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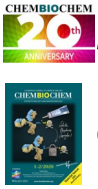

N-Hydroxysuccinimide-Modified Ethynylphosphonamidates Enable the Synthesis of Configurationally Defined Protein Conjugates

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Herein, the application of *N*-hydroxysuccinimide-modified phosphonamidate building blocks for the incorporation of cysteine-selective ethynylphosphonamidates into lysine residues of proteins, followed by thiol addition with small molecules and proteins, is reported. It is demonstrated that the building blocks significantly lower undesired homo-crosslinking side products that can occur with commonly applied succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) under physiological pH. The previously demonstrated stability of the phosphonamidate moiety additionally solves the problem of premature maleimide hydrolysis, which can hamper the efficiency of subsequent thiol addition. Furthermore, a method to separate the phosphonamidate enantiomers to be able to synthesize protein conjugates in a defined configuration has been developed. Finally, the building blocks are applied to the construction of functional antibody–drug conjugates, analogously to FDA-approved, SMCC-linked Kadcyla, and to the synthesis of a functional antibody–protein conjugate.

Introduction

The chemical fusion of proteins with other complex molecules, such as fluorescent dyes, functional peptides, therapeutically active drugs, or even other full-length proteins, can significantly expand their function, and thus, give rise to new tools in the area of cellular biology, medical diagnostics, and targeted therapeutics.[1] Due to the ease of application in modifying unengineered proteins, bifunctional chemical reagents that specifically modify native amino acid residues are extremely powerful compounds for the construction of complex protein conjugates.[2]

Control over the conjugation process can be achieved with hetero-bifunctional molecules that carry functional groups with distinct reactivities, which allows the coupling of two different amino acid residues in a stepwise manner. The prime residues targeted by such hetero-bifunctional crosslinking reagents are typically cysteine and lysine due to their differences in reactivity. The ε-amino group of lysine and the N terminus is commonly addressed with amine-reactive *N*-hydroxysuccinimide (NHS) esters, which demonstrate favorable reaction kinetics at almost neutral pH, together with sufficient selectivity.[3] Most prominently, such NHS esters have been combined with thiol-reactive electrophilic disulfides and various maleimide derivatives to form a reductively cleavable disulfide bond be-

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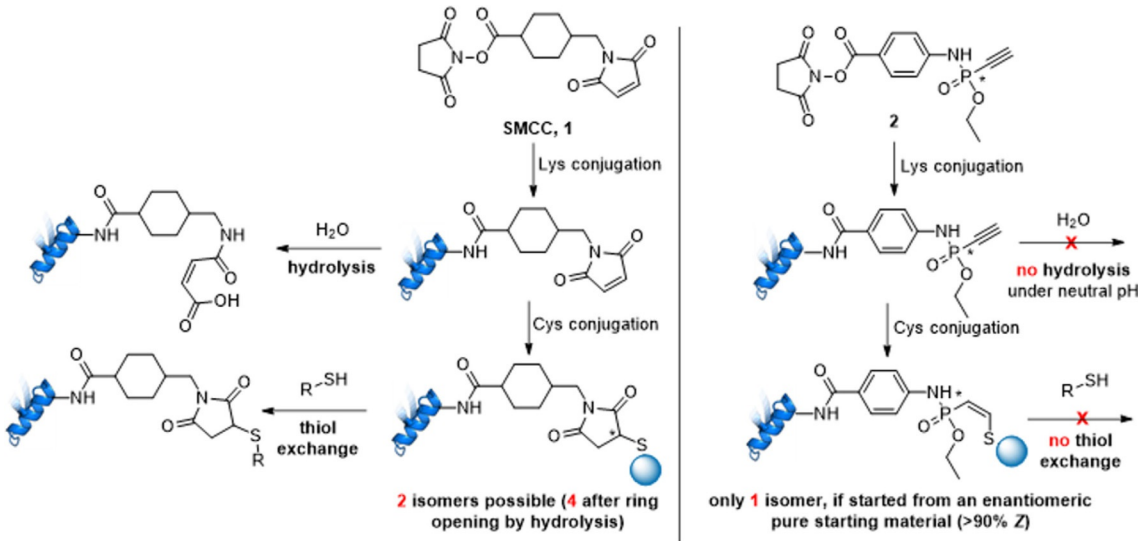


