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Trifunctional Dibromomaleimide Reagents Built Around A Lysine Scaffold Deliver Site-selective Dual-modality Antibody Conjugation

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We describe the synthesis and application of a selection of trifunctional reagents for the dual-modality modification of native, solvent accessible disulfide bonds in trastuzumab. The reagents were developed from the dibromomaleimide (DBM) platform with two orthogonal clickable functional groups built around a lysine core. We also describe the development of an aryl diselenide additive which enables antibody disulfide reduction in 4 minutes and a rapid overall reduction-bridging-double click sequence.

Introduction

Antibody-drug conjugates (ADCs) are amongst the fastest growing classes of drugs for use in oncology. An ADC is an antibody loaded with a cytotoxic payload *via* a chemical linker; combining the potency of the drug with the cellular selectivity of the antibody.[1] There have been 13 ADCs approved by the U.S. Food and Drug Administration (FDA).[2] In addition to ADCs, antibody conjugation is also employed in diverse alternative applications, such as imaging-agents,[3] the half-life extension of antibody fragments,[4] and the construction of bispecifics.[5] The preparation of antibody conjugates is traditionally achieved by the modification of accessible native amino acid residues on the protein's surface, most commonly nucleophilic lysine or cysteine side chains.

Lysine conjugation is prevalent in antibody modification due to the stable amide bonds generated. There are over 80 lysine residues in a typical IgG1 antibody and at least 40 of them are solvent accessible for modification.[6,7] Lysine residues are abundant, and randomly distributed on the antibody. Therefore, conjugation to lysine residues inevitably yields a heterogeneous mixture, which comprises of the antibodies with different numbers of payloads, and at varying conjugation sites.[8] Cysteine conjugation is commonly preferred when site-selective attachment is desired, due to its lower natural abundance. There are 4 interchain solvent-accessible disulfide bonds in total, located in the Fab and hinge region of IgG1 antibody (which represents the dominant isotype used). The conjugation of reactive electrophilic reagents, such as maleimides, to free cysteine residues *via* partial reduction of disulfide bonds commonly results in a mixture with drug-to-antibody ratios (DARs) of 0, 2, 4, 6, 8.[9] Some ADCs with high DARs have been demonstrated to have reduced stability,[10,11] and rapid *in vivo* clearance, due to increased hydrophobicity; ultimately leading to lower tumour-killing effects.[12] To resolve issues caused by conventional cysteine conjugation, antibodies with engineered cysteine residues have been developed.[13] Such mutants can generate ADCs with lower and more controlled DARs, though their preparation is more complex. Selective antibody conjugation can also be achieved *via* the incorporation of unnatural amino acids (UAAs),[14,15] enzyme-directed modification,[16,17,18] or Tub-tag labelling.[19,20,21] Alternatively, site-selective native cysteine conjugation can be achieved by 'bridging' the two cysteines that are released from one disulfide bond.[22,23,24,25] This technique of disulfide bridging can load one drug to each disulfide bond, generating ADCs with a controlled DAR of approximately 4 in IgG1s.[26,27,28] Developed disulfide bridging reagents include next generation maleimides (NGMs),[29,30,31] pyridazinediones (PDs),[32,33,34] bis-sulfones,[35,36,37] divinylpyrimidines,[38,39] divinyltriazines,[39,40] arylenedipropiolonitriles,[41] dichlorotetrazines,[42] and others.[43,44,45,46]

We have previously illustrated dibromomaleimides (DBMs, also known as NGMs), which retain the rapid kinetics and cysteine selectivity of maleimides whilst efficiently inserting into disulfide bonds.[47,48,49] As part of this work, we optimised these reagents to identify that electron-withdrawing groups on the nitrogen of the DBM are crucial for enabling efficient post-conjugation hydrolysis of NGMs to generate robust serum-stable conjugates.[49] However, most antibody conjugation methodologies are designed to attach a single drug, or other functional moiety, which might limit their efficacy and breadth of applications. As an example, for complex diseases, ADCs with more than one cytotoxic payloads are highly sought after, to provide orthogonal modes of action.[50] New methods for dual-modality bioconjugation broadly enable the incorporation of two different functionalities[51,52,53] and disulfide-bridging dual-

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conjugates have shown promising opportunities including the construction of bispecific conjugates, synthetic antibodies, and controlled-loading antibody-payload conjugates.[54,55,56] In this work, we aimed to explore a convenient trifunctional scaffold for DBM reagents, which would facilitate further research and applications of this reagent class. We thus designed a selection of DBMs, built around a lysine core, incorporating two orthogonal clickable handles for selective antibody modification to give dual-modality antibody conjugates. Functionally relevant motifs such as fluorophores and biotin were attached, to demonstrate versatility of the attachment and as a model for payload conjugation. Finally, inspired to explore opportunities to accelerate antibody conjugation protocols, we describe a new aryl selenide additive which catalyses rapid disulfide reduction and enables an overall 20-minute reduction-bridging-double click conjugation sequence.

