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RESEARCH ARTICLE

Isoindolium-Based Allenes: Reactivity Studies and Applications in Fluorescence Temperature Sensing and Cysteine Bioconjugation

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Abstract: The reaction of a series of electron-deficient isoindolium-based allenes with sulfhydryl compounds has been studied, leading to the formation of isoindolium-based vinyl sulfides. The vinyl sulfides generated could be readily converted into the corresponding indanones and amines upon heating at 30 - 70 °C with good yields up to 61%. The thermal cleavage reaction of vinyl sulfides was further studied for developing temperature-sensitive systems. Notably, a novel FRET-based fluorescent temperature sensor was designed and synthesized for temperature sensing at 50 °C, giving a 6.5-fold blue fluorescence enhancement. Moreover, chemoselective bioconjugation of cysteine-containing peptides with the isoindolium-based allenes for the construction of multifunctional peptide bioconjugates was investigated. Thermal cleavage of isoindoliums on the modified peptides at 35 - 70 °C gave indanone bioconjugates with up to >99% conversion. These results indicated the biocompatibility of this novel temperature-sensitive reaction.

Introduction

Development of temperature-sensitive linkers and systems has significant importance in chemical biology and food industry due to their potential applications in a wide range of research fields, such as magnetic hyperthermia (MHT) and photothermal therapy (PPT) for cancer treatment,[1-2] as well as time-temperature indicators (TTIs)[3-4] and smart food packaging[5 -6]. In the biomedical field, temperature-sensitive drug delivery systems combined with hyperthermia treatment (40 - 45 °C) can serve as a promising approach to achieve on-demand drug release. [7-9] For food safety applications, time-temperature indicators (TTIs) with response temperature range within "temperature danger zone" (< 60 °C) can help record the temperature history of perishable food products.[10-11] With the versatile potential applications in different fields of science and technology, the development of innovative temperature-sensitive systems is considered significantly important.

The design principles of temperature-sensitive systems are generally based on the intrinsic properties of thermoresponsive materials and thermal cleavage of chemical bondings. These thermally sensitive units could be disassembled or cleaved in response to changes in temperature that allow controllable drug release[12] and monitoring of thermal history.[13] Over the past years, thermo-labile covalent bonds, such as thermo-labile azo-linkers[14] and thermo-reversible Diels-Alder (DA) reactions[15], have been introduced as thermo-degradable moieties for the development of thermoresponsive systems (Scheme 1A). However, high temperature (> 80 °C) is generally required for effective bond cleavage, which restricts their applications in biological chemistry.[16-17] Despite the diverse applications of the temperature-sensitive systems, the discovery of new classes of thermally cleavable linkers with biocompatibility remains sparse. Therefore, the development of new thermally cleavable linkers that allows covalent bond cleavage under mild conditions and in a tunable temperature range is of great importance.

Recently, we have developed a regio- and chemoselective synthesis of 1*H*-isoindolium-based alkynes and allenes through a facile and novel cascade cyclization/iodination of propargylamine-based 1,6-diynes under mild conditions.[18] Based on this work, we were interested to explore the reactivity of these electron-deficient compounds towards various nucleophiles and their applications in biological chemistry. Herein, we report a regio- and chemoselective reaction of isoindolium-based allenes **1** with sulfhydryl compounds to give isoindolium-based vinyl sulfides **2**, and the novel thermal cleavage reaction of **2** for developing temperature-sensitive systems. As described in Scheme 1B, treatment of isoindolium-based allene **1** with thiol or sulfhydryl-containing peptide/dye affording an isoindolium-based vinyl sulfide **2** which could be converted to the corresponding indanone **3** and amine **4** upon heating at a relatively low temperature range (30 - 70 °C). The applications of vinyl sulfides **2** were demonstrated by the design and synthesis of 1) a FRET-based fluorescent temperature sensor and 2) multi-functionalized cysteine-containing peptide bioconjugates (Scheme 1C).

Results and Discussion

To study the reactivity of the electron-deficient isoindolium-based allenes towards nucleophiles, we initially performed the reaction of allene **1a** with various amines.[19-20] However, the formation of desired adducts were not observed in different reaction conditions (see Table S1 in Supporting Information). To our delight, the isoindolium-based allene **1a** exhibited high chemo

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Supporting information for this article is given via a link at the end of the document.

