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Article

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Chemically induced vinylphosphonothiolate electrophiles for thiol-thiol bioconjugations

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ABSTRACT: Herein we introduce vinylphosphonothiolates as a new class of cysteine-selective electrophiles for protein labeling and the formation of stable protein-protein conjugates. We developed a straightforward synthetic route to convert nucleophilic thiols into electrophilic, thiol-selective vinylphosphonothiolates: In this protocol, intermediately formed disulfides can be chemoselectively substituted with vinylphosphonites under acidic conditions to yield the desired vinylphosphonothiolates. Notably, this reaction sequence enables the installation of vinylphosphonothiolate electrophiles directly on cysteine side chains within peptides and proteins. In addition to labeling the monoclonal antibody trastuzumab with excellent cysteine-selectivity, we applied our protocol for the site-specific conjugation of two proteins with unique cysteine residues yielding a non-hydrolyzable phosphonothiolate-linked diubiquitin and an ubiquitin-α-synuclein conjugate. The latter was recognized as a substrate in a subsequent enzymatic ubiquitination reaction.

Introduction

The development and improvement of chemical methods for the modification of proteins with synthetic molecules such as fluorescent probes and drugs, or even other proteins, is crucial for a plethora of applications in life sciences and pharmacology[1-4]. Many protein conjugation methods make use of electrophilic reagents by addressing nucleophilic amino acids like cysteine[5-8] or lysine[9]. Other strategies involve redox-based reagents for the modification of methionine[10], radical reactions with tryptophan[11] and cycloadditions with unnatural amino acids bearing alkynes, azides[12] or tetrazines[13].

In contrast to exploiting the inherent reactivity of natural amino acid side chains or making use of laboriously introduced unnatural functionalities, a different approach is to transform a given nucleophilic residue into an electrophilic motif (Fig. 1A). This inversion of reactivity allows for subsequent modifications with various nucleophiles including nucleophilic amino acid residues of other proteins. Such approaches enable selective conjugation between two polypeptides bearing only canonical amino acids, yielding homogenous conjugates, which is in high demand for studying various biochemical processes[4, 14].

Due to its distinct reactivity among the canonical amino acids, cysteine is particularly well suited for the selective transformation into an electrophilic motif. A common approach utilizes bis-electrophilic molecules or linkers, such as dibromomaleimides[15, 16], dibromopyridazinediones[16, 17], 3-bromo-2-bromomethyl-1-propenes[18], diselenoesters[19], *N,N'*-ethylene bis(acrylamide)[20], chloroacetaldehyde[21] or palladium oxidative addition complexes[22], which can be attached to cysteines and subsequently reacted with various natural or unnatural nucleophiles.

Alternatively, rather than attaching a preexisting electrophilic functional group, some approaches are known to directly transform the cysteine residue itself into an electrophile. Such approaches are attractive because they allow for a one-pot conjugation between the formed electrophile and a second thiol component without problems of homodimer formations associated with bifunctional linkers. For instance, in a chemoenzymatic protocol, a formyl-glycine generating enzyme converts a former cysteine residue into an electrophilic aldehyde-containing amino acid[23]. In addition, chemical protocols can deliver electrophiles, that can be reacted with canonical nucleophilic amino acids in a selective manner.

The most well known protocol following this concept is the formation of electrophilic disulfides with Ellmann's reagent, which facilitates the formation of disulfide-linked protein-protein conjugates. This strategy has been applied to various protein substrates, especially in the area of chemical ubiquitylation to construct ubiquitinated histones[24], ubiquitin-PCNA[25] and ubiquitin-α-synuclein conjugates[26]. The applicability of this approach is notably limited due to the inherent lability of dislufides in reducing environments[27]. In another approach, a cysteine residue can be chemoselectively converted into an electrophilic dehydroalanine (Dha) by means of a bis-alkylation-elimination protocol using 2,5-dibromohexanediamide[28]. Dha is susceptible to thiol[29] and aza-Michael-addition[30], as well as carbon-based free radical chemistry[31]. Brik and co-workers employed thiol addition of a Cys-peptide to Dha and subsequent

native chemical ligation for the construction of protein-ubiquitin conjugates.[32] Furthermore, they used Dha on ubiquitin for activity-based probes to catch deubiquitinating enzymes (DUBs).[33, 34, 35] However, to the best of our knowledge, no direct protein-protein conjugation with Dha proteins has been reported for non-affinity induced systems (such as the DUB example). A disadvantage for the formation of protein-protein conjugates via Dha can be, that the addition of a nucleophile to Dha results in a mixture of diastereoisomers in the protein backbone.[32] Finally, selenocysteine has also been employed as an electrophile for protein conjugation, when activated by means of oxidation.[36, 37] However, the incorporation of seleno-cysteine into proteins requires additional genetic engineering and is therefore labor intensive.

With the disadvantages of the forementioned methods in mind, we set out to develop a method for the conversion of thiols in cysteine side chains into electrophilic handles based on unsaturated, electrophilic phosphorous(V) compounds.

As such, we herein present a straightforward reaction sequence for the generation of vinylphosphonothiolate electrophiles originating from thiol-containing substrates (Fig. 1B). In this approach, a nucleophilic thiol is formally converted in a chemoselective process into an electrophilic vinylphosphonothiolate.

We have previously shown that phosphonamidates, another class of electrophilic phosphorous(V) reagents, can be generated from vinyl- or alkynyl-phosphonites via Staudinger-phosphonite reactions with azides (Fig. 1C)[8, 38]. In these reactions, the electron-rich double or triple bond in the phosphonites is transformed into an electrophile, inducing reactivity for a subsequent thiol addition. The thiol addition proceeds with excellent chemoselectivity in aqueous buffer, yielding highly stable conjugates, as shown for the construction of stapled peptides[38] and efficacious antibody-drug conjugates[39].

