Supplementary Information

Fluorosulfate as a Latent Sulfate in Peptides and Proteins

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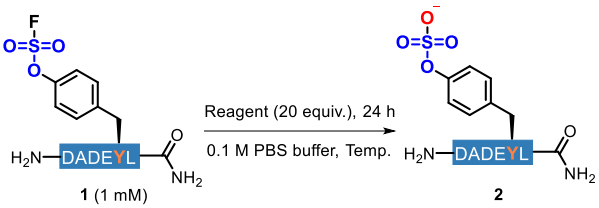
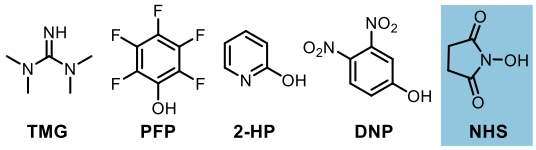
Supplementary Results

Table S1. Initial exploration of the release conditions of fsY



Entry	Reagent	Temp.	Yield of 2[a]
1	-	37 °C	4%
2[b]	-	37 °C	2%
3	TMG	25 °C	2%
4	TMG/PFP	25 °C	1%
5	TMG/2-HP	25 °C	1%
6	TMG/DNP	25 °C	1%
7	NHS	25 °C	61%
8	TMG/NHS	25 °C	56%
9	NHS	37 °C	quant.
10[b]	NHS	37 °C	84%

[a]The yields was calculated by the integral ratio of all emerged peptide peaks.
[b]0.1 M Tris buffer was used as the solvent. quant.: quantitative yield.



The fsY-containing hexapeptide **1** was found to be stable in pH 7.0 phosphate buffer (PBS) or pH 8.0 Tris buffer with negligible hydrolysis of fluorosulfate after 24 hours (Table S1, entry 1-2). Next, we examined reagents that could potentially activate **1** in aqueous solution at pH 7.0. Previously, we found that sulfate diesters with electron-deficient aryl groups were more readily hydrolyzed under basic conditions.[1] Combining this finding with Kim's report that tetramethylguanidine (TMG) promotes the SuFEx coupling between fluorosulfate and nucleophiles in aqueous solution,[2] we wondered if a SuFEx reaction between fsY and electron-deficient phenols could occur in aqueous media to give a transient sulfate diester en route to spontaneous hydrolysis. Unfortunately, there was no sign of the target sulfopeptide **2** either when TMG was used as the sole reagent or when it is combined with co-reagents such as pentafluorophenol (PFP), 2-hydroxypyridine (2-HP), or 2,4-dinitrophenol (DNP) (entry 3-6). To our surprise, *N*-hydroxysuccinimide (NHS) alone was able to convert **1** into **2** in 61% yield at 25 °C in 24 hours, and no other byproducts were detected on HPLC (entry 7). Adding TMG to the reaction did not further improve the yield of **2** (entry 8). The reaction was accelerated when the temperature was elevated to 37 °C, producing **2** quantitatively in 24 hours (entry 9).

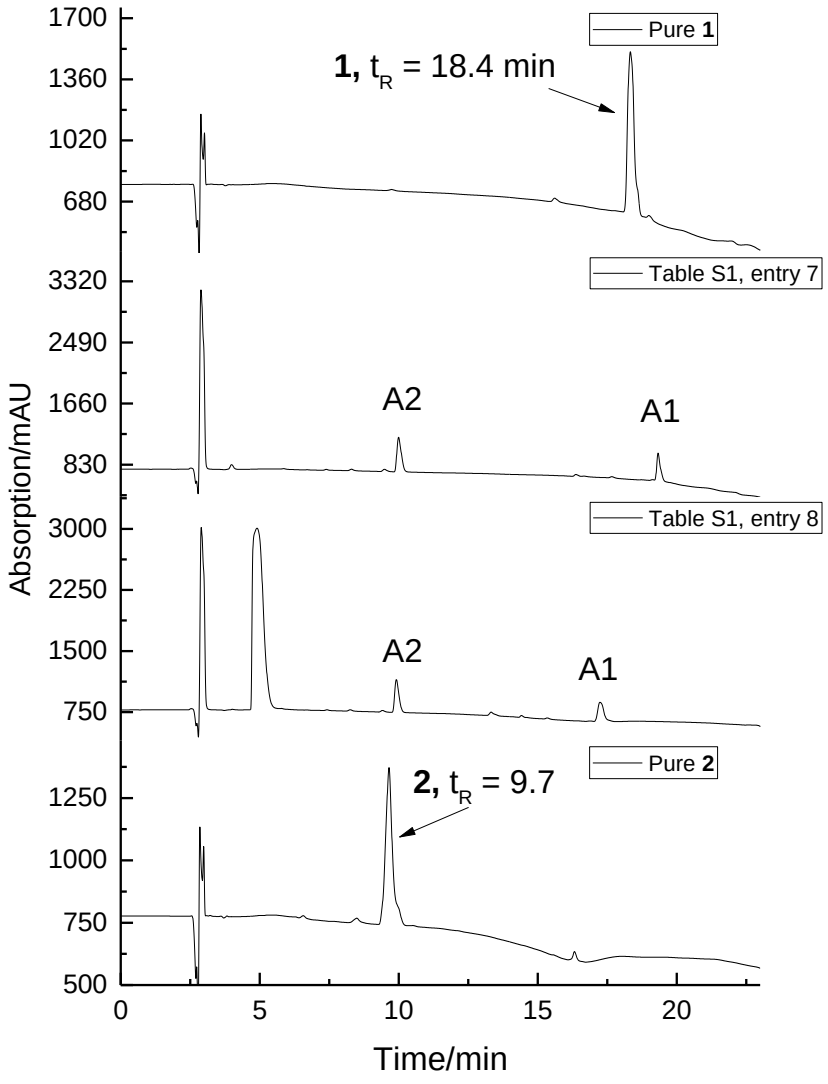
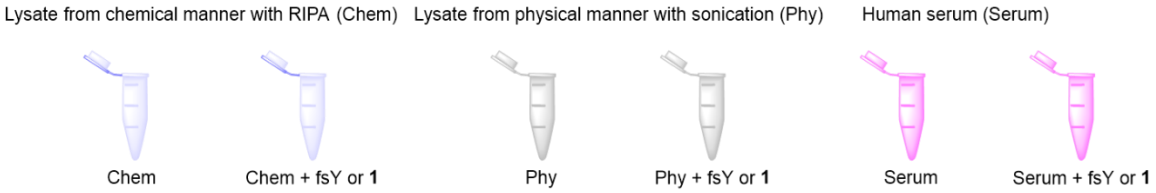


Figure S1. HPLC analysis of the fluorosulfate decaging reaction of **1** shown in Table S1. From top to bottom: analytical HPLC traces of starting material **1**; reaction mixture of Entry 7 (NHS, rt, 24 h); reaction mixture of Entry 8 (TMG+NHS, rt, 24 h); pure product **2**. Yields were calculated using the ratios of the peak integrals based on the following equation: Yield of the reaction = A2/(A1 + A2) * 100%. The HPLC method was set as follows: CH_3CN: 20 mM ammonium acetate buffer mobile phases, linear gradient from 5%~33% CH_3CN from 0-20 min, 20-21 min 33%~95% CH_3CN, and 95% CH_3CN washing for another 2 min. All HPLC traces was monitored by the UV absorbance at 210 nm.

Table S2. Stability test of fluorosulfotyrosine (fsY) and fluorosulfohexpeptide **1** in cell lysate. **a**) Reaction setup. **b**) Recovery yields in different media.

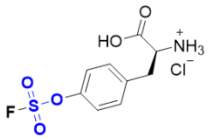
a)



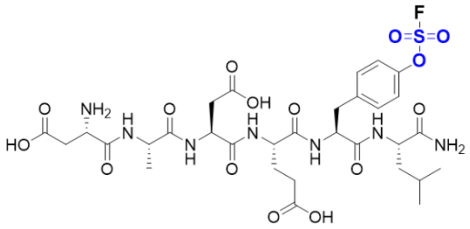
b)

Entry	Substrate	Lysate	Time	fsY	1
1	**fsY**	Phy	48 h	79%	-
2	**fsY**	Chem	48 h	88%	-
3	**fsY**	Serum	12 h	60%	-
4[a]	**1**	Phy	48 h	87%	N.T.
5[a]	**1**	Chem	48 h	82%	N.T.
6[a]	**1**	Serum	12 h	33%	36%

[a]Recovery rate of fsY was reported based on fsY standard (Figure S1).
N.T.: No trace of the peptide **1**.



fsY



1 (TFA complex form)

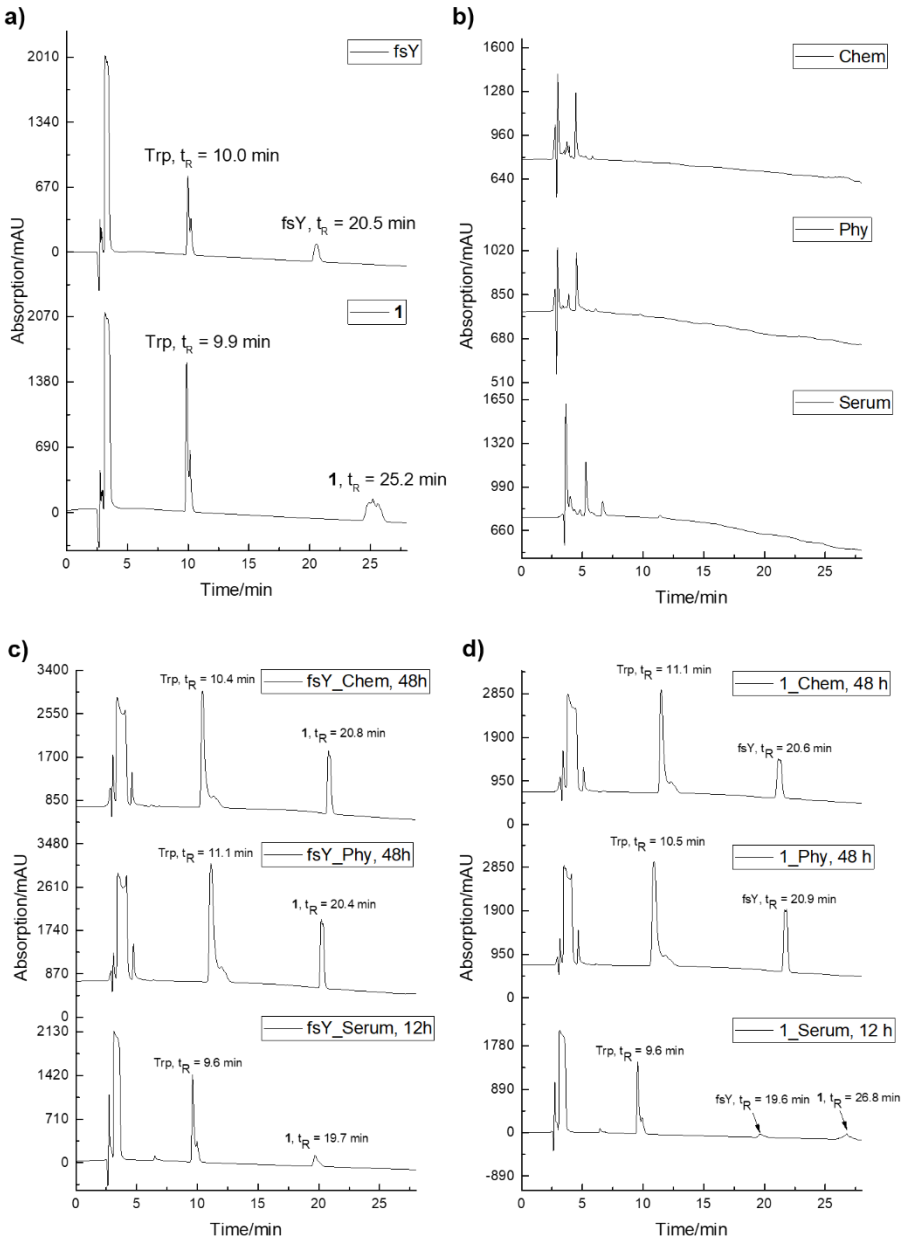
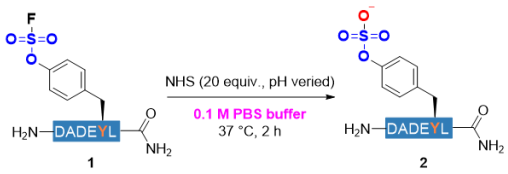


Figure S2. Analytical RP-HPLC verified the stability of fsY and **1** in cell lysate. a) Analytical RP-HPLC traces of fsY or hexpipetide **1**. A known amount of tryptophan (Trp) was co-injected as an internal standard. b) Analytical RP-HPLC trace of cell lysate at 37 °C for 48 h after centrifuge filtration. No signal overlap was observed between the lysate and either Trp or the substrates. c) Analytical RP-HPLC trace of fsY in cell lysate at 37 °C after centrifuge filtration: in Chem lysate for 48 h (top); in Phy lysate for 48 h (middle); in human serum for 12 h (bottom). A known amount of Trp was co-injected as an internal standard. d) Analytical RP-HPLC trace of hexpeptide **1** in cell lysate at 37 °C after centrifuge filtration: in Chem lysate for 48 h (top); in Phy lysate for 48 h (middle); in human serum for 12 h (bottom). A known amount of Trp was co-injected as an internal standard. The stability of the substrate is calculated based on the ratios of the peak corresponding to the substrate or the product to the Trp peak. The ratio of the substrate to Trp is r_1, and the ratio of the product to Trp is r_2. Then the stability (recovery yield) is calculated as $\eta = r_2/r_1 * 100\%$. HPLC method was set as: using CH_3CN: 20 mM ammonium acetate buffer as mobile phase, 0-5 min, 7%~10% CH_3CN; 5-26 min, 10%~27% CH_3CN;26-27 min, 27%~95% CH_3CN; 27-28 min, 95% CH_3CN. All analytical RP-HPLC was monitored under 210 nm wavelength with 10.0 μL injection volume.

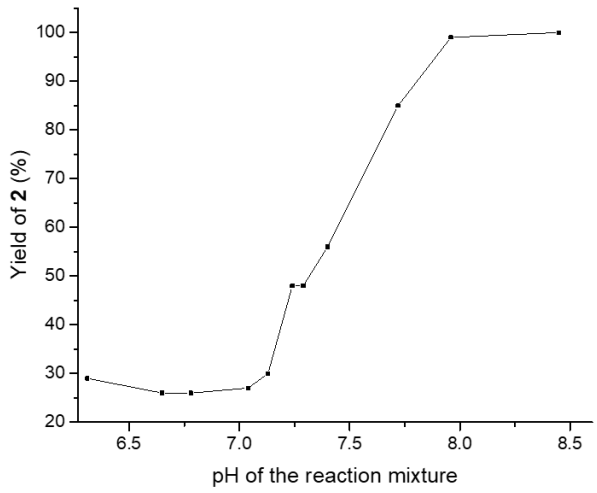
Interestingly, even though **1** was rapidly broken down into amino acids by the proteases in cell lysate and serum, the majority of the resulting fsY can still be detected after 12 hours in serum and after 48 hours in cell lysate (Table S2 and Figure S1), further supporting that fluorosulfate is inert in the complex biological context. It is noteworthy that this result is consistent with a proteome-wide study by Kelly et al. that found covalent modification of proteins by arylfluorosulfate is rare under physiological conditions.[3]

Table S3. pH dependence of the fsY decaging reaction mediated by *N*-hydroxysuccimide (NHS)



pH		Yield of 2
NHS Stock	Reaction	
5.66	6.31	29%
6.50	6.65	26%
9.44	6.78	26%
10.87	6.96	30%
11.05	7.04	27%
11.23	7.13	30%
11.75	7.24	48%
11.85	7.29	48%
12.02	7.40	56%
12.13	7.96	99%
12.42	8.45	quant.

quant.: quantitative yield.



The pH dependence of fsY decaging mediated by NHS provided further insights into the mechanism. The yield of **2** remained low at pH values lower than 7.0 but sharply increased from pH 7.0 to 8.0. This pH dependence correlates well with the pH-induced hydrolysis of NHS (Figure S3).

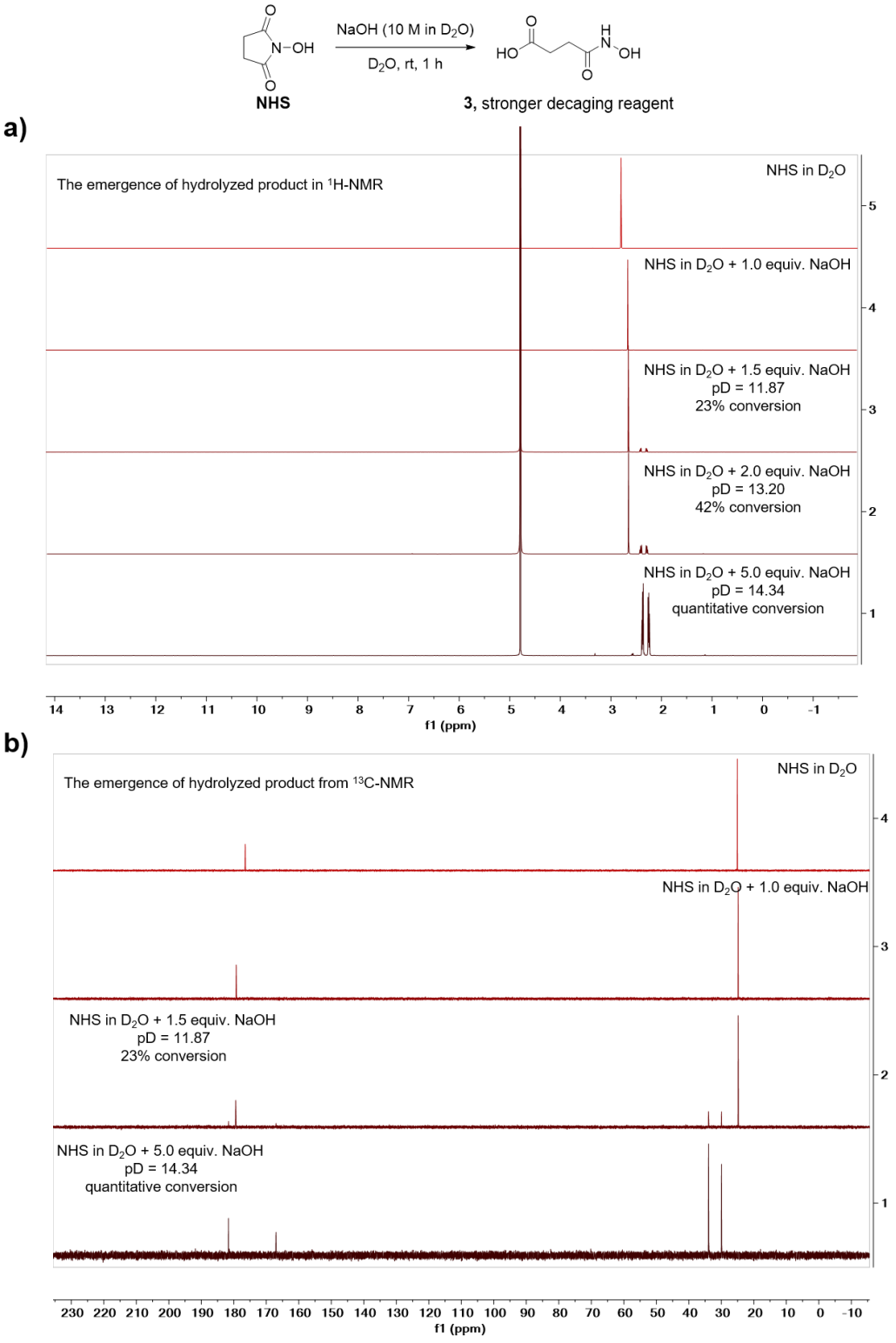


Figure S3. Hydrolysis of NHS in deuterated water by titrating NaOH. **a**) ^{1}H-NMR of the reaction in D_2O. **b**) ^{13}C-NMR of the reaction in D_2O.

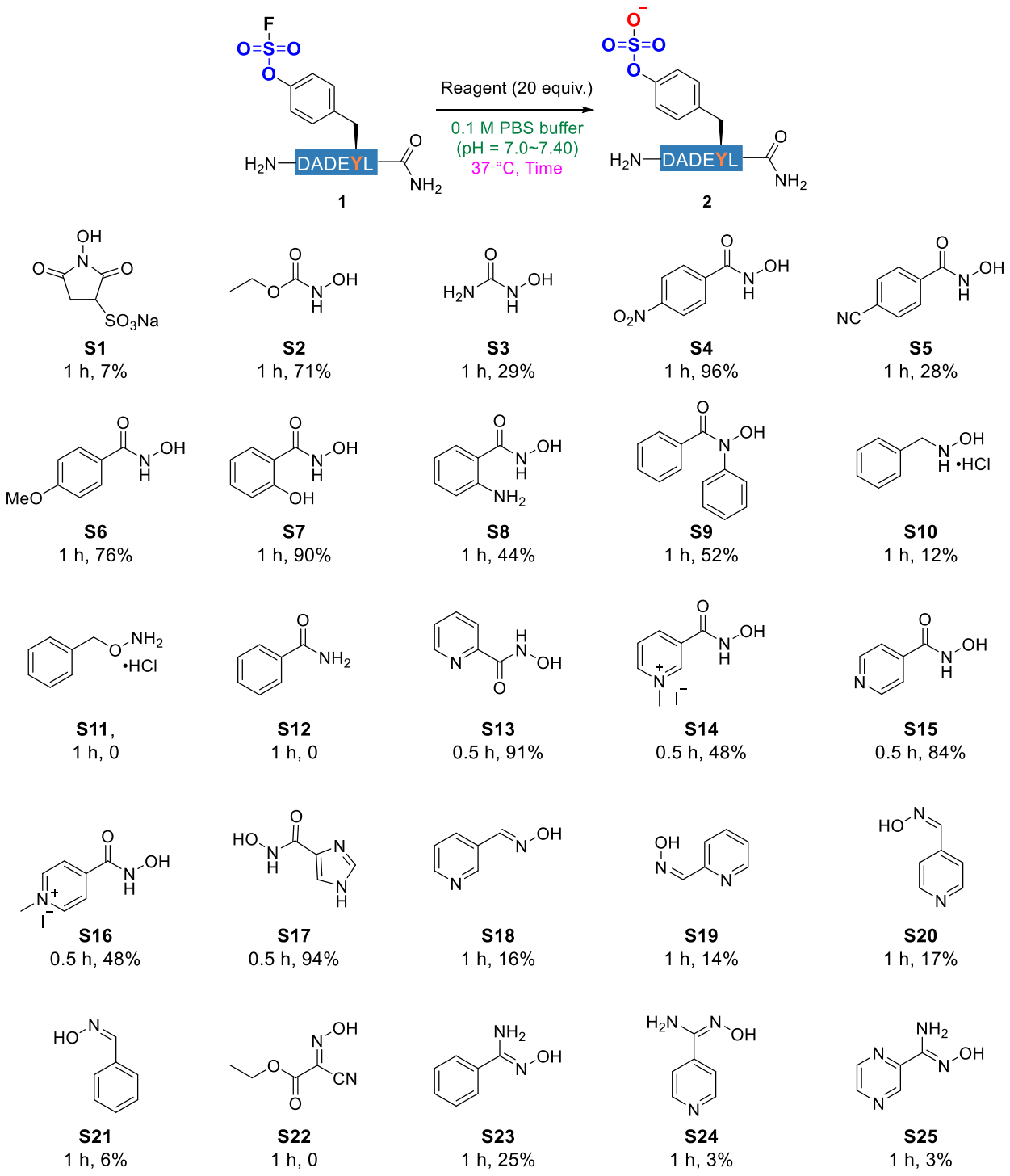
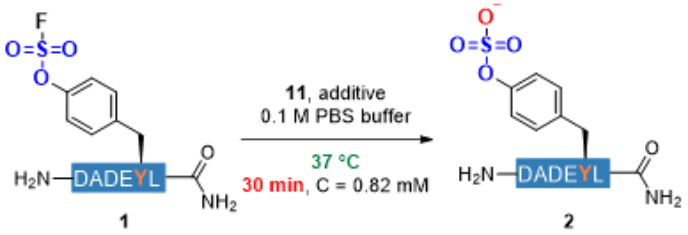
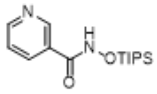


Figure S4. Extended screening of reagents for fsY decaging. Similar to **7**, heteroaromatic hydroxamic acids consisting of pyridine and imidazole structures, e.g., **S13**, **S15**, and **S17**, were also found to result in efficient decaging, producing **2** in 91%, 84%, and 94% yields in 30 minutes, respectively.

Table S4. Decaging of fsY in **1** mediated by silyl-protected reagent **11**.



Entry	Additive	Yield of 2
1	-	0
2	**KF**	41%



11

Potassium fluoride

KF

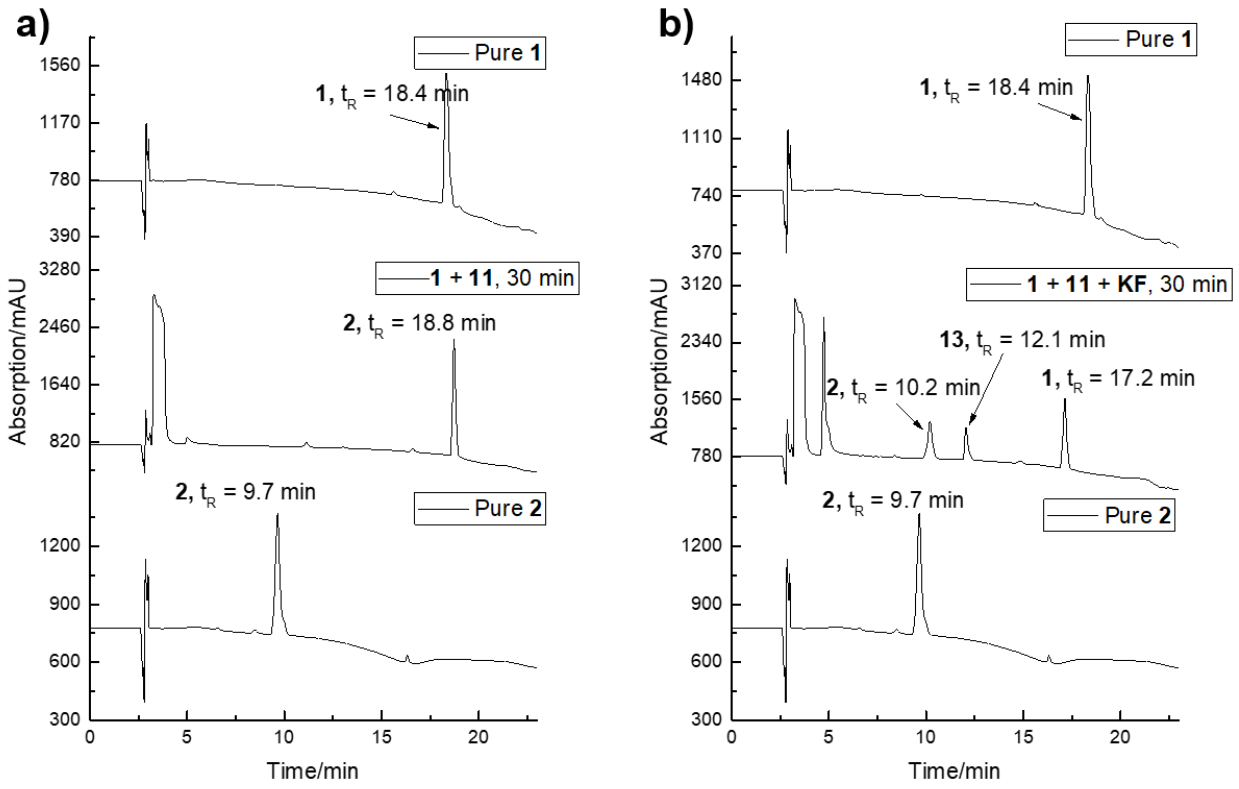
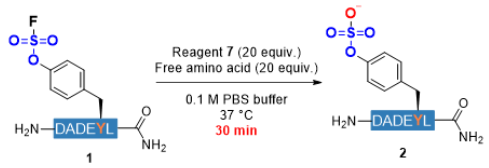
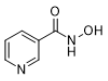


Figure S5. Analytical HPLC analysis of fsY decaging mediated by silyl-protected reagent. **a**) Substrate **1** remained intact when only silyl caged **11** was added. From top to bottom: starting material **1**; **1** treated with **11**; product **2**. **b**) The decaging of fsY occurred in the presence of both caged reagent **11** and potassium fluoride (**KF**). From top to bottom: starting material **1**; **1** treated with **11** and **KF**; and product **2**. HPLC method was set as: CH_3CN: 20 mM ammonium acetate buffer mobile phases, linear gradient from 5%~33% CH_3CN from 0-20 min, 20-21 min 33%~95% CH_3CN, and 95% CH_3CN washing for another 2 min. All HPLC traces was monitored by the UV absorbance at 210 nm.

Table S5. Compatibility test of the decaging reaction of **1** in the presence of free amino acids.



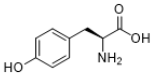
Reagent	pH[a]	Concentration	Yield of 2
7	7.24	0.83 mM	95%
7 + Ala	7.16	0.71 mM	80%
7 + Pro	7.18	0.71 mM	73%
7 + Tyr[a]	7.50	0.71 mM	78%
7 + Arg	7.20	0.71 mM	71%
7 + His	7.15	0.71 mM	71%
7 + His	7.13	0.71 mM	77%
7 + Cys	7.21	0.71 mM	84%
7 + Lys	7.17	0.71 mM	74%
7 + Thr	7.18	0.71 mM	84%
7 + Ser	7.23	0.71 mM	82%



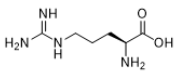
7

L-Alanine
Ala

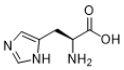
L-Proline
Pro



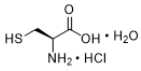
L-Tyrosine
Tyr



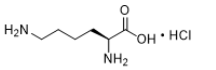
L-Arginine
Arg



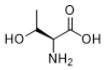
L-Histidine
His



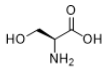
L-Cysteine hydrochloride monohydrate
Cys



L-Lysine monohydrochloride
Lys



L-Threonine
Thr



L-Serine
Ser

[a]Reaction mixture pH was examined after the reaction; [b]Tyrosine in DMSO as white suspension.

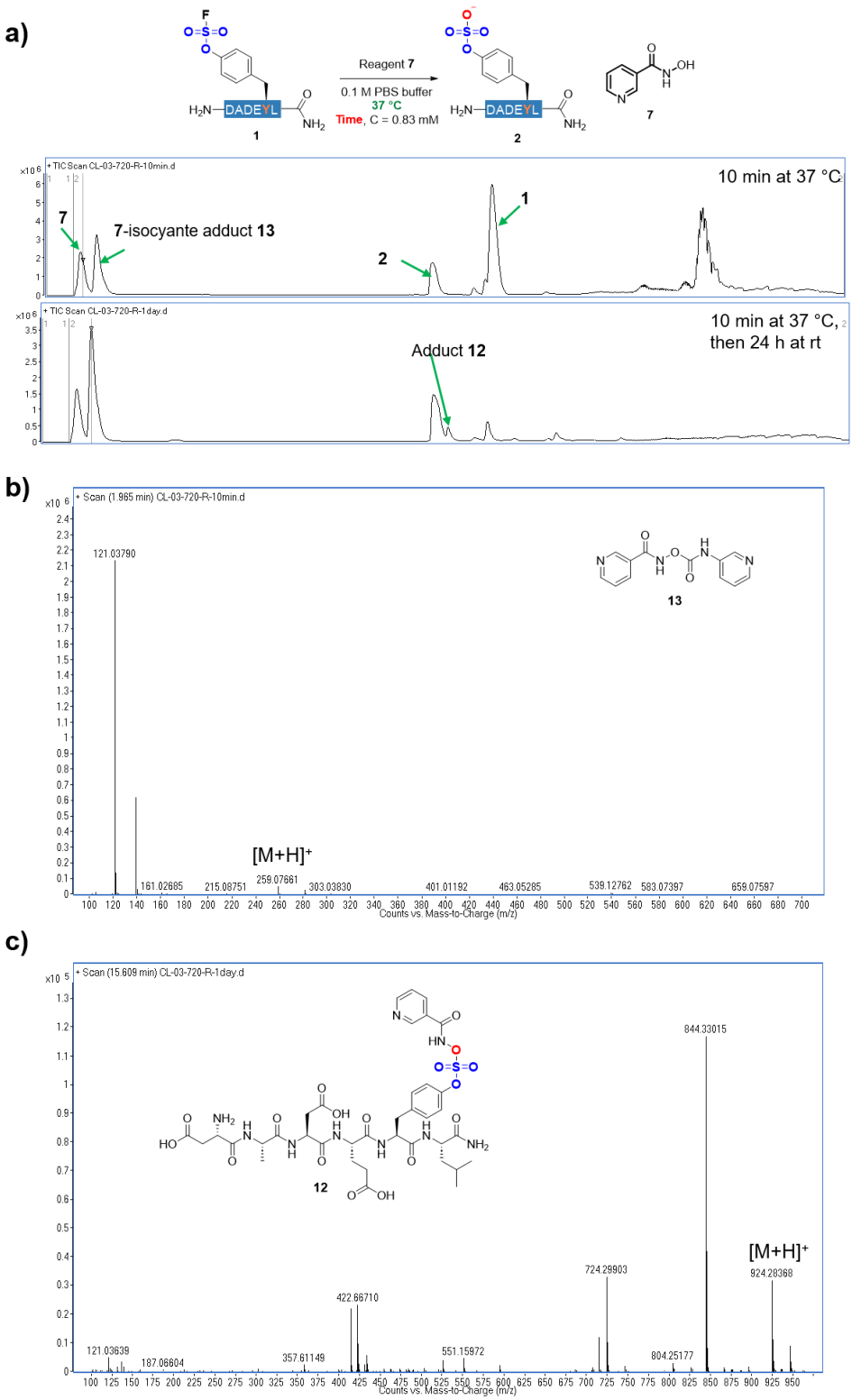


Figure S6. Real-time LC-MS analysis to probe the reaction mechanism. **a**) Total ion chromatogram (TIC) of the reaction after 10 min at 37 °C and then incubated at room temperature for 24 h. **b**) Mass spectrum of **7**-isocyante adduct **13**. c) Mass spectrometry captured **7**-**1** adduct **12**.

