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RESEARCH ARTICLE

Dichloro Butendiamides as Irreversible Site-Selective Protein Conjugation Reagent

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Abstract: We describe maleic-acid derivatives as robust cysteine-selective reagents for protein labelling with comparable kinetics and superior stability relative to predominantly used maleimides. Diamide and amido-ester derivatives proved to be efficient protein-labelling species with a common mechanism in which a spontaneous cyclization reaction occurs upon addition to cysteine. Introduction of chlorine atoms in their structures triggers ring hydrolysis or further conjugation with adjacent residues, which results in conjugates that are completely resistant to retro-Michael reactions in the presence of biological thiols and human plasma. By controlling the microenvironment of the reactive site, we can control selectivity towards the hydrolytic pathway, forming homogeneous conjugates. The method is applicable to several scaffolds and enables conjugation of different payloads. The synthetic accessibility of these reagents and the mild conditions required for fast and complete conjugation together with the superior stability of the conjugates makes this strategy an important alternative to maleimides in the bioconjugation toolbox.

Over the last decade protein site-selective conjugation has become a thriving research field.[1] A plethora of methods have been reported for the construction of protein conjugates with a wide array of applications in chemical biology and medicine.[2] Cysteine[3] (Cys) and lysine[4] (Lys) remain the main target residues in protein modification although recently, methodologies that target alternative residues, such as aspartic/glutamic acid,[5] tryptophan,[6] methionine,[7] serine[8] or tyrosine[9] have been described. Cys-targeting methods are particularly ubiquitous, as they yield structurally homogeneous conjugates in proteins that contain native[10] or genetically engineered free Cys.[11] Multiple strategies have been reported for Cys-selective conjugation, usually based on alkylation[12]/arylation[13] reagents or Michael acceptors.[14] Michael acceptors, particularly maleimides, remain the most commonly used reagent for the construction of conjugates for biological applications[15] because of their associated fast reaction kinetics. In fact, a number of Food and Drug Administration approved conjugates, such as antibody-drug conjugates (ADCs) Brentuximab vedotin[15] and Trastuzumab emtansine[16] or PEGylated conjugate Certolizumab pegol,[17] contain a thio-succinimide adduct derived from maleimide conjugation.[18]

However, it is well known that thio-succinimide adducts can undergo fast and uncontrolled disruptive cleavage by thiol-exchange in plasma, which ultimately compromises the safety and efficacy of the conjugate.[19] Considerable efforts have been reported on how to increase the stability of maleimide-based constructs.[20] The most common approach consists of hydrolyzing the thio-succinimide linkage to form a stable, linear thio-succinamic acid species.[21] Different strategies to control the hydrolysis kinetics have been described. For instance, the addition of electron-withdrawing groups in the payload,[22] tuning the amino-acid environment near the inserted Cys, [21a] or mild ultrasonication[23] can accelerate thio-succinimide hydrolysis. Alternatively, maleimides with different leaving groups attached to the vinylic bond or similar structures have been reported to form conjugates with higher stability based on maleimide or maleamic acid linkages.[24]

There are also acyclic reagents, such as benzoyl acrylic reagents[11, 25] or fumarate derivatives.[26] They produce conjugates with linear linkages, which can present enhanced

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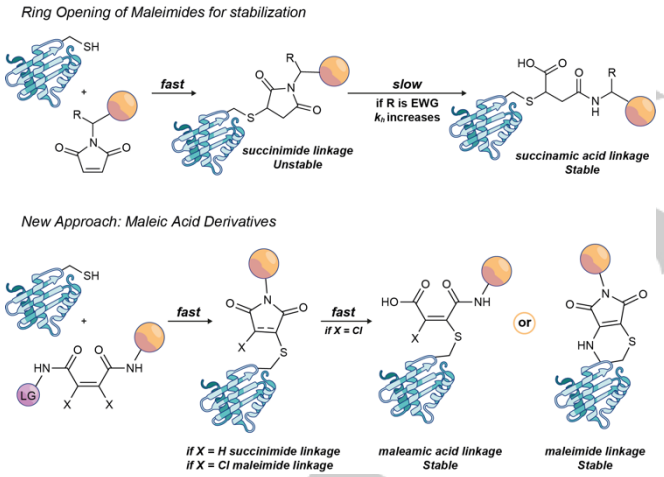
stability in plasma relative to maleimides.[11, 25] Despite these advances, most of these strategies fail to combine fast conjugation kinetics along with the formation of stable conjugates, which is required for biological applications, and where maleimides remain the first choice. The challenge remains to find a general, simple alternative for Cys bioconjugation with comparable kinetics and synthetic accessibility to maleimides, but complete linkage stability.

