdb.rec614)[3] containing 100 µM $CaCl_2$, 200 µM $MgSO_4$, 1x Wolfe's vitamin solution (Sigma), 1% casamino acid solution (Sigma), 1% glycerol and 50 µg/mL kanamycin at 37°C with shaking (150 rpm). Inductions were performed by growing 25 mL cultures to an optical density at 600 nm (OD_{600}) of 0.3-0.6, evenly dividing the culture and adding arabinose to 0.2% to one of the replicates and growing the cells for an additional 17-20 hours in 125 mL glass flask with a breathable membrane on top to facilitate good gas exchange. A final OD_{600} was recorded, cells were pelleted by centrifugation at 5000 rpm for 5 minutes and resuspended in a volume of cold 1x PBS determined by multiplying the final OD_{600} reading by 120 µL. RE1000 lacking the plasmid (grown as described above except without the kanamycin) was also used as a negative control.

1.3 Generation of bacterial lysates

Overnight cultures (15 mL) of bacteria were grown in 50 mL culture tubes in the appropriate media and conditions as listed in Section 1.1. Cultures were pelleted (10 min, 10,000 x g), the supernatant discarded, and resuspended in 1X PBS (2 mL). Bacterial suspensions in PBS

were aliquoted (500 µL) into safe-lock 1.5 mL tubes containing approximately 100 µL of silica-zirconia beads. Bacteria was lysed via bead-beating (3 x 3 minutes, icing in between) using a Bullet Blender. Samples were pelleted (10 min, 10,000 x g) and the lysates were collected as the supernatant and the protein concentration was determined via Piece BCA Protein Assay Kit (ThermoFisher). Cell lysates were stored at -80 °C until immediately before use, and were diluted to a normalized protein concentration of 1.1 mg/mL using PBS.

2) SDS-PAGE supplemental details

2.1 Labeling of lysates with C5a-Fl (S1)

The lysates were incubated with C5a-Fl at various concentration and then analyzed with SDS-PAGE (Figure **S1**).

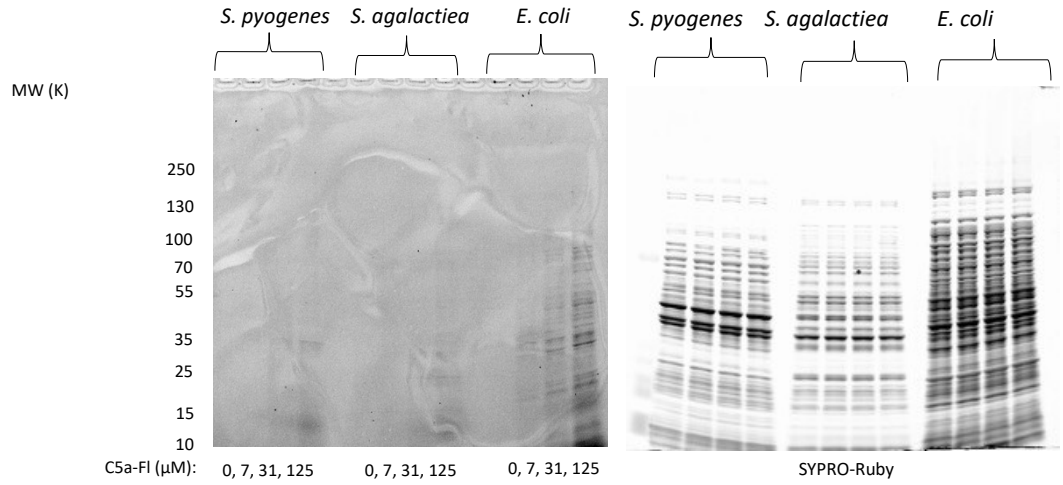


Figure S1. Labeling of *S. Pyogenes, S. agalactiae, and E. coli* lysates *with* **C5a-Fl**

2.2 Human C5a Fusion protein (C5a-Fc) sequence

Sequence of commercially sourced C5a-Fc peptide obtained from the vendor:
lqkkieeiaakykhsvvkkccydgacvnndetceqraarislgprcikafteccvvasqlranishkdmqlgr
Linker(gggsgggs)-Fc(pkssdkthtcppcpapellggpsvflfppkpkdtlmisrtpevtcvvvdvshedpev
kfnwyvdgvevhnaktkpreeqynstyrvvsvltvlhqdwlngkeykckvsnkalpapiektiskakgqprepqvytlppsrdeltk
nqvsltclvkgfypsdiavewesngqpennykttppvldsdgsfflyskltvdksrwqqgnvfscsvmhealhnhytqkslslspgk)

2.3 Confirmation of C5a peptidase activity in whole cells using C5a-Fc (S2, S3 & S4)

To determine an optimum visualization concentration of C5a-Fc in SDS-PAGE analysis, various amounts of C5a-Fc in PBS were prepared and then analyzed using SDS-PAGE (Figure **S1**).

Then, *E. coli* RE1000 with and without scpA at various ODs (50 uL) were incubated with of C5a-Fc (800 ng) in PBS for 1h at 37 °C. Then, the cells were spun down (5000 rcf; 7 min). The supernatant was analyzed by SDS-PAGE (Figure **S2**). Similar experiments were carried out with *S. agalactiae* and *S. pyogenes* (Figure **S3***)*.

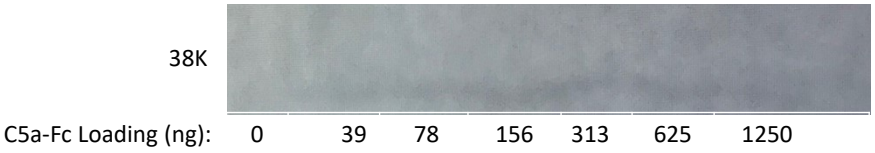


Figure S2 C5a-Fc visualized with Coomassie protein gel stain.

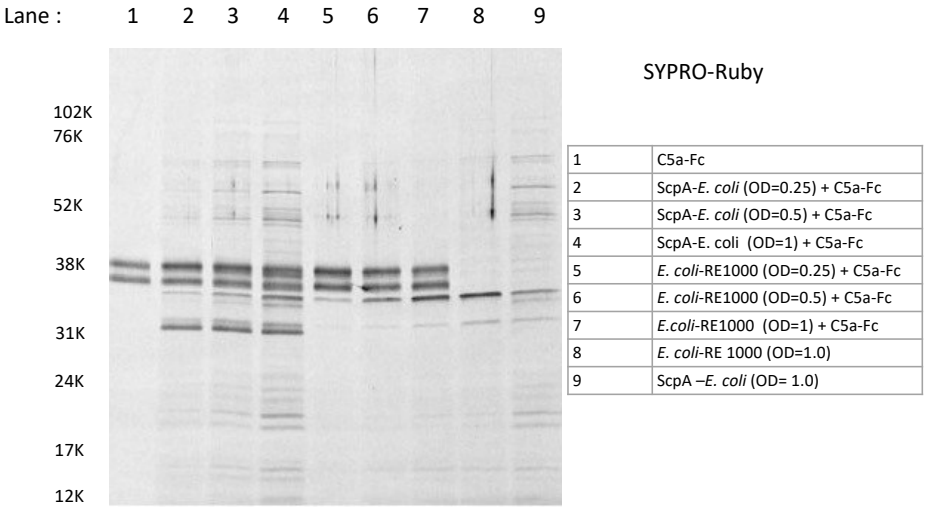
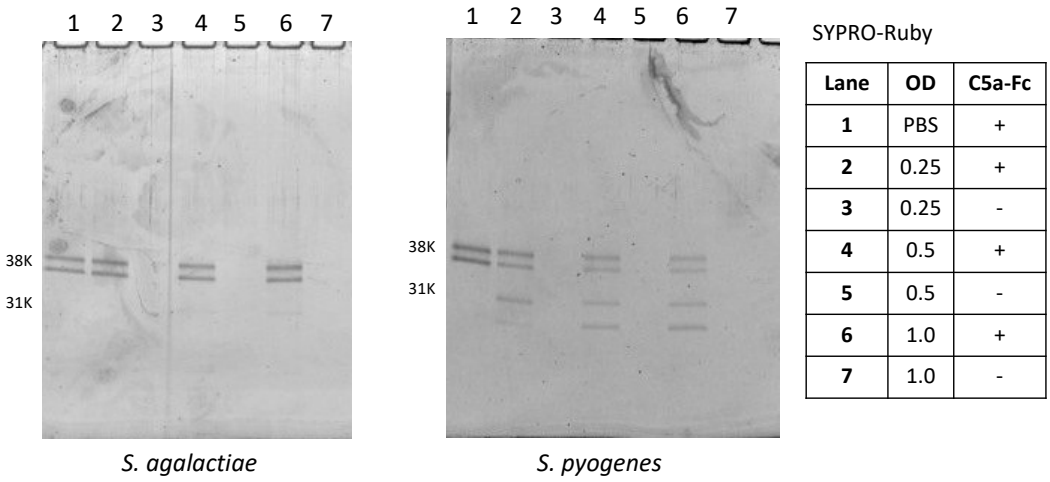


Figure S3 Confirmation of ScpA activity using C5a-Fc in ScpA-*E. Coli.*



Lane	OD	C5a-Fc
1	PBS	+
2	0.25	+
3	0.25	-
4	0.5	+
5	0.5	-
6	1.0	+
7	1.0	-

Figure S4 Confirmation of C5a peptidase activity using C5a-Fc in *S. agalactiae* and *S. pyogenes*

2.4 Inhibition of ScpA activity by C5a-Fl and C5a-Al

E. coli RE1000 with and without scpA at 0.25 OD (50 uL) were incubated with various concentrations of **C5a-Fl** and **C5a-Al** for 1h at 37 °C in PBS. Then, C5a-Fc (800 ng) in DI water was introduced and incubated for 1h at 37 °C. Then, the cells were spun down (5000 ref; 7 min) and the supernatant was analyzed by SDS-PAGE (Figure **S5** and **S6**).

