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Bis-ethynylphosphonamidates as an Modular Conjugation Platform to Generate Multi-Functional Protein- and Antibody-Drug-Conjugates

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In memory of Klaus Hafner

Bis-ethynylphosphonamidates allow for a simple chemoselective addition of two thiol-containing modules in a row. We describe four such bis-electrophiles that carry different functional O-substituents with tunable hydrophilicity and enable further subsequent conjugations, thus facilitating a simple protocol for constructing protein-protein conjugates. An increased spacer length between the two ethynylphosphonamidates simplifies the formation of a conjugate from two bulky proteins. We apply these reagents to obtain homogeneous Antibody-Drug-Conjugates (ADCs) from DM1 and trastuzumab with excellent cytotoxicity and selectivity for the targeted cell line. Moreover, a bis-ethynylphosphonamidate, carrying an additional alkyne for a chemoselective triple conjugation, has been subjected to fluorescent labeling of an ADC specifically at the drug site give an Antibody-Drug-Fluorophore-Conjugate (ADFC), allowing for the observation of intracellular trafficking after ADC uptake into the targeted cell.

Introduction

Recent breakthroughs in chemical modification of complex biomolecules through selective conjugation to cysteine residues highlight the great potential of exploiting the unique properties of the sulfhydryl group to functionalize peptides, proteins, and antibodies.[1–2] Cysteine modification has found a broad range of applications to address urgent problems in chemical biology as well as for the development of innovative new therapeutics.[4–5] The sulfhydryl group in proteins or peptides has been elegantly utilized to study fundamental biological processes, for example, by incorporating fluorescent handles,[6] to control the release of a conjugated protein,[7] for intracellular protein delivery[8] and to incorporate natural post-translational modifications.[9–10]

Another challenge in chemical biology is the site-selective construction of protein-protein conjugates. To ease the access, it is highly desirable to use proteins that are solely composed of canonical amino acids to ensure a broadly applicable method. Hence, the cysteine residue has been widely employed for this purpose. Protein-protein- and protein-polymer-conjugates have been synthesized by pre-modifying one protein via its lysine residues with a thiol-reactive moiety, followed by subsequent reaction with the cysteine residue of a second protein[11–14] or polymer.[15] Although this concept is applied frequently (for example, by using the commercially available succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate, SMCC linker), it suffers from unsatisfactory specificity due to high lysine abundance in native proteins and the required absence from cysteine in the first protein. Therefore, protein-protein-conjugates that rely on low abundant amino acids on both ends are highly desired. Next to a recent report on tyrosine and cysteine,[16] the subsequent conjugation of two cysteine residues has found several applications in chemical biology.[17–20]

Moreover, cysteine modification has been employed for synthesizing peptide- and protein-conjugates in the field of targeted drug delivery. With 7 market approvals within the last two years and 11 marketed products in total, a class that stands out is Antibody-Drug-Conjugates (ADCs). 9 out of the 11 marketed ADCs are linked via Sulfhydryl-Maleimide adducts,[21] even though known to be unstable during blood circulation in some cases.[22–23] We have previously shown that Adcetris, a marketed ADC consisting of the anti CD30 antibody brentuximab, linked via its interchain cysteine residues to the antimitotic drug MMAE by using maleimide technology, loses

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more than 70% of its cytotoxic payload when incubated with rat serum for 3 days at 37 °C.[24]

In addition to recent technical advancements, such as self-hydrolyzing maleimides,[25] in improving maleimide stability,[2] we have been developing a technology to solve the issue of retro-Michael related instability of maleimide-linked drug conjugates based on unsaturated phosphonamidates **1**. Ethynyl-[3] and vinylphosphonamidates[26] have been shown to react highly selectively with cysteine residues on proteins and antibodies (Figure 1). Most importantly, the formed adducts are highly stable in the presence of several matrices such as external thiols and human serum[3] and have been used to construct ADCs with superior serum stability when directly compared to marketed ADCs.[24] N-Hydroxysuccinimide (NHS)- modified phosphonamidates have been further developed for the stable conjugation of thiols and amines and applied to the construction of Protein-Protein conjugates and ADCs.[27] Moreover, diethynyl phosphinates **2** have been used for a subsequent addition of two cysteine residues in a row and rebridging of native protein disulfides after reduction.[28]

In the present work, we extend the repertoire of available unsaturated phosphonamidate reagents to bis-ethynylphosphonamidates **3**, which we apply to construct protein-protein conjugates and ADCs from sulfhydryl containing drugs by the subsequent addition of two thiols in a row. We report four bisamidates that carry different functional *O*-substituents, such as a short ethylene glycol unit as well as a terminal alkyne. Short PEG-chains have been shown to increase water solubility and, therefore, protein conjugation efficiency.[24,27] Moreover, we demonstrate that the additional terminal alkyne can be used for a final incorporation of a third functionality by copper-catalyzed azide-alkyne-cycloaddition (CuAAC), which facilitates the site-specific labeling of an ADC with a fluorescent dye. This allowed us to achieve a direct visualization of an ADC uptake into the targeted cell by fluorescence microscopy.