In order to convert vinylphopshonites into vinylphosphonothiolates, we envisioned electrophilic disulfides as appropriate reagents. This proposal was based on our recent work, in which we could show that electrophilic disulfides react selectively with phosphites to deliver phosphorylated cysteine residues on unprotected peptides.[40] It should be noted that Trishin and co-workers synthesized vinylphosphonothiolates from sulfenyl chlorides and vinylphosphonites in the late 1980s;[41] however, we regarded the synthesis of sulfenyl chlorides as prohibitively challenging on complex substrates like unprotected peptides and proteins. Furthermore, we envisioned that upon installation on activated cysteine disulfide residues, vinylphosphonothiolates could be used as electrophilic handles for subsequent cysteine-selective bioconjugation. Small molecule vinylphosphonothiolates could also be used for cysteine-selective protein labeling, thereby contributing to the toolbox of electrophilic reagents for cysteine modification.

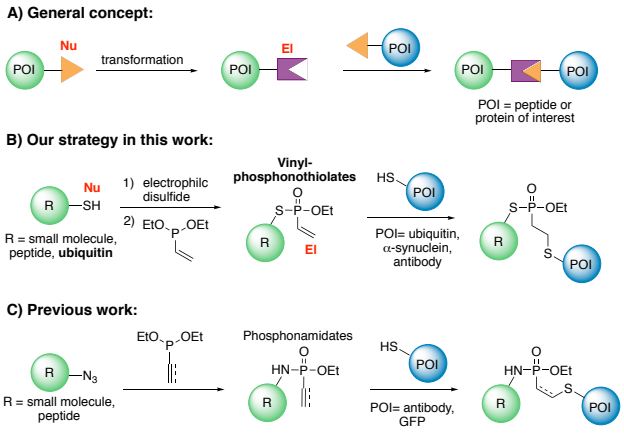


Figure 1: A) The general concept of this work is to convert a nucleophilic, canonical amino acid residue into an electrophilic moiety, thereby inducing reactivity for the subsequent addition of a nucleophilic residue of a second protein. B) Our strategy in this work is to convert an activated cysteine residue (electrophilic disulfide) via the reaction with diethyl-vinylphosphonite into an electrophilic vinylphosphonothiolate, thereby inducing reactivity for a subsequent thiol addition. C) Electrophilic phosphonamidates, generated from azides and vinyl- or ethynylphosphonites, react selectively with cysteine residues on proteins[8, 38, 39]. Nu: nucleophile, El: electrophile, EWG: electron-withdrawing group, R: as specified in scheme. POI: protein of interest.

Results and Discussion

Synthesis of small molecule and peptide vinylphosphonothiolates

At the outset of our studies we developed a synthetic route to access vinylphosphonothiolates from thiol precursors via electrophilic disulfides. For this, we activated small molecule thiols with 2,2'-dithiobis(5-nitropyridine) to obtain the respective electrophilic mixed disulfides **1**-**5**. To investigate the reaction with diethyl-vinylphosphonite we used the ethyl-derivative **1** as a model substrate and employed ^{31}P-NMR as a read-out for reaction optimization (Fig. 2, Fig. S1). We first reacted electrophilic disulfide **1** with one equivalent of vinylphosphonite in DMSO. Complete conversion of **1** was observed in less than one minute upon addition of the vinylphosphonite. The desired vinylphosphonothiolate **6** was isolated next to the side product **13**, identified as the addition product of the 6-nitropyridine-3-thiolate leaving group to **6**, in a ratio of almost 1:1 (Fig. 2A and Fig. S1A). The fact that **13** is formed *in situ*, shows that vinylphosphonothiolates are reactive towards thiols (Fig. 2B). We reasoned that the formation of the re-attack by-product could be circumvented under acidic conditions, since the leaving group would be protonated and thus not reactive anymore as a nucleophile. Indeed, when the reaction was performed in the presence of ten equivalents of trifluoroacetic acid (TFA), the formation of the re-attack product was inhibited (Fig. 2C and Fig. S1B). In addition, we identified diethyl-vinylphosphonate in the reaction mixture, which is the oxidation product of the vinylphosphonite. Due to this partial oxidation in DMSO containing TFA, 1.5-2 equivalents are needed to obtain full conversion of disulfide **1**.

