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Investigating Ugi / Passerini multicomponent reactions for the Site-Selective Conjugation of Native Trastuzumab

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KEYWORDS
Bioconjugation; multicomponent reaction; antibodies; antibody-drug conjugates

ABSTRACT: Site-selective modification of proteins has been the object of intense studies over the past decades, especially in the therapeutic field. Prominent results have been obtained with recombinant proteins, for which site-specific conjugation is made possible by the incorporation of particular amino acid residues or peptide sequences. In parallel, methods for the site-selective and site-specific conjugation of native and natural proteins are starting to thrive, allowing the controlled functionalization of various types of amino acid residues. Pursuing the efforts in this field, we planned to develop a new type of site-selective method, aiming at the simultaneous conjugation of two amino acid residues. We reasoned that this should give higher chances of developing a site-selective strategy compared to the large majority of existing methods that solely target a single residue. We opted for the Ugi four-center three-component reaction to implement this idea, with the aim of conjugating the side-chain amine and carboxylate groups of two neighbouring lysine and aspartate/glutamate. Herein, we show that this strategy can give access to valuable antibody conjugates bearing several different payloads, and limits the potential conjugation sites to only six on the model antibody trastuzumab.

Proteins posttranslational modifications is Nature's way of generating a rich and diverse proteome from a more limited genetic coding capability. First occurrences of intentional, man-made – artificial – proteins modifications using a defined chemical – thus excluding the food-related Maillard reaction for example – could be dated back to the use of formaldehyde in the tanning industry or for the production of toxoids,[1,2] which evolved later on to immunization studies using chemically-modified bovine serum albumin in the 1900s and eventually led to Landsteiner's synthetic haptenes studies.[3,4] The field of protein modification has since largely benefited from the understanding of proteins' and amino acids' structures coupled to the parallel appearance of more efficient and precise analytical tools. This finally resulted in the development of bioconjugation reagents with excellent chemoselectivity towards various amino acids' side chains groups (i.e. residue-selectivity) that translated into major applications, notably in the pharmaceutical field with the generation of protein-fluorophore adducts for trafficking studies, or the polyethyleneglycol chains functionalization (PEGylation) of proteins to give less-immunogenic and more plasma-stable conjugates.[5,6]

However, site selectivity quickly emerged as the main limitation of chemoselective strategies, due to the presence of multiple copies of each type of amino acid residue at the surface of proteins. Statistic conjugation of surface-accessible lysine residues with amine-selective reagents typically results in highly heterogeneous mixtures, containing up to millions of different adducts when large proteins such as antibodies are utilised.[7,8] Each of these adducts possessing distinct physicochemical properties, such chemoselective conjugation necessarily leads to mixtures with different *in-vivo* pharmacokinetic properties along with virtually no reproducibility in batch-to-batch production.[9,10] Regioselective (i.e., site-specific) methods were thus developed and are currently dominated by the use of recombinant proteins, incorporating exogenous amino acid residues – natural or unnatural – or peptide sequences that can be specifically targeted by a tailored reagent or strategy.[11–13] In parallel, site-selective chemical strategies for the conjugation of native and natural proteins have also flourished over the past few years, giving rise to methods targeting various types of amino acids – e.g. lysine, cysteine, tryptophan, tyrosine – that proved to be effective on proteins of all sorts of sizes, including antibodies.[14–28]

With the aim of pursuing the efforts in this field, we could not help but notice that the vast majority of previously reported strategies for the site-selective conjugation of native proteins were focused on the modification of a unique residue. We hypothesized that targeting two different amino acid side chains simultaneously would lower the enormous subset of possibilities given by single-residue bioconjugation techniques, thus increasing our chances of developing a site-selective method by minimising the number

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of potentially reactive sites, a path that has also been successfully explored by others in the meantime.[29] In order to do so, we elected the Ugi four-centre three-component reaction (U-4C-3CR) for the concomitant conjugation of two spatially close lysine and aspartate (or glutamate) residues (Figure 1). This variation of the classical Ugi four-component reaction (U-4CR) employs bifunctional reagents, where two of the four components necessary to the reaction (i.e. an amine, a carboxylic acid, a carbonyl and an isocyanide) are borne by the same molecule. It was thus anticipated that close amine/carboxylate pairs at the surface of the protein would give the expected bis-amide final product in the presence of an aldehyde and an isocyanide, while lysines that are not in the vicinity of a carboxylate moiety could form imines but would not give any productive Ugi reaction.[30,31] A solution of hydroxylamine would then simply be added at the end of the reaction to regenerate the amino groups of these lysine residues that did not partake in the multicomponent reaction. While the Ugi reaction has been the object of extensive studies for the conjugation and stapling of peptides, only a handful of publications reports the use of proteins as coupling partners.[32–36] Most notably, all these strategies use the four-component version of the Ugi reaction (U-4CR) – i.e., they only target a single site at the protein surface, whether it be an amino acid residue (lysines or aspartates / glutamates) or aldehydes from the glycan portion after periodate oxidation – and necessitate excessive amounts of reagents (up to 4000 equivalents) and long reaction times (up to 4 days). In addition, the absence of thorough analytical investigations precludes the conclusion that the Ugi reaction, and not an uncontrolled side reaction, was indeed responsible for the bioconjugation. This aspect is of prime importance in our case, due to the probable competition of the Passerini reaction for the conjugation of aspartate / glutamate residues that could erode the potential site-selectivity of the U-4C-3CR.