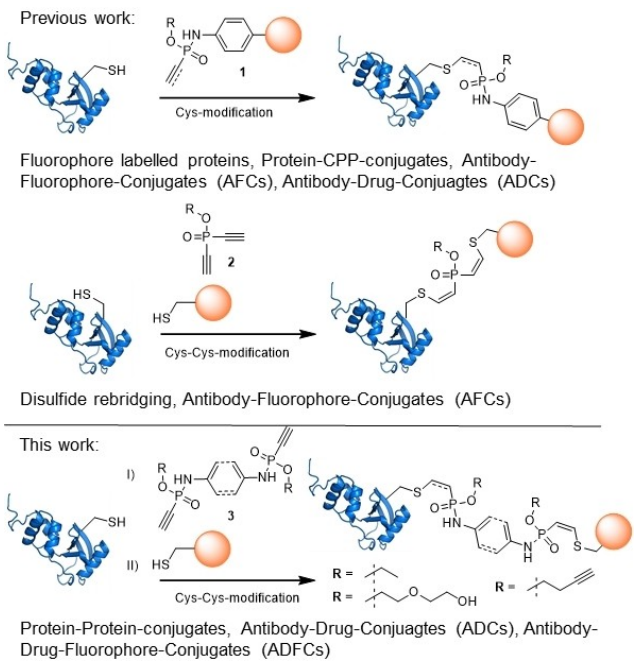


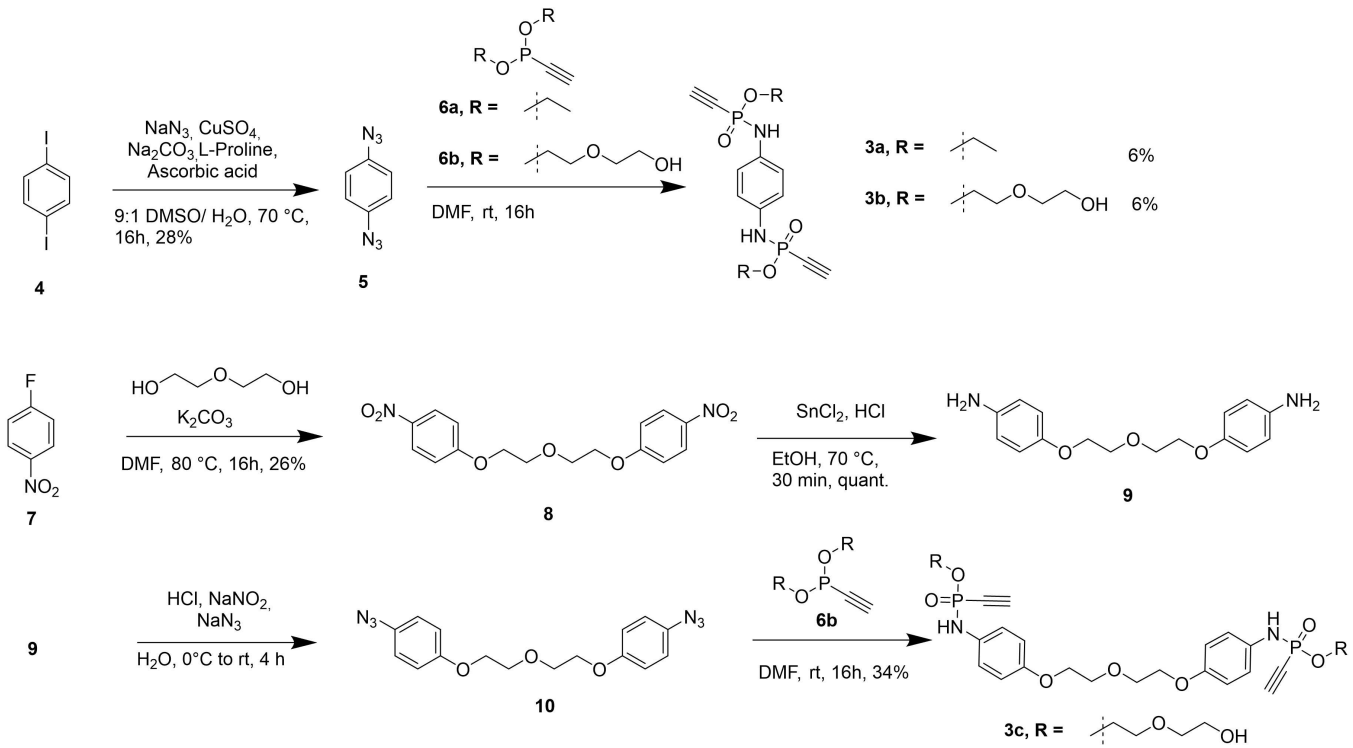
Figure 1. Unsaturated phosphorous(V)-moieties have been previously developed for the selective and stable modification of Cys-residues in proteins and antibodies and for disulfide rebridging. In this work we introduce bis-ethynylphosphonamidates to achieve a simple versatile method for the conjugation of two thiol-containing moieties in a row.

Results and Discussion

Our studies began by establishing a synthesis route towards bis-ethynylphosphonamidates. Based on our previous work, a very modular way of incorporating phosphonamidates is the chemoselective Staudinger-phosphonite reaction (SPhR) from azides with unsaturated phosphonites **6**.[3,29] Due to slightly better yields in the SPhR[3] and our previous observations that unsaturated *N*-phenyl-phosphonamidates react slightly faster with cysteine residues compared to their *N*-alkyl counterparts,[26] we started with the synthesis of 1,4-diazido benzene **5** (Scheme 1). Several attempts to synthesize **5** from 1,4,–phenylenediamin and Azidotrimethylsilane[30] or by diazotization,[31] as described previously, lead to the formation of high amounts of the mono-functionalized azido-aniline. Best conversions to **5** with an isolated yield of 28% were achieved by applying a protocol published by Wang et al.[32] Next, we performed the SPhR between **5** and phosphonite **6a** under our previously established conditions.[3] Accordingly, **6a** was synthesized from Diethylchlorophosphite and used without any further purification. To increase the water-solubility of the bisamidates, which is crucial for the modification of proteins, we also performed the SPhR using phosphonite **6b**.[24] Despite the fact that the SPhR is highly chemoselective, it is also known for the generation of side products such as the reduced amine, resulting from *P-N*-hydrolysis.[29] Moreover, we observed that the monofunctionalized aide intermediates tend to oligomerization by 1,3-dipolar cycloaddition. Together with the fact that the desired bis-ethynylphosphonamidates had to be isolated by preparative reversed-phase HPLC due to a very high affinity of the phosphonamidate moiety to silica gel, we achieved only low yields of 6% for the isolation of compounds **3a** and **3b** after lyophilization.

The desired construction of protein-protein-conjugates via Cys-residues with compounds **3a** and **3b** might be hindered by bulky proteins due to the relatively short linkage between the two Cys-reactive groups. Hence, we also synthesized a bis-ethynylphosphonamidate structure **3c**, carrying a diethylene glycol spacer unit between two *N*-aryl-phosphonamidates. For this, azide **10** was synthesized in three steps from 1-fluoro-4-nitrobenzene **7**. First, nucleophilic aromatic substitution with diethylene glycol in the presence of potassium carbonate led to the formation of the bis-nitro compound **8**, which was further reduced by Tin(II)-chloride to give the diamine **9** before it was transformed into **10** by diazotization in good overall yield. **3c** was afterwards synthesized from the diazide **10** and phosphonite **6b** with 34% isolated yield.

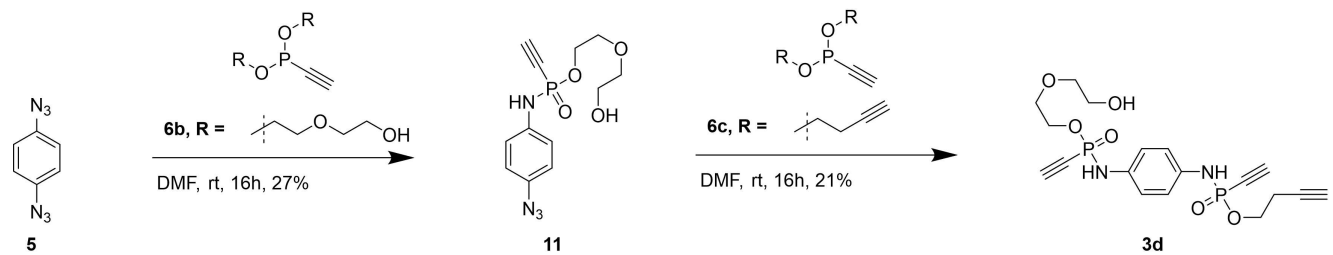
To further expand the versatility of our constructs, we also sought to incorporate a terminal alkyne into one of the *O*-