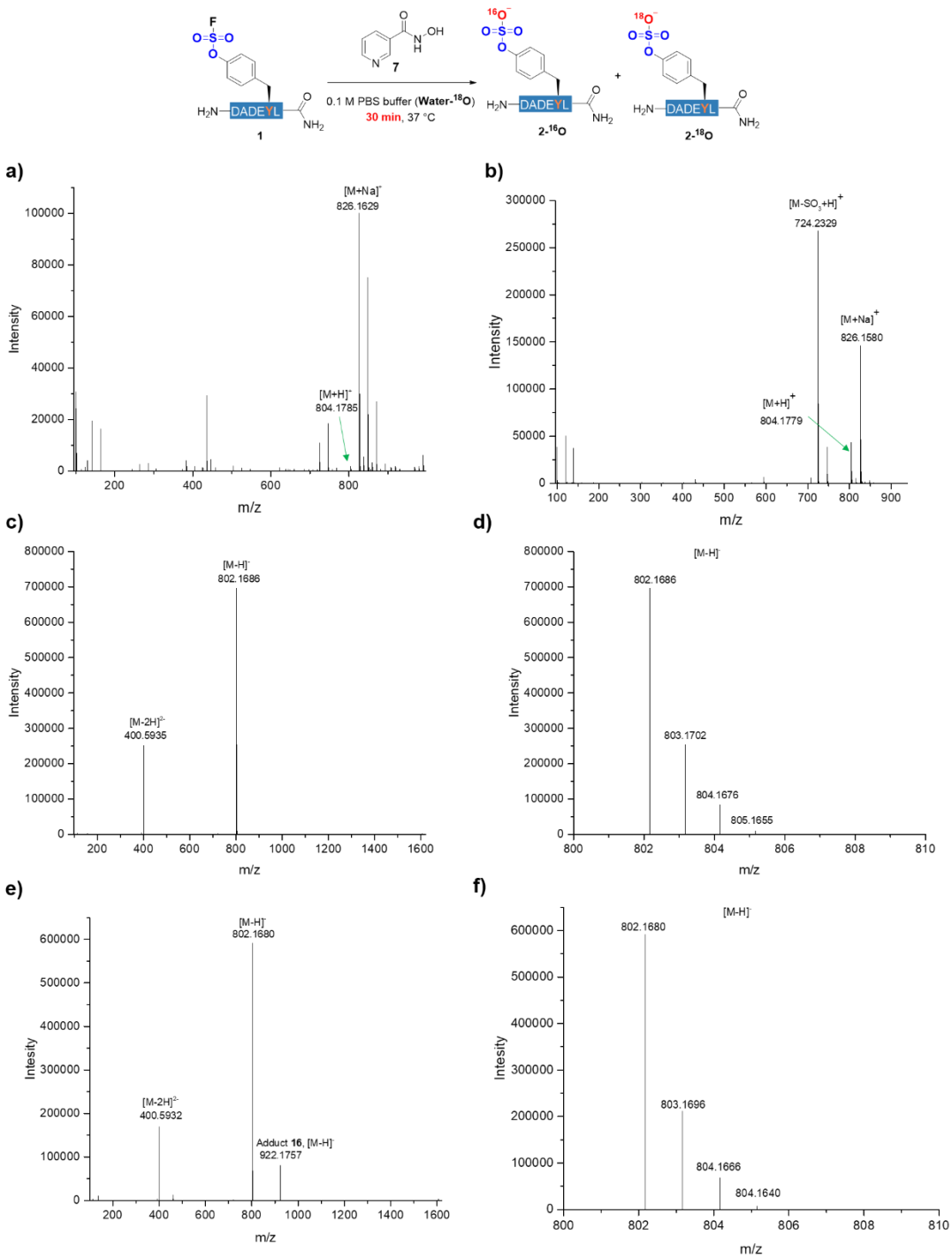


Figure S7. Mass spectra of the $H_2{}^{18}O$ isotope labeling experiment. **a**) Product **2** in $H_2{}^{18}O$ buffer, positive mode. **b**) Decaging reaction mixture containing starting material **1** in $H_2{}^{18}O$ buffer, positive mode. **c**) Product **2** in $H_2{}^{18}O$ buffer, negative mode. **d**) Zoomed-in mass spectrum of product **2** in $H_2{}^{18}O$ buffer, negative mode. **e**) **1** in $H_2{}^{18}O$ buffer, negative mode. **f**) Zoomed-in mass spectrum of **1** in $H_2{}^{18}O$ buffer, negative mode.

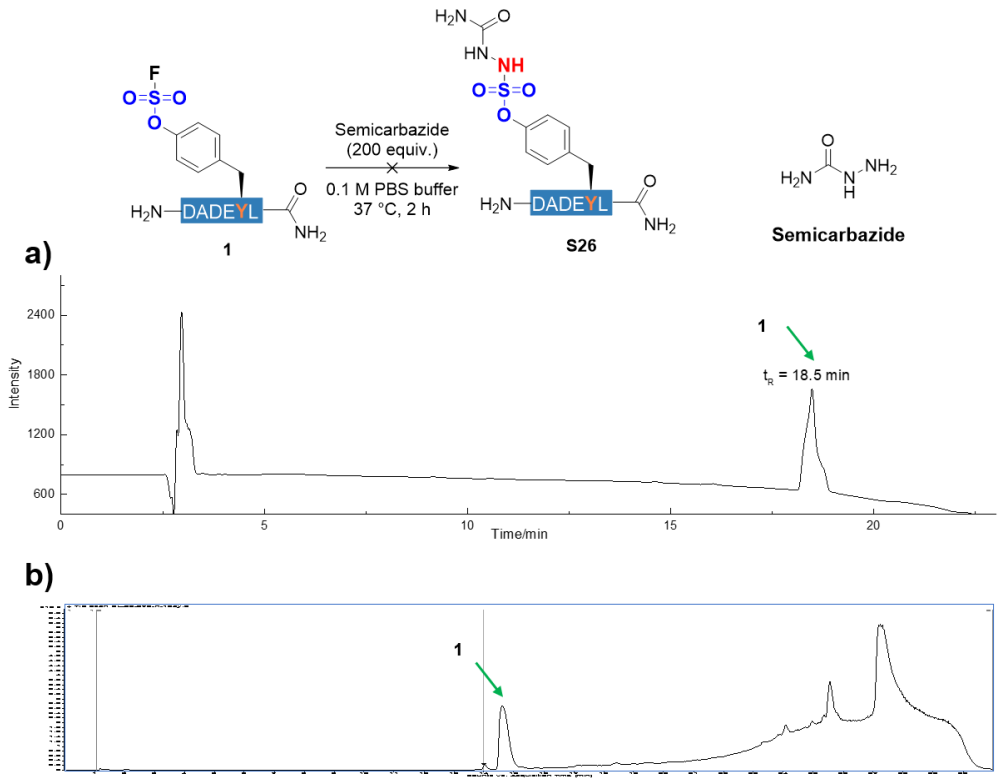
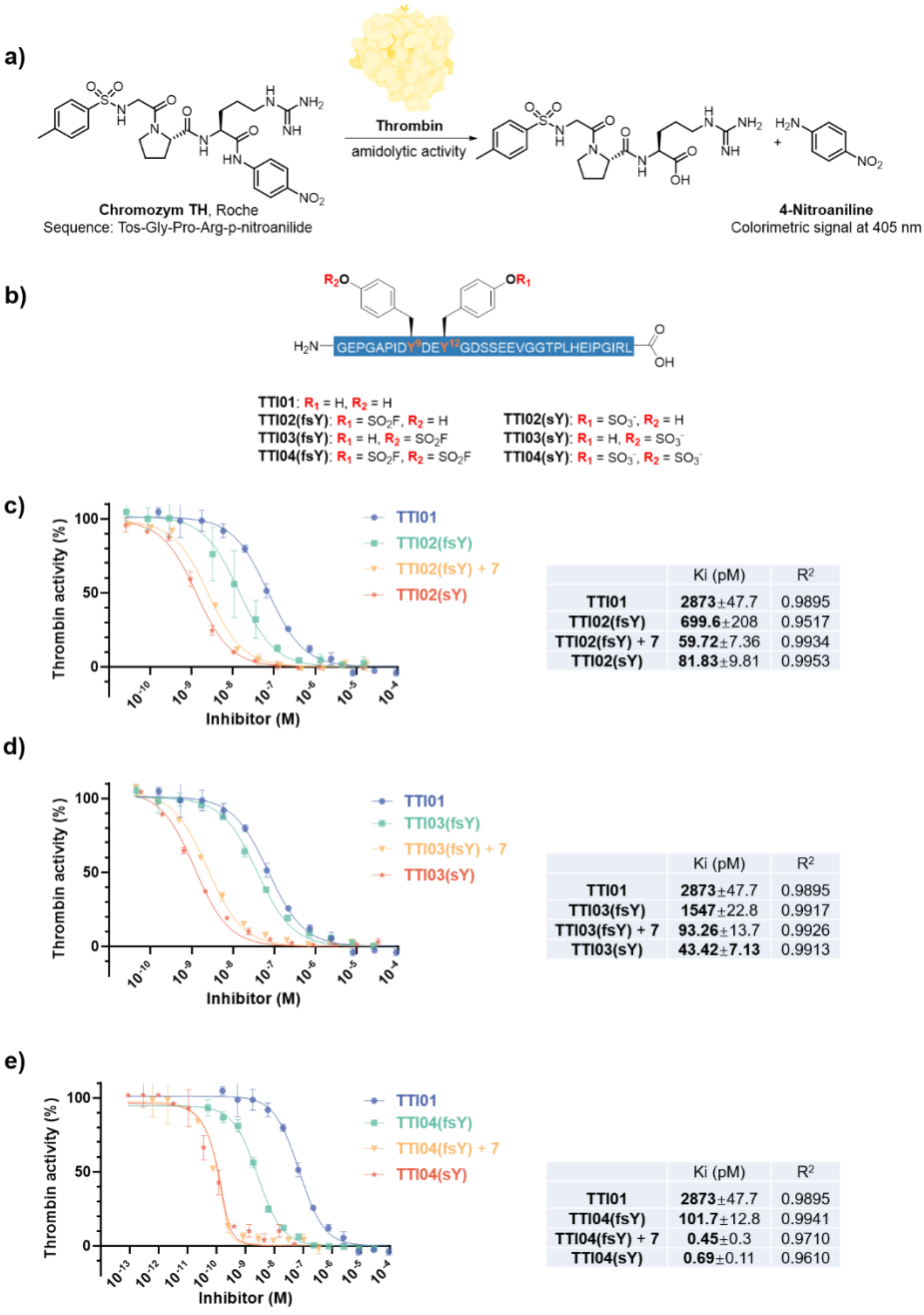


Figure S8. Compatibility of semicarbazide with fsY-containing substrates **1**. **a**) Analytical HPLC trace after incubating semicarbazide with **1** for 2 h. HPLC conditions: CH_3CN: 20 mM ammonium acetate buffer mobile phases, linear gradient from 5%~33% CH_3CN from 0-20 min, 20-21 min 33%~95% CH_3CN, and 95% CH_3CN washing for another 2 min). All HPLC traces was monitored by the UV absorbance at 210 nm. **b**) Total ion chromatogram (TIC) of the reaction mixture after 24 hours.



	Ki (pM)	R^2
TTI01	**2873**±47.7	0.9895
TTI02(fsY)	**699.6**±208	0.9517
TTI02(fsY) + 7	**59.72**±7.36	0.9934
TTI02(sY)	**81.83**±9.81	0.9953

	Ki (pM)	R^2
TTI01	**2873**±47.7	0.9895
TTI03(fsY)	**1547**±22.8	0.9917
TTI03(fsY) + 7	**93.26**±13.7	0.9926
TTI03(sY)	**43.42**±**7.13**	0.9913

	Ki (pM)	R^2
TTI01	**2873**±47.7	0.9895
TTI04(fsY)	**101.7**±12.8	0.9941
TTI04(fsY) + 7	**0.45**±0.3	0.9710
TTI04(sY)	**0.69**±0.11	0.9610

Figure S9. Thrombin inhibition by in situ decaged TTI peptides. **a**) Representative scheme of thrombin amidolytic activity with Chromozym TH was the substrate. **b**) Sequences of TTI peptides. **c**) Inhibition of thrombin by **TTI02(fsY)**, **TTI02(sY)**, and in situ decaged **TTI02(fsY)**. **d**) Inhibition of thrombin by **TTI03(fsY)**, **TTI03(sY)**, and in situ decaged **TTI03(fsY)**. **e**) Inhibition of thrombin by **TTI04(fsY)**, **TTI04(sY)**, and in situ decaged **TTI04(fsY)**.

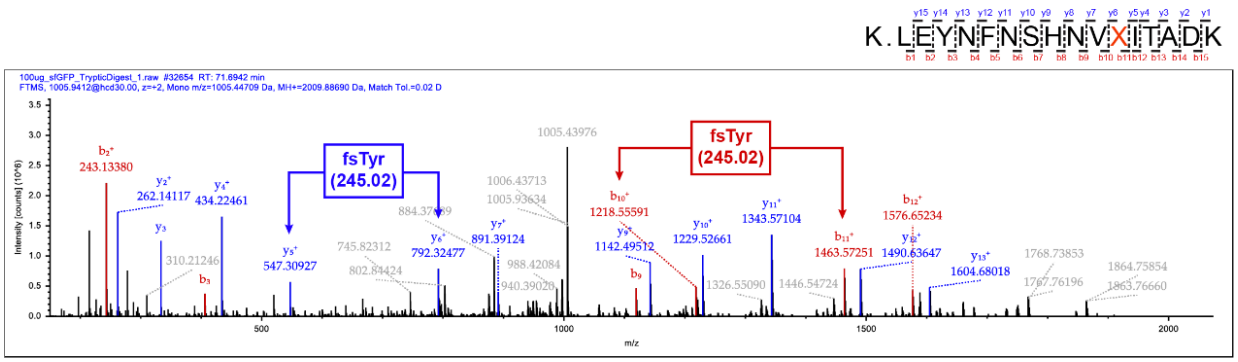


Figure S10. Tandem MS analysis of sfGFP-151-fsY after trypsin digestion and alkylation with iodoacetamide. X in the sequence logo represents fsY.

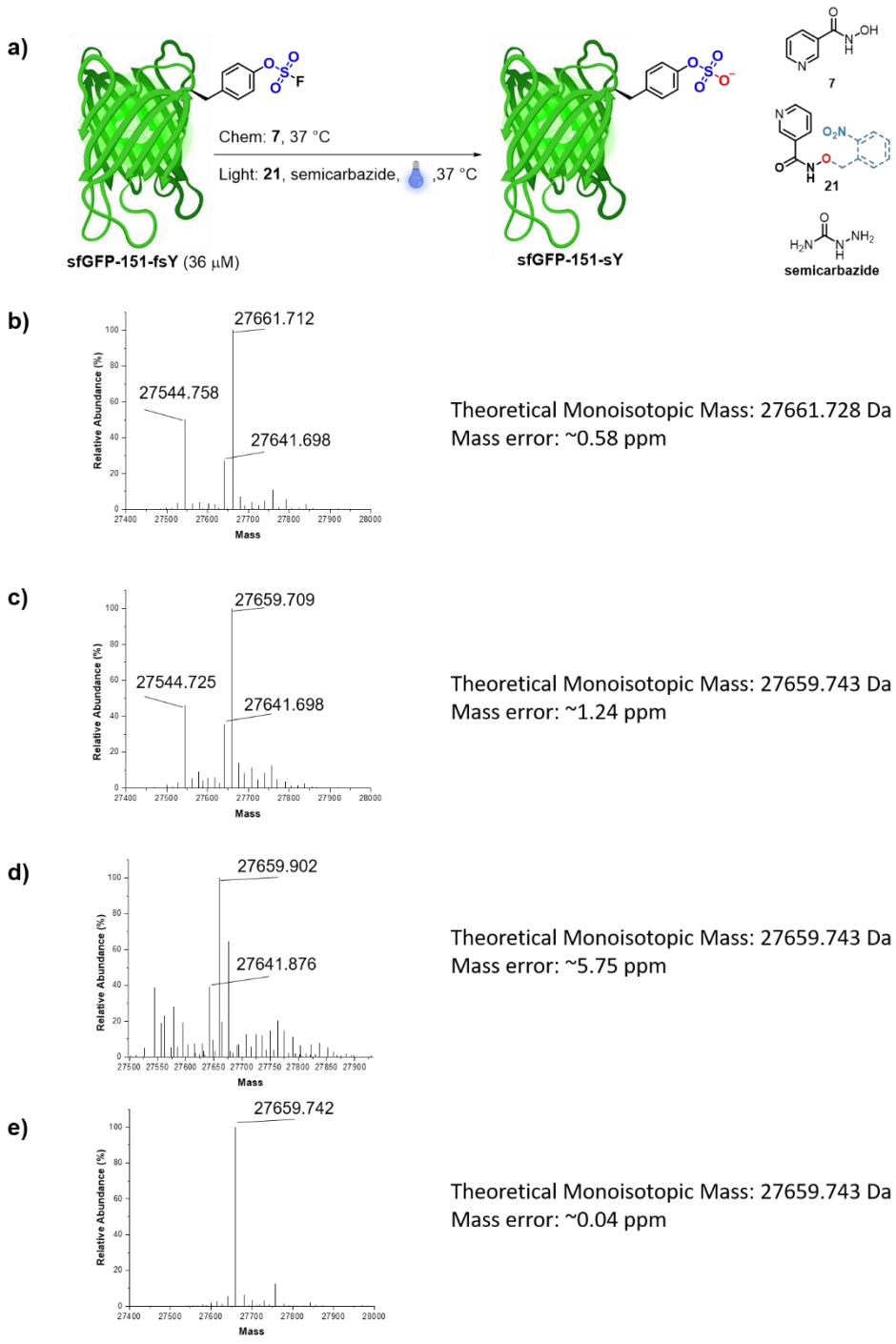


Figure S11. High-resolution Orbitrap mass spectrometry analysis of the decaging of sfGFP-151-fsY. **a**) Reaction scheme of sfGFP-151-fsY decaging using reagent **7** or photocaged reagent **21**. **b**) Mass spectrum of purified sfGFP-151-fsY. **c**) Mass spectrum of sfGFP-151-sY, generated by in situ decaging of sfGFP-151-sY by **7**. **d**) Mass spectrum of sfGFP-151-sY, generated by in situ decaging of sfGFP-151-sY by **21** after it was exposed to 370 nm light. **e**) Mass spectrum of sfGFP-151-sY, expressed with sY incorporated by the ncAA mutagenesis method.

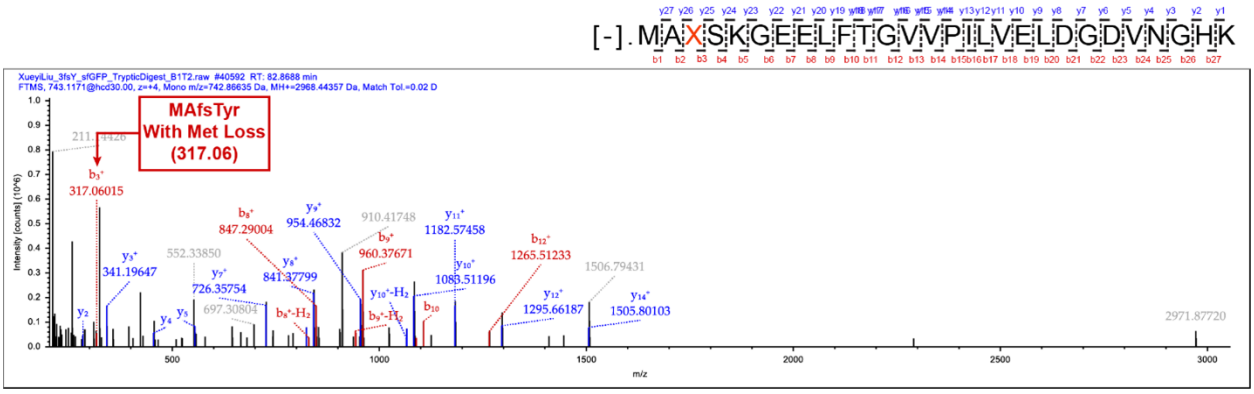


Figure S12. Tandem MS analysis of sfGFP-3-fsY after trypsin digestion and alkylation with iodoacetamide. X in the sequence logo represents fsY.

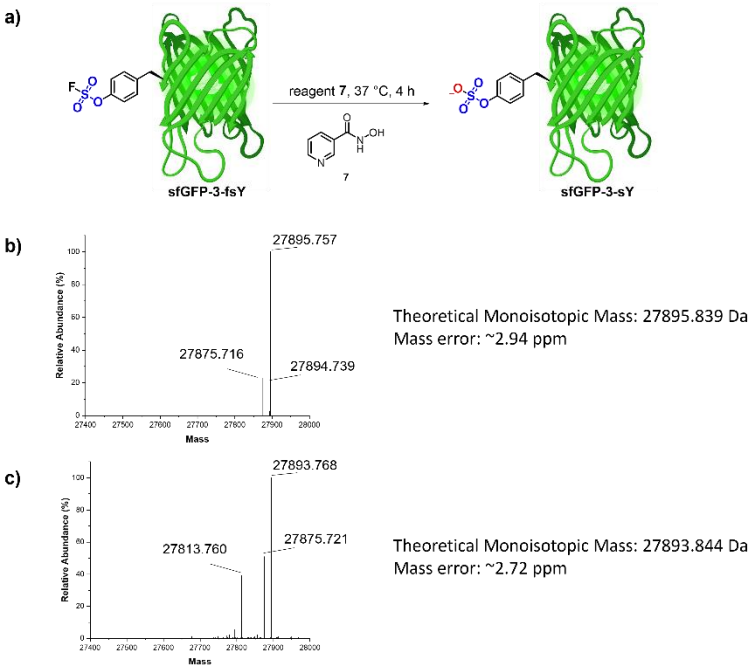


Figure S13. High-resolution Orbitrap mass spectrometry analysis of the decaging of sfGFP-3-fsY. **a**) Reaction scheme of sfGFP-3-fsY decaging using reagent **7**. **b**) Mass spectrum of purified sfGFP-3-fsY. **c**) Mass spectrum of sfGFP-3-sY, generated by in situ decaging of sfGFP-3-sY by **7**.

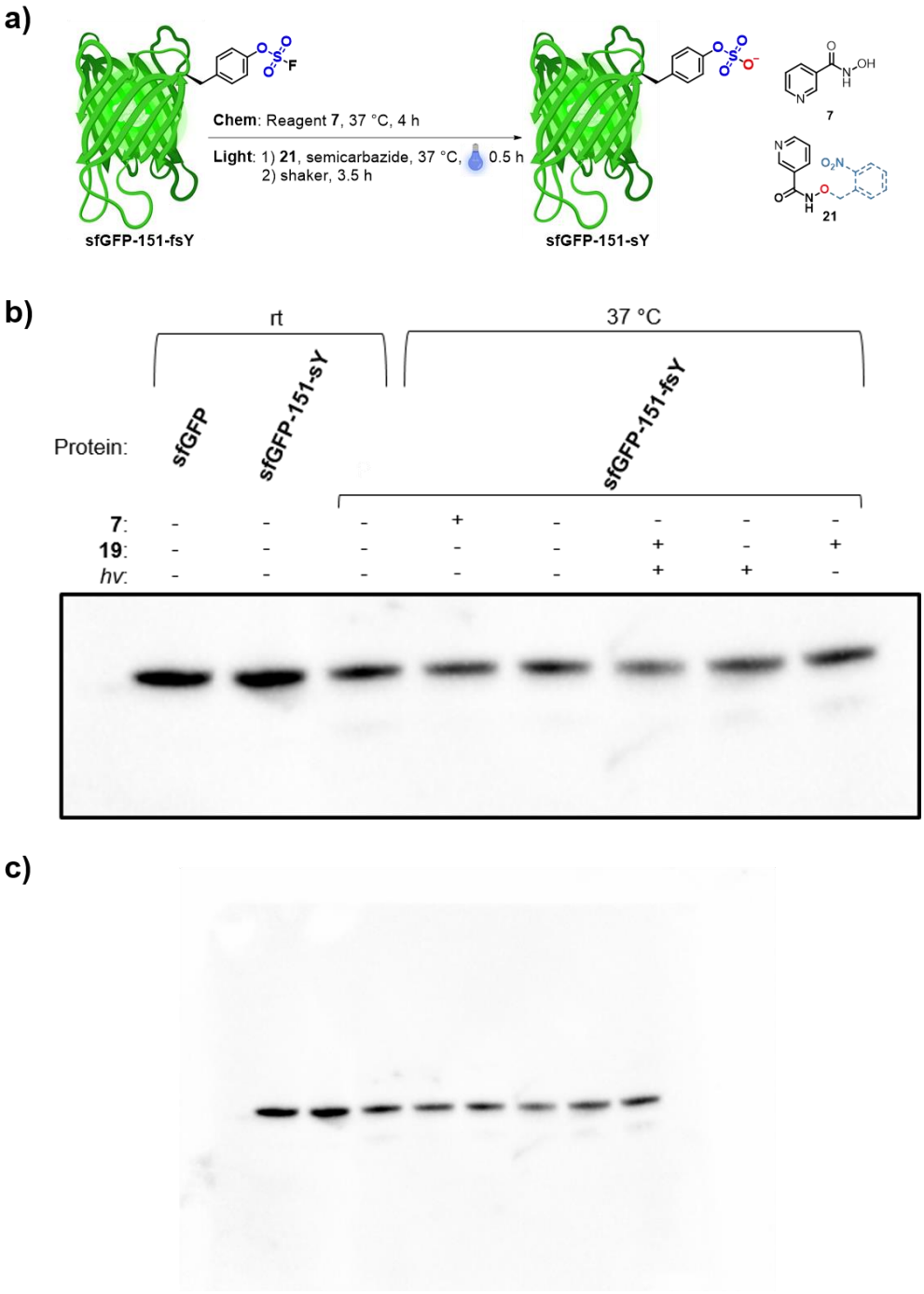


Figure S14. sfGFP-151-fsY and sfGFP-151-sY remained intact in decaging condtions by Anti-His western blot. **a**) Reaction setup for decaging fsY-containing sfGFP (**fsGFP-151-fsY**) in which condition **Chem** with reagent **7** and condition **Light** with photocaged reagent **21**. **b**) Anti-His western blot of above reaction mixture with purified wild type sfGFP, sfGFP-151-fsY and sfGFP-151-sY as control. **c**) Original Anti-His western blot image.

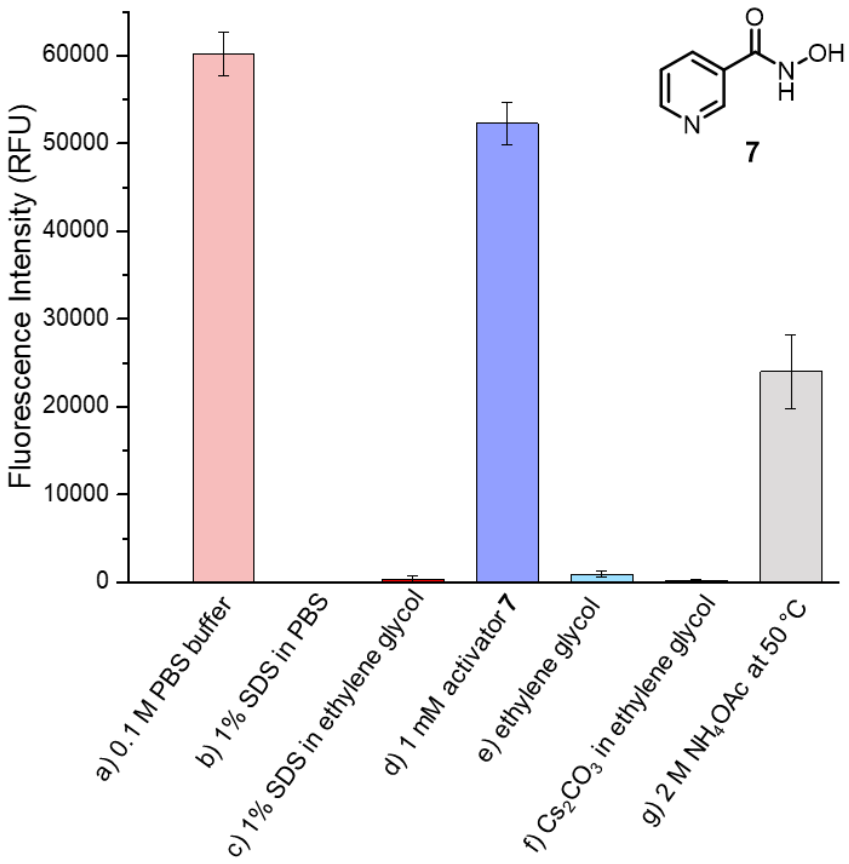


Figure S15. Fluorescence intensity of sf-GFP-151-fsY after being treated with various conditions. Either ethylene glycol alone or Cs_2CO_3 in ethylene glycol resulted in complete protein denaturation. 2 M NH_4OAc also caused partial denaturation of sfGFP. In contrast, reagent **7** did not cause significant protein denaturation. Conditions a and d to f: 4 μL of the sfGFP-151-fsY (10 μg/μL) was mixed with corresponding reagents (final volume is 50 μL) and placed on the shaker at 37 °C for 1 h under the protection of aluminum foil. Conditions b and c: negative control with complete denaturing conditions. 4 μL of the sfGFP-151-fsY (10 μg/μL) was mixed with 1% SDS in 0.1 M PBS buffer or ethylene glycol and placed in the incubator at 95 °C for 5 min. Condition g: the temperature was set as 50 °C, 1 h.

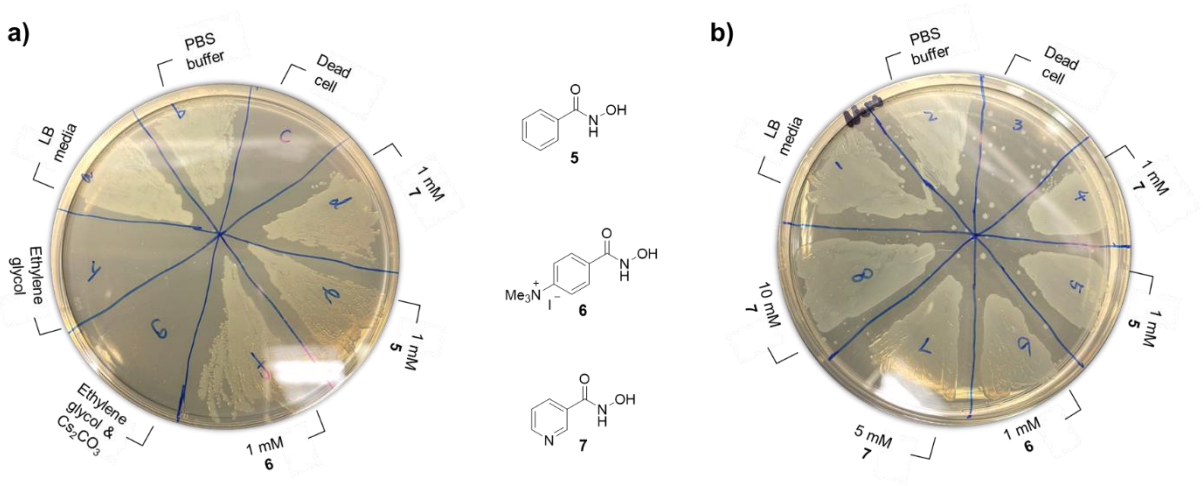


Figure S16. Plating assay to determine *E. coli* (BL21) viability under various fluorosulfate decaging conditions. **a**) Either ethylene glycol alone or Cs_2CO_3 in ethylene glycol resulted in complete cell death when used to decaging fluorosulfate. In contrast, HA reagents **5-7** demonstrated low cytotoxicity. **b**) Both 1 mM and 10 mM of HA reagents **5-7** were well tolerated by *E. coli*. 45 μL *E.coli* cell culture (BL21) was mixed with 5 μL corresponding stock solution and incubated at 37 °C for 1 h. The negative (dead cell) control was generated by treating the cells at 90 °C for 1 h. Subsequently, 5 μL of each above reaction mixture was streaked on the LB-Agar plate and placed in 37 °C overnight.

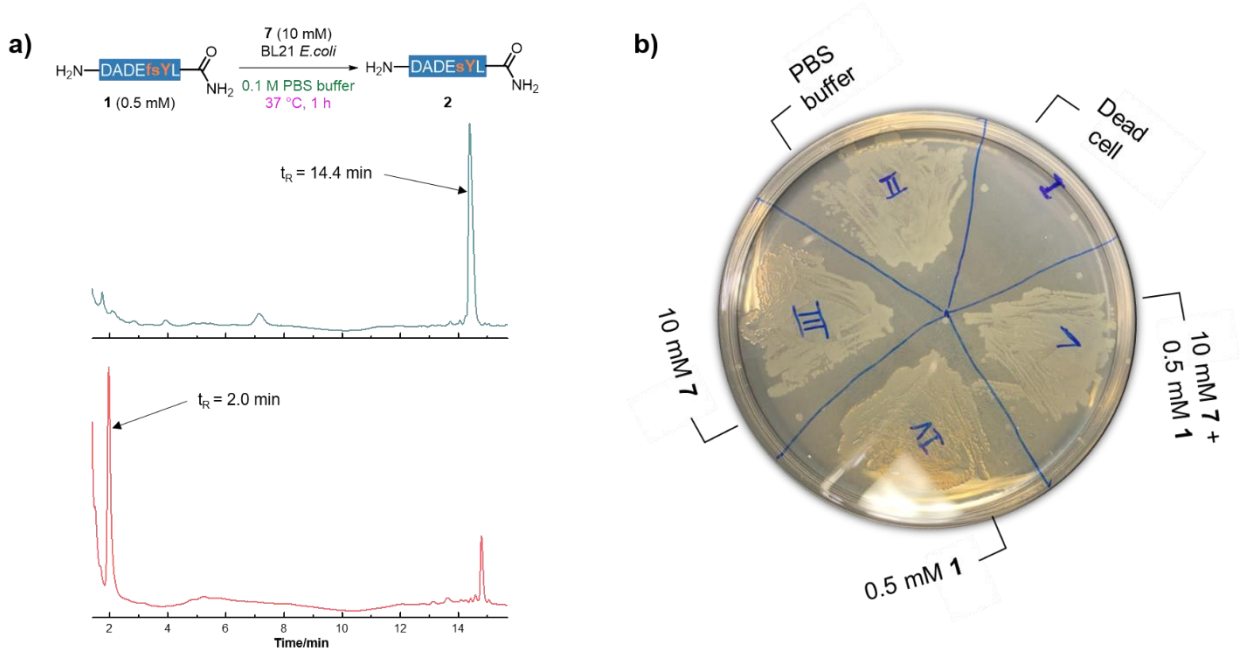


Figure S17. Free peptide **1** decaging in the presence of live *E. coli* cells to mimic the soluble sulfated small molecules and peptides.[4] **a**) The HPLC traces of peptide **1** before (top) and after (bottom) decaging in the presence of live *E. coli* cells, monitored by the UV absorbance at 220 nm. In the presence of *E. coli* cells, **1** was still smoothly converted to the sulfopeptide **2** by reagent **7** in 81% yield. **b**) Plating assay to determine the viability of BL21 *E. coli* cells after the decaging reaction of peptide **1.** After the decaging reaction, the *E. coli* cells showed no sign of reduced viability or growth.

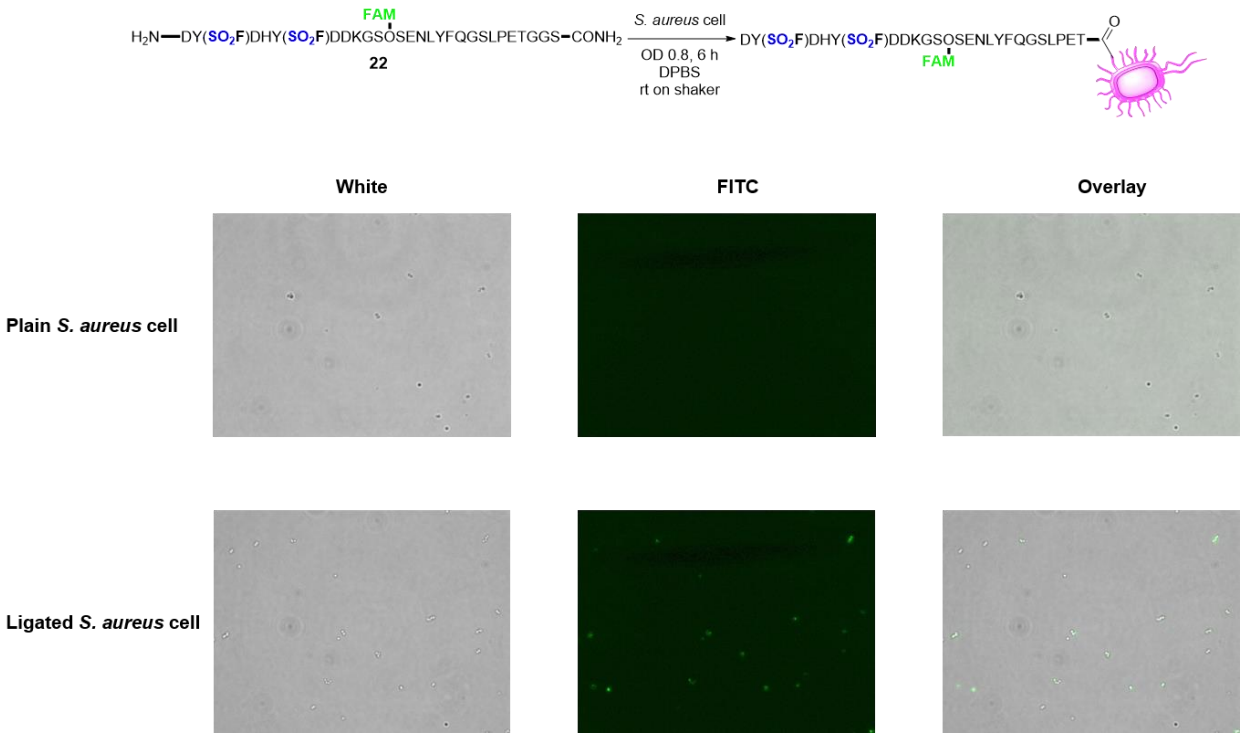
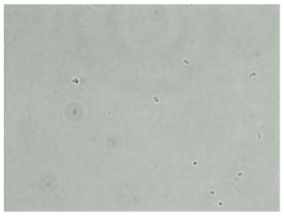
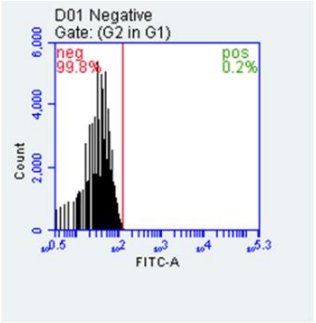


Figure S18. Fluorescent micrograph of the *S. aureus* cells before and after ligation of the 5(6)-carboxyfluorescein (FAM)-labeled peptide **22**.

