Fluorosulfate as a Latent Sulfate in Peptides and Proteins

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ABSTRACT: Sulfation widely exists in the eukaryotic proteome. However, understanding of the biological functions of sulfation in peptides and proteins has been hampered by the lack of methods to control its spatial or temporal distribution in the proteome. Herein, we report that fluorosulfotyrosine can serve as a latent precursor of sulfotyrosine in peptides and proteins, which can be efficiently converted into sulfotyrosine residues by hydroxamic acid activators under physiologically relevant conditions. Photocaging the hydroxamic acid activators further allowed for light-controlled activation of functional sulfopeptides. This work provides a valuable tool for probing functional roles of sulfation in the peptides and proteins.

O-Sulfation of the tyrosine residue is a post-translational modification (PTM) that widely exists in peptides and proteins (Figure 1a), and has been implicated to regulate a variety of biological functions such as immune response, hemostasis, and pathogen evasion.[1] It has been found that about 1% of all tyrosine residues in eukaryotes are sulfated. However, only a small fraction of the sulfoproteome has been annotated.[2-3] A long-standing challenge for studying the sulfoproteome is that sulfation is highly dynamic, often producing a heterogeneous population of various sulfopeptides and sulfoproteins with different sulfoforms.[4] Therefore, a general method to control the sites and stoichiometry of sulfation in the proteomic context would be highly valuable for studying their functional roles in biology.[5]

Caging strategies have been developed for various protein PTMs to probe how these PTMs regulate dynamic cellular events. Although a broad collection of caging groups are available for a variety of PTMs, a caging group that stably protects sulfotyrosine (sY) residues in peptides and proteins and can be efficiently removed under physiological conditions remains elusive.[6] The reasons for such a knowledge gap includes the high energy barrier for chemically activating the sulfate group for coupling chemistries, the liability of sY to acid, heat, and high-energy ionization, and the strong electron-withdrawing propensity of sulfate that renders commonly used benzylic ester caging groups unstable.[7] [8-9] On the other hand, while multiple alkyl and aryl esters have been successfully used as protecting groups of sY in solid-phase peptide synthesis,[10-11] such as 2,2,2-trichloroethyl (TCE),[12-13] 2,2-dichlorovinyl (DCV),[14-15] 2,2,2-trifluoroethyl (TFE),[16] neopentyl,[17-18] and phenyl[19] sulfate diesters, their deprotection conditions (e.g., strong base, hydrogenolysis, etc.) are incompatible with biological systems.

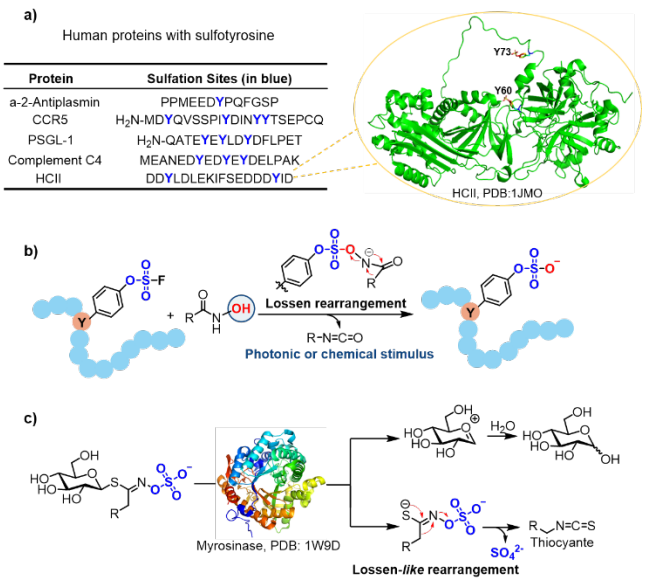
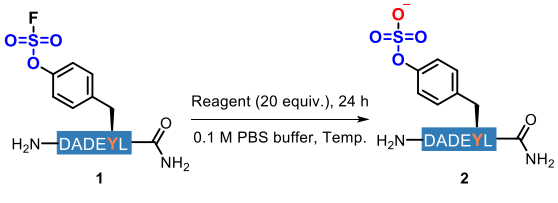


Figure 1. Background and our approach. a) Sulfation widely exists in diverse bioactive peptides and proteins. **b**) In this work, fluorosulfate is incorporated in peptides and proteins as a latent sulfate and can be efficiently converted into sulfate by hydroxamic acid activators under physiologically relevant conditions. **c**) Our approach mirrors the myrosinase-catalyzed Lossen-like rearrangement of glucosinolates in nature.