We hereby report on diamide and amido esters derivatives from maleic acid as new, efficient reagents for Cys-selective protein conjugation. These reagents undergo fast Michael addition reactions with free Cys containing proteins in stoichiometric amounts under physiological conditions. Conjugation occurs through an unprecedented mechanism in which, upon Cys addition, spontaneous cyclization is promoted by attack of an amide moiety to the distal carbonyl group to form a succinimide link (**Scheme 1**). By expanding this approach to 2,3-dichloro but-en-diamides, we can selectively target free Cys residues that form chloro-maleimide intermediate linkages, which quickly undergo hydrolysis. The final conjugates are fully resistant to thiol-exchange and cleavage in the presence of biological thiols and human plasma. Through this approach an array of free Cys containing proteins could be irreversibly tagged, such as ubiquitin (Ub), human serum albumin (HSA) or different antibody fragments, with different tags including azide or alkyne functionalities. The favourable kinetics, stability and stoichiometry of the conjugation makes a flexible platform to access complex constructs, such as ADCs, which is an advantage over currently existing platforms.



Scheme 1. Synthesis of conjugates with linear succinamic acid linkages.

With the goal of finding alternative approaches to access homogeneous conjugates with fully stable linkages in free Cys-containing proteins, we set out to investigate maleic acid derivatives with labile functionalities, which we envisioned could undergo fast Michael addition with thiols under physiological conditions and, upon conjugation, would hydrolyze to form succinamic acid structures or equally stable analogs.

The reactivity of β-mercaptoethanol (BME) was evaluated with different maleic acid derivatives (**1**–**10**, **Figure 1, Conditions A, Figures S3–S6**) to assess their reactivity as Michael acceptors. Having determined the reactivity of **1**–**10** in small molecule experiments, we now exposed these reagents to the presence of a free Cys-containing protein (**Figure 1, Conditions B, Figures S25–S31**). As a model protein we chose Ub, which was engineered with a reactive Cys at position 63 (K63C). Surprisingly, the reactivity of the different species varied depending on the setup. Some species showed high reactivity in small-molecule reactions but no protein conjugation reactivity, whereas others proved to be very efficient protein conjugation reagents but were unreactive in a small molecule setup. In successful conjugations, Cys-selectivity was confirmed after digestion by MS/MS studies (**Figure S82–S83**) and by using Ellman's reagent as a chemical control (**Figure S32**).

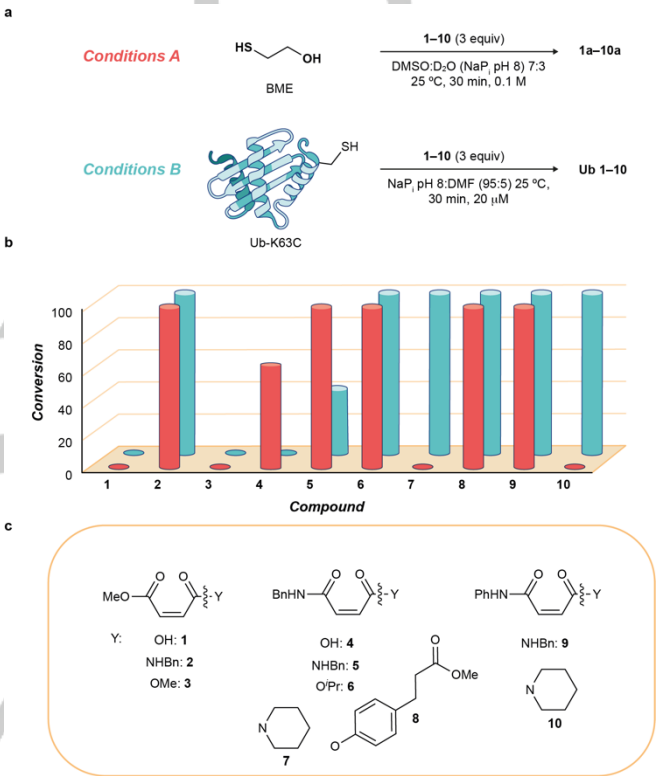


Figure 1. Small molecule and Ub reaction with different maleic acid derivatives. DMF = dimethyl formamide.