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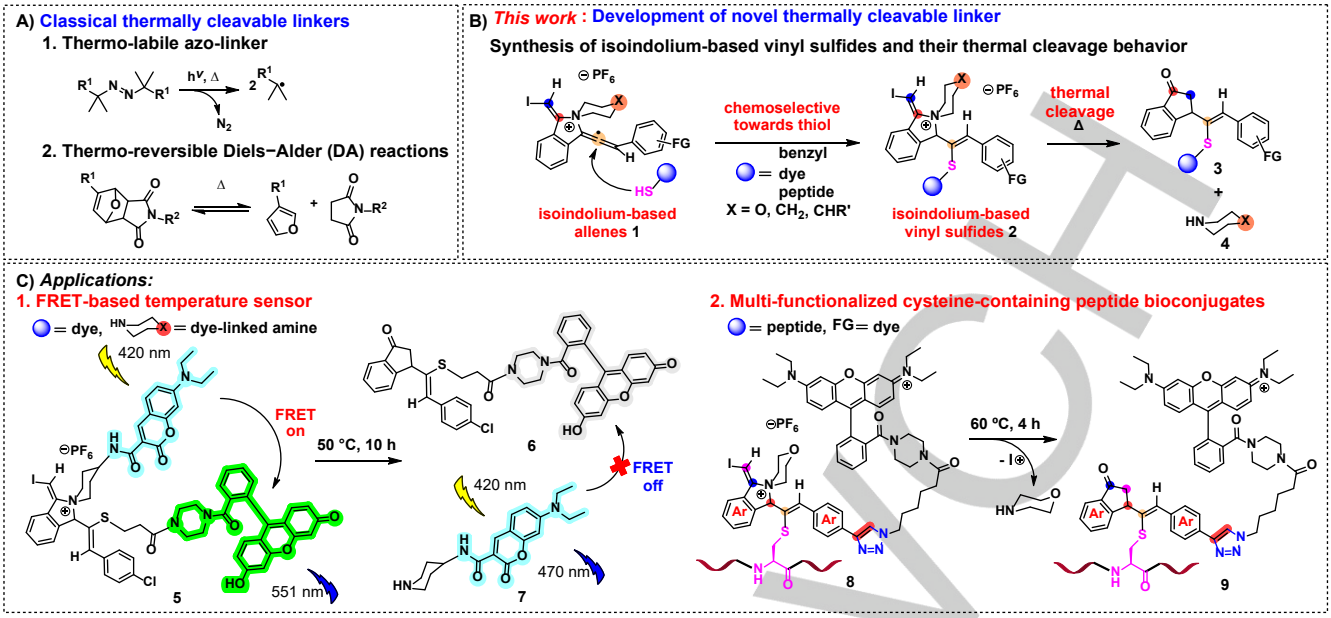
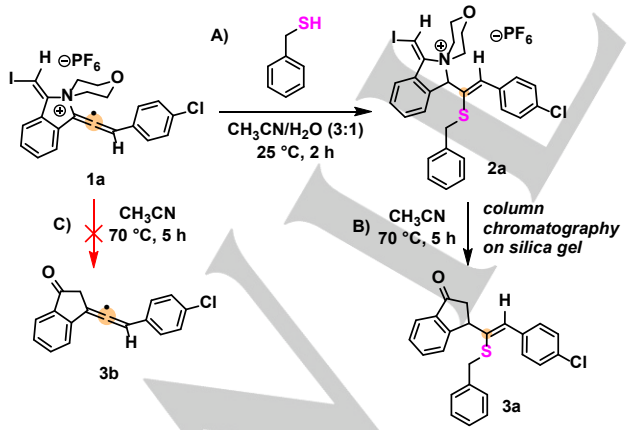


Scheme 1. (A) Classical thermally cleavable linkers; **(B)** The development of novel thermally cleavable linker; **(C)** Applications of novel thermally cleavable linker.

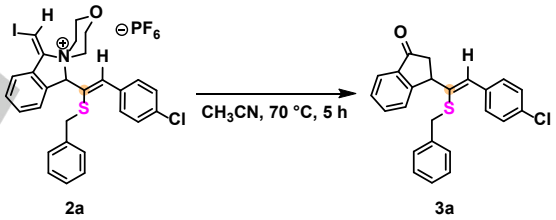
-selectivity towards sulfhydryl group. Treatment of allene **1a** with benzyl mercaptan in CH_3CN/H_2O (3:1) at 25 °C for 2 h resulted in a vinyl sulfide product **2a** in 66% isolated yield (Scheme 2A). In addition, we found that when **2a** was heated at 70 °C in CH_3CN for 5 h, an indanone product **3a** was isolated in 43% yield (Scheme 2B). The structure of **3a** was confirmed by ^1H NMR, ^{13}C NMR and HRMS analysis. (see Figure S3 and S4 in Supporting Information). In contrast, similar formation of the expected indanone **3b** was not observed when heating **1a** under the same reaction conditions (Scheme 2C). These preliminary findings suggested that the paradigm of thermal cleavage could only be observed for vinyl sulfide **2a** and the thermal sensitivity of isoindolium-based vinyl sulfide would be an excellent candidate to develop a class of novel thermally cleavable linker for new temperature-sensitive systems.



Scheme 2. (A) Reaction of electron-deficient allene **1a** with thiols; (B) Thermal cleavage reaction of **2a**; (C) Attempt of thermal cleavage reaction of **1a**.