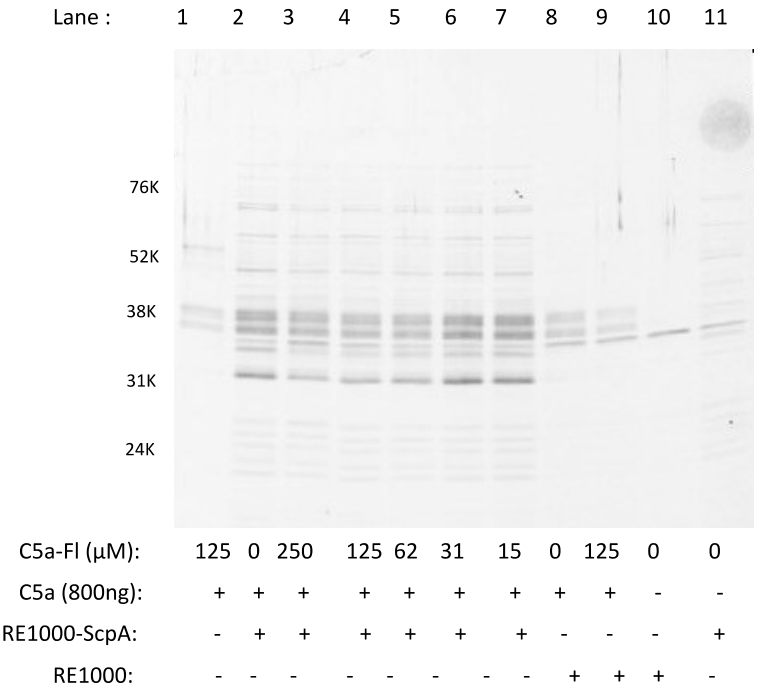


Figure S5 Inhibition of ScpA activity on ScpA-*E. coli* by **C5a-Fl**

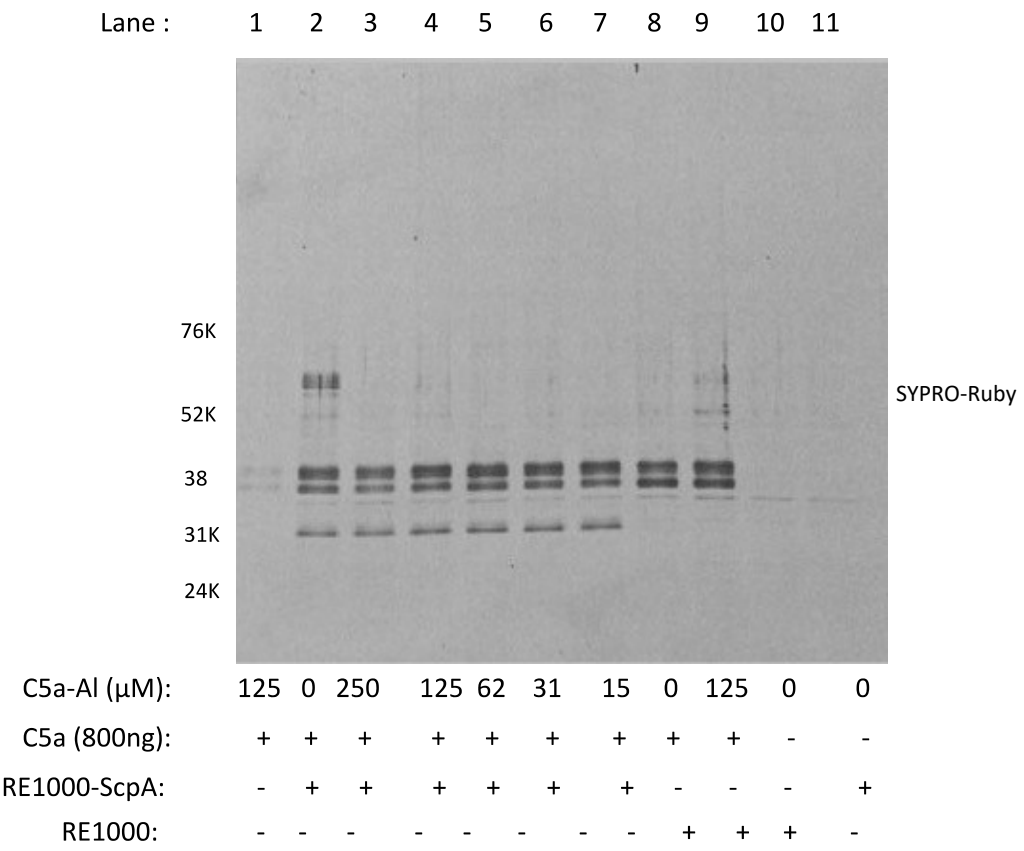


Figure S6 Inhibition of ScpA activity on ScpA-*E. coli* by **C5a-Al**

3) Flow cytometry/FACS experimental details

3.1 Flow cytometry methods

Flow cytometry was performed on either a Sony SH800S cell sorter in analyzer mode or an ACEA Biosciences NovoCyte Flow Cytometer.

Experiments done on a Sony SH800S cell sorter in analyzer mode were done using a 100 μm sort chip. Automatic calibration was performed using Sony Automatic Setup Beads prior to each experiment. Doublet discrimination was performed using forward (FSC) and side (SSC) scatter and probe fluorescence was detected using 488 nm excitation and a 525/50 bandpass filter. RFP and PI fluorescence were detected using 561 nm excitation and a 600/60 bandpass filter. When necessary, compensation was performed manually within the Sony Cell Sorter Software. Data was collected at 1,000-5,000 events per second. “Probe negative” gates were drawn

using unstained (DMSO-only) controls. Following acquisition, data was exported as .fcs files and analyzed in FlowJo10.

Experiments done on an ACEA Biosciences NovoCyte Flow Cytometer were analyzed for FITC fluorescence. 500,000 events were collected for each sample, and PBS was run through the instrument to ensure thorough washing between samples. Data was exported as .fcs and analyzed in FlowJo10. Experiments were run in triplicate.

SYPRO-Ruby stained gels were imaged on Typhoon FLA 9500 (General Electric). GelCode Blue stained gel was imaged using GelDocEZ (Bio-rad Laboratories). Gel image analyses were done using ImageQuant or imageJ software.

3.2 Materials

Cell sorter chips and Automatic Setup Beads were purchased and used as is from Sony Biotechnology. Propidium iodide was purchased from Sigma Aldrich and used as directed. Heat inactivated bovine serum albumin (BSA) and 10X phosphate buffered saline, pH 7.4, SYPRO-Ruby and GelCode Blue were purchased from Fisher Scientific.

3.3 ScpA-*E. coli* labeling protocol with C5a-Fl

E. coli RE1000 with or without scpA were cultured as described and supplemented with either glucose, arabinose, or no additives. Cultures were pelleted and adjusted to an OD of 0.5 in PBS. Either DMSO (0.5 µL) or **C5a-Fl** (0.5 µL, 12.5 mM stock in DMSO) were added to 50 µL aliquots of each cell suspension. Samples were incubated for 1 hour at 37 °C, shaking at 500 rpm. Aliquots were pelleted (5 min, 7000 x g), washed with 1X PBS (50 µL), and resuspended in 1X PBS (0.5 mL) prior to analysis on an ACEA Biosciences NovoCyte Flow Cytometer. Data obtained in triplicate (Figure **S7)**.

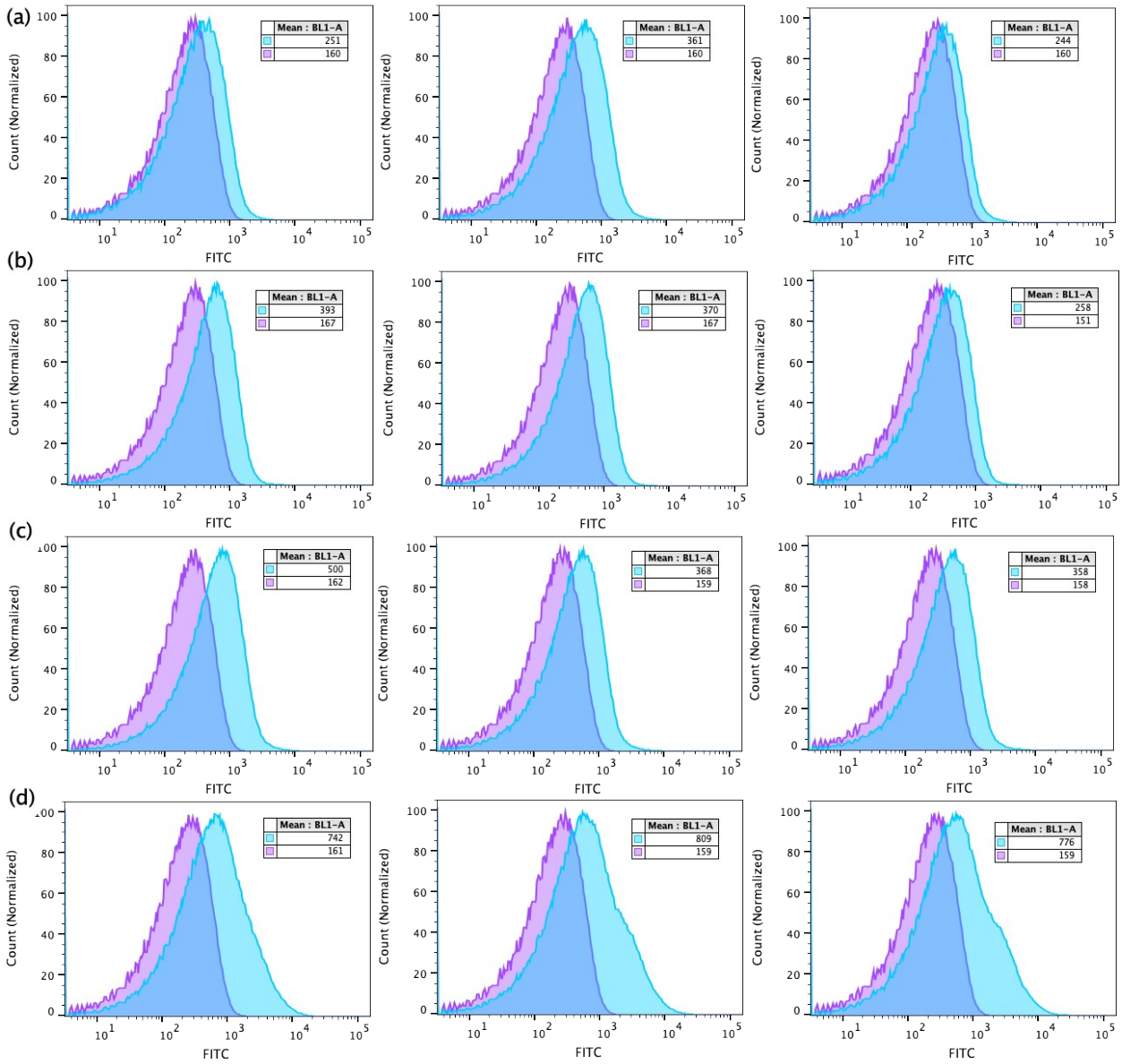


Figure S7. Histograms in triplicate for labeling of (a) RE1000 + glucose (b) RE1000 + arabinose (c) scpA + glucose and (d) scpA + arabinose with 125 µM **C5a-Fl** (blue) compared to DMSO alone (purple).

3.4 Dose dependence and cell viability in monocultures

S. pyogenes (BHI), *S. agalactiae* (BHI), *S. pneumoniae* (TSB + 5% sheep's blood), and *E. coli* Nissle (LB) were cultured overnight at 37 °C, pelleted, washed with 1X PBS, and adjusted to an OD of 0.5 in PBS. Probe stock solutions (0.5 µL; 0, 0.79, 3.13, 12.5 mM in DMSO) were added to aliquots of monocultures (50 µL) and incubated for 1 hour at 37 °C, shaking at 500 rpm. Aliquots were pelleted (5 min, 7000 x g), washed with bovine serum albumin (BSA, 0.1% in 1X

PBS), and resuspended in 1X PBS (0.5 mL) prior to analysis on a Sony SH800S. Data in triplicate shown in **Figure S8**.