Mass analysis of the protein conjugates formed revealed a unique conjugate with a mass of 8754 Da (protein mass + 188 Da) for all the examples. Interestingly, the mass of the newly formed conjugate did not correspond to the addition of the full reagent. Instead, we observed the formation of a thio-succinimide linkage. The origin of such a linkage is through intramolecular attack of the amide to the distal carbonyl group (**Scheme 2a**), which can happen prior or after Cys conjugation. Similar cyclizations are known although never triggered by aqueous solution or thiol conjugation.[27] Furthermore, the cyclization step seems to play a key role in the viability of the conjugation, because only compounds with at least one secondary amide, capable of driving such a cyclization, underwent conjugation. To gain better mechanistic insight, compounds **2**, and **5**–**10** were stirred for 2 h at 25 °C in DMSO/D_2O (NaP_i pH 8, 50 mM) to determine if the cyclization was induced as a result of the pH of the solvent or the thiol addition. Amido esters **2** and **8** cyclized spontaneously in the

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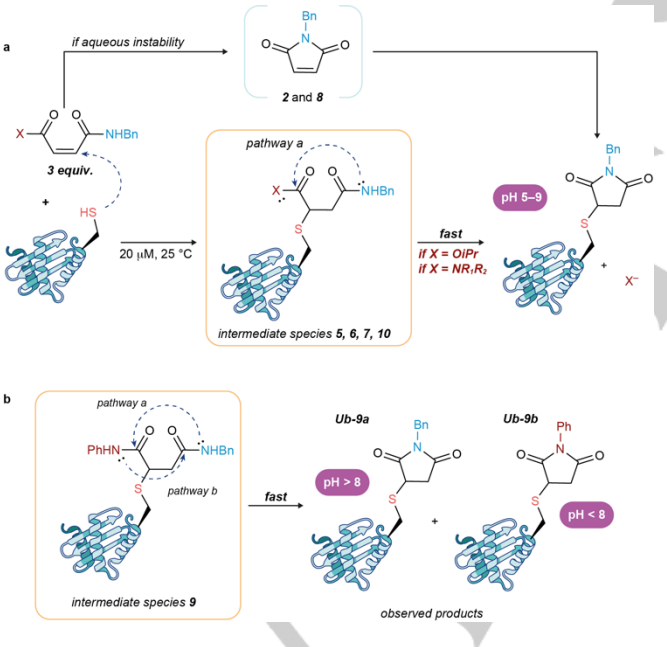
presence of D_2O pH 8 to form a maleimide species (**Figures S1–S2**). However, this was not the general behaviour for amido esters; compound **6** together with diamides **5, 7, 9** and **10** were completely stable under these conditions. This aqueous stability supports the idea that thiol addition in the protein environment is responsible for triggering the cyclization. By using diamide **10** and Ub as a model, the reaction showed great tolerance for pH [5–9, **Figures S33–S37**)] and temperature [25–37 ºC (**Figures S31 and S36**)].

When a reagent with two different secondary amide groups, such as **9**, is conjugated, a regioselectivity issue arises. The presence of two secondary amide moieties allows for two possible cyclizations each driven by one of the amides (**Scheme 2b**), each pathway leads to a different conjugate: **9a** or **9b**. The preferred pathway could be controlled by the pH at which the reaction was done. Under slightly acidic conditions, pH 6–7, the aryl amide group directed cyclization was favored, whereas under basic conditions, pH 8–9, the opposite selectivity was observed (**Figures S38–39**).

