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Herein we introduce 3-vinyl-1,2,4-triazines derivatives as dual-reactive linkers that exhibit selectivity towards cysteine and specific strained alkynes, enabling conjugate addition and inverse electron-demand Diels–Alder (IEDDA) reactions. This approach facilitates site-selective bioconjugation of biologically relevant peptides, followed by rapid and highly selective reactions with bicyclononyne (BCN) reagents.

The reactivity variations among amino acids have been effectively utilised in the realm of selective peptide and protein modifications. Extensive methods have been developed for modification of lysine,^{1,2} cysteine,^{3,4} tryptophan,⁵ methionine,^{6,7} tyrosine,⁸ histidine⁹ and the *N*- and *C*-terminal residues.^{10,11} Among these, cysteine's exceptional nucleophilicity under mild and biocompatible conditions has made it an attractive and dependable candidate for orthogonal manipulation. Numerous reagents have been developed specifically for cysteine modification, encompassing bromomaleimides,¹² vinylsulfones,¹³ and others.^{14–17} Despite the emergence of these advanced cysteine conjugation technologies, maleimide reagents remain a highly favoured choice due to their rapid reaction kinetics.¹⁸ Nevertheless, they exhibit limited chemoselectivity and tend to react with other nucleophilic residues present on the protein surface. Additionally, the thiosuccinimide linkages formed during maleimide conjugation can result in inadequate plasma stability. Premature release of payloads compromises their intended drug delivery properties, leading to off-target effects. To mitigate these detrimental side effects and enhance the therapeutic window of such treatments, the development of more stable linkages becomes crucial. Therefore, there is a need for improved cysteine modification reagents that can address these challenges. In addition to direct modification with payloads, alternative modification strategies introduce functional

Site-selective peptide functionalisation mediated via vinyl-triazine linchpins[†]

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groups capable of performing bioorthogonal click reactions.^{19–21}

Commonly employed bioorthogonal click reactions include the copper-catalysed azide–alkyne cycloaddition (CuAAC),^{22,23} strain-promoted azide–alkyne cycloaddition (SPAAC),²⁴ and inverse electron-demand Diels–Alder (IEDDA) reactions between tetrazines and strained alkynes and alkenes.²⁵ While IEDDA reactions have found utility in modifying various biomolecules, the high reactivity of tetrazine groups can pose challenges due to their decomposition in buffer or reactivity with thiol groups.²⁵ Recently, 1,2,4-triazines have emerged as effective IEDDA reagents, exhibiting a favourable balance between rapid reaction kinetics and reagent stability. They have proven successful in the modification of proteins and oligonucleotides.^{20,21}

Strained alkynes such as bicyclo[6.1.0]non-4-yne (BCN) and dibenzocyclooctyne (DBCO) have been demonstrated to react with unprotected cysteines;^{26–28} however, there are large limitations to the technique stability issues.²⁷ The growing interest in the reactivity of strained alkynes and the stability of the resulting products formed through their click reactions, combined with the appealing nature of cysteine for peptide modifications, has sparked a desire to create selective, dual-reactive linkers capable of connecting the two.²⁹

In our previous study, we introduced the utilisation of vinyl heteroarenes for achieving site-selective protein modification *via* cysteine.¹⁵ This methodology allowed for the selective and stable modification of proteins and antibodies. However, the previous approach required the presence of an external reactive handle for payload attachment, in addition to the use of copper for facilitation of the attachment process (Fig. 1; Previous work).