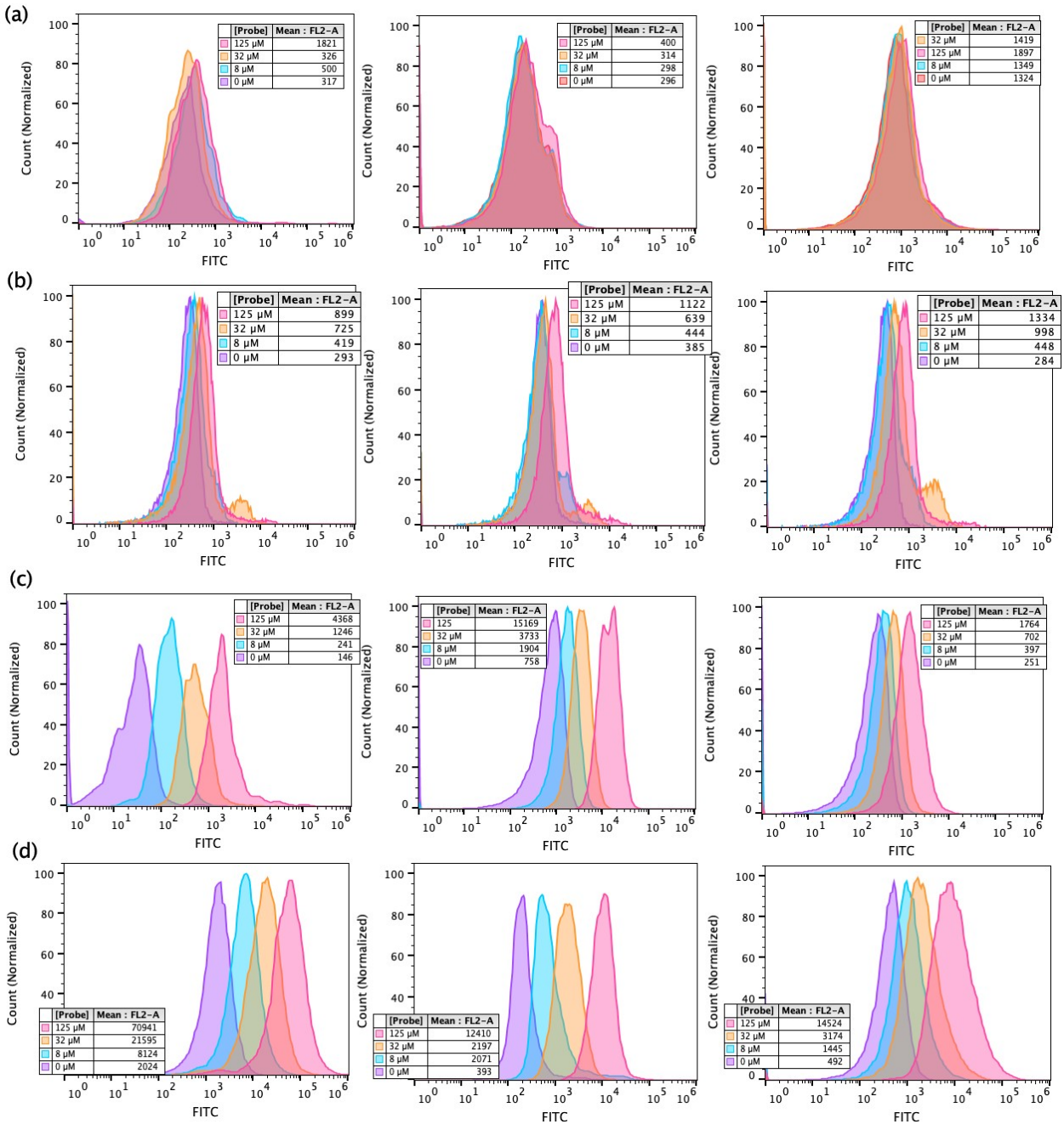


Figure S8. Histograms in triplicate for dose-dependent labeling of (a) *E. coli* Nissle (b) *S. pneumoniae,* (c) *S. agalactiae* and (d) *S. pyogenes* with C5a-Fl.

For cell viability experiments, the procedure was performed as above, with the addition of PI (0.5 µL 20 mM stock solution into 500 µL sample; 20 µM final concentration) and incubation at room temperature for 30 min prior to analysis (**Figure S9**).

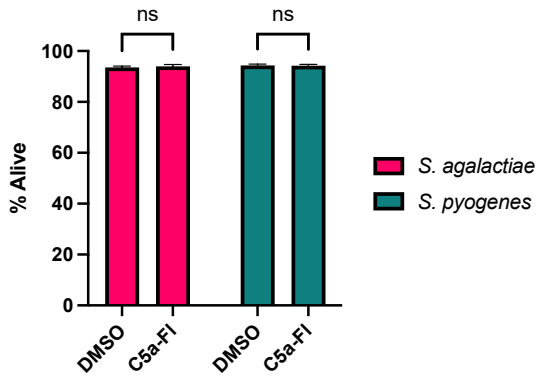


Figure S9. Cell viability analysis *of S. agalactiae* and *S. pyogenes* following labeling with C5a-Fl (125 µM).

3.5 Selectivity in the presence of RFP E. coli

S. pyogenes (BHI), *S. agalactiae* (BHI), and RFP *E. coli* (LB, 50 µg/mL kanamycin) were cultured overnight at 37 °C, pelleted, washed with 1X PBS, and adjusted to an OD of 0.5 in PBS. Simple mixtures were made by combining equal volumes of either *S. pyogenes* or *S. agalactiae* and RFP *E. coli*. DMSO or probe stock solutions (0.5 µL; 12.5 mM probe in DMSO) were added to aliquots of the mixtures (50 µL) and incubated for 1 hour at 37 °C, shaking at 500 rpm. Aliquots were pelleted (5 min, 7000 x g), washed with bovine serum albumin (BSA, 0.1% in 1X PBS), and resuspended in 1X PBS (0.5 mL). Samples were filtered through a 35 µm cell strainer immediately prior to analysis by flow cytometry. Data in triplicate shown in Figure **S10**.

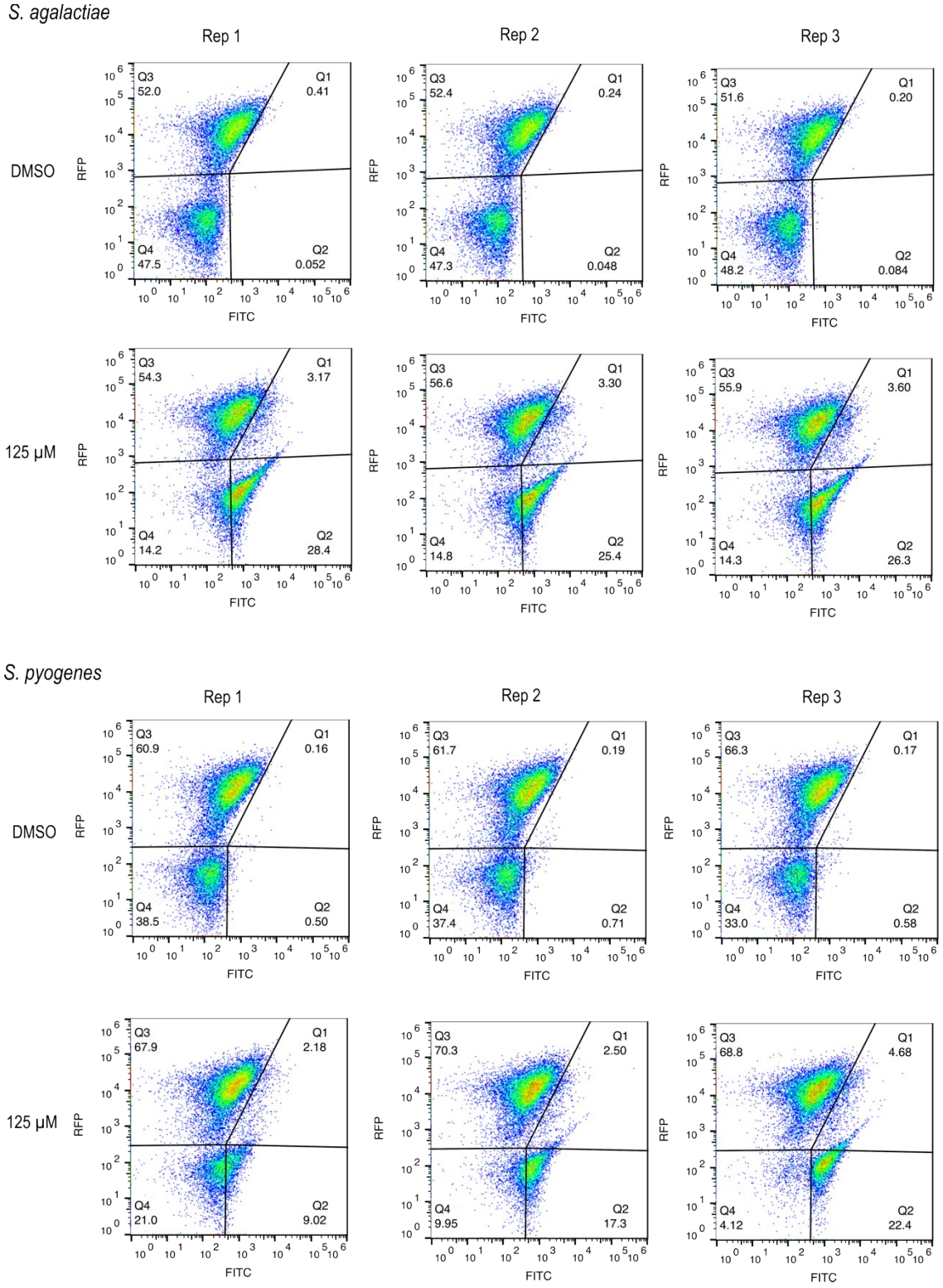


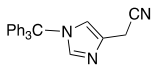
Figure S10. Data in triplicate for selectivity for *S. agalactiae* (top) and *S. pyogenes* (bottom) in the presence of RFP *E. coli.* RFP gating was set based on controls of RFP *E. coli* alone.

4. Materials and methods for chemical synthesis

Chemicals were purchased from Fisher or Sigma Aldrich unless otherwise noted. All reagents were used as received unless otherwise mentioned. Reactions were carried out on bench and dry solvents were obtained from in house drying system. ^{1}H, ^{13}C, and ^{31}P NMR were recorded on an Bruker 400MHz, 100MHz and 162MHz spectrometer, respectively. ^{1}H NMR chemical shifts were determined relative to the signal of a residual protonated solvent, $CDCl_3$ (δ 7.24 ppm) or acetone-d_6 (2.05 ppm) or DMSO-d_6 (2.5 ppm) or MeOH-d_4 (3.31 ppm). ^{13}C NMR chemical shifts were determined relative to the ^{13}C signal of solvent, $CDCl_3$ (δ 77.23 ppm) or DMSO-d_6 (39.52 ppm) or acetone (29.84). ^{31}P NMR signals are externally referenced to 80% phosphoric acid (0 ppm). Mass of the products were measured using direct injects LT-Q instrument using M+H or M+Na solvents. HRMS was obtained from Environmental Molecular Sciences Laboratory user facility. Purity analysis was performed on Shimadzu analytical HPLC system and the chromatogram was processed using LC solution software.