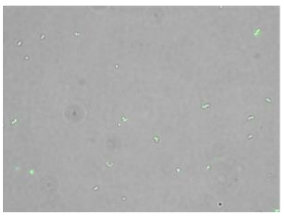
Plain *S. aureus* cell

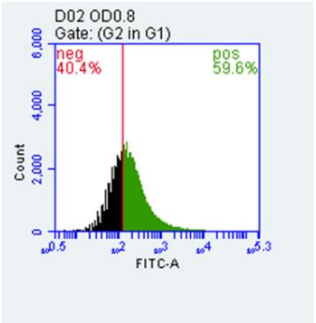




Plot 4, Neg, Gated on (G2 in G1)	% of This Plot	Mean FITC-A
This plot	100.00%	35.27
Pos (124.0/16,777, 215.0)	0.20%	136.82

Ligated *S. aureus* cell





Plot 4, Neg, Gated on (G2 in G1)	% of This Plot	Mean FITC-A
This plot	100.00%	493.93
Pos (124.0/16,777, 215.0)	59.6%	778.53

Figure S19. Flow cytometry of the *S. aureus* cells before and after ligation of the FAM-labeled peptide **22**. Fluorescent images of cell populations are shown on the left as reference.

Supplementary Methods

Material and Equipment

Chemicals were purchased from vendors such as MilliporeSigma, Thermo Fisher Scientific, and Chemical Industry Co., Ltd. (TCI) and used as received. Deuterated solvents were purchased from either Cambridge Isotope Laboratories, Inc. or Thermo Fisher Scientific. Sulfuryl fluoride (Vikane) was purchased from SynQuest Lab. Inc. The organic solvents such as acetonitrile (MeCN), tetrahydrofuran (THF), dichloromethane (DCM), and dimethylformamide (DMF) were purchased from Thermo Fisher Scientific and used after the purification by a dry solvent purification system from Pure Process Technology (PPT). 0.1 M phosphate buffered saline (PBS) buffer was prepared from purchased 20X Modified Dulbecco's PBS buffer (thermos scientific, Lot WE325396) with the pH adjusted to 7.60 by 1 M hydrogen chloride and 1 M sodium hydroxide. Human serum (Fisher BioReagents, Lot: 206088) was used as purchased with subtle pH adjustment. Thin layer chromatography was performed on Merck TLC plates (silica gel 60 F254) and visualized by UV irradiation (254 nm) and by charring with a cerium molybdate solution (0.5 g $Ce(NH_4)_2(NO_3)_6$, 24 g $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 500 mL H_2O, 28 mL H_2SO_4, filtered if necessary). Silica gel chromatography was carried out using an automated flash chromatography (Biotage Isolera One Flash Chromatography Instrument). The reaction Schlenk bottles were flame dried before use. Labconco FreeZone 2.5 L -84C benchtop freeze dryer was used for lyophilization. Gyros Protein Tribute peptide synthesizer was employed for automated peptide synthesis.

Characterization

^{1}H NMR, ^{13}C NMR measurements were conducted in deuterated solvents like deuterated chloroform ($CDCl_3$), deuterated oxide (D_2O), deuterated methanol (CD_3OD), and deuterated dimethyl sulfoxide (DMSO-d6) using a Varian Gemini-600 (600 MHz), Varian Inova-500 (500 MHz) NMR, or Bruker Advance Neo 500 MHz (with Helium CryoProbe) spectrometer. Chemical shifts are in ppm calibrated using the resonances of the residual carbon and proton of the deuterated solvent. High-resolution mass spectrometry was performed on a Micromass LCT ESI-MS and JEOL Accu TOF Dart (positive mode) at the Boston College Mass Spectrometry Facility. Mass spectrometry data for the peptides were obtained using an Agilent 6230 LC-TOF mass spectrometer with an Agilent InfinityLab Poroshell 120 column (2.7 µm, 2.1 x 50 mm, for positive mode) equipped with a Phenonemex Luna 3 µm HILIC 200 Å column (2 x 50 mm, for negative mode). High-resolution mass spectrometry of intact protein was performed on a Orbitrap Fusion

Lumos Tribrid mass spectrometer (Thermo Fisher Scientific, San Jose, California, USA) coupled with a TriVersa NanoMate (Advion, Ithaca, NY).

The following methods were used to analyze the pure samples and real-time reaction monitoring in the LC-MS instrument.

Method A for positive mode, solvent A contains acetonitrile/water/formic acid = 5:95:0.1, solvent B contains acetonitrile/water/formic acid = 95:5:0.1.

Time (min)	A (%)	B (%)	Flow (mL/min)
0.00	95.0	5.0	0.200
5.00	95.0	5.0	0.200
20.00	5.0	95.0	0.200
25.00	5.0	95.0	0.200
26.00	95.0	5.0	0.200
31.00	95.0	5.0	0.200

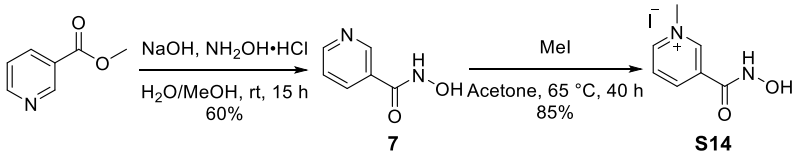
Method B for negative mode, solvent A contains 5 mM ammonium acetate in water, solvent B contains acetonitrile.

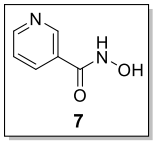
Time (min)	A (%)	B (%)	Flow (mL/min)
0.00	5.0	5.0	0.200
2.00	5.0	95.0	0.200
20.00	90.0	10.0	0.200
25.00	90.0	10.0	0.200

26.00	10.0	90.0	0.200
31.00	10.0	90.0	0.200

Analytical RP-HPLC was performed on an Agilent 1100 Series HPLC system with manual injection. Analytical RP-HPLC was done with a Waters XBridge Peptide BEH C18 column (300 Å, 2.5 μm, 4.6 mm x 150 mm) using a 0.5 mL/min flow rate. Semi-preparative RP-HPLC was achieved using a Waters 1525 binary HPLC pump equipped with a Waters 2489 UV/Visible detector. Semi-preparative HPLC was conducted with a Aglient Prep-C18 column (100 Å, 5 μm, 10 mm x 250 mm) using a 5 mL/min flow rate. Prep RP-HPLC was carried out on Waters 2545 quaternary gradient module with a Waters 2489 UV/visible detector. Prep RP-HPLC was done on a Phenomenex (00G-4055-P0) Jupiter C18 column (300 Å, 10 um, 21.2 mm x 250 mm).

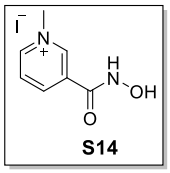
Small Molecule Synthesis





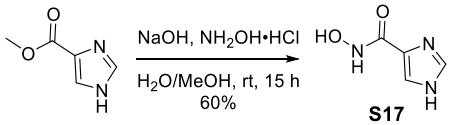
Compound **7** was obtained from the previously reported protocol with slight modification.[5] Hydroxylamine hydrochloride ($NH_2OH{\cdot}HCl$, 1.01 g, 14.58 mmol) was added to the solution of sodium hydroxide (NaOH, 1.17 g, 29.17 mmol) in water (7.5 mL) at room temperature. 5 min later, above reaction mixture was added to methyl nicotinate (1.00 g, 7.29 mmol) in methanol (11.1 mL). 16 h later, TLC showed the complete consumption of the starting material and the reaction mixture was acidified by 5% HCl to be pH = 5~6. The solvent was removed under reduced pressure on rotavopor, diluted with methanol, filtered out the sodium chloride (NaCl) salt, and recrystallized from water to yield 604.47 mg solid of **7** in 60% yield. The obtained analysis results were consistent with literature report. ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.38 (s, 1H), 9.20 (s, 1H), 8.90 (dd,

J = 2.2, 0.9 Hz, 1H), 8.69 (dd, *J* = 4.8, 1.7 Hz, 1H), 8.09 (dt, *J* = 7.9, 2.0 Hz, 1H), 7.49 (ddd, *J* = 7.9, 4.9, 0.9 Hz, 1H). HRMS (DART$^+$): calculated for $[C_6H_7N_2O_2]^+$ $(M+H)^+$ 139.0502, found 139.0510.

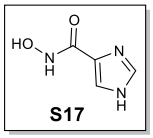


S14

To a flame-dried Schlenck bottle with a stirring bar and reagent **7** (163.4 mg, 1.18 mmol) were added iodomethane (8.21 g, 3.6 mL, 57.83 mmol) and acetone (4.0 mL) subsequently under the protection of nitrogen. The resulting reaction mixture was heated up to 60 °C in the sealed bottle. 40 h later, the reaction mixture was diluted with ethyl ether and significant amount of precipitation was observed. The reaction mixture was filtered and the solid was washed by cold ether, dried, and weighted to give 280.7 mg light yellow solid of **S14** in an 85% yield. ^{1}H NMR (500 MHz, D_2O) δ 9.25 (s, 1H), 9.01 (d, *J* = 6.1 Hz, 1H), 8.82 (d, *J* = 8.1 Hz, 1H), 8.22 (dd, *J* = 8.2, 6.2 Hz, 1H), 4.51 (s, 3H). ^{13}C NMR (126 MHz, D_2O) δ 161.54, 147.48, 144.66, 143.24, 132.06, 128.27, 48.74. HRMS (EST$^+$): calculated for $[C_7H_9N_2O_2]^+$ $(M-I)^+$ 153.0659, found 153.0616.

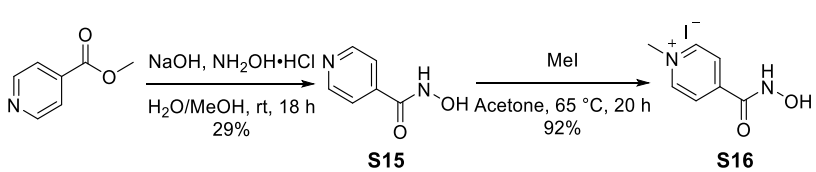


S17

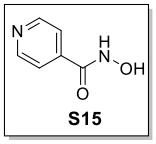


S17

To a solution of sodium hydroxide (NaOH, 1.19 g, 29.74 mmol) in water (14.8 mL) was added hydroxylamine hydrochloride ($NH_2OH{\cdot}HCl$, 843.1 mg, 12.13 mmol) at room temperature. 5 min later, methyl 4-imidazolecarboxylate (1.00 g, 7.93 mmol) was added at the same temperature. The resulting reaction mixture was allowed to stir overnight. 15 h later, the reaction mixture was neutralized to pH = 6~7 by 1 M HCl. White precipitation was generated from the clear solution. The reaction mixture was directly filtered, washed by cold water (Note: too much water washing could cause targeting material loss) and acetone once to dry. The solid was dried over air and weighted to afford 1.49 g white solid of **S17** in a 60% yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.55 (broad, 1H), 10.83 (broad, 1H), 8.94 (broad, 1H), 7.73 (d, *J* = 1.3 Hz, 1H), 7.62 (s, 1H). ^{13}C NMR (126 MHz, DMSO) δ 160.60, 136.06, 134.58, 119.32. HRMS (DART$^+$): calculated for $[C_4H_6N_3O_2]^+$ $(M+H)^+$ 128.0455, found 128.0463.

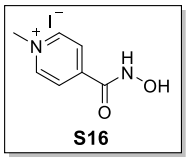


S15 **S16**



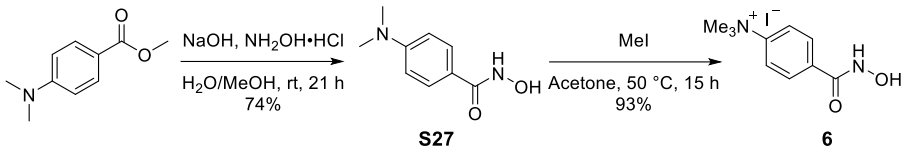
S15

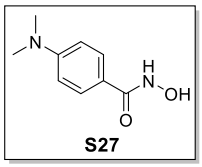
Compound **S15** was obtained from the previously reported protocol with slight modification.[5] Hydroxylamine hydrochloride ($NH_2OH{\cdot}HCl$, 1.01 g, 14.58 mmol) was added to the solution of sodium hydroxide (NaOH, 1.17 g, 29.17 mmol) in water (7.5 mL) at room temperature. 20 min later, above reaction mixture was added to methyl isonicotinate (1.00 g, 7.29 mmol) in methanol (11.1 mL). 18 h later, TLC showed the complete consumption of the starting material and the reaction mixture was acidified by 5% HCl to be pH = 5~6. The solvent was removed under reduced pressure on rotavopor, diluted with methanol, filtered out the sodium chloride (NaCl) salt, and recrystallized from water to yield 296.6 mg solid of **S15** in 29% yield. The obtained was consistent with literature report. ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.51 (s, 1H), 9.29 (s, 0H), 8.73 – 8.67 (m, 1H), 7.70 – 7.60 (m, 1H). HRMS (DART$^+$): calculated for $[C_6H_7N_2O_2]^+$ $(M+H)^+$ 139.0502, found 139.0509.



S16

To a flame-dried Schlenck bottle with a stirring bar and reagent **S15** (52.3 mg, 0.38 mmol) were added iodomethane (2.28 g, 1.0 mL, 16.06 mmol) and acetone (1.0 mL) subsequently under the protection of nitrogen. The resulting reaction mixture was heated up to 60 °C in the sealed bottle. 20 h later, the reaction mixture was diluted with ethyl ether and significant amount of precipitation was observed. The reaction mixture was filtered and the solid was washed by cold ether, dried, and weighted to give 98.0 mg light yellow solid of **S16** in an 92% yield. ^{1}H NMR (500 MHz, D_2O) δ 8.99 (d, J = 6.2 Hz, 2H), 8.32 (d, J = 6.1 Hz, 2H), 4.48 (s, 3H). ^{13}C NMR (126 MHz, D_2O) δ 161.99, 146.48, 146.32, 125.83, 48.52. HRMS (DART$^+$): calculated for $[C_7H_9N_2O_2]^+$ $(M-I)^+$ 153.0659, found 153.0652.

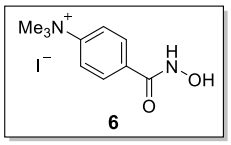




S27

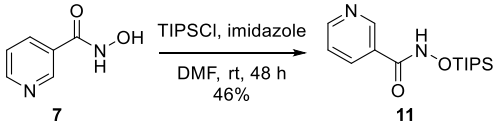
To a solution of hydroxylamine hydrochloride ($NH_2OH{\cdot}HCl$, 1.55 g, 22.32 mmol) in water (4.7 mL) was added sodium hydroxide (NaOH, 1.79 g, 44.64 mmol) at room temperature. There's a significant amount of heat was released. After the reaction mixture cooled back to room temperature, a solution of methyl 4-(dimethylamino)benzoate (2.00 g, 11.16 mmol) in methanol (MeOH, 7.5 mL) was added at the same temperature. The resulting suspension was allowed to stir overnight. 21 h later, the reaction mixture was neutralized to pH = 7 by 10 M HCl. The whole reaction became a gel-like mixture. Ethyl acetate (EA) was used

for dilution and washed by water. The aqueous layer was extracted with EA for another two times. The organic layer was combined, dried over anhydrous sodium sulfate (Na_2SO_4), and concentrated under reduced pressure. The residue was recrystallized from ethanol (20 mL) and the solid was washed by cold ether. The filtrate was concentrated and recrystallized again with ethanol (5 mL) and washed by cold ether. The combined solid was dried over air and weighted to afford 1.49 g brown solid as target compound **S27** in a 74% yield. ^{1}H NMR (600 MHz, CD_3OD) δ 7.68 – 7.53 (m, 2H), 6.76 – 6.64 (m, 2H), 2.99 (s, 6H). ^{13}C NMR (151 MHz, CD_3OD) δ 169.03, 154.31, 129.44, 119.66, 112.20, 40.19. HRMS (DART$^+$): calculated for $[C_9H_{13}N_2O_2]^+$ $(M+H)^+$ 181.0972, found 181.0966.

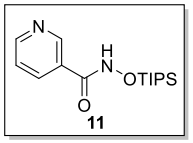


6

To a flame-dried Schlenck bottle with a stirring bar and **S31** (192.4 mg, 1.07 mmol) were added iodomethane (6.43 g, 2.8 mL, 45.29 mmol) and acetone (4.0 mL) subsequently under the protection of nitrogen. The resulting reaction mixture was heated up to 60 °C in the sealed bottle. 15 h later, the reaction mixture was diluted with ethyl ether and significant amount of precipitation was observed. The reaction mixture was filtered and the solid was washed by cold ether, dried, and weighted to give 320.5 mg white solid of **6** in an 93% yield. ^{1}H NMR (600 MHz, D_2O) δ 8.02 – 7.98 (m, 2H), 7.95 (m, 2H), 3.71 (s, 9H). ^{13}C NMR (151 MHz, d_2o) δ 166.33, 148.91, 133.21, 129.18, 120.55, 56.95. HRMS (DART$^+$): calculated for $[C_{10}H_{15}N_2O_2]^+$ $(M-I)^+$ 195.1128, found 195.1124.

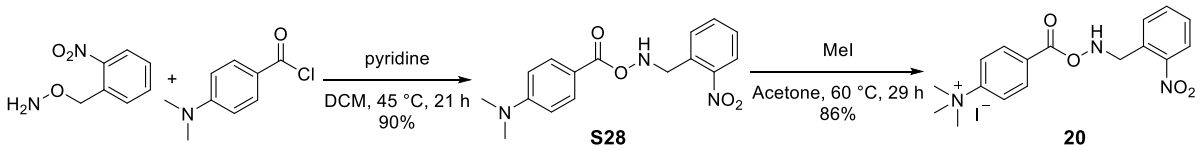


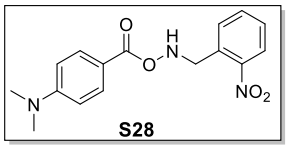
7 **11**



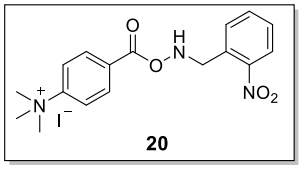
11

To a solution of reagent **7** (100.0 mg, 0.73 mmol) in DMF (6.9 mL) were added triisopropylsilyl chloride (TIPSCl, 279.2 mg, 1.45 mmol) and imidazole (98.6 mg, 1.45 mmol) subsequently at room temperature (rt). 48 h later, the reaction was diluted with EA, washed by water, brine, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The residue was loaded on the Biotage for purification, hexane/EA was used as the mobile phase to afford 97.7 mg product **11** in a 46% yield. ^{1}H NMR (500 MHz, CD_3OD) δ 8.87 (s, 1H), 8.67 (s, 1H), 8.14~8.17 (m, 1H), 7.53 (dd, J = 8.0, 5.0 Hz, 1H), 1.27~1.35 (m, 3H), 1.17 (d, J = 7.7 Hz, 18H). ^{13}C NMR (126 MHz, CD_3OD) δ 167.02, 152.80, 148.74, 136.91, 130.27, 125.20, 18.27, 13.18. HRMS (DART$^+$): calculated for $[C_{15}H_{27}N_2O_2Si]^+$ $(M+H)^+$ 295.1836, found 295.1837.

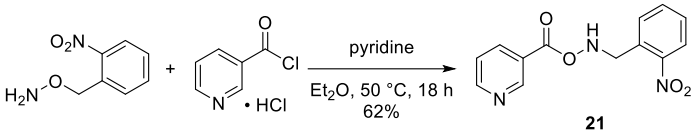


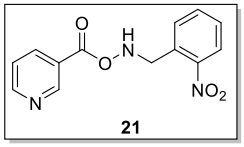


In a flame-dried 25 mL Schlenck bottle with a stirring bar, 4-(dimethylamino)benzoyl chloride (530.8 mg, 2.89 mmol) was dissolved in dichloromethane (DCM, 5.0 mL) which was followed by the addition of dry pyridine (685.9 mg, 701.3 uL, 8.67 mmol) under the protection of argon at room temperature. The reaction mixture turned into an orange-red clear solution quickly. Subsequently, *O*-(2-nitrobenzyl)hydroxylamine was added to the reaction mixture dropwise at the same temperature. The resulting reaction mixture was allowed to be heated up to 45 °C in the sealed bottle (refluxing occurred). 21 h later, the reaction mixture was diluted with ethyl acetate (EA), washed by water, brine, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure on rotavapor. The residue was loaded on the Biotage for purification with hexane/EA as eluent. 42% EA gave 547.6 mg product **S28** in a 90% yield. ^{1}H NMR (600 MHz, CD_3OD) δ 8.08 (dd, *J* = 8.2, 1.3 Hz, 1H), 7.89 (dd, *J* = 7.7, 1.4 Hz, 1H), 7.74 (td, *J* = 7.6, 1.3 Hz, 1H), 7.62 – 7.55 (m, 3H), 6.75 – 6.62 (m, 2H), 5.33 (s, 2H), 3.01 (s, 6H). ^{13}C NMR (151 MHz, CD_3OD) δ 169.25, 154.64, 149.62, 134.62, 133.09, 131.61, 130.23, 129.78, 125.70, 118.92, 112.13, 75.17, 40.16. HRMS (DART$^+$): calculated for $[C_{16}H_{18}N_3O_4]^+$ $(M+H)^+$ 316.1292, found 316.1292.



To a flame-dried Schlenck bottle with a stirring bar and above compound (219.4 mg, 0.70 mmol) were added iodomethane (4.20 g, 1.84 mL, 29.57 mmol) and acetone (1.6 mL) subsequently under the protection of nitrogen. The resulting reaction mixture was heated up to 60 °C in the sealed bottle. 29 h later, the reaction mixture was diluted with ethyl ether and significant amount of precipitation was observed. The reaction mixture was filtered and the solid was washed by cold ether, dried, and weighted to give 273.0 mg pale yellow solid of **20** in an 86% yield. ^{1}H NMR (600 MHz, DMSO-d_6) δ 12.08 (s, 1H), 8.11 – 8.04 (m, 3H), 7.91 (d, *J* = 8.5 Hz, 2H), 7.84 (m, 1H), 7.80 (m, 1H), 7.64 (m, 1H), 5.31 (s, 2H), 3.62 (s, 9H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 162.94, 149.41, 148.06, 133.88, 133.37, 131.16, 130.80, 129.67, 128.86, 124.72, 121.00, 73.38, 56.48. HRMS (DART$^+$): calculated for $[C_{17}H_{20}N_3O_4]^+$ $(M-I)^+$ 330.1448, found 330.1459.





In a flame-dried Schlenck bottle with a stirring bar, *O*-(2-nitrobenzyl)hydroxylamine (245.6 mg, 1.46 mmol) was dissolved in ethyl ether (Et_2O, 6.0 mL) and followed by the addition of dry pyridine (347.6 mg, 4.74 mmol, 383.0 μL) at room temperature under the protection of nitrogen. Subsequently, nicotinoyl chloride hydrochloride (260.0 mg, 1.46 mmol) was added. The resulting reaction mixture was sealed and heated up to 50 °C. 18 h later, the reaction mixture was diluted with dichloromethane (DCM), washed by water and brine, dried over anhydrous Na_2SO_4, concentrated under reduced pressure, loaded on Biotage for purification using hexane/ethyl acetate as the mobile phase. 80% EA provided the 245.6 mg title compound **21** as white solid in a 62% yield. ^{1}H NMR (600 MHz, CD_3OD) δ 8.85 (d, *J* = 2.1 Hz, 1H), 8.68 (dd, *J* = 4.9, 1.7 Hz, 1H), 8.14 (dt, *J* = 8.0, 1.9 Hz, 1H), 8.08 (dd, *J* = 8.3, 1.3 Hz, 1H), 7.86 (d, *J* = 7.7 Hz, 1H), 7.74 (td, *J* = 7.6, 1.3 Hz, 1H), 7.59 (td, *J* = 7.8, 1.4 Hz, 1H), 7.55 – 7.49 (m, 1H), 5.40 (s, 2H). ^{13}C NMR (151 MHz, CD_3OD) δ 153.06, 149.80, 148.84, 136.94, 134.65, 132.64, 131.83, 130.50, 129.66, 125.77, 125.22, 75.16. HRMS ($DART^+$): calculated for $[C_{13}H_{12}N_3O_4]^+$ $(M+H)^+$ 274.0822, found 274.0831.

Peptide Synthesis and Decaging

General Procedure A. Manual solid-phase synthesis of fluorosulfotyrosine (fsY)-containing peptides.

Fluorenylmethyloxycarbonyl-solid phase peptide synthesis (Fmoc-SPPS) was performed manually using a 50 mL Synthware peptide synthesis vessel employing Rink amide resin (0.49 mmol/g) at 100 μmol scale. The resin was preswollen in DMF for 330 min before use. Amino acids were coupled using the following reagents and reaction time: Fmoc-AA-OH (5 equiv.), HBTU (5 equiv.), DIPEA (5 equiv.)-preactivation was done in a 20 mL vial with 3 mL DMF and a clear reaction mixture would be obtained within 2 min which was directly transferred to the peptide synthesizer vessel. The activated amino acid was added to the resin-bound primary amine with nitrogen gas bubbling for a coupling period of 45 min for natural amino acid or 60 min for fluorosulfotyrosine to generate a new amide bond. Removal of the Fmoc group was achieved using 20% (volume percent) 2-methylpiperidine in DMF (3 x 10 min). The resin was washed with DMF between each coupling and Fmoc-removal step (5 x 3 min). After the final Fmoc deprotection step, the resin was washed with DMF (5 x 3 min), dichloromethane (3 x 3 min), and hexane (3 x 3 min)

and dried in air. Cleavage of the crude fsY-containing peptides from the resin and side chain unmasking (except for the fluorosulfate group) was achieved with a cleavage cocktail consisting of TFA:TIPS:H_2O (95:2.5:2.5) for three times and 30 min each. The combined filtrates were concentrated carefully under reduced pressure at 40 °C which were followed by precipitatioin in cold diethyl ether. The resulting suspension was centrifuged at 5000 rpm at 4 °C for 10 min before dumping the ether. Obtained pellet was redissolved in water and filtered for LC-MS verification. The crude was then subjected for semi-prep HPLC purification using solvent A (95% CH_3CN, 5% H_2O, and 0.1% TFA): solvent B (5% CH_3CN, 95% H_2O, and 0.1% TFA) as mobile phase. Appropriate fractions were characterized by LC-MS, combined, concentrated by air stream, and lyophilized. The obtained product was analyzed by HPLC and LC-MS for confirmation.

General Procedure B. Automated solid-phase synthesis of peptides.

Fmoc-SPPS of peptides without the fsY incorporation were performed automatically on the Gyros Protein Tribute peptide synthesizer employing Rink amide resin (0.49 mmol/g) at 100 μmol scale. The resin was loaded in the 10 mL reaction vessel. Subsequently, each amino acid (5 equiv.) mixed with HBTU (5 equiv.) was weighed into the 10 mL vial and sealed.

The working sequence was set as follows:

Swelling with 3 mL DMF for 30 min and then 2 mL DMF washing (6 x 30 sec). 3 mL 20% piperidine solution in DMF for Fmoc removal (3 x 5 min) and 3 mL DMF washing (6 x 30 sec).

Coupling: NMM (0.4 M in DMF, 2.5 mL) was injected into the amino acid and HBTU mixture and then delivered to the reaction vessel. The resulting reaction mixture was shaken for 40 min, followed by the addition of 3 mL 20% piperidine solution in DMF for Fmoc removal (2 x 5 min) and 2 mL DMF washing (6 x 30 sec).

Final Fmoc-removal: 3 mL 20% piperidine solution in DMF for Fmoc removal (8 x 5 min) and 2 mL DMF washing (9 x 30 sec).

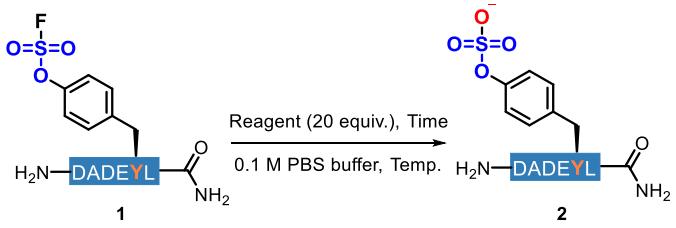
The reaction vessel was next taken out of the synthesizer, washed by 3 mL dichloromethane (3 x 3 min) and hexane (3 x 3 min), and dried in air. The obtained beads with peptides attached on could be used for subsequent fsY incorporation following above procedure. Once the beads were ready for cleavage, TFA:TIPS:H_2O (95:2.5:2.5) cocktail was used to treat the beads three times and 30 min each. The combined filtrates were concentrated carefully

under reduced pressure at 40 °C which were followed by precipitation in cold diethyl ether. The resulting suspension was centrifuged at 5000 rpm at 4 °C for 10 min before dumping the ether. Obtained pellet was redissolved in water and filtered for LC-MS verification. The crude was then subjected for semi-prep HPLC purification using solvent A (95% CH_3CN, 5% H_2O, and 0.1% TFA): solvent B (5% CH_3CN, 95% H_2O, and 0.1% TFA) as mobile phase. Appropriate fractions were characterized by LC-MS, collected, concentrated by air stream, and lyophilized. The obtained product was analyzed by HPLC and LC-MS for confirmation.

Yield Calculation

Yield of purified fsY-containing peptides were calculated from the adjusted resin loading and based on the assumption that all basic side chains, and *N*-terminal amines in the peptides were lyophilized as salts with TFA.

General Procedure C. Screening fluorosulfate decaging reagents



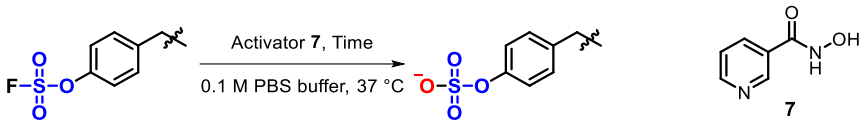
Stock solution preparation and reaction setup:

Fluorosulfohexpeptide **1** (TFA salt from semi-prep RP-HPLC) was dissolved in 0.1 M PBS buffer. The final concentration of **1** was 1 mM in 0.1 M PBS buffer, pH = 7.20~7.40. For reagents with good aqueous solubility, they were dissolved in 0.1 M PBS buffer and the final pH was adjusted by 1 M HCl/1 M NaOH to give 0.1 M final aqueous solution with pH maintained between 7.0~7.40. For reagents with poor aqueous solubility, they were dissolved in DMSO to give 0.1 M corresponding stock solution and used directly in the reaction. The pH of the final reaction mixture was also confirmed to be within the physiological pH range 7.00~7.40.

A 1.5 mL glass vial was equipped with a stirring bar and **1** (1 mM, 150.0 μL, 0.15 μmol). Meanwhile, the stock solutions of reagents and reagents (0.1 M) were also placed at the same temperature (25 °C for Table 1, entry 3-

7; 37 °C for Table 1, entry 8 and Figure 2a, S4) for at least two minutes. Subsequently, reagent stock solution (30.0 μL, 20 equiv.) was added to the stirring vial. The resulting reaction mixture was allowed to stir at the same conditions for certain reaction time (24 h for Table 1; 1 h or 0.5 h for Figure 2a and S4). Then the whole reaction mixture was filtered and subjected for RP-HPLC analysis.

General Procedure D. Decaging of fsY-Containing Peptides.



A solution of fsY-containing peptides (1 mM) and 0.1 M 3-pyridinylhydroxamic acid (**7**) both in 0.1 M PBS buffer was warmed up separately in the warm room (37 °C constant temperature) for 2 min. Next, 20 equiv. of reagent **7** with respect to the substrate was added to the substrate solution with stirring. The reaction was monitored by RP-HPLC until the reaction was complete. Then the reaction was filtered, and then injected directly into semi-prep RP-HPLC for purification using CH_3CN:20mM ammonium acetate buffer as mobile phase. Fractions were analyzed by positive mode LC-MS. Fractions containing the desired product were combined and concentrated using air stream. The resulting residue was redissolved in water and lyophilized. The product was confirmed by LC-MS and RP-HPLC. The pH of the stock solutions of substrates and reagents in 0.1 M PBS buffer were always adjusted between 7.00~7.40.

Yield Calculation

Yield of the purified sY-containing peptides were calculated based on the assumption that all acidic side chains in the peptides were lyophilized as ammonium or acetate salts.

DADEfsYL-NH_2 (1)

The synthesis was performed manually following general procedure A with 408.0 mg resin loading (0.2 mmol). Purification of the titled peptide through the Semi-prep RP-HPLC with eluent A (95% H_2O, 5% CH_3CN, and 0.1% TFA) and B (5% H_2O, 95% CH_3CN, and 0.1% TFA). The running method was set as 0-5 min, 15% B, linear

gradient from 15%~40% B over 15 min. The desired peptide was observed at t_R = 15.0 min and obtained in a 32% yield after twice purification and lyophilization. Analytical RP-HPLC of this material dissolved in 0.1 M PBS buffer (CH_3CN: 20 mM ammonium acetate buffer mobile phases, linear gradient from 5%~33% CH_3CN from 0-20 min, 20-21 min 33%~95% CH_3CN, and 95% CH_3CN washing for another 2 min) showed a single peak at t_R = 18.4 min, monitored by the UV absorbance at 230 nm (Figure S18). HRMS (ESI^+): calculated for $[C_{31}H_{45}N_7O_{15}SF]^+$ $(M+H)^+$ 806.2673, found 806.2140 (Figure S19).

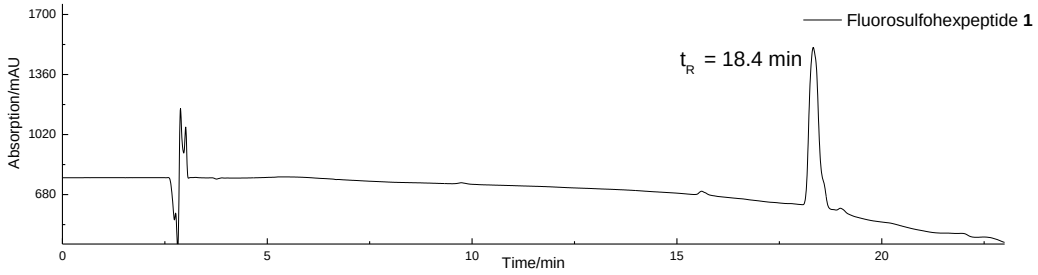


Figure S18: Analytical RP-HPLC chromatogram of purified peptide **DADEfsYL-NH_2** (**1**). Method: CH_3CN: 20 mM ammonium acetate buffer mobile phases, linear gradient from 5%~33% CH_3CN from 0-20 min, 20-21 min 33%~95% CH_3CN, and 95% CH_3CN washing for another 2 min.

