Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Chemically triggered crosslinking with bioorthogonal cyclopropenones

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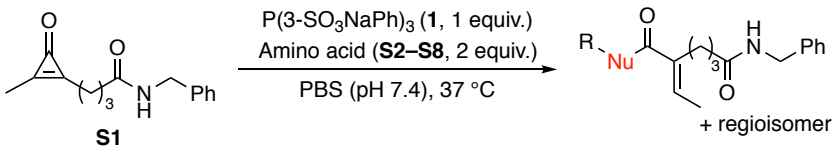
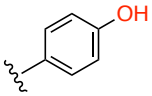
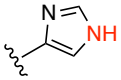
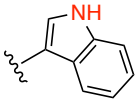
Contents

Table S1. Phosphine-Mediated Crosslinking of CpO with Amino Acids.



S1

Entry	Amino acid	Nucleophile (Nu)	% crosslink 500 μM	% crosslink 4 mM
1	Fmoc-L-Ser-OH **S2**		<1%	1%
2	Fmoc-L-Cys-OH **S3**		5%[a]	8%[a]
3	Fmoc-L-Lys-OH **S4**		<1%	2%
4	Fmoc-L-Tyr-OH **S5**		0%	1%
5	Fmoc-L-Ala-OH **S6**		0%	N.D.
6	Fmoc-L-His-OH **S7**		0%	N.D.
7	Fmoc-L-Trp-OH **S8**		0%	N.D.

[a] Disulfide formation observed over course of reaction

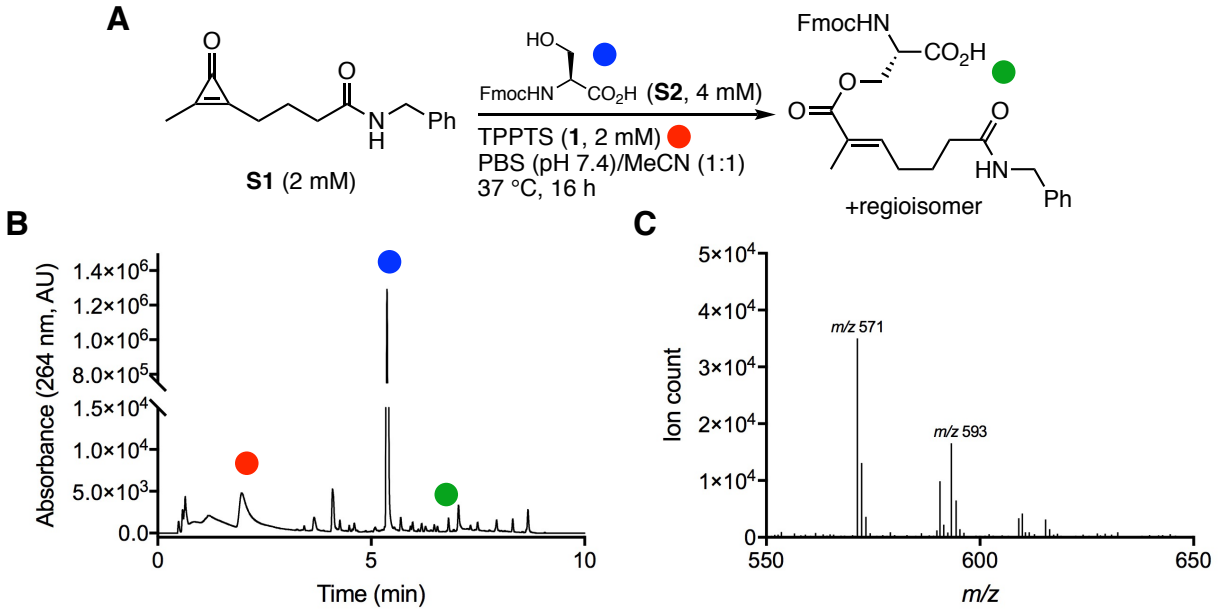


Figure S1. Chemically triggered CpO trapping with serine. (A) CpO **S1** was incubated with phosphine **1** and Fmoc-protected serine **S2**. Conversion to the crosslinked product was monitored via (B) UV absorption spectroscopy and (C) mass spectrometry.

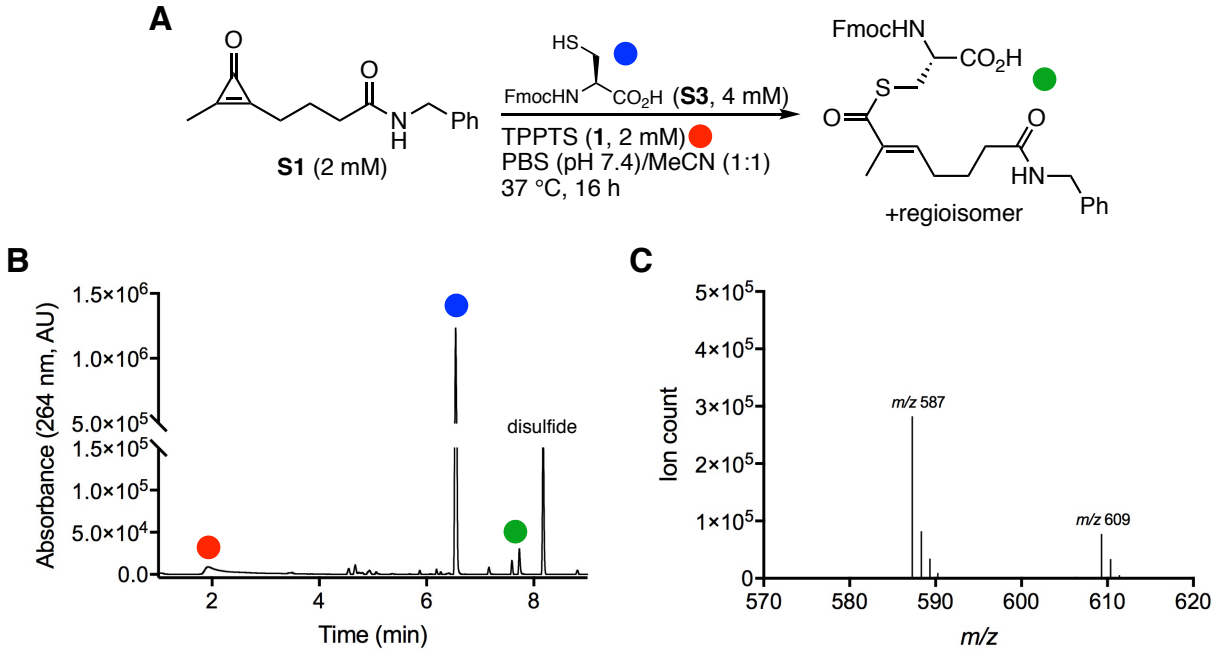


Figure S2. Chemically triggered CpO trapping with cysteine. (A) CpO **S1** was incubated with phosphine **1** and Fmoc-protected cysteine **S3**. Conversion to the crosslinked product was monitored via (B) UV absorption spectroscopy and (C) mass spectrometry.

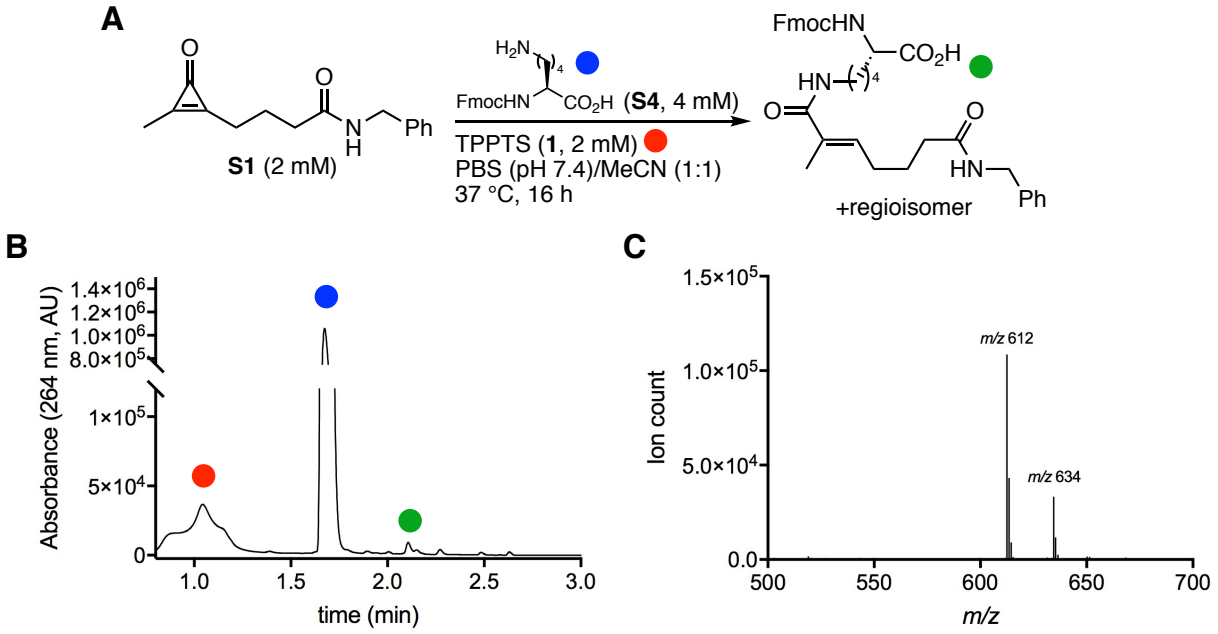


Figure S3. Chemically triggered CpO trapping with lysine. (A) CpO **S1** was incubated with phosphine **1** and Fmoc-protected lysine **S4**. Conversion to the crosslinked product was monitored via (B) UV absorption spectroscopy and (C) mass spectrometry.

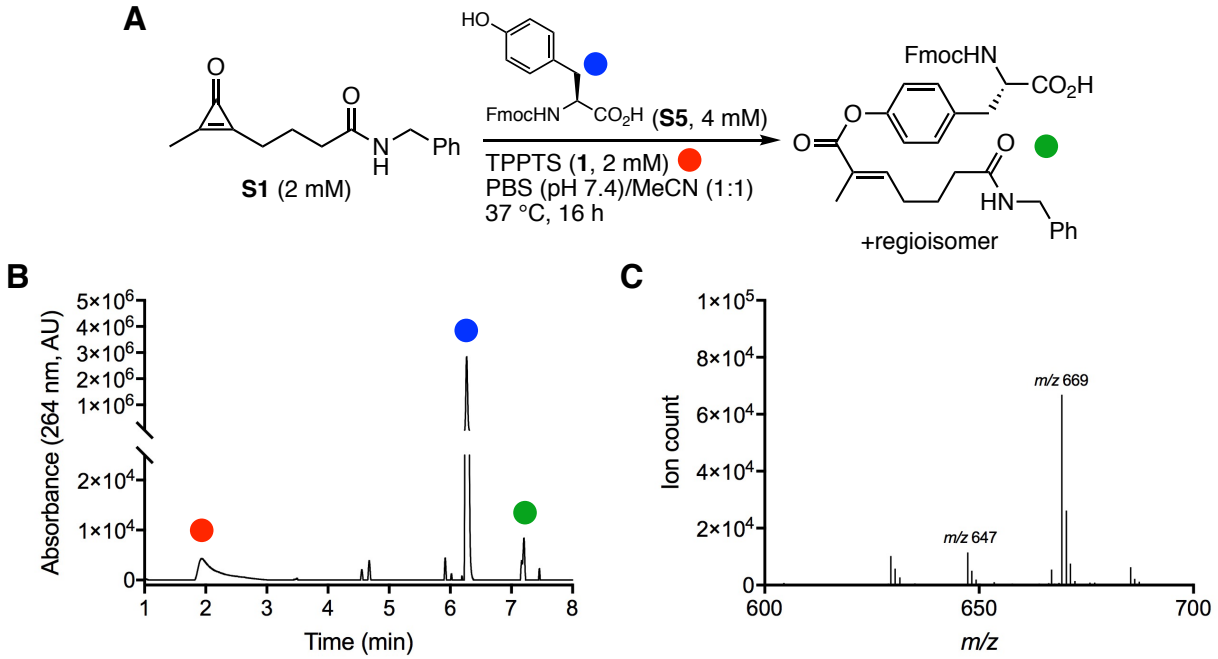


Figure S4. Chemically triggered CpO trapping with tyrosine. (A) CpO **S1** was incubated with phosphine **1** and Fmoc-protected tyrosine **S5**. Conversion to the crosslinked product was monitored via (B) UV absorption spectroscopy and (C) mass spectrometry.