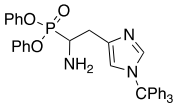
4.1 Detailed Synthetic Procedures

2-(1-trityl-1*H*-imidazol-4-yl)acetonitrile, **2**



Triethyl amine (3.6 mL; 25.6 mmol; 1.1 equiv) was added to a mixture of 2-(1*H*-imidazol-4-yl)acetonitrile **(1)** (2.5 g; 23.3 mmol; 1 equiv) and trityl chloride (7.15 g; 25.6 mmol; 1.1 equiv) in dry DCM (100 mL) under nitrogen atmosphere and stirred for 24h at ambient temperature. Then, volatiles were removed, and the resulting residue was purified by Flash column chromatography using a silica column and EtOAc/hexane (85-95%) gradient to obtain a white solid (7.3 g; 90%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.53 – 7.30 (m, 10H), 7.23 – 7.00 (m, 6H), 6.86 (dd, J = 1.5, 0.8 Hz, 1H), 3.84 (d, J = 0.8 Hz, 2H). ^{13}C NMR (400 MHz, DMSO-d_6) δ 142.0, 138.6, 130.8, 129.2, 128.3, 128.1, 119.5, 118.8, 74.8, 16.6. 74.8, 16.6. LRMS [M+H]: 350.

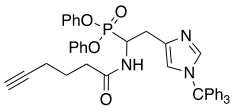
diphenyl (1-amino-2-(1-trityl-1*H*-imidazol-4-yl)ethyl)phosphonate, **3**



2 (1.4 g; 4 mmol) was dissolved in dry DCM (15 mL) and cooled down to -78 °C, and DIBAL in cyclohexane (1.1M; 1.1 equiv; 4 mL) was introduced and stirred at -78 °C for 2 h (yellow solution), then diphenylphosphite (1 mL) was introduced; effervescence and disappearance of yellow color. The reaction mixture was slowly brought to -45 °C over an hour; then allowed to come to RT and stirred for 30 min. Quenched with sat. potassium ammonium tartrate and stirred for 30 min, crystalline emulsion left overnight to separate in the separatory

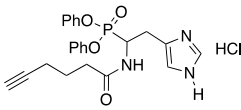
funnel (Note: breaking the emulsion is important in recovering more product). The layers were separated, and the aq. layer was extracted one more time with DCM. The combined DCM layer was dried under vacuum. TLC showed presence of starting material and two spots staining with ninhydrin. The residue was loaded on silicon and eluted with 90-93% hex/EtOAc. Then, eluted with 4-10% DCM/MeOH, the product fractions were combined, and rotary evaporated to obtain pale yellow foaming semi-solid 152 mg (26%). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.41 (d, *J* = 1.4 Hz, 1H), 7.36 – 7.27 (m, 12H), 7.22 – 6.90 (m, 15H), 6.72 (d, *J* = 1.4 Hz, 1H), 3.83 (td, *J* = 10.2, 3.6 Hz, 1H), 3.31 (dddd, *J* = 14.5, 9.8, 3.6, 0.9 Hz, 1H), 2.92 (dt, *J* = 14.7, 10.0 Hz, 1H). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 150.7 (d, *J* = 9.9 Hz), 142.5, 139.1, 137.2 (d, *J* = 17.6 Hz), 129.9, 129.8, 128.2, 125.2 (d, *J* = 2.7 Hz), 120.8 (t, *J* = 3.8 Hz), 119.9 (d, *J* = 1.6 Hz), 75.4, 49.5 (d, *J* = 155.1 Hz), 30.7. ^{31}P NMR (162 MHz, Chloroform-*d*) δ 22.3. LRMS, m/z [M+H]$^{+}$: 586.

diphenyl (1-(hex-5-ynamido)-2-(1-trityl-1*H*-imidazol-4-yl)ethyl)phosphonate, 4



HATU (148 mg, 0.39 mmol, 3 equiv) was added to a solution of hex-5-ynoic acid (44 mg, 43 uL, 0.39 mmol, 3 equiv) and anhydrous DIPEA (68 uL, 0.39 mmol, 3 equiv) in DMF (0.5 mL; anhydrous) and stirred for 10 min. Then, **3** (80 mg, 0.13 mmol; 1 equiv) was added as a solution in DMF (2 X 1 mL) and stirred for 24h. The reaction mixture was diluted with ethyl acetate and washed with water and brine. The resulting solution was dried on anhy. MgSO4, filtered and dried under reduced pressure. The resulting residue was purified by flash column chromatography using EtOAc as an eluent to obtain an off-white solid (73.5 mg; 83%). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.46 (s, 1H), 7.40 (s, 1H), 7.38 – 7.25 (m, 15H), 7.23 – 7.04 7.20 – 7.02 (m, 10H), 6.74 (1H), 5.14 (dtt, *J* = 15.5, 8.9, 4.4 Hz, 1H), 3.37 – 3.06 (m, 2H), 2.32 (t, *J* = 7.4 Hz, 2H), 2.19 (td, *J* = 7.0, 2.7 Hz, 2H), 1.94 (t, *J* = 2.6 Hz, 1H), 1.89 – 1.73 (m, 2H). ^{31}P NMR (162 MHz, Chloroform-*d*) δ 16.6. ^{13}C NMR (101 MHz, Chloroform-*d*) δ 171.8 (d, *J* = 5.0 Hz), 150.5 (dd, *J* = 21.3, 9.9 Hz), 142.4, 138.7, 136.6 (d, *J* = 11.6 Hz), 129.9, 129.8 (d, *J* = 4.5 Hz), 128.2, 125.3 (d, *J* = 13.4 Hz), 120.9 (d, *J* = 4.1 Hz), 120.6 (d, *J* = 4.2 Hz), 119.7, 83.7, 75.5, 69.2, 47.6, 45.9, 35.2, 28.3, 24.2, 18.0. LRMS, m/z [M+H]$^{+}$: 680.

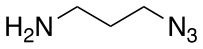
diphenyl (1-(hex-5-ynamido)-2-(1*H*-imidazol-4-yl)ethyl)phosphonate hydrogen chloride, C5a-Al



4 (20 mg, 29.4 μmol) was dissolved in 0.2M HCl in hexafluoro isopropanol[4] (1 mL, prepared by adding conc. HCl with hexafluoro isopropanol) and stirred for 30 min. The volatiles were evaporated and azeotropically dried with ethanol (2X) times

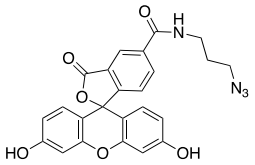
to get an off-white solid, which was then washed with ether to get off-white solid (11 mg, 86%). Note: if the reaction is incomplete, purification can be carried out using a reverse phase flash column chromatography using C18 column with a 95-10% gradient of 0.1% TFA/water and 0.1% TFA/acetonitrile to obtain a glassy white solid (TFA salt). ^{1}H NMR (400 MHz, Acetone-d_6) 8.67 (broad s, 1H), 8.65 (s, 1H), 7.51 (s, 1H), 7.40 – 7.14 (m, 10H), 5.21 – 4.91 (m, 1H), 3.55 (dt, J = 15.1, 4.4 Hz, 1H), 3.40 – 3.21 (m, 1H), 2.30 (m, 3H), 2.09 (m, 2H), 1.76 – 1.55 (m, 2H). ^{31}P NMR (162 MHz, Acetone-d_6) δ 16.6. ^{13}C NMR (101 MHz, Acetone-d_6) δ 172.8 (d, J = 4.5 Hz), 151.7 (d, J = 9.7 Hz), 151.4 (d, J = 9.1 Hz), 134.7, 131.7 (d, J = 20.7 Hz), 130.6, 126.2 (d, J = 1.3 Hz), 126.0, 121.8 (d, J = 4.0 Hz), 121.4 (d, J = 4.4 Hz), 118.3, 84.30, 70.3, 48.2, 46.6, 34.9, 25.9 (d, J = 5.9 Hz), 25.3, 18.2. LRMS, m/z $[M+H]^+$: 536

3-azidopropan-1-amine, 5[5]



A mixture of 3-bromopropylamine hydrogen bromide (5 g, 22.8 mmol) and sodium azide (4.45g, 68.4 mmol) was refluxed in water (40 mL) for 18 h. The resulting mixture was reduced in volume (1/3 to starting volume) and cooled in an ice-bath, then ether (50 mL) was added, and then KOH (6.24g) in water (10 mL) was added slowly with stirring. The resulting biphasic mixture was stirred for 10 min, then organic layer was separated, and the aqueous layer further extracted with ether (2X). The combined organic layer was dried on anhy. $MgSO_4$, filtered and dried under reduced pressure to obtain pale yellow liquid (1.66 g, 73%). ^{1}H NMR (400 MHz, Chloroform-d) δ 3.49 – 3.27 (m, 1H), 2.82 (t, J = 6.8 Hz, 1H), 1.74 (pd, J = 6.7, 2.5 Hz, 1H).

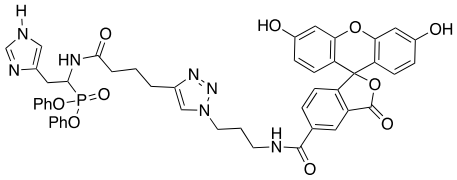
***N*-(3-azidopropyl)-3',6'-dihydroxy-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthene]-5-carboxamide, 6[4]**



A mixture of HATU (380 mg, 1 mmol, 4 equiv), 5-carboxyfluorescein (100 mg, 0.26 mmol), DMSO (2.5 mL) and Et_3N (101 mg, 140 μL, 1 mmol) was stirred at room temperature for 10 min, then 3-azidopropan-1-amine (100 mg, 100 μL, 1 mmol) was introduced, and the resulting mixture was stirred for 24h. The reaction mixture was diluted with ether and 10% NaOH was added, organic layer was separated, the aq. layer was further extracted with ether, combined ether layer was washed with water and brine, dried on $MgSO_4$, filtered, and dried under reduced pressure. The resulting residue was purified by column chromatography to obtain an orangish yellow solid (60 mg, 50%). ^{1}H NMR (400 MHz,

MeOD) δ 8.42 (d, *J* = 1.2 Hz, 1H), 8.20 (dd, *J* = 8.1, 1.6 Hz, 1H), 7.30 (dd, *J* = 8.1, 0.7 Hz, 1H), 6.69 (d, *J* = 2.3 Hz, 2H), 6.58 (s, 2H), 6.54 (dd, *J* = 8.7, 2.4 Hz, 2H), 3.52 (t, *J* = 7.0 Hz, 3H), 3.45 (td, *J* = 6.7, 4.3 Hz, 4H), 1.92 (td, *J* = 6.8, 5.1 Hz, 4H).