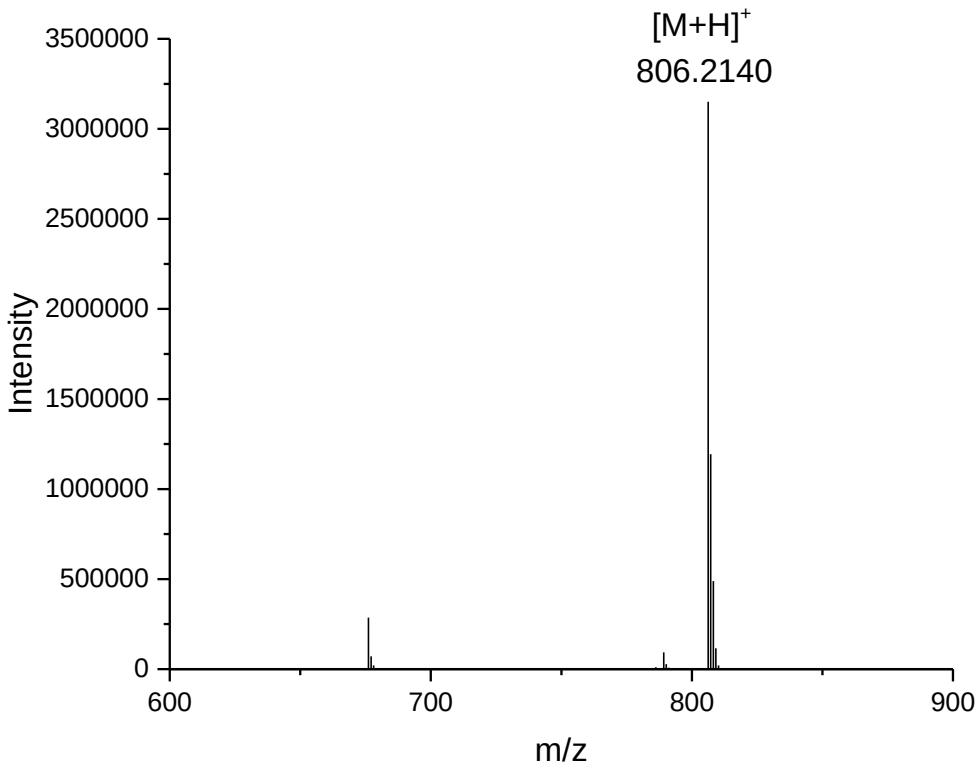


Figure S19. ESI-TOF mass spectrum for peptide **DADEfsYL-NH$_2$** (**1**)

DADEsYL-NH$_2$ (**2**)

Decaging of **1** was performed following general procedure C with 5.3 mg substrate dissolved in 0.1 M PBS buffer with 20 equivalents of 3-PHA (0.1 M stock solution in 0.1 M PBS buffer) as reagent. 2 h later, the reaction was directly filtered and loaded on the Semi-prep RP-HPLC for purification. CH_3CN:20 mM ammonium acetate buffer mobile phase was chosen as the mobile phase, and the method was set as 0-5 min, 5% CH_3CN; linear gradient 5-13min, 5%~19.4% CH_3CN. The desired peptide was afforded with a t_R = 9.5 min. The combined fractions were air concentrated and the residue was lyophilized to provide 6.4 mg white solid with a 93% yield. Analytical RP-HPLC of this material dissolved in 0.1 M PBS buffer (CH_3CN: 20 mM ammonium acetate buffer mobile phases, linear gradient from 5%~35% CH_3CN from 0-20 min, 20-21 min 35%~95% CH_3CN, and 95% CH_3CN washing for another 2 min)

showed a single peak at t_R = 9.7 min, monitored by the UV absorbance at 230 nm (Figure S20). HRMS (ESI^+): calculated for $[C_{31}H_{45}N_7O_{16}NaS]^+$ $(M+Na)^+$ 826.2536, found 826.1847 (Figure S21).

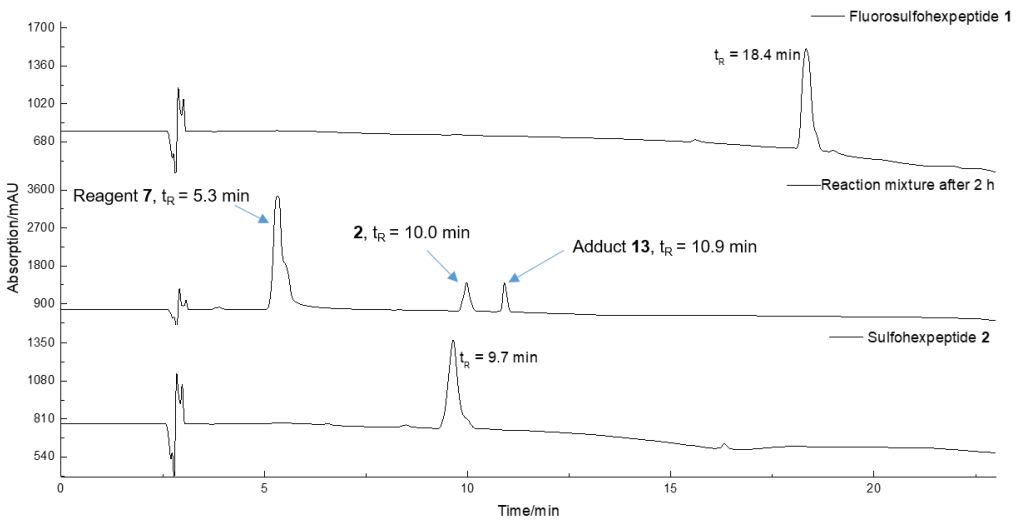


Figure S20. Analytical RP-HPLC chromatograms of the decaging reaction mixture (middle) and purified peptide **DADEsYL-NH$_2$** (**2**, bottom). Method: CH_3CN: 20 mM ammonium acetate buffer mobile phases, linear gradient from 5%~33% CH_3CN from 0-20 min, 20-21 min 33%~95% CH_3CN, and 95% CH_3CN washing for another 2 min.

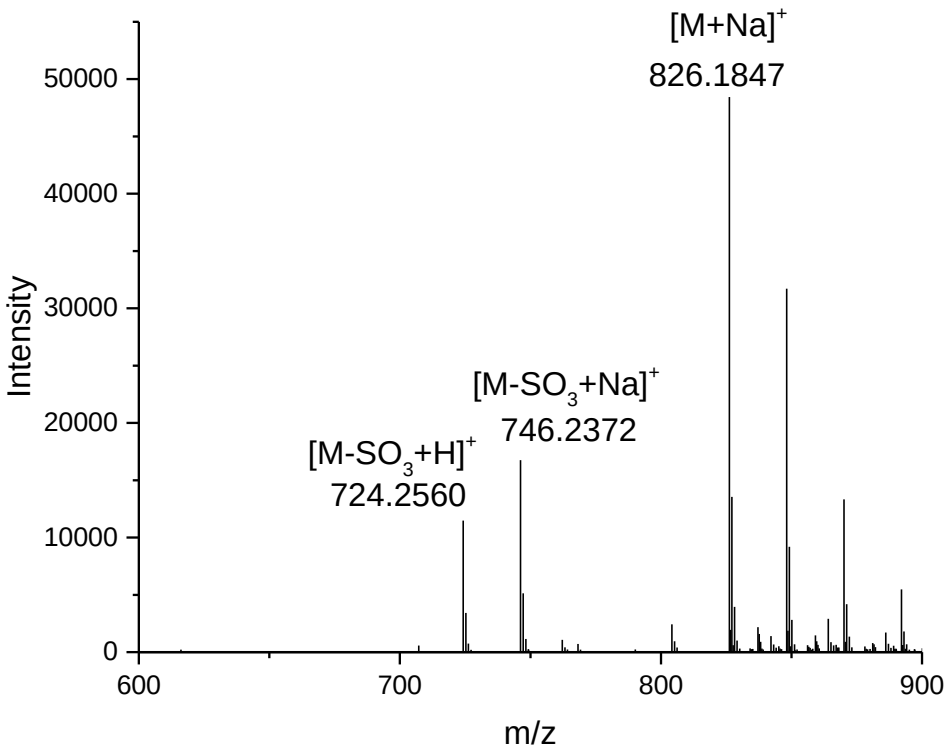


Figure S21. ESI-TOF mass spectrum for peptide **DADEsYL-NH$_2$ (2)**

fsYEfsYLDfsYDF-NH_2 (14)

The synthesis of the title octapeptide was performed manually following general procedure A with 102.0 mg resin loading (0.2 mmol). Purification of the titled peptide through the Semi-prep RP-HPLC with eluent A (95% H_2O, 5% CH_3CN, and 0.1% TFA) and B (5% H_2O, 95% CH_3CN, and 0.1% TFA). The running method was set as linear gradient from 25% to 55% within 30 min. The desired peptide was observed at t_R = 21.5 min and 10.2 mg cotton-like white solid was obtained in a 15% yield after twice purification and lyophilization. Analytical RP-HPLC of this material dissolved in DMSO (CH_3CN: 20 mM ammonium acetate buffer mobile phases, linear gradient from 5%~60% CH_3CN over 40 min) showed a single peak at t_R = 23.4 min, monitored by UV absorbance at 230 nm (Figure S22). HRMS (ESI^+): calculated for $[C_{55}H_{65}F_3N_9O_{23}S_3]^+$ $(M+2H)^+$ 687.1607, found 687.1832 (Figure S23).

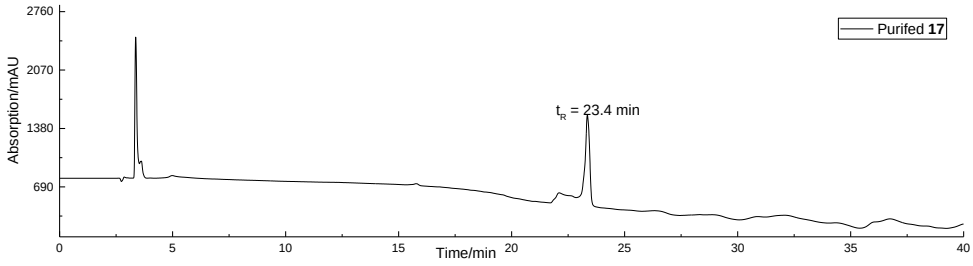


Figure S22. Analytical RP-HPLC chromatogram of purified peptide **fsYEfsYLDfsYDF-NH_2 (17)**. Method: CH_3CN: 20 mM ammonium acetate buffer mobile phases, linear gradient from 5%~60% CH_3CN over 40 min.

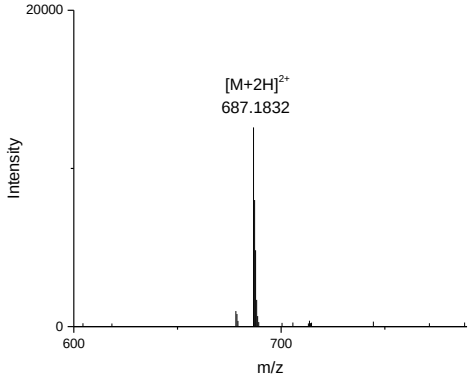


Figure S23. ESI-TOF mass spectrum for peptide **fsYEfsYLDfsYDF-NH_2 (14)**

sYEsYLDsYDF-NH_2 (15)

Decaging of **14** was performed following general procedure D with 0.6 mg substrate (suspension in a mixture of 0.1 M PBS buffer/CH_3CN = 7:3) used with 60 equivalents of 3-PHA (0.1 M stock solution in 0.1 M PBS buffer) as reagent. 3 h later, RP-HPLC showed the reaction reached completion. The reaction mixture was first passed through a PD-10 column (cytiva, Sephadex^{TM} G-25 M) using pure water as eluent to get rid of the excessive amount of reagent and salts. The fraction conotaining targeting sulfate molecule was verified by LC-MS and combined for subsequent semi-prep RP-HPLC purification. CH_3CN: 20 mM ammonium acetate buffer was employed as mobile phases. The running method was set as 0-5 min, 5% CH_3CN, linear gradient from 5%~25% CH_3CN over 15 min. The desired peptide was observed at t_R = 12.5 min and 0.4 mg cotton-like white solid was obtained in a 15% yield after purification and lyophilization. Analytical RP-HPLC of this material dissolved in water (CH_3CN: 20 mM ammonium acetate buffer mobile phases, linear gradient from 5%~60% CH_3CN over 40 min) showed a single peak at t_R = 15.7 min, monitored by UV absorbance at 230 nm (Figure S24). HRMS (ESI^-): calculated for $[C_{55}H_{65}N_9O_{26}S_3]^{2-}$ $(M-2H)^{2-}$ 681.66074, found 681.7419 (Figure S25).

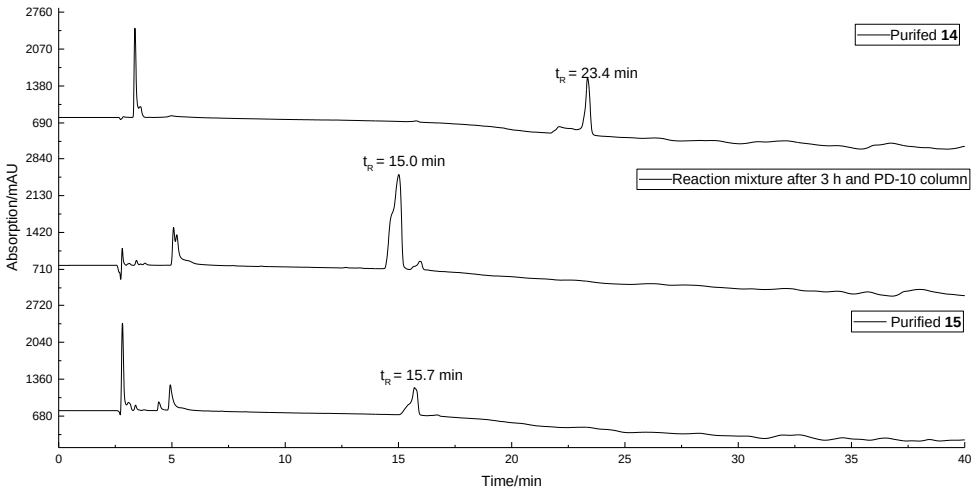


Figure S24: Analytical RP-HPLC chromatogram of reaction mixture after 3 h and passed through a PD-10 column (middle) purified peptide **sYEsYLDsYDF-NH_2** (**15**, bottom). Method: CH_3CN: 20 mM ammonium acetate buffer mobile phases, linear gradient from 5%~60% CH_3CN over 40 min.

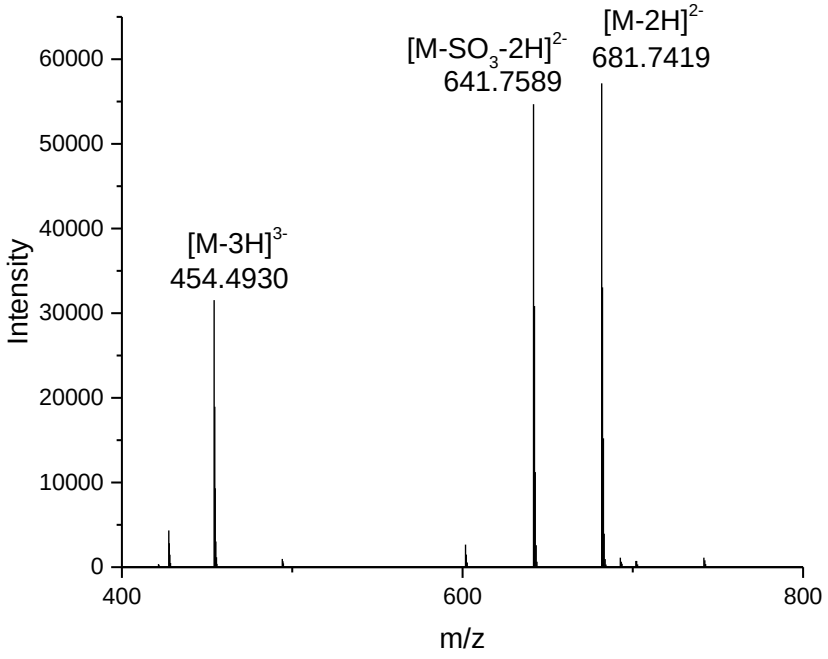


Figure S25. ESI-TOF mass spectrum for peptide **sYEsYLDsYDF-NH$_2$** (**15**)

Biotin-TTPDfsYGHfsYDDKDTLDLNTPVDK-NH$_2$ (**16**)

The synthesis of the title 22mer peptide was separated in two steps. The coupling of the first 14 amino acids (counted from the *C*-terminal) was constructed automatically following general procedure B with 200.0 mg Rink amide resin was loaded After the last coupling step and *N*-terminal Fmoc removal, the resin was washed with DMF, dichloromethane, ethyl ether, hexane subsequently (for each solvent 3 x 3 min), and dried to afford 509.6 mg total resin. A small portion of the resin was cleaved with standard cleavage cocktail, and verified by LC-MS, providing the expected 14-mer signal: HRMS (ESI$^+$): calculated for $[C_{66}H_{112}N_{18}O_{27}]^{2+}$ $(M+2H)^{2+}$ 794.3976, found 794.3710 (Figure S26). 112.6 mg (0.022 mmol, calculated based on the previous total resin obtained) resin was used for the subsequent amino acids incorporation following general procedure A. Purification of the titled peptide through the Semi-prep RP-HPLC with eluent A (95% H_2O, 5% CH_3CN, and 0.1% TFA) and B (5% H_2O, 95% CH_3CN, and 0.1% TFA). The running method was set as: 0-5 min, 15% B; linear gradient 5-35 min, 15%~35% B. The product peak emerged at t_R

= 31.0 min under 210 nm wavelength and appropriate fractions verified by LC-MS were combined. After concentration with air-blowing, the residue was subjected for lyphilization to provide 42.7 mg of the title compound in 60% yield. Analytical RP-HPLC of this material dissolved in water (CH_3CN: 20 mM ammonium acetate buffer mobile phases, linear gradient from 5%~60% CH_3CN over 40 min) showed a single peak at t_R = 18.8 min, monitored by the UV absorbance at 230 nm (Figure S27). HRMS (ESI^+): calculated for $[C_{119}H_{179}F_2N_{30}O_{47}S_3]^{3+}$ $(M+3H)^{3+}$ 971.7229, found 971.6719 (Figure S28).

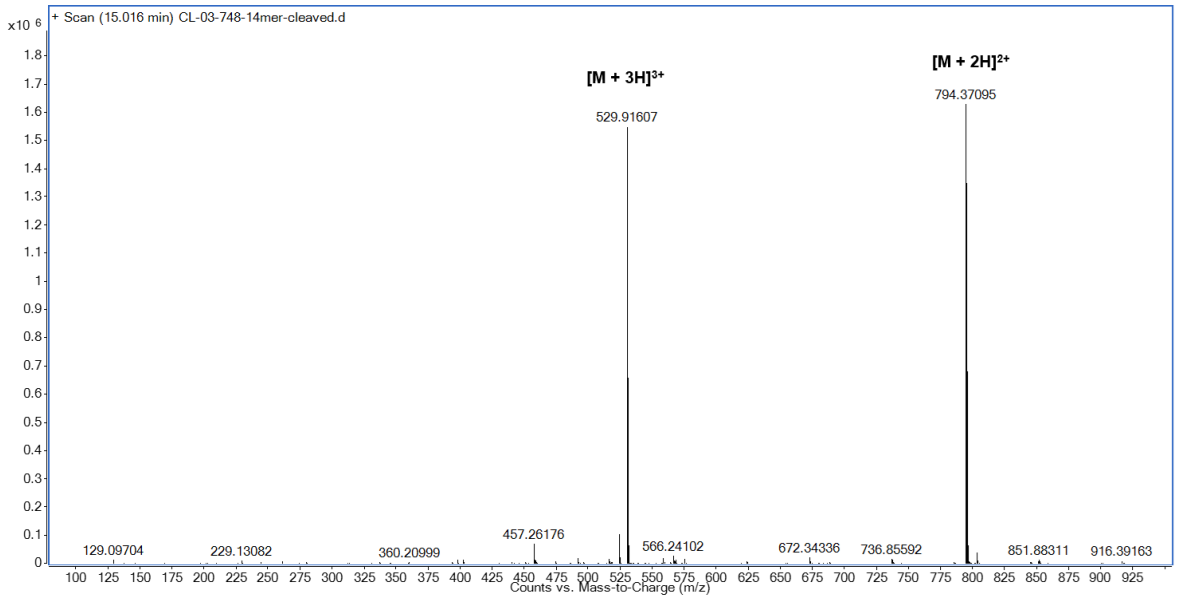


Figure S26. ESI-TOF mass spectrum for peptide **DDKDTLDLNTPVDK-NH_2**

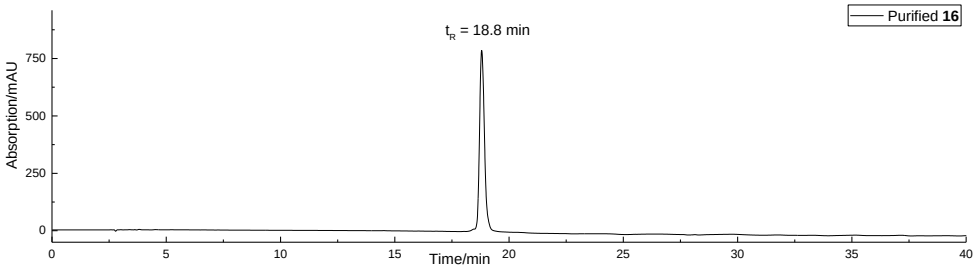


Figure S27. Analytical RP-HPLC chromatogram of purified peptide **Biotin-TTPDfsYGHfsYDDKDTLDLNTPVDK-NH_2** (**16**). Method: CH_3CN: 20 mM ammonium acetate buffer mobile phases, linear gradient from 5%~60% CH_3CN over 40 min.

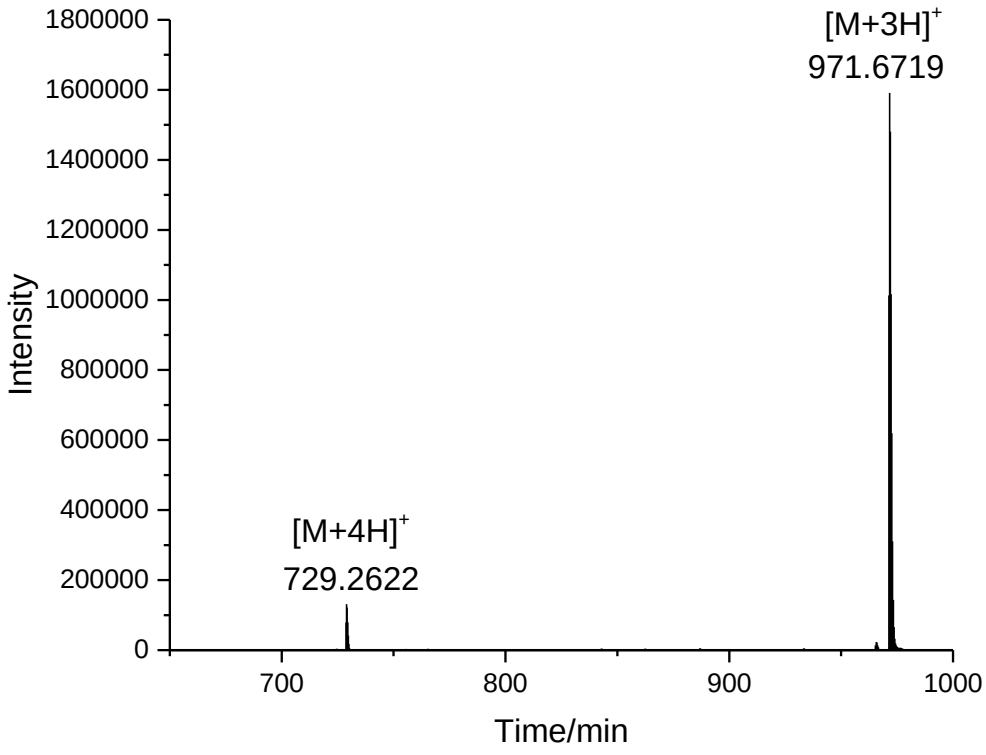


Figure S28. ESI-TOF mass spectrum for peptide **Biotin-TTPDfsYGHfsYDDKDTLDLNTPVDK-NH$_2$** (**16**)

Biotin-TTPDsYGHsYDDKDTLDLNTPVDK-NH$_2$ (17)

Decaging of **16** was performed following general procedure D with 8.8 mg substrate (dissolved in 0.1 M PBS buffer) and 40 equivalents of 3-PHA (0.1 M stock solution in 0.1 M PBS buffer, pH = 7.35) as reagent. 2.5 h later, RP-HPLC showed the reaction reached completion. The reaction mixture was directly filtered and subjected for semi-prep RP-HPLC purification. CH_3CN: 20 mM ammonium acetate buffer was employed as mobile phases. The running method was set as 0-5 min, 5% CH_3CN; linear gradient from 5%~25% CH_3CN over 25 min. The desired peptide was observed at t_R = 13.0 min. Appropriate fractions were verified by LC-MS, combined, dried over air-blowing, lyophilized to give 6.1 mg white solid in a 74% yield. Analytical RP-HPLC of this material dissolved in water (CH_3CN: 20 mM ammonium acetate buffer mobile phases, linear gradient from 5%~60% CH_3CN over 40 min) showed a single peak

at t_R = 15.1 min, monitored by the UV absorbance at 230 nm (Figure S29). HRMS (ESI^-): calculated for $[C_{119}H_{181}N_{30}O_{49}S_3]^{3+}$ $(M+3H)^{3+}$ 970.3924, found 970.3001 (Figure S30).

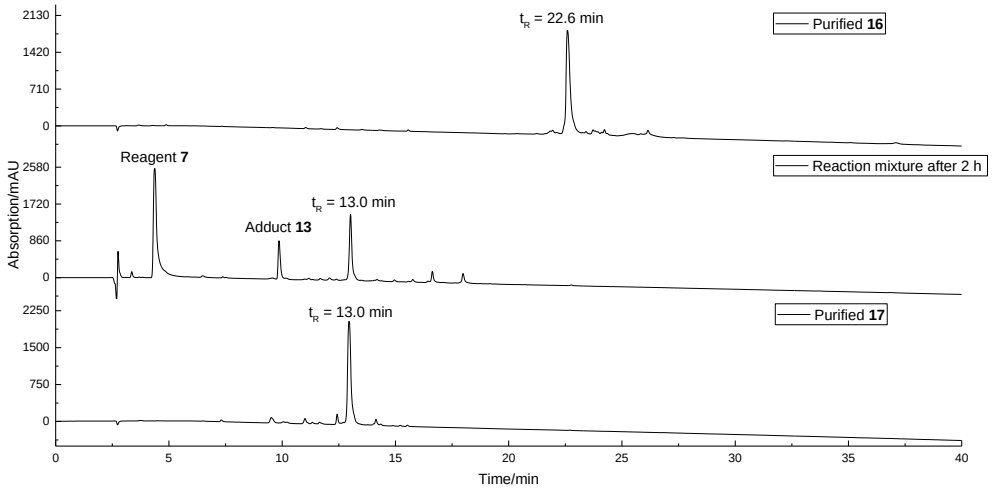


Figure S29. Analytical RP-HPLC chromatogram of purified reaction mixture (middle) peptide **Biotin-TTPDsYGHsYDDKDTLDLNTPVDK-NH_2** (**17**, bottom**)**. Method: CH_3CN: 20 mM ammonium acetate buffer mobile phases, linear gradient from 5%~60% CH_3CN over 40 min.

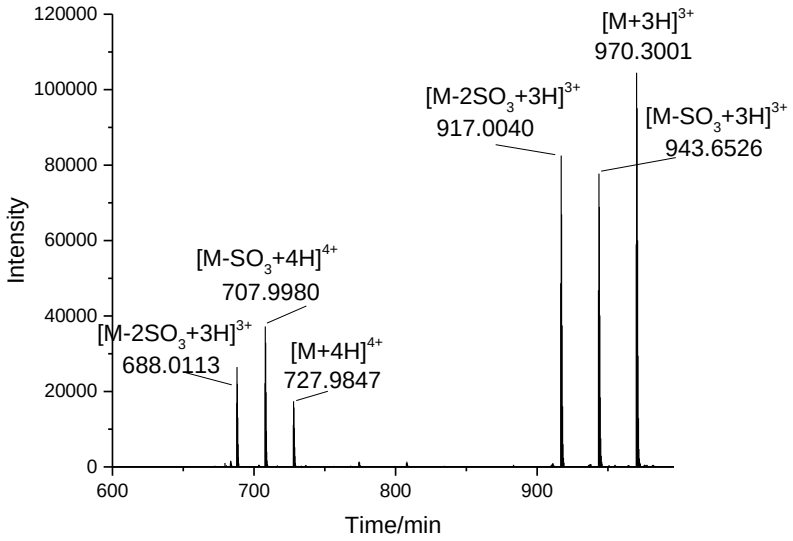
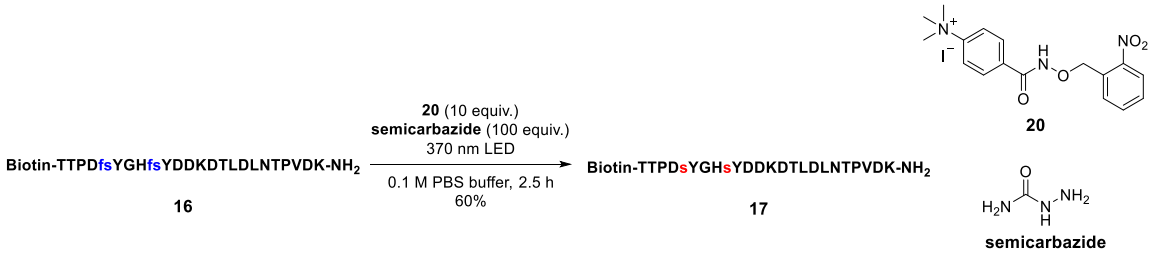


Figure S30. ESI-TOF mass spectrum for peptide **Biotin-TTPDsYGHsYDDKDTLDLNTPVDK-NH_2 (17)**

Light-mediated fluorosulfate decaging of 16



A 1.5 mL transparent glass vial was equipped with a stirring bar and 1 mM stock solution of substrate in 0.1 M PBS buffer (3.2 mg, 1 mM in 0.1 M PBS buffer, 983.1 μL). Reagent **20** (0.1 M in DMSO, 98.3 μL) and semicarbazide stock solution (1 M in 0.1 M PBS buffer, 98.3 μL) were added subsequently at room temperature. Then the whole reaction mixture was placed in the photoreactor to warm up to 37 °C. After two minutes, light irradiation was kept on for another 2.5 hours at the same temperature. The reaction mixture was monitored by analytical RP-HPLC until completion. The reaction mixture was passed through a PD-10 column using 0.1 M PBS buffer as eluent. Appropriate fractions were verified by LC-MS and combined to be dried over air blowing. Lots of salts was observed as residue. Minimum amount of water was used for re-dissolving the mixture, filtered, tested analytical RP-HPLC (Figure S31a), and loaded on semi-prep RP-HPLC purification. CH_3CN: 20 mM ammonium acetate buffer was employed as mobile phases. HPLC method was set as 0-5 min, 5% CH_3CN; linear gradient from 5%~20% CH_3CN over 29 min. The desired peptide was observed at about t_R = 16.0 min. Appropriate fractions were verified by LC-MS, combined, dried over air-blowing, lyophilized to give 1.8 mg white solid in a 60% yield. Analytical RP-HPLC of this material dissolved in water (CH_3CN: 20 mM ammonium acetate buffer mobile phases, linear gradient from 5%~60% CH_3CN over 40 min) showed a single peak at t_R = 15.0 min, monitored by UV absorbance at 230 nm (Figure S31b, bottom). This result was consistent with previously synthesized pure sulfated 22mer (Figure S31b, top). HRMS (ESI^-): calculated for $[C_{119}H_{181}N_{30}O_{49}S_3]^{3+}$ $(M+3H)^{3+}$ 970.3924, found 970.4073 (Figure S32).

The control experiments were performed following the same protocol described above. Analytical RP-HPLC was applied to monitor the reaction mixtures before and after their incubation under 37 °C for 2.5 hours without the UV irritation (in dark, Figure S31c) or the addition of reagent **20** (without **20**, Figure S31d) .

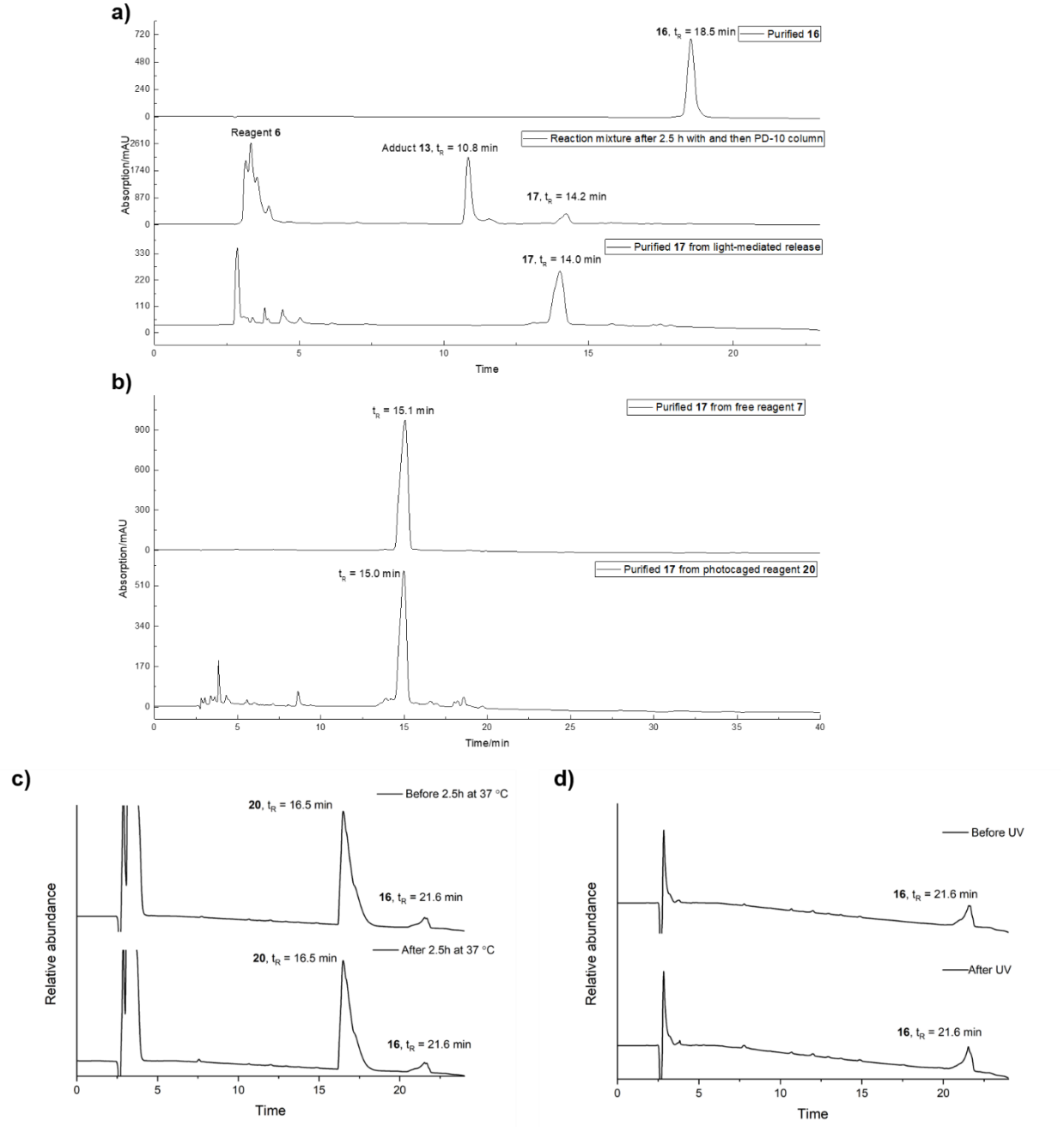


Figure S31. Analytical RP-HPLC chromatogram of **a**) photolytic reaction mixture. Method: CH_3CN: 20 mM ammonium acetate buffer mobile phases, linear gradient from 5%~33% CH_3CN from 0-20 min, 20-21 min 33%~95% CH_3CN, and 95% CH_3CN washing for another 2 min, monitored by the UV absorbance at 230 nm. **b**) Comparison of purified **22** from free (top) or photocaged (bottom) reagent. HPLC method: CH_3CN: 20 mM ammonium acetate buffer mobile phases, linear gradient from 5%~60% CH_3CN over 40 min, monitored by the UV absorbance at 230 nm. Control experiments **c**) without UV irradiation in dark and **d**) without photocaged reagent **20**. Method: CH_3CN: 20 mM ammonium acetate buffer mobile phases, linear gradient from 5%~33% CH_3CN over 40 min, monitored by the UV absorbance at 210 nm.

