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STEFs - Activated Vinylogous Protein-Reactive Electrophiles

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Abstract: We report the synthesis of a class of semi-oxamide vinylogous thioesters that we have designated STEFs and the use of these agents as new electrophilic warheads. This includes preparation of both simple probes that contain this reactive motif as well as its installation on a more complex kinase inhibitor scaffold. A key aspect of STEFs is their reactivity towards both thiol- and amine groups and we substantiate that amine-conjugations in peptidic and proteinogenic samples can be facilitated by initial, fast conjugation to proximal thiol residues. We provide evidence that both the selectivity and the reactivity can be tuned by the structure of STEFs and given the unique ability of this functionality to conjugate via an addition-elimination mechanism, STEFs are electrophilic warheads that could find broad use in chemical biology.

The utility of electrophilic functionalities in chemical biology ranges from proteomic profiling to structure-based drug design. In classic activity-based protein profiling (ABPP), hybrid molecules consisting of an electrophilic warhead and a reporter group are designed to react irreversibly with nucleophilic amino acid residues in the active site of defined classes of enzymes.[1] This method principally allows for labelling of functionally active enzymatic sub-populations and has resulted in a series of important insights, notably in the protease field but also for other enzyme-classes.[2] Generalization of the ABPP concept to electrophiles with broad reactivity has been remarkably productive, e.g. with proteome-wide methods to identify the sub-population of cysteines with enhanced nucleophilic reactivity revealing that hyperreactive cysteines are overrepresented amongst functional and regulatory residues.[3] The broad reactivity required in those studies is enabled by simple thiol-reactive probes such as the iodoacetamide-derivative IA-alkyne (IA-Alk). Potent and broadly reactive electrophiles can however be tempered when placed in molecules with a more complex design, thus imparting enhanced selectivity to the covalent interaction with a biomolecule. Complex natural products that include highly reactive groups are extreme examples of this,[4] but even fragment-sized synthetic molecules with appended electrophiles can display remarkable selectivity.[5] Phenotypic screening using such covalent-fragments has recently been combined with broadly reactive probes to assist target-identification and this appears to be a promising approach to discover new therapeutically relevant proteins that can be addressed using small molecules.[6]

The warhead functionality dictates the overall properties of an electrophilic probe.[7] Of particular relevance is the reactivity (and stability), the nucleophile selectivity: S(cys), N(lys/arg/*N*-term), O(ser/thr/tyr/asp/glu), C(tyr/trp), and the (ir)reversibility of conjugation. New types of reactive groups are of strong interest as they can widen the portion of the proteome that can be addressed with covalent ligands.[7] Here, we report the first biological studies of a class of protein reactive acrylate electrophiles (STEFs – to be further defined below) that function via an addition-elimination mechanism.

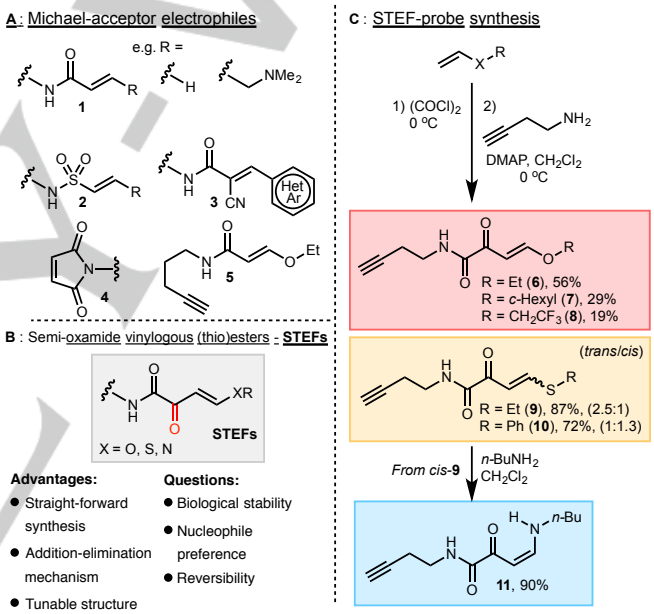

Figure 1. Synthesis and properties of STEFs compared to established Michael-acceptors.