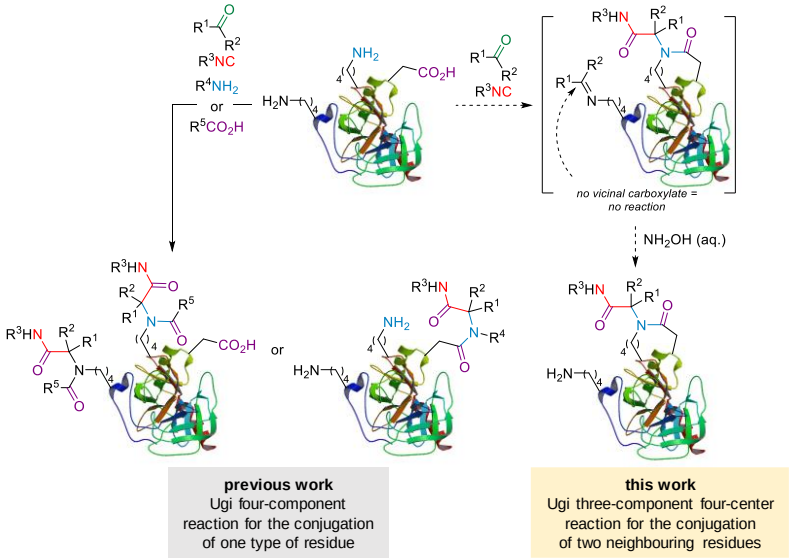

Figure 1: application of the Ugi reaction to the bioconjugation of native proteins

As we ambition to develop a site-selective strategy that could be applied to the conjugation of large proteins, we selected trastuzumab (~150 kDa) as our model protein, a FDA-approved antibody marketed as Herceptin that is used for the treatment of breast cancer by targeting the overexpressed HER2 receptors.[37] We embarked upon the study of the feasibility of our strategy by reacting it with various amounts of the model TAMRA aldehyde **1a** and *tert*-butyl isocyanide **2a** (Figure 2, a). The reaction was set up in phosphate-buffered saline (PBS) buffer (1x, pH 7.5, 137 mM NaCl) – to avoid the use of amine or carboxylate buffers that could compete with the antibody for the Ugi reaction – and average degrees of conjugation (DoC) and conversion rates were determined by native mass spectrometry (native MS) by comparing peak intensities. We were delighted to see that even low concentrations of reagents – from 1.4 mM (20 equiv.) to 2.7 mM (40 equiv.) – gave positive results in just 16 h at 25 °C without the need of any catalyst or imine preformation.[38,39] We also observed a strong influence of the aldehyde-to-isocyanide ratio on the reaction outcome, with better conversion rates and DoC obtained with equimolar or near equimolar ratios. Increasing the amounts of both aldehyde and isocyanide led to higher DoC and conversion rates, however, only to a certain extent: a plateau was reached when large excess were used (i.e. ≥60:120 equiv. **1a**/**2a**), possibly indicating a limit in the number of functionalization sites on trastuzumab. This hypothesis was reinforced when looking at the conjugates distribution: for the highest conversions (i.e. >80%), the maximal DoC value observed was only 6 and represented less than 5% of all conjugates. Excellent conversion could be obtained with a narrower DoC distribution when 45

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equivalents of both **1a** and **2a** were employed, with adducts with DoC > 3 (D4 and D5; Figure 2, b) representing a negligible fraction of all conjugated species. Control experiments were also done at this stage, where trastuzumab was reacted in the absence of either aldehyde **1a** or isocyanide **2a**, leading to no conjugation in both cases and thus proving the multicomponent aspect of the reaction (see ESI and Figure S2 for more details). Prolonging the reaction time up to 2.5 days or heating at 37 °C resulted in improved conversions but extended DoC distribution – up to D4 when a 20:40 **1a**/**2a** ratio was utilised – while performing the reaction at 4 °C had the opposite effect. On the other hand, varying the NaCl concentration or the pH of the buffer solution led to no noticeable change (see ESI and Figure S3 for more details). Thus, optimal conditions for the conjugation of trastuzumab were determined to be 45 equivalents of both **1a** and **2a** in PBS buffer (1x, 137 mM of NaCl, pH 7.5), with a reaction time of 16 hours at 25 °C, offering the best compromise between conversion and DoC distribution.