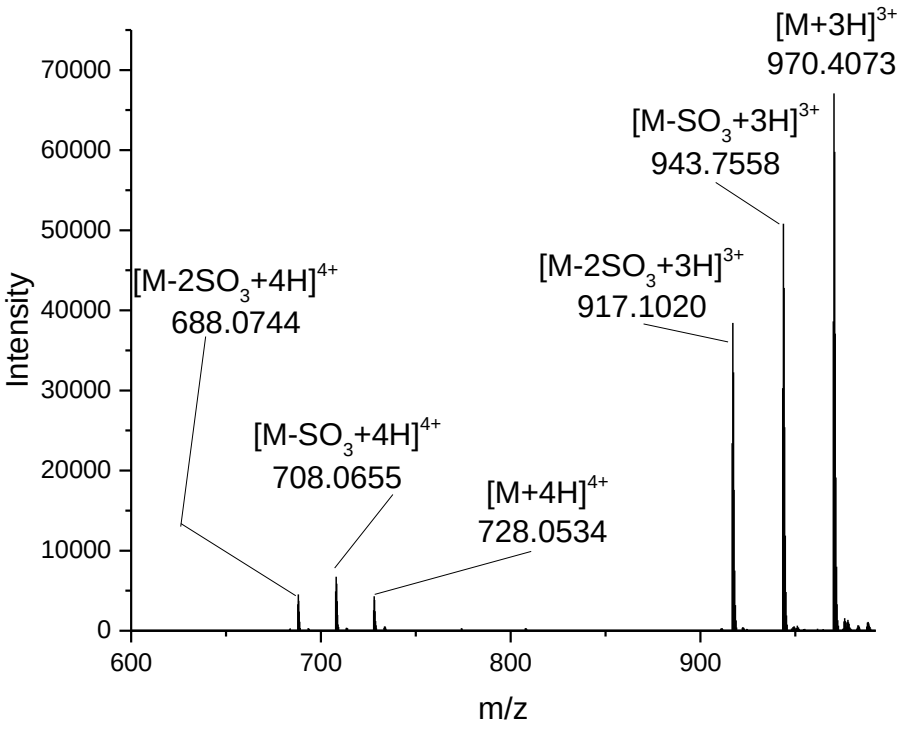


Figure S32. ESI-TOF mass spectrum for peptide **Biotin-TTPDsYGHsYDDKDTLDLNTPVDK-NH$_2$** (**17**) from light-mediated decaging reaction.

GDfsYDSMKEPCFR-NH$_2$ (**18**)

The synthesis of the title 12mer peptide **18** was separated in two steps. The first 10 amino acids (counted from the *C*-terminal) was constructed automatically following general procedure B with 204.0 mg Rink amide resin was loaded After the last coupling step and *N*-terminal Fmoc removal, the resin was washed with DMF and transferred to a Synthware vessel to perform the subsequent amino acids incorporation following general procedure A. Purification of the title peptide through the semi-prep RP-HPLC with eluent A (95% H_2O, 5% CH_3CN, and 0.1% TFA) and B (5% H_2O, 95% CH_3CN, and 0.1% TFA). The running method was set as: 0-5 min, 5% B; linear gradient 5-30 min, 5%~29% B. The product peak emerged at t_R = 26.5 min, monitored by the UV absorbance at 210 nm and appropriate fractions verified by LC-MS were combined. After concentration with air-blowing, the residue was

subjected for lyphilization to provide 50.0 mg of the title compound in 27% yield. Analytical RP-HPLC of this material dissolved in water (CH_3CN: 20 mM ammonium acetate buffer mobile phases, linear gradient from 5%~40% CH_3CN over 20 min; 20-21 min, 40%~95% CH_3CN in 1 min; 21-25 min, 95% CH_3CN) showed a single peak at t_R = 20.5 min under 210 nm wavelength (Figure S33). HRMS (ESI^+): calculated for $[C_{61}H_{92}FN_{17}O_{22}S_3]^{2+}$ $(M+2H)^{2+}$ 764.7869, found 764.7541 (Figure S34).

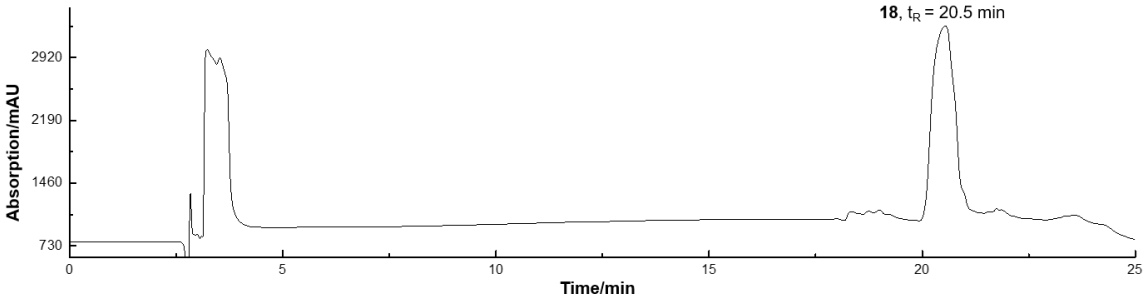


Figure S33. Analytical RP-HPLC chromatogram of purified fluorosulfopeptide **18** (CH_3CN: 20 mM ammonium acetate buffer mobile phases, linear gradient from 5%~40% CH_3CN over 20 min; 20-21 min, 40%~95% CH_3CN in 1 min; 21-25 min, 95% CH_3CN, monitored at 210 nm).

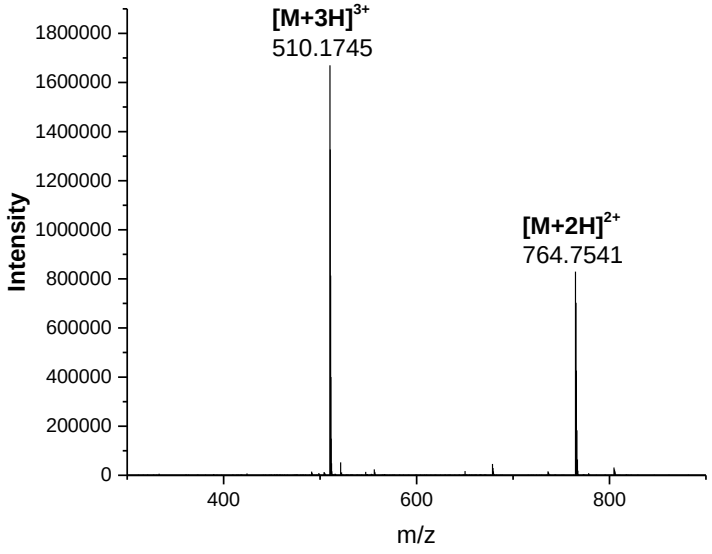


Figure S34. ESI-TOF mass spectrum for peptide **GDfsYDSMKEPCFR-NH_2** (**18**)

GDsYDSMKEPCFR-NH_2 (19)

Decaging of **18** was performed following general procedure C with 1.05 mg substrate dissolved in 0.1 M PBS buffer (561.2 μL) with 20 equivalents of 3-PHA (112.3 μL, 0.1 M stock solution in 0.1 M PBS buffer) as promoting reagent. 1 h later, the reaction was directly filtered and loaded on the Semi-Prep RP-HPLC for purification. CH_3CN:20 mM ammonium acetate buffer mobile phase was chosen as the mobile phase, and the method was set as 0-5 min, 5% CH_3CN; linear gradient 5-13min, 5%~25% CH_3CN. The desired peptide was afforded with a t_R = 21.0 min based on the UV absorbance at 210 nm. The appropriate fractions verified by LC-MS were combined fractions, concentrated by air blowing, and the residue was lyophilized to provide 0.72 mg white solid with an 81% yield. Analytical RP-HPLC of this material dissolved in 0.1 M PBS buffer (CH_3CN: 20 mM ammonium acetate buffer mobile phases, linear gradient from 5%~33% CH_3CN from 0-20 min; 20-21 min, 33%~95% CH_3CN; 21-23 min, keep washing with 95% CH_3CN for another two minutes) showed a single peak at t_R = 15.5 min, monitored by the UV absorbance at 210 nm (Figure S35, bottom). HRMS (ESI^+): calculated for $[C_{61}H_{93}N_{17}O_{22}S_3]^{2+}$ $(M+Na)^{2+}$ 763.7891, found 763.7057 (Figure S36).

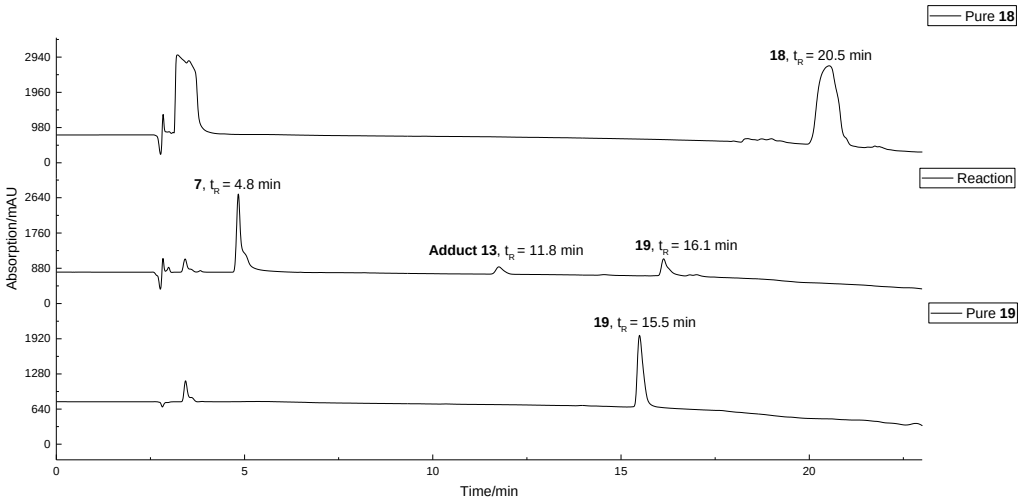


Figure S35. Analytical RP-HPLC chromatogram of purified fluorosulfopeptide **18** (top), decaging reaction of **18** with reagent **7** (middle), and purified sulfopeptide **19** (bottom) (CH_3CN: 20 mM ammonium acetate buffer mobile phases, linear gradient from 5%~33% CH_3CN from 0-20 min; 20-21 min, 33%~95% CH_3CN; 21-23 min, keep washing with 95% CH_3CN for another two minutes)

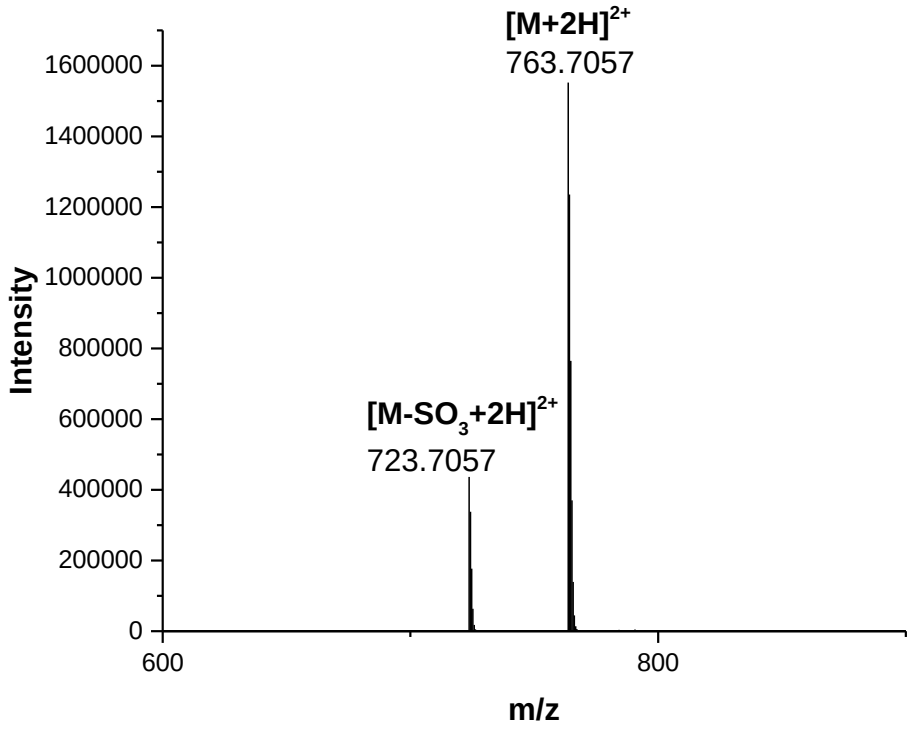


Figure S36. ESI-TOF mass spectrum for peptide **GDsYDSMKEPCFR-NH$_2$** (**19**)

GEPGAPIDYDEYGDSSEEVGGTPLHEIPGIRL-OH (**TTI01**)

The peptide **TTI01** were synthesized according to the Fmoc-based SPPS outlined in the general procedure B using the Fmoc-L-OH loaded Wang resin (0.025 mmol). After cleavage, the crude product was purified by HPLC and lyophilized to afford a white powder (45.0 mg, 0.0134 mmol, 54 % isolated yield). Purification of the **TTI01** through the semi-prep RP-HPLC with eluent A (95% H_2O, 5% CH_3CN, and 0.1% TFA) and B (5% H_2O, 95% CH_3CN, and 0.1% TFA). The running method was set as linear gradient from 15% to 35% within 30 min. The desired peptide was observed at t_R = 19.6 min. Analytical HPLC of this material dissolved in water showed a single peak at t_R = 9.3 min, monitored by the UV absorbance at 210 nm. Method for analytical HPLC was set as: A (95% H_2O, 5% CH_3CN, and

0.1% formic acid): B (5% H_2O, 95% CH_3CN, and 0.1% formic acid) as mobile phase, linear gradient from 5%~95% B from 0-12 min, 12-16 min with 95% B, and Agilent InfinityLab Poroshell 120 column (2.7 µm, 2.1 x 50 mm) was used (Figure S37). ESI-MS calculated for **TTI01** ($C_{147}H_{221}N_{37}O_{54}$): $[M + 3H]^{3+}$ m/z = 1123.8562; found: 1124.4307 (Figure S38).

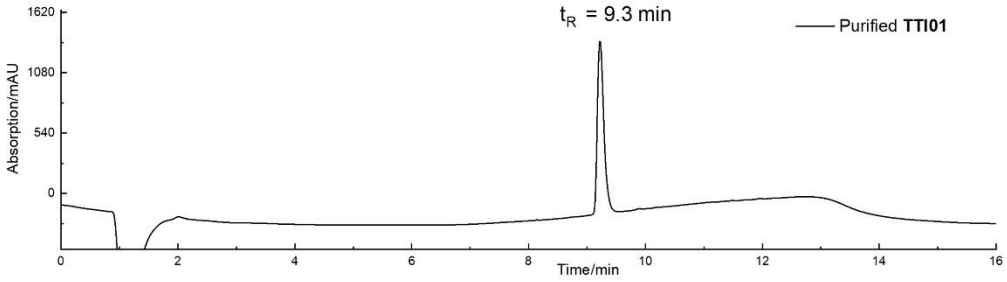


Figure S37. Analytical RP-HPLC chromatogram of (A (95% H_2O, 5% CH_3CN, and 0.1% formic acid): B (5% H_2O, 95% CH_3CN, and 0.1% formic acid) as mobile phase, linear gradient from 5%~95% B from 0-12 min, 12-16 min with 95% B) purified peptide **TTI01**.

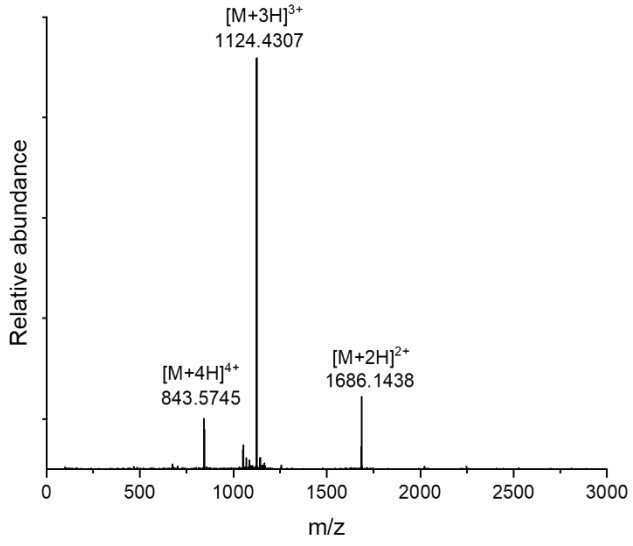


Figure S38. ESI-TOF mass spectrum for peptide **TTI01**.

GEPGAPIDfsYDEYGDSSEEVGGTPLHEIPGIRL-OH [**TTI02(fsY)**]

The synthesis of peptide **TTI02(fsY)** was separated in two steps. The coupling of first 20 amino acids (counted from the C-terminal) was constructed automatically following general procedure B with Fmoc-L-OH loaded Wang resin (0.025 mmol). After the last coupling step and *N*-terminal Fmoc removal, the resin was washed with DMF and transferred to a plastic vessel to perform the subsequent amino acids incorporation following general procedure A. Purification of the title peptide through the semi-prep RP-HPLC with eluent A (95% H_2O, 5% CH_3CN, and 0.1% TFA) and B (5% H_2O, 95% CH_3CN, and 0.1% TFA). The running method was set as: linear gradient 0-30 min, 20%~35% B. The product peak emerged at t_R = 26.5 min, monitored by the UV absorbance at 210 nm and appropriate fractions verified by LC-MS were combined. After concentration with air-blowing, the residue was subjected for lyophilization to provide 18.9 mg of the title compound in 22% yield. Analytical RP-HPLC of this material dissolved in water (mobile phase A: 95% H_2O, 5% CH_3CN, and 0.1% formic acid); mobile phase B:5% H_2O, 95% CH_3CN, and 0.1% formic acid; linear gradient from 5%~95% B from 0-12 min, 12-16 min with 95% B, and Agilent InfinityLab Poroshell 120 column) showed a single peak at t_R = 9.0 min, monitored by the UV absorbance at 210 nm (Figure S39). ESI-MS calculated for **TTI02(fsY)** ($C_{147}H_{220}FN_{37}O_{56}S$): $[M + 3H]^{3+}$ m/z = 1151.1737; found: 1151.7231 (Figure S40).

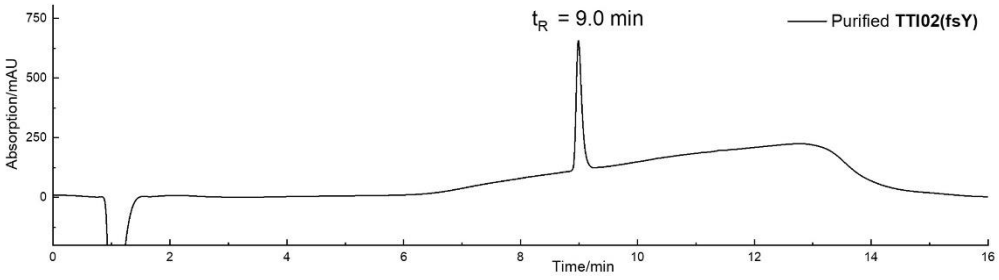


Figure S39. Analytical RP-HPLC chromatogram of purified peptide **TTI02(fsY)**. Method: A (95% H_2O, 5% CH_3CN, and 0.1% formic acid): B (5% H_2O, 95% CH_3CN, and 0.1% formic acid) as mobile phase, linear gradient from 5%~95% B from 0-12 min, 12-16 min with 95% B.

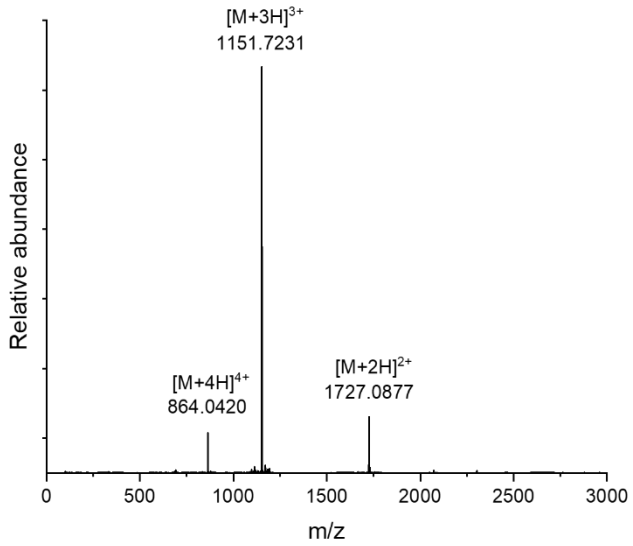


Figure S40. ESI-TOF mass spectrum for peptide **TTI02(fsY)**

GEPGAPIDYDEfsYGDSSEEVGGTPLHEIPGIRL-OH [**TTI03(fsY)**]

The synthesis of peptide **TTI03(fsY)** was separated in two steps. The coupling of first 20 amino acids (counted from the C-terminal) was constructed automatically following general procedure B with Fmoc-L-OH loaded Wang resin (0.025 mmol). After the last coupling step and *N*-terminal Fmoc removal, the resin was washed with DMF and transferred to a plastic vessel to perform the subsequent amino acids incorporation following general procedure A. Purification of the title peptide through the semi-prep RP-HPLC with eluent A (95% H_2O, 5% CH_3CN, and 0.1% TFA) and B (5% H_2O, 95% CH_3CN, and 0.1% TFA). The running method was set as: linear gradient 0-30 min, 20%~35% B. The product peak emerged at t_R = 19.7 min, monitored by the UV absorbance at 210 nm and appropriate fractions verified by LC-MS were combined. After concentration with air-blowing, the residue was subjected for lyophilization to provide 22.0 mg of the title compound in 26% yield. Analytical RP-HPLC of this material dissolved in water (mobile phase A: 95% H_2O, 5% CH_3CN, and 0.1% formic acid); mobile phase B:5% H_2O, 95% CH_3CN, and 0.1% formic acid; linear gradient from 5%~95% B from 0-12 min, 12-16 min with 95% B, and Agilent InfinityLab

Poroshell 120 column) showed a single peak at t_R = 9.1 min, monitored by the UV absorbance at 210 nm (Figure S41). ESI-MS calculated for **TTI03(fsY)** ($C_{147}H_{220}FN_{37}O_{56}S$): $[M + 3H]^{3+}$ m/z = 1151.1737; found: 1151.7433 (Figure S42).

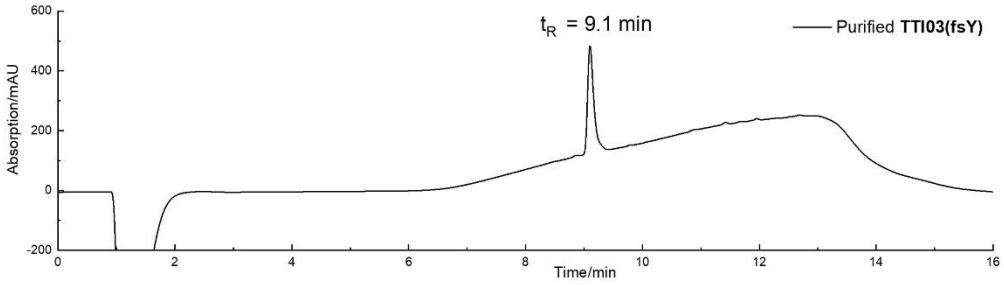


Figure S41. Analytical RP-HPLC chromatogram of purified peptide **TTI03(fsY)**. Method: A (95% H_2O, 5% CH_3CN, and 0.1% formic acid): B (5% H_2O, 95% CH_3CN, and 0.1% formic acid) as mobile phase, linear gradient from 5%~95% B from 0-12 min, 12-16 min with 95% B.

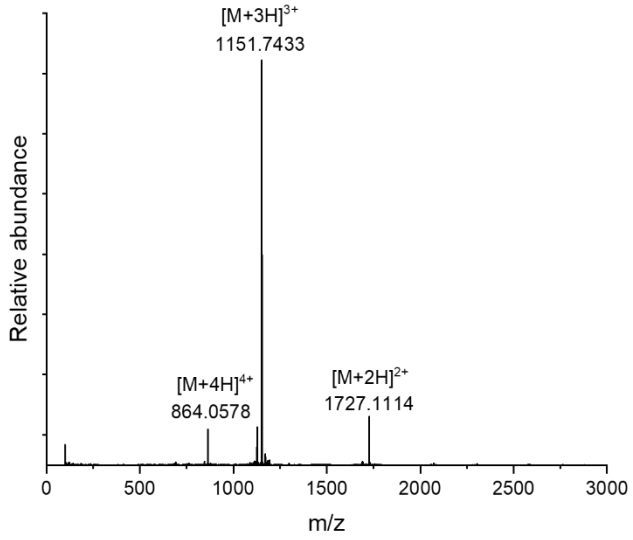


Figure S42. ESI-TOF mass spectrum for peptide **TTI03(fsY)**

GEPGAPIDfsYDEfsYGDSSEEVGGTPLHEIPGIRL-OH [**TTI04(fsY)**]

The synthesis of peptide **TTI04(fsY)** was separated in two steps. The coupling of first 20 amino acids (counted from the C-terminal) was constructed automatically following general procedure B with Fmoc-L-OH loaded Wang resin (0.025 mmol). After the last coupling step and *N*-terminal Fmoc removal, the resin was washed with DMF and transferred to a plastic vessel to perform the subsequent amino acids incorporation following general procedure A. Purification of the title peptide through the semi-prep RP-HPLC with eluent A (95% H_2O, 5% CH_3CN, and 0.1% TFA) and B (5% H_2O, 95% CH_3CN, and 0.1% TFA). The running method was set as: linear gradient 0-30 min, 20%~35% B. The product peak emerged at t_R = 21.7 min, monitored by the UV absorbance at 210 nm and appropriate fractions verified by LC-MS were combined. After concentration with air-blowing, the residue was subjected for lyophilization to provide 7.4 mg of the title compound in 8% yield. Analytical RP-HPLC of this material dissolved in water (mobile phase A: 95% H_2O, 5% CH_3CN, and 0.1% formic acid); mobile phase B:5% H_2O, 95% CH_3CN, and 0.1% formic acid; linear gradient from 5%~95% B from 0-12 min, 12-16 min with 95% B, and Agilent InfinityLab Poroshell 120 column) showed a single peak at t_R = 9.4 min under 210 nm wavelength (Figure S43). ESI-MS calculated for **TTI04(fsY)** ($C_{147}H_{219}F_2N_{37}O_{58}S_2$): $[M + 3H]^{3+}$ m/z = 1178.4911; found: 1179.0600 (Figure S44).

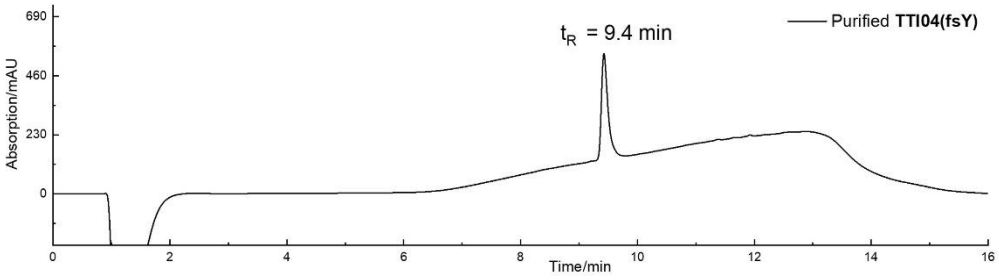


Figure S43. Analytical RP-HPLC chromatogram of purified peptide **TTI04(fsY)**. Method: A (95% H_2O, 5% CH_3CN, and 0.1% formic acid): B (5% H_2O, 95% CH_3CN, and 0.1% formic acid) as mobile phase, linear gradient from 5%~95% B from 0-12 min, 12-16 min with 95% B.

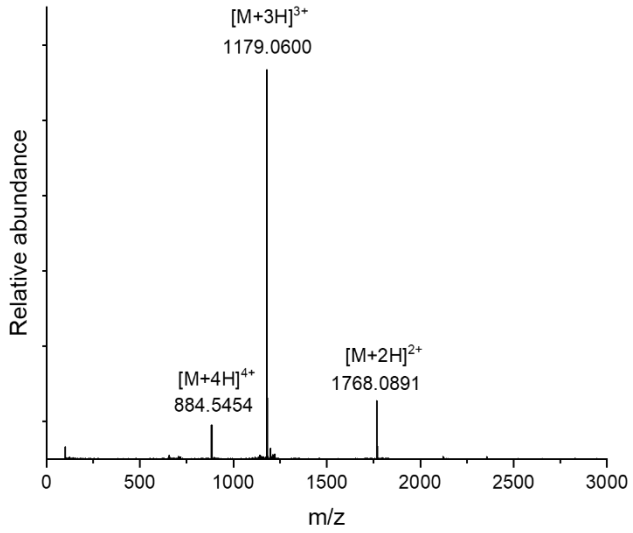


Figure S44. ESI-TOF mass spectrum for peptide **TTI04(fsY)**

GEPGAPIDsYDEYGDSSEEVGGTPLHEIPGIRL-OH [**TTI02(sY)**]

To a prewarmed 0.1 M PBS solution of **TTI02(FsY)** (1.2 mg, 1.0 equiv., 5.78×10^{-4} mmol, 578.0 μL, pH 7.2, 37 °C), a solution of **7** (0.1 M, 20.0 equiv., 1.16×10^{-3} mmol, 115.6 μL) was added. The resulting mixture was vigorously stirred at 37 °C for 3 h to give **TTI02(sY)** (Figure S45a). The reaction mixture was monitored by RP-HPLC and loaded directly on the semi-prep HPLC for purification with CH_3CN:20mM ammonium acetate buffer as mobile phase. After purification and lyophilization, **TTI02(sY)** white powder (1.1 mg, 3.18×10^{-4} mmol, 55 % isolated yield) was obtained. Purification of the **TTI02(sY)** through the Semi-prep RP-HPLC with CH_3CN and 20 mM ammonium acetate buffer. The running method was set as linear gradient from 15% to 55% CH3CN within 30 min. The desired peptide was observed at t_R = 10.6 min. Analytical HPLC of this material dissolved in water (CH_3CN: H_2O mobile phases, 0.1% TFA, linear gradient from 5%~35% CH_3CN over 30 min) showed a single peak at t_R = 16.7 min,

monitored by UV absorbance at 210 nm (Figure S45b). ESI-MS calculated for **TTI02(sY)** ($C_{147}H_{221}N_{37}O_{57}S$): $[M - 3H]^{3-}$ m/z = 1148.5084, found: 1149.2087 (Figure S46).

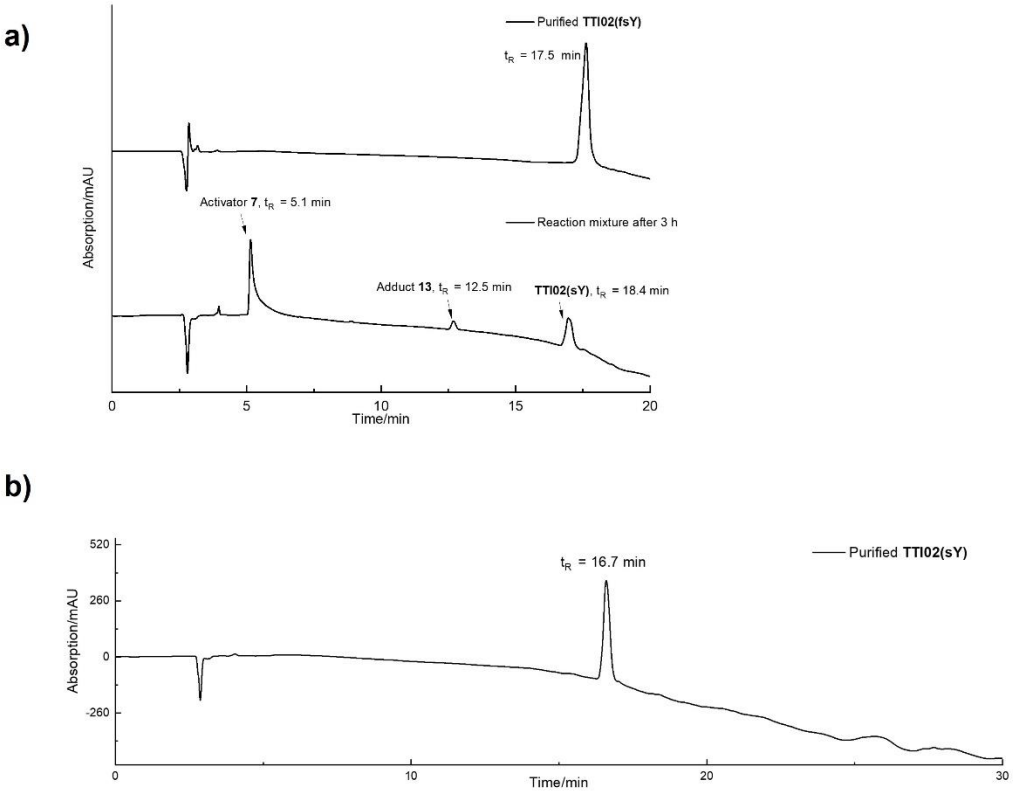


Figure S45. **a**) Analytical RP-HPLC chromatogram of decaging reaction mixture. Method: CH_3CN: 20 mM ammonium acetate buffer mobile phases, linear gradient from 5%~35% CH_3CN over 20 min. **b**) Analytical RP-HPLC chromatogram of purified **TTI02(sY)**. Method: CH_3CN: 20 mM ammonium acetate buffer mobile phases, linear gradient from 5%~35% CH_3CN over 30 min.