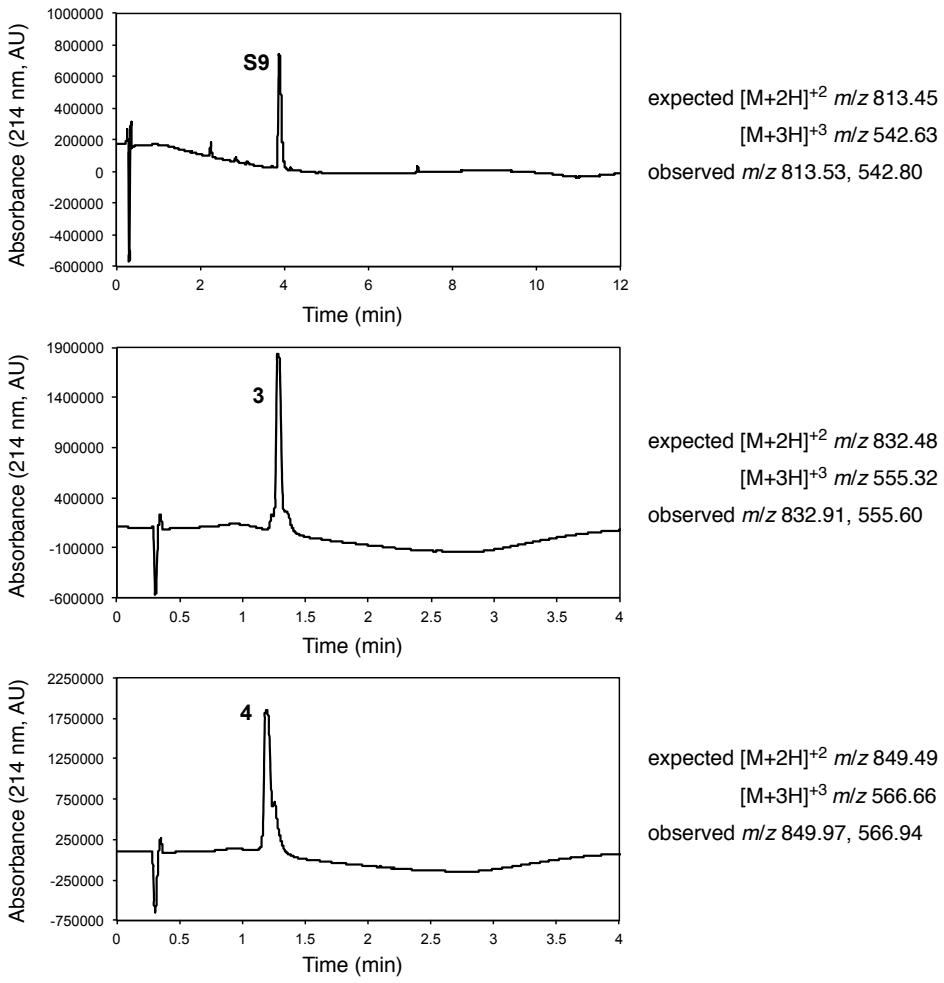


Figure S5. LC-MS characterization of SmBiT-CpO peptides **S9**, **3**, and **4**. The peptides were synthesized on solid phase and purified via HPLC.

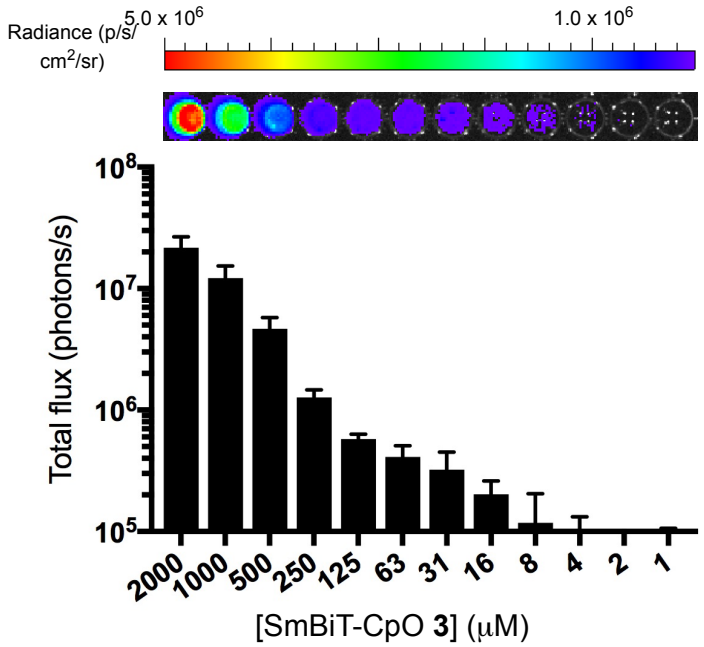


Figure S6. SmBiT-CpO **3** and LgBiT associate to form a functional, light-emitting enzyme. Furimazine (50X dilution, Promega) was added to wells containing SmBiT-CpO **3** (1–2000 μM) and LgBiT (40 pM). Light emission was quantified and error bars represent the standard error of the mean for n = 3 experiments. Sample images are shown.

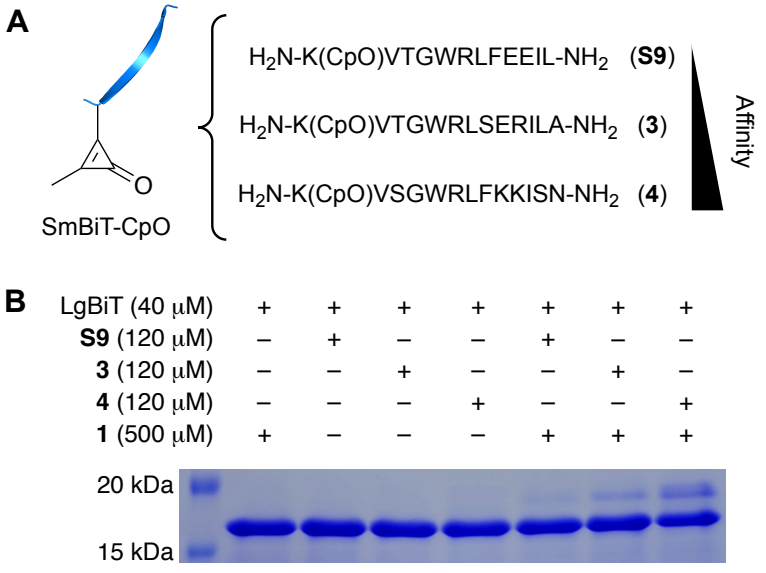


Figure S7. Phosphine-triggered crosslinking tracks with affinity. (A) SmBiT-CpO peptides (covering a range of LgBiT affinities[2]) were accessed via SPPS. (B) Crosslinking is most pronounced with high affinity interactions. LgBiT (40 μM) and SmBiT-CpO peptides (120 μM) were incubated with phosphine **1** (500 μM) at 37 ºC for 4 h. Samples were analyzed by SDS-PAGE. The crosslinking yields (3-20%) were determined by ImageJ analysis.

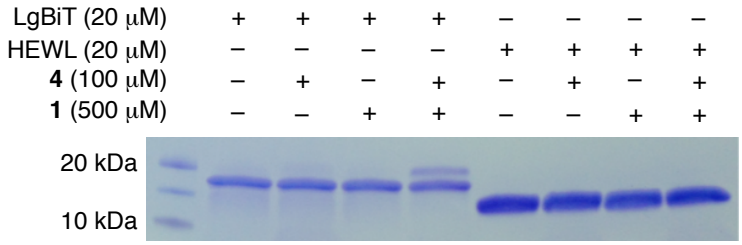


Figure S8. SmBiT-CpO **4** does not form covalent adducts with HEWL. LgBiT (20 μM) or HEWL (20 μM), SmBiT-CpO **4** (100 μM), and phosphine **1** (500 μM) were added to PBS (pH 7.4). Samples were incubated at 37 °C for 16 h, then denatured with urea (1.6 M final concentration) and analyzed by SDS-PAGE. The crosslinking yield (20%) was determined by ImageJ analysis.

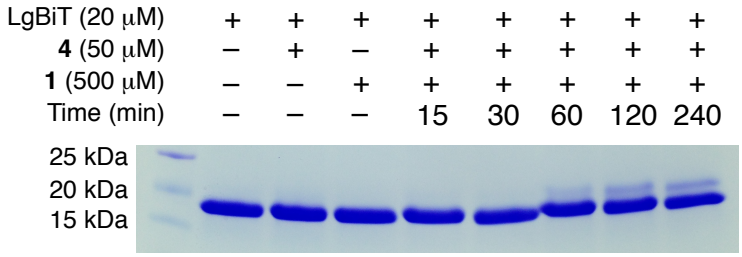


Figure S9. Crosslinking is time-dependent with phosphine **1**. LgBiT (20 μM) and SmBiT-CpO **4** (50 μM) were combined in PBS (pH 7.4). Phosphine **1** (500 μM) was added, and the reactions were incubated at 37 °C for 15–240 min. Reactions were then quenched with H_2O_2 (1% final concentration) prior to SDS-PAGE analysis. Crosslinking yields (1-4%) were determined by ImageJ analysis.

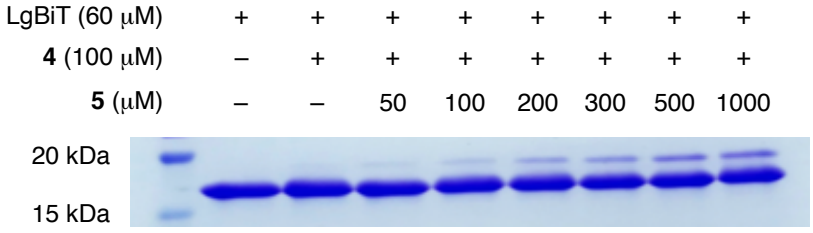


Figure S10. Crosslinking is dose-dependent with phosphine **4**. LgBiT (60 μM) and SmBiT-CpO **5** (100 μM) were combined in PBS (pH 7.4). Phosphine **5** (50 – 1000 μM) was added, and the reactions were incubated at 37 °C for 30 min. Reaction samples were denatured with urea (1.6 M final concentration) prior to SDS-PAGE analysis. Crosslinking yields (1-15%) were determined by ImageJ analysis.

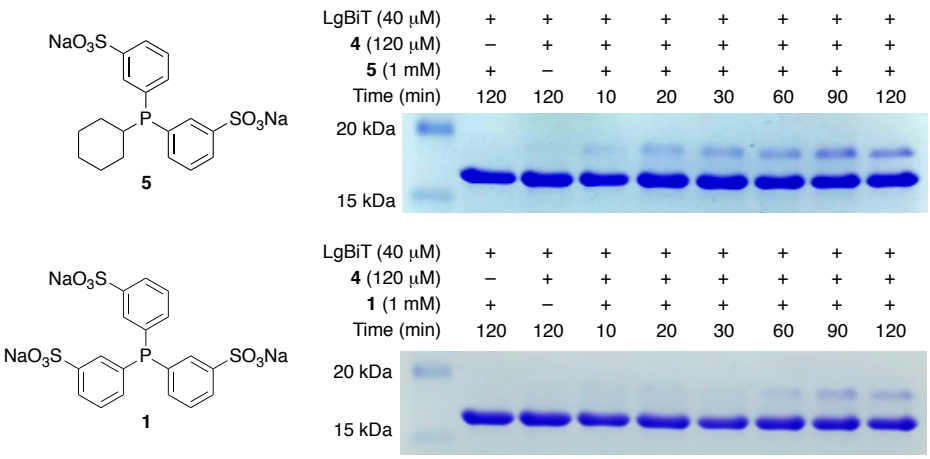


Figure S11. Rapid crosslinking observed with more nucleophilic phosphine **5**. (top) LgBiT (40 μM) and SmBiT-CpO **4** (120 μM) were combined in PBS (pH 7.4). Phosphine **5** (1 mM) was added, and the reactions were incubated at 37 °C for 10–120 min. (bottom) LgBiT (40 μM) and SmBiT-CpO **4** (120 μM) were combined in PBS (pH 7.4). Phosphine **1** (1 mM) was added, and the reactions were incubated at 37 °C for 10–120 min. For (A)-(B), reaction samples were denatured with urea (1.6 M final concentration) prior to SDS-PAGE analysis.