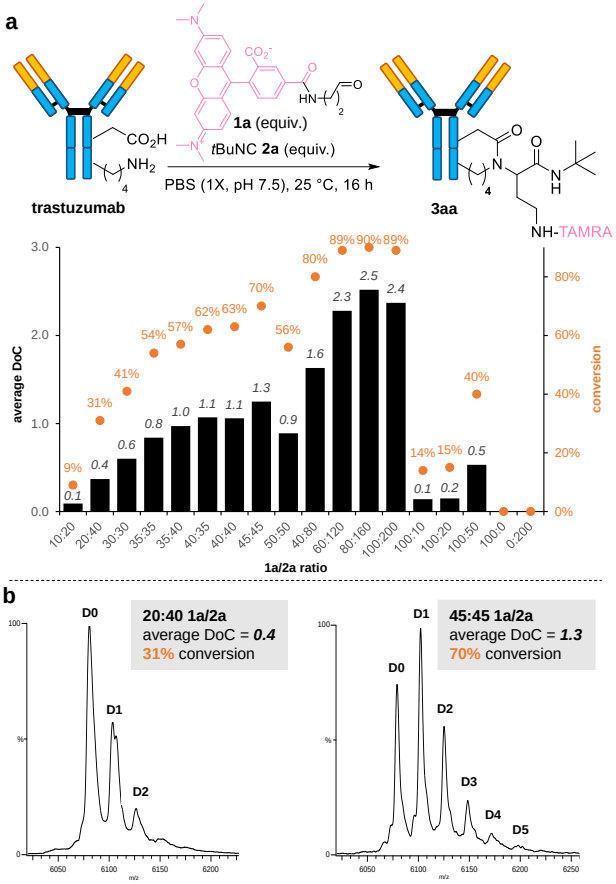

Figure 2: Ugi four-centre three-component reaction on trastuzumab. **(a)** Influence of aldehyde **1a** to isocyanide **2a** ratio on trastuzumab's average DoC, determined by native MS. **(b)** Native mass spectra of the resulting functionalized trastuzumab after deglycosylation (zoom on the 24+ charged state); reaction conditions: **1a** (20 or 45 equiv.), **2a** (40 or 45 equiv.), PBS 1x (137 mM NaCl, pH 7.5), 25 °C, 16 h; conversion = 100 - %D0.

With this set of conditions in hands, we then turned our attention towards the development of a 'plug-and-play' version of our strategy,[15,40,41] by first incorporating an azide group onto trastuzumab – via our multicomponent strategy – that could then be easily functionalized by strain-promoted azide-alkyne cycloaddition (SPAAC) with various payloads (Figure 3, a). For practical synthetic reasons, it was found easier to have the azide group borne by the aldehyde component and azide-containing aldehyde **1b** was thus easily synthesized in three steps from commercially available tetraethylene glycol and reacted with **2a** and trastuzumab under our optimal conditions. The resulting conjugate **3ba** was then purified and subjected to SPAAC with the bicyclononyne (BCN) derivative **4a**, equipped with a cyanine-5 fluorophore, delivering the functionalized adduct **5** whose average DoC was determined by native MS. It is important to stress that partial precipitation was always observed due to the hydrophobic nature of the cyanine-5 payload, thus slightly impacting the average DoC and conversion rate values.
Screening different aldehyde-to-isocyanide ratios, we found that conversion and average DoC values were slightly lower with **1b** than with **1a** in general (Figure 3, b). However, DoC distribution turned out to be better, with D4 being the highest conjugated species observed across all conducted experiments and representing <8% of all conjugates. Stability studies of conjugate **5** (see ESI and Figure S4 for more

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details), conducted for five days in different media, showed no apparent decomposition in human plasma and a good stability in PBS at various pH.

