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Site-selective *S-gem*-difluoroallylation of unprotected peptides with 3,3-difluoroallyl sulfonium salts†

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Bench-stable 3,3-difluoroallyl sulfonium salts (DFASs), featuring tunable activity and their editable C- β and *gem*-difluoroallyl group, proved to be versatile fluoroalkylating reagents for site-selective *S-gem*-difluoroallylation of cysteine residues in unprotected peptides. The reaction proceeds with high efficiency under mild conditions (ambient temperature and aqueous and weak basic conditions). Various protected/unprotected peptides, especially bioactive peptides, are site-selectively *S-gem*-difluoroallylated. The newly added *gem*-difluoroallyl group and other functional groups derived from C- β of DFASs are poised for ligation with bio-functional groups through click and radical chemistry. This stepwise “doubly orthogonal” modification of peptides enables the construction of bioconjugates with enhanced complexity and functionality. This proof of principle is successfully applied to construct a peptide–saccharide–biotin chimeric bioconjugate, indicating its great potential application in medicinal chemistry and chemical biology.

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Introduction

The tactical introduction of fluorine functionalities into organic molecules has emerged as one of the most powerful tools in modern drug discovery¹ and advanced functional materials,² because of the unique properties of fluorine atom(s) that often change the physiochemical and biological properties of organic molecules.³ In this context, the site-selective fluorofunctionalization of peptides has long been used to alter their acidity, basicity, hydrophobicity, geometry conformation, and bioavailability.⁴ Furthermore, owing to the absence of fluorine atoms in native biomolecules, such as peptides, fluorine functionalities can serve as probes to investigate the protein–ligand interaction and the instant conformational changes of peptides and proteins *via* ¹⁹F NMR.⁵ As a result, there is a high demand for the site-selective fluorination of peptides in peptidomimetics (Fig. 1a).

Among the 20 proteinogenic amino acids, cysteine, with its sulfhydryl side chain, exhibits superior nucleophilicity compared to other amino acids, rendering it the primary choice

for site-selective modification, including fluorination, with no doubt, of unprotected peptides.⁶ To date, the site-selective fluorination on cysteine residues has demonstrated diverse applications^{7–13} in chemoproteomic profiling of cysteines,⁷ ¹⁸F-PET molecular imaging,⁸ protein–ligand interaction,⁹ and efficient bioconjugation.¹¹ Nonetheless, the site-selective fluorination on cysteine residues of an unprotected peptide is severely constrained to only a few fluoroalkylating reagents, including the Togni reagent,¹⁴ Umemoto reagent,¹⁵ perfluoroarenes,^{10–13} and perfluoroalkyl halides compounds¹⁶ (Fig. 1b). This limitation is even more pronounced when considering fluoroalkylating reagents for other less nucleophilic amino acid residues, such as tryptophan,¹⁷ tyrosine,¹⁸ histidine, and dehydroalanine.¹⁹ Consequently, the restricted fluorination toolbox with limited fluorine space, perfluoroalkyl groups mostly, for peptides is insufficient to explore the unique “fluorine effect” in peptidomimetics.

To bridge the gap, we envisioned that the bench-stable 3,3-difluoroallyl sulfonium salts (DFASs) (Fig. 1c)^{20,21} could be beneficial for site-selective fluoroalkylation of peptides, because such a type of fluoroalkylating reagent features a flexible molecular scaffold to be readily edited, satisfying varieties of demands for peptide research: (1) the electrophilicity of DFAS could be tuned by the substituents on the sulfur atom, allowing for highly chemical selectivity toward cysteine without affecting other nucleophilic residues or enabling potential proximity-driven reactivity; (2) the β -C of DFASs could be readily modified with bioorthogonal functional groups, such as alkynyl, azide, or useful probes (*e.g.*, dyes, biomarkers); (3) upon