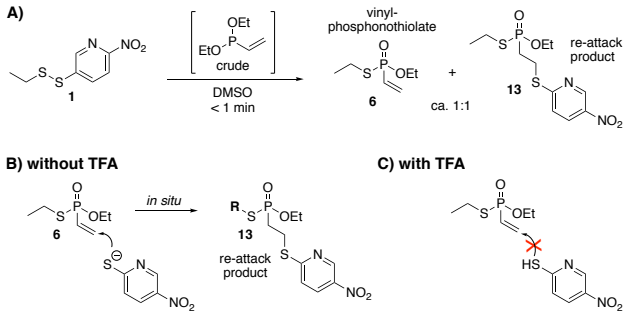
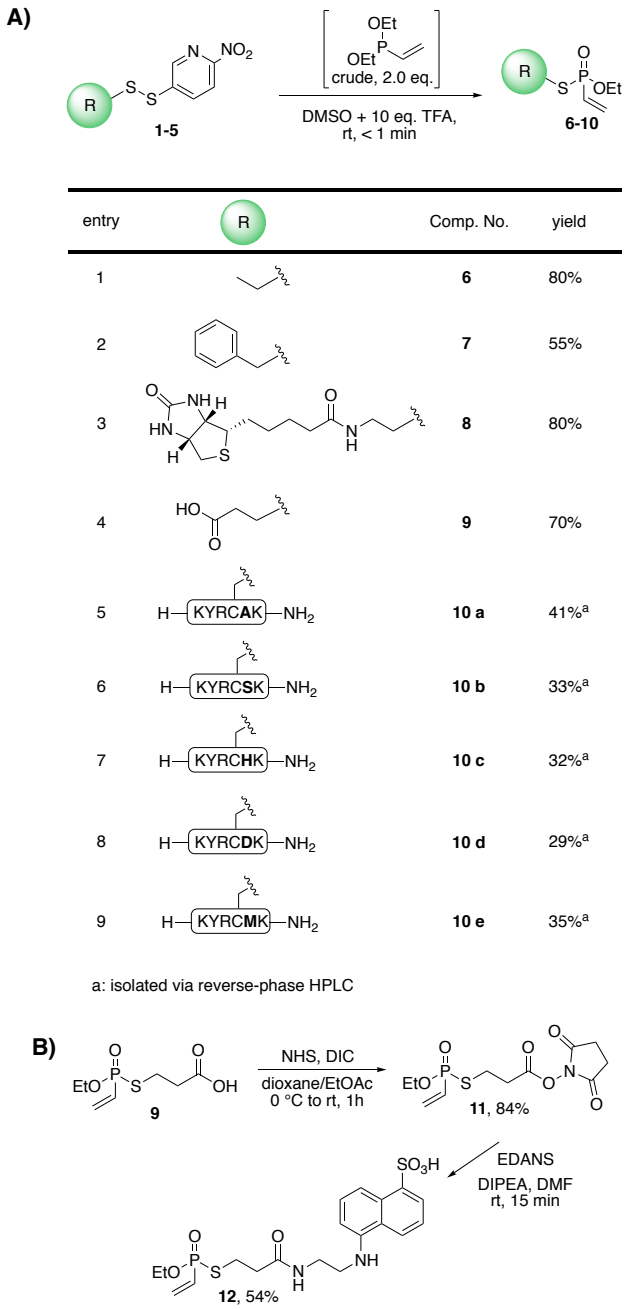


Figure 2: A) The reaction of electrophilic disulfide **1** and crude vinylphopshonite (formed from commercially available diethyl chlorophosphite and vinylmagnesium bromide as previously reported[38]) in DMSO gives a mixture of the desired vinylphosphonothiolate **6** and re-attack side-product **13**. B and C) The *in situ* formed re-attack of the thiolate leaving group to the product can be inhibited in the presence of TFA.

Using these optimized reaction conditions (two equivalents of phosphonite in DMSO and ten equivalents TFA) we synthesized vinylphosphonothiolate derivatives **6**-**10** in good yields, starting from the respective electrophilic disulfides **1**-**4** (Fig. 3A). The carboxylic acid derivative **9** can be used as a modular building block in amide couplings as shown for the synthesis of the fluorescent EDANS-derivative **12** (Fig. 3B). We also installed the vinylphosphonothiolate moiety on cysteine-residues on model peptides with the sequence KYRCXK, whereas X are different amino acids as specified in Fig. 3A. The amino acid X was varied to probe the chemoselectivity of the reaction. The vinylphosphonothiolate peptides **10a**-**10e** could be obtained in 29-41% isolated yield after HPLC purification. This result demonstrates that it is possible to install vinylphosphonothiolates chemoselectively on a cysteine side chain in an unprotected peptide and that the reaction proceeds chemoselectively in the presence of other functional groups. The main side product in this reaction is reduced cysteine peptide (ca. 10-14% isolated yield) (Table S2).

Thiol addition to vinylphosphonothiolates

With the isolated vinylphosphonothiolates in hand, we next set out to investigate their reactivity towards thiols. Since we aimed at using vinylphosphonothiolates for the synthesis of protein-protein conjugates, we were particularly interested in the kinetics and selectivity of the thiol addition, as well as in the stability of the vinylphosphonothiolates before and after thiol-addition. In a first model reaction, we reacted benzyl derivative **7** with reduced glutathione at pH 8.5, containing 10% DMF to ensure solubility (Fig. 4). We observed the formation of **14** as the single product, which could be isolated in 77% yield by HPLC. Similarily, we reacted purified peptides **10a**-**10e** with reduced glutathione in aqueous buffer and observed clean conversion to the desired thiol-addition products and no side products as analyzed by UPLC-MS and ^{31}P-NMR (Fig. S8).



entry	R	Comp. No.	yield
1		**6**	80%
2		**7**	55%
3		**8**	80%
4		**9**	70%
5	H—KYRC**A**K—NH_2	**10 a**	41%[a]
6	H—KYRC**S**K—NH_2	**10 b**	33%[a]
7	H—KYRC**H**K—NH_2	**10 c**	32%[a]
8	H—KYRC**D**K—NH_2	**10 d**	29%[a]
9	H—KYRC**M**K—NH_2	**10 e**	35%[a]

a: isolated via reverse-phase HPLC

Figure 3: A) Synthesis of vinylphosphonothiolates from electrophilic disulfides. In this table, isolated yields after silica gel chromatography are given, or for peptides after HPLC purification. B) Carboxylic acid derivative **9** was further functionalized into a fluorescent EDANS derivative **12** via amide coupling.

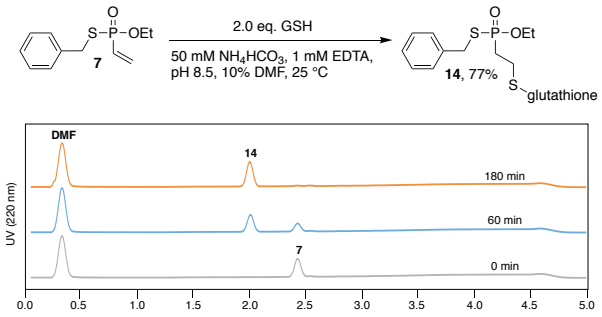


Figure 4: Thiol addition of glutathione (GSH) to **7** (10 mM) gives thiol-conjugate **14**. The reaction was monitored by LC-UV (220 nm).