Another appealing aspect of this conjugation reaction is the controlled release of small molecules in a particular microenvironment of the protein sequence. Upon cyclization, an amine (diamides) or alcohol (amido-esters) molecule is released, which could open new approaches for monitoring reactions (release of fluorophores), Cys-targeted decaging of payloads or site-directed bifunctionalization of proteins. We are currently investigating this directed release in our laboratories and will be reported in due course.



Scheme 2. Regioselectivity of the cyclization step with compound **9**.

The thio-succinimide linkages obtained are known to hydrolyze over time under physiological conditions to give a linear and stable *N*-substituted thio-succinamic acid linkage, but the kinetics of hydrolysis is extremely slow and often yields mixtures of conjugates. Harsh hydrolyzing conditions, such as high pH and temperature, could ultimately lead to the complete hydrolysis to succinamic acid linkages, but such conditions are not compatible or desirable with proteins because they can result in unfolding and loss of functional properties. To address this problem, we focused on accelerating the hydrolysis of the linkages created by the conjugation of maleic acid diamides.

Aqueous instability of dichloro maleimides[24c] suggests that hydrolysis kinetics of maleimides can be exponentially increased by the introduction of halogens into the ring. This led us to think that, by introducing chlorine substituents into the alkene scaffold of maleic acid diamides, the stability of the compounds and their reactivity would not be affected, although now instead of obtaining conjugates with succinimide linkages, chloro-maleimide linkages would be generated, which would quickly hydrolyze to stable linear chloro maleamic acid species. By following reported procedures[28] we synthesized symmetrical di-chloro butendiamides (**11**–**14, Figure 2a**) and, to our delight, their reactivity still included a cyclization step which yielded a cyclic chloro-maleimide linkage with enhanced hydrolysis kinetics. Addition of **11** (3 equiv) to a solution of Ub resulted in complete conjugation (**Figure 2**) after 30 min at 25 ºC. The cyclic intermediate was observed if the reaction mixture was analyzed shortly after addition of the reagent (**Figure S71**). No trace of cyclic maleimide linkage and only linear chloro maleic acid linkage was noted after 30 min at different temperatures (25–37 ºC) and pH [pH 5–9 (**Figures S67–S70**)], which further supports the hypothesis of rapid hydrolysis rates of the linkage. The mechanism associated with this conjugation is similar to that of dibromomaleimides[23] in that the linkages obtained are similar, but an additional cyclization step and much more favourable hydrolysis kinetics, makes this approach advantageous to obtain homogenous, stable maleamic acid linkages.

The generality of the method was demonstrated by conjugation of **7** and **14** to alternative free Cys containing proteins. Under similar conditions, HSA, the N-terminal domain of phage repressor R434 and a camelian nanobody targeted at amyloid precursors (HET nanobody)[29] were effectively conjugated (**Figures S40–S42**) to **7**. In all examples Michael addition/cyclization mechanism to yield a succinimide was observed, which supports the generality of the mechanism in different protein sequence microenvironments. Analysis of the circular dichroism (CD) profiles of the conjugates relative to native proteins confirmed that overall tertiary structure of the proteins was maintained throughout conjugation (**Figures S85–S86**).

An array of di-chloro but-en-diamide analogs with different functionalities were synthesized (compounds **11**–**14**). Alyphatic **11** and aromatic **12** amide species could be accessed as well as amides with an incorporated alkyne **13** or azide **14** functionality. These new functionalities were incorporated into the protein structure to enable further functionalization through copper(I)-catalyzed alkyne-azide cycloadditon. Compounds **12–14** showed similar reactivity to **11** upon reaction with Ub to form stable, linear chloro maleamic acid linkages (**Figures S44–S46**). However, when compound **14** was tested with different proteins, upon conjugation-cyclization, different reactivity was observed for some of these proteins. In Ub, C2Am[30] or peptide ASCATN the expected sequential Michael addition-cyclization-hydrolysis occurs to give a final conjugate with a chloro-maleamic acid linkage. When the scope was expanded and proteins like HSA, H3K4C or a full-length IgG antibody Gemtuzumab, a second alternative pathway, in which a second Michael addition from an adjacent residue takes place, occurs instead of the hydrolysis step (**Figure 2b**). This alternative pathway forms cyclic maleimide

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linkages with two coordination sites to the protein. Both linkages cannot undergo retro-Michael addition and form stable conjugates, which was demonstrated by analyzing the stability of **Ub-14**; the conjugate remained stable in buffer, and no degradation was observed after 48 h at 37 ºC in glutathione (1 mM) or human plasma (**Figures S73–S74**).