Scheme 1. Principle of the linkage of a lysine residue and a thiol with succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (**1**; left) and the NHS-functionalized phosphonamidate **2** (right). In contrast to maleimides, phosphonamidates are not prone to hydrolysis and thiol exchange,[12c,23] and can deliver configurationally defined conjugates if starting from enantiomerically pure phosphonamidates.

tween the two reactants[4] or generate a succinimidyl–thioether bond with increased stability, respectively.[5]

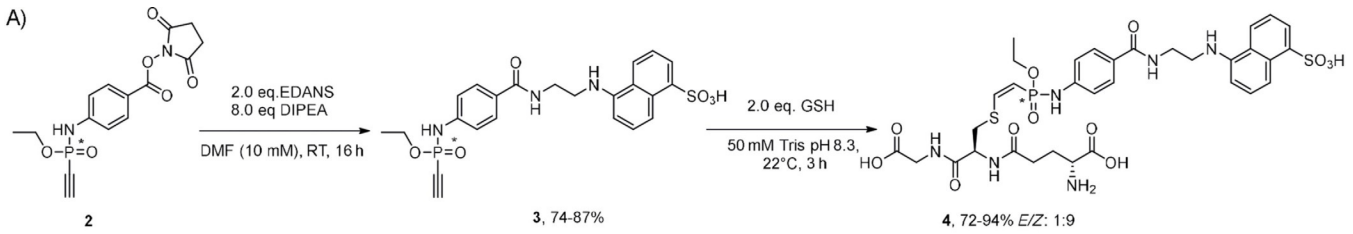
Despite being the most widely used cysteine conjugation method, maleimide chemistry is associated with several drawbacks.[6] First, maleimide conjugates are prone to hydrolysis, forming the ring-opened maleamic acid moiety, which is unreactive to sulfhydryl groups (Scheme 1). This is especially problematic in the context of hetero-bifunctional crosslinking reagents because maleimides that hydrolyze during the installation step on the protein by NHS chemistry can diminish the overall conjugation yield for the final conjugate.[2,7] Furthermore, the succinimidyl–thioether bond is prone to undergo the retro-Michael reaction, re-forming the unconjugated maleimide, which can, in turn, conjugate to other external thiols thereafter (Scheme 1, left).[8] This thiol exchange reaction is especially problematic in the context of targeted therapeutics because severe side effects can arise from premature drug loss from the targeting unit during circulation in the blood stream.[9] A strategy to overcome this issue is to hydrolyze the maleimide residue after thiol conjugation to the open-ring form, which is not susceptible to thiol exchange.[10] However, this strategy might be limited by incomplete hydrolysis and the formation of constitutionally and stereoisomeric mixtures.[11] Furthermore, we and others have observed that maleimides can cross-react with other nucleophilic amino acid residues, such as lysine.[12]

A maleimide-based hetero-bifunctional crosslinker that is known for limited hydrolysis and thiol exchange, due to its sterically hindered cyclohexyl moiety, is **1**,[2b,7] which is today's most widely used reagent for the stepwise conjugation of complex protein structures.[2a] Among others, it has been applied to the synthesis of peptide–protein conjugates,[13] hapten–carrier complexes,[14] antigen–antibody[15] and enzyme–antibody fusions,[16] therapeutically relevant immunotoxins,[17] and antibody–drug conjugates (ADCs).[18] A major drawback of **1** is its hydrophobicity, which often requires the addition of co-solvents in the first reaction step. Although this problem has been partially solved by the incorporation of a sulfonic acid moiety at the succinimidyl leaving group,[19] the remaining linker hydrophobicity in the final product can still cause aggregation and precipitation, which has been described as one of the main issues in the context of ADCs.[20] Substitution of the central cyclohexane ring with a 1,3-dioxane structure was shown to enhance the hydrophilicity of the final conjugate.[21]