Scheme 1. Synthesis of bis-ethynylphosphonamidates **3a**–**3c**. The symmetric compounds have been synthesized from diazidophenyls via Staudinger-phosphonite reaction with the previously described phosphonites **6a** and **6b**.

substituents of the bis-ethynylphosphonamidates to generate the modular building block with the structure of **3d** (Scheme 2). This structure can be used for a chemoselective triple conjugation of two thiol-containing moieties, followed by the incorporation of a third functional entity utilizing CuAAC. To incorporate only one alkyne handle into a bis-ethynylphosphonamidate structure with two *O*-substituents, we performed two SPhRs in a row with two different phosphonites. At first, conditions were screened to mono-functionalize the bisazide **5** and generate compound **11**. Best yields were achieved with 1.3 equivalents of the phosphonite and decreased reaction times. Longer reaction times lead to decomposition of the product **11**, by the above described 1,3-dipolar cycloaddition. With **11** in hand, we synthesized compound **6c**, a novel phosphonite from 3-butyn-1-ol, carrying a terminal alkyne for further modifications by CuAAC. **6c** had to be directly used further in the next step after synthesis and purification due to its high tendency to oxidize when exposed to air. SPhR between **11** and **6c** yielded the desired **3d**, which could be isolated by preparative HPLC. Phosphonamidates are chiral and formed as racemic mixtures from SPhR, as previously reported.[27] Accordingly, compounds **3a**–**3d** exist as a mixture of diastereoisomers, which could not be separated.

With compounds **3a**–**d** in hand, we proceeded with the construction of protein-protein dimers from two single cysteine-containing proteins. Since compound **3a** turned out to be insoluble at higher concentrations under aqueous conditions, we excluded **3a** from the reactions with proteins. We started the investigations by screening different equivalents of **3b** in the reaction with a single addressable cysteine-containing protein eGFP C70M S147C[3] (Figure 2, I). We applied our



Scheme 2. Synthesis of unsymmetric bis-ethynylphosphonamidate **3d** via two subsequent Staudinger-phosphonite reactions in a row with two different phosphonites.

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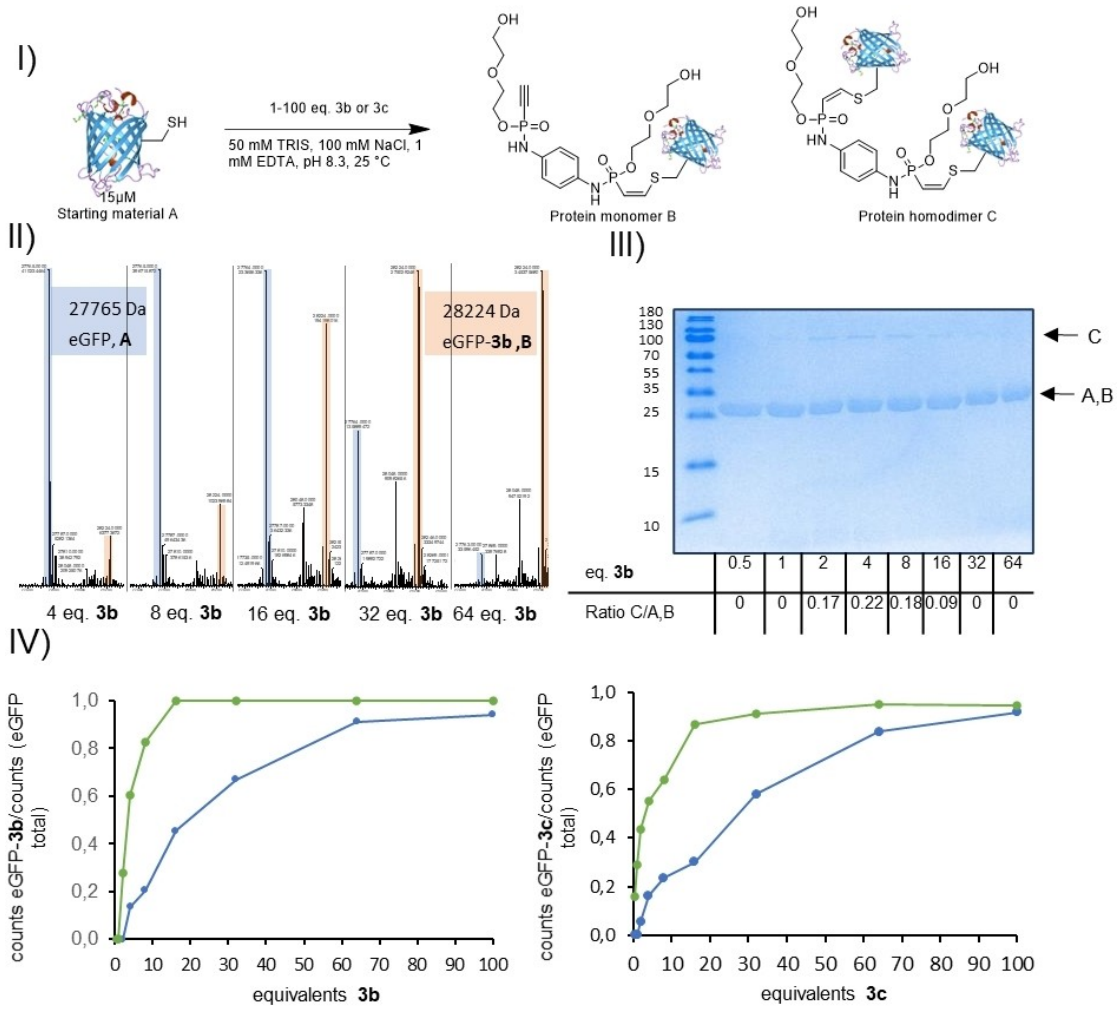

Figure 2. Conjugation of bis-ethynylphosphonamidates **3** to eGFP. I) Scheme for the conjugation of **3b** to eGFP **A** to yield a protein monomer **B** after a single addition to **3b** and a homodimer **C** after double addition. Double bonds are drawn in a *Z*-orientation, since it has been previously shown that this orientation is highly preferred over *E*.[3] Various equivalents were screened to maximize the yield of the desired product **B**. II) Intact protein LC-MS of the starting material incubated for 1 hour with increasing amounts of **3b**. Note: **C** was not detectable via LC-MS III) SDS-PAGE to assses the amount of homodimer **C**. IV) Amount of **B** after 1 h incubation (blue) and overnight (green) with varying equivalents of **3b** (left), **3c** (right), analyzed via LCMS.

previously described conditions of slightly basic pH[3] at a protein concentration of 15 μM and analyzed the reaction mixtures via intact protein ESI/MS after 1 h reaction time (Figure 2, II). It was observed that more equivalents of **3b** lead to increasing amounts of the modified protein **B**. The highest degree of modification was observed when 64 equivalents of **3b** were used in the reaction. Unfortunately, we were unable to observe the protein homodimer **C** by MS, which is expected to form via double addition of the eGFP to **3b**, especially when fewer equivalents are used in the reaction. Therefore, we also performed analyses by SDS-PAGE (Figure 2, III), where a band of higher molecular weight appeared with increasing equivalents of **3b** and a maximal ratio of 0.22 between protein dimer and monomers when 4 eq. of **3b** were used in the reaction. The band of the homodimer **C** further diminished again when even more equivalents of **3b** were used, most likely in favor of the modified monomer **B**, as observed by MS (Figure 2, II). We envision that a heterodimer of two different cysteine-containing proteins will be more useful for most applications in chemical biology. Hence, we carefully screened different equivalents of **3b** and **3c** in the reaction with eGFP and estimated the ratio of unmodified protein **A** and modified protein monomer **B** with the MS-intensities after 1 h of reaction and overnight (Figure 2, IV). More than 80% conversion was reached with 64 equivalents after one hour and fewer than 16 equivalents after overnight reaction for both compounds.