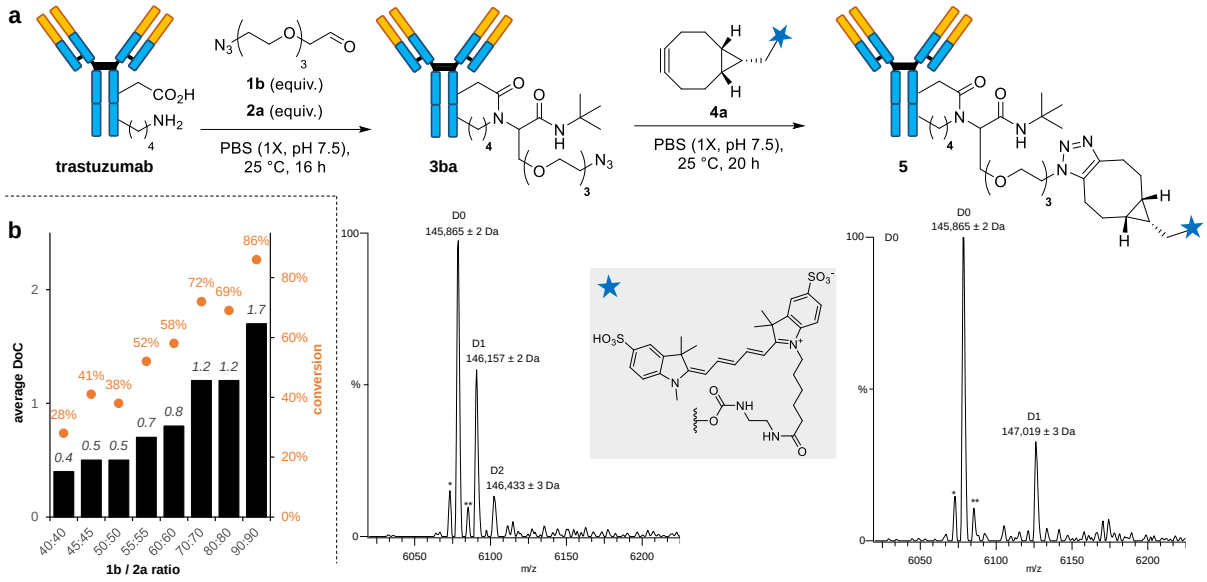

Figure 3: 'Plug-and-play' strategy: **(a)** conjugation of trastuzumab with the azide-containing aldehyde **1b** and isocyanide **2a** followed by functionalization with strained alkyne **4a** and native mass spectra of the different adducts after deglycosylation (zoom on the 24+ charged state – reaction conditions corresponding to the spectra: "plug step": **1b** (45 equiv.), *t*BuNC **2a** (45 equiv.) in PBS 1x (137 mM of NaCl, pH 7.5), 25 °C, 16 h; "play step": **4a** (10 equiv.) in PBS 1x (137 mM of NaCl, pH 7.5), 25 °C, 20 h); **(b)** optimization of the "plug step" (average DoC and conversion rates determined by native MS analysis of the corresponding conjugates **5** after deglycosylation).

Having developed a set of robust and modular conditions leading to stable constructs, the versatility of our strategy at both the plug and the play stages was then evaluated by screening various isocyanides and diversely functionalized strained alkynes. As shown in Figure 4, the reaction proceeded smoothly and gave better average DoC values with commercially available isopropyl isocyanide **2b**, cyclohexyl isocyanide **2c** and ethyl isocyanoacetate **2e**, while diethyl isocyanomethylphosphonate **2f** led to no visible conjugation. Different isocyanides were also newly synthesized with the aim of incorporating other reactive groups onto trastuzumab: **2g** was accessed in a single step from **2e**, while isocyanides **2h-2m** were easily generated from the known benchstable *p*-nitrophenol carbonate **2n**.[42] Mixed results were obtained for the conjugation of trastuzumab with this set of compounds however, with **2i** being the only isocyanide to give acceptable conversion and average DoC values. Overall, small and apolar isocyanides seem to work best for this transformation, which could possibly indicate that reactive conjugation sites are located in hydrophobic pockets.

As for the play stage, starting from conjugate **3bc**, complete conversions for the SPAAC reaction were consistently observed with BCN motifs, allowing the introduction of various payloads onto the antibody: mass spectrometry and NMR tags (**4b** and **4d**), iminobiotin (**4c**), or 20-mer oligonucleotides (**4e** and **4f**). Switching to dibenzocyclooctyne (DBCO) groups, we successfully generated the antibody-drug conjugate (ADC) **5g** by incorporating the monomethyl auristatin E (MMAE) drug (**4g**), connected to the strained alkyne scaffold by a cleavable valine-citruline linker. Analysis by native MS after purification by gel filtration chromatography showed an incomplete conversion during the SPAAC reaction but an average drug-to-antibody ratio (DAR) of 1.4 – a higher value than previously noted for **5**, which could be explained by an absence of precipitation during the SPAAC with **4g**. The cytotoxicity of the ADC **5g** was assayed on the HER2-positive SK-BR-3 and the HER2-negative MDA-MB-231 cancer cell lines, using the FDA-approved trastuzumab emtansine (T-DM1) as a benchmark. Gratifyingly, **5g** was found to be at least as active and selective as T-DM1, with comparable EC_{50} values on HER2-positive cells and a lack of apparent toxicity on HER2-negative cells (Figure 4, e and Figure S6).