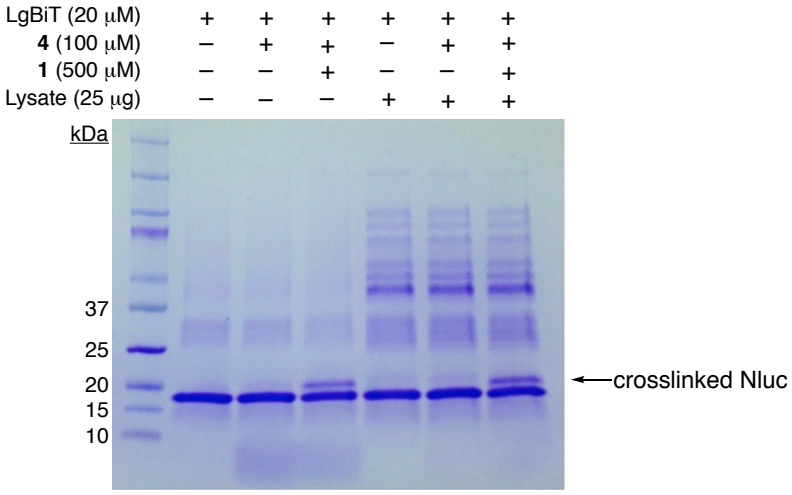


Figure S12. Crosslinking observed in bacterial lysate using phosphine **1**. LgBiT (20 μM) and SmBiT-CpO **4** (100 μM) were incubated in the presence or absence of bacterial lysate (25 μg) and phosphine **1** (500 μM). Reactions were incubated at 37 °C for 4 h, then denatured with urea (1.6 M final concentration) and analyzed by SDS-PAGE. Crosslinking yields in the presence (20%) and absence (19%) of lysate were determined by ImageJ analysis.

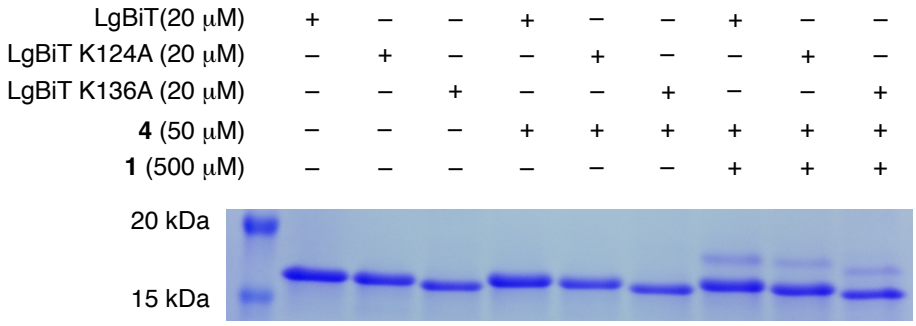


Figure S13. LgBiT residues K124 and K136 are not necessary for crosslinking. WT LgBiT, or LgBiT mutants K124A or K136A (20 μM) were combined with SmBiT-CpO **4** (50 μM) and phosphine **1** (500 μM). Samples were incubated at 37 °C for 4 h, then denatured with urea (1.6 M final concentration) and analyzed by SDS-PAGE. Crosslinked products were observed with both mutants. The crosslinking yields for LgBiT (15%) and the K124A (11%) and K136A (14%) mutants were determined by ImageJ analysis.

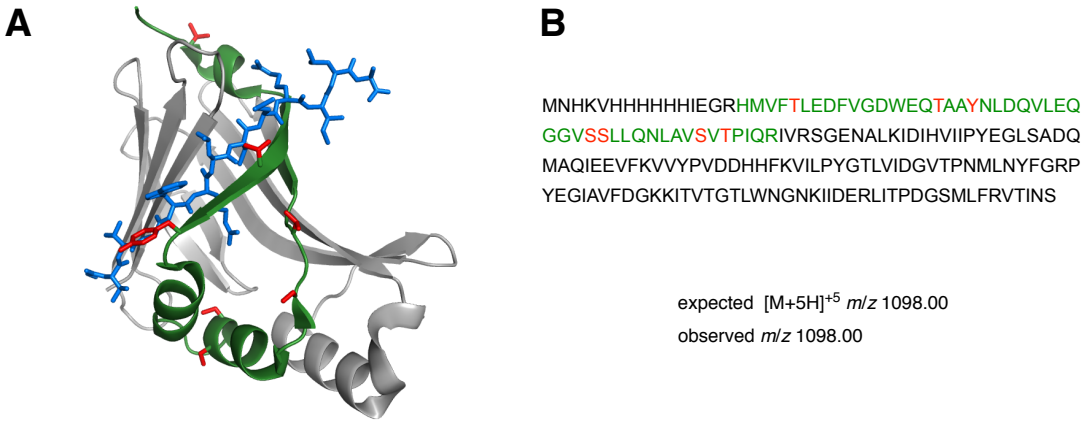


Figure S14. Site of Nluc crosslink analyzed via tryptic digest. (A) A peptide matching residues 1–43 (green) showed a mass shift corresponding to the addition of the first 6 residues of SmBiT-CpO peptide **4** (blue). Residues bearing potential trapping sidechains are highlighted in red. B) Primary sequence of Nluc in the pCOLD construct. The residues 16–64 are highlighted in green. Potential nucleophilic side chains within the sequence are highlighted in red.

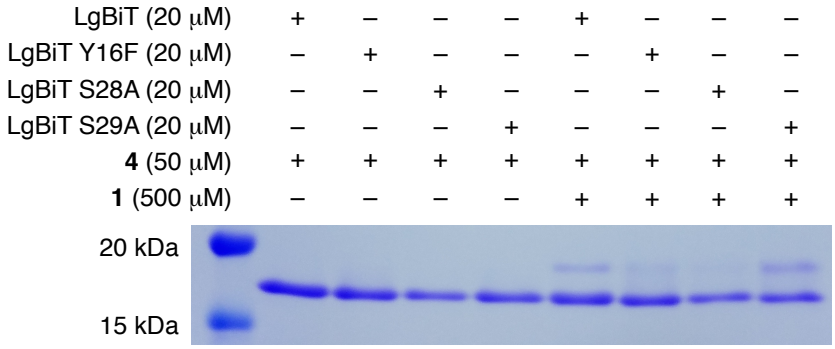


Figure S15. Diminished crosslinking is observed with LgBiT mutants Y16F and S28A. WT LgBit, or LgBiT mutants comprising Y16F, S28A, or S29A (20 μM) were combined with SmBiT-CpO **4** (50 μM) and phosphine **1** (500 μM), then incubated at 37 ºC for 4 h. Samples were denatured with urea (1.6 M final concentration), then analyzed by SDS-PAGE. The crosslinking yields for LgBiT (12%) and the Y16F (6%), S28A (5%), and S29A (22%) mutants were determined by ImageJ analysis.

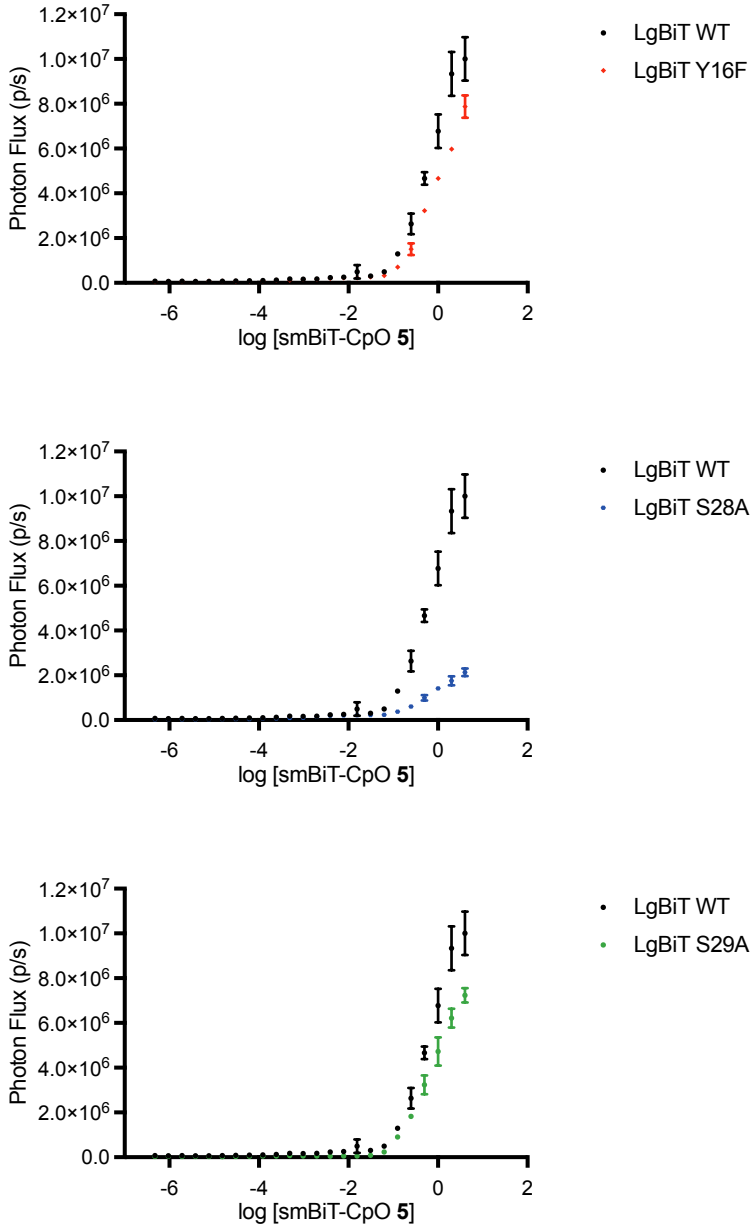


Figure S16. Dissociation constants for SmBiT-CpO **4** and either native (WT) or mutant LgBiTs. Apparent K_D values for mutants Y16F and S29A are comparable to WT. Error bars represent standard error of the mean for n = 3 experiments.

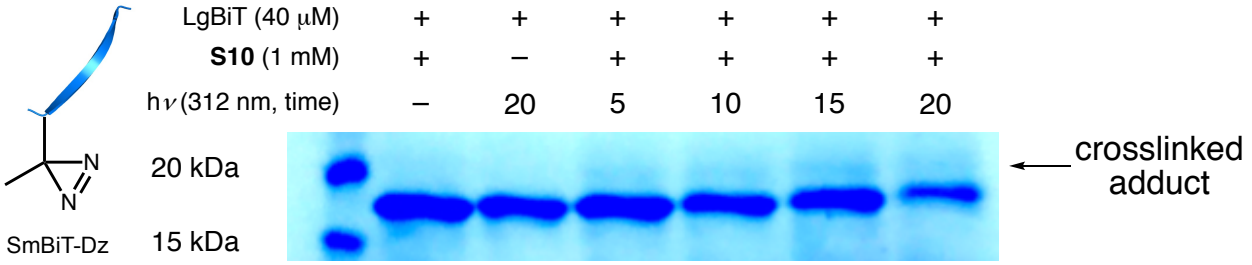


Figure S17. Prolonged UV irradiation promotes photocrosslinking. LgBiT (40 μM) was combined with SmBiT-Dz **S10** (1 mM) in PBS (pH 7.4). The samples were spun down and left to equilibrate (10 min) at room temperature. The samples were then placed on a transilluminator (312 nm) for the indicated time. All reaction samples were denatured with urea (1.6 M final concentration) prior to SDS-PAGE analysis. Crosslinking was further confirmed via mass spec analysis. LgBiT degradation was observed upon prolonged UV exposure. Crosslinking yields ranged from 1–5%.