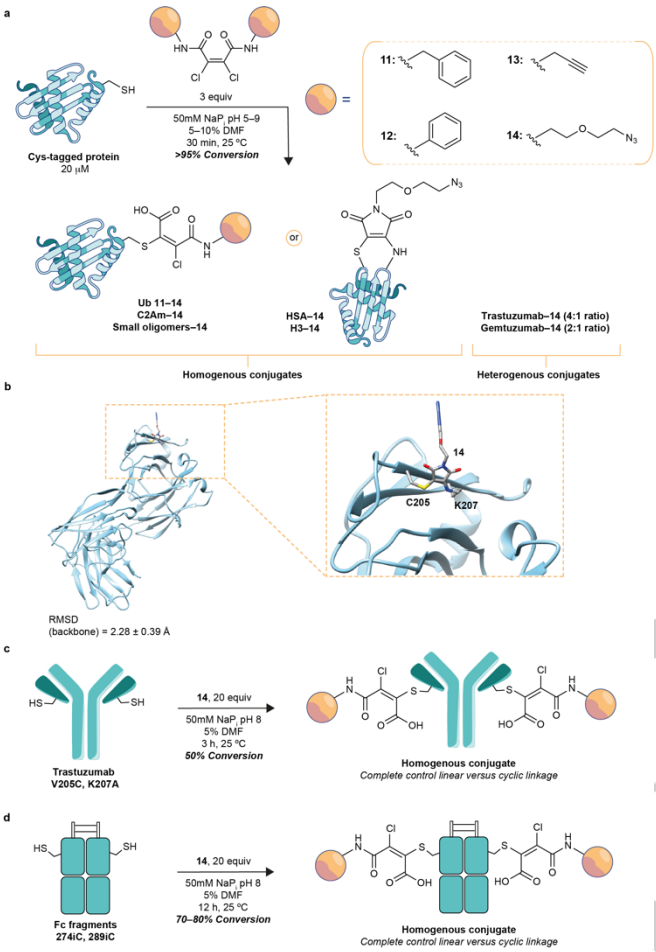

Figure 2. a, Conjugation reaction of reagents **11**–**14** with different Cys-containing proteins. **b**, Representative snapshot derived from 0.5 μs MD simulations on conjugate HER2-**14.** The RMSD value of the protein, relative to the first frame, is also shown. **c**, Conjugation reaction of Trastuzumab V205C, K207A with **14**. **d**, Conjugation reaction of Fc fragments 274iC and 289iC with **14**.

The enhanced plasma stability displayed by the conjugates formed from the reaction of Cys with reagents **11**–**14** has potential for the construction of stable ADCs. However, because two alternative linkages can be formed, this could constitute an issue regarding homogeneity of the final construct. Next, we analysed the ratio between cyclic and linear conjugates using LCMS. The conjugate derived from Gemtuzumab V205C showed a mixture of species, [ratio 2:1 cyclic vs linear (**Figure S53**)], but all others formed selectively one of the two potential linkages. Conjugation of HER2 targeted IgG Trastuzumab V205C with **14** led to a similar result (ratio 4:1, **Figure S54**).

To explain this result at the atomic level, we performed molecular dynamics (MD) simulations on the Fab fragment of HER2 conjugated to compound **14** (**Figures 2b** and **S87**) and examined the distance from all lysine residues to the reactive carbon (C–Cl). According to these calculations, lysine at position 207 had the smallest average distance and was presumably the residue that directed the second nucleophilic attack to form the cyclic derivative. The simulations indicated also that the incorporation of this cyclic derivative into Fab does not significantly disrupt its structure (**Figure 2b**). The involvement of K207 in the formation of the cyclic linkage was further confirmed by MS/MS studies on Trastuzamab V205C-**14** (**Figure S84**). By mutating lysine 207 into an alanine, the new Trastuzumab V205C+K207A was conjugated to **14** and this time complete selectivity to the linear linkage was obtained (**Figure S55**). This result supported the idea that nearby lysines residues were the driving factors for the formation of the cyclic linkage, allowing us to predict the final linkage formed and if mixtures were to arise, avoid the formation of the cyclic species by introducing cysteines in the appropriate place or mutating the problematic lysine residues. We found, however, that mutation of lysine 207 in Trastuzumab V205+K207A reduces the nucleophilicity of cysteine 205 and only ~50% conjugation is achieved under the previously described conditions (**Figure S55**).