Recently, we introduced unsaturated phosphonamidates for the selective and irreversible modification of cysteine residues in proteins and antibodies.[12c,22] We described the synthesis of NHS-modified ethynylphosphonamidate building block **2** and demonstrated that hydrophilic diethylene glycol substitution at the phosphonamidate ester residue could drastically increase the water solubility.[12c,23] Here, we applied these building blocks for the synthesis of ADCs and antibody–protein conjugates and compared those to state-of-the-art **1**. In contrast to the maleimide residue within **1**, the phosphonamidates are stable to hydrolysis under NHS conjugation conditions.[12c] We demonstrate that ethynylphosphonamidates exhibit a higher cysteine selectivity and thereby reduce undesired homo-crosslinking side products at physiological pH (7.4). Furthermore, we showed that NHS-phosphonamidates were able to deliver configurationally defined conjugates, if the enantiomers were separated beforehand, giving rise to linker molecules that enabled the construction of stable, polar, and more homogeneous conjugation products (Scheme 1).

Results and Discussion

We started our investigations by preparative chiral HPLC separation of the two enantiomers of compound **2** that originated from the previously described Staudinger–phosphonite reaction of diethyl ethynylphosphonite and 4-azidobenzoic acid



B)

Starting material	$[\alpha]_D^{24}$ **2**	Yield **3** [%]	Yield **4** [%]	*E/Z* **4**	%*de* **4**
(−)-**2**	− 30.1	87	74	1:9	>99
(+)-**2**	+28.6	74	72	1:9	>99
(±)-**2**	-/-	75	94	1:9	0

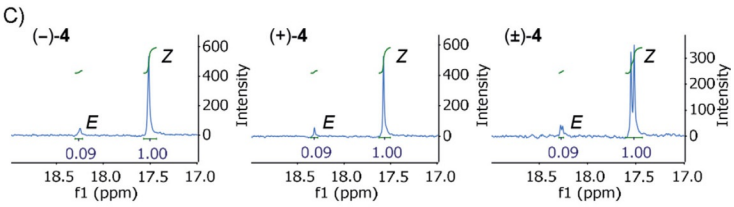


Figure 1. Enantiomerically purified **2** can yield a configurationally defined conjugate after amine–thiol conjugation. A) Synthetic scheme for the attachment of EDANS to enantiomerically pure and racemic **2** followed by glutathione addition. DIPEA: *N*,*N*-diisopropylethylamine. B) Table of yields of products isolated for EDANS intermediate **3** and yields of product isolated, *E*/*Z* ratio, and diastereomeric excess of cysteine adduct **4**. C) Section of the ^{31}P NMR spectra of **4**, which has been synthesized from (−)-**2** (left), (+)-**2** (middle), and racemic **2** (right).

NHS ester.[12c] After successful separation with a Chiralpak IA column, eluting with 30% isopropanol in hexane, both enantiomers (+)- and (−)-**2**, as well as the racemic mixture, were reacted with the 5-[(2-aminoethyl)amino]naphthalene-1-sulfonic acid (EDANS) amine under basic conditions and the functional fluorescent phosphonamidates **3** were isolated in very good yields. Next, L-glutathione was added to the triple bond and the corresponding products **4** were subjected to HPLC and ^{31}P NMR spectroscopic analysis (Figure 1A). Similar to our previous observations,[12c] we found that more than 90% of the *Z* isomer formed upon thiol addition (Figure 1B and Figure S1 in the Supporting Information). In addition, the *Z* isomers that originated from the reaction of the enantiomerically pure phosphonamidates (+)- and (−)-**2** displayed a single resonance in the ^{31}P NMR spectrum at $\delta = 17.58$ and 17.51 ppm, respectively. In contrast, two resonances in the same shift range were observed for the compound that originated from the racemic mixture (Figure 1C). These observations clearly demonstrate that enantiomerically pure ethynylphosphonamidates can deliver configurationally defined conjugates after cysteine addition, with more than 90% of conjugates in the *Z* configuration.