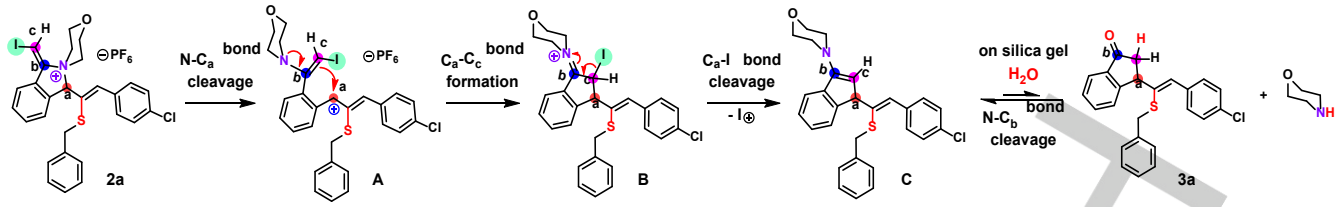
To get insight of the thermal cleavage of the isoindolium-based vinyl sulfide, we investigated the influence of temperature and solvent on the thermal cleavage of **2a** (Table 1). As mentioned, **3a** could be obtained in 61% yield when the reaction was carried out at 70 °C (Table 1, entry 1). When the reaction temperature was reduced to 60 °C and 50 °C, the yield of **3a** significantly decreased to 43% and 12%, respectively (Table 1, entries 2-3). Heating of **2a** at 40°C and 30 °C gave a trace amount of product

Table 1. Condition screening of thermal cleavage of **2a**.[a]



Entry	Solvent	Temp (°C)	Yield of **2a** (%)[b]
1	CH_3CN	70	61(43[c])
2	CH_3CN	60	43
3	CH_3CN	50	12
4	CH_3CN	40	trace
5	CH_3CN	30	trace
6	CH_3CN	25	0
7	DCE	70	54
8	THF	70	35
9	DMF	70	18
10	CH_3CN/H_2O (3:1)	70	50

[a] Reactions were performed with **1a** (0.05 mmol) in solvent (2 mL) for 5 h. [b] Yield was determined by ^1H NMR analysis using 1,3,5-trimethoxybenzene as the internal reference. [c] Isolated yield.



Scheme 3. Proposed pathway of the thermal cleavage reaction.

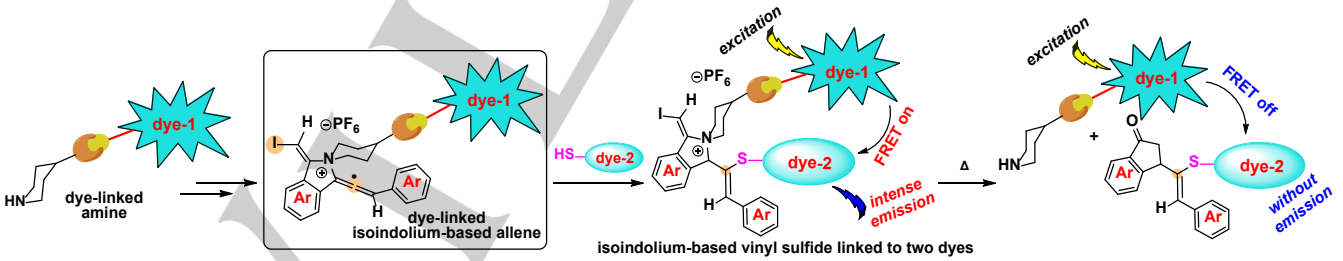
3a (Table 1, entries 4-5). The formation of **3a** was not observed after stirring of **2a** at 25 °C for 5 h (Table 1, entry 6), indicating that isoindolium-based vinyl sulfide **2a** was stable without heating. In addition, comparable yield (54%) of **3a** could be obtained in DCE (Table 1, entry 7) while lower product yields were obtained in THF and DMF in 35% and 18% yield, respectively (Table 1, entries 8-9). To examine the compatibility with biological systems, the reaction was carried out in CH_3CN/H_2O (3:1). Product **3a** was achieved in 50% yield, revealing that this reaction was compatible with aqueous solvent (Table 1, entry 10).

On the basis of the experimental results, a proposed reaction pathway accounting for this thermal cleavage reaction is outlined in Scheme 3. Initially, a N-C_a bond cleavage of **2a** leads to an enamine intermediate **A**, which undergoes a nucleophilic attack leads to a C_a-C_c bond formation to give an iminium intermediate **B**. Subsequent C_a-I bond cleavage of **B** to give enamine **C**.[21] The enamine **C** is reactive to electrophiles. Thus, indanone **3a** was obtained after column chromatography on silica gel, together with the release of amine.