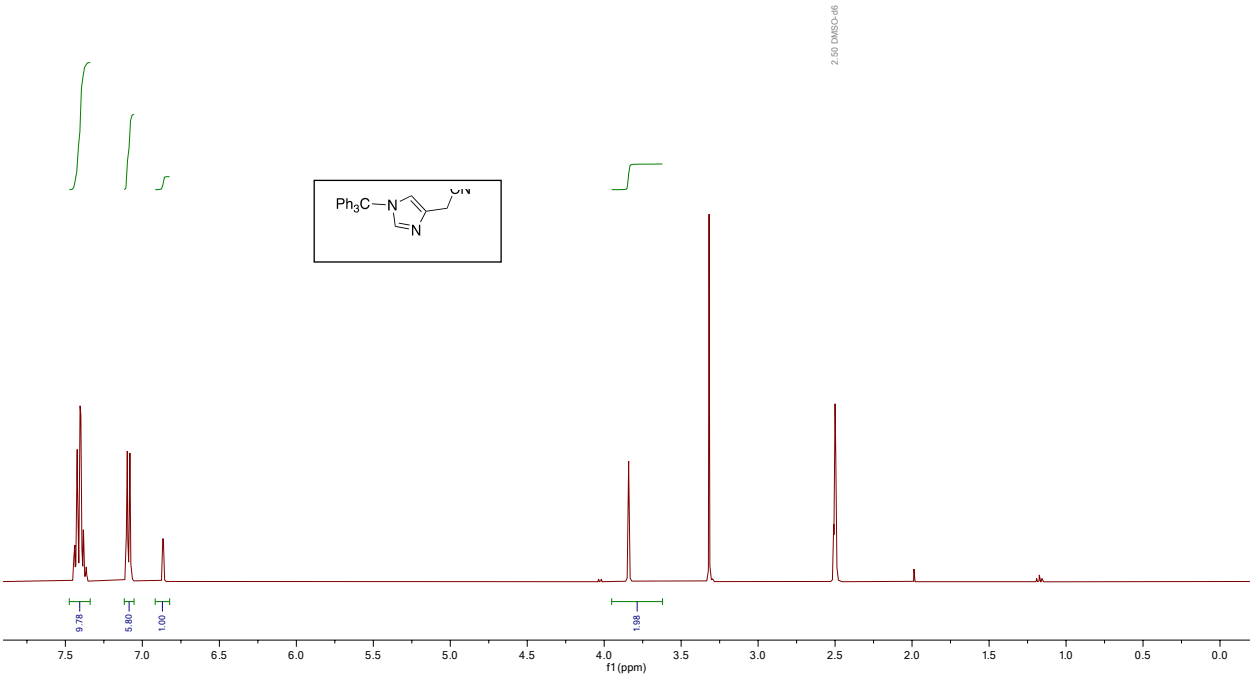
diphenyl (1-(4-(1-(3-(3',6'-dihydroxy-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthene]-5-carboxamido)propyl)-1*H*-1,2,3-triazol-4-yl)butanamido)-2-(1*H*-imidazol-4-yl)ethyl)phosphonate, C5a-Fl



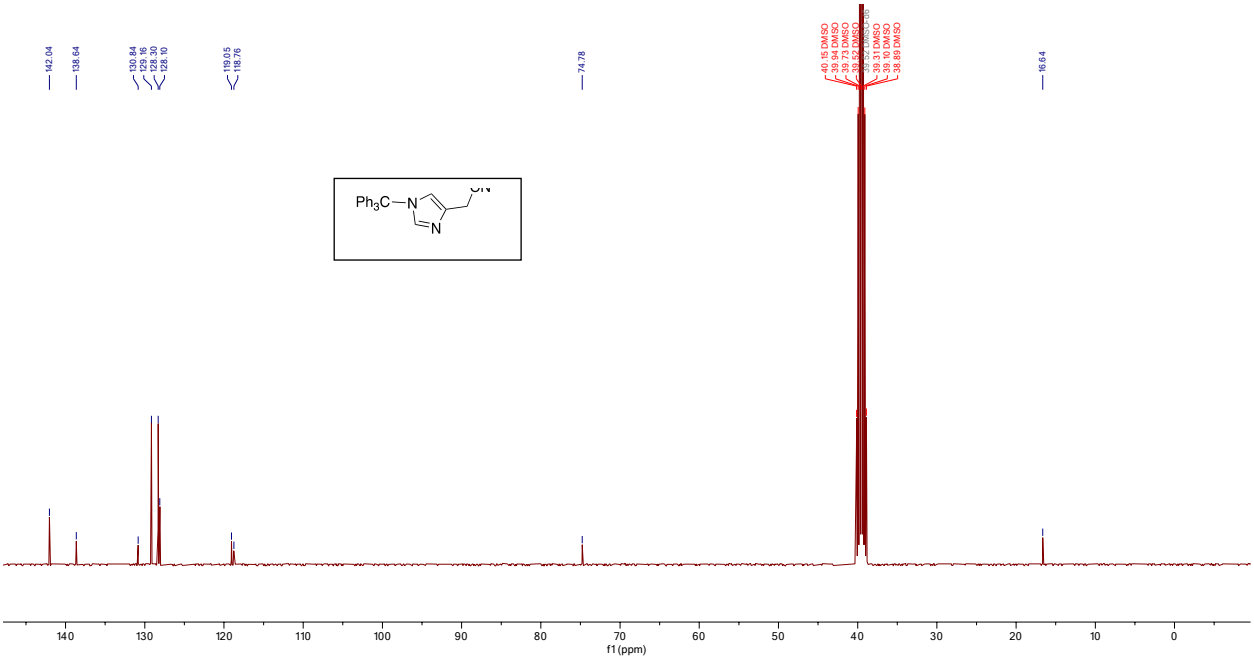
A mixture of **C5a-Al** (2 mg, 4.2 μmol) and azide **6** (2 mg, 4.4 μmol, 1.05 eq) were stirred in degassed THF/H_2O (v/v 80:20, 200 μL). Then, sodium ascorbate (2 equiv, 8.4 μmol in 20 uL water) and $CuSO_4$ (1 equiv, 4.2 μmol in 20 μL water) were introduced subsequently. The vial was flushed with N_2 and allowed to stir over night (≈17h) at ambient temperature. Then, THF was removed under reduced pressure. The resulting mixture was diluted with DMSO and purified by flash column chromatography using a reverse phase C18 column with (95-10%) 0.1% TFA/water and 0.1% TFA/acetonitrile gradient to obtain yellow film (2 mg, 50%). ^{1}H NMR (400 MHz, MeOD) δ 8.86 (d, *J* = 1.4 Hz, 1H), 8.42 (d, *J* = 16.6 Hz, 1H), 8.25 – 8.09 (m, 1H), 7.78 (s, 1H), 7.50 – 7.05 (m, 16H), 6.71 (d, *J* = 2.2 Hz, 3H), 6.65 – 6.47 (m, 4H), 5.30 – 5.09 (m, 1H), 4.49 (t, *J* = 6.7 Hz, 2H), 3.54 – 3.39 (m, 3H), 2.59 (t, *J* = 7.4 Hz, 2H), 2.25 (d, *J* = 7.2 Hz, 3H), 1.96 – 1.76 (m, 2H), 1.37 (dd, *J* = 6.6, 3.6 Hz, 2H). ^{31}P NMR (162 MHz, MeOD) δ 15.6. Purity, 97% by HPLC (20 min, water/acetonitrile; Retention time: 10.75 min; see spectral section), LRMS $[M+H]^+$ m/z: 897; $[M+2H]^{2+}$ m/z: 449 (eee spectral section). HRMS calculated for $[C_{47}H_{43}N_7O_{10}P]$ 896.280906; found 896.28061.

4.2 NMR, Mass spectral and HPLC data

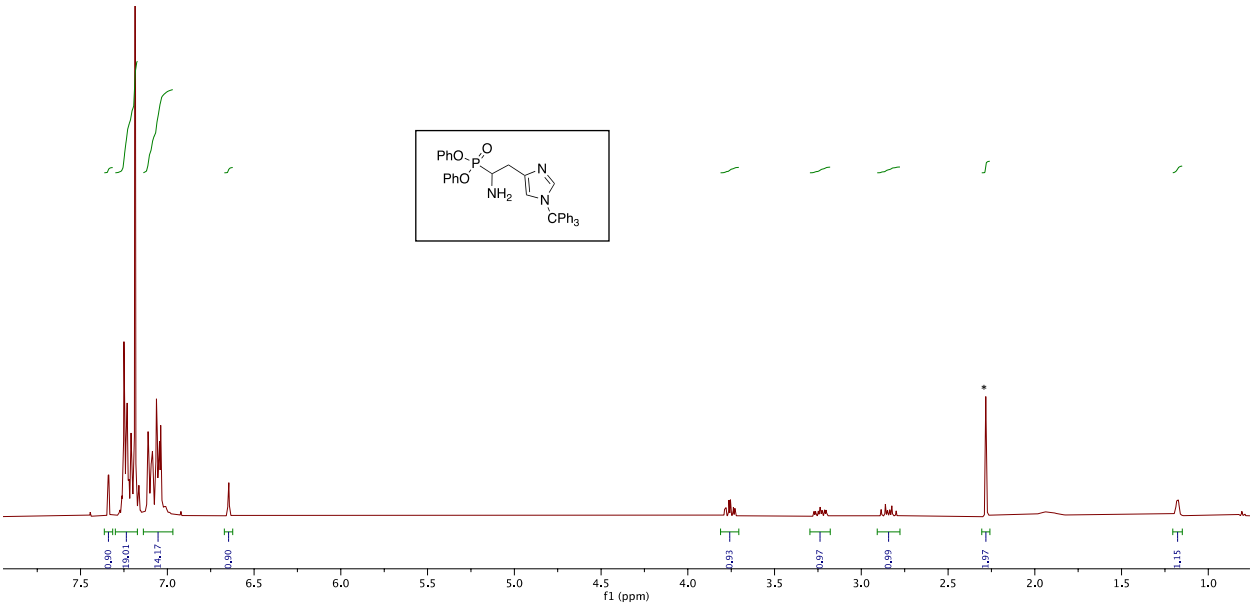
^{1}H NMR ($CDCl_3$)



^{13}C NMR ($CDCl_3$)