In 2014, Sharpless et al. reported the reactivity of fluorosulfate in Sulfur(VI) Fluoride exchange (SuFEx) reaction, which has found broad utilities in organic chemistry, polymer science, and chemical biology.[20-23] Compared to other halogen-substituted sulfate derivatives, fluorosulfate not only has a size closest to that of sulfate, but is also far less

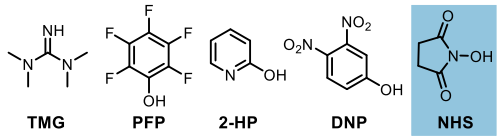
electrophilic due to the π-donation from fluorine to sulfur.[24] As a result, fluorosulfate has demonstrated excellent metabolic stability in vivo.[25] While reactivities of fluorosulfate with cellular nucleophiles have been reported, these examples all require the close spatial proximity through ligand-receptor binding.[7, 26-27] The chemical inertness of fluorosulfate has allowed its tyrosine derivative, L-fluorosulfotyrosine (fsY), to be incorporated into peptides and proteins via solid-phase peptide synthesis[11, 28] and non-canonical amino acid (ncAA) mutagenesis.[7, 26] Herein, we demonstrate that fluorosulfate can serve as a latent sulfate in sulfopeptides and sulfoproteins and can be efficiently converted into sulfate (hereafter denoted as "decaging") by hydroxamic acid activators under physiologically relevant conditions. Mechanistic studies revealed an unusual Lossen rearrangement pathway of fluorosulfate activation and decaging (Figure 1b) that is analogous to the Myrosinase-mediated Lossen-like rearrangement of glucosinolate in nature (Figure 1c). It is noteworthy that a recent report by Kelly et al. showed that fsY could be converted into sY in synthetic peptides, but the reaction required strongly basic conditions and was incompatible with physiological peptides and proteins.[28]

Table 1. 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.

[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.



Our investigation began with confirming the stability of fsY in various aqueous environments such as buffer solution, cell lysate, and serum. 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 1, entry 1-2). 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 S1 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.[29]

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.[19] Combining this finding with Kim's report that tetramethylguanidine (TMG) promotes the SuFEx coupling between fluorosulfate and nucleophiles in aqueous solution,[30] 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 activator or when it is combined with co-activators such as pentafluorophenol (PFP), 2-hydroxypyridine (2-HP), or 2,4-dinitrophenol (DNP) (Table 1, 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 (Table 1, entry 7). Adding TMG to the reaction did not further improve the yield of **2** (Table 1, entry 8). The reaction was accelerated when the temperature was elevated to 37 °C, producing **2** quantitatively in 24 hours (Table 1, entry 9).

We wondered if the hydroxamic acid (HA) motif of NHS is the reactive center that mediated the decaging reaction (Table S3 and Figure S3). Indeed, we found that the hydrolysis product of NHS, *N*-hydroxylsuccinic acid monoamide (**3**), provided **2** in 57% yield in one hour, indicating its superior nucleophilicity towards fluorosulfate (Figure 2a). Encouraged by this finding, we examined other HA derivatives (Figure 2b, S4). Acetohydroxamic acid (**4**) promoted the reaction to 78% over one hour. Good yield (95%) of **2** was obtained using aromatic benzohydroxamic acid (**5**) as the activator under the same condition. The highest efficiency was observed when the cationic HA **6** and heteroaromatic HA **7** were used, achieving quantitative conversion in 30 minutes. Other non-HA α-nucleophile activators such as oxime **8**[31], 2-aminoxime **9**, and 1-hydroxybenzotriazole (**10**)[32] resulted in lower reaction efficiency. In contrast, triisopropylsilyl ether (TIPS)-masked HA **11** showed no reactivity until potassium fluoride (KF) was added to remove the TIPS protecting group (Table S4 and Figure S5), confirming that HA is the reactive center for fluorosulfate activation. It is also noteworthy that the decaging reaction mediated by **7** proceeded with no detectable side reaction in the presence of 20 equivalents of nucleophilic amino acids including lysine, histidine, and tyrosine (Table S5), highlighting the high selectivity of **7** to fluorosulfate.