After we demonstrated that chiral NHS-modified ethynylphosphonamidate **2** was able to deliver conjugates in a defined configuration, we continued with the evaluation of the lysine reactivity on proteins. The monoclonal antibody trastuzumab was chosen as a functional, therapeutically relevant protein structure. Different equivalents of the NHS esters **1** and **2** and more hydrophilic diethylene glycol substituted **5** were added to the antibody at a concentration of 1 mg mL^{-1} in phosphate-buffered saline (PBS) at pH 7.4, containing 10% of DMSO to ensure solubility for all of the compounds (Figure 2A). After purification to remove excess reagent, the reaction products were analyzed by means of MS to determine the average degree of modification (Figure S2). We observed a linear correlation between the equivalents we applied to the antibody and the degree of protein modification for all of the tested reagents. It turned out that 20 equivalents of the modification reagents led to an average degree of 3.5 modifications per antibody in case of compound **1**. Under the same conditions, NHS esters **2** and **5** gave only 2.1 and 1.2 modifications per antibody, respectively. We attribute this observation to increased hydrolysis rates of the more electron-deficient aromatic NHS esters **2** and **5**. To compensate for this, we also performed the reaction at higher antibody concentrations of 4 mg mL^{-1}. Here, both compounds gave a good degree of modification of 4.6 for compound **2** and almost 4 for compound **5**, upon applying 20 equivalents of reagent (Figure 2B). However, in case of **1**, the formation of a white precipitate was observed and no antibody could be detected during MS analysis. Therefore, we also analyzed all reactions by means of SDS-PAGE to visualize intermolecular covalent crosslinks that might originate from unspecific reactions of the thiol-reactive moiety with amino acids other than cysteine. Indeed, we observed an increasing amount of high-molecular-weight bands with increasing equivalents of maleimide compound **1** under our tested reaction conditions. These bands were absent for all tested ethynylphosphonamidate reactions, even at high antibody concentrations of 4 mg mL^{-1} and 20 equivalents of reagents (Figures 2C and S3).

Next, we proceeded with the addition of a thiol to the installed sulfhydryl acceptors. Recently, we have shown that ethynylphosphonamidate linkages exhibit excellent properties for the construction of ADCs, such as high serum stabilities and in vivo efficacy.[23] As trastuzumab, modified with **1** and the thiol-containing drug DM1, is one of the five FDA-approved ADCs, named trastuzumab–emtansine (brand name Kadcyla),[18,24] we aimed to reconstruct this ADC by applying the conjugation conditions described in the previous paragraph in PBS.[25]

In our case, we treated the trastuzumab antibodies that had previously been incubated with 5, 10, and 20 equivalents of **1**, **2**, and **5** at a concentration of 4 mg mL^{-1} with 10 equivalents of DM1 with respect to the antibody. According to our previously optimized conditions,[23] we performed the thiol addition to trastuzumab–**2** and trastuzumab–**5** at pH 8.5, whereas phys-