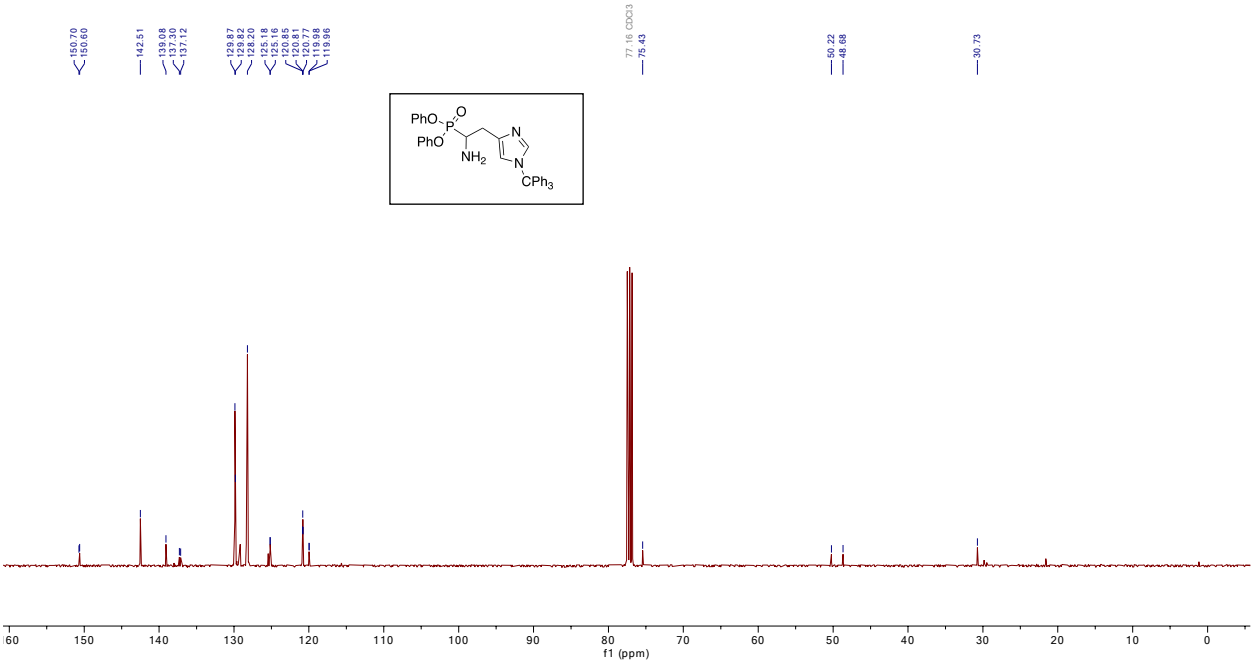


^{1}H NMR ($CDCl_3$)

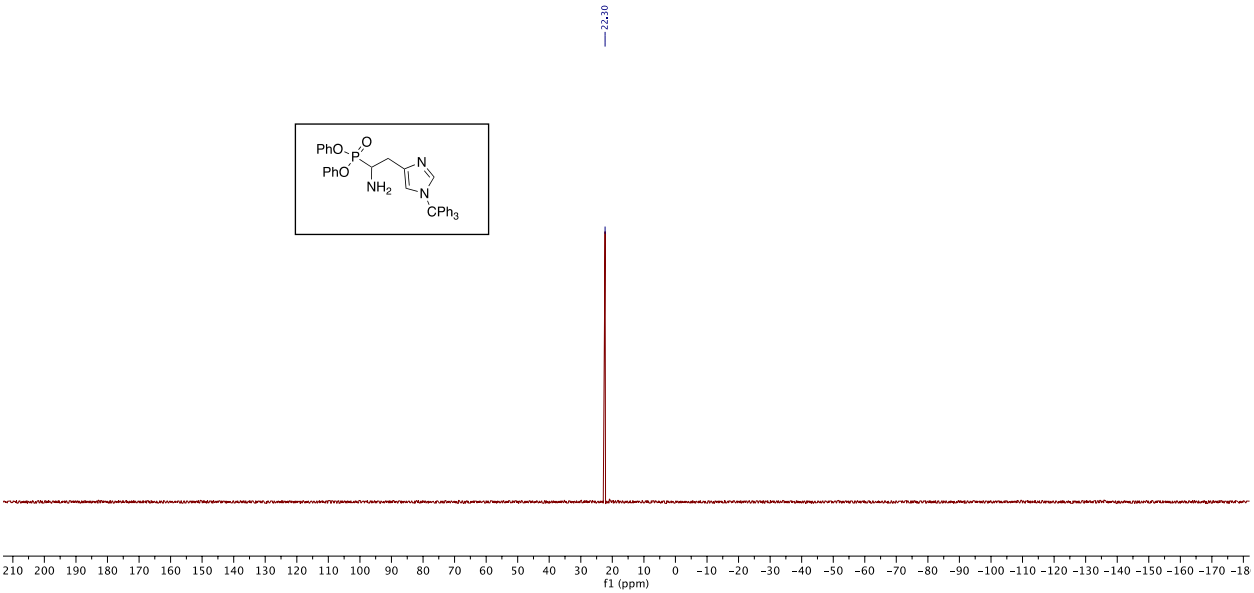


*residual toluene

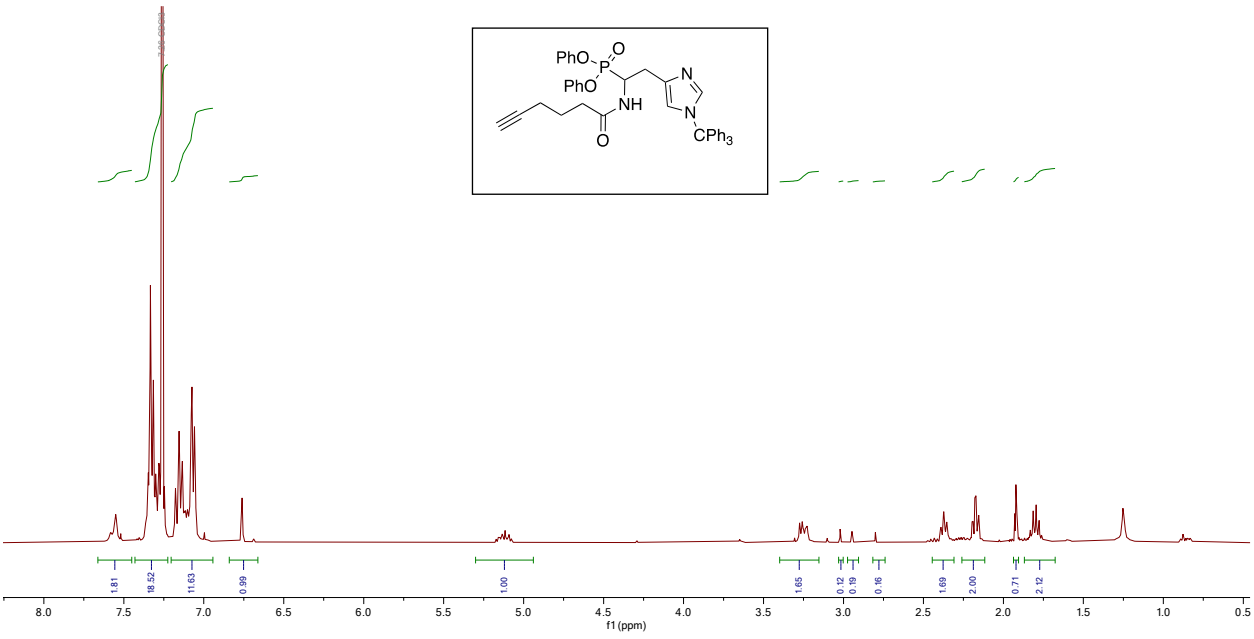
^{13}C NMR ($CDCl_3$)



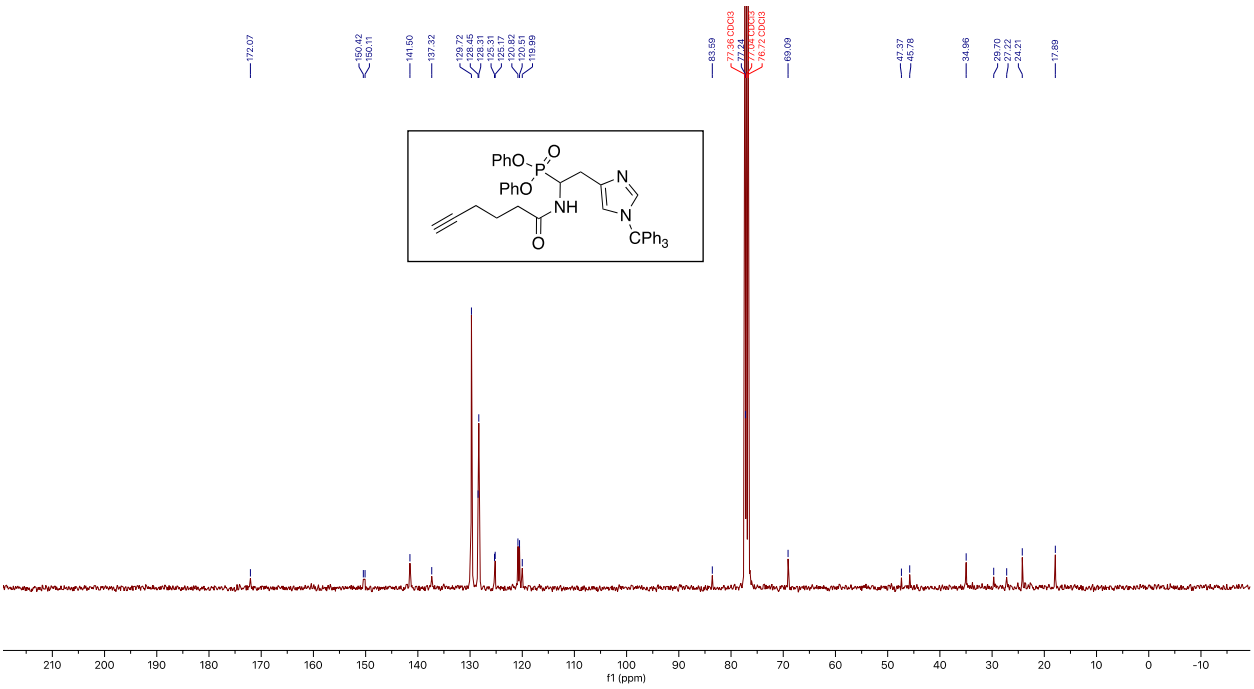
^{31}P NMR ($CDCl^3$)



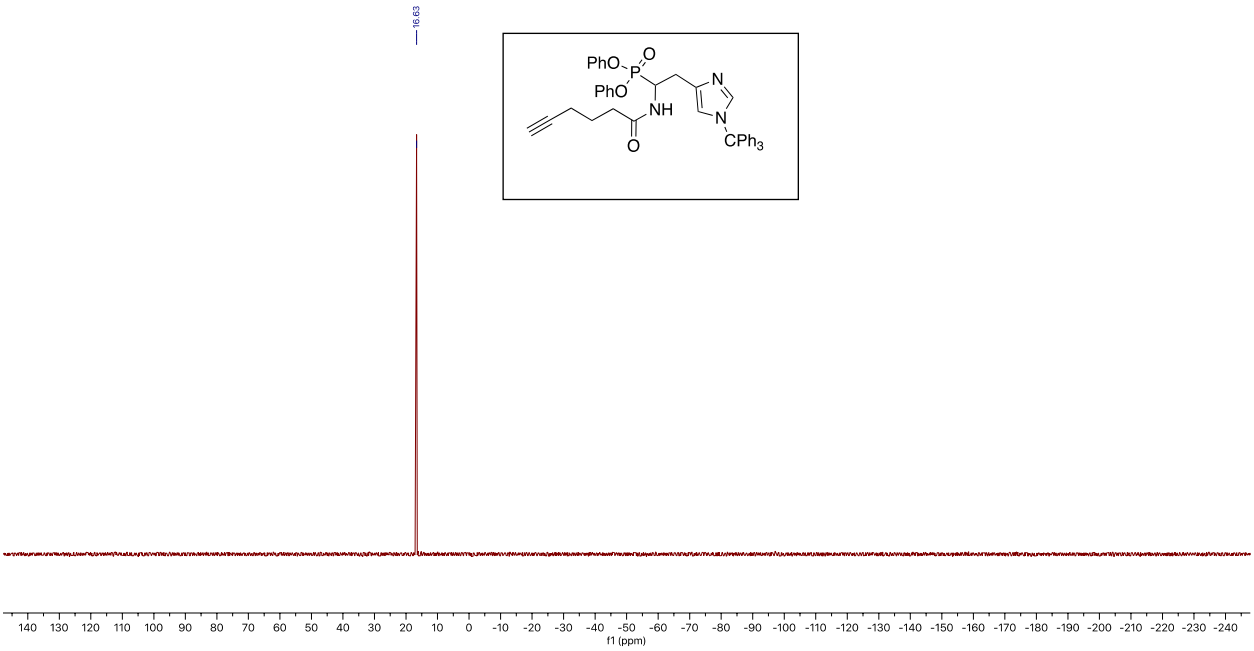
^{1}H NMR ($CDCl_3$)