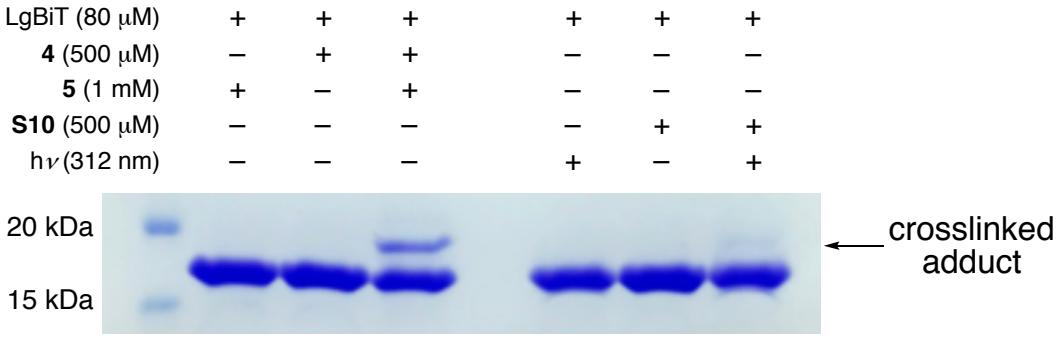


Figure S18. CpO chemical crosslinking is more efficient than diazirine photocrosslinking. LgBiT (80 μM) was combined with either SmBiT-CpO **4** (500 μM) or SmBiT-Dz **S10** (500 μM) in PBS (pH 7.4). For chemical crosslinking samples (lanes 2–4), phosphine **5** (1 mM) was added, and the reactions were incubated at 37 °C for 30 min. For photocrosslinking reactions (lanes 6–8), samples were irradiated with UV light (312 nm) for 10 min. All reaction samples were denatured with urea (1.6 M final concentration) prior to SDS-PAGE analysis. The crosslinking yields for SmBiT-CpO (22%) and SmBiT-Dz (1%) were determined by ImageJ analysis.

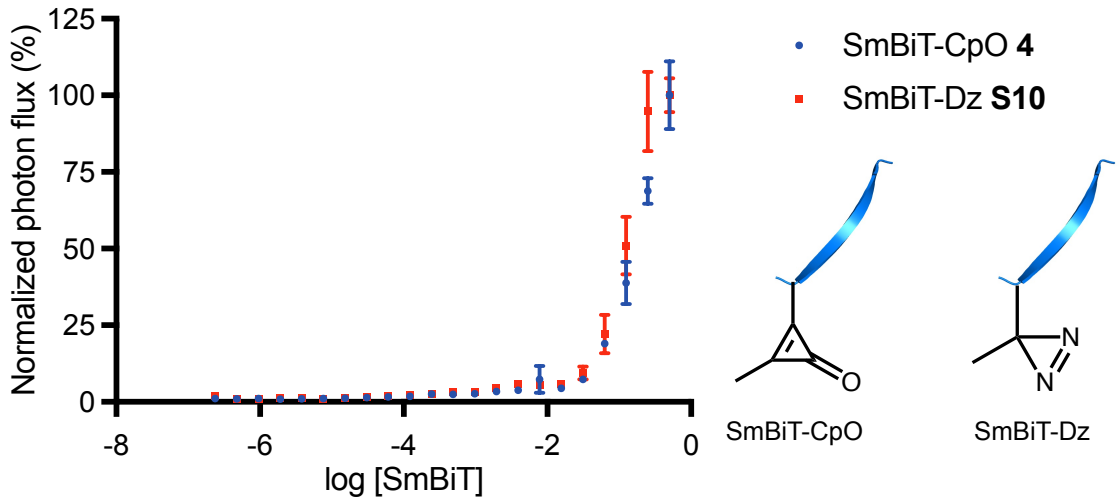


Figure S19. Dissociation constants for native (WT) LgBiT and either SmBiT-CpO **4** or SmBiT-Dz **S10**. Apparent K_D values for both SmBiT peptides are comparable. Error bars represent standard error of the mean for n = 3 experiments.

Materials and Methods

General Information

All reagents and solvents were used as received, unless otherwise specified. Anhydrous organic solvents were prepared by degassing with argon and passing through two 4 x 36 in. columns of anhydrous neutral A2 (8 x 12 mesh; LaRoche Chemicals; activated at 350 °C for 12 h under a flow of argon). Column chromatography was carried out using Silicycle 60 Å (230–400 mesh) silica gel. Thin-layer chromatography (TLC) was carried out with Agela Technologies 200 mm silica gel MF254 plates, and plates were visualized using UV light or $KMnO_4$ stain. Organic solutions were concentrated under reduced pressure using a Büchi rotary evaporator. HPLC purifications were performed on an Agilent Technologies 1260 Infinity II equipped with a multiple wavelength detector, using an Agilent Eclipse XDB-C18 semiprep column (9.4 x 250 mm, 5 µm) and a 3 mL/min flow rate.

^{1}H, ^{13}C, and ^{31}P NMR spectra were obtained using either a Bruker DRX400 or a Bruker AVANCE600 instrument equipped with a cryo probe. ^{1}H NMR spectra were acquired at 600 MHz, ^{13}C NMR spectra were acquired at 150 MHz, and ^{31}P NMR spectra were acquired at 162 MHz. Spectra were internally referenced to residual solvent signals ($CDCl_3$ was referenced to 7.27 ppm for ^{1}H and 77.16 ppm for ^{13}C, $(CD_3)_2SO$ was referenced to 2.50 ppm for ^{1}H and 39.52 ppm for ^{13}C, D_2O was referenced to 4.79 ppm for ^{1}H). All spectra were acquired at 298 K. Chemical shifts are reported in ppm, and coupling constants (*J*) are reported in Hz. Mass spectra were acquired at the University of California, Irvine Mass Spectrometry Facility.

LC-MS Analysis of CpO Trapping with Amino Acids

Trapping of phosphine-activated cyclopropenone (CpO) **S1** was performed with amino acids **S2**–**S8**. Conversion to crosslinked products was analyzed by LC-MS. Each amino acid (10 µL of a 5 mM stock in 50% MeCN/PBS, 0.5 mM final concentration) was combined with a solution CpO **S1** (2.5 µL of a 10 mM stock in 50% MeCN/PBS, 0.25 mM final concentration) and phosphine **1** (2.5 µL of a 10 mM stock in 50% MeCN/PBS, 0.25 mM final concentration). The resulting solution was diluted to 100 µL with PBS (12 mM, pH 7.4). Reactions were incubated at 37 °C overnight. Samples were then analyzed on a Waters ACQUITY UPLC with an ACQUITY QDa Mass Detector, using a C18 column and eluting with a gradient of 10–90% MeCN/H_2O (containing 0.1% formic acid) over 5 min with a flow rate of 0.5 mL/min.

Trapping reactions with amino acids **S2**–**S5** were also examined at higher concentrations. Amino acids **S2**–**S5** (200 µL of 10 mM stock solutions in 50% MeCN/PBS, 4 mM final concentration) were combined with CpO **S1** (100 µL of a 10 mM stock in 50% MeCN/PBS, 2 mM final concentration) and phosphine **1** (100 µL of a 10 mM stock in 50% MeCN/PBS, 2 mM final concentration). The resulting solutions were then diluted to 500 µL with PBS (12 mM, pH 7.4). Reactions were incubated at 37 °C overnight. Samples were analyzed on a Waters ACQUITY UPLC with an ACQUITY QDa Mass Detector, using a C18 column and eluting with a gradient of 10–90% MeCN/H_2O (containing 0.1% formic acid) over 15 min with a flow rate of 0.5 mL/min.

Percent crosslinking was calculated by comparing the integrations of the Fmoc-protected amino acids and product using UV absorbance at 264 nm (λ_{max} for product). The ratio was scaled to account for the stoichiometric ratio of the amino acid (2 equivalents).

$$\%\ \text{crosslink} = \frac{I_P}{I_{aa} + I_P} \times 100 \times 2$$

I_P = product integration at 264 nm
I_{aa} = Fmoc-protected amino acid integration at 264 nm

Molecular Cloning and Plasmid Construction

LgBiT point mutants (Y16F, S28A, S29A, K124A, and K136A) were constructed using overlapping circular polymerase extension cloning (CPEC).[1] Two PCR reactions (PCR1 and PCR2) were used to assemble the insert containing the mutation of interest, using primers listed in Table S2. The insert was then integrated into a linearized pCOLD vector using primers outlined in Table S3. The resulting plasmid was transformed into BL21 *E. coli* for protein expression and purification.

Forward primers for each mutant and Insert-For1 (for Y16F, S28A, and S29A) or Insert-For2 (for K124A and K136A) (PCR1), along with reverse primers for each mutant and Insert-Rev1 (for Y16F and S29A), Insert-Rev2 (for S28A), or Insert-Rev3 (for K124A and K136A) (PCR2), were used to construct individual halves of each mutant gene product. The PCR1 and PCR2 reactions were assembled using appropriate Insert-For and Insert-Rev to give the fully constructed mutant gene product (Tables S2 and S3). PCR products from all insert reactions were analyzed using 1% agarose gels and GelRed™ (Biotium, Inc.) staining. The bands of interest were excised, and DNA was isolated following incubation at 65 ºC for 20 min in ADB buffer (400 µL, Zymo Research Company). DNA products were purified using a ZymoPURE Plasmid Miniprep Kit (Zymo Research Company), eluting with Nanopure water (30 µL).

The constructed mutant gene products were ultimately inserted into a pCOLD vector backbone. The pCOLD vector was linearized by amplification using primers pCOLD-vector-For1 and pCOLD-vector-Rev (for Y16F and S29A), pCOLD-vector-For2 and pCOLD-vector-Rev (for S28A), or pCOLD-vector-For3 and pCOLD-vector-Rev2 (for K124A and K136A, Table S3). Q5® High-Fidelity DNA Polymerase (New England BioLabs) was used in the amplifications. The linearization reaction was analyzed and purified as above. The resulting linear vector was combined in a 4:1 volumetric ratio with the constructed mutants described above. The insert and vector were combined via CPEC using the following conditions: 1X Q5® High-Fidelity DNA Polymerase reaction buffer, 1X Q5® High GC Enhancer buffer, dNTPs (0.8 mM), and Q5® High-Fidelity DNA Polymerase (1 U) in a total volume of 50 µL. The following thermal cycling conditions were used: initial denaturation at 95 °C for 60 s, then 24 cycles of denaturation (95 °C, 30 s), annealing (60 °C for insert construction, 68 ºC for vector linearization and full vector amplification), and extension (72 °C, 60 s for insert construction, 72 ºC, 180 s for vector linearization and full vector amplification). The fully constructed plasmid was

purified using a ZymoPURE Plasmid Miniprep Kit (Zymo Research Company) and eluted with Nanopure water (10 µL). The full elution volume was used for transformation into TOP10 *E. coli* cells via electroporation.

Primer Lists

All primers were purchased from Integrated DNA Technologies, Inc. (San Diego, CA) and are written in the 5'→3' direction. Bases highlighted in red denote the target position for site-directed mutagenesis.

Table S2. Forward and reverse primers used to generate mutant LgBiT plasmids.

Forward Primers	
Y16F	GAACAGACAGCCGCCTTTAACCTGGACCAAG
S28A	CTTGAACAGGGAGGTGTGGCGAGTTTGCTGCAGAATC
S29A	CTTGAACAGGGAGGTGTGTCCGCGTTGCTGCAGAATC
K124A	CGTGTTCGACGGCAAAGCGATCACTGTAACAGGGACC
K136A	CTGTGGAACGGCAACGCGATTATCGACGAGCG
Reverse Primers	
Y16F	CTTGGTCCAGGTTAAAGGCGGCTGTCTGTTC
S28A	GATTCTGCAGCAAACTCGCCACACCTCCCTGTTCAAG
S29A	GATTCTGCAGCAACGCGGACACACCTCCCTGTTCAAG
K124A	GGTCCCTGTTACAGTGATCGCTTTGCCGTCGAACACG
K136A	CGCTCGTCGATAATCGCGTTGCCGTTCCACAG

Table S3. Primers used to generate gene inserts and vector backbones.