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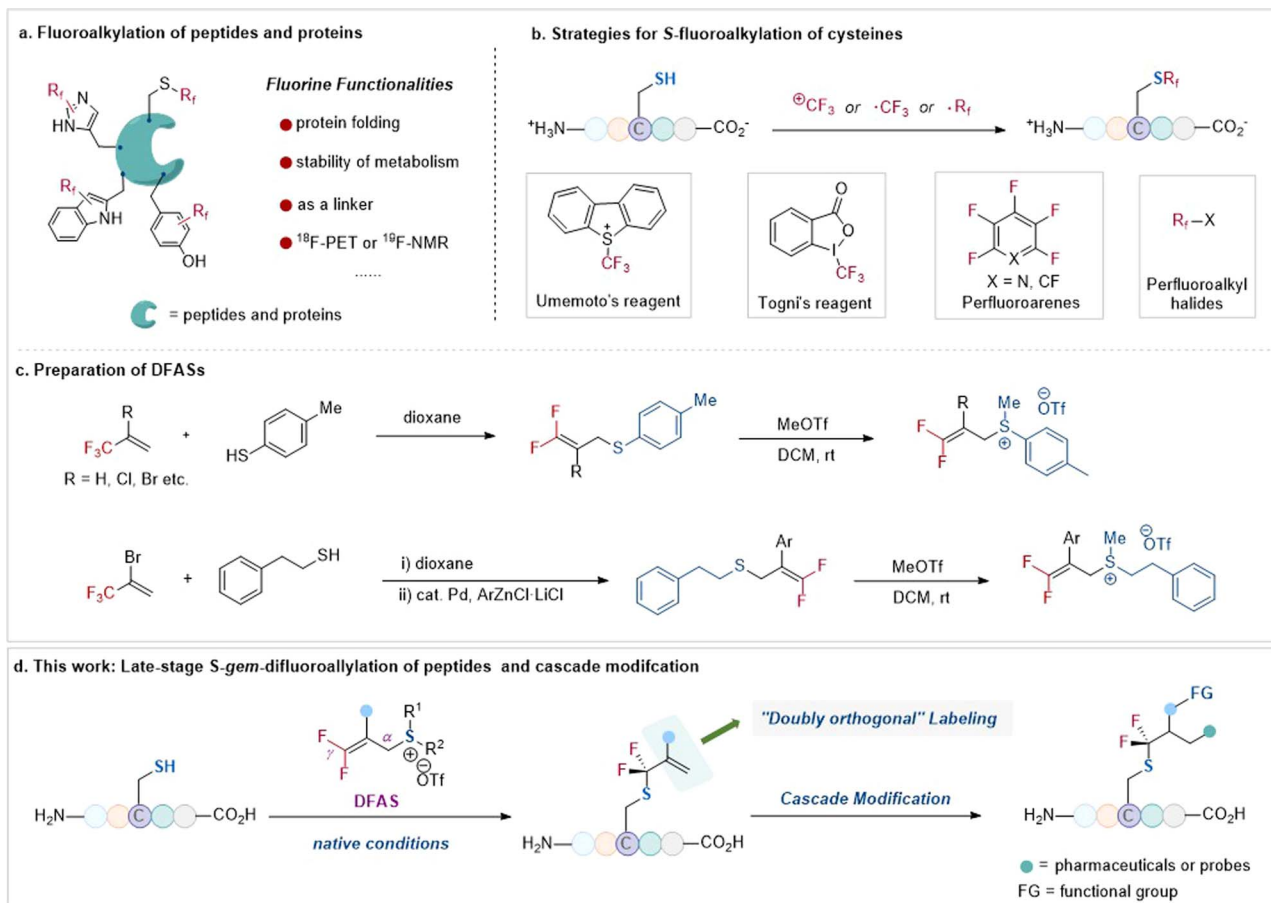


Fig. 1 Strategies for S-fluoroalkylation of cysteines and late-stage S-gem-difluoroalkylation of peptides and cascade modification (this work).

reacting with a peptide, the newly added *gem*-difluoroalkyl group is a good handle for successive "doubly orthogonal" modifications (Fig. 1d).

Results and discussion

To test our hypothesis, we chose protected cysteine **1a** and DFAS **2a** as the model substrates (Table 1). As depicted in Fig. 1c, DFASs could be easily prepared from inexpensive and bulk chemical feedstock 3,3,3-trifluoropropene (TFP) or 2-bromo-3,3,3-trifluoropropene through a two or three-step procedure (for details, see ESI†).^{20,21} A total conversion of **1a** was observed when the reaction was carried out with **1a** (1.0 equiv.) and **2a** (1.0 equiv.) in the presence of Na_2CO_3 (1.0 equiv.) in DMSO/ H_2O (1/1, v/v) at room temperature for 1 h (entry 1). Although moderate regioselectivity (**3a/4a** = γ/α = 3.3 : 1) was obtained, this positive result encouraged us to improve the regioselectivity. We envisioned that the introduction of an aryl group at the β -position of **2a** would increase the γ -selectivity, due to the ability of the aromatic ring to activate the *gem*-difluoroalkene, thus enabling attack of the thiol group on cysteine to the carbon-carbon double bond easier. An improved γ -selectivity (γ/α = 10 : 1) along with a 79% yield of **3b** was obtained when **2b** was used (entry 2). Replacing the aryl group on the sulfur atom