Using the fluorescent EDANS derivative **12** we investigated the reaction kinetics of the thiol addition by monitoring the addition of glutathione with fluorescent HPLC. We observed clean conversion to the thiol addition product and deduced second order rate constants of 0.0021 $M^{-1}s^{-1}$ (at pH 7.4) and 0.074 $M^{-1}s^{-1}$ (at pH 8.5) (Fig. S3). These rates are in the range of other typically applied protein modification reactions[3] and allow installation of vinylphosphonothiolates on proteins at dilute conditions.

When using vinylphosphonothiolates for protein modification, the stability of these reagents in aqueous media under buffered conditions is of high importance. In aqueous solution at pH 7.4, more than 90% of vinylphosphonothiolate **12** remained intact after 90 hours, both at r.t. and at 37 °C (Fig. S2). In contrast, at pH 8.5 at 37 °C, we observed ca. 40% hydrolysis of the P-S bond after 90 hours. Notably, vinylphosphonothiolates show excellent stability under acidic conditions and can be stored at 4 °C for several months in isolated form and as solutions in DMSO without any observable decomposition.

We also monitored the stability of isolated vinylphosphonothiolate peptides **10a**-**10e** in aqueous buffers at pH 7.4 or 8.5. Depending on the pH we observed P-S hydrolysis and in addition a migration of the vinyl-*O*-ethylphosphate group to other nucleophilic residues (Fig. S7). As a consequence of these two processes ca. 50% of peptides **10a**-**10e** are decomposed after 24 h at neutral pH (Fig. S7C). However, when the purified vinylphosphonothiolate peptides **10a**-**10e** are directly reacted with reduced glutathione in aqueous buffer, clean conversion into the thiol addition product to the vinylphosphonothiolate is observed (as mentioned above) and no migration of the P(V) moiety to other residues (Fig. S8).

Next we investigated the stability of the thiol-addition products. For this, we made use of a previously reported fluorescence-quenching assay[8]. Generally, the conjugates show very good stability in buffer, in Hela cell lysate and in human serum, which is crucial for applications in biological systems (Fig. S4). Furthermore, the conjugates are stable in the presence of reducing agents and excess free thiols, and in contrast to our previously reported phosphonamidates[8, 38] also under acidic conditions (1 N HCl, pH 0).

Encouraged by these results, we probed the applicability of vinylphosphonothiolates for the labeling of cysteine-residues on proteins in general and antibodies in particular. We reacted the therapeutically relevant, monoclonal IgG antibody trastuzumab with biotin derivative **8**, following a previously described reduction-alkylation protocol[8] (Fig. 5A). After reduction with DTT, the antibody was incubated with **8** at 14 °C for 16 hours at either pH 7.4 or 8.5. Analysis by intact protein MS revealed a labeling degree of 1.3 and 3.1 biotins per antibody at pH 7.4 and pH 8.5, respectively using 100 equivalents of biotin derivative **8** at an antibody concentration of 5 mg/ml.

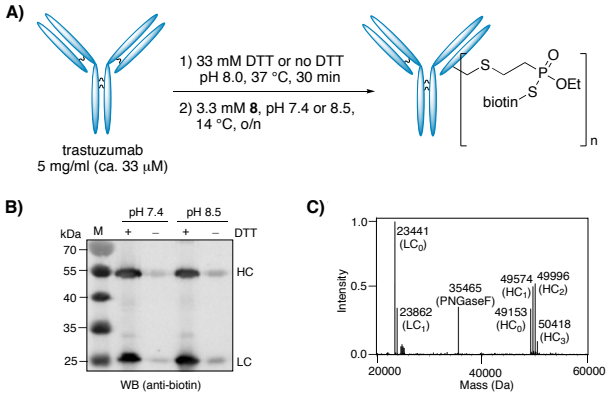


Figure 5: A) Labeling of IgG antibody trastuzumab with 100 eq. biotin-vinylphosphonothiolate **8** at pH 7.4 and 8.5 gives a modification degree of n= 1.3 and 3.1 biotins per antibody, respectively. B) Western-Blot (anti-biotin). C) Deconvoluted MS spectra of trastuzumab labelled with **8** at pH 8.5. LC: light chain, HC: heavy chain, subscripts: number of modification.

In a control reaction without DTT reduction of disulfides, no antibody labeling was observed, thus demonstrating excellent cysteine-selectivity of vinylphosphonothiolates (Fig. 5B, Fig. S5 & S6). This finding is in line with previously published results from our group, where we could show that electrophilic phosphonamidates show excellent cysteine-selectivity as well[8, 39]. Taken together, these results demonstrate that vinylphosphonothiolates are suitable reagents for the cysteine-selective labeling of biomolecules such as antibodies to generate stable bioconjugates.

Vinylphosphonothiolates for protein-protein conjugation

Other than using small molecule vinylphosphonothiolates for protein labeling, we wanted to apply the chemoselective induction of electrophilicity on amino acid side chains to the conjugation of two proteins. Hereby, we focused on the synthesis of biologically relevant ubiquitin-protein conjugates. Ubiquitination is one of the largest reversible post-translational modification of proteins, regulating various cellular processes from protein degradation and trafficking to DNA repair and transcription[42]. Ubiquitin chains are usually connected through the C-terminus of a distal ubiquitin to any of seven available lysine residues by E1, E2 and E3 enzymes via an isopeptidic bond at the proximal ubiquitin[42, 43]. Studying the biological function of different ubiquitin chain linkages as well as probing the impact of ubiquitination on substrate proteins constitutes a major challenge in current research. Consequently, there is a great need for homogeneous site-specific ubiquitinated substrates for functional investigations[44, 45]. Inspired by the work of A. Brik[21, 32, 46, 47], A. Marx[48], M. R. Pratt[26] and E. Strieter[14, 49] in the field of chemical ubiquitination we wanted to apply our vinylphosphonothiolate conjugation strategy to generate non-hydrolyzable homogenous ubiquitin-protein conjugates. Within this goal, we particularly

aimed at synthesizing diubiquitin and an ubiquitin-α-synuclein conjugate.

