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Chemistry–A European Journal

Research Article
doi.org/10.1002/chem.202303242

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Hot Paper

Site-Selective Protein Conjugation by a Multicomponent Ugi Reaction

Ilias Koutsopetras,[a] Valentine Vaur,[a] Rania Benazza,[b, c] Hélène Diemer,[b, c] Charlotte Sornay,[a] Yağmur Ersoy,[d] Léa Rochet,[e] Carmen Longo,[d] Oscar Hernandez-Alba,[b, c] Stéphane Erb,[b, c] Alexandre Detappe,[f] Arne Skerra,[d] Alain Wagner,[a] Sarah Cianferani,[b, c] and Guilhem Chaubet*[a]

The chemical bioconjugation of proteins has seen tremendous applications in the past decades, with the booming of antibody-drug conjugates and their use in oncology. While genetic engineering has permitted to produce bespoke proteins featuring key (un−)natural amino acid residues poised for site-selective modifications, the conjugation of native proteins is riddled with selectivity issues. Chemoselective strategies are plentiful and enable the precise modification of virtually any residue with a reactive side-chain; site-selective methods are less common and usually most effective on small and medium-sized proteins. In this context, we studied the application of the Ugi multicomponent reaction for the site-selective conjugation of amine and carboxylate groups on proteins, and antibodies in particular. Through an in-depth mechanistic methodology work supported by peptide mapping studies, we managed to develop a set of conditions allowing the highly selective modification of antibodies bearing N-terminal glutamate and aspartate residues. We demonstrated that this strategy did not alter their affinity toward their target antigen and produced an antibody-drug conjugate with subnanomolar potency. Excitingly, we showed that the high site selectivity of our strategy was maintained on other protein formats, especially on anticalins, for which directed mutagenesis helped to highlight the key importance of a single lysine residue.

Introduction

Selectivity is an essential aspect of chemical reactivity. In the course of a reaction leading to the possible formation of several structurally related products, selectivity can be defined as the preferred formation of one over the others. This can take on several forms: favoring one isomer over another (i.e., stereoselectivity) or one reactive chemical group over another (i.e., chemoselectivity) and, if multiple copies of this chemical group are present on the molecule, favoring one site – one position – over all the others (i.e., regioselectivity).[1–6] Since a lack in selectivity may result in complex mixtures of unwanted products, the art of chemical synthesis relies on the development of reaction conditions that will maximize the sole formation of the desired compound. While this can be a challenging task, small molecules possess a limited number of reactive sites. Coupled with a wide range of reaction conditions and reagents available to the experimenter – a plethora of organic solvents, different techniques (e.g., electrochemical, photoredox, air-free), a temperature scale spanning ~400 °C, use of extremely reactive chemicals (strong oxidants/reducers, acids/bases), etc. –, several approaches can be used to address selectivity issues in classical synthetic chemistry. However, this diversity in tunable reaction parameters shrinks when transitioning to the field of chemical biology and protein conjugation, most notably. Indeed, handling proteins comes with several constraints: not only the molecules are far bigger in size, thus increasing the number of potential reactive sites, but their sensitivity imposes a narrower liberty of action to the chemobiologist: water is the solvent by default, with only limited amounts of a handful of miscible organic co-solvents being tolerated; the temperature scale is more restricted, usually from 4 °C to 37 °C; the choice of reagents and techniques is limited by both the fragility of biomolecules and the aqueous environment. However, despite these restrictions – or thanks to them, constraints being "conductive to creativity"–,[7] numerous chemo- and site-selective approaches have been developed over the past decades for the chemical conjugation of all sorts of

[a] I. Koutsopetras, V. Vaur, C. Sornay, Dr. A. Wagner, Dr. G. Chaubet
Bio-Functional Chemistry (UMR 7199), LabEx Medalis, University of Strasbourg, 74 Route du Rhin, 67400 Illkirch-Graffenstaden, France
E-mail: chaubet@unistra.fr

[b] R. Benazza, H. Diemer, Dr. O. Hernandez-Alba, S. Erb, Dr. S. Cianferani
Laboratoire de Spectrométrie de Masse BioOrganique (LSMBO), Université de Strasbourg, CNRS, IPHC UMR 7178, 67000 Strasbourg, France

[c] R. Benazza, H. Diemer, Dr. O. Hernandez-Alba, S. Erb, Dr. S. Cianferani
Infrastructure Nationale de Protéomique ProFI – FR2048, 67087 Strasbourg, France

[d] Y. Ersoy, C. Longo, Prof. Dr. A. Skerra
Lehrstuhl für Biologische Chemie, Technische Universität München, Emil-Erlenmeyer-Forum 5, 85354, Freising, Germany

[e] L. Rochet
Department of Chemistry, University College London, London, UK

[f] Prof. A. Detappe
Institut de Cancérologie Strasbourg Europe, Strasbourg, France

Supporting information for this article is available on the WWW under https://doi.org/10.1002/chem.202303242