Insert Construction and Amplification Primers	
Insert-For1	CTTCCACTTTTTCCCGCGTTTTCGCAGAAAC
Insert Rev1	GGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAA AAAACTTGATTTG
Insert Rev2	CAAATAAAAAAATCCCCGCCAAATGGCAGGGATCTTAG ATTC
Insert-For2	CCAGTGTAGTAAGGCAAGTCCCTTCAAGAGTTATCGTT GATACCCCTCGTAG
Insert-Rev3	GGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAA AAAACTTGATTTG
Vector Linearization	
pCOLD-vector-For1	CCGTAAAGCACTAAATCGGAACCCTAAAGGGAGCCCCC GATTTAGAG

pCOLD-vector-Rev1	GAAAAAGTGGAAGCGGCGATGGCGGAGCTGAATTACATTC
pCOLD-vector-For2	GGCGGGGATTTTTTTATTTGTTTTCAGGAAATAAATAATCGATC
pCOLD-vector-For3	CCGTAAAGCACTAAATCGGAACCCTAAAGGGAGCCCCCGATTTAGAG
pCOLD-vector-Rev2	TGAAGGGACTTGCCTTACTACACTGGATATGCGCTAGCACATCAAATTG

Protein Expression and Purification

E. coli TOP10 cells expressing plasmids pZER09 (pCOLD-WT_LgBiT), pDR15 (pCOLD-LgBiT K136A), pDR16 (pCOLD-LgBiT K124A), pSN09 (pCOLD-LgBiT Y16F), pSN10 (pCOLD-LgBiT S28A), or pSN11 (pCOLD-LgBiT S29A) were used to inoculate 50 mL of Luria-Bertani broth (LB) containing ampicillin (100 µg/mL). After overnight incubation at 37 °C with shaking (225 rpm), 15 mL of each starter culture was used to inoculate 1 L of LB containing ampicillin (100 µg/mL). The cultures were incubated at 37 °C with shaking until an OD_{600} of ~0.6 was achieved. The cultures were then chilled in an ice bath for 20 min. IPTG was added (1 mM final concentration) to induce protein expression, and cultures were incubated at 16 °C with shaking (225 rpm) for 20 h.

Cells were collected via centrifugation at 4000 rpm for 20 min (4 °C). The supernatant was decanted and the pellets were suspended in 25 mL of 50 mM phosphate buffer (pH 7.8). Protease inhibitor cocktail (Sigma-Aldrich) and phenylmethylsulfonyl fluoride (PMSF, Gold Biotechnology, 500 µM final concentration) were added to each suspension. Each sample was sonicated, and the lysate was centrifuged at 10000 rpm for 40 min (4 °C). The clarified lysates were filtered using 0.45 µm filters (Olympus). Profinity™ IMAC resin (BioRad, 5 mL bed volume) was added to each clarified lysate, and the mixtures were rocked at 4 °C for 30 min. For each sample, the resin was rinsed with wash buffer (50 mM phosphate, 20 mM imidazole, pH 7.8, 50 mL), and proteins were isolated with elution buffer (50 mM phosphate, 250 mM imidazole, pH 7.8, 3 x 5 mL). The fractions were analyzed via SDS-PAGE, and those containing the desired protein were combined and concentrated via spin filter centrifugation (3 kDa MW cutoff). Protein concentrations were determined using a Pierce™ BCA protein assay kit (Thermo Fisher) or by absorbance at 280 nm using a JASCO V730 UV-Vis spectrophotometer and an extinction coefficient of 19940 M^{-1} cm^{-1} (calculated using Expasy ProtParam).

Peptide K_D Measurements

All bioluminescence imaging experiments were performed in black 96-well plates (Greiner Bio One). Plates containing luminescent reagents were imaged in a light-proof chamber with an IVIS Lumina (Xenogen) CCD camera chilled to –90 ºC. The stage was kept at 37 ºC during the imaging session, and the camera was controlled using Living Image software. The exposure time was set to 30 s, with data binning levels set to medium. Regions of interest were selected for quantification and total flux values were analyzed using Living Image software.

Peptide affinities for LgBiT were measured as previously reported.[2] SmBiT-CpO **3** (1–2000 μM) or **4** (0.5 nM – 2 mM) was combined with native LgBiT (WT, 40 pM) or mutant LgBIT (40 pM) and samples were incubated at room temperature for 30 min. Furimazine (Promega, 100 μL of a 50X dilution of the commercial stock) was then added, and bioluminescence output was measured.

Crosslinking Reactions with Split Nluc

All crosslinking experiments were carried out in PBS (pH 7.4) at 37 °C. For chemical crosslinking experiments, LgBiT (10–80 μM final concentration) or hen egg-white lysozyme (HEWL, 20 μM) was combined with SmBiT-CpO peptides **3**, **4**, and **S9** (50 μM–1 mM). Phosphine **1** (500 μM–1 mM) or **5** (1 mM) was then added, and the reactions were incubated at 37 °C for 10 min–16 h.

For photocrosslinking reactions, LgBiT (80 μM final concentration) was combined with SmBiT-Dz peptide **S10** (500 μM) and incubated at room temperature for 10 min. The samples were irradiated with UV light (312 nm for 10 min) using an FBTIV-816 Transilluminator (Fisher Scientific).

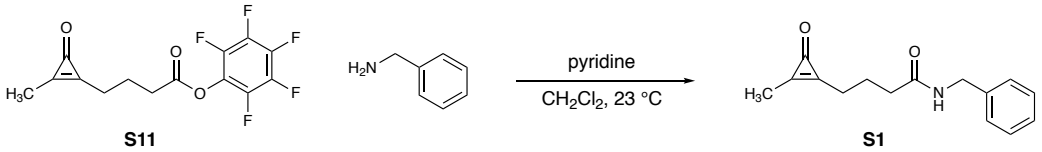
All samples were denatured with urea (1.6 M final concentration) for 30 min at 25 ºC, then analyzed by SDS-PAGE (BioRad 4–20% gels, 130 V, 4 ºC, 70–80 min). Gel staining was accomplished using Coomassie Blue. In Figure S9, excess phosphine was quenched with H_2O_2 (1% final concentration) prior to SDS-PAGE analysis. The percentages of crosslinked products were determined via gel scanning and calculating the area of protein bands using ImageJ software.

Crosslink Analysis via Trypsin Digestion

LgBiT (20 μM) was incubated with SmBiT-CpO **4** (100 μM) and phosphine **1** (500 μM) for 4 h at 37 ºC. Samples were then analyzed via SDS-PAGE. Bands corresponding to the crosslinked product were excised and destained (50 mM NH_4HCO_3 in 50% MeCN, 1 mL). The destain buffer was removed, and the sample was washed with MeCN (1 x 1 mL). After drying, the samples were incubated with Trypsin Gold, Mass Spectrometry Grade (Promega, 50 ng/μL, 1 μL) at 37 °C overnight. Peptides were eluted in 0.1% formic acid and analyzed via LC-MS.

Synthetic Procedures

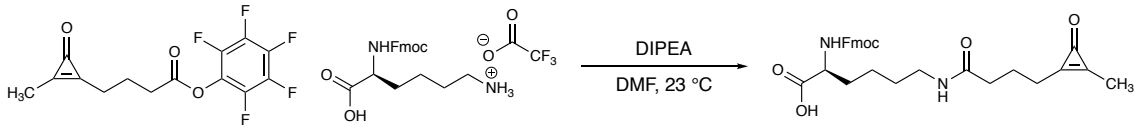
Compounds **5**[3], **S11**[4], and **S12**[5] were synthesized as previously described.



S11 **S1**

***N*-Benzyl-4-(2-methyl-3-oxocycloprop-1-en-1-yl)butanamide (S1)**

To a flame-dried round-bottom flask under a nitrogen atmosphere was added **S11** (192 mg, 0.600 mmol), followed by anhydrous CH_2Cl_2 (6 mL), anhydrous pyridine (72 µL, 0.89 mmol), and benzylamine (33 µL, 0.30 mmol). The solution was stirred at 23 ºC for 16 h, then diluted with H_2O (25 mL) and washed with 1 M HCl (5 mL). The layers were separated, and the aqueous layer was extracted with CH_2Cl_2 (3 x 10 mL). The organic layers were combined, dried with $MgSO_4$, filtered, and concentrated *in vacuo*. The crude residue was passed through a silica plug (eluting with 0–100% acetone in EtOAc), then further purified by HPLC (eluting with 10–90% MeCN + 0.1% TFA in H_2O + 0.1% TFA over 15 min). The desired fractions were collected and lyophilized to give **S1** as a colorless oil (18 mg, 25%). ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.31–7.29 (m, 2H), 7.26–7.23 (m, 3H), 6.74 (br s, 1H), 4.41 (d, *J* = 5.8 Hz, 2H), 2.62 (t, *J* = 6.9 Hz, 2H), 2.38 (t, *J* = 7.0 Hz, 2H), 2.24 (s, 3H), 2.02 (quint, *J* = 7.0 Hz, 2H). ^{13}C NMR ($CDCl_3$, 150 MHz) δ 171.8, 160.9, 160.4, 156.9, 138.4, 128.8, 128.0, 127.6, 43.7, 35.0, 25.2, 22.1, 11.4. HRMS (ESI^+) calculated for $C_{15}H_{17}NO_2Na$ $[M+Na]^+$ *m/z* 266.1157, found 266.1149.

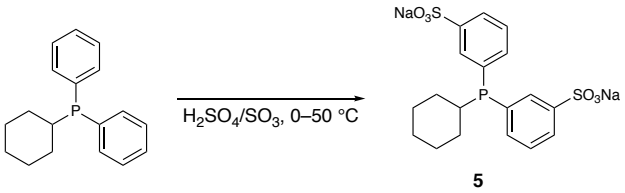


S11 **S12** **2**

Cyclopropenone amino acid (2)

To a flame-dried round-bottom flask under a nitrogen atmosphere was added a solution of **S11** (298 mg, 0.932 mmol) in anhydrous DMF (2 mL), followed by DIPEA (0.97 mL, 5.6 mmol). A solution of **S12** (886 mg, 1.84 mmol) in anhydrous DMF (2 mL) was added dropwise, and the reaction was stirred at room temperature overnight. The solution was transferred to a separatory funnel containing 1 M HCl (~100 mL) and extracted into CH_2Cl_2 (3 x 20 mL). The combined organic layers were dried over $MgSO_4$, filtered, and concentrated *in vacuo*. The residue was diluted with toluene (100 mL) and concentrated *in vacuo* to remove residual DMF. This dilution-concentration sequence was repeated twice. The resulting residue was purified by flash column chromatography (eluting with a gradient of 2–10% MeOH in CH_2Cl_2) to give **2** as an off-white solid (0.27 g, 0.54 mmol). This material was used in solid-phase peptide synthesis without further purification. A small portion was further purified by HPLC (40–90% MeCN + 0.1% TFA in H_2O + 0.1% TFA over 15 min) for characterization. The desired fractions were collected and lyophilized. ^{1}H NMR (600 MHz, $(CD_3)_2SO$) δ 7.89 (d, *J* =

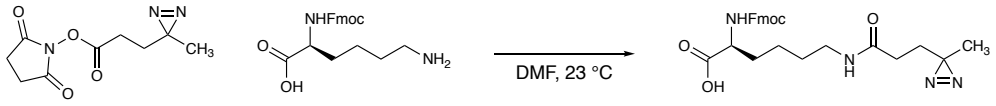
7.5 Hz, 2H), 7.83 (t, *J* = 5.3 Hz, 1H), 7.72 (d, *J* = 7.2 Hz, 2H), 7.61 (d, *J* = 8.0 Hz, 1H), 7.42 (t, *J* = 7.4 Hz, 2H), 7.33 (t, *J* = 7.4 Hz, 2H), 4.27 (d, *J* = 7.1 Hz, 2H), 4.22 (t, *J* = 7.1 Hz, 1H), 3.90 (td, *J* = 9.2, 4.7 Hz, 1H), 3.04–3.00 (m, 2H), 2.56 (t, *J* = 7.1 Hz, 2H), 2.22 (s, 3H), 2.14 (t, *J* = 7.4 Hz, 2H), 1.83 (quint, *J* = 7.2 Hz, 2H), 1.72–1.67 (m, 1H), 1.62–1.56 (m, 1H), 1.43–1.27 (m, 4H). ^{13}C NMR (150 MHz, $(CD_3)_2SO$) δ 174.0, 171.1, 160.6, 158.1, 157.3, 156.2, 143.8, 140.7, 127.6, 127.1, 125.3, 120.1, 65.6, 53.7, 46.7, 38.2, 34.4, 30.4, 28.7, 25.3, 23.1, 21.9, 11.1. HRMS (ESI$^+$) calculated for $C_{29}H_{32}N_2O_6Na$ $[M+Na]^+$ 527.2158 *m*/*z*, found 527.2162.