First, we applied the previously optimized protocol for the installation of the vinylphosphonothiolate moiety to a ubiquitin mutant, carrying a single cysteine (G76C) (Fig. 6A). We chose Ellmann's reagent to activate the cysteine-residue in this case because 2,2'-dithiobis(5-nitropyridine) is poorly soluble in aqueous buffer and gave incomplete conversion. Addition of 100 equivalents vinylphosphonite to activated ubiquitin **16** in DMSO containing 1 % TFA at room temperature resulted in full conversion and yielded vinylphosphonothiolate ubiquitin **17**, as confirmed by ESI-MS. Crude vinylphosphonothiolate ubiquitin **17** was purified by semipreparative HPLC and could be isolated in 24% yield. The reaction can also be performed at 60 °C with only 50 eq. of the vinylphosphonite. In this case, full conversion is achieved in only 4 hours. As mentioned before, excess vinylphosphonite oxidizes to vinylphosphonate in DMSO; however, the latter does not react with **16**. In addition, non-activated disulfide-ubiquitin **15** does not react with the vinylphosphonite at r.t., as confirmed by ESI-MS. The conversion of an activated disulfide to vinylphosphonothiolates by means of the reaction with vinylphosphonite requires dry conditions; hence the applicability of this method is limited to protein substrates, which are stable under these conditions (in DMSO + 0.1% TFA) and can subsequently be refolded.

Next, we probed protein-protein conjugation in a model system between vinylphosphonothiolate-Ub **17** and an eGFP cysteine mutant (C70M S147C) and observed more than 80% conversion after 24 hours at pH 8.5 (Fig. S24). At pH 8.5 the reaction is significantly faster than at pH 7.4, which is consistent with the small molecules studies described above. Furthermore, we investigated the stability of unconjugated vinylphosphonthiolate-ubiqutin **17** in aqueous buffer and, similar to the observed behavior of small molecule **12**, observed *P-S* bond hydrolysis over time at pH 8.5, as well as to some extent at pH 7.4 (Fig. S23). Because of the *P-S* bond hydrolysis, the free thiol ubiquitin reacts with intact **21** to form C-terminal ubiquitin dimers. Having this observation in mind, we decided to perform protein conjugations with **17** at pH 8.0, which constitutes a compromise of stability and reactivity. Next, vinylphosphonothiolate-Ub **17** was subjected to the conjugation with UbK48C mutant **18** as well as the α-synucleinK6C mutant **20** (Fig. 6B). The reactions were monitored by SDS-PAGE and intact protein MS, which indicated the formation of covalent protein-protein conjugates. For both conjugation reactions, maximal conversion was obtained after 24 hours (Fig S27 & S30). The conjugates DiUb(K48) **19** and Ub-α-synuclein **21** were purified by size exclusion chromatography and isolated in 16% and 27% yields, respectively. For both protein conjugates **19** and **21** the yields are based on pure product-containing fractions; mixed fractions after size-exclusion chromatography are not accounted for the isolated yield.

For both conjugates **19** and **21**, the site-selective linkages to UbG76C-phosphonothiolate were confirmed by LC-MS/MS analysis after tryptic digest of the purified conjugates (Fig. S9 & S10). In line with the above described observations for the vinylphosphonothiolate-peptides (Fig. S8), we also did not observe P(V)-transfer conjugation products on protein level.

Purified thiol conjugates **19** and **21** showed excellent stability in aqueous buffer and could be stored for several months at 4 °C without any sign of decomposition. To further verify the utility of phosphonothiolate-linked conjugates, we demonstrated that DiUb(K48) conjugate **19** is stable in the presence of deubiquitinating enzyme USP2-CD (Fig. S36); that it is recognized by an α-poly-Ub antibody (Fig. 6B); and that it shows similar elipticity in CD spectra compared to the wildtype diubiquitin (K48 amide linkage) (Fig. S11).

Finally, to prove that the non-hydrolyzable phosphonothiolate-linked ubiquitinated proteins can be recognized by ubiquitin binding proteins, we mixed α-synuclein-ubiquitin conjugate **21** with the enzymes Ubc1 (E2-25k) and E1 to enable enzymatic ubiquitination (Fig. 6C). The ubiquitin conjugating enzyme Ubc1 catalyzes the formation of K48-linked ubiquitin chains from mono ubiquitin [50, 51]. To prevent polyubiquitination, we employed the K48M ubiquitin mutant **22**. After 24 h incubation at 37 °C we could detect the formed di-ubiquitinated α-synuclein **23** by ESI-MS, demonstrating that the ubiquitinating enzymes indeed recognize our phosphonothiolate-linked ubiquitin substrate. Using a specific anti-polyUb(K48) antibody, we could additionally confirm by Western-Blot that conjugate **23** is linked via the K48 residue.