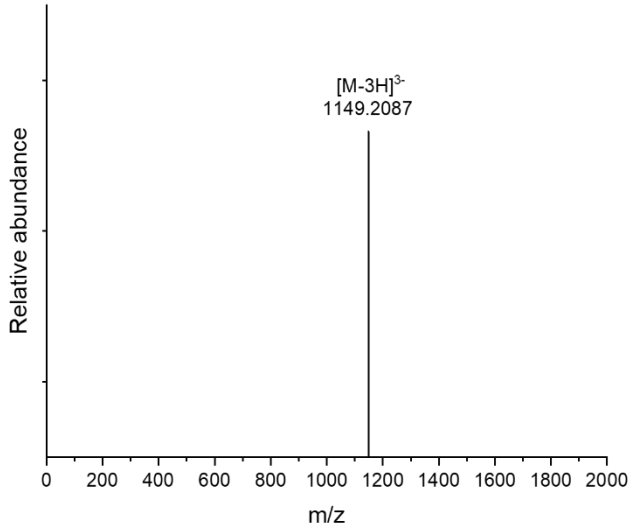


Figure S46. ESI-TOF mass spectrum for peptide **TTI02(sY)**

GEPGAPIDsYDEsYGDSSEEVGGTPLHEIPGIRL-OH [**TTI03(sY)**]

To a prewarmed 0.1 M PBS solution of **TTI03(fsY)** (1.04 mg, 3.01×10^{-4} mmol, 300.0 μL, pH 7.2, 37 °C), a solution of **7** (0.1 M, 20.0 equiv., 6.02×10^{-3} mmol, 60.0 μL) was added. The resulting mixture was vigorously stirred at 37 °C for 3 h to give **TTI03(sY)** (Figure S47a). The reaction mixture was monitored by RP-HPLC and loaded directly on the Semi-prep HPLC for purification with CH_3CN:20mM ammonium acetate buffer as mobile phase. After purification and lyophilization, **TTI03(sY)** white powder (0.6 mg, 1.73×10^{-4} mmol, 57 % isolated yield) was obtained. Purification of the **TTI03(sY)** through the Semi-prep RP-HPLC with eluent A (CH3CN) and B (20 mM ammonium acetate buffer). The running method was set as linear gradient from 15% to 55% within 30 min. The desired peptide was observed at t_R = 10.7 min. Analytical HPLC of this material dissolved in water (CH_3CN: 20 mM ammonium acetate buffer mobile phases, linear gradient from 5%~35% CH_3CN over 30 min) showed a single peak at t_R = 17.0 min, monitored by UV absorbance at 210 nm (Figure S47b). ESI-MS calculated for TTI03(sY) ($C_{147}H_{221}N_{37}O_{57}S$): $[M - 3H]^{3-}$ m/z = 1148.5084, found: 1148.8703 (Figure S48).

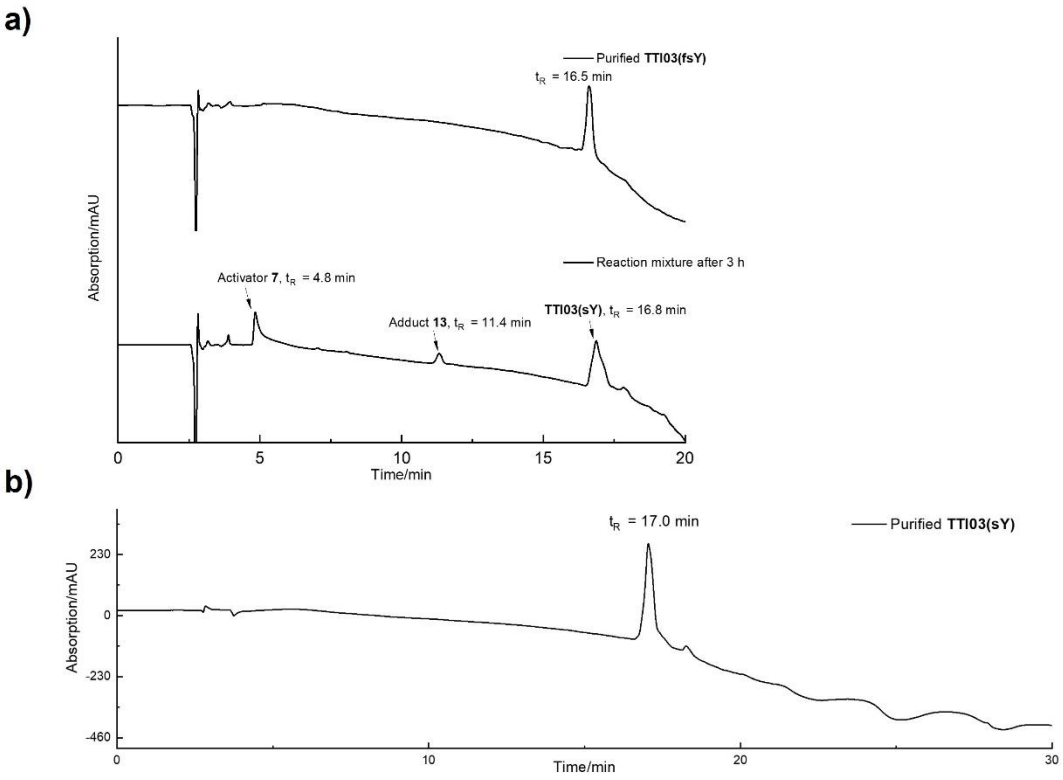


Figure S47. **a)** Analytical RP-HPLC chromatogram of decaging reaction mixture. Method: CH_3CN: 20 mM ammonium acetate buffer mobile phases, linear gradient from 5%~35% CH_3CN over 20 min. **b**) Analytical RP-HPLC chromatogram of purified **TTI03(sY)**. Method: CH_3CN: 20 mM ammonium acetate buffer mobile phases, linear gradient from 5%~35% CH_3CN over 30 min.

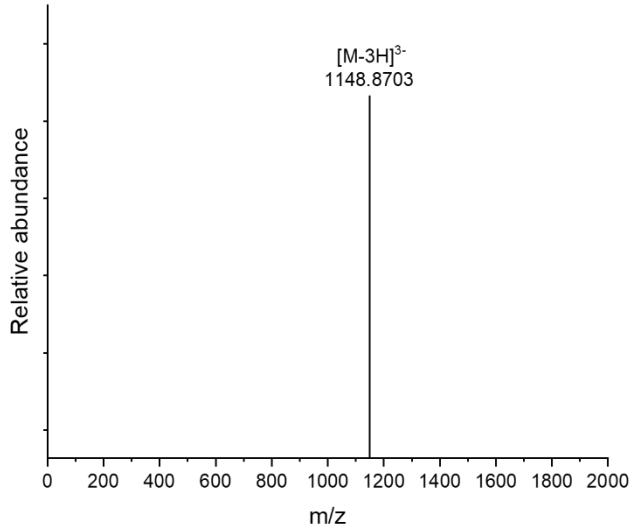


Figure S48. ESI-TOF mass spectrum for peptide **TTI03(sY)**

GEPGAPIDsYDEsYGDSSEEVGGTPLHEIPGIRL-OH [**TTI04(sY)**]

To a prewarmed 0.1 M PBS solution of **TTI04(fsY)** (1.14 mg, 3.22×10^{-4} mmol, 321.0 μL, pH 7.2, 37 °C), a solution of **7** (0.1 M, 20.0 equiv., 6.4×10^{-4} mmol, 64.0 μL) was added. The resulting mixture was vigorously stirred at 37 °C for 4 h to give **TTI04(sY)** (Figure S49a). The reaction mixture was monitored by RP-HPLC and loaded directly on the Semi-prep HPLC for purification with CH3CN:20mM ammonium acetate buffer as mobile phase. After purification and lyophilization, **TTI04(sY)** white powder (0.4 mg, 1.13×10^{-4} mmol, 35 % isolated yield) was obtained. Purification of the TTI04(sY) through the Semi-prep RP-HPLC with eluent A (CH_3CN) and B (20 mM ammonium acetate buffer). The running method was set as linear gradient from 15% to 55% within 30 min. The desired peptide was observed at t_R = 9.9 min. Analytical HPLC of this material dissolved in water (CH_3CN: 20 mM ammonium acetate buffer mobile phases, linear gradient from 5%~35% CH_3CN over 30 min) showed a single peak at t_R = 17.1 min under 210 nm wavelength (Figure S49b). ESI-MS calculated for **TTI04(sY)** ($C_{147}H_{221}N_{37}O_{60}S_2$): $[M - 3H]^{3-}$ m/z = 1175.1607, found: 1175.5287 (Figure S50).

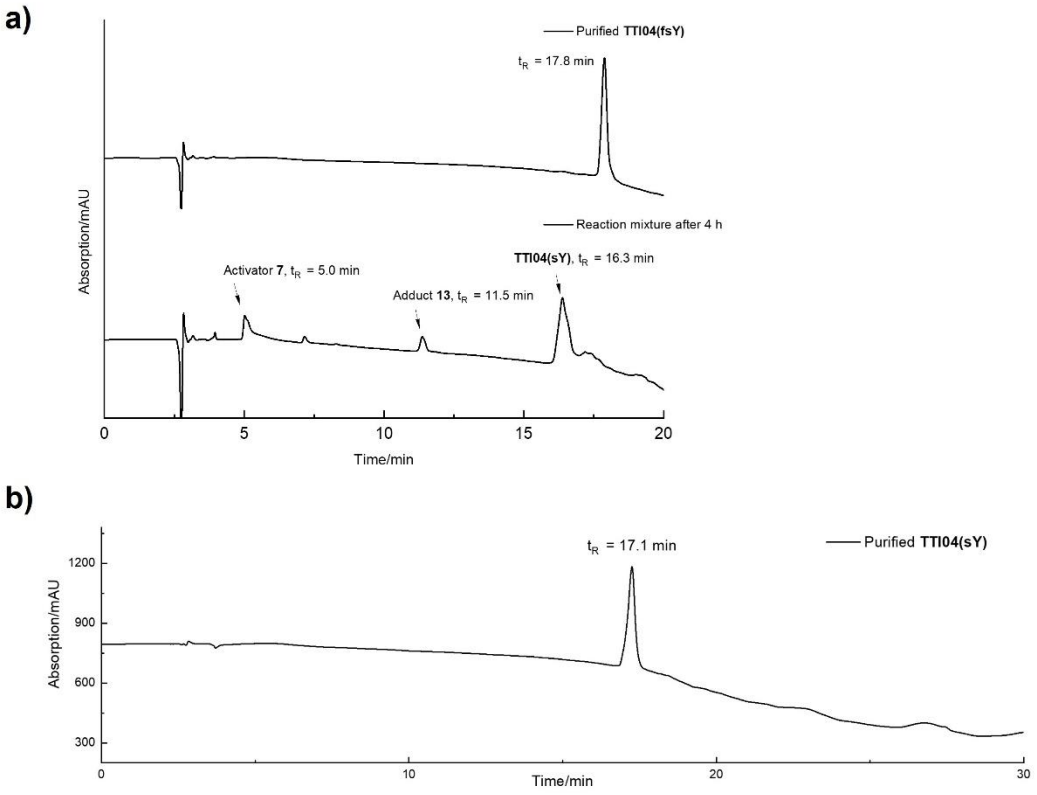


Figure S49. **a)** Analytical RP-HPLC chromatogram of decaging reaction mixture. Method: CH_3CN: 20 mM ammonium acetate buffer mobile phases, linear gradient from 5%~35% CH_3CN over 20 min. **b**) Analytical RP-HPLC chromatogram of purified **TTI04(sY)**. Method: CH_3CN: 20 mM ammonium acetate buffer mobile phases, linear gradient from 5%~35% CH_3CN over 30 min.

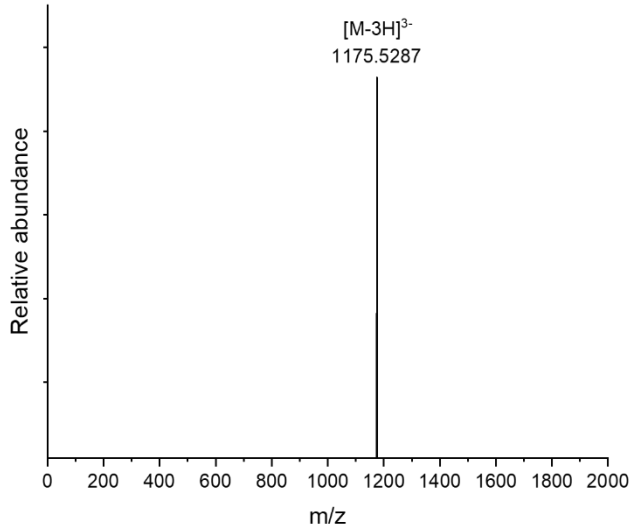


Figure S50. ESI-TOF mass spectrum for peptide **TTI04(sY)**

DfsYDHfsYDDKGSO(FAM)SENLYFQGSLPETGGS-NH_2 (22)

The synthesis of the title 27mer peptide **22** was separated in two steps. The first 22 amino acids (counted from the *C*-terminal) were constructed automatically following general procedure B with 713.0 mg Rink amide resin (0.3 mmol) was loaded. Among them, easily removed 4-methyltrityl (Mtt) group was used for the side-chain protection of L-ornithine. After the last coupling step and *N*-terminal Fmoc removal, the resin was washed with DMF, dichloromethane, ethyl ether, hexane subsequently (for each solvent 3 mL x 3 min), and dried to afford 3.56 g total resin. 1.78 g dried resin were weighted out and transferred to a Synthware vessel to perfrom the subsequent amino acids incorporation following general procedure A. Before the last Fmoc-removal step, TFA:TIPS:DCM = 1:2:97 cocktail was applied to the beads 8 mL x 30 min x 3 times to remove the Mtt protection. Then, the resin was washed by DCM (5 mL x 3 min x 2), methanol (5 mL x 3 min x 2), DCM (5 mL x 3 min x 2), 1% DIPEA in DMF (5 mL x 5 min x 2), and DMF (5 mL x 3 min x2). The fluorescently labeling 5/6-Carboxyfluorescein (FAM, 564.5 mg, 1.50 mmol) was preactivated with *N, N, N′,N′*-tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU, 568.9 mg, 1.50 mmol) and *N,N*-diisopropylethylamine (DIPEA, 193.9 mg, 261.3 μL, 1.50 mmol) and then transferred to the resin in the Syntheware tube to perform coupling for 2 hours under nitrogen bubling. Then standard washing

(DMF 3 min x 3), Fmoc removal (20% 2-methylpiperidine 5 min x 3), final deprotection and cleavage (TFA:TIPS:H_2O = 95:2.5:2.5, 30 min x 3), concentration under vaccum, precipitation out of ether. The crude was finally dissolved in a mixture of CH_3CN and water, filtered. Purification of the title peptide through the Prep RP-HPLC with eluent A (95% H_2O, 5% CH_3CN, and 0.1% TFA) and B (5% H_2O, 95% CH_3CN, and 0.1% TFA). The running method was set as: 0-5 min, 5% B; linear gradient 5-30 min, 5%~45% B. The product peak emerged at t_R = 30.5 min, monitored by the UV absorbance at 210 nm and appropriate fractions verified by LC-MS were combined. After concentration with air-blowing, the residue was subjected for lyphilization to provide 89.0 mg of the title compound in 18% yield. Analytical RP-HPLC of this material dissolved in water (CH_3CN: 20 mM ammonium acetate buffer mobile phases, 0-5min, 5% CH_3CN; 5-40 min, linear gradient from 5%~55% CH_3CN over 35 min) showed a single peak at t_R = 24.0 min under 230 nm wavelength (Figure S51). HRMS (ESI^+): calculated for $[C_{150}H_{194}F_2N_{34}O_{59}S_2]^{2+}$ $(M+2H)^{2+}$ 1759.1329, found 1759.2822 (Figure S52).

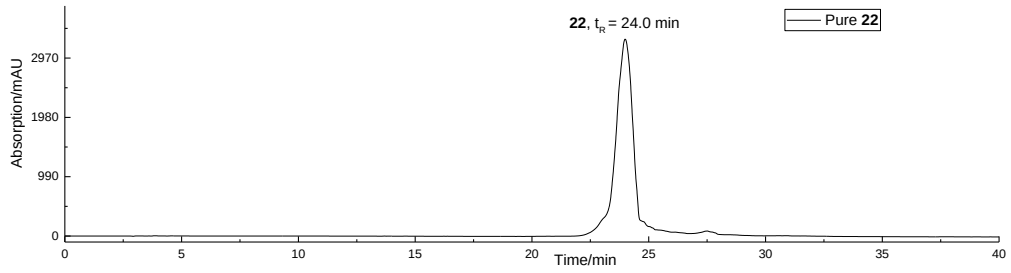


Figure S51. Analytical RP-HPLC chromatogram of purified fluorosulfopeptide with fluorescent label **22** (CH_3CN: 20 mM ammonium acetate buffer mobile phases, 0-5min, 5% CH_3CN; 5-40 min, linear gradient from 5%~55% CH_3CN over 35 min, monitored by the UV absorbance at 210 nm).

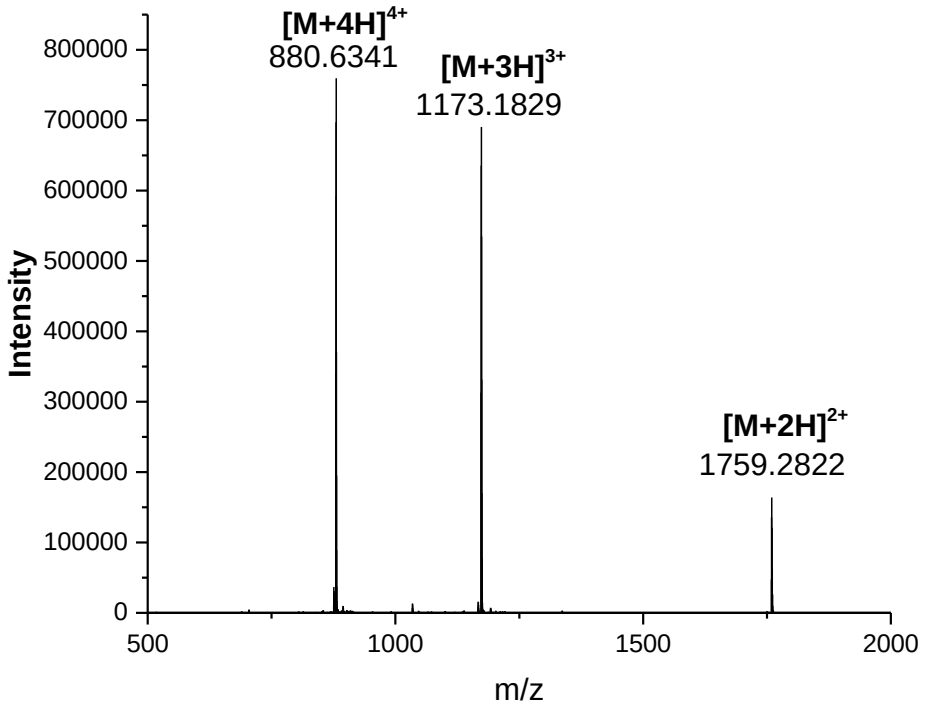


Figure S52. ESI-TOF mass spectrum for peptide **DfsYDHfsYDDKGSO(FAM)SENLYFQGSLPETGGS-NH_2 (22)**

Protein Expression via ncAA mutagenesis for fsY incorporation.

Plasmids construction

The pUltra-Opt-MmPylT-TAGc, pEvolT5-EcY-sfGFP151TAG and pEvolT5-EcY-sfGFP3TAG were used as previously reported.[6] The pUltra-FsTyrRS-OptMmPylT containing FsTyrRS/tRNA pair for fsY incorporation was constructed by insertion of mutated fragment of *Methanosarcina mazei* PylRS (A302I, L305T, N346T, C348I, Y384L and W417K) into the plasmid pUltra-Opt-MmPylT-TAGc via Gibson Assembly.[7]

Fluorescence visualization of cells after sfGFP expression

E. coli B95 cells containing plasmids (1. pET22b-T5.lac-sfGFP, 2. pUltra-FsTyrRS-OptMmPylT, and 3. pET22b-T5.lac-sfGFP-151TAG) were grown in LB medium at 37 °C overnight. Then, the cell culture was diluted 100-fold with LB with corresponding antibiotics and cultured in the shaker at 37°C. When the OD600 of the cell

culture reached 0.6, protein expression was induced by the addition of 1 mM IPTG and/or non-cannonial amino acids (1. +/-IPTG; 2. +IPTG, +/-fsY). After growth 16 hours at 30 °C, cell pellets from 1 mL cell culture were collected by centrifugation at 4,750 × g for 10 min at 4 °C and resuspended in 1 mL PBS buffer. Then, 100.0 µL resuspension culture transferred to 96-well black clear bottom assay plates. The sfGFP fluorescence was measured using a bioreader (SpectraMax GeminiEM, Molecular Devices, Excitation: 488/10, Emission: 534/10, cutoff: 515). The normalized fluorescence was obtained by dividing by OD600 value.

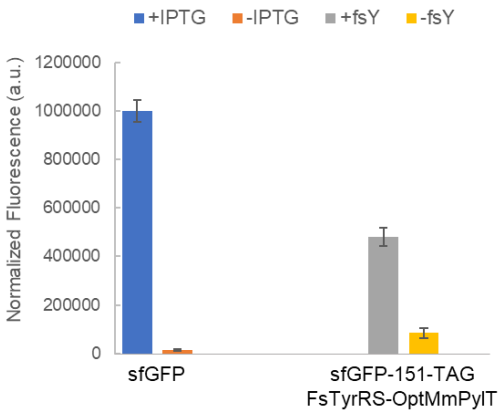


Figure S53. Normalized sfGFP fluorescence. Left: sfGFP-wt expression in the presence/absence of IPTG; Right: sfGFP-151-fsY expression in the presence of IPTG and in the presence/absence of fsY.

Expression of sfGFP-151-fsY and sfGFP-3-fsY

E. coli B95 cells, cotransformed with pUltra-FsTyrRS-OptMmPylT and pET22b-T5.lac-sfGFP-151TAG (or pET22b-T5.lac-sfGFP-3TAG) were grown in LB medium at 37 °C overnight. Then, the cell culture was diluted 100-fold with 50 mL LB with antibiotics (1 µg/mL Spectinomycin and 3 ug/mL Ampicillin) and cultured in the shaker at 37°C. When the OD600 of the cell culture reached 0.6, protein expression was induced by the addition of 1 mM IPTG and fsY. After growth 16 hours at 30 °C, the protein was extracted and purified following the general procedure below.

Expression of sfGFP-151-sY

C321-ATMY cells, cotransformed with pBK-sulfo-VGM-CSK and pET22b-T5.lac-sfGFP-151TAG were grown in LB medium at 37 °C for 18 hours.[8] Then, the cell culture was diluted 100-fold with 50 mL LB with antibiotics (1 µg/mL Chloramphenicol, Kanamycin and Spectinomycin) and cultured in the shaker at 37°C. When the OD600 of the

cell culture reached 0.6, protein expression was induced by the addition of 1 mM IPTG and sulfotyrosine (sY). After growth 16 hours at 30 °C, the protein was extracted and purified following the general procedure below.

Expression of sfGFP-wt

E. coli B95 cells, transformed pET22b-T5.lac-sfGFP were grown in LB medium at 37 °C overnight. Then, the cell culture was diluted 100-fold with 50 mL LB with antibiotics (3 ug/mL Ampicillin) and cultured in the shaker at 37°C. When the OD600 of the cell culture reached 0.6, protein expression was induced by the addition of 1 mM IPTG. After growth 16 hours at 30 °C, the protein was extracted and purified following the general procedure below.

The general protocol of protein purification

Cell pellets containing specific protein were collected by centrifugation at 4,750 × g for 10 min at 4 °C and resuspended in cool lysis buffer (5 mL B-Per, 0.5 μL universal nuclease and 50.0 μL protease inhibitor). The cool mixture was put in 4 °C shaker for 1 hour and then centrifuged at 4,750 × g for 10 min. The supernatant was collected and dilute with 100 mL equilibrium buffer (20 mM Na_2HPO_4, 300 mM NaCl, 10 mM imidazole, pH 7.4). The equilibrium solution was loaded on chromatography column with pre-equilibrated Ni-NTA agarose resin (1 mL) and washed with 150 mL of wash buffer (20 mM Na_2HPO_4, 300 mM NaCl, 25 mM imidazole, pH 7.4). After the addition of 3 mL elution buffer (20 mM Na_2HPO_4, 300 mM NaCl, 300 mM imidazole, pH 7.4), the fractions of eluates with fluorescence were collected and subjected to concentration and buffer exchange with 0.1M PBS buffer using MilliporeSigma™ Amicon™ Ultra-4 Centrifugal Filter. The protein was confirmed by SDS-PAGE and High-resolution Mass spectrometric analysis (Figure S54).

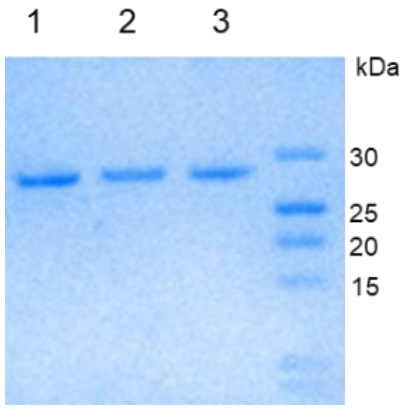


Figure S54. SDS-PAGE analysis of purified sfGFP-wt (lane 1), sfGFP-151-sY (lane 2) and sfGFP-151-fsY (lane 3).

Detailed Procedure for Supplementary Results

Stability test of fluorosulfotyrosine (fsY) and fluorosulfohexpeptide 1 in cell lysate

Cell lysate preparation and reaction setup:

HEK-293T cells were split in two different plates (100 mm dish) in adherent culture and grown in 37 °C incubator. the cell monolayer was gently washed by PBS buffer after 48 h. Cell lysate prepared by both chemical and physical methods. The chemical method involves homogenizing cells in RIPA buffer. Cell debris was removed centrifugation to afford a Chem cell lysate (pH = 7.01). In a similar manner, cell lysate can also be prepared by physically disrupting the cell membrane via 4 min sonication (rest for 10 s for every minute) in 4 °C cold room to afford a Phy cell lysate (pH = 7.41). Human serum (1.00 mL) pH was adjusted to 7.40 by 0.1 M PBS buffer (pH = 3.17, 60.0 μL) and used without any further treatment.

In a 200 μL Eppendorf tube, freshly prepared stock solution of fsY (50 mM in DMSO, 2.0 μL) or **1** (50 mM in DMSO, 2.0 μL) was added to the cell lysis solution (98.0 μL). The resulting mixture was sealed and placed on the shaker in the 37 °C constant warm room. Meanwhile, a parallel control with only cell lysate was also set up at the same conditions. After 48 hours of incubation, the control cell lysate was directly transferred into the Microcon-10 kDa centrifuge filter and filtered with 15000 rpm for 15 min at 4 °C. For the reaction containing the substrates, a stock solution of tryptophan (Trp, 50 mM in DMSO, 2.0 μL, well-mixed before use) was added as internal standard and flicked to mix them well. The resulting mixture was transferred for centrifuge filtration with Microcon-10kDa centrifuge filter with 15000 rpm for 15 min at 4 °C. The filtrate was subjected for analytical RP-HPLC and high-resolution LC-MS (Figure S1).

The pH dependence of the fsY decaging reaction mediated by NHS

Stock solution preparation and reaction setup:

Substrate **1** was dissolved in 0.1 M PBS buffer at 1 mM, pH = 6.70. NHS was weighted and dissolved in small amount of 0.1 M PBS buffer, and the final pH and concentration were adjusted to 5.66 to 12.42 by 1 M HCl and NaOH.

In a 1.5 mL vial with a stirring bar, stock solution of **1** (1 mM, 150.0 μL, 0.15 μmol) and NHS (0.1 M, 30.0 μL, 30 μmol) was added subsequently. The resulting reaction mixture was allowed to stir at the 37 °C for 2 h and its pH was measured then filtered, and subjected for analytical RP-HPLC.

Hydrolysis of NHS in deuterated water by titrating NaOH

Reaction set up:

In a 4 mL glass vial equipped with a stirring bar, NHS (10.9 mg, 0.094 mmol) was dissolved in deuterated water (D_2O, 932.9 μL) at room temperature. A stock solution (10 M in D_2O, 21.4 μL, 1.0 equivalent) was added and the resulting reaction mixture was allowed to stir at the same temperature for 1 h. Then the reaction was transferred for NMR tests. Same set up for 1.5, 2.0, and 5.0 equivalents of NaOH. Detected pD = pH + 0.4, and pH was obatined from the pH meter.[9]

Decaging of fsY in 1 mediated by the silyl-protected reagent 11

Stock solution preparation and reaction setup:

Stock solution of the substrate and the silyl-protected reagent were freshly prepared. Fluorosulfohexpeptide **1** was dissolved in 0.1 M PBS buffer (1 mM, pH = 7.23). The reagent **11** were dissolved in DMSO to afford 0.1 M stock solution of it. Potassium fluoride was dissolved in 0.1 M PBS buffer to give 1.0 M stock solution of it.

In a 1.5 mL vial with a stirring bar, stock solution of **1** (0.15 μmol, 150.0 μL) was added and warmed up in the 37 °C for 2 min. Simultaneously, stock solutions of the silyl-protected reagents **11** and additives KF were also warmed up to the same temperature. When only the reagent **11** (0.1 M, 30.0 μL, 20 equiv.) was added, there's no trace of the released product (Table S4, entry 1). When both the reagent **11** (0.1 M, 30.0 μL, 20 equiv.) and additive potassium fluoride (KF, 1.0 M, 3.0 μL) were added, a 41% yield of **2** was observed in 30 minutes.

Compatibility test of the decaging reaction of 1 in the presence of free amino acids

Stock solution preparation and reaction set up:

Fluorosulfohexpeptide **1** (3.3 mg) was dissolved in 0.1 M PBS buffer (pH = 7.64, 3.60 mL) to provide 1 mM stock solution of **1**, pH = 7.31. Reagent **7** was dissolved in 0.1 M PBS buffer with 1 M HCl and NaOH for pH adjustment to afford 0.1 M stock solution of **7**, pH = 7.39. All the free amino acids except tyrosine was dissolved in 0.1 M PBS buffer with 1 M HCl and NaOH solution for pH adjustment to give 0.1 M final concentration of the free amino acids and pH was maintained between 7.0-7.4. Tyrosine showed a very poor solubility even in DMSO. A suspension of tyrosine (0.1 M in DMSO) was made and used directly in the reaction.

To a solution of **1** in 0.1 M PBS buffer (1 mM, 150.0 μL) were added reagent **7** (0.1 M, 30.0 μL, 20 equiv.) and free amino acid stock solution (0.1 M, 30.0 μL, 20 equiv.) in the warm room at 37 °C. The resulting reaction mixture was allowed to stir for 30 min, filtered, and subjected for analytical HPLC.

LC-MS monitoring of $H_2{}^{18}O$ isotope labeling experiment

Stock solution preparation and reaction setup:

0.1 M PBS buffer ($H_2{}^{18}O$) was freshly prepared with 10.2 mg of Na_2HPO_4 and 3.5 mg of NaH_2PO_4 in $H_2{}^{18}O$ (97 atom %). 0.1 M stock solution of **1**, **2**, and **7** in DMSO was also prepared.

To a 1.5 mL vial equipped with a stirring bar and 0.1 M PBS buffer ($H_2{}^{18}O$, 177.1 μL) was added stock solution of **1** in DMSO (0.1 M, 1.0 μL) at 37 °C. The resulting reaction mixture was allowed to stir for 30 min, filtered, and subjected for LC-MS analysis.

Similarly, to a 1.5 mL vial equipped with a stirring bar and 0.1 M PBS buffer ($H_2{}^{18}O$, 177.1 μL) were added stock solution of **2** and **7** in DMSO (0.1 M, 1.0 μL) in the warm room at 37 °C. The resulting reaction mixture was allowed to stir for 30 min, filtered, and subjected for LC-MS analysis.

Compatibility of semicarbazide with fsY-sontaining substrates 1

Reaction set up:

To a solution of **1** in 0.1 M PBS buffer (1 mM, 0.15 μmol, 150.0 μL) was added the stock solution of semicarbazide (1 M in 0.1 M PBS buffer, pH = 7.38, 30 μmol, 30.0 μL) in the warm room at 37 °C. The resulting reaction mixture was allowed to stir for 2 h, filtered and subjected for analytical HPLC. After another 22 h standing at room temperature, the sample subjected for LC-MS analysis.

Thrombin inhibition by in situ decaged TTI peptides

Tos-Gly-Pro-Arg-*p*-nitroanilide (Cayman Chemicals) was used as the chromogenic substrate for the inhibition of the amidolytic activity of human-α-thrombin (Invitrogen). The assays were performed in the Tris buffer (50mM Tris-HCl pH 8.0, 50 mM NaCl), which contains 100 mM substrate and various concentrations of the TTI peptides. After the addition of activation buffer (1 mg/mL BSA, 0.2 nM human-α-thrombin), The 96-well microtiter plates were incubated at 37 °C for 45 min and then monitored UV-Vis absorbance at 405 nm on a multi-mode microplate reader. (The data were corrected by subtracting the absorbance value of corresponding background). All measurements were repeated three times independently and then fitted with the Morrison equation (Williams and Morrison, 1979). Inhibition constants (K_i) and standard errors were calculated using this equation by Prism 6 (GraphPad Software).

Thrombin inhibition assay of **TTI04(fsY)** with photocaged **21** at specific concentration

To a prewarmed 0.1 M PBS solution of **TTI04(fsY)** (0.05 mg, 1.45×10^{-5} mmol, 50.0 μL, pH 7.2, 37 °C), a solution of **21** (0.1 M, 40.0 equiv., 2.90×10^{-4} mmol, 5.8 μL) and semicarbazide (1 M, 400 equiv., 2.90×10^{-3} mmol, 5.8 μL) were added. The resulting mixture was vigorously stirred at 37 °C and 370 nm UV irradiation for 4 h to give **TTI04(sY)**. The peptide substrate **TTI04(fsY)**, **21** + UV, **TTI04(fsY)** + **21**, **TTI04(fsY)** + UV, **TTI04(fsY)** + **21** + UV reaction mixture were diluted to 3.7 nM (peptide concentration) for thrombin inhibition activity test.

Tandem MS analysis of sfGFP-151-fsY and sfGFP-3-fsY

100 μg sfGFP-151-fsY (or sfGFP-3-fsY) was added in 100.0 μL of 100% solution of trichloroacetic acid in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH = 7.50). Vortex the mixture vigorously and then freeze at -80 °C for 1 hour. Then, the thawed sample was centrifuged at 15000 rpm for 10 min. After the removal of supernatant, the pellet was resuspended in 500.0 μL of cool acetone. Afterwards the mixture was centrifuged at 15000 rpm for 10 min. The pellet was saved and dried. Then, the pellet was resuspended in 30.0 μL of 8 M urea in PBS buffer. After the addition of 70.0 μL of 100 mM ammonium bicarbonate and 1.5 μL of 1 M DTT, the sample was incubated at 65 °C for 15 min. Then, 2.5 μL of 500 mM iodoacetamide was added and incubated at room temperature for 30 min before the addition of 120.0 μL PBS buffer. 4.0 μL trypsin solution (0.5 μg/μL in trypsin buffer) and 2.5 μL of 100 mM $CaCl_2$ were added to digest the protein. After the overnight incubation at 37 °C, the digestion was quenched by 10.0 μL of formic acid. Then the sample was centrifuged at 15000 rpm for 15 min. The supernatant was collected and underwent mass spectra analysis.

LC-MS/MS analysis was performed on an Orbitrap Exploris 240 mass spectrometer with Xcalibur v4.4 (Thermo Scientific) coupled to a Dionex Ultimate 3000 RSLCnano system. The MS sample was desalted on a SepPak C18 cartridge (Waters) and dried on Speedvac. Desalted peptides were resuspended in Buffer A (100% H_2O, 0.1% formic acid) and 5.0 μL of the resulting solution were injected onto a 4 cm Acclaim PepMap 100 C18 column. Then peptides were eluted onto an Acclaim PepMap RSLC and separated with a 1-hour gradient from 5% to 25% of Buffer B (20% H_2O, 80 % CH_3CN, 0.1% formic acid) in Buffer A at a flow rate of 0.3 μL/min. The spray voltage was set to 2.1 kV. One full MS1 scan (120,000 resolution, 350-1800 m/z, RF lens 65%, AGC target 300%, automatic maximum injection time, profile mode) was obtained every 2 secs with dynamic exclusion (repeat count 2, duration 10 s), isotopic exclusion (assigned), and apex detection (30% desired apex window) enabled. A variable number of MS2 scans (15,000 resolution, AGC 75%, maximum injection time 100 ms, centroid mode) were obtained between each MS1 scan based on the highest precursor masses, filtered for monoisotopic peak determination, theoretical precursor isotopic envelope fit, intensity (5E4), and charge state (2-6). MS2 analysis consisted of the isolation of precursor ions (isolation window 2 m/z) followed by higher-energy collision dissociation (HCD) (collision energy 30%). The MS data was analyzed by Proteome Discoverer V2.4 software package and searched using the SequestHT and Percolator Algorithms. Trypsin was specified as the protease with a maximum of 2 missed cleavages. Peptide precursor mass tolerance was set to 10 ppm with a fragment mass tolerance of 0.02 Da. Fluorosulfotyrosine (+82.048), oxidation of

methionine (+15.995) as well as acetylation (+42.011) and/or methionine-loss (+131.040) of the protein N-terminus were set as dynamic modifications. Cysteine alkylation (+57.021) was set as a static modification. The false discovery rate (FDR) for peptide identification was set to 1%. The mass-spectrometry data were collected as two technical replicates from one biological replicate.