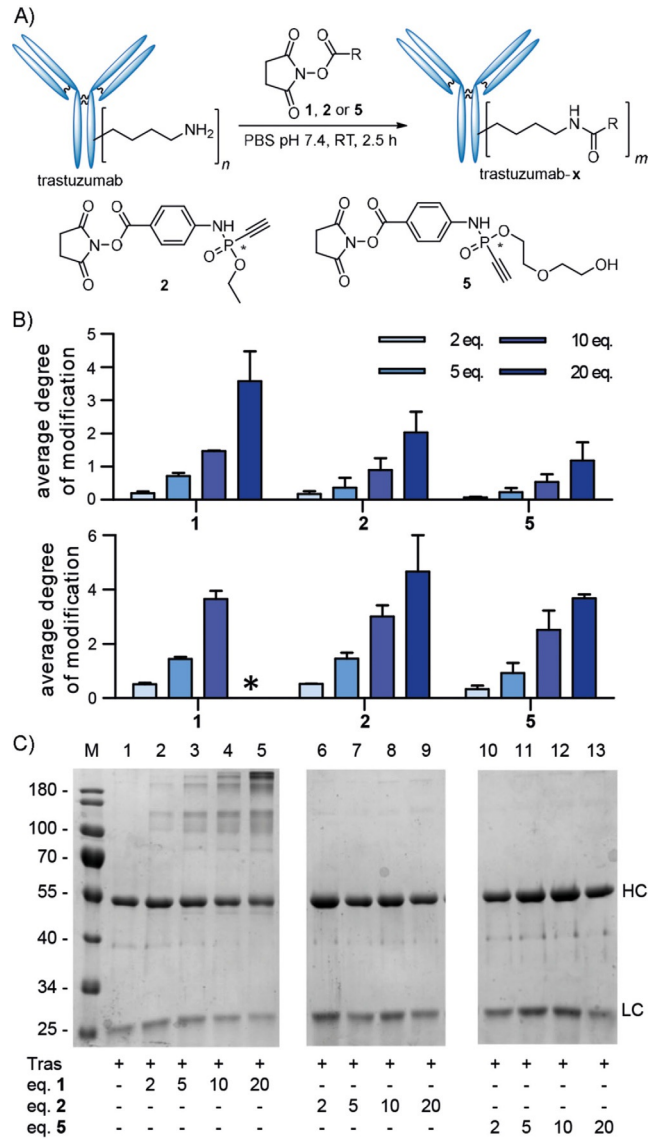


Figure 2. Addition of different equivalents of NHS esters **1**, **2**, and **5** to trastuzumab and analysis by means of MS and SDS-PAGE. A) Synthetic scheme. B) Estimation of the degree of antibody modification by means of MS analysis after incubation with 2, 5, 10, and 20 equivalents of the three compounds. Top: 1 mg mL^{-1} antibody was used in conjugation, bottom: 4 mg mL^{-1}. Shown are mean and error from two independent experiments. *No protein masses detected. C) SDS-PAGE analysis of the antibodies treated at 1 mg mL^{-1} with 2, 5, 10, and 20 equivalents of the three compounds.

iological pH was used in the case of trastuzumab–**1**. Afterwards, we purified the ADCs by means of size-exclusion chromatography (SEC) to remove excess toxin and analyzed the drug-to-antibody ratio (DAR) of the constructs by means of MS (Figure 3 A and C). Here, we faced severe aggregation and solubility issues for the ADCs that had been constructed from 20 equivalents of compounds **1** and **2** at pH 7.4. Hence, we were unable to measure the DAR by means of MS. We attribute this observation to the undesired crosslinking that we have observed previously for compound **1** and to the fact that hydrophobic DM1 in combination with the rather hydrophobic molecules **1** and **2** drastically increases the antibody's tendency to aggregate, especially for the higher DAR species.[26] Antibodies treated with 5 or 10 equivalents showed better behavior in terms of aggregation; however, here it was not possible to achieve higher drug loadings than that of 1 or 1.5 for **1** and **2**, respectively, under the conjugation conditions that have been applied here. Again, decreasing the antibody concentration to 1 mg mL^{-1} improved the conjugation efficiency and led to a DAR of 2 if 20 equivalents of **1** were used, but only to a DAR of 1.2 for compound **2** (Figure 3 B). No aggregation or precipitation, even at higher concentrations, were observed if the more polar di(ethylene glycol)-substituted phosphonamidate **5** was applied for the fusion of trastuzumab to DM1. These results are in line with our previous observations that di(ethylene glycol) substitution at the phosphonamidate core can drastically improve the aqueous solubilization behavior of lipophilic drug molecules.[23]

After extensive characterization of the synthesized ADCs by means of MS, SDS-PAGE, SEC, and hydrophobic-interaction chromatography (HIC; Figures S5–S8), we validated the functionality of the synthesized ADCs. Therefore, we tested three trastuzumab–DM1 conjugates, synthesized from compounds **1**, **2**, and **5** with similar DARs of 1.4 to 1.6 in a HER2-based cell viability assay with a Her2-overexpressing cell line (SK-BR-3) and a Her2-negative cell line as a control (MDA-MB-468). Antibody concentrations leading to 50% maximal growth inhibition (IC_{50}) were measured to be 14.9 ng mL^{-1} for trastuzumab–**1**–DM1, 16.3 ng mL^{-1} for trastuzumab–**2**–DM1, and 19.8 ng mL^{-1} for trastuzumab–**5**–DM1 (Figure 3 D). These values are very similar to previously determined inhibition constants of trastuzumab emtansine of 11 ng mL^{-1} with SK-BR-3 cells.[27] As an additional control, trastuzumab was treated under the conjugation conditions with DM1 only, without prior installation of a thiol acceptor to rule out any unspecific binding of the drug to the antibody. As expected, this antibody did not show any effect on the Her2-positive cell line after purification from excess drug. Notably, no cytotoxicity for any of the constructs was observed on the Her2-negative cell line.