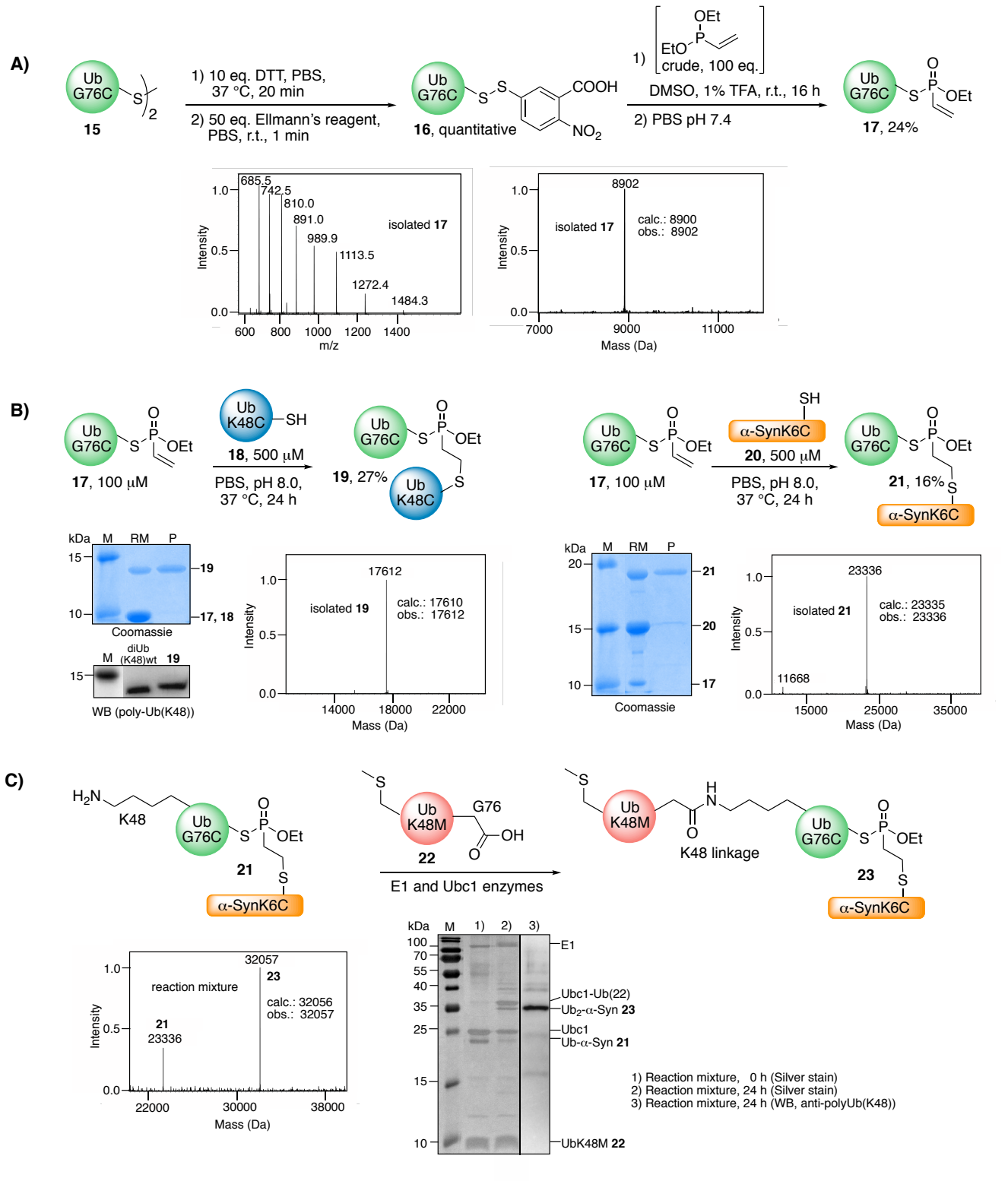


Figure 6: Vinylphosphonothiolates as cysteine-selective linkers for protein-protein conjugation. A) Synthesis of vinylphosphonothiolate ubiquitin **17** from cysteine mutant **15** via Ellmann's activated ubiquitin **16**. Shown are raw and deconvoluted MS spectra of isolated product **17**. DTT: dithiothreitol, TFA: trifluoroacetic acid. B) Protein conjugation of vinylphosphonothiolate ubiquitin **17** to UbK48C **18** and α-synucleinK6C **20**. Shown are MS spectra of the isolated products and SDS-gels of the isolated products and the reaction mixtures. WB (anti-polyUb(K48) antibody) analysis reveals, that the phosphonothiolate-linked diUb **19** is linked via K48. RM: reaction mixture, P: product. C) Enzymatic ubiquitination of α-synuclein-ubiquitin conjugate **21** at position K48 by E1 and Ubc1 enzymes. UbK48M mutant **22** was used as a dead-end building block to avoid poly-ubiquitination. Shown are a MS spectrum of the reaction mixture and a SDS-gel (silver stain) of the reaction mixture at time 0 and 24 hours. Additionally, an anti-polyUb(K48) WB supports that the ubiquitins in **23** are linked via the K48 residue.

Conclusions

Herein, we presented vinylphosphonothiolates as a new class of cysteine-reactive reagents for protein labeling and protein-protein conjugation. Vinylphosphonothiolates can be generated in good yields even on unprotected peptides following a straightforward protocol from vinylphosphonites and thiol-containing molecules via intermediately formed electrophilic disulfides, thereby inducing reactivity for a thiol addition. Importantly, performing the phosphonite-disulfide reaction under acidic conditions circumvents the *in situ* re-attack of the thiolate leaving group. Excellent cysteine-selectivity of vinylphosphonothiolates could be demonstrated by labeling the monoclonal antibody trastuzumab. Importantly, we showed that vinylphosphonothiolate electrophiles could be installed site-selectively on a cysteine residue in a single cysteine-containing ubiquitin mutant. The resulting electrophilic ubiquitin-vinylphosphonothiolate was conjugated to Cys-containing mutants of α-synuclein and ubiquitin, generating nonhydrolyzable conjugates, which were accepted as substrates for subsequent enzymatic ubiquitynation. Taken together, this method enables the conjugation of two biomolecules containing single free cysteines in a chemoselective and site-selective manner. We believe that the herein presented formal attachment of electrophilic moieties to a former Cys-residue will find use for the generation of homogenous peptide and protein conjugates containing only canonical amino acids and in particular for the formation of non-hydrolyzable protein-ubiquitin conjugates.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.
Detailed experimental procedures, organic synthesis, compound characterizations, protein chemistry (PDF).