After having a protocol for constructing modified protein monomer **B** with **3b** and **3c** established, we moved on with the synthesis of a protein heterodimer **D** by adding a single cysteine-containing ubiquitin variant G76C, which we have previously introduced[20] (Figure 3, I). A simple gel filtration step was suitable for removing excess reagents **3** and delivered construct B, ready for the second protein addition. For this, we screened different equivalents of ubiquitin in the reaction with **eGFP-3b** and **eGFP-3c** under our typical cysteine modification conditions at slightly basic pH and a constant concentration of

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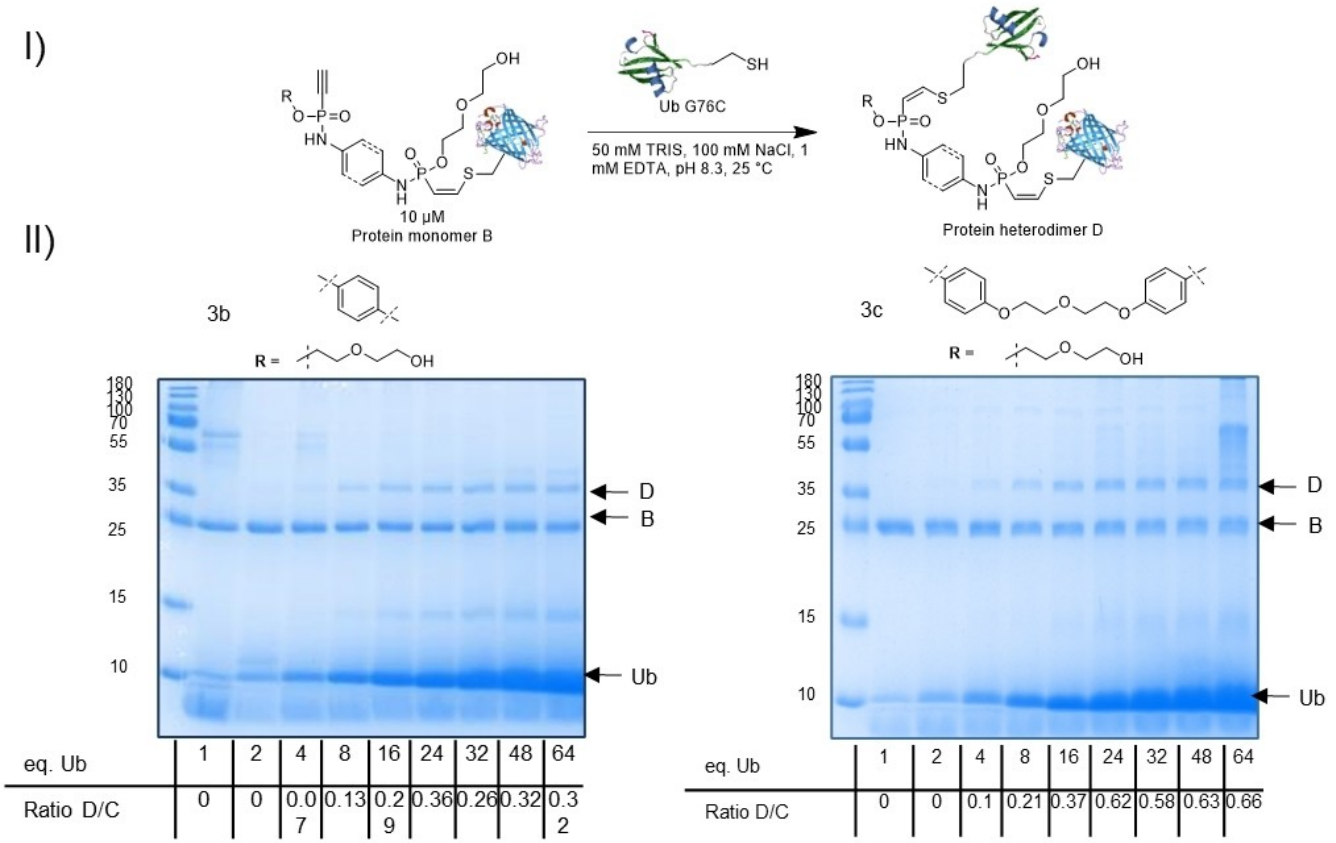

Figure 3. I) Scheme for the conjugation of eGFP-**3** to a single cysteine containing Ubiquitin mutant (G76C). Compounds **3b** and **3c** have been used to construct the eGFP-Ubiquitin hetero-protein-protein conjugate **D**. eGFP monomers **B** have been synthesized with 100 eq. **3** prior reaction in order to ensure full conversion without any undesired homodimer **C** formation. II) SDS-Page analysis of the eGFP monomers **B** synthesized from **3b** (left) and **3c** (right) incubated with different equivalents of Ubiquitin.

10 µM eGFP and analyzed the reaction mixtures after overnight incubation at room temperature using SDS-PAGE and MS (Supporting Information). A maximal conversion of around 30% to the desired hetero-protein-dimer **D** according to the band intensity in the SDS-PAGE analysis was observed in this setting when using 48 equivalents of ubiquitin and eGFP-**3b** (Figure 3, II). Using even more equivalents did not have any further effect on the outcome of this experiment. We attribute this relatively low conversion to the steric hindrance of the two proteins. To further prove this hypothesis, we conducted the same experiments using the longer linker system of **3c**, which allows for more degrees of freedom as well as space between the two proteins during the conjugation process. Indeed, linker **3c** increased the conversion of **B** to **D** to roughly 60%, and the maximal conversion was already reached by using 24 equivalents of ubiquitin (Figure 3, II). Taken together, these experiments clearly show that bis-ethynylphosphonamidates can be used for a simple two-step conjugation protocol of two different cysteine-containing proteins in a row, with only one simple gel-filtration step in between.