In addition, we analyzed the constructs by means of HIC, which is a method that enables separation of different ADC species based on the overall hydrophilicity.[28] As expected, we observed a broad peak in the HIC chromatogram, due to the broadly homogenous ADC species that typically arise from lysine conjugation.[29] This peak was clearly shifted towards shorter retention times for more polar trastuzumab–**5**–DM1 relative to those of the other two tested linker systems (Figure S8). Because it is known that more hydrophilic ADCs are, in general, less prone to undesired aggregation behavior,[30] we believe that compound **5** is able to address current issues associated with the hydrophobic linker used in trastuzumab–emtansine.[31]

Finally, we also wanted to apply our phosphonamidate building blocks to a more challenging thiol-containing molecule and aimed for the synthesis of an antibody–protein conjugate. We chose our previously established eGFP mutant C70M S147C, which carries only one addressable cysteine residue, and incubated it in a 3.5-fold excess with trastuzumab that was preactivated with 20 equivalents of **5** at a concentration of

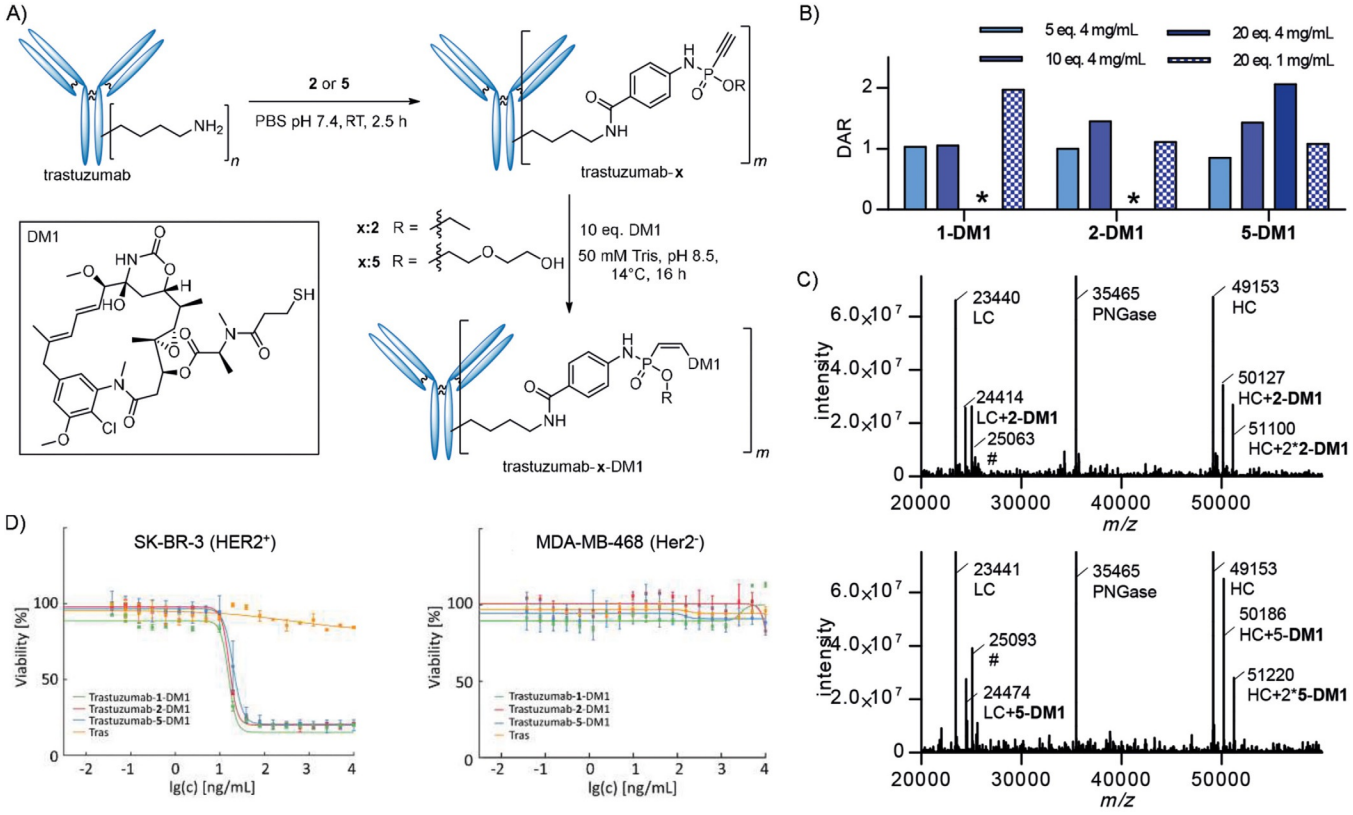


Figure 3. Synthesis of efficacious ADCs from trastuzumab; **1**, **2**, or **5**; and DM1. A) Synthetic scheme for ADC synthesis from **2** or **5**. B) DAR estimation of the ADCs by means of MS after the synthetic procedure, applying a protocol of 4 mg mL^{-1} antibody and 5, 10, or 20 equivalents of **1**, **2**, and **5**, followed by incubation with 10 equivalents of DM1. DM1 conjugation was performed in a buffer containing 50 mM Tris at pH 8.5 for trastuzumab–**2** and trastuzumab–**5** and in PBS at pH 7.4 for trastuzumab–**1**. Dashed bar: 1 mg mL^{-1} antibody and 20 equivalents. *No protein masses detected. C) MS analysis of the ADCs. Light (LC) and heavy chain (HC) after DM1 conjugation (top: trastuzumab–**2**–DM1, bottom trastuzumab–**5**–DM1). # half-masses of the HC signals, deconvolution artefacts. D) Cell viability assay of three ADCs from **1** (green), **2** (red), and **5** (blue) and a control in which trastuzumab was incubated with DM1 only (orange). All constructs were purified by means of SEC before analysis. Constructs were tested on an antigen-positive cell line (SK-BR-3) and an antigen-negative cell line (MDA-MB-468).