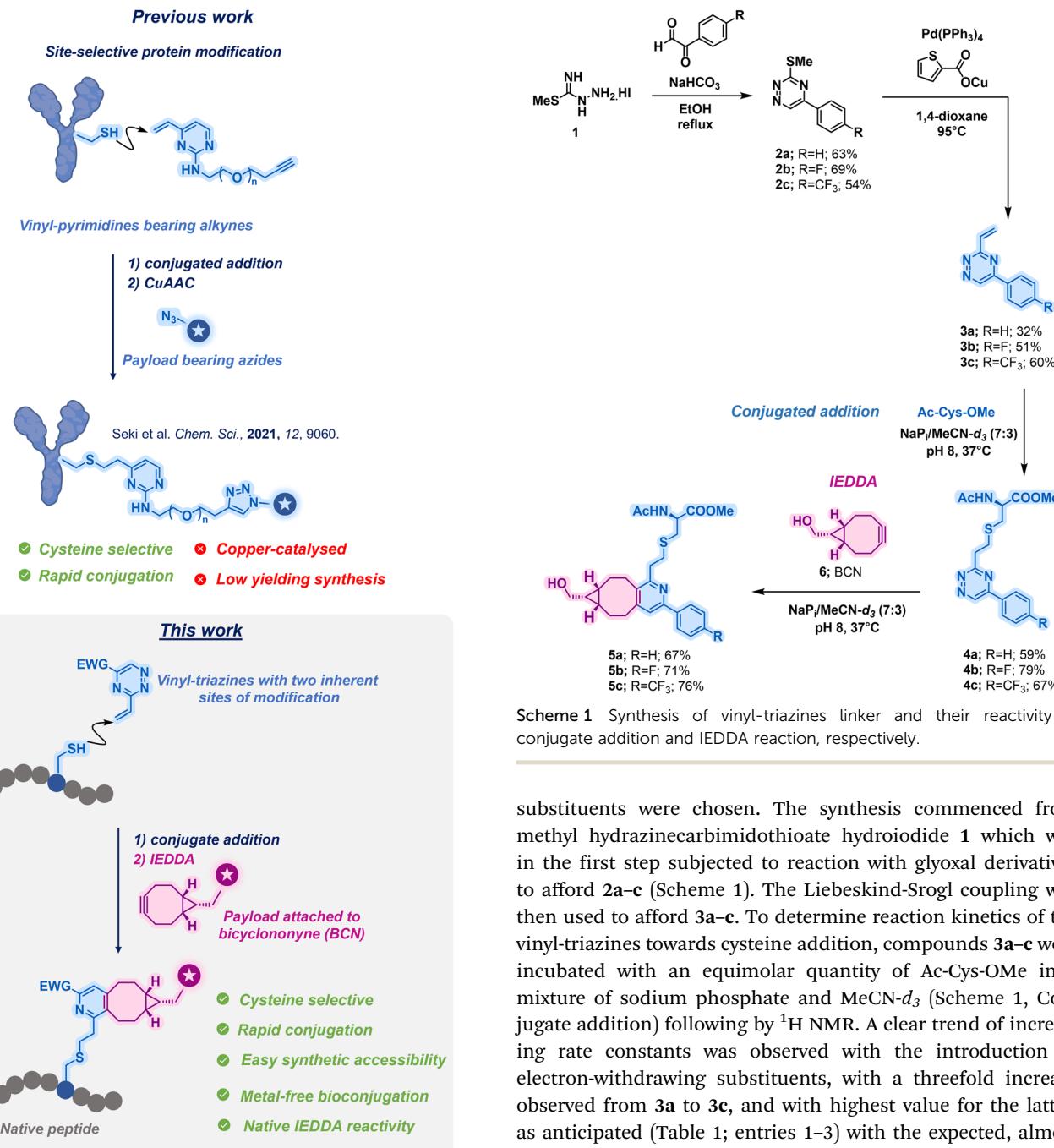
Herein, we present a distinct approach by employing 3-vinyl-1,2,4-triazines (vinyl-triazines thereafter) as dual-reactive linkers. This innovative strategy involves the initial vinyl conjugation *via* cysteine followed by a rapid IEDDA reaction of the triazine. Through this process, a diverse range of functionalised biomolecules can be efficiently generated (Fig. 1; This work).

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Scheme 1 Synthesis of vinyl-triazines linker and their reactivity in conjugate addition and IEDDA reaction, respectively.

substituents were chosen. The synthesis commenced from methyl hydrazinecarbimidothioate hydroiodide 1 which was in the first step subjected to reaction with glyoxal derivatives to afford 2a-c (Scheme 1). The Liebeskind-Srogl coupling was then used to afford 3a-c. To determine reaction kinetics of the vinyl-triazines towards cysteine addition, compounds 3a-c were incubated with an equimolar quantity of Ac-Cys-OMe in a mixture of sodium phosphate and MeCN-*d*₃ (Scheme 1, Conjugate addition) following by ¹H NMR. A clear trend of increasing rate constants was observed with the introduction of electron-withdrawing substituents, with a threefold increase observed from 3a to 3c, and with highest value for the latter, as anticipated (Table 1; entries 1–3) with the expected, almost full conversion (95%) after less than 2 hours under tested conditions. The rate constants obtained are comparable to those commonly observed bioconjugations.³¹ Importantly, after

Fig. 1 Previous work vs. this work.