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Author Contributions

A. L. B., S. S. and C. P. R. H. have designed the research and have written the manuscript. A. L. B. investigated small molecule and peptide vinylphosphonothiolates. S. S. has conducted experiments involving ubiquitin.
All authors have given approval to the final version of the manuscript. / ‡These authors contributed equally.

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Notes

The technology described in the manuscript is part of a pending patent application by C.P.R. H., A.-L. B., Marc-André Kasper, Stephen Byrne, Jonas Helma-Smets, Heinrich Leonhardt, Tina Stoscheck, Marcus Gerlach and Dominik Schumacher.

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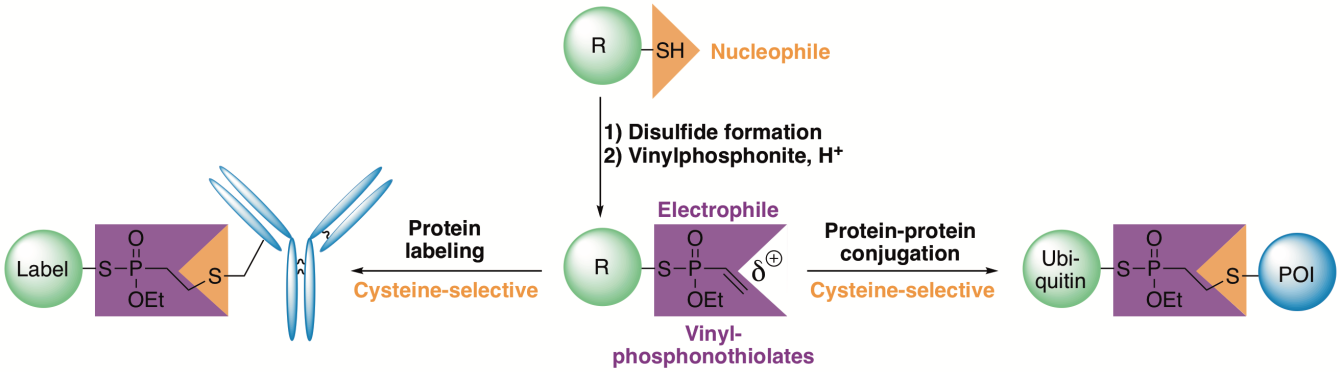

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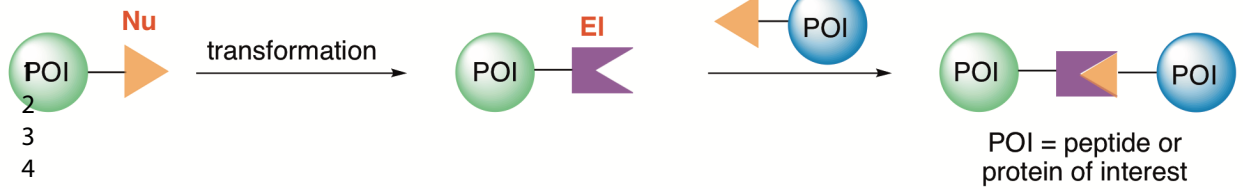
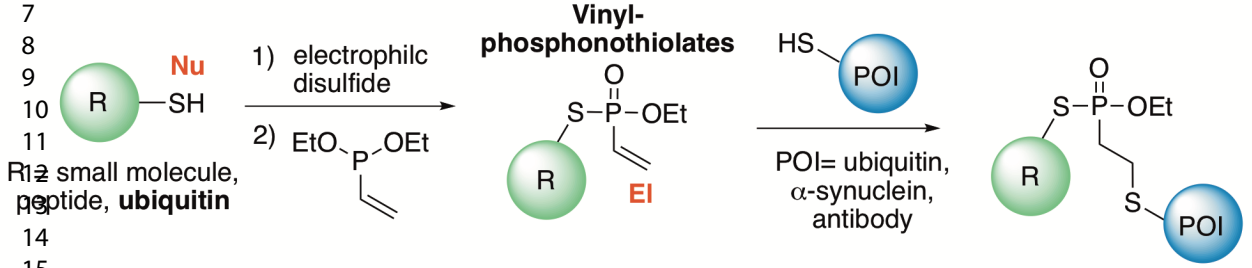


B) Our strategy in this work:



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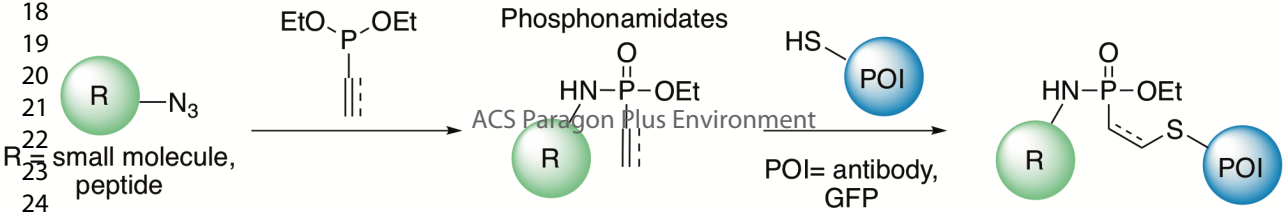
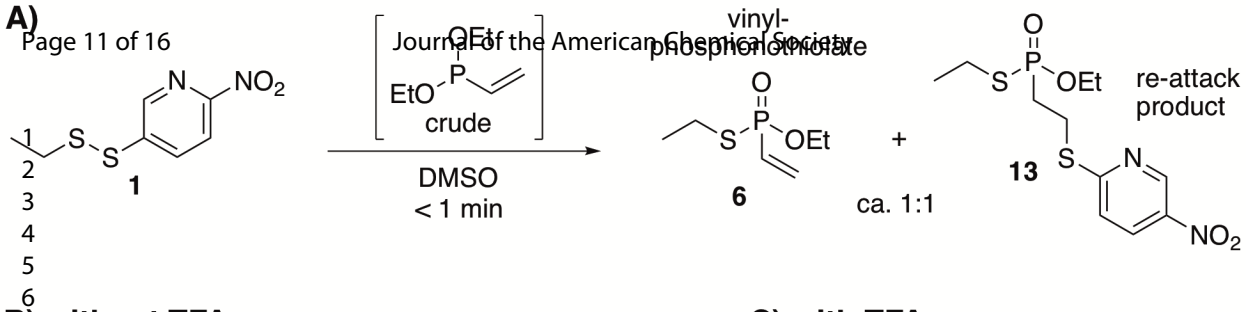
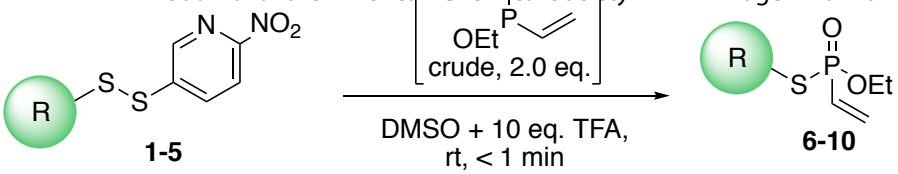