with an alkyl group could exclusively provide **3b** without observation of the side product **4b** (entry 3). However, the absence of the aryl group at the β -position did not affect the regioselectivity and reaction efficiency (entry 4), demonstrating the critical role of the substituent on the sulfur atom. We ascribed this observation to the decreasing leaving ability of the sulfonium salt by using an alkyl group on the sulfur atom, which provides a driving force to produce the γ -selectivity through an $\text{S}_{\text{N}}2'$ pathway. Considering that the introduction of an additional aryl group at the β -position of DFAS could improve the structural diversity for the cascade modification of the peptides, we used **2c** for further optimization. Further optimization of the reaction conditions showed that 0.5 equiv. of Na_2CO_3 was enough to promote the reaction (entry 5), and DMF is the optimal co-solvent, providing **3b** in almost quantitative yield (95%) (entry 6, for the examination of other solvents, see Table S3†). The competitive experiments between **1a** and other amino acids bearing a nucleophilic side chain with **2c** exhibited superior S-selective *gem*-difluoroalkylation (entries 7–14). In particular, tyrosine and lysine did not interfere with the reaction (entries 7 and 12), providing an opportunity for late-stage S-gem-difluoroalkylation of cysteine residues in peptides and proteins. We also found that the reaction is very fast and could completely afford **3b** within 4 min, in sharp contrast to

Table 1 Optimization of the reaction conditions^a

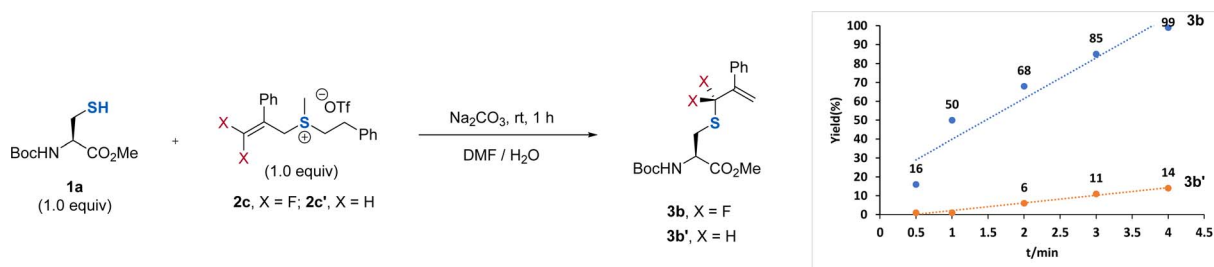
<p>2a, \bullet = H \bullet = <i>p</i>Me-C₆H₄</p> <p>2b, \bullet = Ph \bullet = Ph</p> <p>2c, \bullet = Ph \bullet = PhCH₂CH₂</p> <p>2d, \bullet = H \bullet = PhCH₂CH₂</p>					
Entry	2, x	AA (y)	Solvent	3 and 4	
1	2a, 1.0	— (0)	DMSO	3a/4a = 77/23	3.3 : 1
2	2b, 1.0	— (0)	DMSO	3b/4b = 79/8	10 : 1
3	2c, 1.0	— (0)	DMSO	3b/4b = 92/—	>99 : 1
4	2d, 1.0	— (0)	DMSO	3a/4a = 93/—	>99 : 1
5	2c, 0.5	— (0)	DMSO	3b/4b = 90/—	>99 : 1
6	2c, 0.5	— (0)	DMF	3b/4b = 95/—	>99 : 1
7	2c, 0.5	Boc-Tyr-OMe (1.0)	DMF	3b/4b = 99/—	>99 : 1
8	2c, 0.5	Boc-His-OMe (1.0)	DMF	3b/4b = 99/—	>99 : 1
9	2c, 0.5	Boc-Trp-OMe (1.0)	DMF	3b/4b = 90/—	>99 : 1
10	2c, 0.5	Boc-Ser-OMe (1.0)	DMF	3b/4b = 95/—	>99 : 1
11	2c, 0.5	Pro-OMe (1.0)	DMF	3b/4b = 96/—	>99 : 1
12	2c, 1.5	Boc-Lys (1.0)	DMF	3b/4b = 95/—	>99 : 1
13	2c, 1.5	Boc-Met (1.0)	DMF	3b/4b = 90/—	>99 : 1
14	2c, 1.5	Boc-Gln (1.0)	DMF	3b/4b = 99/—	>99 : 1