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proteins. On the chemoselective front, lysine and cysteine are the most studied residues, due to both the solvent accessibility and high nucleophilic character of their side-chain functional groups, for which a plethora of strategies and reagents has been developed.[8] For α- and ε-amino groups, their conjugation relies essentially on 1,2-nucleophilic addition to reactive carbonyls and activated esters in particular, as best epitomized by *N*-hydroxysuccinimide esters, the reference standard of lysine conjugation.[9] For thiols, most commonly used strategies employ either 1,4-nucleophilic addition – e.g., to maleimides – or direct nucleophilic substitution of alkyl halides – e.g., iodoacetamide.[10,11] Besides these two residues, several other conjugation methods have been developed to target other reactive functionalities, resulting in chemoselective strategies for almost all proteinogenic α-amino acids equipped with a reactive side-chain.[12–22] With the aim of minimizing the heterogeneity of chemoselective approaches – especially in the case of lysine conjugation, where dozens of copies of the residue are present at the surface of the protein –, site-selective, *viz.* regioselective, methods have also been developed.[23] Focusing strictly on chemical approaches for the conjugation of natural and native proteins, chemists addressed this challenge in different ways: by relying on kinetic control and template-directed approaches;[24–27] by developing bespoke reagents;[28,29] or by taking advantage of key reactivity and structural features, as it is the case for *N*-terminus-selective strategies.[30–34] The majority of these techniques have been reported in the last two decades, emphasizing how flourishing the field is and how proteins are seen as an exciting playground for synthetic chemists seeking new challenges. In this context, we previously reported the use of multicomponent reactions for the conjugation of trastuzumab, a therapeutic monoclonal antibody (mAb) belonging to the family of immunoglobulins G and currently used in clinics to fight HER2-positive breast cancer.[35] Due to their large size – 150 kDa on average –, mAbs are inherently more complicated to conjugate in a site-selective manner than their smaller fragments (e.g., F(ab')$_2$, Fab, scFv, V$_H$H) via a chemical approach. Our strategy arose from the observation that almost all conjugation reactions concentrate on single-residue modification and that strategies targeting two residues concomitantly were scarce in comparison. However, such an approach could drastically reduce the number of potential conjugation sites and hence increase our chances to develop a site-selective strategy. In this regard, we decided to evaluate the Ugi four-center three-component reaction (U-4C-3CR) as a new conjugation tool. We managed to demonstrate that this approach was applicable to various carbonyl-isocyanide combinations, and showed in particular that the addition of aldehyde **1a** and cyclohexyl isocyanide **2a** to a solution of trastuzumab in PBS led to the cross-conjugation of lysine K126 and either aspartate D122 or glutamate E123 (undistinguishable, Figure 1). However, this "inter-residue" U-4C-3CR was also accompanied with competing single-residue modifications, involving aspartate/glutamate – whose conjugation was caused by the mechanistically related tricomponent Passerini reaction – and *N*-terminal E1 and D1 residues, labelled after an "intra-residue" U-4C-3CR involving their α-amine as the amine component instead of the ε-amine of a lysine residue. Even though six conjugation sites ended up being detected under the conditions employed, far from the site-selectivity we had initially imagined, this unexpected *N*-terminus reactivity urged us to delve into an in-depth study of this multicomponent reaction, in the hope of identifying key parameters that could favor one site and/or one of the two competing multicomponent reactions. In order to do so, we opted for the following protocol: trastuzumab conjugates would first be produced through multicomponent conjugation with varying parameters (i.e., nature of reagents, pH, temperature, reaction time, concentration, buffer, co-solvent) before being analyzed by native mass spectrometry – to determine both the conversion, defined as the percentage of conjugated protein, and the average degree of conjugation (avDoC), defined as the average number of label per protein – and LC/MS-MS after their tryptic digestion, to identify the conjugation sites by peptide mapping. In cases where differences in molecular weights between all protein species would be too small after the Ugi conjugation, a second functionalization step by strain-promoted azide-alkyne cycloaddition (SPAAC) would be used to increase the molecular weight of the molecular labels, thereby necessitating the presence of an azide group on either the aldehyde or the isocyanide component.

Results & Discussion

We first embarked upon this methodological work by selecting our previously developed model conditions – i.e., trastuzumab (10 mg/mL), aldehyde **1a** (45 equiv.), cyclohexyl isocyanide **2a** (45 equiv.), DMSO/PBS 1X (7:93, v/v), pH 7.5, 25 °C, 16 h – in order to validate the repeatability and reproducibility of our initial results. While no issue was encountered during this first step, the subsequent SPAAC with strained alkyne **BCN-Cy5** (20 equiv., 16 h, 25 °C, same solvent and pH as for the conjugation step) was often accompanied with partial loss of the resulting conjugates. We attributed this issue to the aromatic nature of the cyanine-5 fluorophore, getting potentially adsorbed onto the tube's walls. This variation in yield also translated to marked fluctuations in conversion and average degree of conjugation, conjugates with higher degrees of conjugation (DoC) being presumably more prone to adsorption. This is best depicted by the scatter plot on the top left-hand corner in Figure 1, representing the results of 14 replicates of this reaction conducted by one experimenter (CS) and showing a broad distribution of avDoC (black dots) and conversion values (grey dots) – the colored dot indicates the only sample analyzed by peptide mapping, with the identified conjugation sites listed in the yellow box (in bold for Ugi sites, in italics for Passerini sites). While the conversion and avDoC values should only depend on the first conjugation step, any issue with the subsequent SPAAC will inevitably lead to a loss of information about the exact nature and number of conjugation sites and thus to a lack of repeatability.