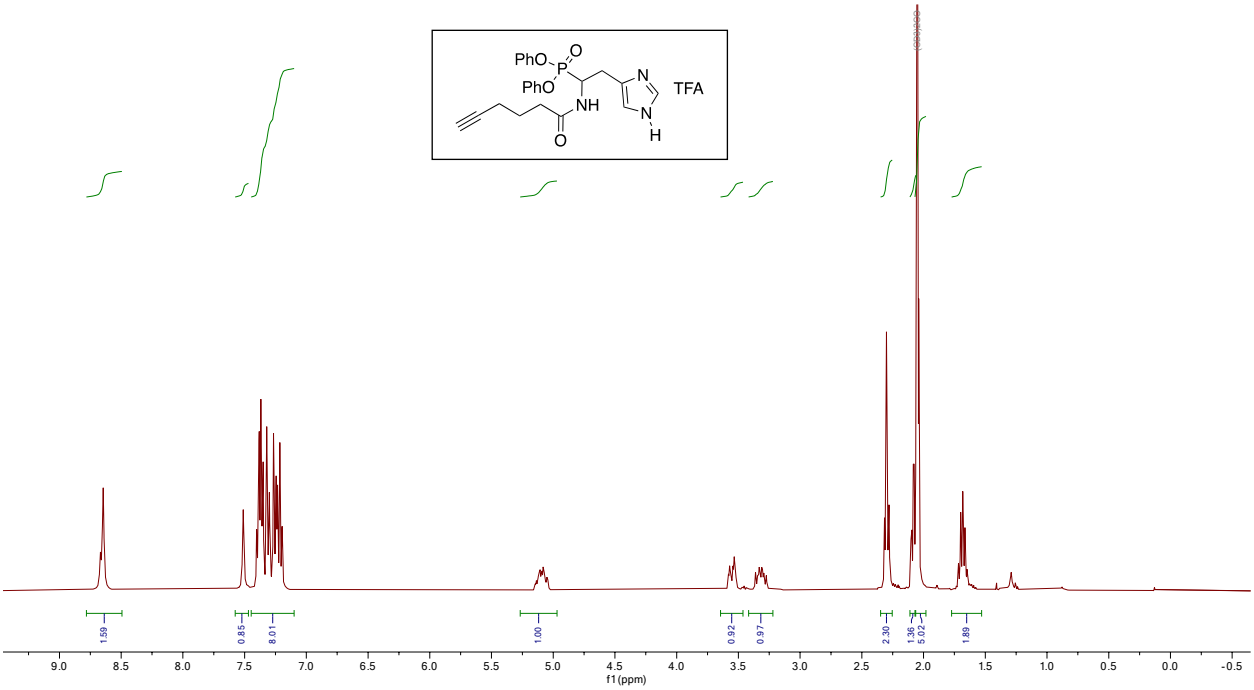
^{13}C NMR ($CDCl_3$)



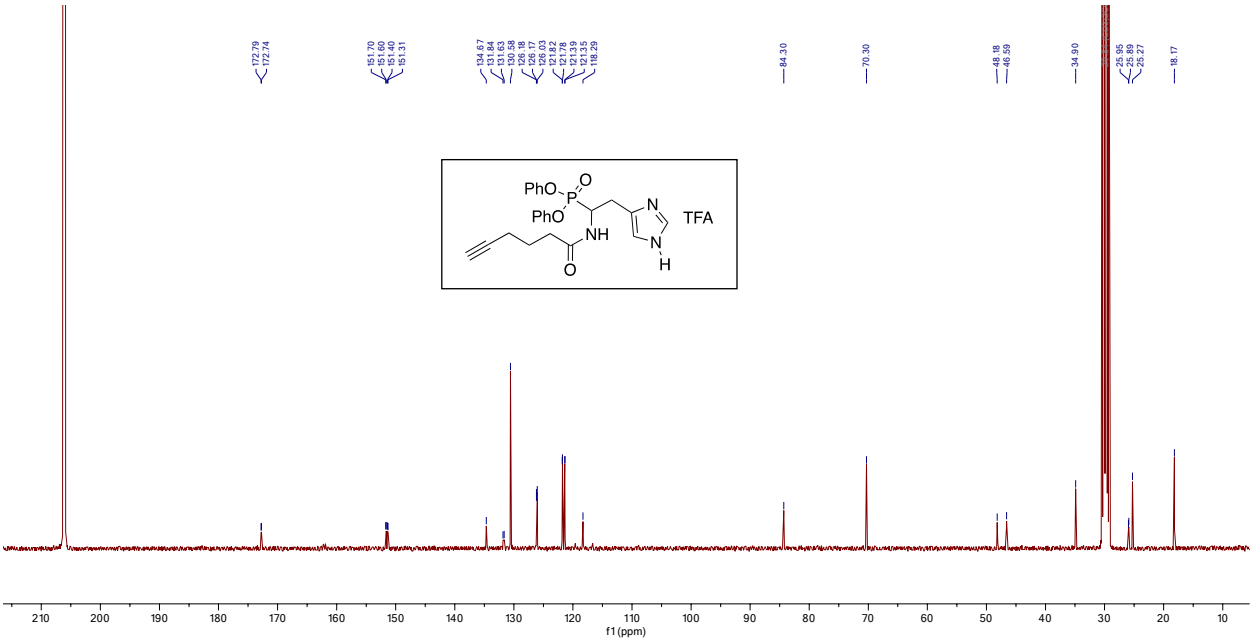
^{31}P NMR ($CDCl_3$)



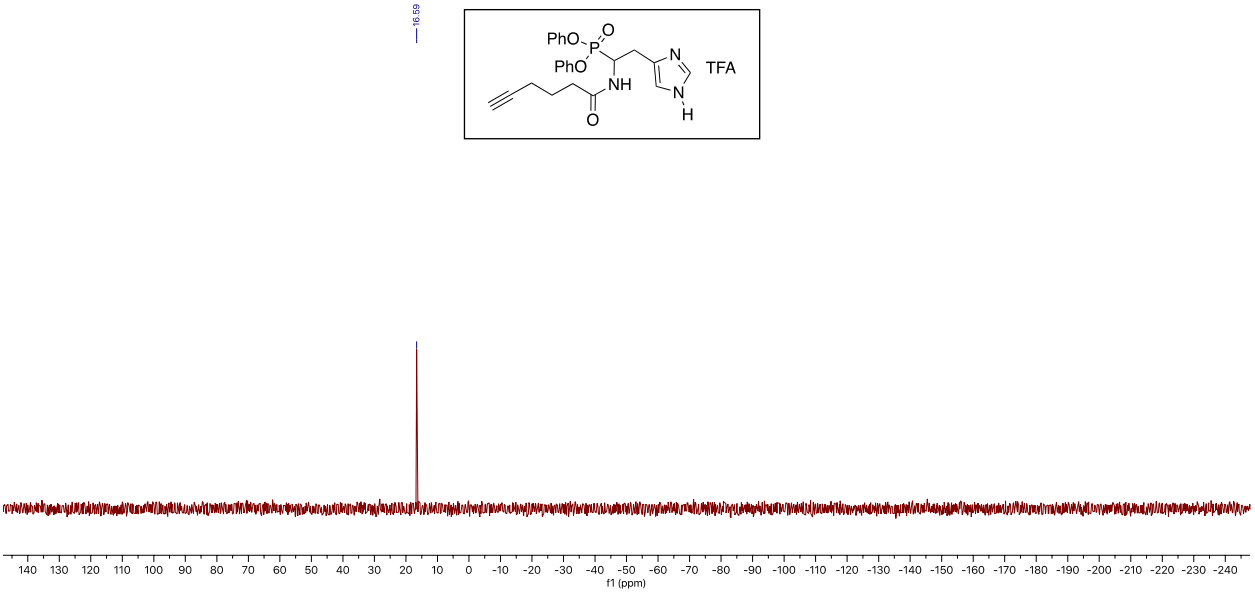
^{1}H NMR (Acetone-d_6)



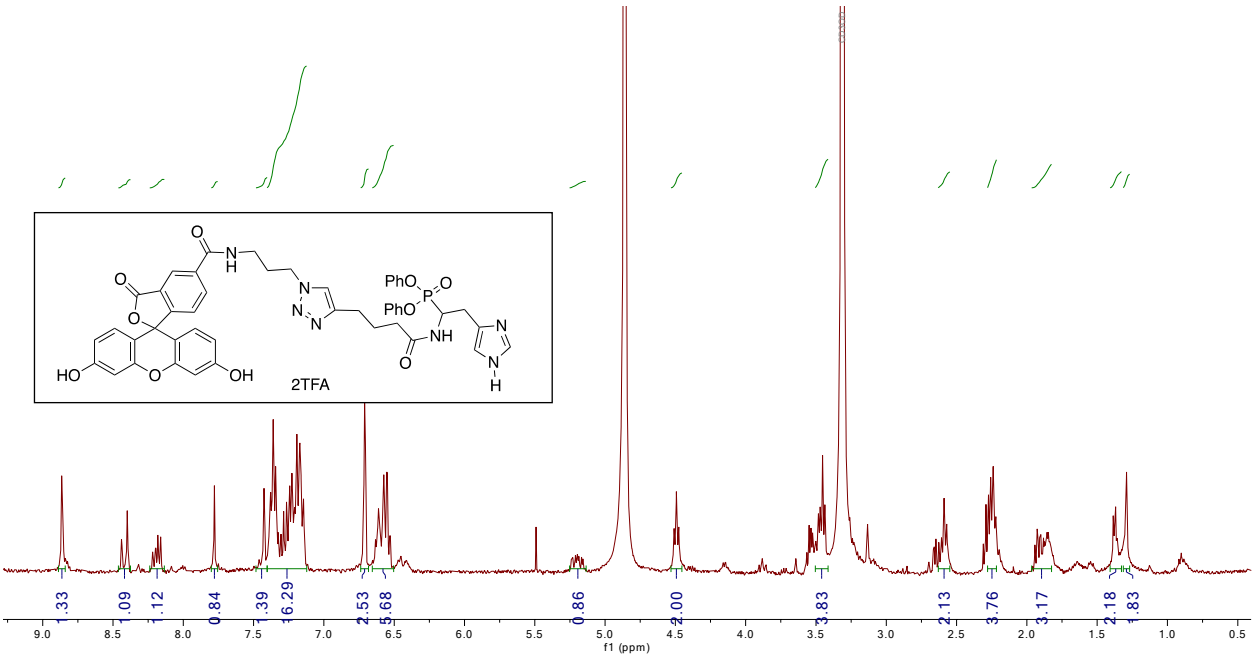
^{13}C NMR (Acetone-d_6)