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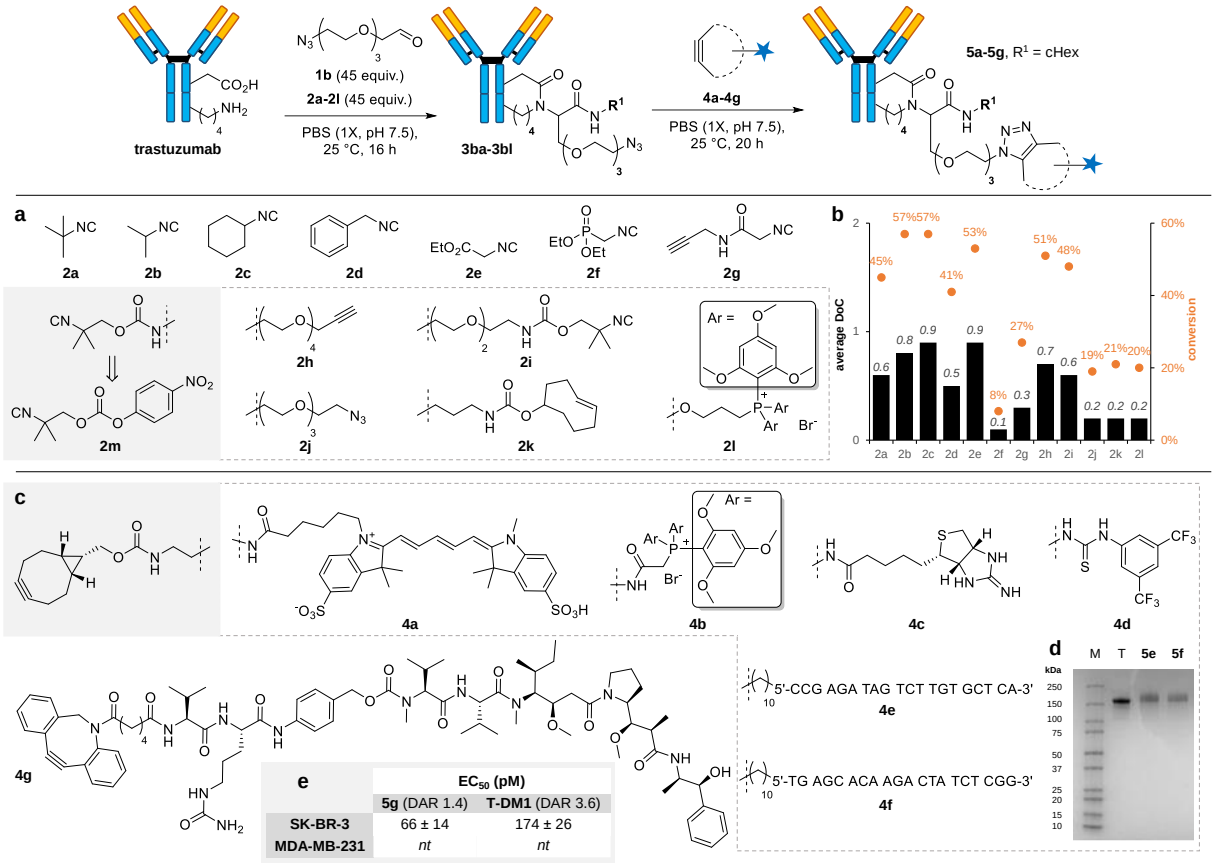

Figure 4: Isocyanide and strained alkynes scope. **(a)** Isocyanide scope. Reaction conditions: *i*) **1b** (45 equiv.), **2a-2l** (45 equiv.), PBS 1x (137 mM NaCl, pH 7.5), 25 °C, 16 h; *ii*) **4a** (10 equiv.), PBS 1x (137 mM NaCl, pH 7.5), 25 °C, 20 h. **(b)** Conversion and average DoC values obtained with **2a-2l**. **(c)** Strained alkynes scope. Reaction conditions: *i*) **1b** (45 equiv.), **2c** (45 equiv.), PBS 1x (137 mM NaCl, pH 7.5), 25 °C, 16 h; *ii*) **4a-4g** (10 equiv.), PBS 1x (137 mM NaCl, pH 7.5), 25 °C, 20 h. **(d)** Denaturing SDS PAGE analysis of conjugates **5e** and **5f** (M: molecular weight marker; T: trastuzumab): the multiple bands correspond to the different DoC. **(e)** Cytotoxicity studies of the ADC **5g** on SK-BR-3 and MDA-MB-231 cancer cell lines (*nt* = non-toxic) – EC_{50} ± SD values from two independent experiments.

While the plug-and-play strategy offers a simple and convenient way for the post-derivatization of trastuzumab conjugate **3bc**, the use of isocyanides **2g**-**2k**, bearing another reactive group, gives an excellent opportunity for dual, and potentially orthogonal, functionalization. We hypothesized that such a double derivatization could be done by either: *i*) SPAAC followed by copper(I)-catalysed azide alkyne cycloaddition (CuAAC), by combining aldehyde **1b** with isocyanides **2g** or **2h**; *ii*) SPAAC followed by a [4+1] or a [4+2] cycloaddition reaction, with **1b** and **2i** or **2k** respectively; or *iii*), in a non-orthogonal manner, with a double SPAAC, using **1b** and **2j**, for the addition of two equivalents of the same strained alkyne payload. In the latter case, double functionalization of conjugate **3bj** with complete conversion was successfully conducted with alkynes **4a** and **4c**, allowing the incorporation of two identical payloads per conjugation site (Figure 5, b). However, orthogonal dual labelling, as detailed in *i*) and *ii*), was only met with failure despite our numerous attempts (see ESI for more details), urging us to seek an alternative route towards this goal. We thus elected aldehyde **1a** and isocyanide **2j** to first conduct the multicomponent conjugation step, giving access to azide-containing conjugate **3aj**. Subsequent SPAAC reaction with alkyne **4c** resulted in complete conversion to adduct **5j**, successfully incorporating both a fluorescent TAMRA probe and an iminobiotin (Figure S10). While only alkyne **4c** was evaluated in this case, the excellent results obtained with such a strategy should easily give access to a plethora of dually functionalized conjugates by simply varying the alkyne component. This strategy thus joins the limited existing group of site-selective dual conjugation methods applicable to native proteins, and most notably, represents the first one to not target cysteine residues to the best of our knowledge.[15,43,44]