The first parameter we tweaked was thus the structure of the molecule borne by the cycloalkyne scaffold, and selected

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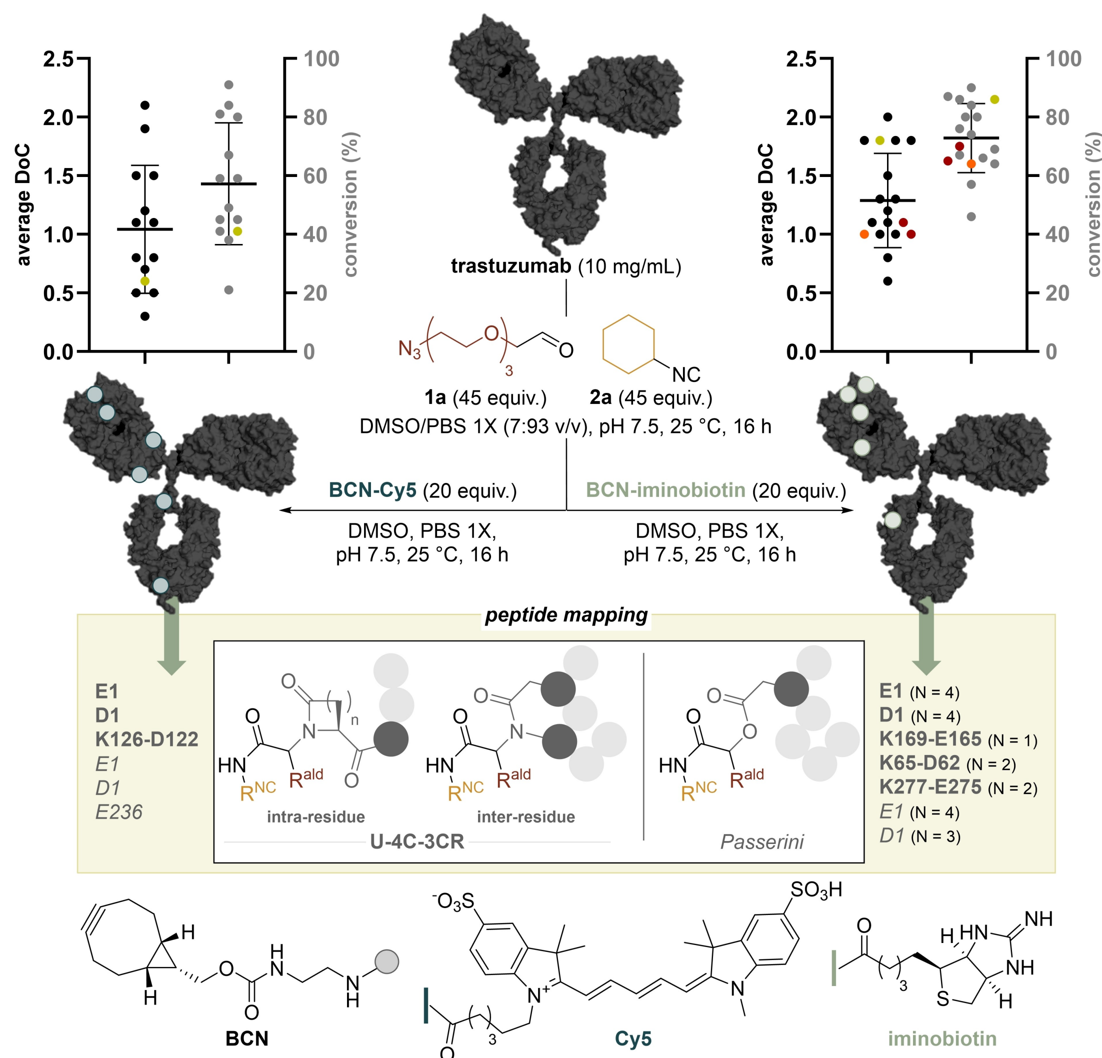

Figure 1. Overview of the multicomponent conjugation of trastuzumab using azide-containing aldehyde 1 a and cyclohexyl isocyanide 2 a followed by SPAAC with two different BCN reagents. The reproducibility of these two-step procedures (N = 14 for BCN-Cy5, N = 18 for BCN-iminobiotin) is given in the form of scatter plots with mean and standard deviations; the samples analysed by peptide mapping are highlighted in colours (i.e.: • = CS; • = VV; • = IK). The structures of the three adducts formed by either U-4C-3CR or Passerini reactions are given; the identity of the conjugation sites (in bold for U-4C-3CR, in italics for Passerini reaction) are listed, as well as the number of replicates (N).

BCN-iminobiotin as our new SPAAC partner. We were pleased to no longer notice loss of material in all repeated and reproduced reactions (N = 18; three different experimenters: CS, VV, IK), which resulted in better conversion and avDoC on average as well as narrower distributions (Figure 1, scatter plot top right-hand corner). We next selected four samples from this series, made by the three different experimenters, for peptide mapping analyses. Three of them shared similar conversions (i.e., IK1 = 70 %; IK2 = 65 %; VV = 64 %) and avDoC (IK1 = 1.1; IK2 = 1.0; VV = 1.0), while the fourth sample showed increased values (CS = 86 % conv., avDoC = 1.8). Even though small discrepancies were found between these new samples and our previously reported data, Passerini- and Ugi-modified E1 and D1 residues were systematically detected in all samples. Importantly, even though samples VV and IK2 shared identical conversion and avDoC values, *N*-terminal selective conjugation (i.e., **E1**, **D1**, and *E1* as the sole modifications detected) was detected in IK2 sample only. While such discrepancies could indicate a lack of reproducibility, it could also be that inter-residue U-4C-3CR accounts for only a small fraction of all conjugation sites, leading to detection issues – all the more so when part of the conjugates are lost in the process, as observed with BCN-Cy5.