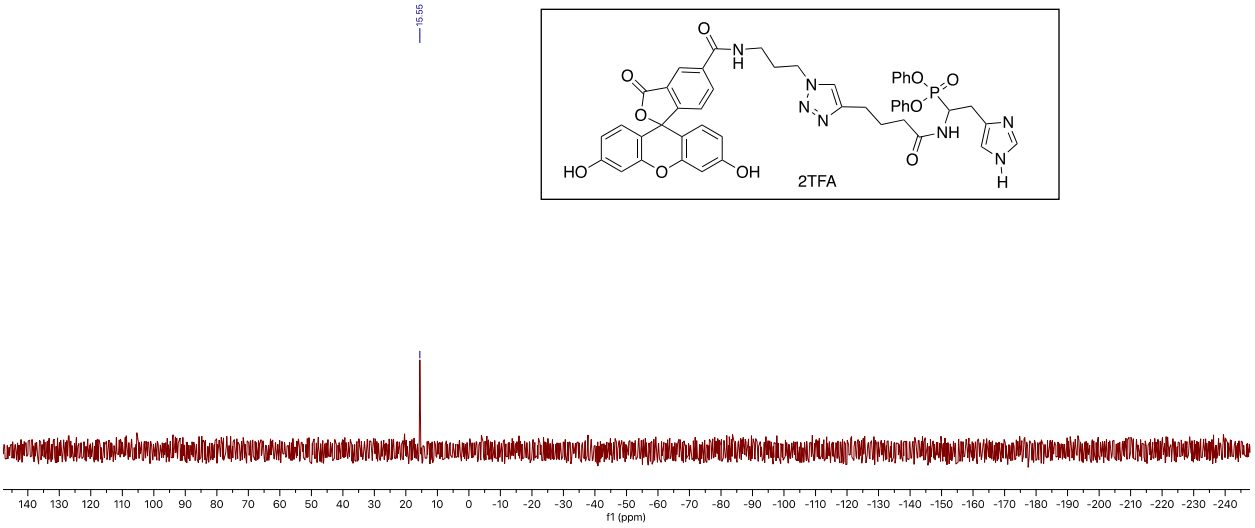
^{31}P NMR (Acetone-d_6)



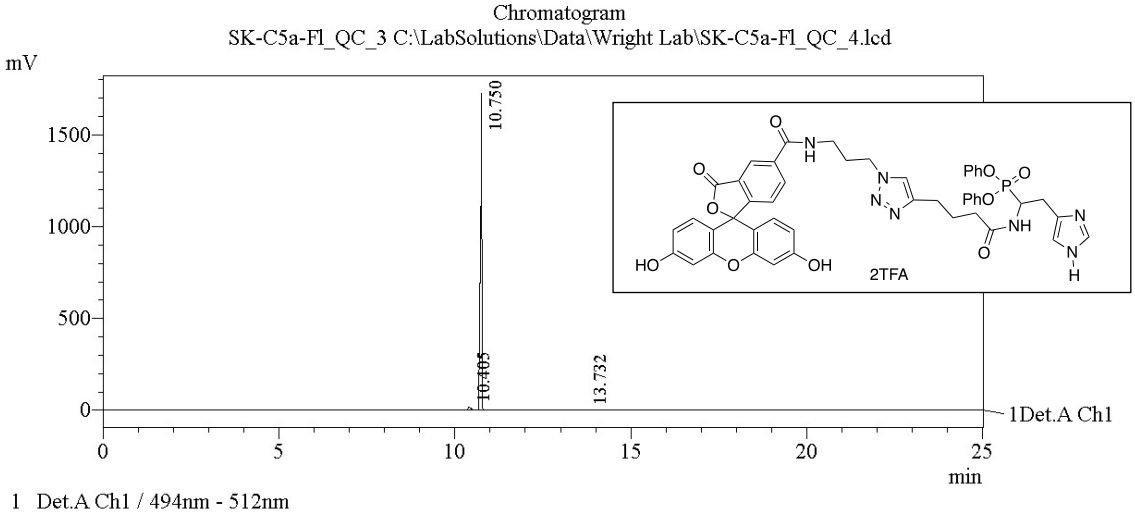
^{1}H NMR (MeOH-d_4)

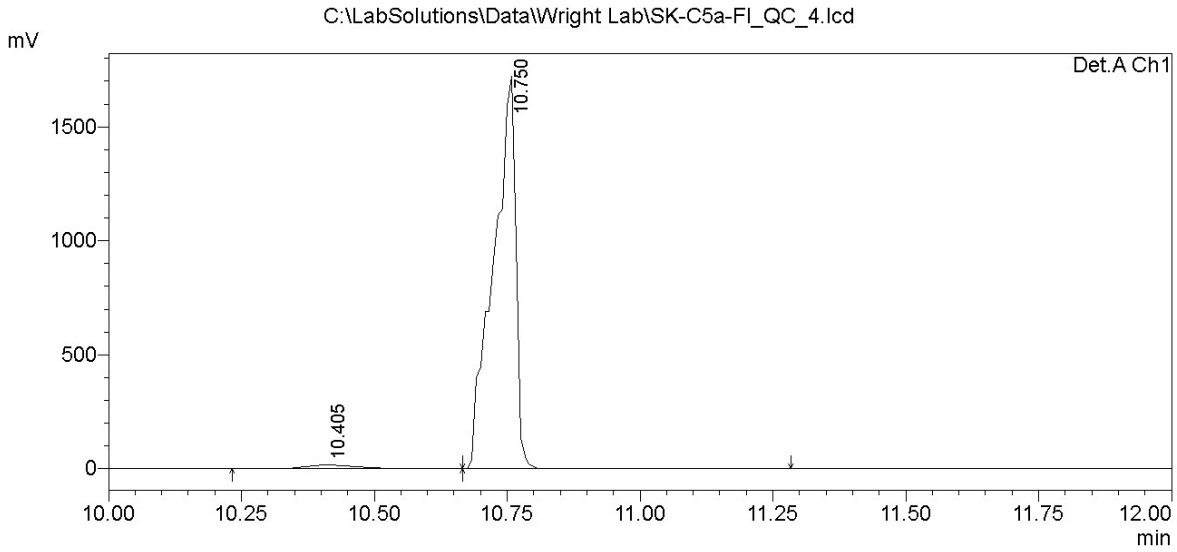


^{31}P NMR (MeOH-d_4)



C5a-Fl HPLC analysis



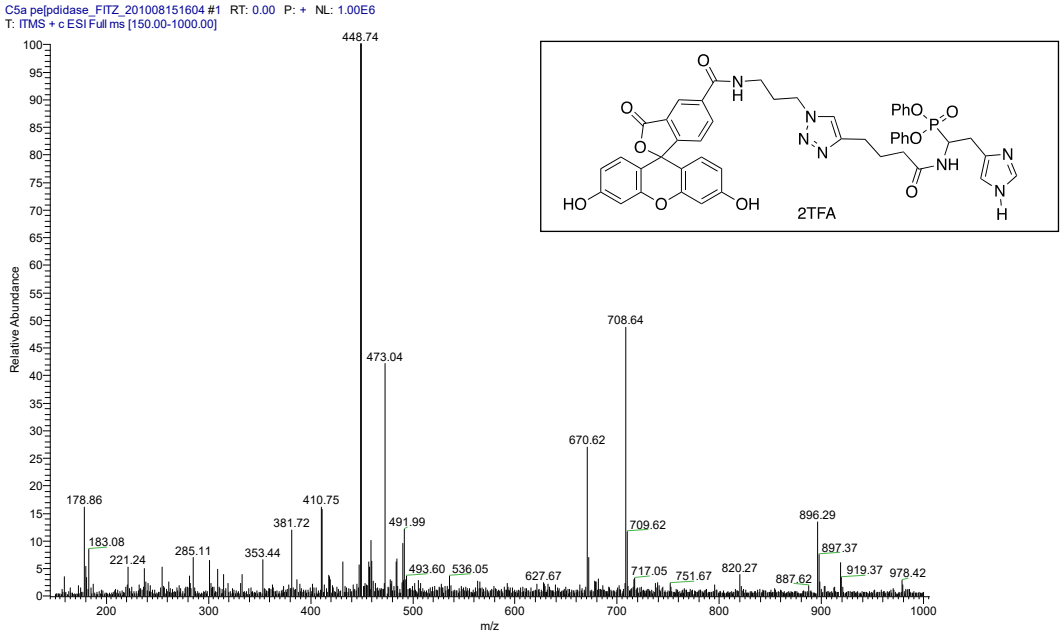


PeakTable

Detector A Ch1 494nm - 512nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	10.405	120606	16914	2.342	1.222
2	10.750	5006699	1366332	97.217	98.702
3	13.732	22713	1049	0.441	0.076
Total		5150017	1384295	100.000	100.000

ESI -LRMS



5. References

[1] Elmore, J. R.; Dexter, G. N.; Salvachua, D.; Martinez-Baird, J.; Hatmaker, E. A.; Huenemann, J. D.; Klingeman, D. M.; Peabody, G. L. t.; Peterson, D. J.; Singer, C.; Beckham, G. T.; Guss, A. M., Production of itaconic acid from alkali pretreated lignin by dynamic two stage bioconversion. *Nat Commun* **2021,** *12* (1), 2261.

[2] Egbert, R. G.; Rishi, H. S.; Adler, B. A.; McCormick, D. M.; Toro, E.; Gill, R. T.; Arkin, A. P., A versatile platform strain for high-fidelity multiplex genome editing. *Nucleic Acids Res* **2019,** *47* (6), 3244-3256.

[3] M9 Salts. *Cold Spring Harbor Protocols* **2006,** *2006* (1).

[4] Palladino, P.; Stetsenko, D. A., New TFA-free cleavage and final deprotection in fmoc solid-phase peptide synthesis: dilute HCl in fluoro alcohol. *Org Lett* **2012,** *14* (24), 6346-9

[5] Onizuka, K.; Shibata, A.; Taniguchi, Y.; Sasaki, S., Pin-point chemical modification of RNA with diverse molecules through the functionality transfer reaction and the copper-catalyzed azide-alkyne cycloaddition reaction. *Chem Commun (Camb)* **2011,** *47* (17), 5004-6.