4 mg mL^{-1} (Figure 4A). After conjugation and purification by means of SEC to remove unconjugated eGFP, we could observe successful conjugation by means of SDS-PAGE (Figure 4B) and MS. MS analysis clearly showed that an eGFP–LC adduct had formed. Because unconjugated phosphonamidates were still detected on the HC and LC of the antibody after the conjugation reaction, it should be noted that the conjugation yield was lower than those in previous conjugation experiments with small-molecule thiols. However, successful conjugation was additionally confirmed by means of immunostaining experiments with the trastuzumab–eGFP constructs after fixation of either SK-BR-3 or MDA-MB-468 cells with paraformaldehyde. Here, excellent target selectivity for the Her2-receptor on the outer cell membrane could be observed for the SK-BR-3 cell line (Figures 4C and S9). This experiment again shows that antibody modification with NHS ester **5** does not affect the antibody's performance and enables the fusion of a full-length protein with retained functionality.

Conclusion

NHS-modified ethynylphosphonamidates were applied to the sequential coupling of lysine residues in protein structures with thiol-containing small molecules and proteins. Furthermore, enantiomerically pure NHS-modified ethynylphosphonamidates enabled the synthesis of a conjugate with a single enantiomer on phosphorus. Considering the high *Z* selectivity of thiol addition, this gives rise to more defined reaction products, in terms of stereochemistry, that would not be possible with standard maleimide chemistry because a stereocenter is formed upon cysteine addition. The hetero-bifunctional reagent was also used in the synthesis of a phosphonamidate-linked analogue of the FDA-approved ADC Kadcyla, in which the NHS reaction was performed at physiological pH. Although these conditions delivered a lower DAR than that of the ADC currently available on the market, we were able to demonstrate the function in cell viability assays and an increase in ADC polarity, if ethylene glycol substituted phosphonamidate **5** was used. Finally, a challenging protein–antibody conjugate was prepared and the functionality of both parts was demon-