In order to test our hypothesis, we next turned our attention towards time course experiments, hoping to determine whether the conjugation of certain sites might be favored over shorter reaction times (Figure 2). From these experiments, we could first see that conversion and avDoC values varied accordingly, following the same trend, with a rather rapid progression in the first 4 h before plateauing after 8 h, reaching the highest values detected in the previous sample CS. Interestingly, only *N*-terminal conjugation – on both E1 and D1 residues through Passerini and Ugi reactions – could be detected by peptide mapping for reaction times ≤ 4 h, with inter-residue conjugation appearing only

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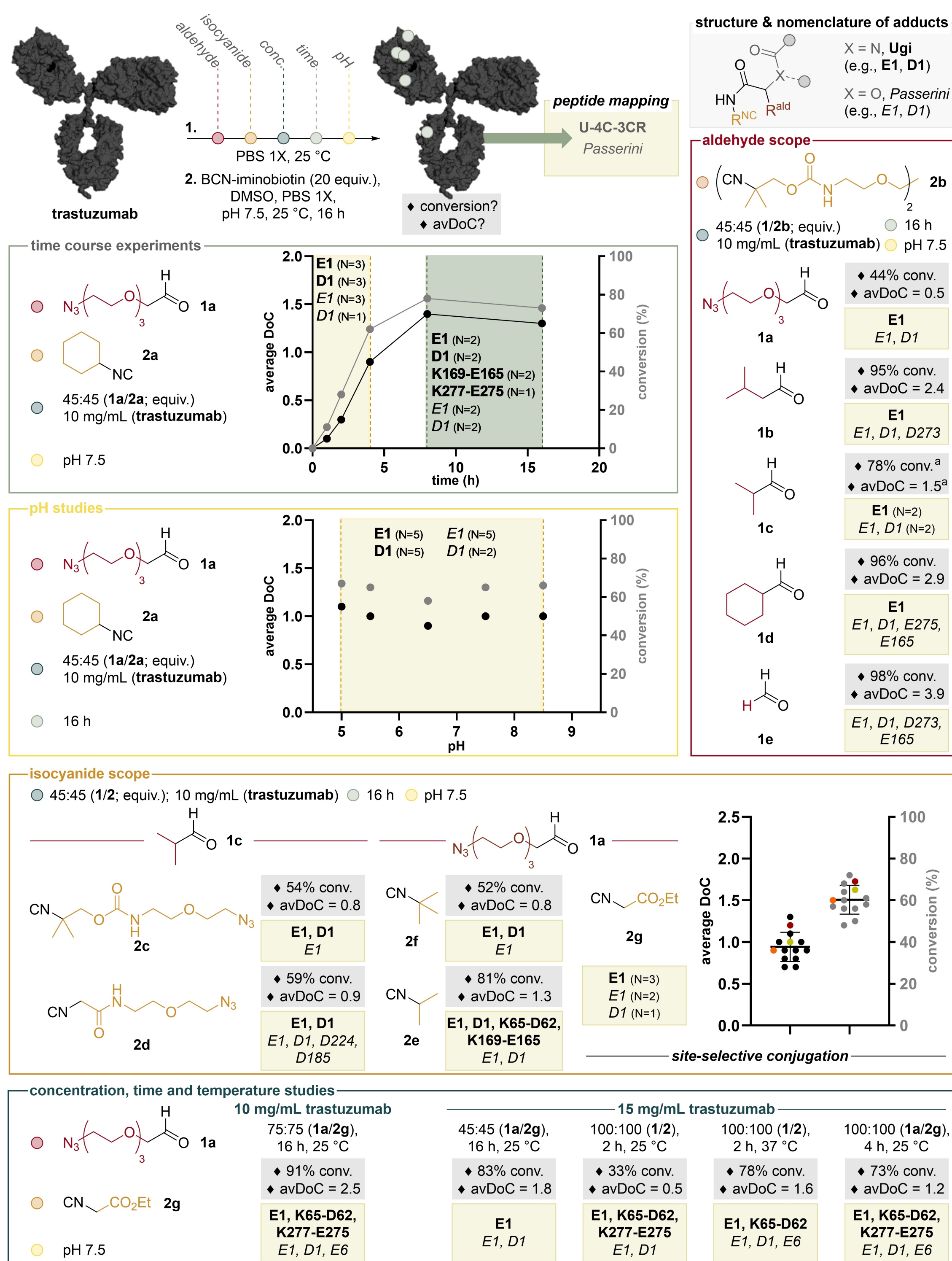

Figure 2. Methodology work studying the influence of reaction time, pH, concentration and reagent's structures on the efficiency of trastuzumab's multicomponent conjugation (as determined by conversion and avDoC) and the identity of the conjugation sites. The latter are listed in **bold** for U-4C-3CR or in *italics* for Passerini reaction; the number of replicates N is indicated whenever available. The reproducibility of the conjugation reaction employing isocyanide **2g** and aldehyde **1a** (N = 14) is given in the form of scatter plots with mean and standard deviations; the three samples analysed by peptide mapping are highlighted in colours (i.e.: • = CS; • = VV; • = IK). [a]: average of two independent experiments (i.e., CS: 84% conv., avDoC = 1.6; VV: 72% conv.; avDoC = 1.3).

after 8 h of incubation. This would tend to confirm that the latter mechanism accounts for only a small fraction of the conjugation sites detected for av. DoC < 1.0, and that the site-selectivity depends heavily on the conversion of the reaction.

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As a further evidence, pre-incubation with *N*-termini selective 2-pyridinecarboxaldehyde resulted in a substantial decrease of the conjugation efficiency (see SI).[36] As *N*-terminal conjugation can be favored by variations in pH due to the difference in pK_a between α- and ε-amines, we then studied its influence on the outcome of the conjugation. Surprisingly, little to no change was detected between all experiments: almost identical conversion and avDoC were obtained, with a maintained *N*-terminal selectivity. While this demonstrates that the intra-residue U-4C-3CR occurs preferentially over its inter-residue variant with this reagent combination, competition with Passerini reaction could not be suppressed.