Acrylamides (**1**) are among the most used electrophilic warheads and their reactivity can be modulated by substitution at the β-carbon (Fig. 1A).[8] Similar to other classes of Michael acceptors such as vinyl sulfones, Knoevenagel adducts, and maleimides (**2**-**4**), these functionalities typically have selectivity for thiol-conjugation and have found extensive use both as broadly reactive probes for profiling and as warheads in highly specific bioactive compounds. In contrast, β-heteroatomsubstituted acrylamides, such as vinylogous carbamates (exemplified by **5**), undergo mechanistically distinct reactions with nucleophiles resulting in displacement of the heteroatom via an addition-elimination pathway – a vinylic substitution reaction[9] – but the low chemical reactivity of these systems limits their biological applications. We hypothesized that additional activation by an electron withdrawing group could bring the reactivity into a domain with potential biological relevance (Fig. 1B). We have chosen to

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designate these activated vinylogous compounds as STEF-electrophiles (STEFs) after Stachel and Effenberger who provided the first examples of their preparation.[10,11] Several groups, notably Tietze[12], have demonstrated the applicability of STEF-compounds as synthetic intermediates, e.g. as chelating substrates in metal-catalyzed cycloaddition reactions,[13] but their performance in a biological context has to the best of our knowledge not been investigated. More than anything, we were fascinated by the possibility that biological nucleophiles could undergo either reversible or irreversible conjugation to STEFs depending on the residue and specific molecular context.