To gain insight into the reaction mechanism, the reaction with **1** as the substrate and **9** as the activator was monitored using liquid chromatography mass spectrometry (LC-MS) to capture the reaction intermediates (Figure 2b and Figure S6). An adduct (**12**) of **7** and **1** was detected, confirming the nucleophilic coupling between the HA activator and the substrate. Surprisingly, an isocyanate adduct **13** was also detected within 10 minutes at 37 °C, suggesting an uncommon intramolecular Lossen rearrangement pathway. To further probe this possibility, we performed the decaging reaction of **1** by **7** in the buffer prepared exclusively using $H_2{}^{18}O$. This reaction yielded **2** that contained no ^{18}O isotope (Figure 2c and Figure S7), suggesting that the conversion of fluorosulfate into sulfate is not through direct hydrolysis. These results further support a Lossen rearrangement mechanism.[33-34] Such a pathway is similar to the myrosinase-catalyzed Lossen-like rearrangement of glucosinolate in *Brassia* plants,[35] in which an inorganic sulfate and isothiocyante are generated from a thiohydroximate-*O*-sulfate intermediate (Figure 1c).

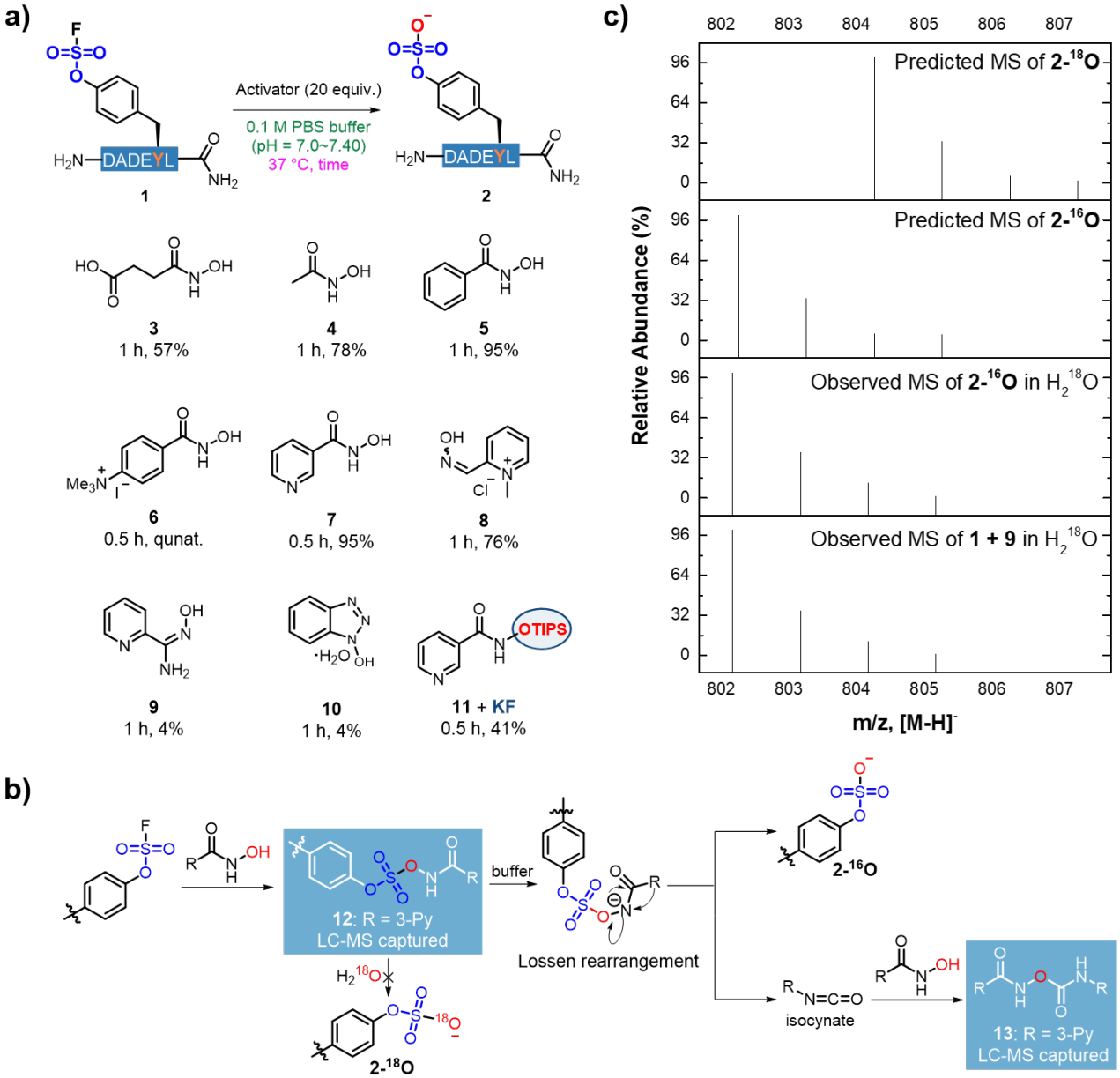


Figure 2. **Activator screen and mechanistic investigation**. **a**) A variety of hydroxamic acid activators were investigated for their ability to activate fluorosulfate in model peptide **1**. **b**) Real-time LC-MS reaction monitoring identified two activator **7** adducts **12** and **13**, suggesting a Lossen rearrangement mechanism. **c**) No ^{18}O-labeled products were found from the reaction in $H_2{}^{18}O$ buffer, suggesting that the sulfate product was not generated from direct hydrolysis.