Pursuing this methodology effort, we next varied the structure and identity of both the carbonyl and isocyanide components. On the one hand, five different aldehydes were evaluated in combination with bis-isocyanide **2b** that we had previously identified as an effective reagent.[35] While stark variations in conversion and avDoC were seen between all of these experiments, it also led to more diverse Passerini adducts as evidenced by peptide mapping studies, culminating in undetected Ugi modification when formaldehyde **1e** was employed. Interestingly, isobutyraldehyde **1c** led again to *N*-terminal selective conjugation, with excellent conversion and an almost perfect inter-operator reproducibility, demonstrating once more the robustness of our approach from both conjugation and analytical perspectives. Unfortunately, in an effort to improve these conditions by incorporating an azide group onto the isocyanide to restore a plug-and-play strategy, we noticed that even the slightest structural change had a detrimental effect on either the efficiency or the selectivity of the reaction (Figure 2, isocyanides **2c** and **2d**). On the other hand, varying the isocyanide component while keeping aldehyde **1a** as a carbonyl source resulted this time in clear fluctuations in the Ugi sites but in Passerini adducts essentially restricted to *N*-terminal glutamate and aspartate residues. Of the three isocyanides evaluated, ethyl isocyanoacetate **2g** (VV sample: 60% conversion, avDoC = 0.9) was the only one showing an outstanding single-residue selectivity, with the sole modification of E1. Even more remarkable, this was accompanied by an absence of Passerini side reaction, making these conditions not only site- and residue-selective but also "mechanism selective", favouring only the Ugi reaction over the Passerini. Given the importance of these results, we once again evaluated their reproducibility (N = 14, four different experimenters – i.e., VV, N = 7 replicates; LR, N = 4; IK, N = 2; CS, N = 1 –, see scatter plot in Figure 2). We were delighted to observe even less pronounced variations than before with cyclohexyl isocyanide (i.e., $\sigma_{conv.} = 7\%$ and $\sigma_{avDoC} = 0.2$ with ethyl isocyanoacetate *versus* $\sigma_{conv.} = 12\%$ and $\sigma_{avDoC} = 0.4$ with cyclohexyl isocyanide, respectively) and an average efficiency of the conjugation (i.e., average conv. = 60% and average avDoC = 0.9) matching perfectly that of the VV sample analysed by peptide mapping (Figure 2, orange dot in the scatter plot). To test our previous hypothesis that site-selectivity depends on the conversion of the reaction, we analysed two other samples from this set of replicates by peptide mapping, with conversions > 60% (i.e., CS: 65% conv., avDoC = 1.0, and IK: 69% conv., avDoC = 1.2). This led to the detection of Passerini adducts, however only circumscribed to the *N*-termini, but with a maintained E1 single-site selectivity for the U-4C-3CR, highlighting once again the favored reactivity of *N*-terminal carboxylates. In an attempt to push the conversion further, we also evaluated the impact of an increased concentration in either the antibody or in both carbonyl and isocyanide. On the one hand, increasing the amount of equivalents of both aldehyde **1a** and ethyl isocyanoacetate **2g** to 75:75 resulted in an almost complete conversion but at the expense of site-selectivity; on the other hand, switching from 10 to 15 mg/mL in trastuzumab successfully resulted in an increased conversion (83%) with an avDoC of 1.8 and a maintained *N*-terminal selectivity. Any further attempt at increasing both concentration parameters (i.e., 15 mg/mL trastuzumab and 100:100 **1a**/**2g**) or the temperature to accelerate the reaction resulted in non-selective U-4 C-3CR even for shorter reaction times and lower conversions. Given the proximity of the *N*-termini to the paratope region, we wanted to assess whether our conjugation could affect the affinity of trastuzumab for its HER2 target. In order to do so, we used the LigandTracer technology, allowing to study the kinetics of antibody binding in real-time and with living cells, only requiring fluorescently labelled conjugates.[37] Starting from our E1-selective Ugi-conjugated trastuzumab, we thus engaged it in a SPAAC step with a BCN-fluorescein partner, effectively delivering the fluorescent conjugate **T$_{Ugi}$-Fluo** (Figure 3). Gratifyingly, the latter showed rate constants of association and dissociation identical to those of the control FITC-labeled trastuzumab **T-Fluo** on HER2-positive SKBR-3 cells (i.e., $k_{on} = 2.3\times10^4\ M^{-1}.s^{-1}$, $k_{off} = 1\times10^{-5}\ s^{-1}$, $K_D = 0.45$ nM versus $k_{on} = 2.0\times10^4\ M^{-1}.s^{-1}$, $k_{off} = 1.37\times10^{-5}\ s^{-1}$, $K_D = 0.69$ nM, respectively), perfectly in line with previously reported values.[38] As further evidence of a maintained affinity, the ADC **T$_{Ugi}$-DM1** was produced by SPAAC, using a BCN-DM1 strained alkyne (avDAR = 1.0, 60–65% conversion, 75% yield due to partial precipitation of the ADC). This ADC was tested in cell viability assays on both HER2-positive and HER2-negative cell lines (i.e., SKBR-3 and MDA-MB-231, respectively) against the marketed ADC Kadcyla, comprising the same linker-drug payload, but produced through stochastic lysine conjugation (avDAR = 3.5).[39] Despite a 3-fold difference in avDAR, our ADC **T$_{Ugi}$-DM1** showed a potency comparable to that of Kadcyla, with a subnanomolar toxicity on SKBR-3 cell line, three orders of magnitude higher than that observed on MDA-MB-231 (Figure 3; see SI for more details).