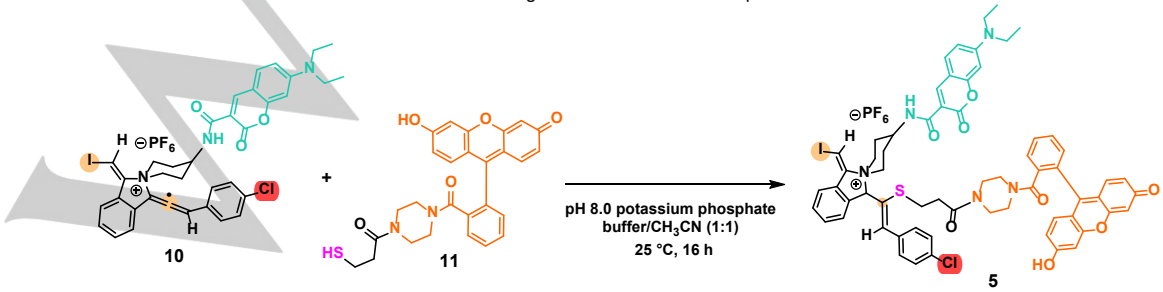
Fluorescence detection and sensing techniques are essential tools with a diversity of applications in various scientific fields due to their high sensitivity and operation simplicity.[22] In particular, fluorescence resonance energy transfer (FRET) has been a powerful technique in the studies of biomolecules. FRET refers to a non-radiative energy transfer from the excited-state donor fluorophore to the proximal ground-state acceptor fluorophore. FRET-based fluorescent probes rely on a distance-dependent energy transfer process, which display an inherent sensitivity and selectivity to the environment conditions. [23-25] Due to the easy elaboration of the allene and amine moieties of the isoindolium-based allene **1**, we hypothesized that a FRET dye pair could be linked by the reaction of a dye-linked isoindolium-based allene with a dye-linked thiol, giving an isoindolium-based vinyl sulfide linked to two dyes as a FRET-based fluorescent probe. Along with our interest in the development of fluorescent sensors,[26-29] we envisioned that thermal cleavage of the fluorescent isoindolium-based vinyl sulfide giving a dye-linked indanone and dye-linked amine would achieve a novel FRET-based temperature sensing system (Scheme 4).

In line with our previous studies on FRET-based probes,[29] coumarin and fluorescein were chosen as the fluorophores for the synthesis of the FRET-based sensor. Considering the spectral overlap between the emission wavelength of coumarin and the excitation wavelength of fluorescein, we envisioned that FRET effect would occur upon linkage of these two fluorophores. Thus, coumarin-linked allene **10** and fluorescein-linked thiol **11** were synthesized (see details in Supporting Information), respectively. Coumarin-linked allene **10** was treated with fluorescein-linked thiol **11** in potassium phosphate buffer (pH 8.0)/CH_3CN (1:1) at room temperature for 16 h, affording vinyl sulfide **5** as confirmed by HRMS analysis (Scheme 5).

We examined the photophysical properties of coumarin-linked allene **10**, fluorescein-linked thiol **11** and vinyl sulfide **5** (Figure 1). Coumarin-linked allene **10** displayed blue fluorescence at 470 nm, while fluorescein-linked thiol **11** showed very weak



Scheme 4. Our design of the FRET-based temperature sensor.



Scheme 5. Preparation of fluorescent vinyl sulfide **5**.

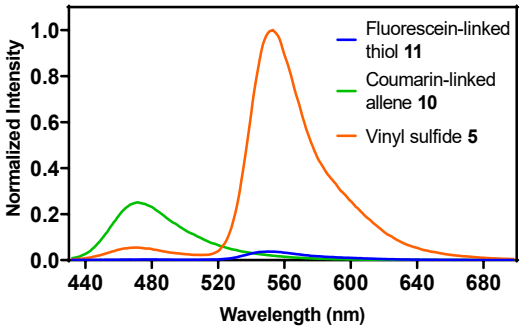


Figure 1. Fluorescence spectra of coumarin-linked allene **10**, fluorescein-linked thiol **11** and vinyl sulfide **5** upon excitation at 420 nm (2 μM in DMSO).

green fluorescence at 551 nm. Upon excitation at 420 nm (λ_{ex} for coumarin), vinyl sulfide **5** exhibited weak fluorescence intensity at 470 nm but intense fluorescence signal at 551 nm, indicating that FRET has occurred. As vinyl sulfide **5** has exhibited distinct FRET effect, we suggested that coumarin-linked allene **10** acted as a donor fluorophore (D) and fluorescein-linked thiol **11** served as an acceptor fluorophore (A) in the FRET-based system.

Next, the fluorescence response of vinyl sulfide **5** upon heating was investigated. Given the inherently thermal sensitivity of **5**, we proposed that heating of **5** would afford cleavage product **6** with the release of coumarin-linked amine **7** in which the coumarin donor and the fluorescein acceptor were separated, and hence no FRET was observed (Figure 2A). The thermal cleavage reaction of vinyl sulfide **5** was conducted by heating at 50 °C in potassium phosphate buffer (pH 8.0)/CH_3CN (3:1) for 10 h. The presence of cleavage products **6** and **7** were confirmed by HRMS analysis of the crude reaction mixture (Figure 2B). During the reaction, the fluorescence signal was measured at each time point for time course experiments. The results showed that there was an increase in blue fluorescence at 470 nm and a decrease in green fluorescence at 551 nm, indicating that FRET was quenched due to the cleavage of linkage between the donor and acceptor upon the thermal cleavage of vinyl sulfide **5** (Figure 2C).