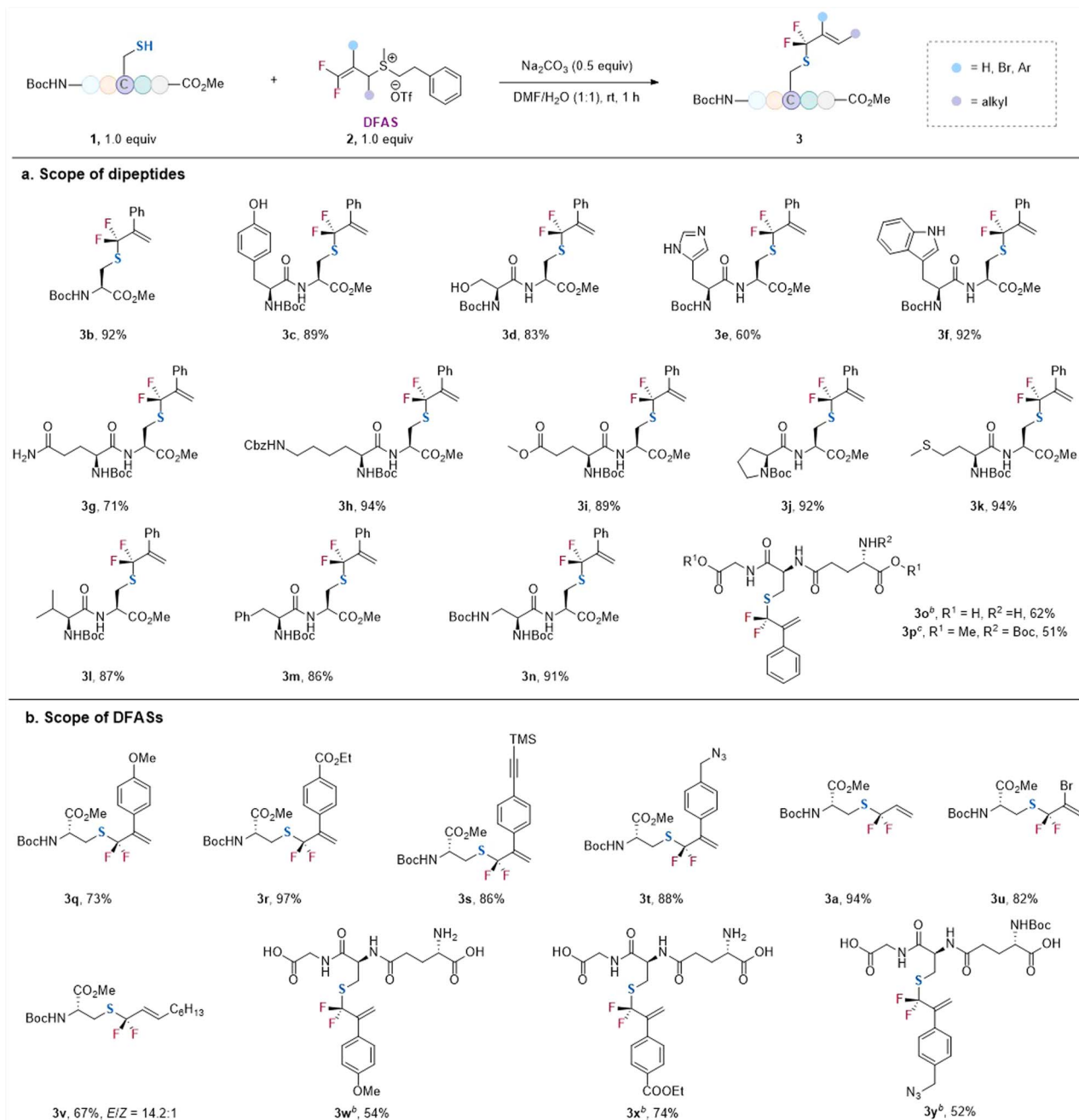
^a Reaction conditions (unless otherwise specified): **1a** (0.1 mmol, 1.0 equiv.), **2** (0.5–1.5 equiv.), solvent (1 mL), and H₂O (1 mL), rt, 1 h. ^b Determined by ¹⁹F NMR using fluorobenzene as an internal standard.

the non-fluorinated sulfonium salt **2c'**, which provided the corresponding nonfluorinated product in only 14% yield (Fig. 2), thus demonstrating the unique fluorine effect on the reaction.