To initiate the investigations, a collection of vinyl-triazines was synthesised. It was crucial to evaluate the influence of triazine ring electronics, as the introduction of electron-withdrawing groups was expected to enhance the conjugate addition rate of cysteine groups, as well as the rate of IEDDA reaction with strained alkynes, being influenced *via* previous work looking at IEDDA rates.³⁰ Furthermore, the vinyl group was attached on the electron deficient 3-position to increase the propensity towards conjugate addition. For this reason, highly electronegative 4-fluorophenyl and 4-trifluoromethylphenyl

Table 1 Second order rate constant for conjugate addition (CA) and IEDDA reaction

Entry	Starting compound	Reaction	Rate constant [$\times 10^{-3} \text{ M}^{-1} \text{ s}^{-1}$]
1	3a	CA	77.4 \pm 0.0003
2	3b	CA	49.0 \pm 0.0057
3	3c	CA	237 \pm 0.136
4	4a	IEDDA	2.43 \pm 0.0044
5	4b	IEDDA	3.75 \pm 0.0008
6	4c	IEDDA	1.64 \pm 0.0021

24 hours of incubation under the same reaction conditions, minimal reactivity with lysine was observed for the most reactive **3c**, which indicates a high selectivity of these scaffolds towards cysteine.

After establishing the fast bioconjugation kinetics with cysteine, it was crucial to evaluate the reactivity of the compounds towards IEDDA reaction with BCN reagents. To investigate this, compounds **4a–c** were incubated with equimolar amounts of **6** under identical solvent conditions as previously described (Scheme 1, IEDDA).

Consistent with the trends observed in literature for IEDDA reaction rates,³² the rate of reaction was found to increase as expected with greater electron deficiency, with compound **4b** exhibiting the highest rate constant (Table 1; entries 4–6).

The potential cross-reactivity of DBCO, another popular strained-alkyne reagent, with the vinyl-triazines was then investigated with no reaction observed for **4c** at 37 °C (Fig. S17–S19, ESI†). This selectivity is attributed to the increased steric bulk of DBCO. Such encouraging findings allows for the opportunity to selectively form dual functionalisation of molecules, using functional groups such as azides that would react with DBCO and then introducing BCN to react with the vinyl-triazines.

Having established the viability of triazines for selective cysteine modification and their reactivity with BCN, the stability of the resulting thioether linkages followed. To evaluate their stability, model substrates (thioethers **7a–c**; Fig. 2) were synthesised from vinyl-triazines **3a–c**. As a benchmark, the maleimide adduct **8** was also synthesised. These model compounds were subjected to incubation at pH 7.4 and 37 °C for 10 days in the presence of excess dithioerythritol. Analysis by ¹⁹F NMR revealed remarkable stability for compounds **7a–c**, with less than 5% degradation observed over the 10-day period (Fig. 2). In contrast, thiosuccinimide **8** was significantly less stable, with over 60% degradation observed within the same timeframe. The improved stability of the vinyl-triazine