We further explored the fluorescence response of vinyl sulfide **5** at different pH values. Thermal cleavage reactions of **5** were performed at pH 7.0 and 6.0, respectively, under the same reaction conditions. Fluorescence intensity changes could be significantly observed (Figure 2D and 2E), indicating that the reaction proceeded well within the physiological pH range. As shown in Figure 2F, the value of F_{470}/F_{551} increased over time. Notably, higher fluorescence intensity ratio between 470 and 551 nm were observed at lower pH (6.0). The highest value of F_{470}/F_{551} (0.369) was achieved when the reaction was conducted at pH 6.0 upon heating at 50 °C for 10 h, giving a 6.5-fold blue fluorescence enhancement. These results could be attributed to the inherent sensitivity of enamine to acidic medium. Slightly acidic pH conditions facilitated the hydrolysis of enamine to ketone **6**, and thus the release of coumarin-linked amine **7** was favored. The color changes of the crude reaction mixture from green to blue could be easily recognized under UV light (Figure 2G). In contrast, control experiment of performing the reaction at 25 °C for 10 h gave negligible fluorescence intensity changes (see Figure S4 in Supporting Information). This result demonstrated that vinyl sulfide **5** was heat-sensitive and the increased fluorescence signal ratio was originated from the thermal cleavage reaction.

These results suggested that the isoindolium-based vinyl sulfide **5** bearing a FRET dye pair exhibited temperature sensing ability. Heating promoted the release of coumarin-linked amine **7**

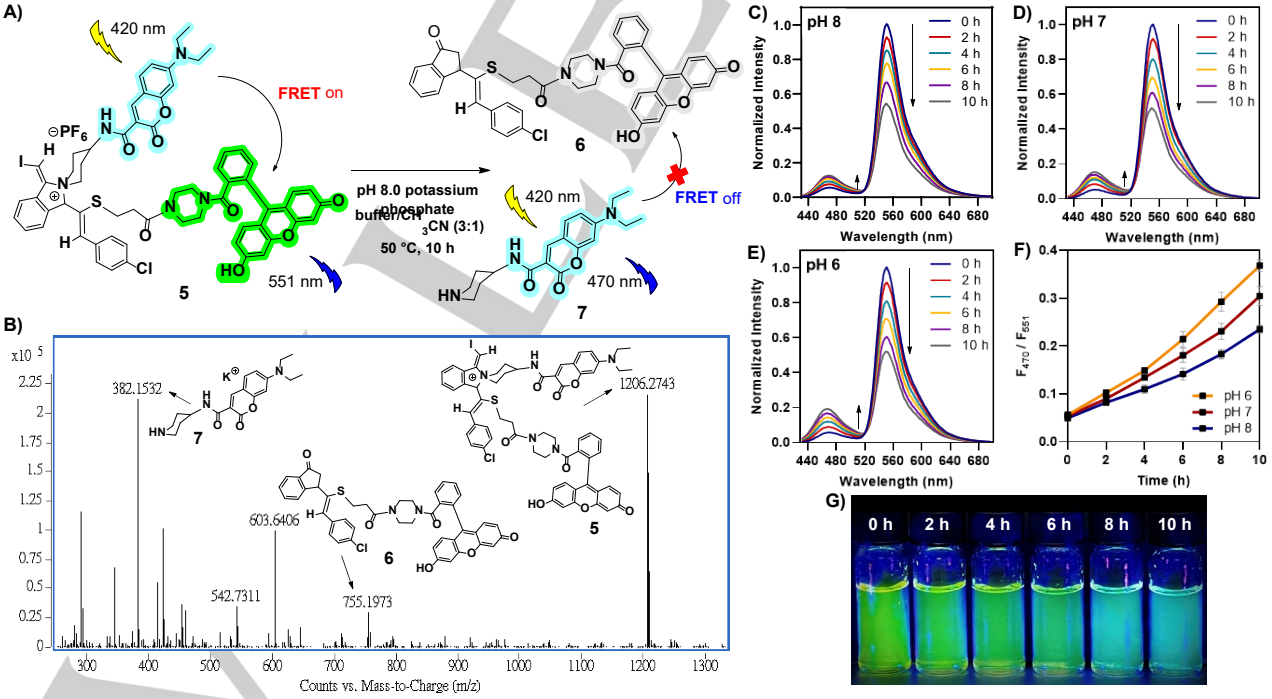
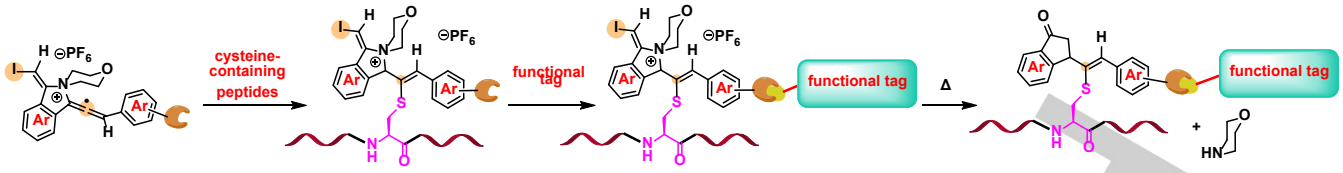


Figure 2. (A) Thermal cleavage of vinyl sulfide **5** and FRET effect; **(B)** HRMS analysis of the reaction mixture of thermal cleavage of vinyl sulfide **5**; Normalized fluorescence spectra of thermal cleavage reaction of **5** at **(C)** pH 8.0; **(D)** pH 7.0; **(E)** pH 6.0; **(F)** Fluorescence intensity ratio (F_{470}/F_{551}) responses of **5** over time at different pH values. **(G)** Fluorescence images of the mixtures of thermal cleavage reaction at different time points under a 365 nm UV lamp (pH 6.0, 3 μM in DMSO).