Having demonstrated that our newly developed site-selective conjugation strategy could lead to valuable trastuzumab conjugates, we finally concluded our in-depth study by evaluating its application to other proteins. Selecting mAbs with *N*-terminal D1 and E1 – i.e., bevacizumab and ramucirumab –, we assessed three of our best carbonyl/isocyanide combinations and found similar results (Figure 4). Using cyclohexyl isocyanide **2a** and aldehyde **1a**, bevacizumab reacted in an almost identical manner as trastuzumab, which also translated in comparable modification sites. Using the same reagent combination, ramucirumab conjugation found to be more sluggish but still proceeded with high site-selectivity.

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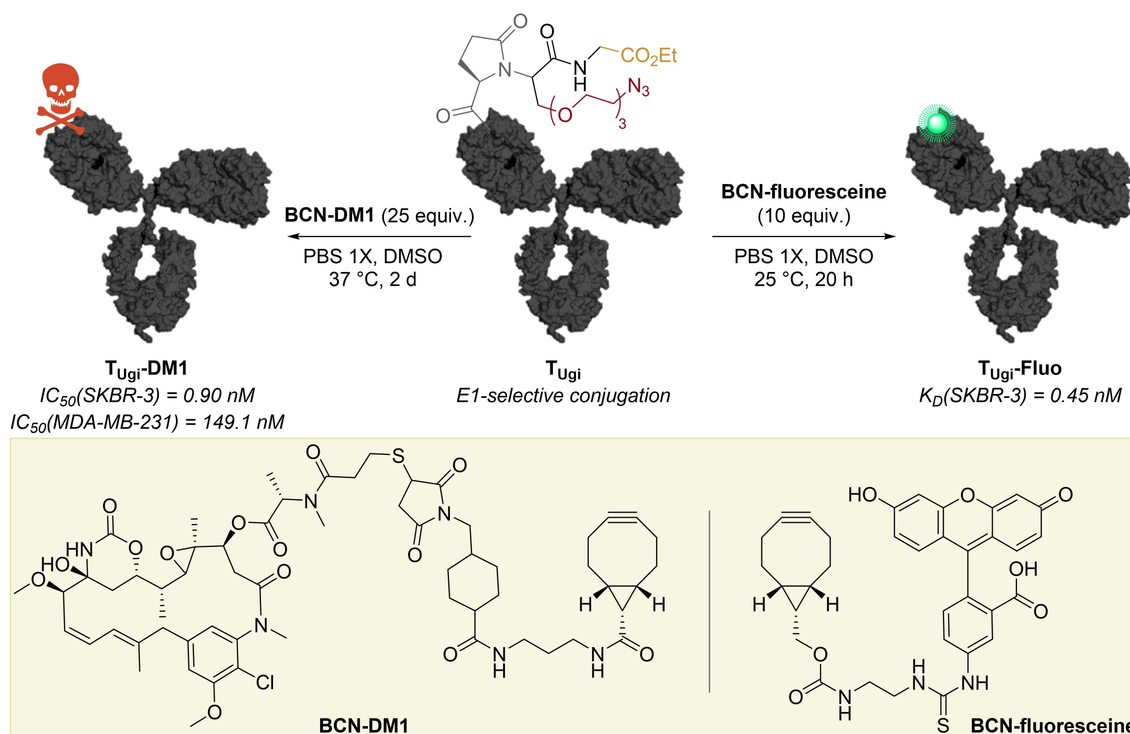

Figure 3. Applications of our E1-selective Ugi-conjugated trastuzumab T_{Ugi}. SPAAC was used for the production of the ADC **T_{Ugi}-DM1**, which proved to be highly potent and selective of HER2-positive SKBR-3 cells, and of the fluorescent conjugate **T_{Ugi}-Fluo**, which was used to determine the real-time binding kinetics of trastuzumab on living cells using the LigandTracer technology.

Switching to ethyl isocyanoacetate **2g**/aldehyde **1a** or isocyanide **2b**/isobutyraldehyde**1c** combinations gave *N*-terminal selectivity in all cases, in perfect coherence with what had been observed on trastuzumab (see SI). It is also worth stressing at this stage that this approach could be applied to the smaller antibody F(ab′)$_2$ fragments, with similar efficiency and conjugation sites being observed compared with whole antibody (see SI). Our conjugation strategy proved also to be tolerant to mAbs deprived of *N*-terminal carboxylates. Rituximab (*N*-ter Q1 on both chains) was successfully conjugated with excellent conversion and avDoC under our first set of optimized conditions; peptide mapping studies highlighted two inter-residue Ugi conjugation sites – i.e., K278-E276 and K169-E165 – along with one Passerini site – i.e., E165 –, all of which having been previously detected on other mAbs during the course of this work, suggesting that multicomponent reaction conjugation tends to work only on a handful of hot-spot reactive sites.

While mAbs gave excellent results, we were surprised to notice that several other smaller proteins proved to be unreactive under the same conditions. Indeed, in spite of all our efforts, none of the following proteins led to noticeable conjugation: albumin (neither bovine nor human), α-chymotrypsin, myoglobin, lysozyme, ubiquitin. In most if not all cases, the unconjugated protein proved to be the major species detected by nMS, sometimes accompanied with small amounts of higher molecular-weight species whose masses did not match those of the expected adducts (see SI). In parallel, we were delighted to notice that anticalin proteins performed efficiently in this multicomponent conjugation reaction (Figure 4).[40] Anticalins are alternative binding proteins derived by protein engineering from the human lipocalins and have been extensively studied as binding proteins in the field of oncology and diagnostics.[41] For this work, we employed a recently developed anticalin directed against the human transmembrane glycoprotein CD98hc, dubbed D11vs.[42] After a short optimization step, D11vs led to full conversion, with an avDoC of 1.3 under our optimal conditions, when using aldehyde **1a** and cyclohexyl isocyanide **2a** albeit in a 35:35 equivalent ratio in order to minimize over-conjugation. Peptide mapping studies allowed the identification of only two conjugation sites: the Passerini-modified residue D2 and the U-4C-3CR-conjugated residues K46 and E44. Lowering reagents concentration down to a 25:25 equivalent ratio allowed to increase single conjugation but at the expense of only partial conversion, typically ~90%. Batch-to-batch reproducibility was once again demonstrated as well as consistency in peptide mapping results, with the same conjugation sites being detected in triplicate experiments performed on two different batches of D11vs. In an attempt to further validate these results, we investigated three D11vs mutants: D11vs(N△6), with a truncated *N*-terminal region; D11vs(K46R), where the key lysine residue was replaced with an arginine, which should not participate in a U-4C-3CR while maintaining a positive charge in this crucial region; D11vs(N△6/K46R), the double mutant featuring both alterations.