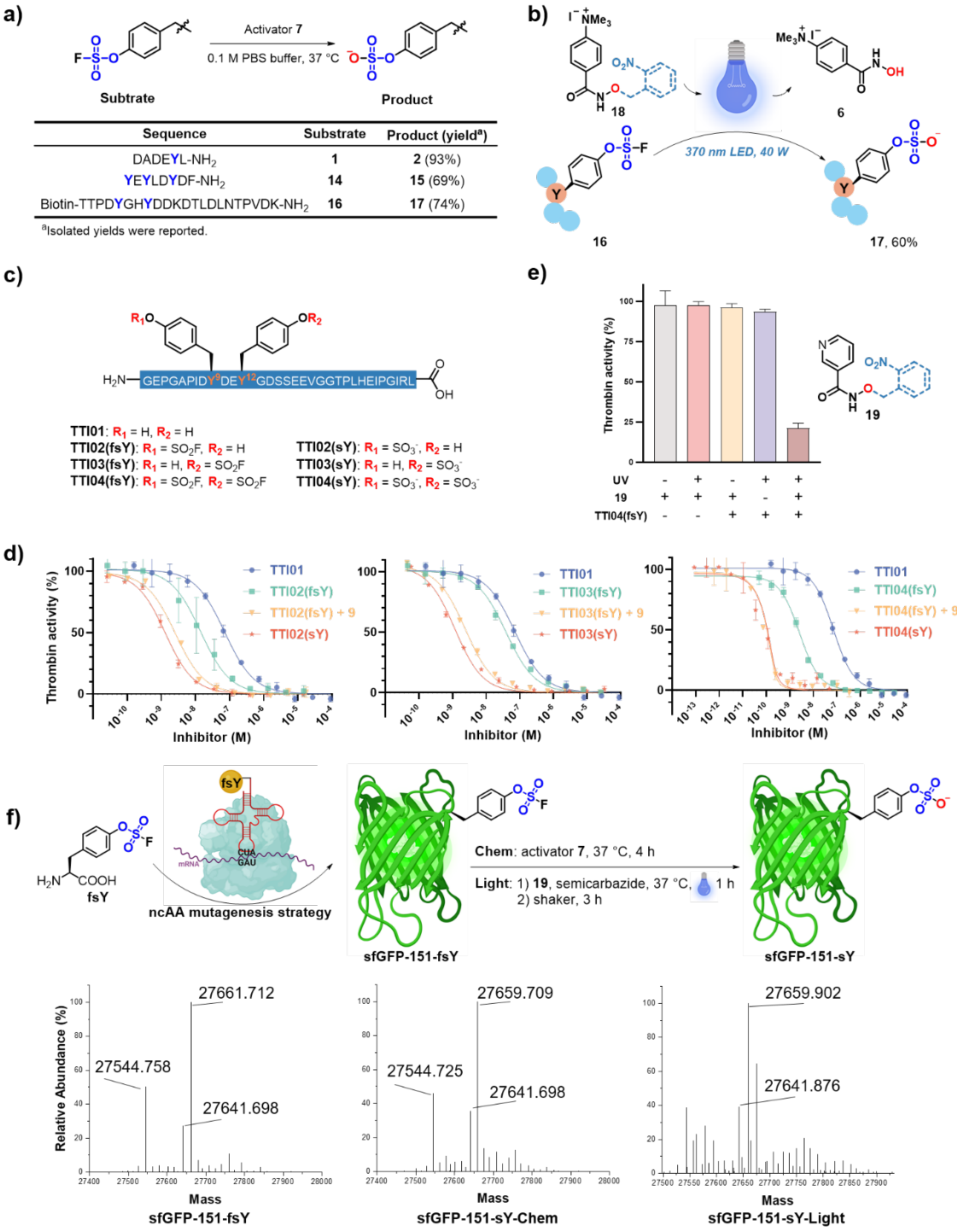


Figure 3. **Activation and decaging of fluorosulfate-containing peptides and proteins under physiologically relevant conditions**. **a**) Fluorosulfate activation and decaging in fsY-containing synthetic peptides. **b**) Light-mediated release of C5a 22mer peptide from caged activator **18**. **c**) TTI peptide sequences and sulfation patterns. **d**) Thrombin inhibition assay of TTI peptides. Data were fitted to the Morrison inhibition model and error bars represent the standard deviation of three independent measurements. **e**) Light-mediated activation and decaging of fluorosulfate-containing TTI peptide **TTI04(fsY)** regulates its sulfation-dependent thrombin inhibitory activity. **f**) Fluorosulfate activation and decaging in fsY-containing protein sfGFP-151-fsY and its corresponding high-resolution mass spectrometry. Left: before the reaction, purified starting material sfGFP-151-fsY (expected exact mass: 27661.739 Da, observed mass: 27661.712 Da) was observed. Middle: after the reaction with free activator **7**, the substrate was fully converted and the decaged product sfGFP-151-sY-chem (expected exact mass: 27659.743 Da, observed mass: 27659.709 Da) was observed. Right: after the reaction with caged activator **19**, the substrate was fully converted and the decaged product sfGFP-151-sY-light (expected exact mass: 27659.743 Da, observed