Scheme 6. Our design of cysteine modification of peptides using electron-deficient allenes and the construction of multi-functionalized peptides.

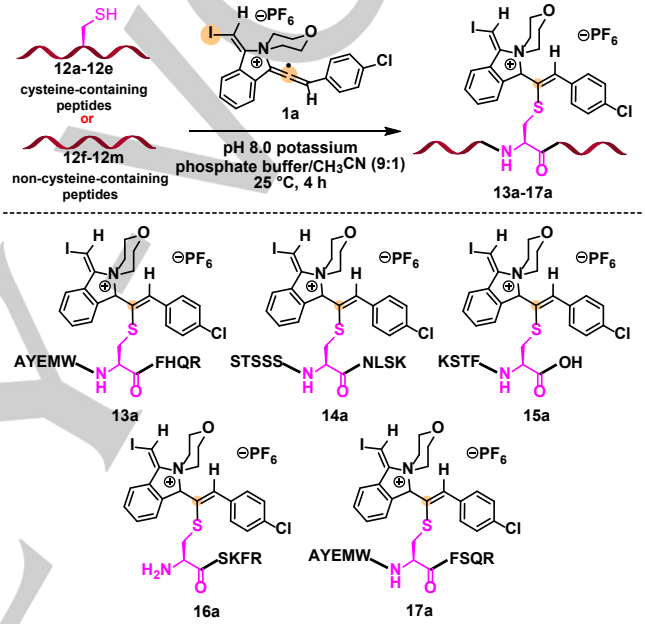
resulting in a 6.5-fold blue fluorescence enhancement, in which irreversible color changes could be observed from the cumulative effect of temperature and time. We envisioned that this novel approach would serve as a potential candidate for novel time-temperature indicators.

Site-selective modification of peptides/proteins has been well developed as a powerful tool for biological studies and development of targeted therapeutics.[30] The low abundance (~ 1.7%), unique reactivity of the sulfhydryl group and ease of incorporation by site-directed mutagenesis allow cysteine to serve as an ideal residue for peptides/proteins labeling.[31-50] In our previous works on modification of cysteine-containing peptides and proteins, we found that electron-deficient alkynones and isoxazoliniums were excellent reagents due to their outstanding electrophilicity.[36a],[37c] Along with our ongoing interest in the development of selective bioconjugations,[36-37],[51-52] we envisioned that the isoindolium-based allene **1**, which exhibited excellent electrophilicity towards sulfhydryl group, would be a useful handle for chemoselective modification of cysteine-containing peptides. Furthermore, functional tags (such as dyes) could be attached to the modified peptides by bioorthogonal click reaction, resulting in multi-functionalized peptide bioconjugates (Scheme 6).

With the idea in mind, we explored the potential applicability of the isoindolium-based allenes in cysteine bioconjugation. We began our study on the bioconjugation between cysteine-containing peptides **12a-12e** and isoindolium-based allene **1a**. Under the optimized reaction conditions (see Table S2 in Supporting Information), treatment of cysteine-containing peptide AYEMWCFHQR **12a** (0.1 mM) with **1a** (5 equiv) in potassium phosphate buffer (pH 8.0)/CH_3CN (9:1) at 25 °C for 4 h afforded modified peptide **13a** in 95% conversion with all other amino acid residues remaining intact as confirmed by LC-MS/MS analysis (Table 2, entry 1). In addition, treatment of cysteine-containing peptides STSSSCNLSK **12b**, KSTFC **12c**, CSKFR **12d** and AYEMWCFSQR **12e** with **1a**, respectively, affording modified peptides **14a**, **15a**, **16a** and **17a** in excellent conversions between 85% and 98% (Table 2, entries 2-5). To verify the cysteine selectivity of the modification, peptides **12f-12m** without free cysteine residue were used to react with **1a** (Table 2, entries 6-13). Notably, no bioconjugation was observed, indicating that the modification was highly chemoselective towards the thiol moiety of cysteine residue despite the presence of other nucleophilic residues, such as *N*-terminus, lysine, histidine, tryptophan and methionine, *etc*.

Next, we investigated the scope of isoindolium-based allenes **1** for the cysteine bioconjugation. Various allenes **1b-1g** bearing different substituents (including F, CF_3, CN, alkynyl and methyl) were treated with cysteine-containing peptides **12a** and **12b,** respectively, providing the corresponding modified peptides **13b-13g** and **14b-14g** in good to excellent conversions (Table 3, entries 2-7; see Table S4 in Supporting Information). Moreover, alkyne-functionalized peptide **13f** could be labeled with fluorescent rhodamine dye to give a multi-functionalized fluorescent peptide **8** by Cu(I)-catalyzed azide-alkyne Huisgen cycloadditions in 62% conversion.