5

Sodium 3,3'-(cyclohexylphosphanediyl)dibenzenesulfonate (5)

Compound **5** was prepared using a previously reported procedure,[6] with some modifications. To a round-bottom flask under a nitrogen atmosphere was added oleum (18–24% free SO_3, 1.25 mL) and the solution was cooled to 0 °C. Cyclohexyldiphenylphosphine (268 mg, 1.00 mmol) was added in portions over 5 min, and the resulting solution was allowed to warm to room temperature. The solution was then heated at 50 °C for 4 hours until starting material was consumed. The reaction was diluted with cold water (70 mL), then triisooctylamine (10 mL) and toluene (10 mL) were added. The resulting biphasic mixture was stirred for 1 h at room temperature. In order to separate the product from impurities, the crude mixture was extracted at different pH levels. To the mixture was added a deoxygenated 5% NaOH solution until pH 3 was reached. The solution was transferred to a separatory funnel and the aqueous layer was discarded. Additional deoxygenated 5% NaOH solution was added to the separatory funnel containing the organic layer with periodic agitation until the aqueous layer reached pH 6. The aqueous layer was then discarded. To the separatory funnel containing the organic layer was carefully added a deoxygenated 5% NaOH solution with periodic agitation until the aqueous layer reached pH 8. The organic layer was then discarded and the aqueous layer was collected and concentrated *in vacuo*. The conversion was measured by ^{31}P NMR (86% desired product, 14% corresponding oxide). This material was further purified as needed by HPLC (10–50% MeCN in H_2O over 15 min) to obtain the desired product as a white crystalline solid (trace amounts of phosphine oxide present in ^{1}H NMR). ^{1}H NMR (D_2O, 600 MHz) δ 7.98 (d, *J* = 7.4 Hz, 2H), 7.82 (d, *J* = 7.7 Hz, 2H), 7.70 (t, *J* = 7.4 Hz, 2H), 7.54 (t, *J* = 7.7 Hz, 2H), 2.50–2.45 (m, 1H), 1.74–1.66 (m, 5H), 1.38–1.32 (m, 2H), 1.26–1.15 (m, 3H). ^{31}P NMR (162 MHz, D_2O) δ –2.2. ^{13}C NMR (D_2O, 150 MHz) δ 142.7 (d, *J* = 7.4 Hz), 136.9 (d, *J* = 11.2 Hz), 136.5 (d, *J* = 16.5 Hz), 130.2 (d, *J* = 21 Hz), 129.4 (d, *J* = 6.7 Hz), 126.3, 34.2 (d, *J* = 4.5 Hz), 29.0 (d, *J* = 13.6 Hz), 26.1 (d, *J* = 11.8 Hz), 25.9. HRMS (ESI–) calculated for $C_{18}H_{20}NaO_6PS_2$ $[M+Na]^-$ *m/z* 449.0275, found 449.0277.



S13

Diazirine amino acid (S13)

To a flame-dried round-bottom flask under an argon atmosphere was added succinimidyl 4,4'-azipentoate (38 mg, 0.17 mmol) and Fmoc-Lys-OH (93 mg, 0.25 mmol). Anhydrous DMF (0.56 mL) was added, and the solution was stirred at ambient temperature overnight. The reaction was diluted with H_2O (20 mL) and extracted with Et_2O (4 x 20 mL). The organic layers were combined and washed with H_2O (2 x 20 mL) and sat. LiCl (1 x 20 mL). The organic layers were then dried with $MgSO_4$, filtered, and concentrated *in vacuo* to give compound **S13** as a white solid (61 mg, 75%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.76 (d, *J* = 7.5 Hz, 2H), 7.60 (t, *J* = 7.4 Hz, 1.5 H), 7.52–7.56 (m, 0.5 H, rotamer), 7.39 (t, *J* = 7.4 Hz, 2H), 7.30 (t, *J* = 7.4 Hz, 2H), 5.83 (br s, 0.4 H, rotamer) 5.77–5.72 (m, 1H), 5.65 (d, *J* = 7.8 Hz, 0.7 H), 4.51 (br s, 0.4 H, rotamer) 4.44–4.34 (m, 3H), 4.21 (t, *J* = 6.9 Hz, 1H), 3.30–3.22 (m, 2H), 1.97 (t, *J* = 7.6 Hz, 2H), 1.94–1.87 (m, 1H), 1.81–1.77 (m, 1 H), 1.75 (t, *J* = 7.6 Hz, 2 H) 1.59–1.51 (m, 2H), 1.49–1.36 (m, 2H), 0.98 (s, 3H). ^{13}C NMR ($CDCl_3$, 150 MHz) δ 174.8, 172.3, 156.5, 144.0 (rotamer), 143.8, 141.5, 127.9, 127.3, 125.3, 120.2, 67.3, 53.6, 47.3, 39.3, 31.9, 30.8, 29.0, 25.6, 22.3, 20.0. HRMS (ESI$^+$) calculated for $C_{26}H_{30}N_4O_5Na$ $[M+Na]^+$ 501.2114 *m*/*z*, found 501.2117.

SmBiT peptides: H_2N-K(CpO)VTGWRLFEEIL-NH_2 (S9), H_2N-K(CpO)VTGWRLSERILA-NH_2 (3), H_2N-K(CpO)VSGWRLFKKISN-NH_2 (4), and H_2N-K(Dz)VSGWRLFKKISN-NH_2 (S10).

To a fritted glass reservoir was added NovaPEG Rink Amide resin (0.46 mmol/g, 110–220 mg, 0.05–0.10 mmol respectively). Throughout the synthesis, the resin was agitated by bubbling N_2 through the reservoir. The resin was initially swelled and washed with DMF (2 x 15 mL, 15 min per cycle), then treated with 20% 4-methylpiperidine/DMF (10 mL, 20 min) for Fmoc removal. After the first deprotection step, the resin was washed with DMF (3 x 10 mL, 5 min). Protected amino acids were then coupled in the following manner: Fmoc-protected amino acid (0.30 mmol), HCTU (120 mg, 0.30 mmol), and diisopropylethylamine (160 µL, 0.89 mmol) were dissolved in 3.0 mL of DMF. The solution was mixed thoroughly, incubated at room temperature for ~3 min, and then added to the deprotected resin. Each coupling step was performed at room temperature for a minimum of 45 min. The resin was then washed with DMF (3 x 10 mL, 5 min). Subsequent Fmoc deprotection was achieved by incubating the resin with 10 mL of 20% 4-methylpiperidine/DMF (20 min). The resin was then washed with DMF (3 x 10 mL, 5 min) prior to the next coupling step. Repeated cycles of amino acid coupling and deprotection were used to achieve the desired peptides. After the final cycle, the resin was washed with CH_2Cl_2 (3 x 10 mL, 5 min). Peptides were then isolated by incubating the resin with 95:2.5:2.5 trifluoroacetic acid (TFA):triisopropylsilane:H_2O (2 x 5 mL, 30 min). The cleavage solutions were combined, concentrated *in vacuo*, and treated with 40 mL cold diethyl ether. The precipitate was then collected via centrifugation (3000 x *g*, 5 min) and dried under vacuum. The

peptides were purified by HPLC (eluting with 40–90% MeCN containing 0.1% TFA over 15 min), and the purified products were lyophilized. Product identity and purity were assessed via LC-MS. Peptide stocks were prepared in PBS (pH 7.4), and concentrations were determined using a Pierce™ BCA protein assay kit (Thermo Fisher) or by absorbance at 280 nm using an extinction coefficient of 5500 M^{-1} cm^{-1} (calculated using Expasy ProtParam).

References

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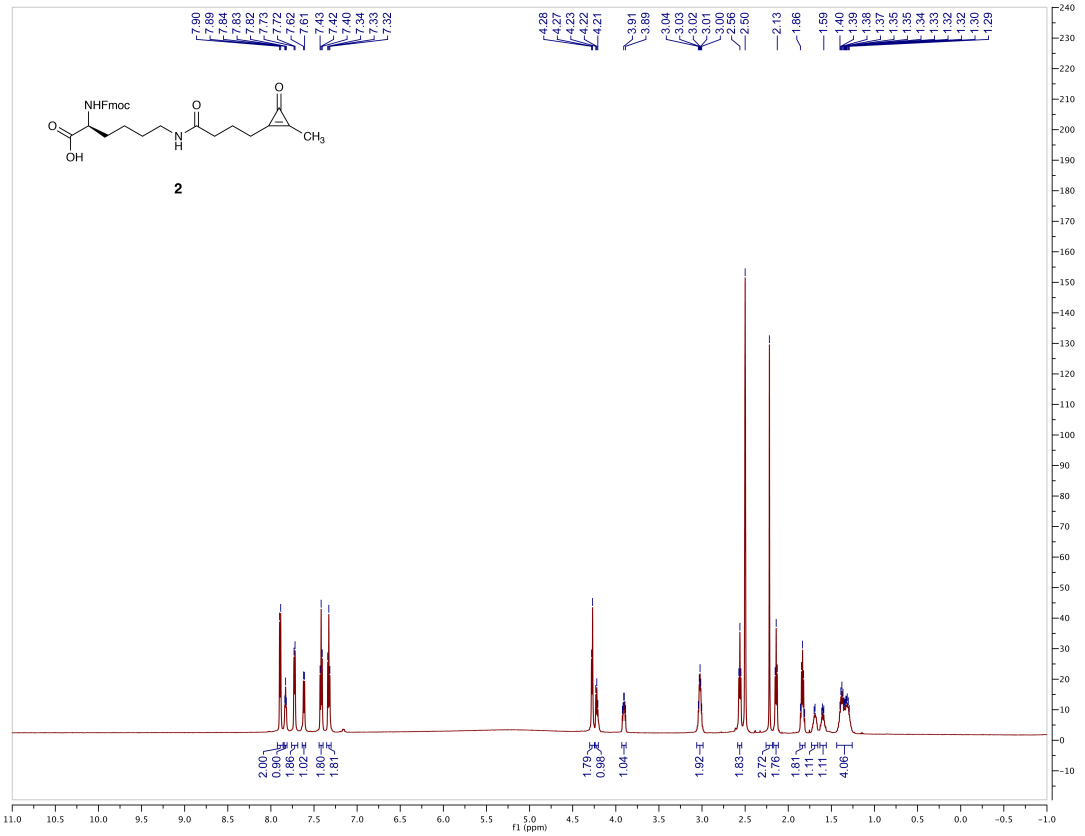
6. Mika, L. T.; Orha, L.; Farkas, N.; Horváth, I. T. *Organometallics* **2009**, *28*, 1593–1596.