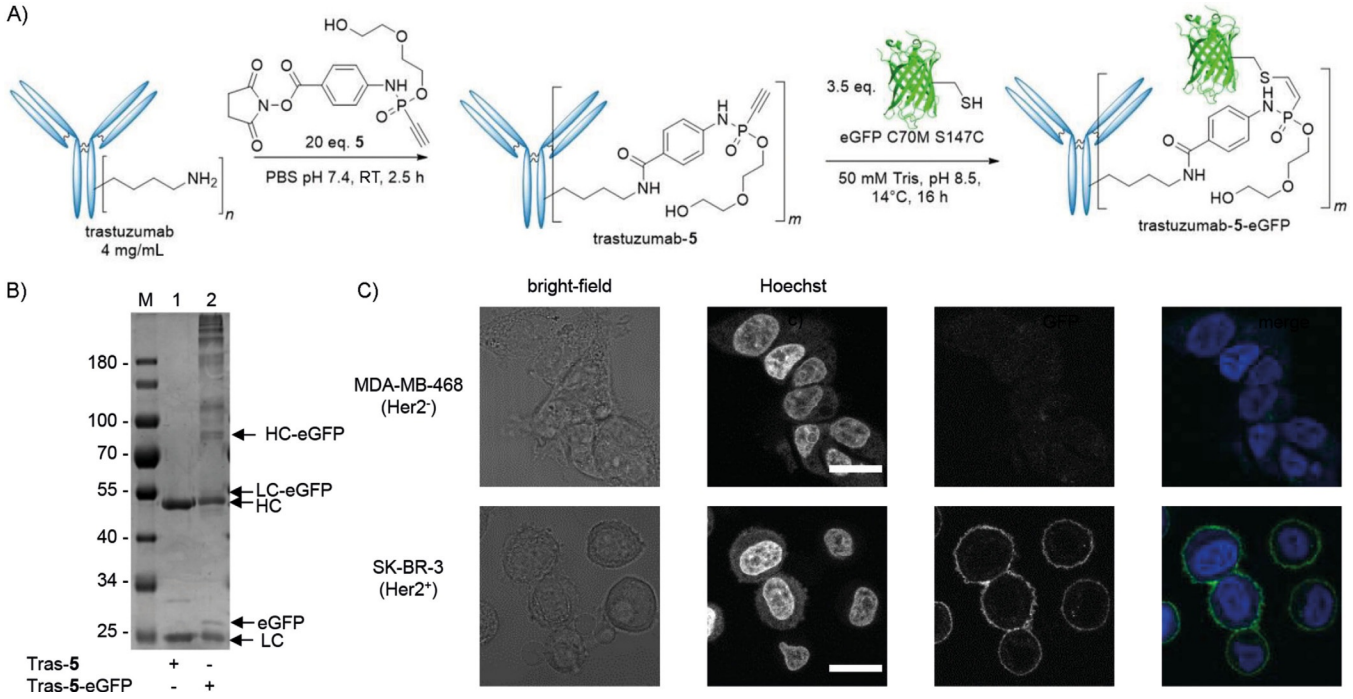


Figure 4. Synthesis and evaluation of a functional antibody–protein conjugate from trastuzumab and eGFP with the aid of compound **5**. A) Synthetic scheme. B) SDS-PAGE analysis of the purified conjugate. C) Immunostainings of fixed cells, either overexpressing the cell surface receptor Her2 (SK-BR-3) or exhibiting low Her2 expression levels (MDA-MB-468). The merged images show the Hoechst signal in blue and the eGFP signal in green. Scale bar: 20 μm.

strated by means of fluorescence microscopy. Taken together, NHS-modified ethynylphosphonamidates have revealed their potential to improve current issues associated with **1**-based linker systems, such as homo-crosslinking due to unspecific maleimide addition under certain labeling conditions.

Keywords: antibody–drug conjugates · bioconjugation · cysteine-selective linkers · protein conjugation · synthesis design

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Conflict of Interest

The technology described in the manuscript is part of a pending patent application by M.-A.K., D.S., J.H., H.L., and C.P.R.H.

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