With the optimized conditions in hand, we examined the reaction of **2c** with a variety of cysteine-containing dipeptides (Scheme 1a). The N-terminal and C-terminal of the peptides were protected for easy separation of the resulting *gem*-difluoroallylated products. Generally, high efficiency and excellent γ -selectivity ($\gamma/\alpha > 99 : 1$) were observed. The reaction exhibited excellent functional group tolerance. The amino acid residues bearing an unprotected nucleophilic side chain, such as tyrosine (**3c**), serine (**3d**), histidine (**3e**), tryptophan (**3f**), and glutamine (**3g**), did not affect the reaction, without observation of the *gem*-difluoroallylation side products resulting from their nucleophilic side chains. Additionally, protected lysine (**3h**), glutamic acid (**3i**), proline (**3j**), methionine (**3k**), valine (**3l**),

phenylalanine (**3m**), and glycine (**3n**) were all compatible with the reaction. Notably, unprotected glutathione (GSH), one of the most important natural antioxidants, underwent the *S*-selective *gem*-difluoroallylation smoothly ($\gamma/\alpha > 99 : 1$) (**3o**). In contrast, the protected GSH led to a slightly lower yield when the reaction was carried out on a one mmol scale (**3p**). The reaction was not restricted to **2c**, as DFASs **2** bearing different substituents at the β -position proceeded the reaction efficiently with excellent regioselectivity ($\gamma/\alpha > 99 : 1$) (Scheme 1b). For instance, the electronic nature of the aryl substituent did not affect the regioselectivity ($\gamma/\alpha > 99 : 1$) (**3q**, **3r**), even for the modification of free GSH (**3w–3y**). However, a higher yield was obtained for the aryl group bearing an electron-withdrawing group (**3r**) because of its more substantial inducing effect to activate the alkene. Additionally, the introduction of bioorthogonal groups, such as alkyne (**3s**) and azide (**3t**), also provided good opportunities for cascade modification of the peptides. Replacing the aryl group

Fig. 2 Kinetic studies of the reaction of **1a** with **2c** or **2c'**.



Scheme 1 *S-gem*-Difluoroallylation of cysteine-containing dipeptides and tripeptides with DFAS 2. ^aReaction conditions: 1 (0.2 mmol, 1.0 equiv.), 2 (1.0 equiv.), Na₂CO₃ (0.5 equiv.), H₂O (2 mL), DMF (2 mL), rt, 1 h. ^b2.5 equiv. Na₂CO₃ was added. ^cThe reaction was carried out on a 1 mmol scale. All reported yields are isolated yields.

at the β -position of DFAS with bromide furnished the corresponding product efficiently (**3u**). Notably, an alkyl group could also be introduced into the alkene with *E*-configuration as the major product (**3v**), demonstrating the generality of the protocol. This modification may also provide the potential to be used as a lipidation analog.²²

Encouraged by the excellent performance of DFASs in achieving regio/chemo-selective *S-gem*-difluoroallylation of cysteine-containing dipeptides and tripeptides, particularly unprotected GSH, a wide range of unprotected cysteine-

containing bioactive oligopeptides were examined, providing the corresponding *S-gem*-difluoroallylated peptides in moderate to good yields without observation of γ -selective allylation side products ($\gamma/\alpha > 99:1$) (Scheme 2). Cyclic peptide RGD, the most common peptide motif responsible for cell adhesion to the extracellular matrix,²³ underwent the *S*-selective *gem*-difluoroallylation smoothly (**6a**, **6b**). Notably, the introduced azide moiety provides an excellent opportunity for downstream biorthogonal conjugation to achieve new interesting bioactive molecules or probes (**6b**). Acyclic peptides from pentapeptide to





Scheme 2 Late-stage *S*-gem-difluoroallylation of unprotected cysteine-containing peptides with DFASs 2. ^a Reaction conditions: 5 (0.02 mmol, 1.0 equiv.), 2 (1.0 equiv.), Na_2CO_3 (1.5 equiv.), H_2O (1 mL), DMF (1 mL), rt, 1 h. ^b The reaction was carried out on a 0.04 mmol scale. ^c ^{19}F NMR yield. ^d (i) 5 (0.02 mmol, 1.0 equiv.), TCEP·HCl (1.5 equiv.), Na_2CO_3 (3.0 equiv.), H_2O (1 mL), DMF (1 mL), rt, 1 h; (ii) 2 (2.0 equiv.), rt, 1 h.