NMR Spectra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7.33
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3.02
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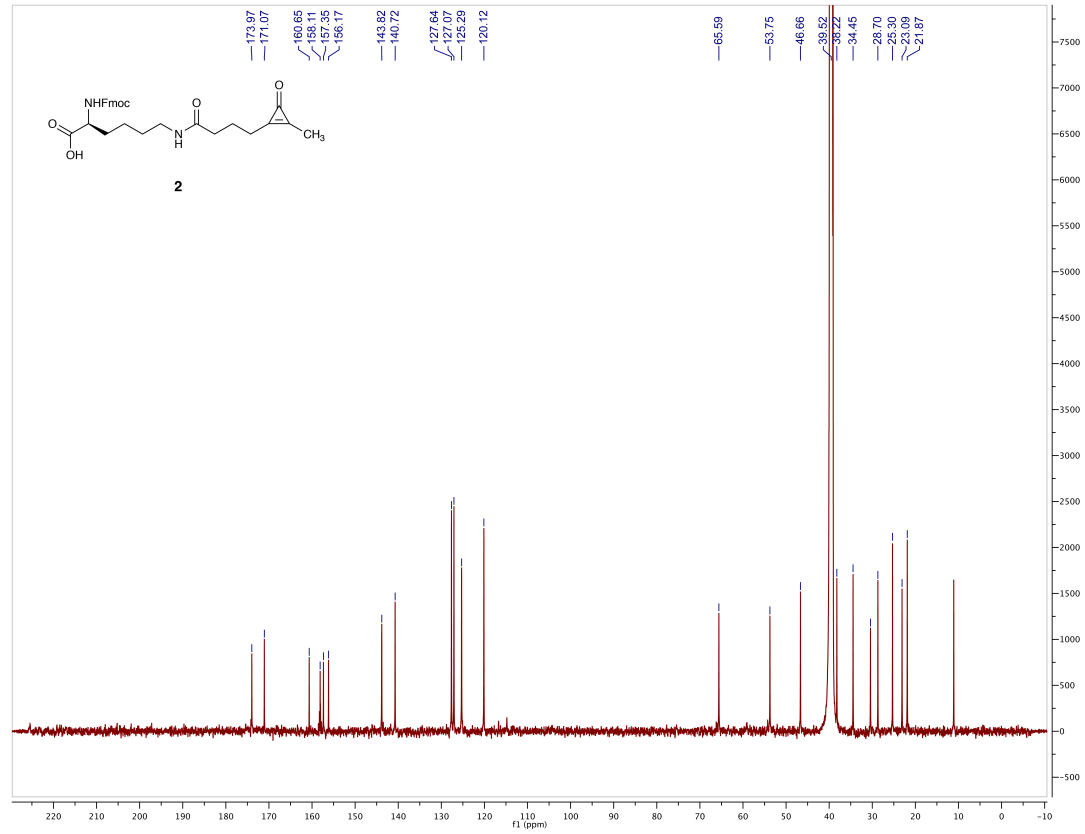
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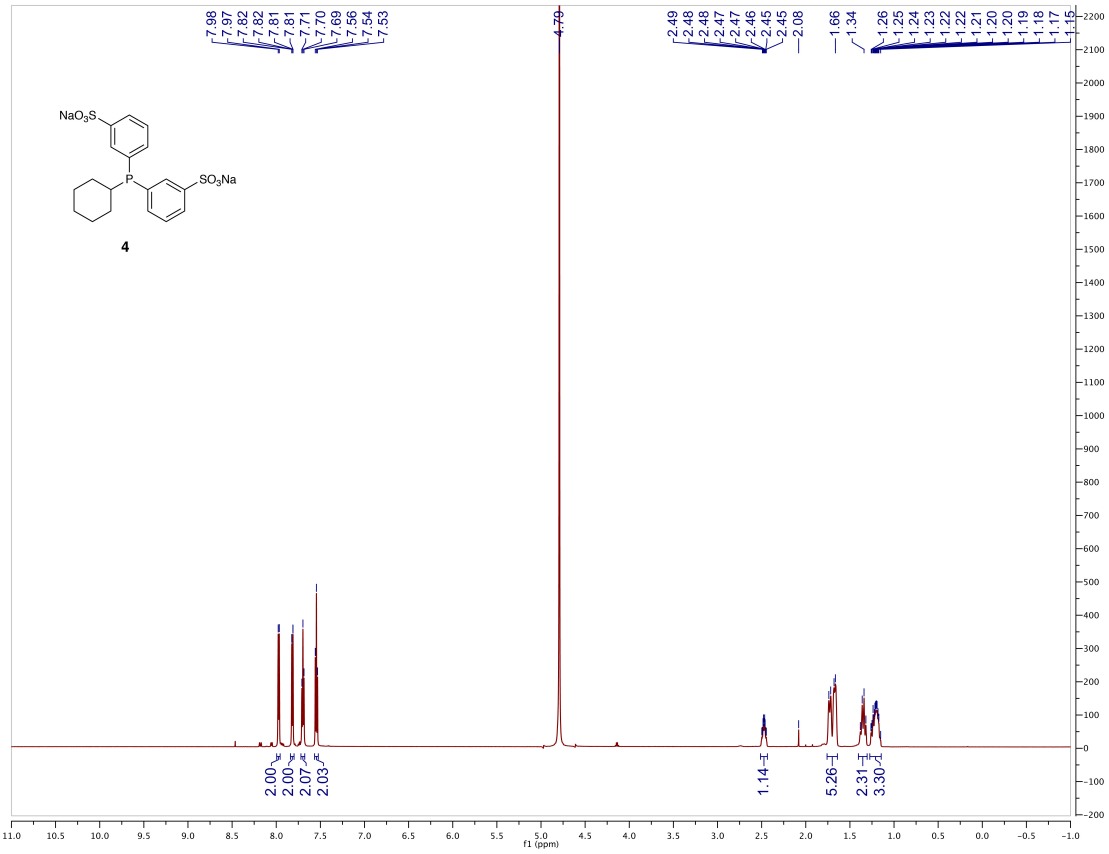
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1.20
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1.17
1.15
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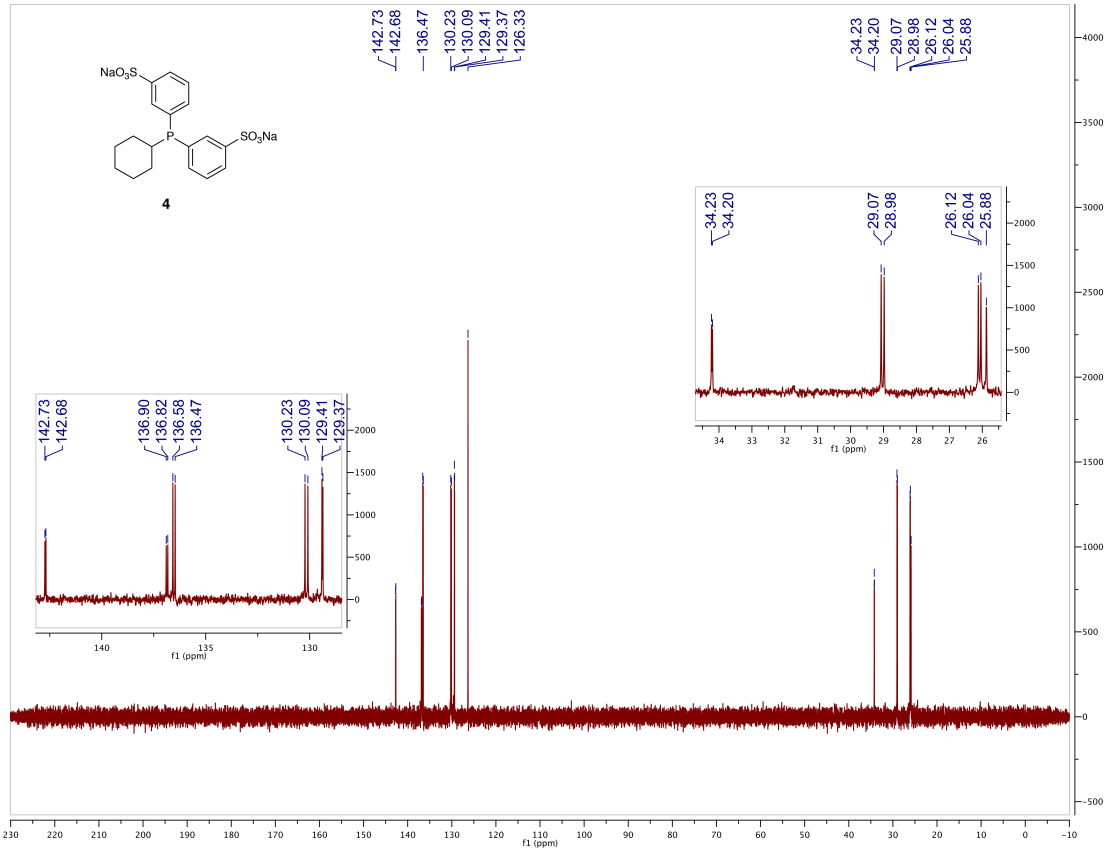
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SO3Na
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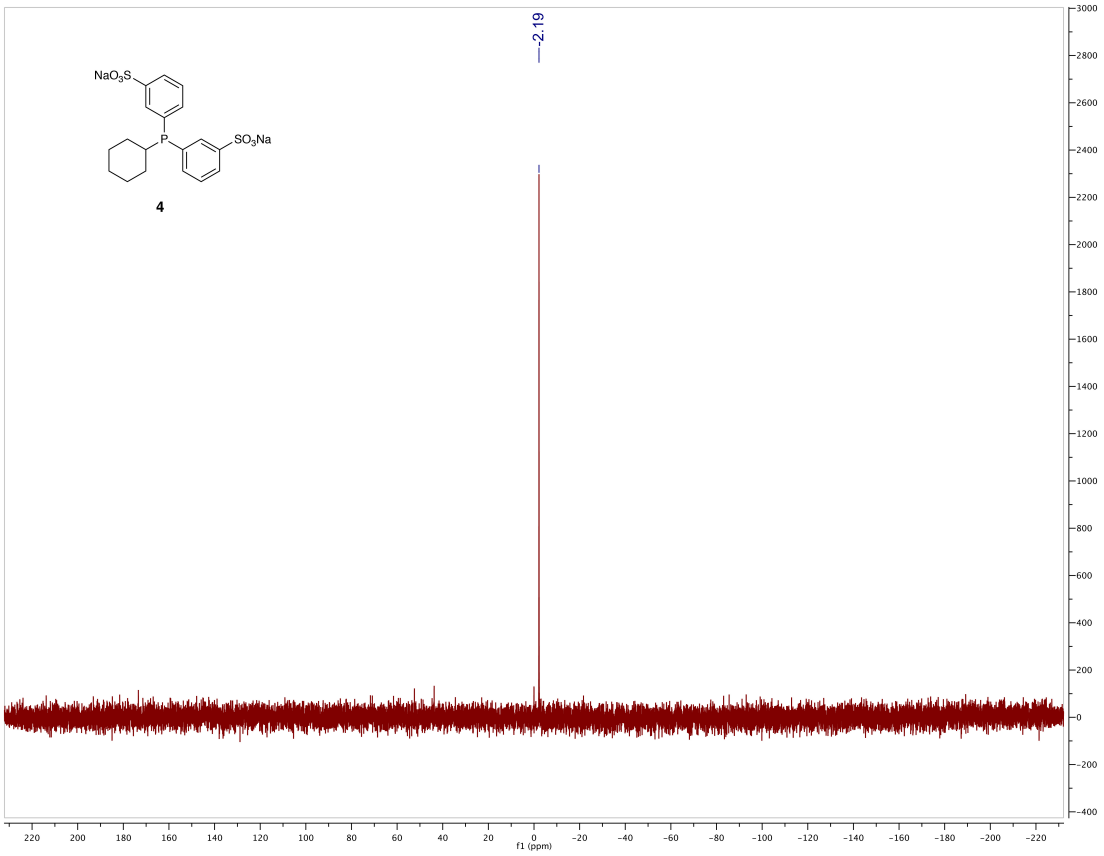