Moreover, we also used compounds **3a–d** for the construction of Antibody-Drug-Conjugates (Figure 4). We have recently shown that ethynylphosphonamidates exhibit excellent properties for the linkage of cytotoxic drugs to antibodies since only minor reagent excess is required. Most importantly, the conjugates have a high stability in the presence of serum.[24] Kadcyla, one of the 11 marketed ADCs, uses the antineoplastic maytansine derivative DM1, which is connected to the lysine residues of the monoclonal HER2-addressing antibody trastuzumab.[33–34] Due to a great number of potential modification sides, Lysine conjugation is typically yielding in heterogeneous ADC mixtures of up to 4.5 million unique molecules.[35] Since DM1 itself is carrying a thiol, which is connected to lysine residues of trastuzumab in Kadcyla, we sought to apply our novel linker systems described herein to connect the thiol of DM1 to the interchain-cysteine residues of the antibody, similar as performed for ADCs connected to auristatins, such as Adcetris.[36] Since humanized monoclonal antibodies carry only 4 interchain cysteine residues, which can be selectively modified to give a maximum of 8 drug molecules per antibody,[37] this strategy allows for the construction of an ADC from trastuzumab and DM1 that is much more homogeneous than Kadcyla. Homogeneous ADC based on DM1 have been previously shown to improve the therapeutic activity.[38]

To avoid interference with other cysteine residues of the antibody and to circumvent undesired disulfide rebridging

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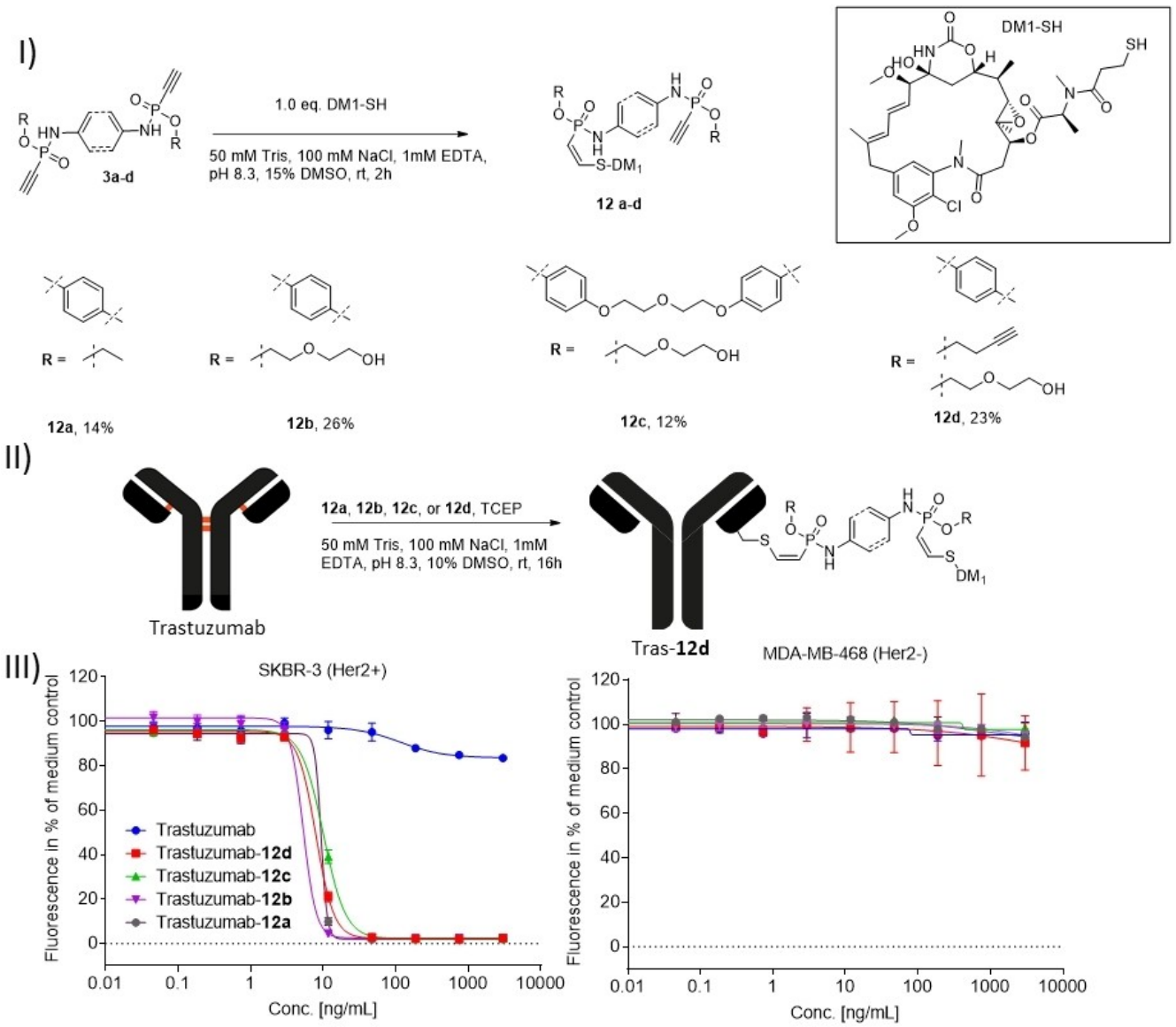

Figure 4. Bis-ethynylphosphonamidates **3a–d** for the construction of Cys-linked trastuzumab-DM1 ADCs. I) Scheme for the synthesis of DM1-adducts to **3a–d** to synthesize Cys-reactive DM1-constructs **12a–d**. Stated are isolated yields after preparative HPLC. II) Scheme for a one-pot conjugation of **12a–d** to the anti-Her2 monoclonal antibody trastuzumab. III) Cytotoxicity of the 4 constructs and an unconjugated trastuzumab control (blue) on an antigen-positive cell-line (SKBR3, left) and an antigen negative cell line MDA-MB-468 (right).

reactions,[39] we first synthesized DM1 adducts from the Bis-phosphonamidates **3a–d** by using one equivalent of DM1 per phosphonamidate and isolated the DM1 mono-adducts **12a–d** via preparative HPLC (Figure 4, I). Yields were slightly impaired by the formation of DM1 homodimers. It should be noted that compound **12d** most likely exists as a mixture of two regioisomers, with the DM1 added either to the phosphonamidate moiety carrying the alkyne or the ethylene glycol. Afterwards, we used those constructs to synthesize trastuzumab-DM1 conjugates via the interchain disulfide bonds of the antibody. To do so, we applied a previously established one-pot reduction and alkylation protocol in the presence of TCEP.[24] Remarkably, we were able to generate very homogeneous ADCs, which mainly consist of only one fully modified DAR8 species, exemplified via HIC-chromatography and MS analysis after antibody reduction and deglycosylation (Supporting Information). With the four ADCs in hand, only differing in the linker structure, we conducted cell-cytotoxicity studies using a Her2-overexpressing cell line (SK-BR-3) and a Her2-negative cell line as a control (MDA-MB-468). While the unconjugated trastuzumab antibody only had a minor growth-inhibiting effect, all four trastuzumab-DM1 conjugates showed strong cytotoxicity with IC_{50} values of 9.3 ng mL^{-1} for trastuzumab-**12a**, 5.3 ng mL^{-1} for trastuzumab-**12b**, 10.1 ng mL^{-1} for trastuzumab-**12c**, and 7.9 ng mL^{-1} for trastuzumab-**12d** in the Her2-positive cell line (Figure 4, III) with no overt differences attributable to the different ADC-linkers. Those values are in the range of previously reported data from Kadcyla on the same cell-line.[40] Excellent