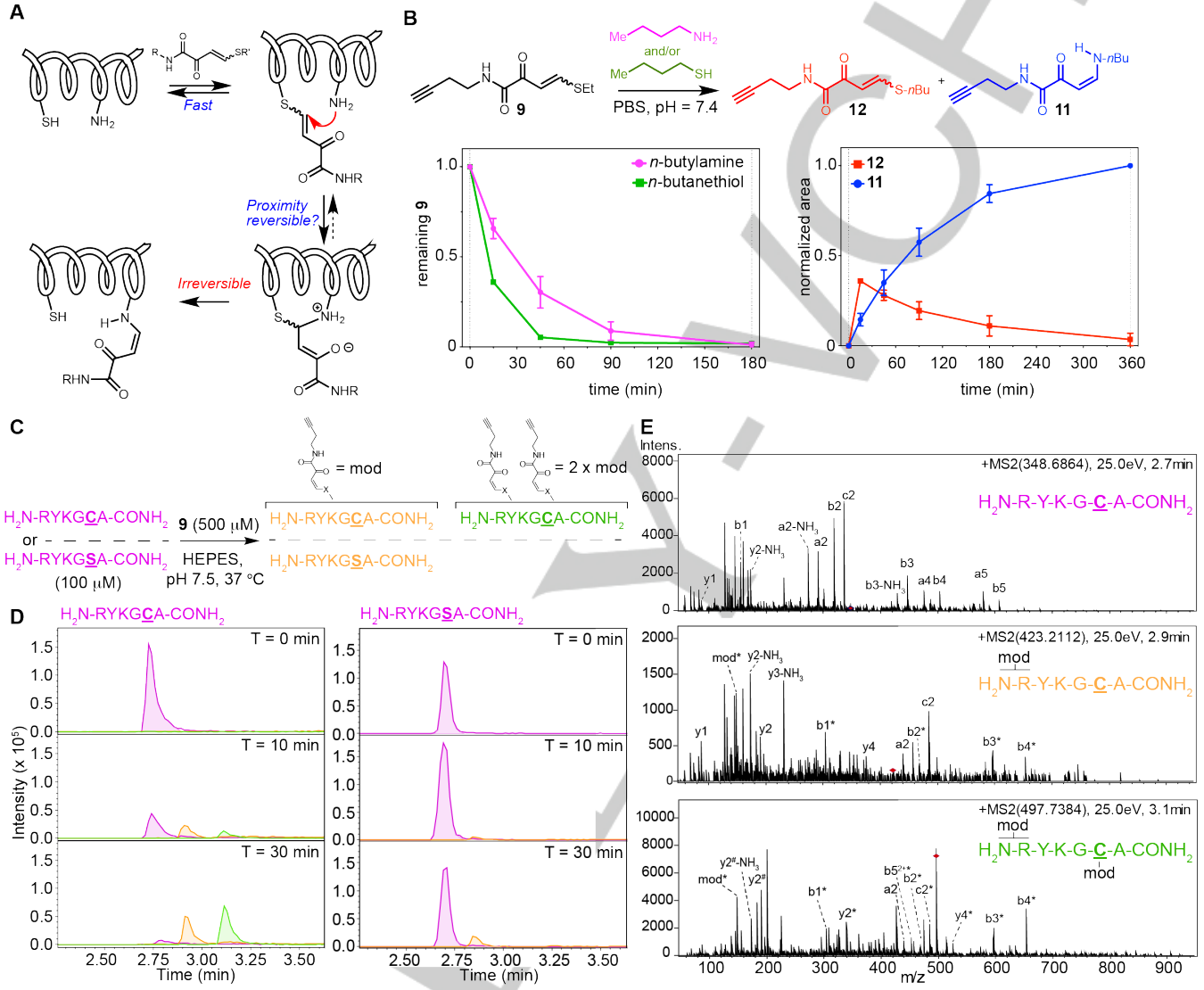

Figure 2. Stability and *in vitro* reactivity of STEFs. A) Model of proposed STEF-reactivity enabling cysteine-directed conjugation to proximal amines. B) Studies of the relative reactivity of STEF-probe **9** with thiols and amines *in vitro* analyzed by HPLC. Probe **9** reacts fast and reversibly with thiols and irreversibly with amines. All data points represent mean ± s.d. (n=2) and are normalized to the peak area of probe **9** at time zero (left) or the peak area of **11** at time 360 min (right). C-E) Comparative reactivity of probe **9** towards model hexapeptides that differ by either cysteine or serine at position 5. C) Experimental setup. D) LC-MS traces of the reaction mixtures at different timepoints. E) MS-Fragmentation spectra from the reaction of probe **9** with the cysteine-containing peptide. b_n^*-ion series (b_1^*-b_4^*) indicate a single STEF-modification present on the N-terminal amine/arginine sidechain (b_1^*-ion). The doubly-modified peptide show y_2^*- and y_4^*-ions, that indicate additional modification at the cysteine residue. The mass of the STEF-modification (m=150.0555) is observed in both modified peptides which suggest instability to fragmentation. This could further explain the presence of unmodified cysteine residues ($y_2^\#$, $y_2^\#$-NH_3) in the doubly-modified peptides.

Starting from either vinyl ethers or vinyl sulfides, we prepared a small collection of alkyne-tagged STEF-probes (**6-10**) by initial reaction with oxalyl chloride[14,15] followed by DMAP-catalyzed trapping with but-3-yn-1-amine to provide a biorthogonal handle (Fig. 1C). All compounds were formed in reasonable yields without extensive optimization, however we observed significant differences in reactivity correlating to the relative electron deficiency of the starting olefin. In this study, we have focused our attention on the simplest disubstituted olefin-scaffolds, although we note that more complex STEFs having additional substituents in the α and β-positions can also very likely be prepared.[14] Probes **6-8** were isolated as single geometric isomers whereas sulfides

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9-**10** were formed as mixtures of *cis* and *trans*-isomers, albeit separable by standard flash chromatography. Finally, we performed an exchange of the thiol moiety in **9** with *n*-butylamine under preparative conditions to afford enamide **11** in good yield. Interestingly, this exchange was found to be stereoconvergent to afford exclusively the *cis*-isomer, irrespective of the stereochemical constitution of the sulfide-precursor (Fig. 1C). To ascertain the potential of STEFs as biologically relevant electrophiles, we first studied their stability. Alkoxy substituted probes (**6**-**8**) were found to degrade upon storage even at -20 °C and we therefore did not further consider their use. In contrast, vinyl sulfides such as **9** and **10** were stable in neutral buffer as well as under both acidic and basic conditions. Enamide **11** was also stable under neutral and basic conditions, however under acidic conditions (pH < 4) the compound degraded over the course of hours, presumably by acid-catalyzed hydrolysis (Fig. S1, Supporting Information).