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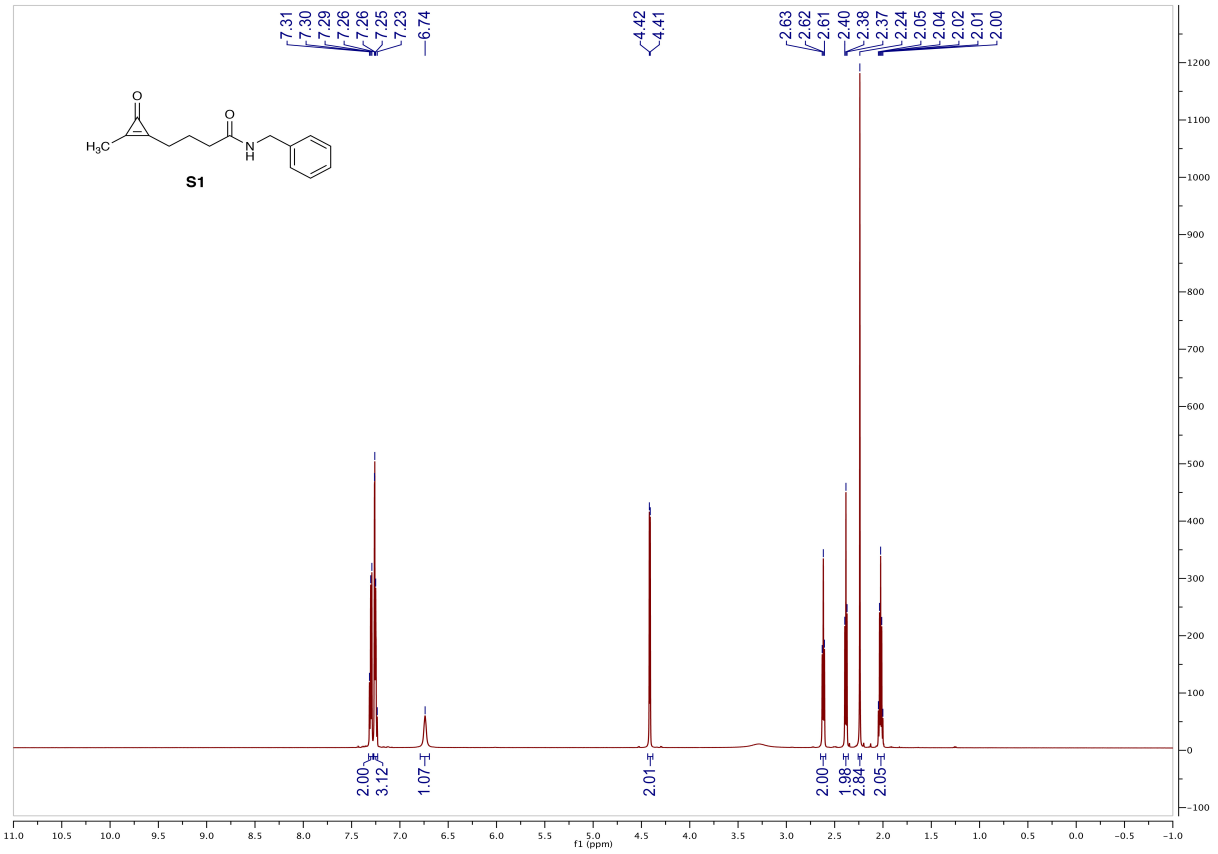
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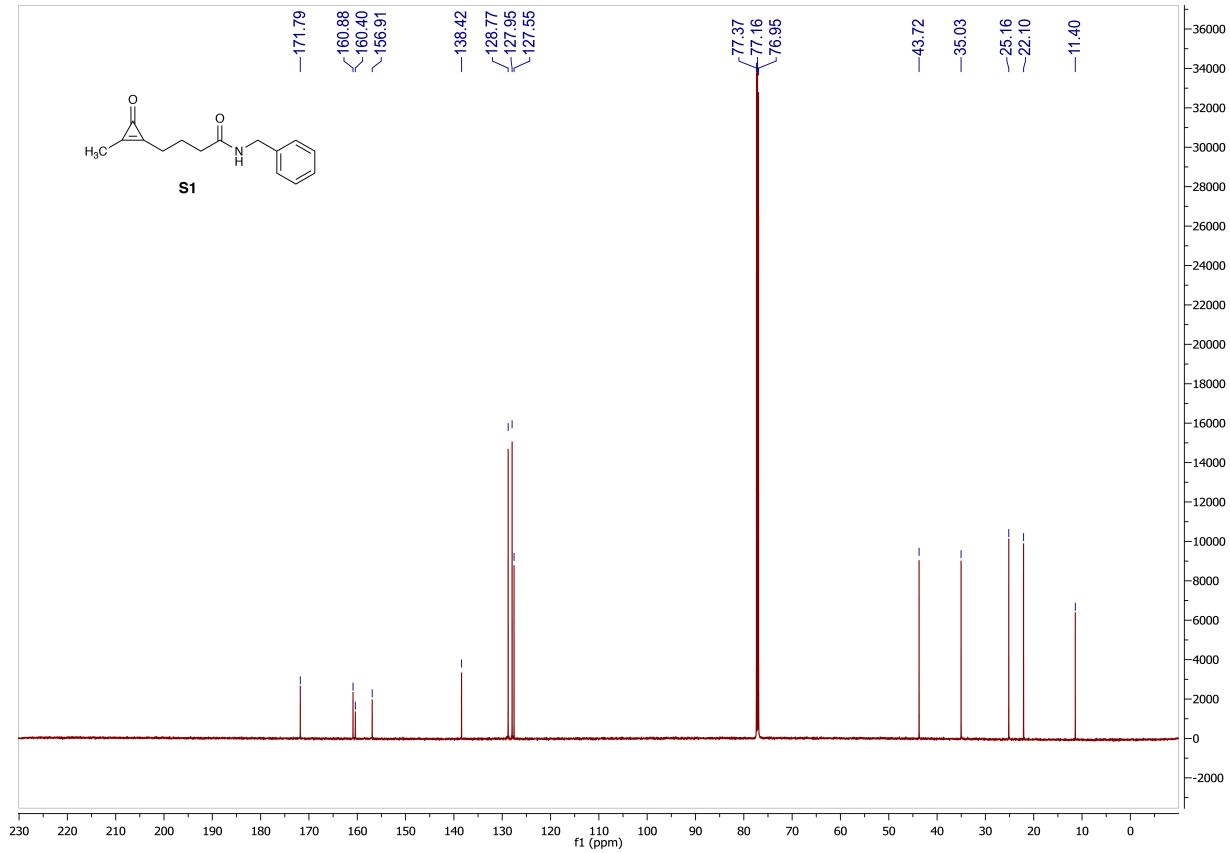
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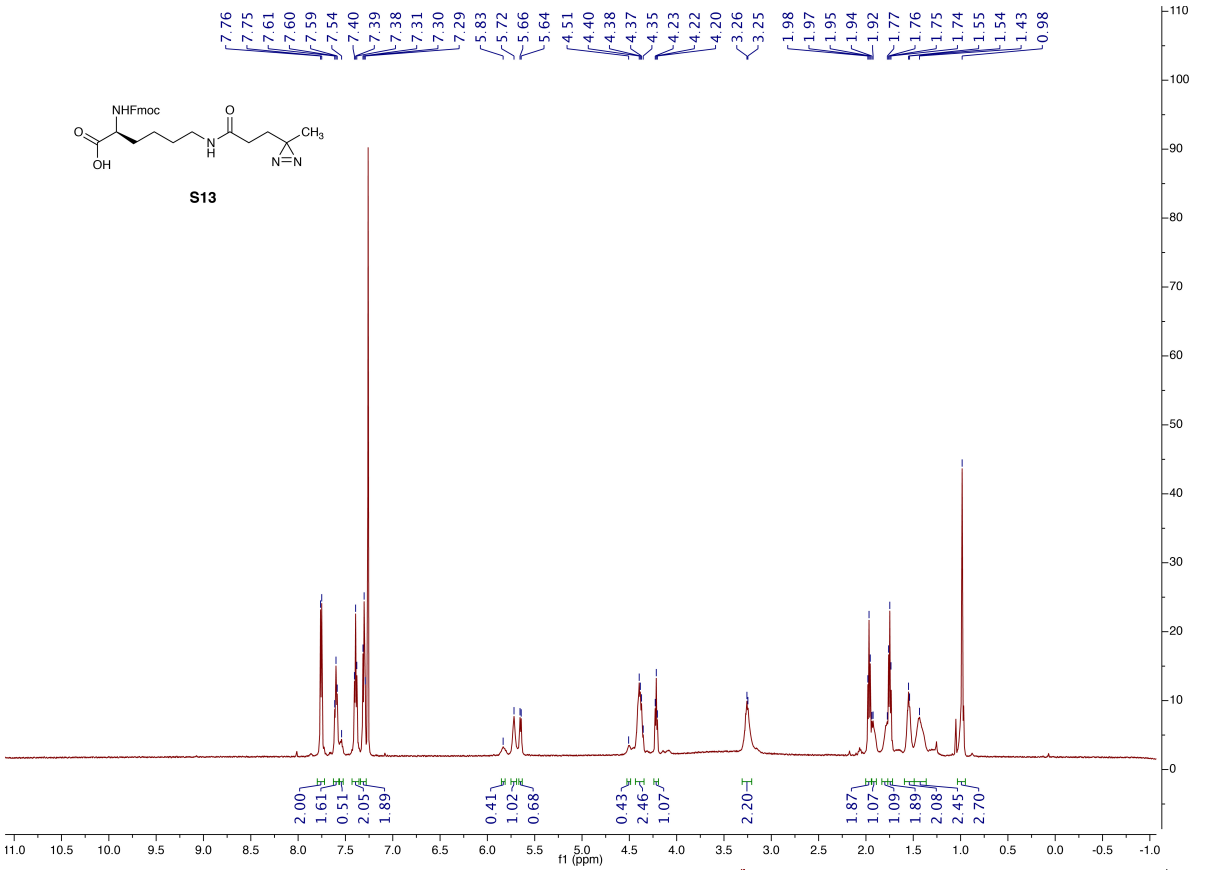
S1
f1 (ppm)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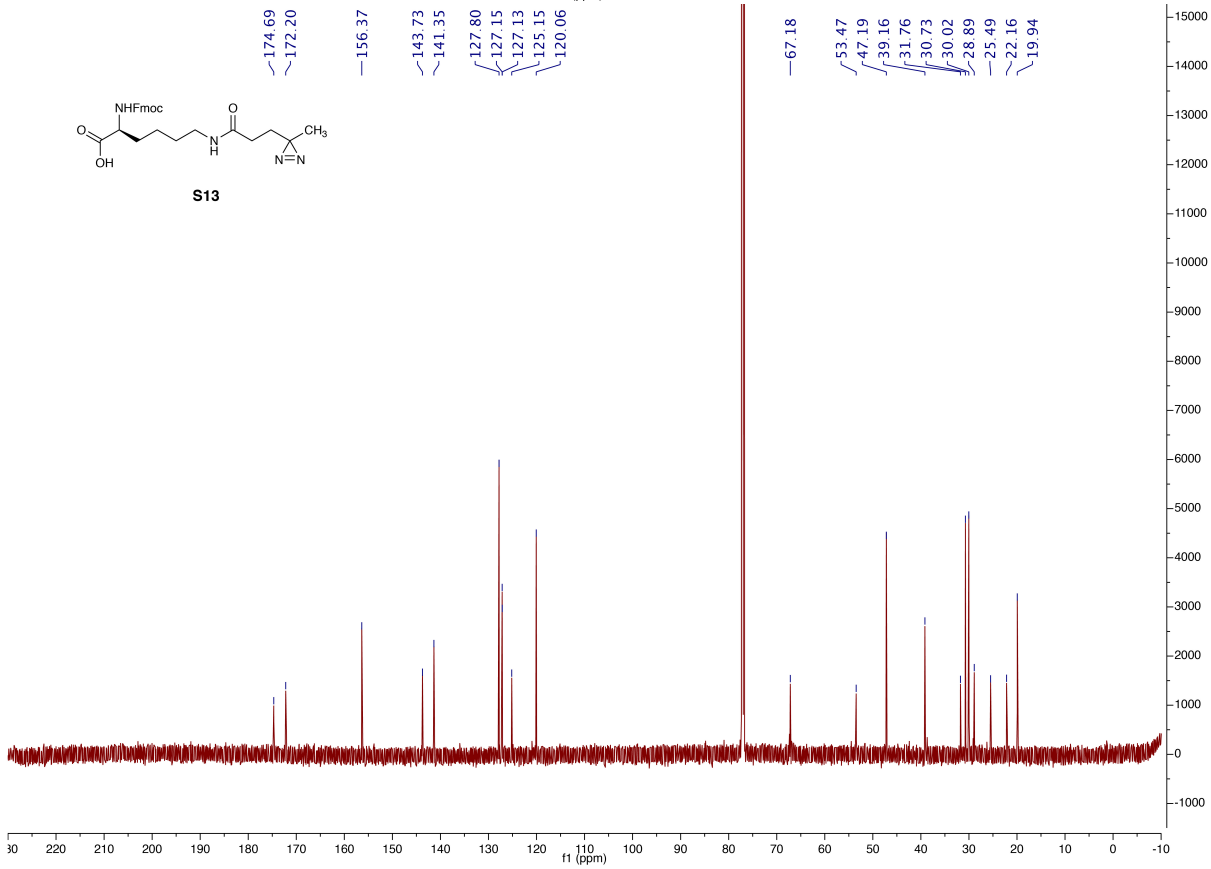
0.51
2.05
1.89
0.41
1.02
0.68
0.43
2.46
1.07
2.20
1.87
1.07
1.09
1.89
2.08
2.45
2.70
f1 (ppm)


174.69
172.20
156.37
143.73
141.35
127.80
127.15
127.13
125.15
120.06
67.18
53.47
47.19
39.16
31.76
30.73
30.02
28.89
25.49
22.16
19.94
NHFmoc
O
OH
N
H
CH3
N=N
S13
f1 (ppm)