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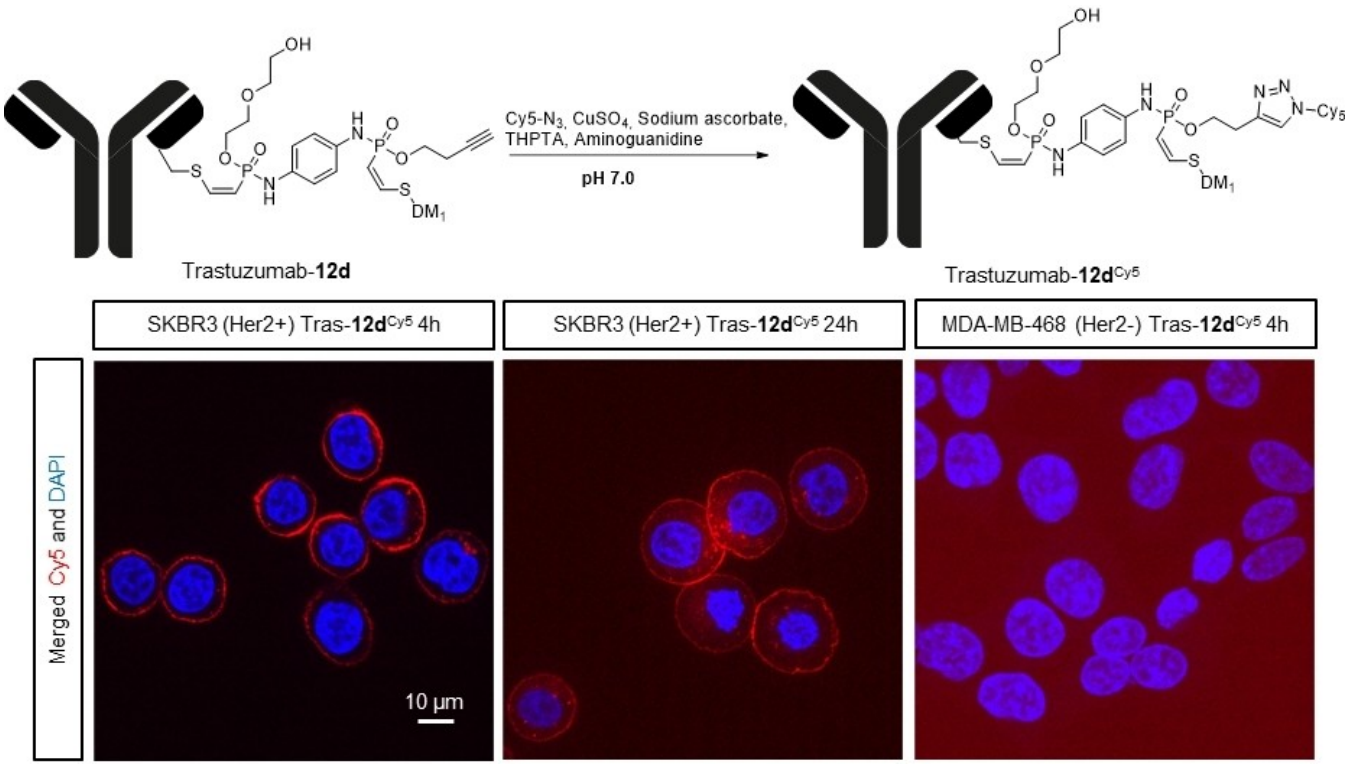

Figure 5. Further labeling of the ADC, synthesized from **12 d** with a fluorescent Cy5 label via CuAAC to construct an Antibody-Drug-Fluorophore-Conjugate (ADFC). To simplify the scheme, only one regioisomer of trastuzumab-**12 d** is shown. A molecule, where the trastuzumab is added to the phosphonamidate, carrying the alkyne and the DM1 to the phosphonamidate carrying the ethylene glycol is likewise possible. The labeled constructs have been used to demonstrate selective labeling and uptake of the ADFC into an antigen positive cell line. Membrane staining could be observed after 4 hours (left) and intracellular Cy5 after 24 h (mid). No cellular staining was observed on the antigen negative cell line (right).

target selectivity of the ADCs was further demonstrated by the lack of cytotoxicity on target-negative MDA-MB-468 cells.

To further demonstrate the versatility of the building-block **3 d**, we created an Antibody-Drug-Fluorophore-Conjugate (ADFC) from the ADC trastuzumab-**12 d** by performing a chemoselective triple conjugation modifying it via CuAAC with the azido modified fluorescent dye Cy5 (Figure 5). The addition of the Cy5 fluorophore allows the direct observation of ADC uptake into the targeted cell via fluorescence microscopy. In contrast to previously described investigations,[41] our method yields highly precise conjugates since neither drug nor fluorophore is conjugated via lysine residues. To demonstrate the functionality of our ADFCs, we incubated Her2 positive SKBR-3 cells with this construct and performed cellular imaging using spinning disk confocal microscopy. While mostly membrane staining was observed after 4 h, the ADC uptake could be directly visualized from the intracellular fluorescence after 24 hours. As expected, no fluorescent signal was observed on the non-targeted cell line (Figure 5). The same attachment site of drug and fluorophore in the ADFC, enabled by compound **3 d**, allows for direct observation of intracellular drug trafficking, even after ADC uptake and lysosomal degradation[42] and will be part of our future studies.

Conclusion

In summary, we developed novel bis-ethynylphosphonamidate building blocks suitable for a simple subsequent chemoselective conjugation of two thiols in a row. We demonstrated that the unique structure of the phosphonamidates facilitates the incorporation of hydrophilic diethylene glycol substituents that allow for the simple construction of protein-protein conjugates from two cysteine-containing proteins. Increasing the distance between the two ethynylphosphonamidate moieties by introducing a short ethylene glycol chain further simplifies the conjugation of two bulky proteins. Furthermore, we applied the building blocks to constructing ADCs from the thiol-containing antiproliferative drug DM1 and trastuzumab. In contrast to Kadcyla, an approved ADC built from the same antibody and drug, our conjugates exhibit an excellent homogeneity by full alkylation of eight possible cysteine sites. Finally, we applied an alkyne modified bis-ethynylphosphonamidate building block to a versatile chemoselective triple conjugation of an antibody, a drug, and a fluorophore, leading to an ADFC with great potential of studying the intracellular trafficking of a drug after intracellular ADC uptake.