A)



entry	R	Comp. No.	yield
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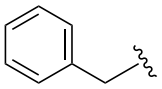
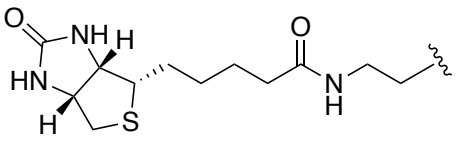
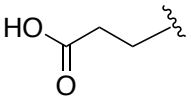
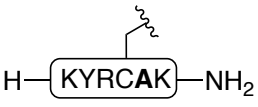
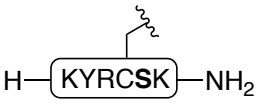
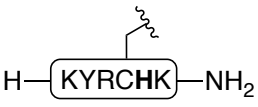
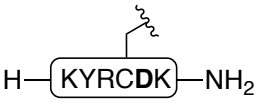
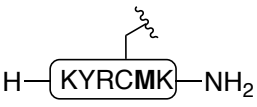
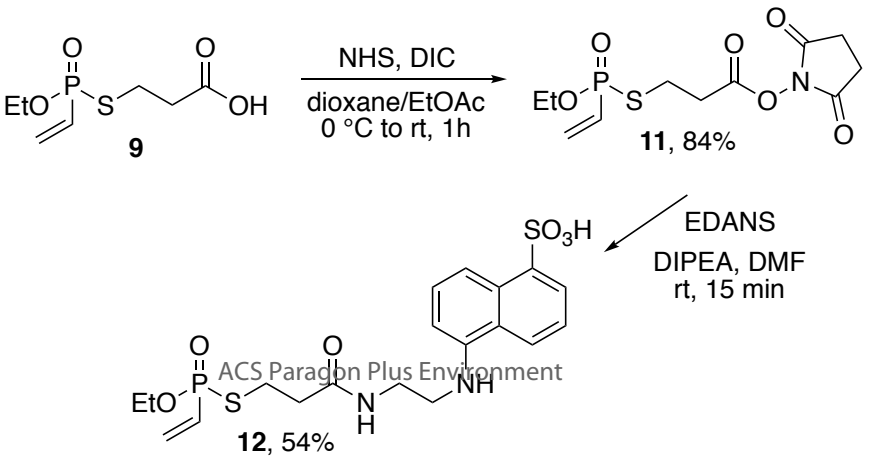
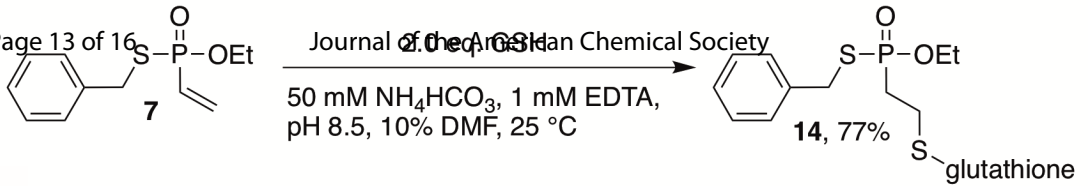
2		**7**	55%
3		**8**	80%
4		**9**	70%
5		**10 a**	41%[a]
6		**10 b**	33%[a]
7		**10 c**	32%[a]
8		**10 d**	29%[a]
9		**10 e**	35%[a]

a: isolated via reverse-phase HPLC

B)

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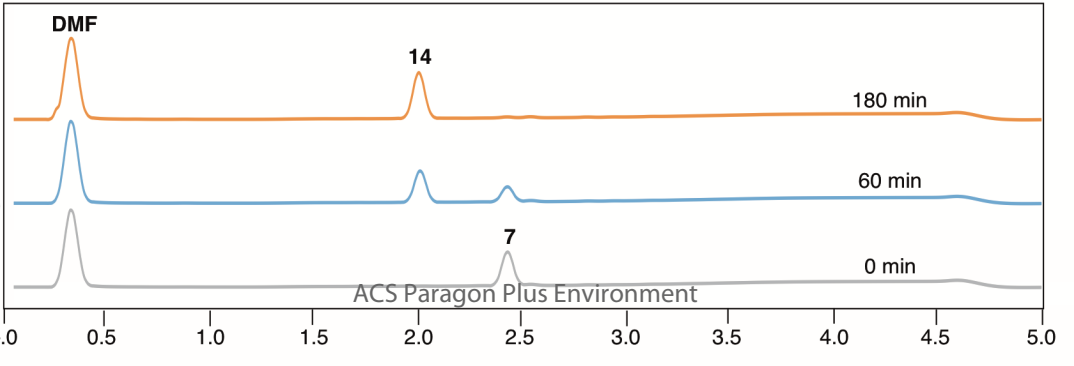
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