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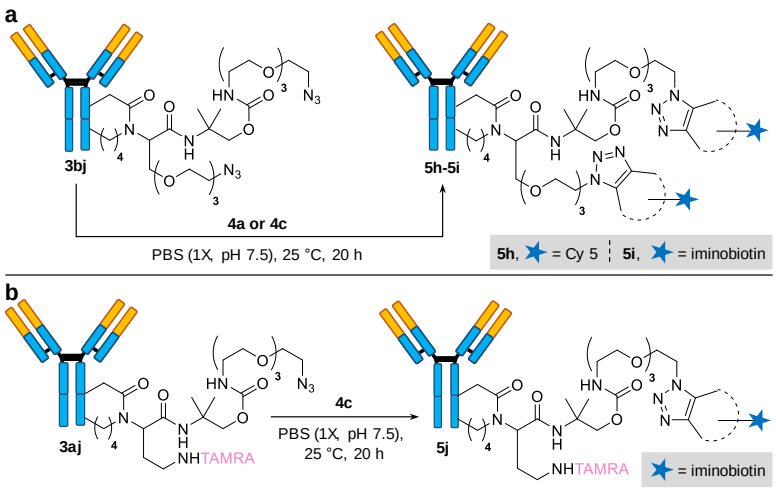

Figure 5: Dual functionalization studies. **(a)** Homo functionalization by double SPAAC reaction; reaction conditions: **4a** or **4c** (20 equiv.), PBS 1x (137 mM NaCl, pH 7.5), 25 °C, 20 h. **(b)** Hetero functionalization; reaction conditions: **4c** (20 equiv.), PBS 1x (137 mM NaCl, pH 7.5), 25 °C, 20 h.

Having screened various isocyanides and investigated the diversity of payloads that could be incorporated at the play stage of our 'plug-and-play' strategy, we set out to study the aldehyde scope of this transformation, utilising **2i** as a model isocyanide. The previously mentioned TAMRA aldehyde **1a** and commercially available aldehydes **1c**-**1r** were thus evaluated and a strong influence of stereoelectronic effects on average DoC and conversion values were observed (Figure 6, a). Degrees and patterns of substitution were found to play an important role as exemplified by linear valeraldehyde **1c** giving poor results compared to its branched analogue **1d**, or by pivaldehyde **1g**, showing a limited reactivity as opposed to isobutyraldehyde **1e** or methyl isopropyl ketone **1f**. Interestingly, cyclohexanecarboxaldehyde **1h** yielded better conversion and narrower DoC distribution (D1 to D5) than **1e**, a feature already observed when isocyanides **2b** and **2c** were compared. More polar aldehydes **1i**-**1l** gave contrasted results: while both glucose and glutaraldehyde failed to conjugate trastuzumab, formaldehyde and glyceraldehyde performed very well, even though the latter gave mass spectra of poor quality, possibly indicating side reactions. Screening aromatic aldehydes, the previously reported 2-pyridinecarboxyaldehyde **1m** was the only one to give positive results,[19] the five others (**1n**-**1r**) leading at best to mediocre conversions and DoC values. While this could be explained by a lack of solubility for compounds **1q** and **1r**, the inefficacy of **1n** and **1o** is puzzling, especially considering their known use for the bioconjugation of proteins.[45] Taken together, these results tend to confirm our hypothesis that the reactive bioconjugation sites could be located in hydrophobic clefts, explaining why small and flexible apolar carbonyls and isocyanides seem to be the best fit for this reaction.