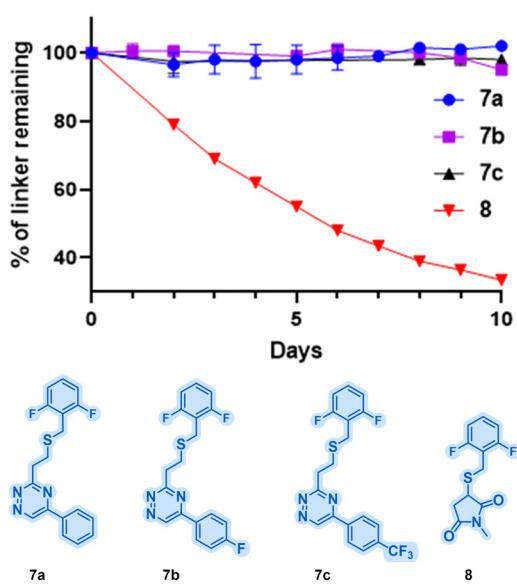
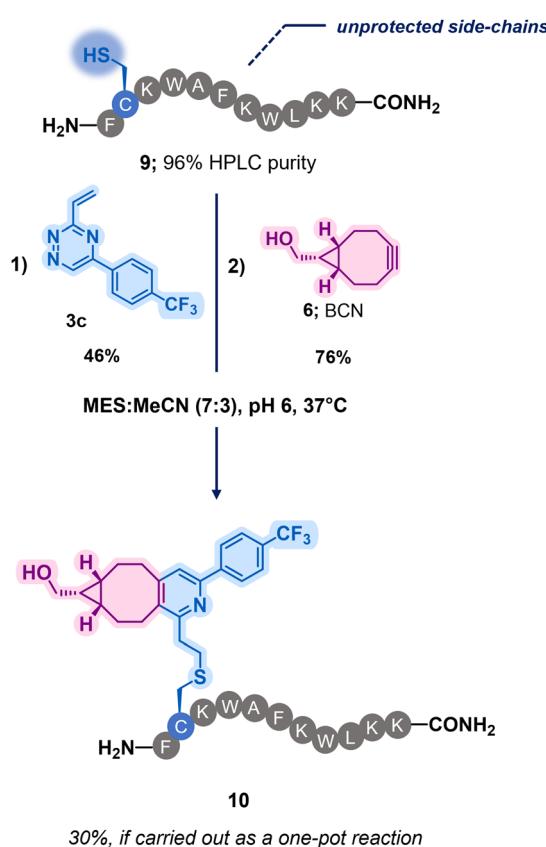


Fig. 2 Stability study of vinyl-triazine linkers **7a–c** vs. maleimide **8**.

conjugates highlights the tremendous potential of this method for biological applications.

To assess the robustness of these dual reactive vinyl-triazine linkers, a bioactive peptide substrate was chosen (Scheme 2 and Scheme S2, ESI†). The design of the peptide **9** was inspired by antimicrobial peptides previously reported in the literature and modified in both a two and one-pot fashion (Scheme 2 and Scheme S2–S4, ESI†). Pleasingly, a single modification event was observed for the reaction between the most reactive vinyl-triazine **3c** and peptide **9**, demonstrating an exclusive selectivity in presence of various lysine residues, free N-terminal amine as well as C-terminal amide. The modified peptide **S4** was then incubated with BCN **6** yielding compound **10** in 5 hours (Scheme 2). This highlights the effectiveness of this technique and its potential applications to append biologically active peptides with payloads through BCN derivatives.

To summarise, we have successfully established a remarkably orthogonal bioconjugation approach utilising dual-reactive 3-vinyl-1,2,4-triazines that display selectivity towards cysteine residues. This methodology enables the generation of modified peptides incorporating a stable triazine handle, which readily reacts with BCN derivatives in an inverse electron-demand Diels–Alder (IEDDA) reaction. Importantly, the observed rate constants for these reactions are comparable to widely employed techniques for protein modification. This technique was used to selectively modify a cysteine in an antimicrobial peptide in the presence of



Scheme 2 Application of the dual orthogonal conjugation on a model peptide **9**.

many lysine residues and a free *N*-terminus allowing for a controlled reaction with **6** which could enable the introduction of different payloads. Following application of the method in protein and antibody modifications is currently a subject of our interest.

J. D. S. was involved in conceptualization, investigation, data curation, formal analysis, methodology, and writing – original draft. H. S. was involved in conceptualization. S. K. was involved in peptide investigation, visualization and writing – editing and review. L. Z. was involved in investigation. T. D. and T. S. were involved in conceptualisation and supervision. D. R. S was involved in conceptualization, supervision, project administration and funding acquisition.

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Conflicts of interest

There are no conflicts to declare.

Notes and references

‡ pH 6 buffer was used due to the solubility of the peptide dependant on acidic pH.

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