Interestingly, D11vs(N△6) led to a similar conjugation profile, with the inter-residue Ugi conjugation being the sole

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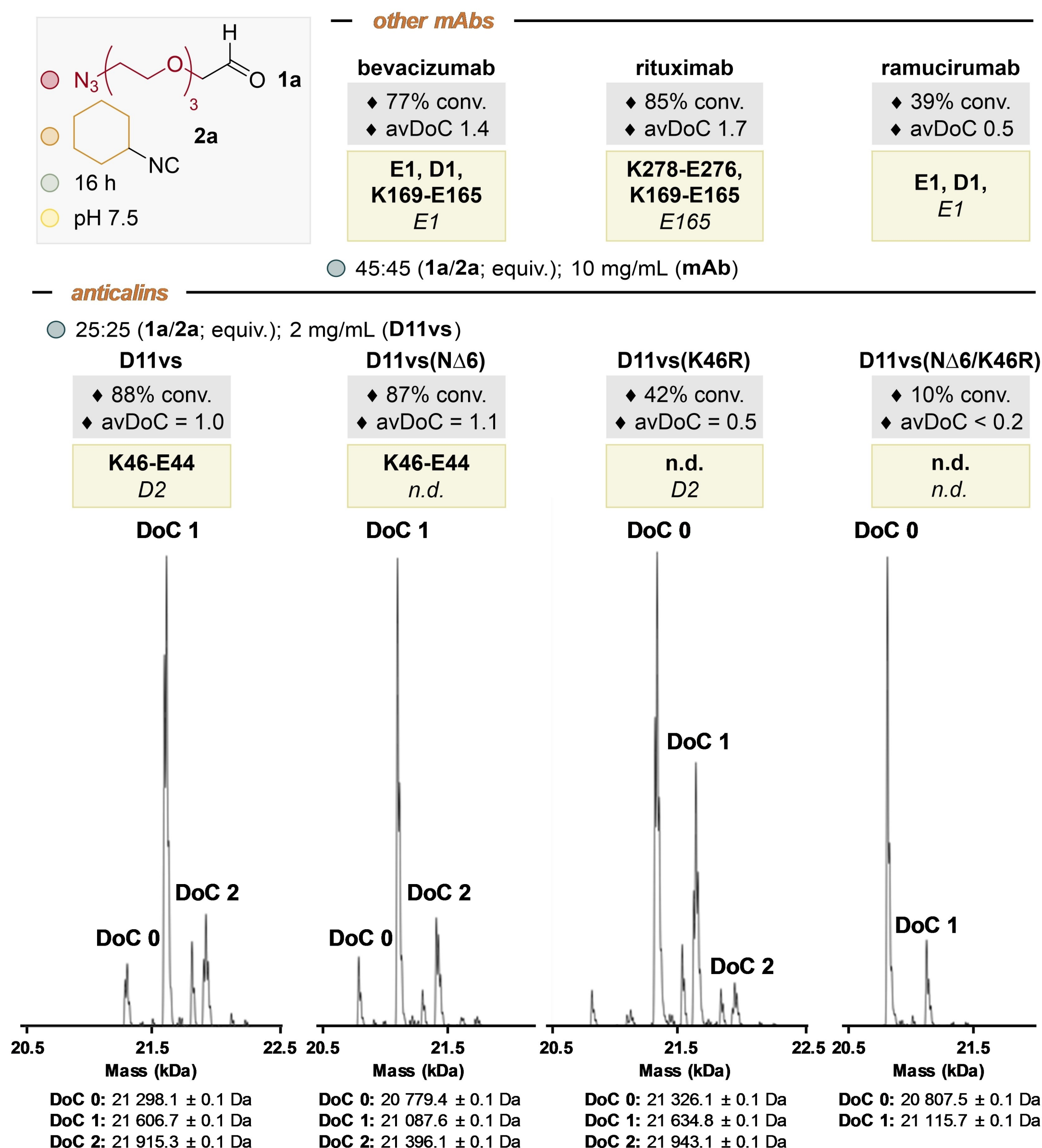

Figure 4. Application of our optimised conditions to other antibodies or an anticalin protein with the corresponding avDoC and conversion values of the transformation and the identities of the conjugation sites, detected by peptide mapping. These are listed in **bold** for U-4C-3CR or in italics for Passerini reaction.

site identified by peptide mapping. This would tend to show that the Passerini reaction is rather marginal in comparison with the U-4C-3CR, or at least occurs at a slower rate, a hypothesis further reinforced by the low level of conjugation observed with the K46R mutant. The high degree of site-selectivity of our method was evidenced by the observation that conjugation was barely detectable on the double mutant anticalin D11vs(N△6/K46R), which lacks the two reactive sites that were previously identified.