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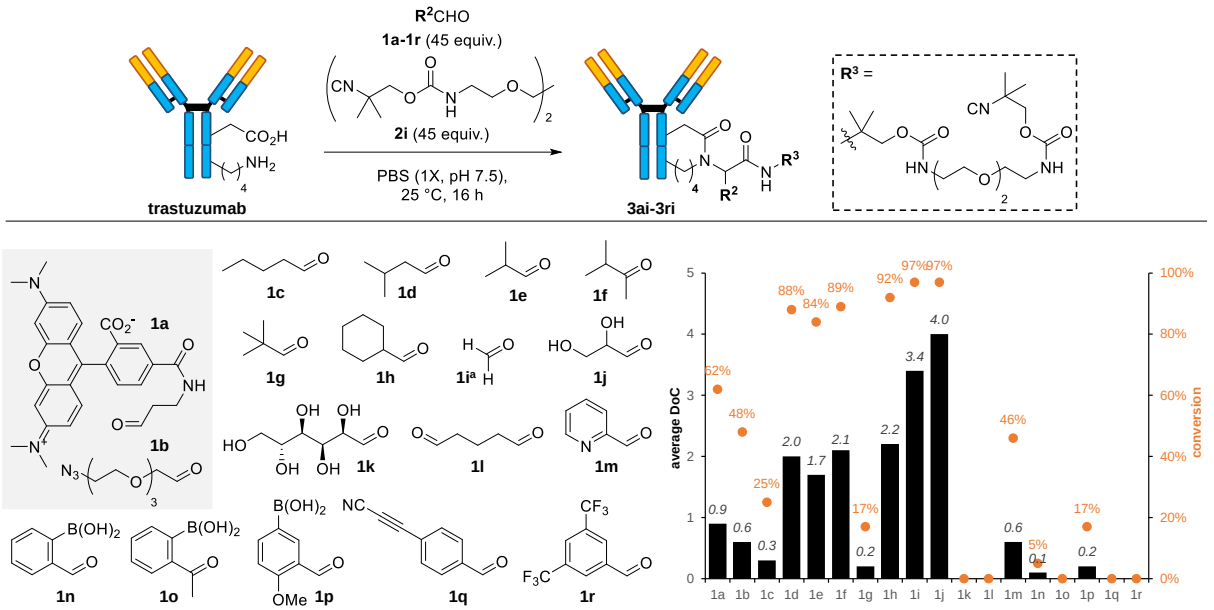

Figure 6: Aldehyde and protein scopes of the multicomponent bioconjugation reaction. **(a)** Aldehyde scope. Reaction conditions: **1a**-**1r** (45 equiv.), **2i** (45 equiv.), PBS 1x (137 mM NaCl, pH 7.5), 25 °C, 16 h; [a]: 5 equiv. used

Having developed an efficient, operationally simple and quick multicomponent bioconjugation strategy that tolerates various reactive groups and substrates, we turned to LC-MS/MS studies to validate the mechanism of the bioconjugation reaction and localize the exact conjugation sites in order to find out whether our strategy could be coined site-specific. Peptide mapping was thus conducted on three separate batches that were prepared and purified in different manners. A first batch of **5a** with an average DoC of 0.6 was generated from conjugate **3bc** after SPAAC with the BCN-cyanine-5 **4a** and analysed without further purification (protein sequence coverage: 100%). Due to the presence of unconjugated trastuzumab yielding peptides that outcompeted the conjugated fragments for mass detection, two enriched batches were produced by two similar but distinct routes (protein sequence coverage: 31-48%). A first strategy utilized conjugate **5c** – synthesized from **3bc** and the BCN-biotin derivative **4c**. Trypsin digestion followed by affinity purification of the digestate on streptavidin column gave a first enriched sample. A second strategy inverted the order of events: conjugate **3bc** was digested before the functionalization with strained alkyne **4c**. The resulting mixture of peptides was then enriched in the same manner as for the first strategy (see ESI for more details). Taken altogether, these analyses highlighted six conjugation sites, only two of which being detected in all three batches: the *N*-terminal glutamate (E1) of the heavy chain, and the *N*-terminal aspartate (D1) of the light chain. Remarkably, both of these residues partook in two different types of multicomponent reaction: an intramolecular U-4C-3R, yielding β- and γ-lactams (from D1 and E1 respectively), and a Passerini reaction, involving only the side chains carboxylate functions (detected only in the two enriched samples for D1, but in all three batches for E1). The four other conjugation sites were all detected in only a single batch and could thus not be cross-validated. Three of them were found to correspond to glutamate residues modified by a Passerini reaction: the light-chain E195, and the heavy-chain E236 and E348. The expected inter-residue U-4C-3R was finally found to be responsible for the fourth conjugation site detected, located on the light chain and involving the side chains of K126 and either D122 or E123 (undistinguishable from each other). While such inter-chain conjugation could impact the protein's folding state, analyses of native trastuzumab and conjugate **3bc** by circular dichroism spectroscopy showed no influence of the chemical conjugation on the antibody's secondary structure (see ESI). Interestingly, the heavy-chain *C*-terminal lysine (K478) did not react in either a U-4C-3R or a Passerini reaction, and was found to be unaffected. Finally, no intermolecular reactions (i.e. cross-linking between two antibodies) could ever be detected, which could be explained by the high-dilution conditions coupled with the prohibitive steric repulsion that is required to drive the reaction between two proteins in the absence of a chemical spacer or linker.

While the number of modification sites on trastuzumab is certainly too high to call this strategy site-specific, the *N*-terminal modification of both chains coupled to the excellent aspartate/glutamate selectivity and the first example of a dual residue modification represent extremely interesting features

that could open new avenues towards the development of native antibodies site-specific conjugation strategies.