To find suitable positions to genetically encode a reactive Cys that allow complete conversion, yet selective formation of the stable linear linkage, we produced Fc fragments of an IgG with Cys insertions at different positions in the absence of nearby Lyz residues (239iC, 268iC, 274iC, 289iC and 442iC). Under identical reaction conditions with **14**, the Fc fragments with 274iC and 289iC reacted completely and selectively formed the linear linkage (**Figures S56–S60**). These simple maleic acid derivatives enable installation of modifications on antibodies at specific sites to form homogenous and stable antibody-conjugates.

To compare the kinetics of conjugation between maleic acids and maleimides, an equimolar mixture of **11** and its analogous maleimide reagent were conjugated to Fc fragment 289iC. Under the same conditions, **11** and maleimide showed comparable conjugation kinetics as a 1:1 mixture of Fc-**11** and Fc-maleimide constructs were obtained (**Figure S81**). However, when we compared the stability of the constructs in the presence of GSH (1mM) after 66 h at 37 ºC, the constructs (trastuzumab-**11**, Fc 274iC-**11**, Fc 289iC-**11**) conjugated to **11** showed higher stability than the corresponding species conjugated with maleimide (**Figures S75–S80**). While the conjugates formed with reagent **11** remain intact in the presence of a thiol, constructs built using maleimides form a mixture of hydrolyzed maleimide and unconjugated antibody. This data shows a key advantage of our reagents to build stable conjugates and illustrates how enhanced hydrolysis kinetics of the cyclic linkages allows the formation of fully stable species.

In summary, we describe the protein conjugation reactivity of a series of maleic acid derivatives and identify amido esters and diamides as viable conjugation reagents to produce succinimide linkages through a new stepwise mechanism. The strategy was optimized to use dichloro butendiamides, which promote a further hydrolysis step to form completely stable linkages to proteins. This latter species shows comparable conjugation kinetics relative to maleimides yet benefits from superior stability. The general mechanism described composes a Michael addition followed by spontaneous cyclization to form cyclic linkages similar to those obtained by conjugation to maleimides. The presence of chlorine atoms in these cyclic linkages promotes subsequent hydrolysis or to give completely stable linkages. Nearby nucleophilic residues can promote an alternative mechanism

where a second nucleophilic attack leads to the formation of a cyclic linkage, but this pathway can be controlled and depleted if undesired conjugate mixtures are obtained. The approach allows for the creation of conjugates with azide/alkyne moieties and opens up possibilities to create tailored conjugation for a number of relevant proteins, including nanobodies or IgGs relevant for the formation of ADCs.

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Keywords: bioconjugation • cysteine • irreversible • maleimides • Michael addition

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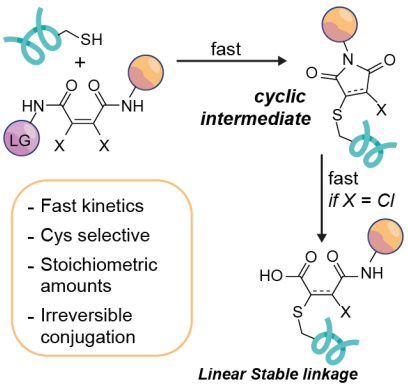

Tag It Irreversibly. We describe maleic acid derivatives as cysteine-selective reagents for protein labelling with comparable kinetics and superior stability to existing reagents. A novel mechanism is described to readily access stable chloro-maleamic acid. The mild conditions, fast kinetics and irreversible conjugation make this method an appealing alternative to existing cysteine-targeted methods.

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