tridecapeptide were suitable substrates (**6c–6f**). Notably, non-peptide bearing the nucleophilic side chain-containing amino acid residues, including lysine, histidine, tyrosine, and tryptophan, was applicable to the reaction (**6d**), thus demonstrating the generality of the current protocol. The Tyr-amyloid P component (**6e**) and β -amyloid peptide fragment (**6f**) proceeded smoothly. The low isolated yield of **6c** is because of its decomposition upon purification. However, its ^{19}F NMR yield was high (72%). We also examined disulfide-bridged bioactive peptides oxytocin (**6g**, **6h**), the “best-understood neuropeptide”,²⁴ and

eptifibatide (**6i**), a glycoprotein inhibitor. Treatment of these cyclic peptides with tris(2-carboxyethyl)phosphine (TCEP)²⁵ to break their disulfide bonds, followed by the reaction of DFAS with the freshly generated thiol group, furnished the *S*-gem-difluoroallylated efficiently (**6g–6i**), featuring the synthetic simplicity and high efficiency without removing the excess TCEP of this two-step, one-pot procedure. The excellent compatibility of DFAS with TCEP would significantly expand its applicability in site-selective fluorination of peptides. Most importantly, the newly formed two *gem*-difluoroallyl handles



Scheme 3 Synthetic applications of the *gem*-difluoroallylated peptides and "doubly orthogonal" modification.

would enhance the chemical space to create more structurally diversified structures of medicinal and chemobiological interest. Moreover, this cysteine-selective *gem*-difluoroallylation has proven effective at low concentrations, as low as 50 μM , thereby providing a promising tool for modifying proteins (Table S7†).

To demonstrate the synthetic utility of the protocol, we explored the potential to construct more intricate bioconjugates by utilizing the newly added bioorthogonal groups. As shown in Scheme 3a, azide-containing RGD **6b** could be easily ligated

with a dansyl fluorophore or an anti-tumor drug, camptothecin, through a ring-strain promoted click reaction²⁶ with the carbon-carbon double bond intact. The newly formed *gem*-difluoroallyl group can also be a good synthetic handle for bioconjugation *via* a radical pathway (Scheme 3b). For example, *gem*-difluoroallylated GSH **3p** underwent radical addition smoothly when treated with readily available biotin-derived redox-active ester²⁷ in the presence of Hantzsch ester (HE) under blue light irradiation. Similarly, the bioactive molecule lithocholic acid derivative (**10b**) and carbohydrate (**10c**) could

also be easily introduced into the peptide. Encouraged by the bioorthogonal properties between the azide and alkenes, we turned our attention to the sequential “doubly orthogonal” peptide modification (Scheme 3c). Modified GSH **3y**, featuring an azide group, initially underwent click reaction²⁸ with biotin derivative **7c** efficiently. Subsequently, treatment of the “single orthogonal” modified peptide **11** with monosaccharide-derived redox-active ester under irradiation led to the “doubly orthogonal” modified peptide **12** with a synthetically reasonable yield. This flexible “doubly orthogonal” peptide modification enables the convenient construction of bioconjugates with multiple functional elements, thus meeting various demands of peptidomimetics research.

Conclusions

In conclusion, we have developed a highly chemo- and regio-selective method for late-stage *gem*-difluoroallylation of cysteine residues in unprotected peptides with versatile DFASs. The protocol features high biocompatibility, mild reaction conditions, and synthetic simplicity. Particularly, the tunable activity of DFAS, as well as the biorthogonal property of the newly added functionalities and the *gem*-difluoroallyl group, allows for stepwise “doubly orthogonal” modification of peptides to meet various demands in peptidomimetics research. This proof of principle has been successfully applied to a wide range of oligopeptides, including bioactive peptides, such as GSH, RGD, and β -amyloid peptide fragment, showing great potential to explore the unique fluorine effect of the CF₂ group in medicinal chemistry and chemical biology, leading to the discovery of new bioactive molecules.

Data availability

All experimental data, procedures for data analysis, and pertinent data sets are provided in the ESI.†

Author contributions

X. Z. and J.-X. R. conceived and designed the experiments. X. Z. directed the project. J.-X. R. performed the experiments. M. Z. and X.-T. F. prepared some starting materials. J.-X. R., F.-P. F., and H.-Y. Z. analyzed the data of ESI.† X. Z. wrote the paper. All authors discussed the results and commented on the manuscript.

Conflicts of interest

There are no conflicts to declare.

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