High-resolution Orbitrap mass spectrometry analysis of sfGFP-151-fsY and sfGFP-3-fsY decaging

Decaging with free reagent **7**: To a 500 µL Eppendorf tube with fsGFP-151-fsY or fsGFP-3-fsY (299.7 µL, 1 µg/µL in 0.1 M PBS buffer) was added reagent **7** (10 mM in 0.1 M PBS buffer, 33.0 µL, 30 equiv.). The resulting reaction mixture was covered with aluminum foil and placed on 37 °C warm room shaker. 4 h later, the reaction was purified by the following sample preparation for Orbitrap MS analysis procedure.

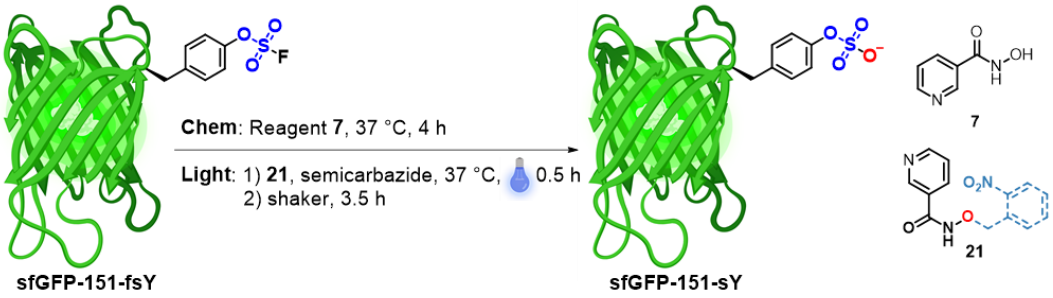
Decaging with photocaged reagent **21** and semicarbazide: To a 500 µL Eppendorf tube with fsGFP-151-fsY (299.7 µL, 1 µg/mL in 0.1 M PBS buffer) was added reagent **21** (10 mM in 0.1 M PBS buffer, 33.0 µL, 30 equiv.) and additive semicarbazide (1 M in 0.1 M PBS buffer, 3.3 µL, 300 equiv.). The resulting reaction mixture was irradiated with 370 nm LED light (40 W, 100%) for 1 h. Subsequently, the whole setup was covered with aluminum foil and placed on 37 °C warm room shaker. After another 3 h, the reaction was purified by the following sample preparation for Orbitrap MS analysis procedure.

Sample preparation for Orbitrap MS analysis**:** The samples were cleaned up with PD-10 desalting columns packed with Sephadex G-25 resins **(**Cytiva Life Sciences) followed by applying Amicon Ultra 0.5 mL Contrifugal filter with 10K cut-off (EMD Millipore, Billerica-MA, USA) with water.

Orbitrap MS analysis: The samples were directly detected on an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific, San Jose, California, USA) coupled with a TriVersa NanoMate (Advion, Ithaca, NY). Data acquisition was performed in positive ion mode. MS scans were acquired from 500-1500 *m/z* at a resolution of 240,000 @200 *m/z* with an AGC target of 100% and a maximum injection time of 100 ms. For each scan 10 µscans were recorded.

Orbitrap MS data processing: MS data were deconvoluted on a Thermo Scientific BioPharma Finder software Version 5.0. Deconvolution Algorithm was set to Xtract (Isotopically Resolved). Output Mass Range was defined from 27,000 to 29,000. The monoisotopic masses of neutral mass (M) were set as Output Mass.

Anti-His western blot analysis of sfGFP-151-fsY decaging



Expt.	I	II	III	IV	V
Activator	7	- (PBS)	21	- (DMSO)	21
hv	-	-	+	+	-

Reaction setup for Expt. I and II: To a 200 µL Eppendorf tube with sfGFP-151-fsY (1 µg/µL, 30.0 µL) was added reagent **7** stock solution (10 mM in 0.1 M PBS buffer, 3.3 µL, 30 equiv.) or PBS buffer (0.1 M, 3.3 µL). The resulting reaction mixture was covered with aluminum foil and placed on 37 °C warm room shaker. 4 h later, the reaction was used directly in the western blot experiment.

Reaction setup for Expt. III and IV: To a 200 µL Eppendorf tube with sfGFP-151-fsY (1 µg/µL, 30.0 µL) was added reagent **21** stock solution (10 mM in DMSO, 3.3 µL, 30 equiv.) or DMSO (3.3 µL). The resulting reaction mixture was irradiated with 370 nm LED light (40 W, 100%) for 0.5 h. Subsequently, the whole setup was covered with aluminum foil and placed on 37 °C warm room shaker. After another 3.5 h, the reaction was directly used in the western blot experiment.

Reaction setup for Expt. V: To a 200 µL Eppendorf tube with sfGFP-151-fsY (1 µg/µL, 30.0 µL) was added reagent **21** stock solution (10 mM in DMSO, 3.3 µL, 30 equiv.). The whole setup was covered with aluminum foil and placed in the same photoreactor as Expt. III and IV. After the reaction mixture was irradiated with 370 nm LED light (40 W, 100%) for 0.5 h, the resulting reaction mixture was kept in dark and placed on 37 °C warm room shaker. 3.5 h later, the reaction was used directly in the western blot experiment.

Protocol for Anti-His western blot:

Western blot was used to confirm the presence of a polyhistidine tag in the reporter protein before and after the releasing reaction to confirm the compatibility of the conditions with protein.[10] Purified wild-type superfolder green fluorescent protein (sfGFP), sY (sulfotyrosine) or fsY (fluorosulfotyrosine)-incorporated mutant of sfGFP reporter proteins, and reaction mixture of fsY-incorporated sfGFP in different releasing conditions were resolved by LDS-PAGE using a freshly purchased Novex™ Tricine 16% gel (Thermo Fisher Scientific) in Tricine running buffer for 90 min at 120 V. The protein was transferred to a PVDF membrane (Life Technologies) using a Trans-Blot Turbo Transfer System 15 (BioRad) in freshly prepared Towbin transfer buffer (at 12 V for 30 min, twice). After complete transfer, membrane was blocked in 10 mL 5% milk in TBAT at 4 °C with constant agitation. Membrane was subsequently incubated in 1:3000 anti-HisTag mouse mAb (Invitrogen, MA1-21315, in 5% TBST) overnight. Next, the membrane was washed three times (10 min per wash) with TBST at room temperature (rt). Afterwards, the membrane was soaked in a 1:6000 dilution of chicken anti-mouse secondary antibody (Invitrogen, SA1-72021, in 5% milk TBST) mixture for 2 h at room temperature. The membrane was washed and activated using SuperSignal West Dura Kit (Thermo Fisher Scientific). The activated blot was imaged on the ChemiDoc MP imaging system (BioRad).

Note: The Towbin transfer buffer recipe is 14.4 g glycine and 3.0 g Tris base in 100 mL MeOH and 900 mL water. The recipe of TBST (Tris-buffered saline with 0.1% Tween® 20 detergent) is 8.0 g sodium chloride (NaCl), 0.2 g potassium chloride (KCl), and 2.3 g Tris base was added into 1000 mL water with 1 mL Tween® 20 detergent.

Decaging of fsY on the surface of live *S. aureus* cells

a) Sortase A-mediated ligation of peptide 22 on live *S. aureus* cells.

From an overnight culture of *Staphylococos aureus*, 500 μL culture sample was transferred to a 17 × 100 mm culture tube with 4.5 mL tryptic soy broth (TSB) medium added. After the cells grew to OD 1.0 at 37 °C, the cells were washed three times with Dulbecco's phosphate-buffered saline (DPBS, catalog number: 14040117 from gibco) and pelleted by centrifugation (5 min, 4000 rpm). The pellet was resuspended in DPBS buffer (4.95 mL), followed by the addition of peptide **22** stock solution (50 μL, 0.2 mM in DMSO). The resulting mixture was covered with aluminum foil and shaking at 250 rpm for 6 h at room temperature. The above sample was washed by DPBS buffer (3 mL × 6 times) until the supernatant was transparent and then resuspended in the same buffer (1 mL).

b) Fluorescence microscopy and flow cytometry characterizations.

For fluorescence microscopy, 2.9 μL of the above cell suspension was placed on a glass slide. The white field and fluorescent images were obtained on Zeiss microscope equipped with filter set 44 (excitation: BP 475/40, emission: BP 530/50). The images were captured using the 100x oil immersion with 1000 ms exposure time. All the images were further processed using Image J software with same parameters (Figure S18).

The fluorescence from the cells was quantified using flow cytometry analysis (Figure S19). Cells were diluted 10^6 cfu in DPBS and analyzed on Becton Dickinson Accuri C6 Plus (BD Biosciences). To enrich for single cells, a side scatter threshold trigger (SSC-H) was applied. To gate for single bacterial cells, we first selected events that appeared on the center of the FSC-A vs. SSC-A plot, then selected events along the diagonal of the FSC-H vs FSC-A plot. Events that appeared on the edges of the fluorescence histogram were excluded.

c) LC-MS characterization of the decaging reaction on the live cell surface.

From an overnight culture of *S. aureus*, 50 μL of culture sample was transferred to a 17 × 100 mm culture tube with 4.5 mL TSB medium added (two identical batches are performed). After the cells grew to OD 0.1 in the same medium (5 mL) at 37 °C, the cells were washed three times with TSB medium and pelleted by centrifugation (5 min, 4000 rpm). The cells were then resuspended in TSB medium (4.95 mL), followed by the addition of 27-FAM peptide stock solution (50 μL, 0.2 mM in DMSO). The resulting mixture was covered with aluminum foil and shaking for 6 h at 37 °C.

The above two batches were combined and washed by 0.1 M PBS buffer (3 mL × 6 times) until the supernatant was transparent and then resuspended in 0.1 M PBS buffer (1.0 mL). The cells were subsequently used for decaging reaction in 0.1 M PBS buffer (55.6 μL of 0.1 M reagent **7** stock solution was added to the mixture, 37 °C for 2 h on shaker with aluminum foil cover**.** A sample of 20 μL of this cell suspension was plated on agar plate for cell viability test, with the heat-killed *S. aureus* (98 °C, 20 min) used as a control (Figure 55). The rest of cell suspension was washed by TEV cleavage buffer (50 mM Tris, 0.5 mM EDTA, 1 mM DTT, pH = 8.0) three times before as pelleted by centrifugation (5 min, 8000 rpm). The pellet was then suspended in 1.0 mL of TEV cleavage buffer. 50 μL of TEV protease (7 μM) was added to the rest of the cells, and incubate at 30 °C overnight or 3 h. The samples were flicked every 30 min to ensure good mixing. The cleaved peptide **24** was filtered using a 50K centrifugal filter. The sample was centrifuged at maximum speed (7830 rpm) for 15 minutes at room temperature and washed

with 100 μL water twice. The flow-through was lyophilized for two days and redissolved in 30 μL of water. And 27 μL of this solution was injected into the LC-MS. As a control, peptide **22** in 90 μL TEV cleavage buffer was subjected to 10 μL of TEV protease (7 μM), and incubate at 30 °C overnight or 3 h. 10 μL of the resulting peptide **23** was injected into the LC-MS. LC-MS condition for analyzing **23** and **24** in positive mode, solvent A contains acetonitrile/water/formic acid = 5:95:0.1, solvent B contains acetonitrile/water/formic acid = 95:5:0.1.

Time (min)	A (%)	B (%)	Flow (mL/min)
0.00	95.0	5.0	0.200
3.00	95.0	5.0	0.200
13.00	5.0	95.0	0.200
18.00	5.0	95.0	0.200
20.00	95.0	5.0	0.200
25.00	95.0	5.0	0.200

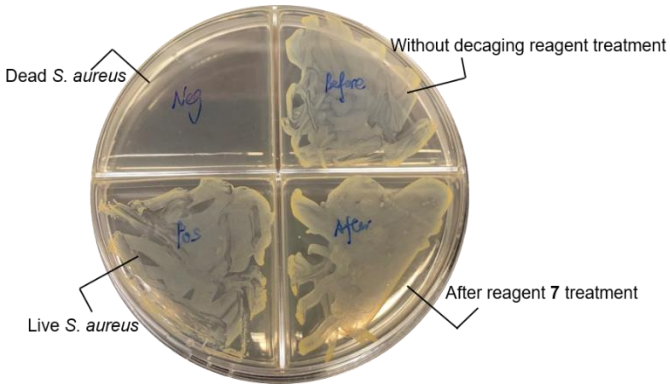
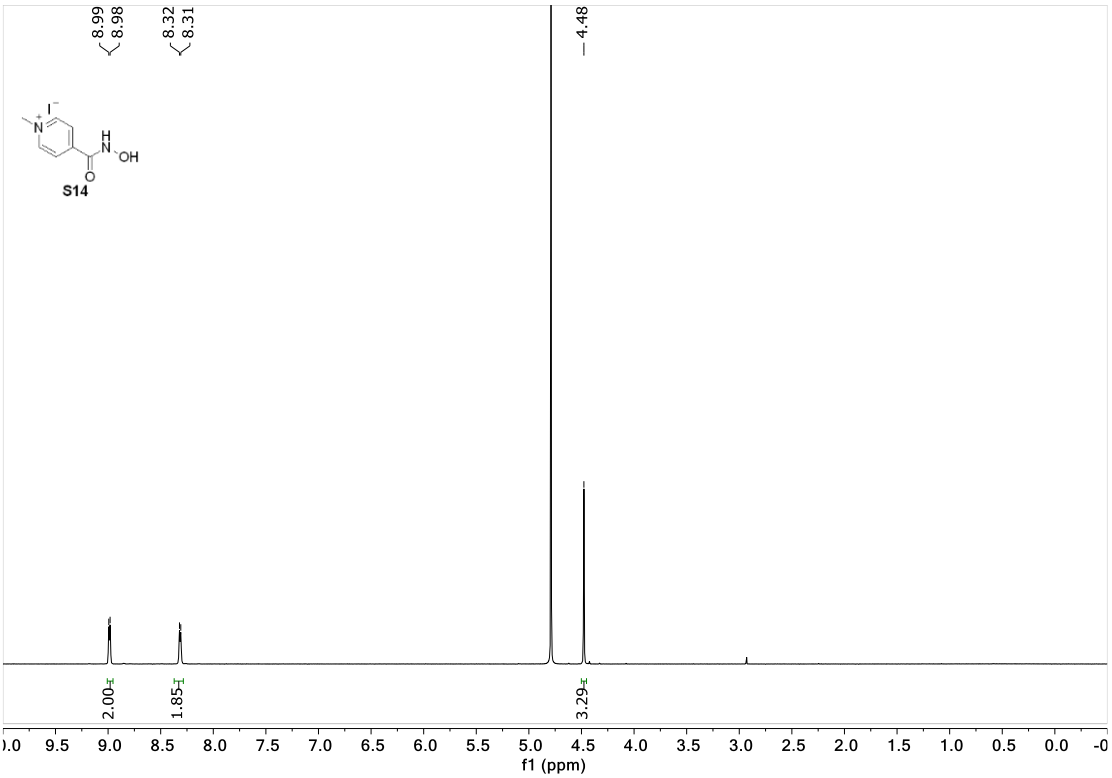
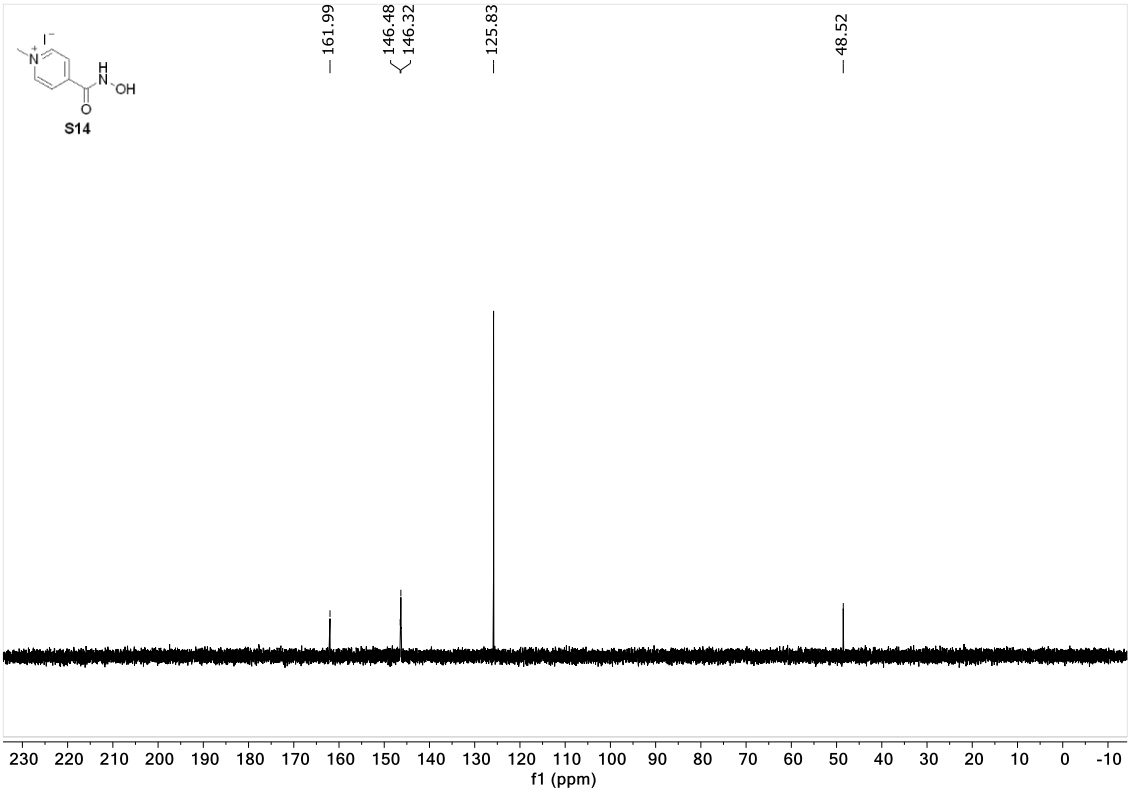
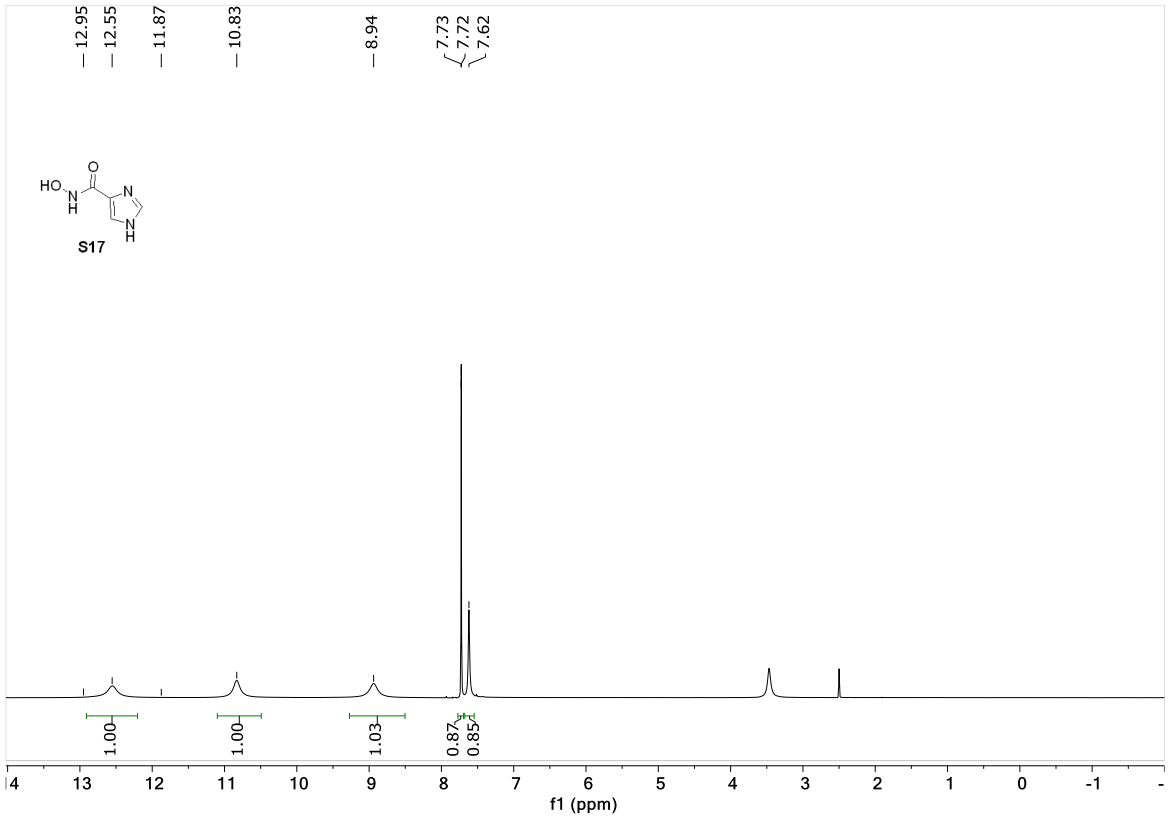


Figure S55. Plating assay to determine *E. coli* (BL21) viability under the decaging reaction