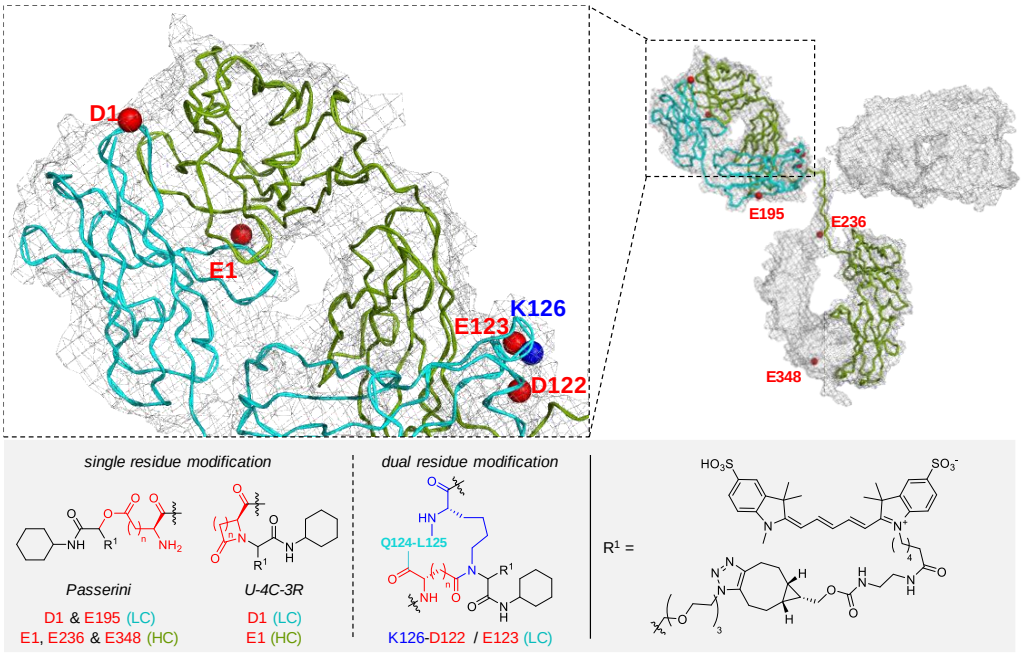

Figure 7: Trastuzumab conjugation sites determined by peptide mapping

In conclusion, we reported the use of a new multicomponent strategy for the conjugation of trastuzumab. By combining commercially available – or easily accessible – aldehydes and isocyanides, this modular approach gives a facile and rapid access to functionalized antibodies incorporating various payloads. Most notably, this strategy was used to synthesize an ADC that retained its selectivity towards HER2-positive cancer cell lines and was found to be as active as the benchmark T-DM1. Two reactions were proved to compete with each other in this conjugation process, the expected Ugi four-centre three-component reaction (U-4C-3R) and the Passerini reaction, both of which occurred at the *N*-terminal aspartate and glutamate residues. While not being fully effective at the present, such an approach could be an excellent stepping stone towards the development of a yet unreported *N*-terminal-Asp/Glu selective strategy. Moreover, this strategy represents the first example of an aspartate/glutamate selective conjugation method that is not using activating agents for their intermediate conversion to activated esters – strategies that often yield undesired protein cross-linking. Overall, these results offer a new approach towards a site-specific bioconjugation strategy that could be applied to native proteins and highlight the potential of multicomponent reactions for this purpose.

Supporting Information

The Supporting Information includes: Experimental procedures and characterization, additional information, Figures S1–S12, Tables S1–S4, NMR and native MS spectra of newly synthesized compounds.

Author Information

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Author Contributions

The work was conceived by G.C. All reactions were carried out by C.S. Cytotoxic studies were done by I.D. Native mass spectrometry analyses and peptide mapping were performed by S.H., S.E, A. E. and T. B. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Conflicts of interest

There are no conflicts of interest to declare.

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Investigating Ugi / Passerini multicomponent reactions for the Site-Selective Conjugation of Native Trastuzumab

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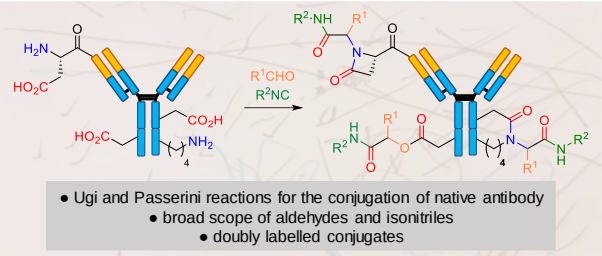

Abstract. Ugi and Passerini reactions were found to be effective strategies for the conjugation of native trastuzumab. The use of functionalized aldehydes and isocyanides allows for the smooth introduction of various payloads onto the antibody, including the cytotoxic drug MMAE.