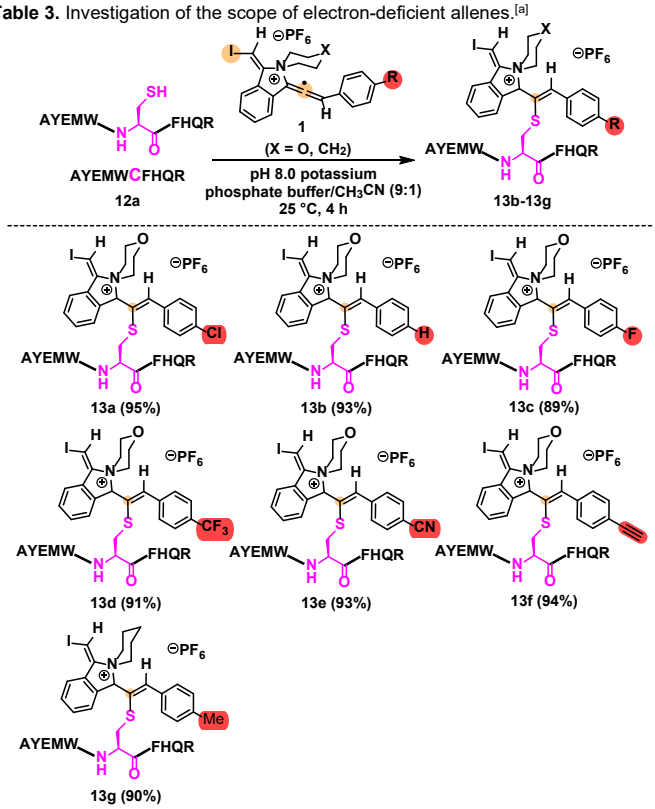
Table 2. Investigation of the cysteine selectivity. [a]



Entry	Peptide	Conversion of peptide (%)[b]
1	AYEMW**C**FHQR **12a**	95
2	STSSS**C**NLSK **12b**	96
3	KSTF**C** **12c**	85
4	**C**SKFR **12d**	94
5	AYEMW**C**FSQR **12e**	98
6	STSSSANLSK **12f**	0
7	STSSSHNLSK **12g**	0
8	AYEMWSFHQR **12h**	0
9	WSKFR **12i**	0
10	YSKFR **12j**	0
11	PSKFR **12k**	0
12	GSKFR **12l**	0
13	ISKFR **12m**	0

[a] Peptides **12a-12m** (1 mM), allene **1a** (5 equiv) in 50 mM pH 8.0 potassium phosphate buffer/CH_3CN (9:1) at 25 °C for 4 h. [b] Conversion was determined by LC–MS analysis.

Table 3. Investigation of the scope of electron-deficient allenes.[a]

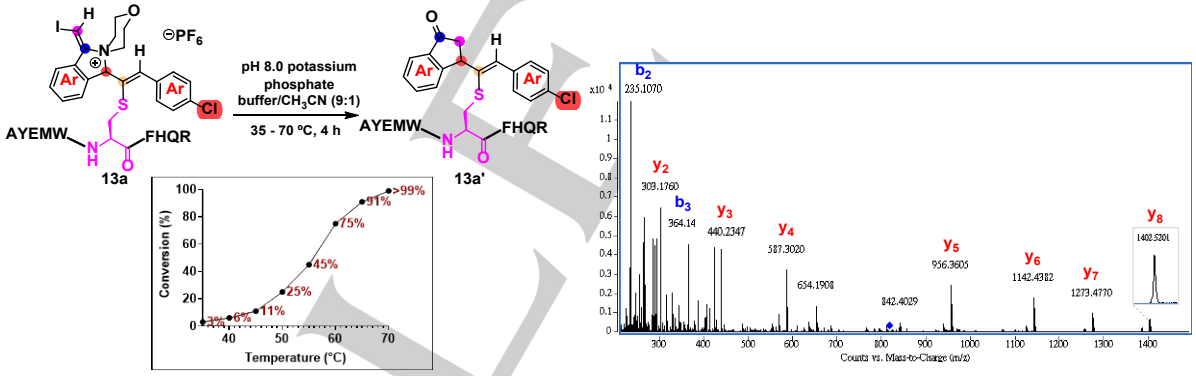


[a] AYEMWCFHQR **12a** (1 mM), allene **1** (5 equiv) in 50 mM pH 8.0 potassium phosphate buffer/CH_3CN (9:1) at 25 °C for 4 h. [b] Conversion was determined by LC–MS analysis.