We hypothesized that thiol-STEFs (**9** and **10**) under physiological conditions would undergo reversible conjugation with thiol nucleophiles (e.g. cysteine-residues) and that subsequent reactions with amine nucleophiles could be irreversible. The preparation of **11** partially illustrates the latter reactivity albeit in organic solvent. In the event that thiol-exchange is fast, such reactivity principally enables cysteine-directed conjugation to proteinogenic amine nucleophiles and, as consequence of the addition-elimination mechanism, the thiol-conjugated probe will remain bound during irreversible amine-conjugation (Fig. 2A). To investigate these interesting and possibly unique aspects of STEFs, probe **9** (100 μM) was first treated with an excess of either *n*-butylamine or *n*-butanethiol in 30% CH_3CN/PBS (Fig. 2B). HPLC-analysis demonstrated that while both conditions could convert **9**, this occurred faster with *n*-butanethiol (Fig. 2B, left). Similar to the reaction in organic solvent, **11** was formed selectively from a mixture of *cis*/*trans*-isomers of **9** (Fig. S2). Next, we co-incubated the nucleophiles and monitored formation of the respective products, **11** and **12**. This experiment strongly indicate initial formation of **12** which subsequently convert to **11** (Fig. 2B, right). In accord with our initial hypothesis concerning the irreversibility of amine conjugations, incubation of pure **11** with a large excess of *n*-butanethiol did not result in any conversion (Fig. S1).