NMR Spectrum of New Compounds



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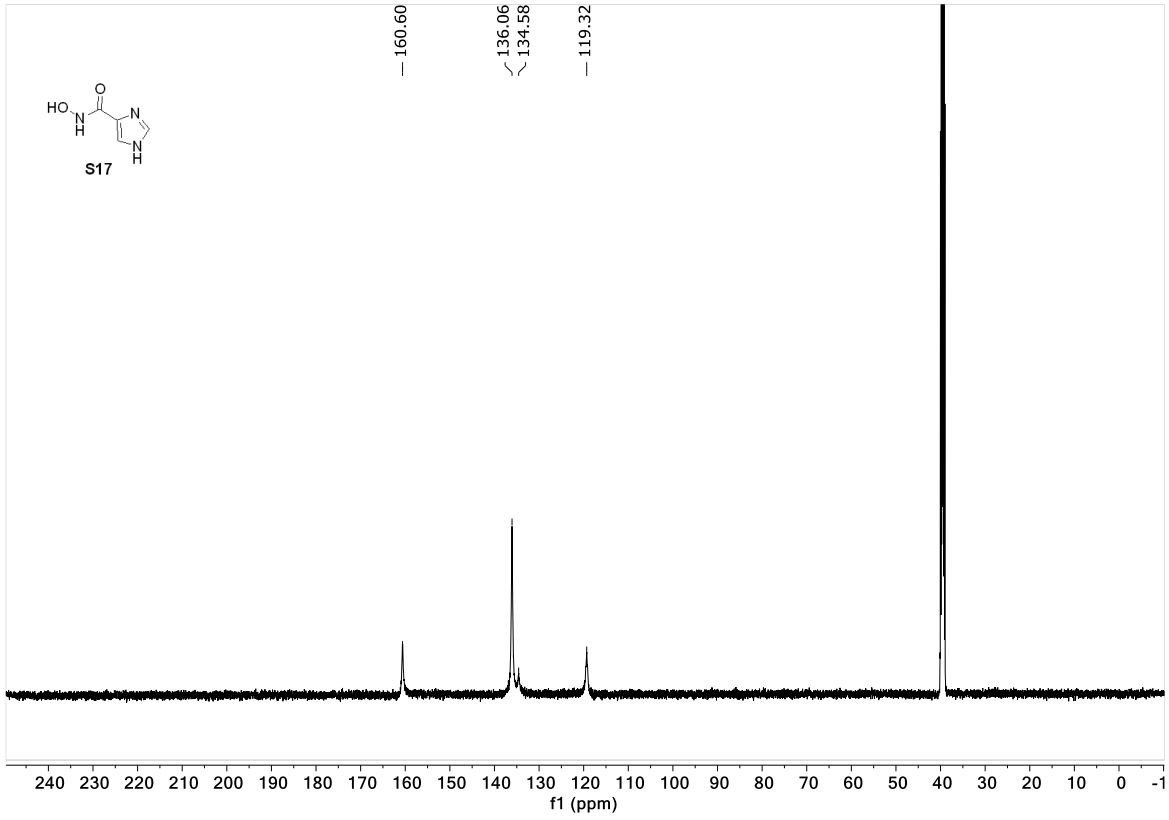
11.87
10.83
8.94
7.73
7.72
7.62
HO
N
H
O
N
N
H
S17
1.00
1.00
1.03
0.87
0.85
f1 (ppm)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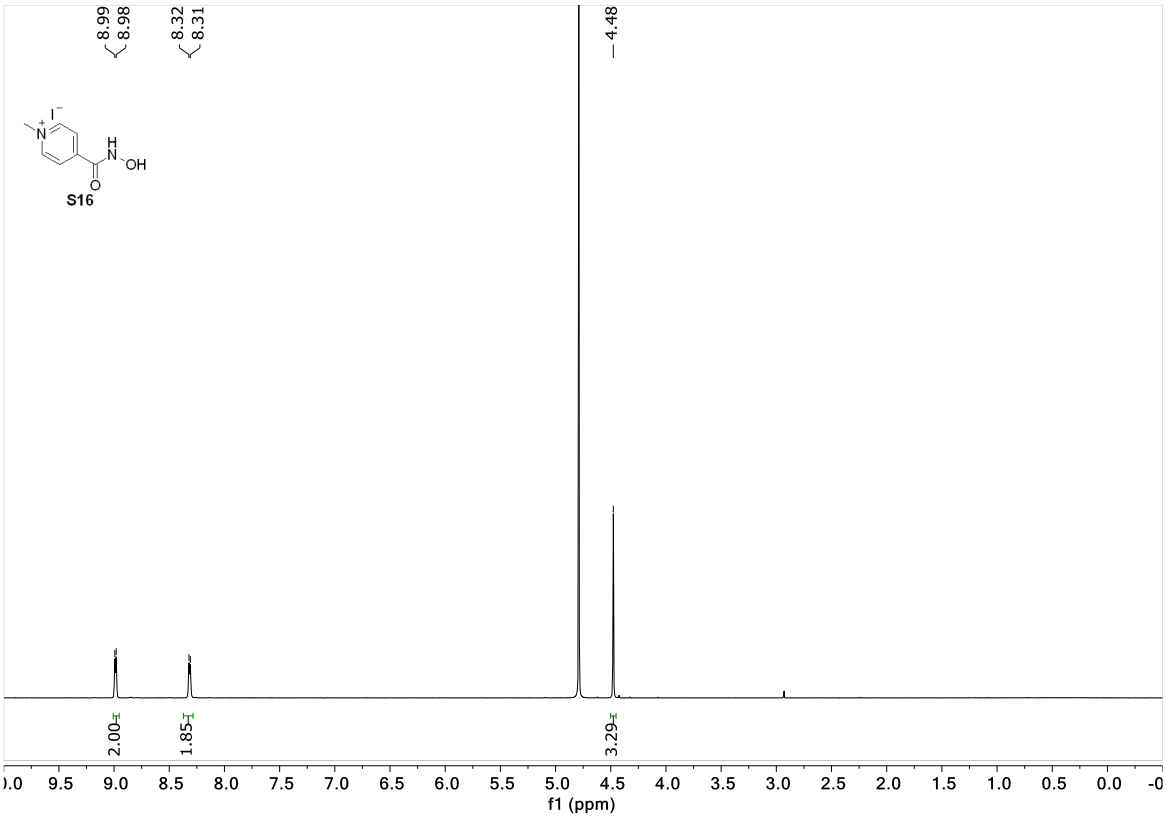
HO
N
H
O
N
N
H
S17
f1 (ppm)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4.48
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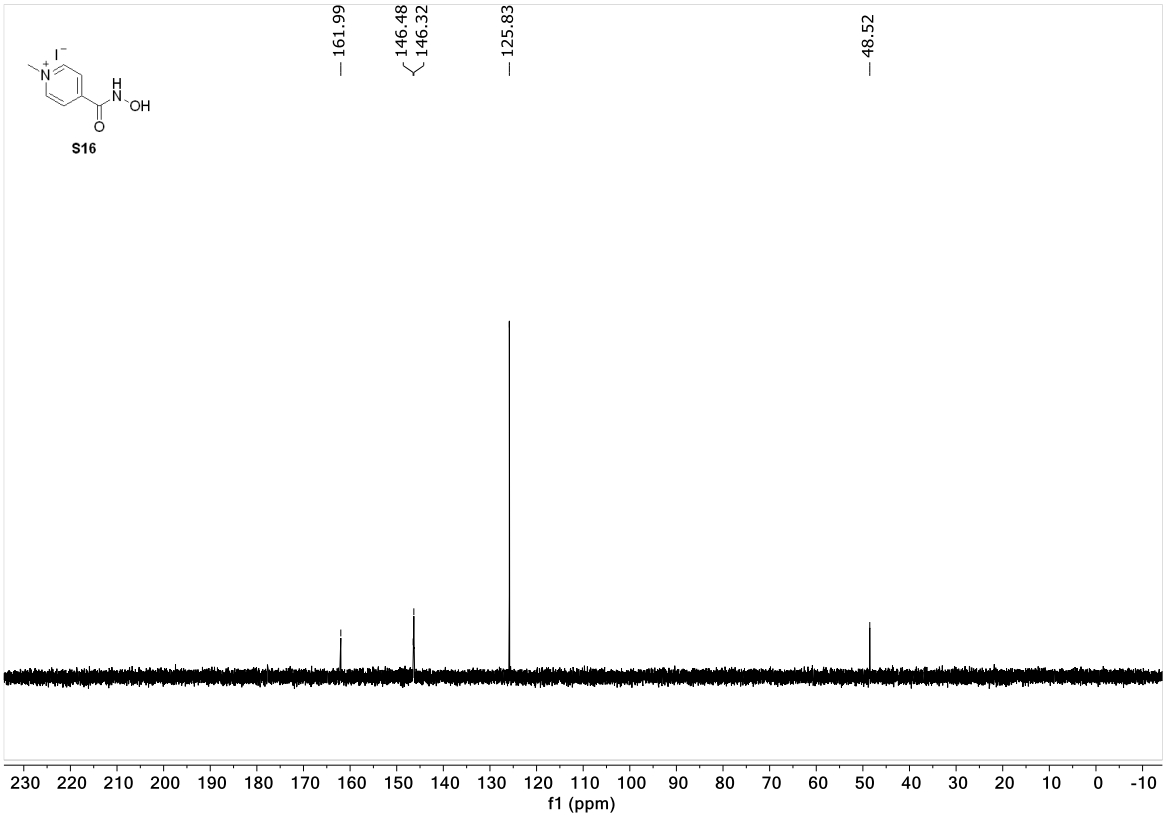
2.00
1.85
3.29
f1 (ppm)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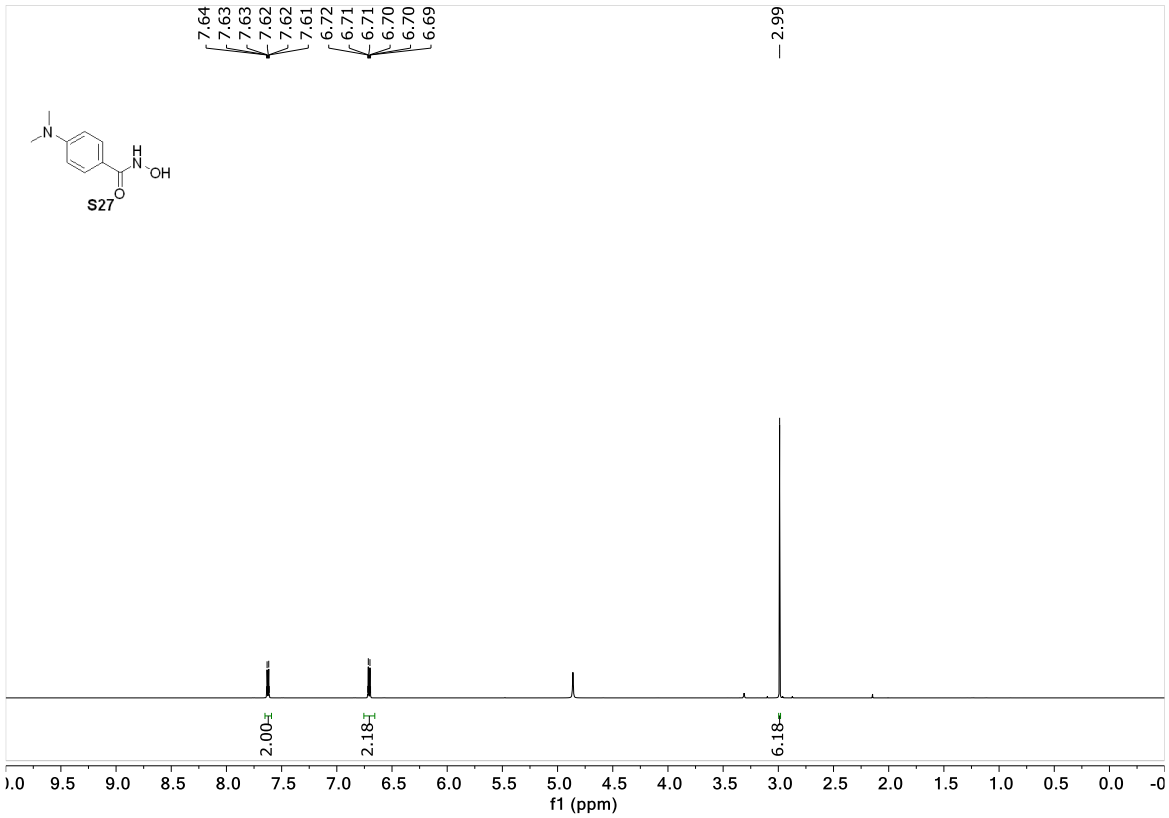
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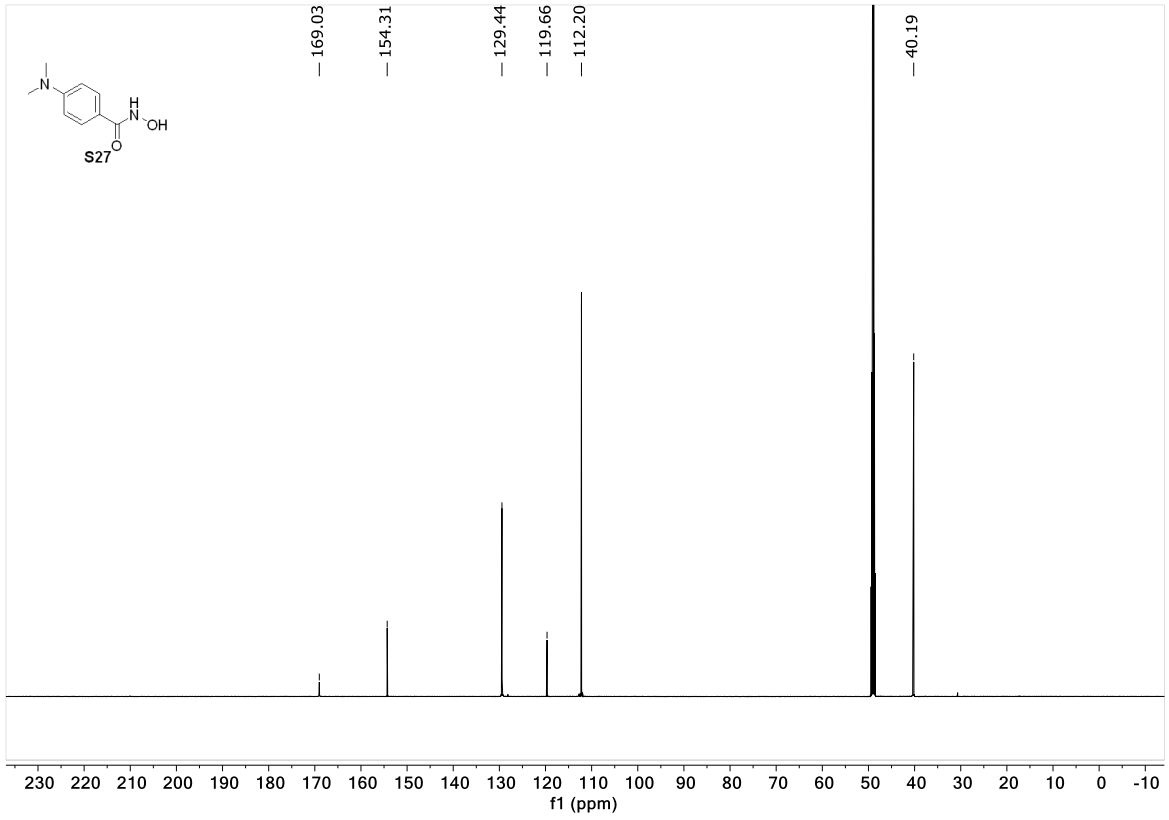
2.00
2.18
6.18
f1 (ppm)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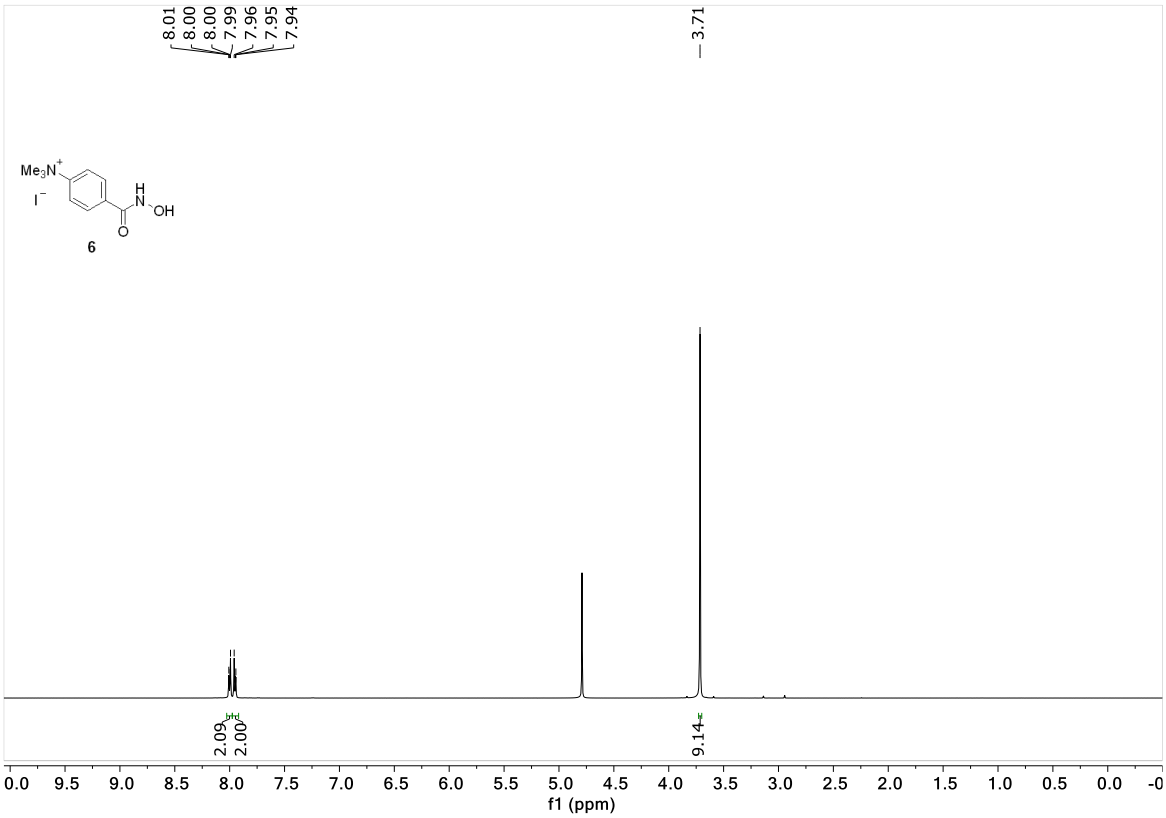
112.20
40.19
S27
f1 (ppm)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7.96
7.95
7.94
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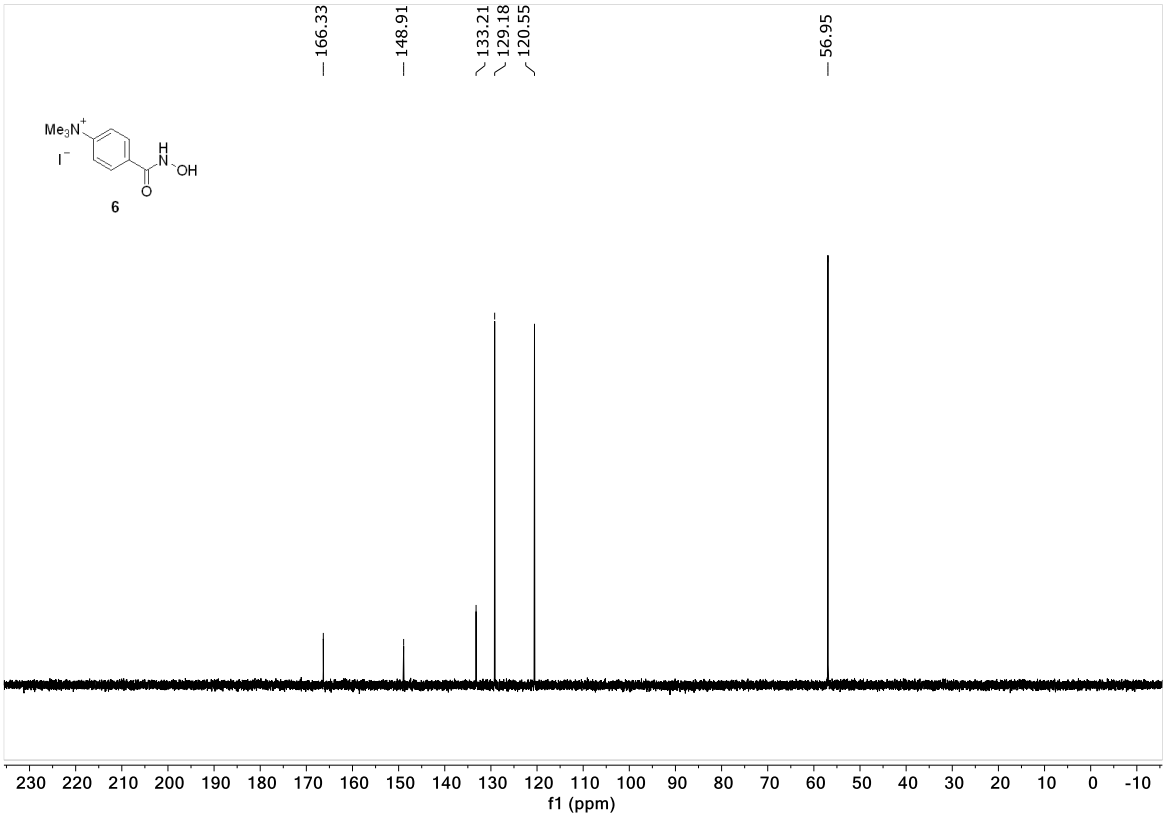
Me3N+
I−
H
N
OH
O
6
2.09
2.00
9.14
0.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0
f1 (ppm)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148.91
133.21
129.18
120.55
56.95
Me3N+
I−
H
N
OH
O
6
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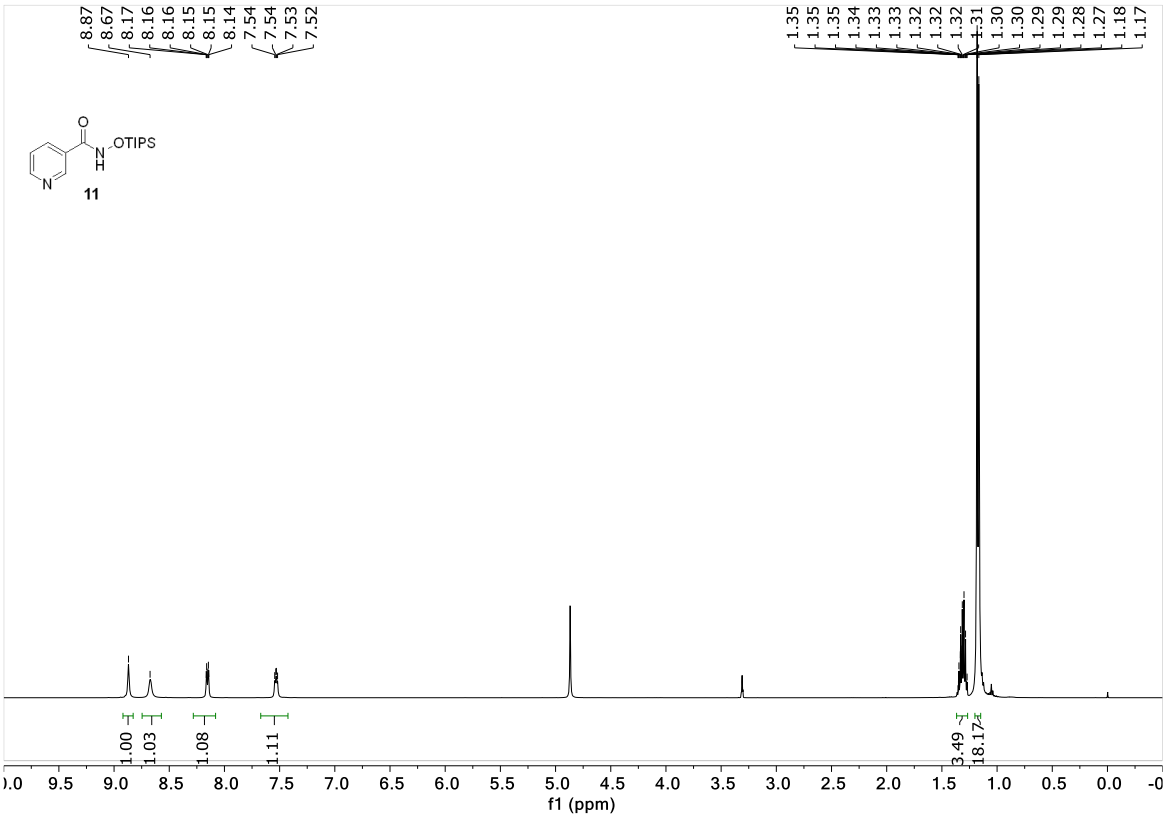
f1 (ppm)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7.54
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1.34
1.33
1.33
1.32
1.32
1.32
1.31
1.30
1.30
1.29
1.29
1.28
1.27
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1.17
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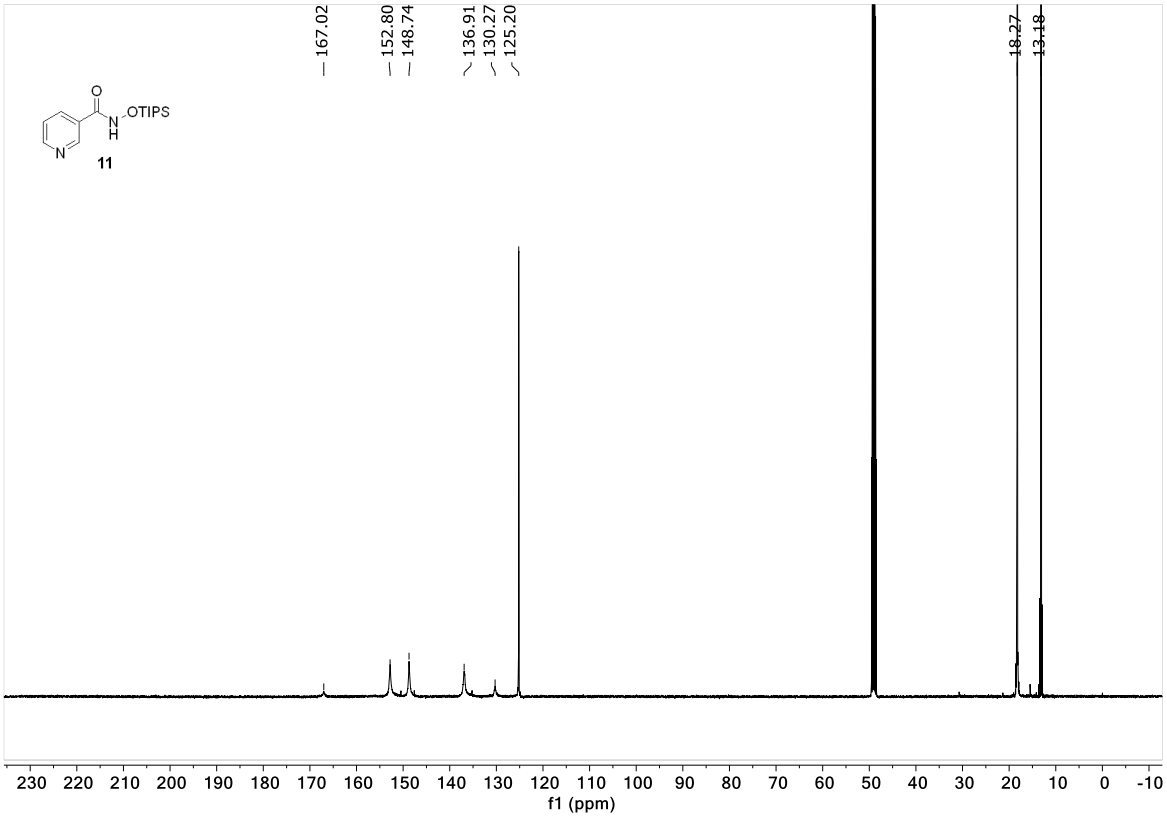
1.03
1.08
1.11
3.49
18.17
f1 (ppm)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11
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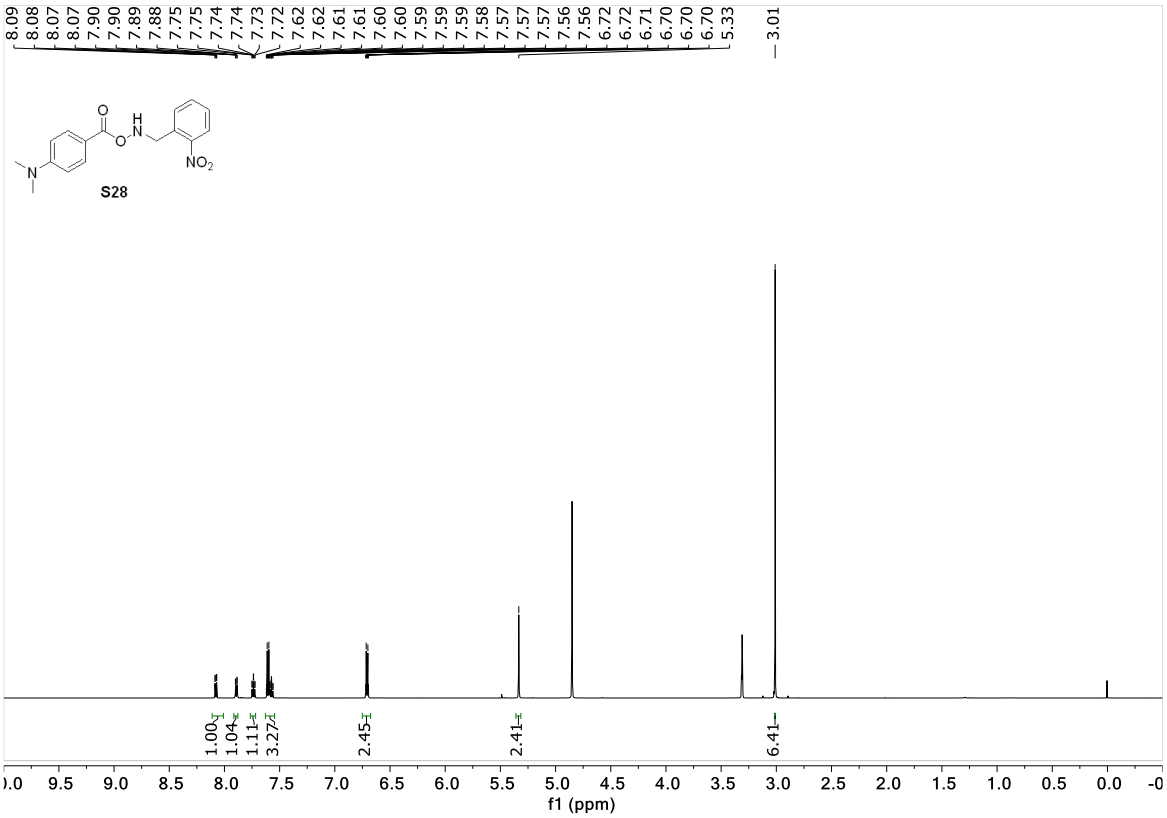
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7.57
7.57
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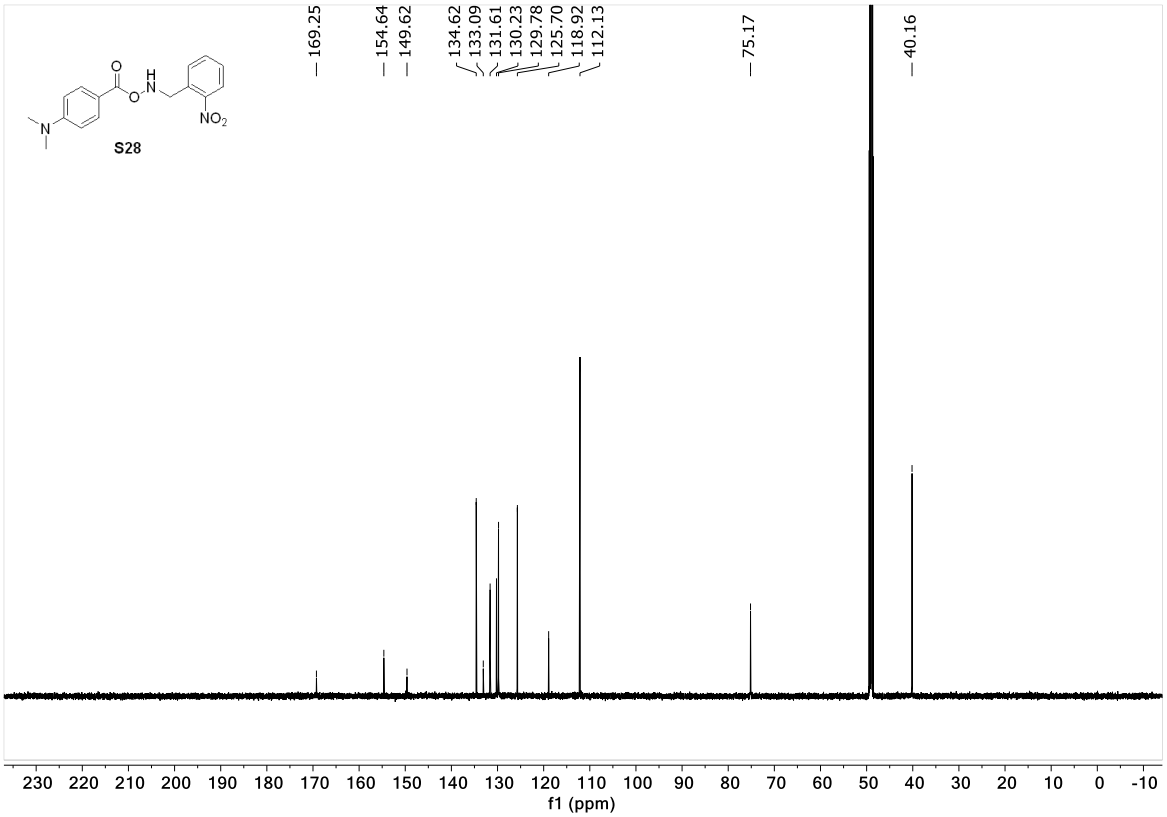
f1 (ppm)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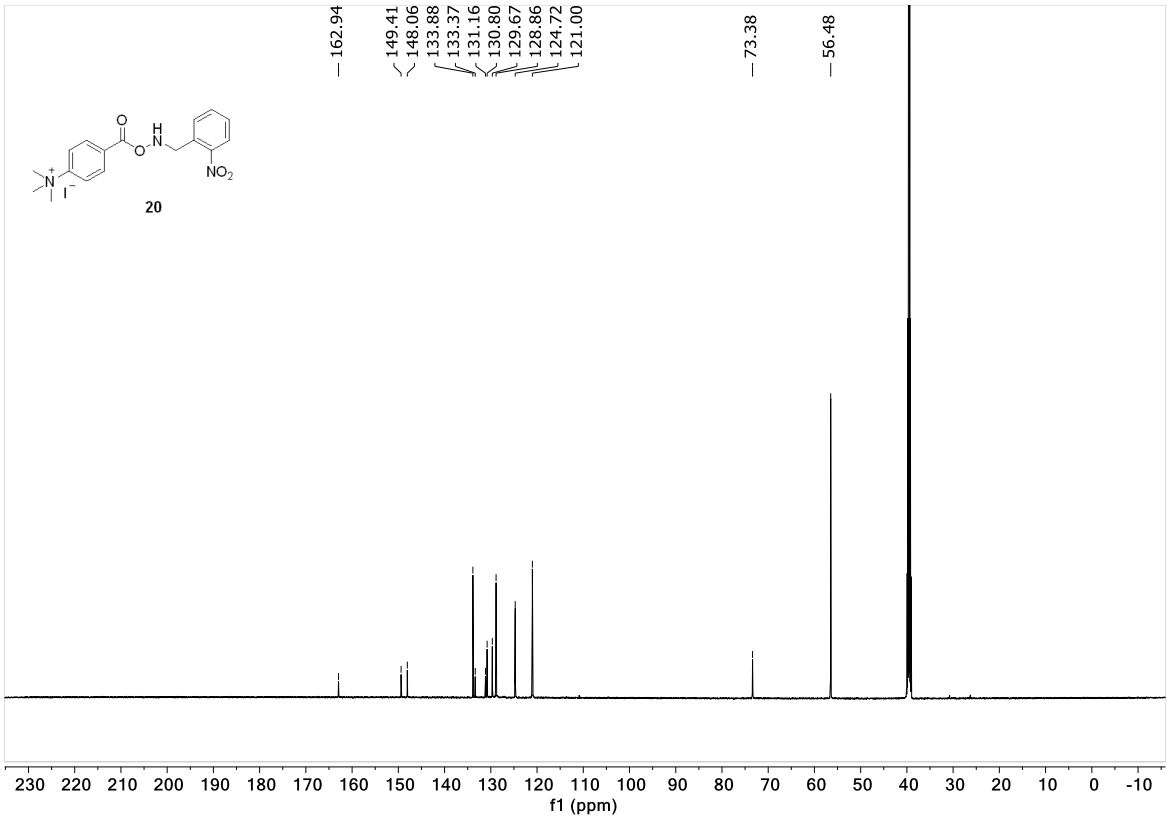
S28
f1 (ppm)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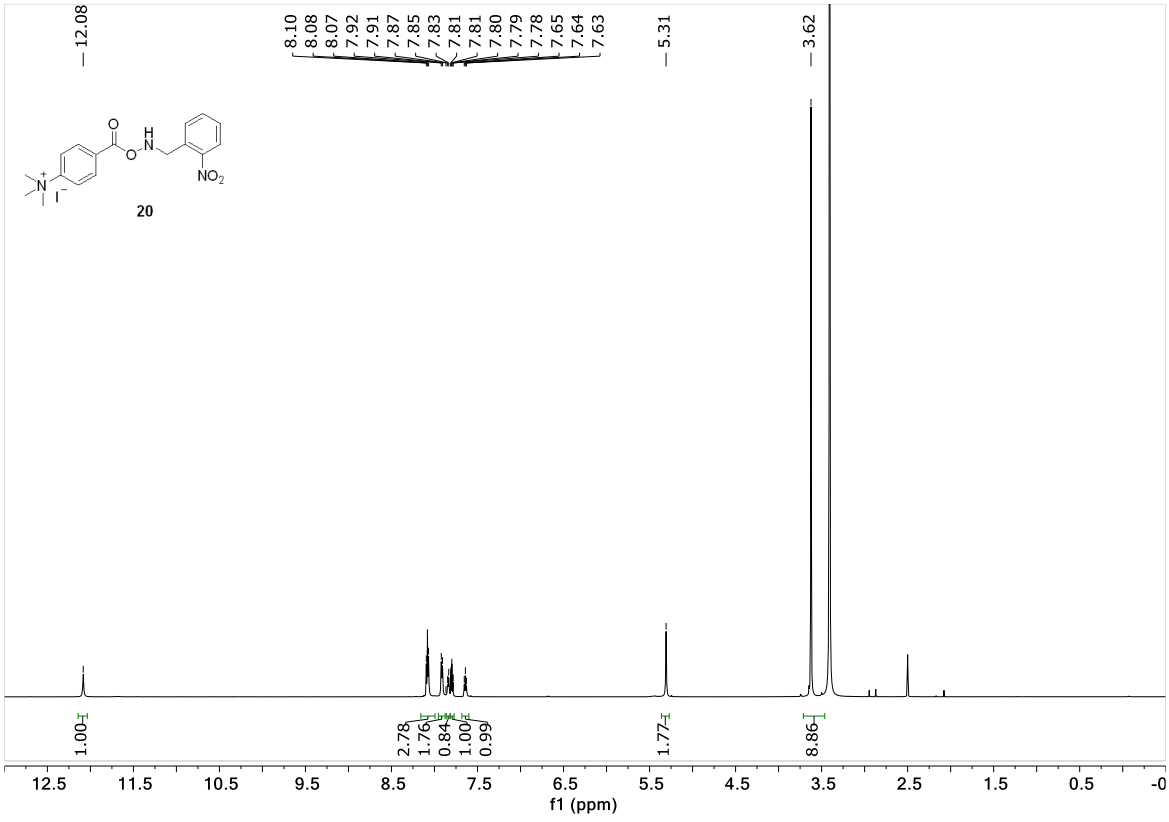
20
f1 (ppm)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7.78
7.65
7.64
7.63
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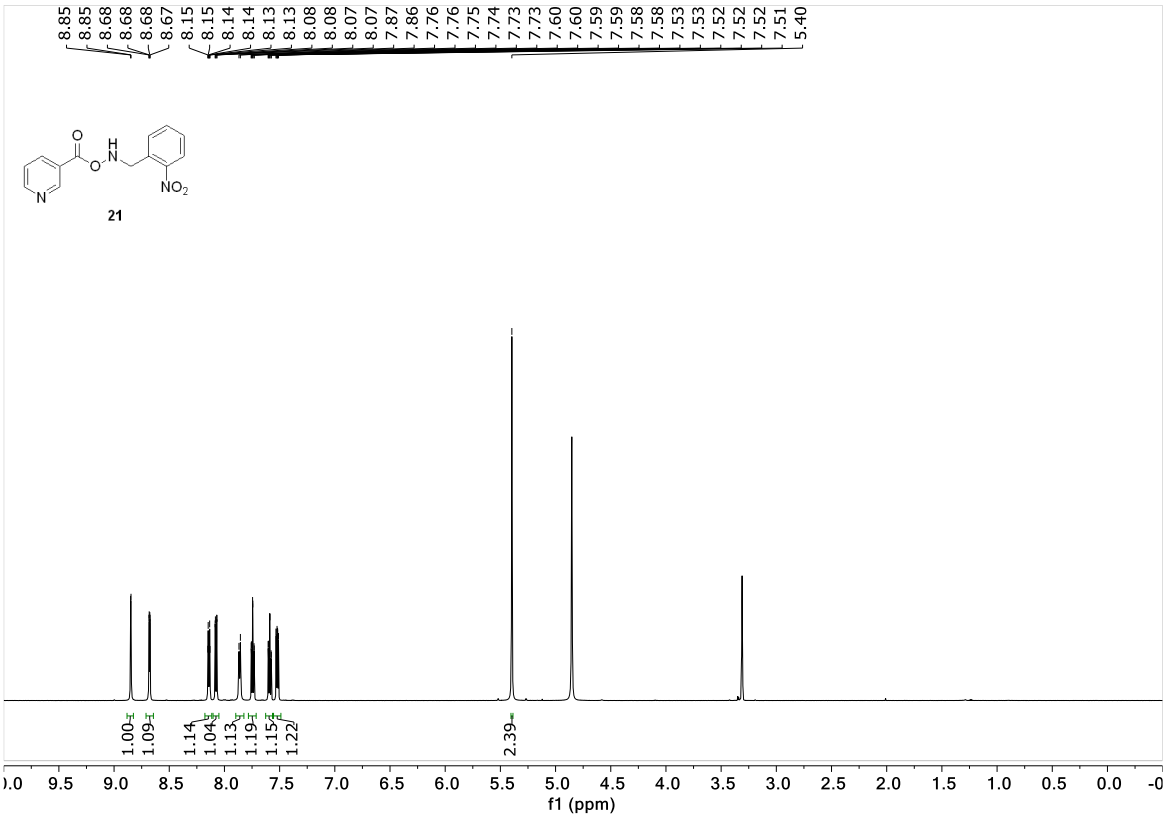
f1 (ppm)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5.40
O
H
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1.22
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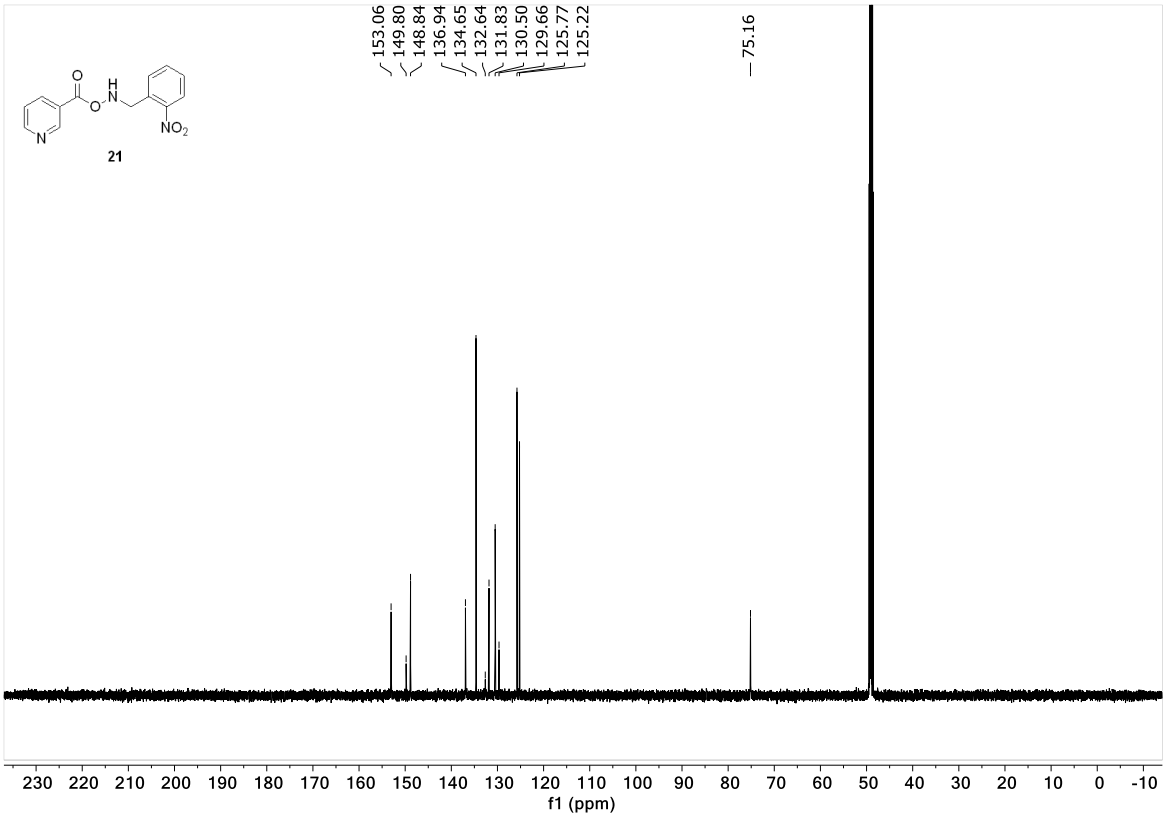
f1 (ppm)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125.22
75.16
O
H
N
O
N
NO2
21
f1 (ppm)

Additional References

(1) Liu, C.; Yang, C.; Hwang, S.; Ferraro, S. L.; Flynn, J. P.; Niu, J. A General Approach to *O*-Sulfation by a Sulfur (VI) Fluoride Exchange Reaction. *Angew. Chem. Int. Ed.* **2020,** *132*, 18593-18599.

(2) Choi, E. J.; Jung, D.; Kim, J. S.; Lee, Y.; Kim, B. M. Chemoselective Tyrosine Bioconjugation through Sulfate Click Reaction. *Chem. Eur. J.* **2018,** *24*, 10948-10952.

(3) Chen, W.; Dong, J.; Plate, L.; Mortenson, D. E.; Brighty, G. J.; Li, S.; Liu, Y.; Galmozzi, A.; Lee, P. S.; Hulce, J. J.; Cravatt, B. F.; Saez, E.; Powers, E. T.; Wilson, I. A.; Sharpless, K. B.; Kelly, J. W. Arylfluorosulfates inactivate intracellular lipid binding protein (s) through chemoselective SuFEx reaction with a binding site Tyr residue. *J. Am. Chem. Soc.* **2016,** *138*, 7353-7364.

(4) Matsubayashi, Y.; Sakagami, Y. Phytosulfokine, sulfated peptides that induce the proliferation of single mesophyll cells of Asparagus officinalis L. *Proc. Natl. Acad. Sci. U.S.A.* **1996,** *93*, 7623-7627.

(5) Mulcahy, C.; Dolgushin, F. M.; Krot, K. A.; Griffith, D.; Marmion, C. J. Synthesis, characterisation and speciation studies of heterobimetallic pyridinehydroxamate-bridged Pt (II)/M (II) complexes (M= Cu, Ni, Zn). Crystal structure of a novel heterobimetallic 3-pyridinehydroxamate-bridged Pt (II)/Cu (II) wave-like coordination polymer. *Dalton Trans.* **2005**, 1993-1998.

(6) Chatterjee, A.; Sun, S. B.; Furman, J. L.; Xiao, H.; Schultz, P. G. A versatile platform for single-and multiple-unnatural amino acid mutagenesis in *Escherichia coli*. *Biochemistry* **2013,** *52*, 1828-1837.

(7) Wang, N.; Yang, B.; Fu, C.; Zhu, H.; Zheng, F.; Kobayashi, T.; Liu, J.; Li, S.; Ma, C.; Wang, P. G.; Wang, Q.; Wang, L. Genetically encoding fluorosulfate-L-tyrosine to react with lysine, histidine, and tyrosine via SuFEx in proteins *in vivo*. *J. Am. Chem. Soc.* **2018,** *140*, 4995-4999.

(8) Liu, C. C.; Schultz, P. G. Recombinant expression of selectively sulfated proteins in *Escherichia coli*. *Nat. Biotechnol.* **2006,** *24*, 1436-1440.

(9) Glasoe, P. K.; Long, F. Use of glass electrodes to measure acidities in deuterium oxide1, 2. *J. Phys. Chem.* **1960,** *64*, 188-190.

(10) Italia, J. S.; Peeler, J. C.; Hillenbrand, C. M.; Latour, C.; Weerapana, E.; Chatterjee, A. Genetically encoded protein sulfation in mammalian cells. *Nat. Chem. Biol.* **2020,** *16*, 379-382.