mass: 27659.902 Da) was observed. The 27544.758 Da and 27544.725 Da mass peaks correspond to the misincorporation of glutamine into sfGFP at position of 151 (expected exact mass: 27544.804 Da), a known byproduct of the ncAA mutagenesis method.[36-37] The observed 27641.698 Da and 27641.876 Da mass peak corresponds to an intramolecular reaction of fluorosulfotyrosine with nucleophilic residue (expected exact mass: 27641.732).

With the optimized conditions in hand, we examined the decaging of various fluorosulfate-containing peptides mediated by **7** under physiological pH. Besides **1**, which was decaged to produce **2** in 93% yield, an octapeptide **14** consisting three fsY residues and a C5a 22-mer[38] peptide **16** containing two fsY residues were decaged to yield the corresponding sulfopeptides in 69% and 74% isolated yield, respectively (Figure 3a). In addition to the reagent-mediated decaging, we also wondered if fluorosulfate decaging can be achieved in a light-mediated fashion. To this end, we prepared the 2-nitrobenzyl-caged activator **18** and demonstrated that it could activate and decage the fsY residues in **16** after exposed to 370 nm UV light irradiation, affording the corresponding sulfopeptide **17** in 60% isolated yield over 2.5 hours (Figure 3b). Semicarbazide was added to scavenge the nitrosobenzaldehyde produced in the light-mediated reaction (Figure S8).[39]