Experimental Section

General Method for the conjugation of 3 to eGFP C70M S147C

eGFP C70M S147C has been expressed and purified as previously described.[3] Potential eGFP disulfide dimers, which might have been formed upon storage have been reduced before the reaction, as follows: An Eppendorf-tube was charged with eGFP C70M S147 in PBS (100 μL, 2.54 mg/mL, 91 μM) and a solution of DTT (1.41 μL, 0.65 M, 100 eq.) in TRIS buffer was added and the solution was incubated at 37 °C for 30 minutes. Excess DTT removal and buffer exchange to a solution containing 50 mM TRIS, 10 mM NaCl, 1 mM EDTA (pH 8.8) was conducted afterwards using 2 ml Zeba™ spin desalting columns with 7 K MWCO (Thermo Fisher Scientific, USA). Subsequently, 10.14 μL of the eGFP was transferred into Eppendorf-tubes. Afterwards, a solution of **3** in TRIS Buffer was added (1.74 mM, 0.5–100 eq., 0.26 μL–52.6 μL). The eGFP concentration was kept identical for all vials (15 μM), independently of the volume of linker added by the addition of appropriate volumes of TRIS buffer solution. Subsequently, the solution was incubated at 25 °C for 1 h or overnight.

General procedure for the addition of Ubiquitin G76C to bisphosphonamidate modified eGFP

Ubiquitin G76C has been expressed and purified as previously described.[20] Potential Ubiquitin disulfide dimers, which might have been formed upon storage have been reduced before the reaction, as follows: An Eppendorf-tube was charged with Ub G76C in PBS (1250 μL, 4.0 mg/mL, 455 μM) and a solution of DTT (87.81 μL, 0.65 M, 100 eq.) in TRIS buffer was added and the solution was incubated at 37 °C for 30 minutes. Excess DTT removal and buffer exchange to a solution containing 50 mM TRIS, 10 mM NaCl, 1 mM EDTA (pH 8.8) was conducted afterwards using 2 mL Zeba spin desalting columns with 7 K MWCO (Thermo Fisher Scientific, USA). After this step, the Ubiquitin solution in TRIS-buffer was added to the eGFP-**3** mono-conjugates (3.78 mg/mL, 426 μM, 1.37 μL–136.95 μL, 1–100 eq.). The eGFP concentration was kept identical for all samples (3 μM), independently of the volume of added Ub. Subsequently, the solution was incubated overnight at 25 °C.

General Method for the conjugation of the DM1 constructs 12 a–d to trastuzumab to achieve DAR8

50 μL of the trastuzumab solution of 10.0 mg/mL (66.6 μM) in P5-conjugation buffer (freshly prepared 100 mM NH_4HCO_3 pH 8.3) were mixed with 3.33 μL of a 10 mM TCEP solution in P5-conjugation buffer (10 eq.). Directly afterwards, 1 μL of a 40 mM solution of the DM1 constructs **12 a**–**d** dissolved in DMSO (1.5 eq. per free Cys) were added. The mixture was shaken at 350 rpm and 25 °C for 16 hours. The reaction mixtures were purified by preparative size-exclusion chromatography with a 25 ml Superdex™ 200 Increase 10/300GL (Cytiva, Sweden) and a flow of 0.8 ml/min eluting with sterile PBS (Merck, Germany). The antibody containing fractions were pooled and concentrated by spin-filtration (Amicon® Ultra- 2 mL MWCO: 30 kDa, Merck, Germany).

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Competing interest

The authors declare competing financial interests: A.V, I.M., J.H., D.S. and M.-A.K. are employers of Tubulis. C.P.R.H. is a co-founder and advisor of Tubulis. The technology described in the manuscript is part of a pending patent application by M.-A.K., D.S., J.H. and C.P.R.H.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: Alkynes · Antibodies · Conjugation · Drug delivery · Protein modifications

[1] P. Ochtrop, C. P. R. Hackenberger, *Curr. Opin. Chem. Biol.* **2020**, *58*, 28–36.
[2] J. M. J. M. Ravasco, H. Faustino, A. Trindade, P. M. P. Gois, *Chem. Eur. J.* **2019**, *25*, 43–59.
[3] M.-A. Kasper, M. Glanz, A. Stengl, M. Penkert, S. Klenk, T. Sauer, D. Schumacher, J. Helma, E. Krause, M. C. Cardoso, H. Leonhardt, C. P. R. Hackenberger, *Angew. Chem. Int. Ed.* **2019**, *58*, 11625–11630; *Angew. Chem.* **2019**, *131*, 11751–11756.
[4] S. B. Gunnoo, A. Madder, *ChemBioChem* **2016**, *17*, 529–553.
[5] P. M. S. D. Cal, G. J. L. Bernardes, P. M. P. Gois, *Angew. Chem. Int. Ed.* **2014**, *53*, 10585–10587; *Angew. Chem.* **2014**, *126*, 10758–10760.
[6] C. Canovas, P.-S. Bellaye, M. Moreau, A. Romieu, F. Denat, V. Goncalves, *Org. Biomol. Chem.* **2018**, *16*, 8831–8836.
[7] Y. Zhang, X. Zhou, Y. Xie, M. M. Greenberg, Z. Xi, C. Zhou, *J. Am. Chem. Soc.* **2017**, *139*, 6146–6151.
[8] A. F. L. Schneider, A. L. D. Wallabregue, L. Franz, C. P. R. Hackenberger, *Bioconjugate Chem.* **2019**, *30*, 400–404.
[9] T. Schlatzer, J. Kriegesmann, H. Schröder, M. Trobe, C. Lembacher-Fadum, S. Santner, A. V. Kravchuk, C. F. W. Becker, R. Breinbauer, *J. Am. Chem. Soc.* **2019**, *141*, 14931–14937.
[10] J. Bertran-Vicente, M. Penkert, O. Nieto-Garcia, J.-M. Jeckelmann, P. Schmieder, E. Krause, C. P. R. Hackenberger, *Nat. Commun.* **2016**, *7*, 12703.
[11] S. Yoshitake, Y. Yamada, E. Ishikawa, R. Masseyeff, *Eur. J. Biochem.* **1979**, *101*, 395–399.
[12] D. J. Betting, K. Kafi, A. Abdollahi-Fard, S. A. Hurvitz, J. M. Timmerman, *J. Immunol.* **2008**, *181*, 4131–4140.
[13] F. Dosio, P. Brusa, L. Cattel, *Toxin Rev.* **2011**, *3*, 848–883.
[14] M.-A. Kasper, M. Gerlach, A. F. L. Schneider, C. Groneberg, P. Ochtrop, S. Boldt, D. Schumacher, J. Helma, H. Leonhardt, M. Christmann, C. P. R. Hackenberger, *ChemBioChem* **2020**, *21*, 113–119.