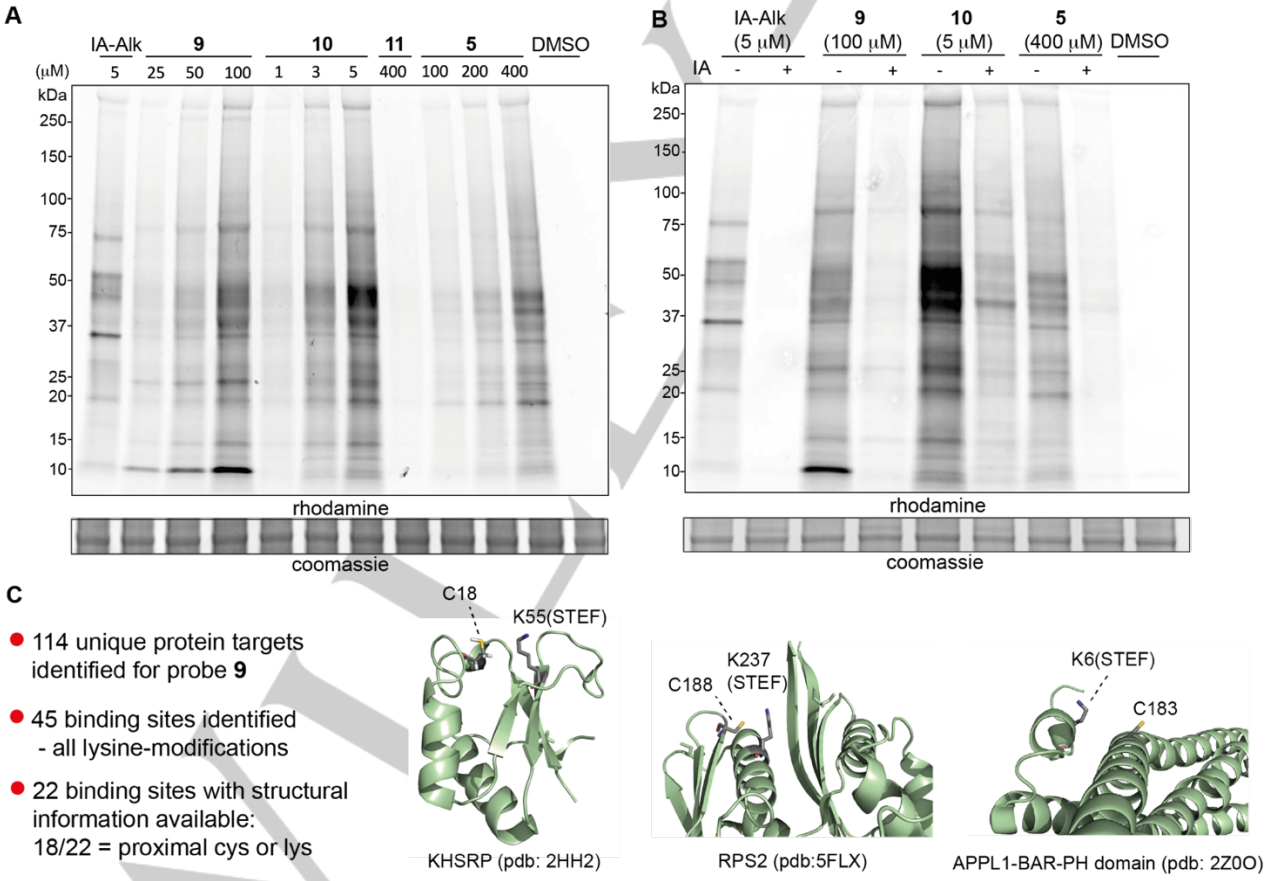

Figure 3. A) Proteome labelling by STEFs. MCF7 cell lysates were treated with STEFs (**9**-**11**), **5**, IA-alkyne (IA-Alk) or DMSO for 2 h, followed by click chemistry with rhodamine-azide, SDS-PAGE and in-gel fluorescence scanning. Lower panel shows Coomassie blue staining and serves as loading control. B) Proteome labelling by STEFs following cysteine-blockade with iodoacetamide. MCF7 cell lysate were pre-incubated with iodoacetamide (20 mM) before probe incubation (2 h). C) Affinity isolation of STEF-binding proteins and examples of binding sites identified. See Table S1 and S2, Supporting Information for all data. More examples of binding sites, see Fig. S9 and Fig. S10.

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Further increasing complexity, we incubated two short peptides with **9** and used UPLC-MS/MS to monitor formation of potential conjugates (Fig. 2C-E, Supporting information Fig. S3-7). The two peptides were designed to differ only by cysteine vs. serine at position 5 and to contain three different amine nucleophiles (*N*-term/Arg/lys). In the presence of 500 µM of **9**, we observed full conversion of the cysteine-containing peptide after 30 min and formation of new peptides with masses matching single and double modifications, respectively (Fig. 2C-D). Fragmentation analysis (Fig. 2E, Supporting Information Fig. S3-5) revealed the single STEF-modification to be present at the *N*-terminal amine/arginine-side chain and the doubly-modified peptide to have an additional modification at the cysteine residue. In contrast, the serine-containing peptide barely converted under identical conditions resulting in low quantities of a peptide carrying a STEF-modification at the *N*-terminal amine/arginine sidechain (Fig. 2C, Supporting Information Fig. S6-7). Collectively, these and the prior experiments suggest that STEFs have an intrinsic ability for fast and reversible thiol conjugation to form intermediates that may be irreversibly intercepted by amine-nucleophiles with favorable proximity and reactivity.[16] Next, we tested the reactivity and selectivity of STEFs in proteomic samples by gel-based analysis. MCF7 cell lysates were treated with STEFs (**9**-**11**, 1-400 µM, 2 h) and subjected to copper-catalyzed azide-alkyne cycloaddition (CuAAC) with rhodamine-azide. The probe-labelled proteins were separated by SDS-PAGE and visualized by in-gel fluorescence scanning (Fig. 3A). We observed a clear concentration-dependent labelling of several proteins by probe **9** and **10**, with probe **10** displaying markedly enhanced reactivity. Despite the significant structural similarity of **9** and **10**, different labelling patterns were apparent, e.g. a ~10-kDa protein was strongly labelled by probe **9** and not by **10**. As expected, probe **11** showed no protein labelling and the simple vinylogous carbamate **5** (Fig. 1A) showed 4- to 80-fold reduced reactivity compared to probe **9** and **10**, respectively. The labelling patterns of STEFs were largely distinct from the thiol-selective probe IA-alkyne (IA-Alk, Fig. 3A). Next, lysates were pre-treated with iodoacetamide before exposure to STEFs (Fig. 3B). As expected, this completely blocked IA-alkyne labelling but, interestingly, also strongly reduced labelling with STEFs, especially with probe **9**. This data further supports a role for cysteine residues as direct targets and, as suggested by the previous experiments, as intermediary conjugation-sites towards irreversible amine-conjugations. In order to probe these questions, we sought the specific protein targets of **9**. We performed an affinity enrichment and mass spectrometry workflow using a cleavable azido-azo-biotin tag[17] conjugated to probe-treated MCF-7 lysates. Biotinylated proteins were enriched by streptavidin agarose resin and eluted with sodium dithionite and resolved with SDS-PAGE (Fig. S8, Supporting Information). Protein bands were excised, digested with trypsin and the released peptides were analyzed by nanoLC-MS/MS. We identified 114 protein targets (≥2 unique peptides of each target captured in three separate replicates) of probe **9** (Supporting Information Figure S8 and Table S1). DMSO-treated negative controls did not result in any protein identifications using these cut-off criteria. Unfortunately, these experiments did not result in identification of specific conjugation sites of probe **9** which may be due to the observed (Fig. 2A, Fig. S1) instability of *N*-linked STEF-conjugates under the strongly acidic conditions needed for the proteomics analysis. Consequently, we devised an alternative sample processing scheme using milder acidic conditions throughout the analysis together with pull-down of peptides instead of entire proteins. To our delight, this resulted in the identification of a series of direct binding sites for probe **9,** which in all cases were at lysine residues (Table S2, Supporting Information). Compared with the former experiment, 17 proteins were in common and an additional 15 were identified. These two types of experimental approaches have different advantages. Protein pull-down can give protein hits even if the probed peptide sequence in question isn't suitable for MS detection, or if the probe is unstable during the procedures. Peptide enrichment, on the other hand, might capture conjugation sites sitting in inaccessible positions of proteins, and has the clear advantage of offering direct evidence of the modification site. The majority of the binding sites, for which high-resolution structural information is available, did indeed feature a cysteine-residue in close proximity to the STEF-bound lysine in accordance with the previous experiments (Fig. 3C, Fig. S9, Supporting Information). In addition, we identified a series of sites where the STEF-bound lysine residue was in close association with another lysine-residue (Fig. 3C, Fig. S10), potentially constituting another favored binding motif.