The sulfation patterns of tsetse fly anticoagulant peptide from tsetse thrombin inhibitor (TTI)[40] have been found to play a critical role in its thrombin inhibitory activities. We used the TTI peptides as a model system to probe the utility of the HA activators in controlling the bioactivities associated with sulfation under physiologically relevant conditions. First, TTI peptides consisting of fsY residues at position 9 and 12, **TTI02(fsY)**, **TTI03(fsY)**, and **TTI04(fsY)**, were subjected to the standard decaging conditions mediated by **7**. These reactions reached quantitative conversion within four hours, and produced the corresponding sulfopeptides **TTI02(sY)**, **TTI03(sY)**, and **TTI04(sY)** in 55%, 57%, and 35% isolated yield after HPLC purification, respectively (Figure 3c). Next, we used a standard human α-thrombin activity assay with Chromozym TH as the substrate to determine the inhibitory effects of the fsY-containing TTI peptides (latent) and the sY-containing TTI peptides (active).[41-42] Although the latent TTI peptides still exhibited minor inhibitory effects compared to the non-sulfated control **TTI01** (K_i = 2873 pM), with K_i values of 700 pM, 1547 pM, and 102 pM for **TTI02(fsY)**, **TTI03(fsY)**, and **TTI04(fsY)**, respectively, the active TTI peptides demonstrated significantly higher potencies, with K_i values of 82 pM, 43 pM, and 0.69 pM for **TTI02(sY)**, **TTI03(sY)**, and **TTI04(sY)**, respectively (Figure 3d and Figure S9).[43] The latent TTI peptides that were decaged in situ by adding activator **7** into the assay all showed similar inhibitory effects as the purified active TTI peptides (Figure 3c). These results confirmed that fluorosulfate can serve as an effective latent sulfate in peptides, and can be facilely decaged in aqueous solution at neutral pH. The two orders of magnitude difference in the K_i values between the latent and active TTI peptides provided a large window for light-controlled decaging. For example, while the latent **TTI04 (fsY)** remains inactive for thrombin inhibition at 3.7 nM in the presence of 2-nitrobenzyl protected activator **19** in dark, after irradiation thrombin activity was reduced to 21% (Figure 3e). Notably, no change in thrombin activity was observed in the absence of the TTI peptide or the activator, suggesting that the reduction of the thrombin activity was caused by the inhibition by the newly decaged latent TTI peptide.

While sY has been successfully incorporated into bacterial[44] and mammalian[45] proteins via the ncAA mutagenesis strategy, incorporation of caged sY that enables controlled release of sulfate post-translationally remained elusive. The small size of fluorine atom allows fsY to be facilely incorporated into proteins as a ncAA.[26, 46] Following the procedure established by Wang et al.,[26] we cloned the fsY-specific aminoacyl tRNA synthetase FsTyrRS and an optimal pyrrolysyl tRNA into plasmids for fsY incorporation into proteins. A sfGFP gene containing a TAG codon at position 151 was co-transformed along with the genes containing the FsTyrRA/tRNA pair into B95 E. coli cells. The targeted sfGFP-151-fsY was successfully expressed in a 12 mg/L yield. Tandem MS results verified the incorporation of fsY at the TAG-specified position-151 (Figure S10).[26, 47] Next, to probe the transformation from fluorosulfate to sulfate in sfGFP-151-fsY, as well as the integrity of the resulting sulfoprotein, we performed whole protein intact mass analyses of sfGFP-151-fsY before and after decaging using high-resolution Orbitrap mass spectrometry, which is capable of achieving sub-5 ppm mass accuracy[48] and can confidently resolve the 1.996 Da mass shift after decaging. Indeed, the Orbitrap mass spectrometry confirmed the decaging of sfGFP-151-fsY into sfGFP-151-sY by **7** (Figure 3f and Figure S11). Using this technique, we also confirmed the light-mediated decaging of sfGFP-151-fsY by the photocaged activator **19** (Figure 3f), highlighting the potential of our approach for the spatiotemporal release of caged sulfoproteins (Figure S12).

In conclusion, we demonstrated that fluorosulfate is a physiologically compatible latent sulfate in peptides and proteins. Fluorosulfate is stable in neutral aqueous buffers, cell lysates, and serum, and can be efficiently converted into sulfate by HA-derived activators under physiologically relevant conditions via a Lossen rearrangement pathway. Combined with the facile incorporation of fluorosulfate-containing amino acid fsY via solid-phase peptide synthesis and ncAA mutagenesis approaches, our reported approach can be applied to studying a wide range of sulfopeptides and sulfoproteins in their physiological states. Moreover, the easily modified, readily accessible, and diverse HA derivatives provide a vast playground for future studies on spatiotemporally controlling the functions of sulfated molecules in vitro and in vivo.

ASSOCIATED CONTENT

Supporting information including the supplementary figures and tables, characterization data and $^{1}H/^{13}C$-NMR spectra, and detailed experimental protocols is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

CCR5, C-C chemokine receptor type 5; PGSL-1, P-selectin glycoprotein ligand-1; HCII, heparin cofactor II.

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