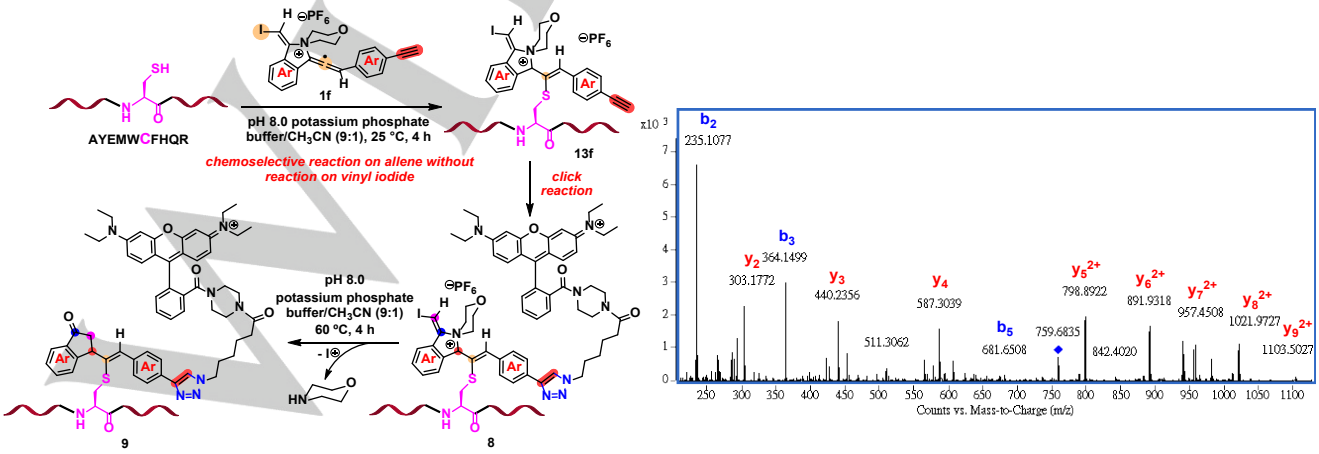
We studied the thermal cleavage reaction of allene-modified peptide **13a** to **13a'** at different temperatures (Scheme 7). According to the LC-MS analysis, formation of product **13a'** (1635 Da) was observed upon heating of modified peptide **13a** (1831 Da) at 35 °C for 4 h. The loss of molecular mass (196 Da) at the cysteine residue indicated the removal of iodide and amine moieties. The efficiency of thermal cleavage was increased to 75% conversion when the temperature was elevated to 60 °C. Upon heating of **13a** at 70 °C, excellent conversion (>99%) of cleavage product was observed. According to the LC-MS/MS analysis, only the cysteine residue on the peptide was modified with all other residues remained intact. These results were consistent with different allene-modified peptides (See Table S5 and Figure S7 in Supporting Information).

In addition, heating of rhodamine-linked peptide **8** (2471 Da) at 60 °C afforded cleavage product **9** (2275 Da) in 94% conversion. The fluorescent label was retained, revealing that the rhodamine tag on the modified peptide did not affect the thermal cleavage reaction and remained stable during the reaction (Scheme 8).

The studies well demonstrated the high efficiency of allenes **1** for chemoselective modification of cysteine-containing biomolecules. Functional tags could be attached to the modified peptides by further bioorthogonal click reaction, which allowed the construction of multi-functionalized bioconjugates. The utility of the thermal cleavage reaction of isoindoliums on the modified peptides enabled the release of amine components in response to changes in temperature. It is envisaged that this novel approach would be interesting for the further development of temperature-sensitive drug delivery system.



Scheme 7. Study of thermal cleavage reaction of allene-modified peptide **13a** to **13a'** at different temperatures.



Scheme 8. Copper(I)-catalyzed azide-alkyne cycloadditions of modified peptide **13f** with rhodamine azide and thermal cleavage of rhodamine-linked peptide **9**.

Conclusion

In summary, we have first developed a novel thermal cleavage reaction of isoindolium-based vinyl sulfides. Heating of isoindolium-based vinyl sulfides at a temperature range of 30 °C to 70 °C resulted in thermal cleavage reaction, giving indanones and amines as the cleavage products. We further demonstrated the utility of this thermal cleavage behavior of isoindolium-based vinyl sulfides by the development of a FRET-based temperature sensor and multi-functionalized cysteine-containing peptide bioconjugates. This work provided a new class of thermal cleavage reaction. It is envisioned that this class of novel thermal cleavable linker can be employed to different fields of research area, including chemical biology, drug delivery and food safety.

Acknowledgements

We are grateful for the financial support from Hong Kong Research Grants Council (PolyU 153001/17P), the Shenzhen Science and Technology Innovation Commission (JCYJ20170818104257975), the State Key Laboratory of Chemical Biology and Drug Discovery, Dean's Reserve (P0034752: ZVST), and The Hong Kong Polytechnic University (P0043816: WZ0Y).

Keywords: allenes • thermal cleavage reactions • chemoselectivity • cysteine • FRET

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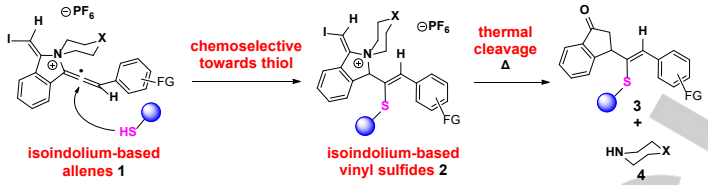

The reactivity of a series of electron-deficient isoindolium-based allenes **1** with sulfhydryl compounds has been studied. The vinyl sulfides **2** generated were converted into indanones **3** and amines **4** upon heating at 30 - 70 °C with good yields. The applications of the vinyl sulfides **2** included design and synthesis of a novel FRET-based fluorescent temperature sensor and chemoselective cysteine bioconjugation providing multifunctional peptide bioconjugates.