[15] A. V. Yurkovetskiy, M. Yin, N. Bodyak, C. A. Stevenson, J. D. Thomas, C. E. Hammond, L. Qin, B. Zhu, D. R. Gumerov, E. Ter-Ovanesyan, A. Uttard, T. B. Lowinger, *Cancer Res.* **2015**, *75*, 3365–3372.
[16] M. J. Lobba, C. Fellmann, A. M. Marmelstein, J. C. Maza, E. N. Kissman, S. A. Robinson, B. T. Staahl, C. Urnes, R. J. Lew, C. S. Mogilevsky, J. A. Doudna, M. B. Francis, *ACS Cent. Sci.* **2020**, *6*, 1564–1571.
[17] C. Chatterjee, R. K. McGinty, B. Fierz, T. W. Muir, *Nat. Chem. Biol.* **2010**, *6*, 267.
[18] F. Meier, T. Abeywardana, A. Dhall, N. P. Marotta, J. Varkey, R. Langen, C. Chatterjee, M. R. Pratt, *J. Am. Chem. Soc.* **2012**, *134*, 5468–5471.
[19] R. Meledin, S. M. Mali, S. K. Singh, A. Brik, *Org. Biomol. Chem.* **2016**, *14*, 4817–4823.
[20] A. Baumann, S. Schwagerus, K. Broi, K. Kemnitz-Hassanin, C. Stieger, N. Trieloff, P. Schmieder, C. Hackenberger, *J. Am. Chem. Soc.* **2020**, *142*, 9544–9552.
[21] S. J. Walsh, J. D. Bargh, F. M. Dannheim, A. R. Hanby, H. Seki, A. J. Counsell, X. Ou, E. Fowler, N. Ashman, Y. Takada, A. Isidro-Llobet, J. S. Parker, J. S. Carroll, D. R. Spring, *Chem. Soc. Rev.* **2021**, *50*, 1305–1353.
[22] C. Wei, G. Zhang, T. Clark, F. Barletta, L. N. Tumey, B. Rago, S. Hansel, X. Han, *Anal. Chem.* **2016**, *88*, 4979–4986.
[23] B. Rago, L. N. Tumey, C. Wei, F. Barletta, T. Clark, S. Hansel, X. Han, *Bioconjugate Chem.* **2017**, *28*, 620–626.
[24] M.-A. Kasper, A. Stengl, P. Ochtrop, M. Gerlach, T. Stoschek, D. Schumacher, J. Helma, M. Penkert, E. Krause, H. Leonhardt, C. P. R. Hackenberger, *Angew. Chem. Int. Ed.* **2019**, *58*, 11631–11636; *Angew. Chem.* **2019**, *131*, 11757–11762.
[25] R. P. Lyon, J. R. Setter, T. D. Bovee, S. O. Doronina, J. H. Hunter, M. E. Anderson, C. L. Balasubramanian, S. M. Duniho, C. I. Leiske, F. Li, P. D. Senter, *Nat. Biotechnol.* **2014**, *32*, 1059.
[26] M.-A. Kasper, M. Glanz, A. Oder, P. Schmieder, J. P. von Kries, C. P. R. Hackenberger, *Chem. Sci.* **2019**, *10*, 6322–6329.
[27] M.-A. Kasper, M. Gerlach, A. F. L. Schneider, C. Groneberg, P. Ochtrop, S. Boldt, D. Schumacher, J. Helma, H. Leonhardt, M. Christmann, C. Hackenberger, *ChemBioChem* **2020**, *21*, 113.
[28] C. E. Stieger, L. Franz, F. Körlin, C. P. R. Hackenberger, *Angew. Chem. Int. Ed.* **2021**, *60*, 15359–15364.
[29] M. R. J. Vallée, L. M. Artner, J. Dernedde, C. P. R. Hackenberger, *Angew. Chem. Int. Ed.* **2013**, *52*, 9504–9508; *Angew. Chem.* **2013**, *125*, 9682–9686.
[30] O. Plietzsch, C. I. Schilling, T. Grab, S. L. Grage, A. S. Ulrich, A. Comotti, P. Sozzani, T. Muller, S. Bräse, *New J. Chem.* **2011**, *35*, 1577–1581.
[31] Z. Jaafar, S. Chniti, A. Ben Sassi, H. Dziri, S. Marque, M. Lecouvey, R. Gharbi, M. Msaddek, *J. Mol. Struct.* **2019**, *1195*, 689–701.
[32] Y. Wang, D. Wang, C. Xu, R. Wang, J. Han, S. Feng, *J. Organomet. Chem.* **2011**, *696*, 3000–3005.
[33] P. M. LoRusso, D. Weiss, E. Guardino, S. Girish, M. X. Sliwkowski, *Clin. Cancer Res.* **2011**, *17*, 6437–6447.
[34] G. D. Lewis Phillips, G. Li, D. L. Dugger, L. M. Crocker, K. L. Parsons, E. Mai, W. A. Blättler, J. M. Lambert, R. V. J. Chari, R. J. Lutz, W. L. T. Wong, F. S. Jacobson, H. Koeppen, R. H. Schwall, S. R. Kenkare-Mitra, S. D. Spencer, M. X. Sliwkowski, *Cancer Res.* **2008**, *68*, 9280–9290.
[35] L. Chen, L. Wang, H. Shion, C. Yu, Y. Q. Yu, L. Zhu, M. Li, W. Chen, K. Gao, *mAbs* **2016**, *8*, 1210–1223.
[36] S. M. Ansell, *Blood* **2014**, *124*, 3197–3200.
[37] S. O. Doronina, B. E. Toki, M. Y. Torgov, B. A. Mendelsohn, C. G. Cerveny, D. F. Chace, R. L. DeBlanc, R. P. Gearing, T. D. Bovee, C. B. Siegall, J. A. Francisco, A. F. Wahl, D. L. Meyer, P. D. Senter, *Nat. Biotechnol.* **2003**, *21*, 778–784.
[38] T. H. Pillow, J. Tien, K. L. Parsons-Reponte, S. Bhakta, H. Li, L. R. Staben, G. Li, J. Chuh, A. Fourie-O'Donohue, M. Darwish, V. Yip, L. Liu, D. D. Leipold, D. Su, E. Wu, S. D. Spencer, B.-Q. Shen, K. Xu, K. R. Kozak, H. Raab, R. Vandlen, G. D. Lewis Phillips, R. H. Scheller, P. Polakis, M. X. Sliwkowski, J. A. Flygare, J. R. Junutula, *J. Med. Chem.* **2014**, *57*, 7890–7899.
[39] S. L. Kuan, T. Wang, T. Weil, *Chem. Eur. J.* **2016**, *22*, 17112–17129.
[40] T. T. Junttila, G. Li, K. Parsons, G. L. Phillips, M. X. Sliwkowski, *Breast Cancer Res. Treat.* **2011**, *128*, 347–356.
[41] S. Knutson, E. Raja, R. Bomgarden, M. Nlend, A. Chen, R. Kalyanasundaram, S. Desai, *PLoS One* **2016**, *11*, e0157762–e0157762.
[42] S. C. Alley, N. M. Okeley, P. D. Senter, *Curr. Opin. Chem. Biol.* **2010**, *14*, 529–537.

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