Conclusions

In conclusion to this methodology work, we varied all conjugation parameters of our previously developed Ugi conjugation reaction in order to study their impact on the selectivity of our approach on several proteins. We proved that mAbs responded well to this approach, with a handful of key reactive sites being systematically detected. Importantly, we managed to refine some conditions to allow the *N*-terminal-selective conjugation of aspartate and glutamate residues, culminating in the precise modification of a single site through an intra-residue U-4C-3CR when commercial ethyl isocyanoacetate was used. This approach offers an interesting alternative to related strategies targeting precise *N*-terminal residues, such as

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glycine,[43] proline,[44–46] or cysteine,[47–51] by being applicable to the site-selective conjugation of native antibodies.

Intriguingly, our optimized strategy proved to be applicable only to a limited set of proteins, in particular an anticalin for which a high degree of site-selectivity was nevertheless consistently observed, suggesting that only precise arrangements of lysine and aspartate/glutamate residues lead to successful conjugation. Our advice to the curious reader who would be tempted to evaluate our site-selective method to their proteins would be to first go with equimolar amounts of **1a** and **2a** – adjusting the number of equivalents to the protein's molecular weight and concentration – in a ~90:10 v/v mixture of PBS 1X and DMSO, for 16 hours at pH 7.5 and 25 °C, before refining the conditions further in case of positive results. In a near future, we will continue exploring the site-selectivity avenues opened by this Ugi reaction, in the hope of better deciphering its mechanism and possibly developing a predictive tool able to foresee the "Ugiability" of any given protein.

Experimental Section

Representative experimental procedures

Ugi reaction on proteins (General procedure). To a solution of a protein (1 equiv., 50 μL in PBS 1x, pH 7.4–67 μM for mAbs, 68.5 μM for $F(ab')_2$, 67.7 μM for BSA, 60.8 μM for α- chymotrypsin; 64.8 μM for myoglobin, 61.8 μM for lysozyme, 132.4 μM for ubiquitin, 2 $mg.mL^{-1}$ for all anticalins) was added aldehyde (as a 0.1 M solution in DMSO) and isocyanide (as a 0.1 M solution in DMSO). The reaction mixture was then incubated for 16 h at 25 °C, after which a 50 wt% solution of hydroxylamine in H_2O (5 μL, 730 equiv.) was added. The resulting solution was incubated for 1 h at 25 °C, before the excess of reagent was removed by gel filtration chromatography using Bio-spin P-30 or P-6 columns pre-equilibrated with PBS 1×, pH 7.5 to give a solution of protein-azide which was further derivatized.

SPAAC reaction (General procedure). To a solution of protein-azide in PBS 1x was added a BCN-derivative (as a 0.1 M solution in DMSO, 20 equiv.). The resulting solution was incubated for 16 h at 25 °C, before the excess of reagent was removed by gel filtration chromatography using Bio-spin P-30 or P-6 columns pre-equilibrated with PBS 1×, pH 7.5 to give a solution of conjugated protein that was further analyzed by native mass spectrometry.

Funding Sources

This work was supported by the European Union's Horizon 2020 Research and Innovation Programme Marie Sklodowska Curie Action ITN under Grant Agreement No 859458 (IK, RB, YE, CL, LR), the "Agence Nationale de la Recherche" (BioconjUgi – ANR-19-CE07-0045; Conformabs – ANR-21-CE29-0009-01), the Région Grand-Est and LabEx MEDALIS (CS), the CNRS, the University of Strasbourg, the French Proteomic Infrastructure (ProFI; ANR-10-INBS-08-03) and the Interdisciplinary Thematic Institute IMS (Institut du Médicament Strasbourg), as part of the ITI 2021–2028 supported by IdEx Unistra (ANR-10-IDEX-0002), SFRI-STRAT'US project (ANR-20-SFRI-0012).

Supporting Information

The authors have cited additional references within the Supporting Information (Ref. [52–54]).

Author Contributions

I. K.: Conceptualisation; Data curation; Formal analysis; Investigation; Methodology; Validation; Writing – review & editing. V. V.: Conceptualisation; Data curation; Formal analysis; Investigation; Methodology; Validation; Writing – review & editing. R. B.: Data curation; Formal analysis; Investigation; Methodology; Validation; Visualization. H. D.: Data curation; Formal analysis; Investigation; Methodology; Validation; Visualization. C. S.: Conceptualisation; Data curation; Formal analysis; Investigation; Methodology; Validation; Writing – review & editing. Y. E.: Data curation; Formal analysis; Investigation; Resources; Validation; Writing – review & editing. L. R.: Data curation; Formal analysis; Investigation; Validation. C. L.: Data curation; Formal analysis; Investigation; Resources. O. H.-A.: Project administration; Resources; Supervision. S. E.: Data curation; Formal analysis; Investigation; Methodology; Validation; Visualization. A. D.: Resources; Writing – review & editing. A. S.: Funding acquisition; Methodology; Project administration; Resources; Supervision; Writing – review & editing. A. W.: Funding acquisition; Methodology; Project administration; Supervision; Writing – review & editing. S. C.: Funding acquisition; Methodology; Project administration; Resources; Supervision; Writing – review & editing. G. C.: Conceptualisation; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Supervision; Validation; Visualization; Resources; Writing – original draft.

Acknowledgements

We thank Dr. Constantin Semenchenko (Syndivia SAS) for cytotoxicity assays. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD045387.[52]

Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are openly available in ProteomeXchange at https://proteomecentral.proteomexchange.org/cgi/GetDataset?ID=PXD045387, reference number 45387.

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Chemistry Europe
European Chemical Societies Publishing

Keywords: Bioconjugation · chemical biology · multicomponent reactions · site-selectivity · proteins

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Manuscript received: October 3, 2023
Accepted manuscript online: December 5, 2023
Version of record online: January 24, 2024

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