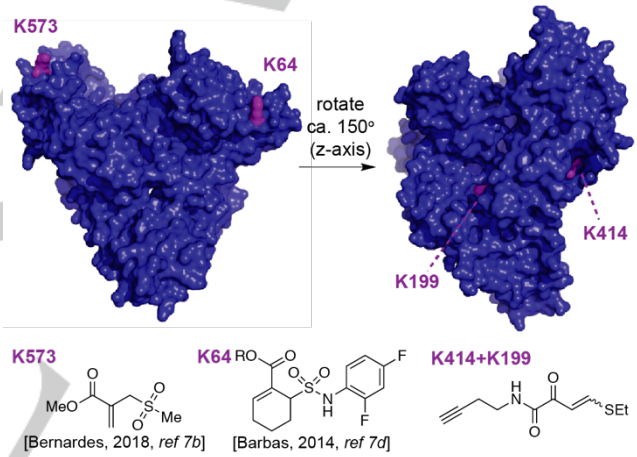

Figure 4. Crystal structure of HSA (pdb: 1BM0) with indicated lysine residues (purple) targeted by electrophilic probes. STEF-probe **9** preferentially targets K414 and K199 (Table S3, Supporting Information).

Finally, to assess whether STEFs have potential for use in bioconjugations, we tested their reactivity in a well-known context, human serum albumin (HSA). Recently reported probes have been demonstrated to target specifically K573[7b] and K64[7d] in this system (Fig. 4). Interestingly, based on the number of mass spectral scans, we detect two major conjugation-sites of probe **9**, K414 and K199, that are both located in shallow hydrophobic groves (Fig. 4B and Table S3, Supporting Information) and not in proximity to the free cysteine (Cys34) present in HSA. Collectively, our experiments with STEF-probe **9** demonstrate that while cysteine-proximity appears to define the major lysine-reactivity determinant, other favored target-motifs exist suggesting that this class of reagents have potential to be uniquely tuned for selective bioconjugations.

In conclusion, we have reported the use of STEFs as new electrophilic motifs in various scenarios and demonstrated that thiol-STEFs are both stable in physiological buffer and capable of conjugation to proteins. We have provided evidence of structure-dependent selectivity in proteomic labelling with simple STEF-

probes and we have laid support for the ability of these electrophiles to conjugate to proteinogenic amine-nucleophiles via intermediacy of a reversibly bound thiol-conjugate even in highly complex samples. In addition to the small probes studied here, we have also demonstrated that it is chemically feasible to install STEFs onto more complex molecules, such as analogs of the EGFR-inhibitor, afatinib (see Supporting Information) which presents another direction for future studies.

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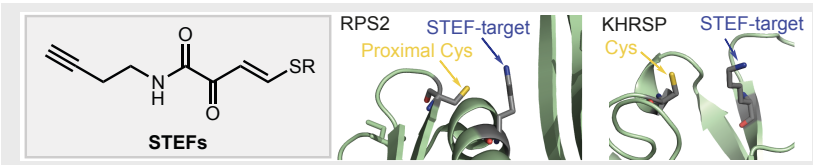

S-Marks the spot. A new class of activated vinylogous thioesters, called STEFs, were evaluated for use as biological electrophiles. These compounds e.g. enable cysteine-directed lysine conjugations via consecutive addition-elimination reactions.

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