Results and Discussion

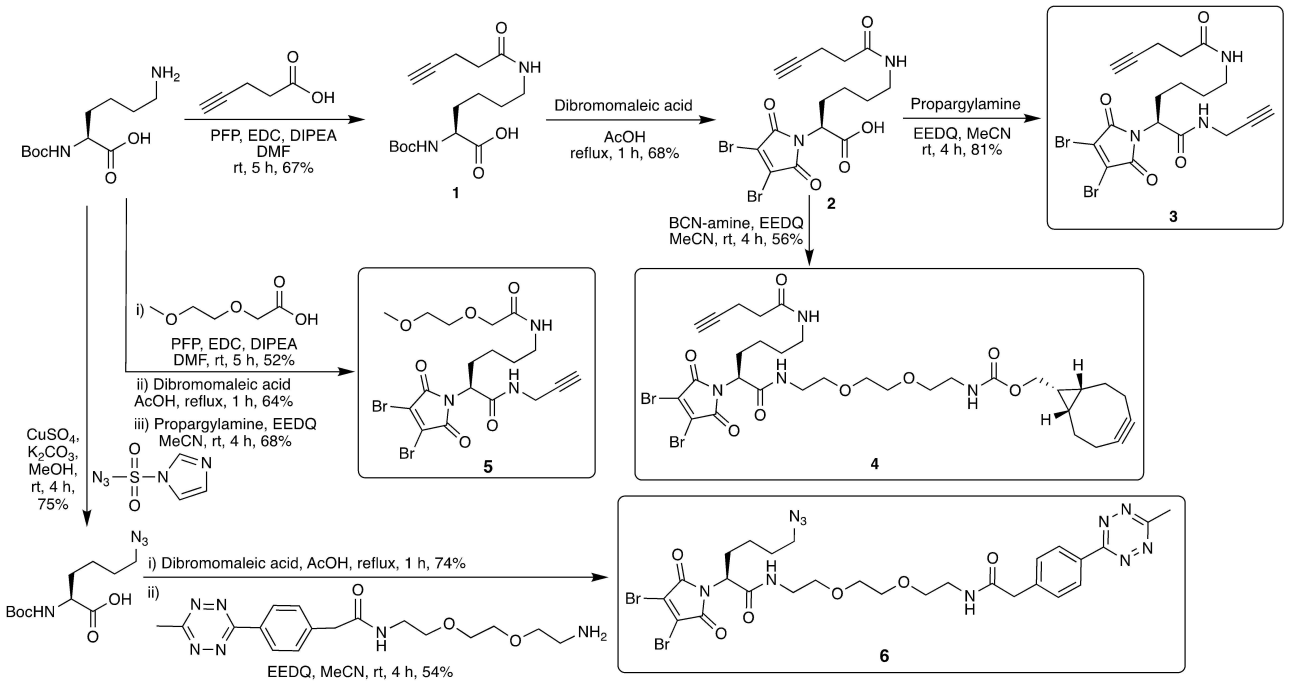
Design of a DBM platform for trifunctionalisation

We envisaged that a lysine core could be conveniently exploited for the syntheses of a selection of dual-modality DBM reagents. The N-terminus could be functionalised with the DBM, whilst the side-chain amine and the C-terminus could undergo amide coupling separately to introduce clickable handles. The use of an amino-acid core in this way would also ensure rapid post-conjugation hydrolysis of the DBM conjugates, due to the electron-withdrawing effect of the amide.[49,57,58] Linear alkyne, strained alkyne, azide, and tetrazine were considered as clickable handles, as they could undergo copper(I)-catalysed alkyne-azide cycloaddition (CuAAC), strain-promoted azide-alkyne cycloaddition (SPAAC) and inverse electron demand Diels-Alder (IEDDA) reactions.[59,60]

Using Boc-lysine as the starting point, 4-pentynoic acid was coupled to the side-chain amine, loading a linear alkyne to afford scaffold **1** (Scheme 1). DBM-alkyne **2** was then accessed by a one-pot Boc-deprotection and condensation with dibromomaleic acid. Finally, the carboxylic acid was coupled to either propargylamine to give bis-alkyne **3**, or BCN-amine to yield orthogonally clickable DBM-alkyne-BCN reagent **4**. This same synthetic strategy was employed to access DBM-alkyne-PEG **5** simply substituting in 2-(2-methoxyethoxy)acetic acid in the initial coupling reaction. This represents a model reagent for the attachment of PEG to antibody conjugates, which has been reported to aid hydrophilicity and therapeutic indexes in ADCs,[61] or for half-life extension by PEGylation.[62,63] Finally, DBM-azide-tetrazine **6** was prepared by converting the lysine side-chain amine to an azide *via* diazo-transfer, then DBM attachment and coupling of a tetrazine-amine (Scheme 1). Overall, by starting with a Boc-lysine scaffold, this represents an extremely efficient strategy for the syntheses of a range of clickable DBM reagents.

Functionalisation of trastuzumab Fab

The monoclonal antibody trastuzumab is an FDA-approved therapeutic for HER2+ breast cancers.[64] The Fab fragment of trastuzumab was selected for initial functionalisation, as it contains a single disulfide bond and such Fab conjugates are of clinical interest due to the potential for enhanced tumour penetration and tunable half-lives for drug delivery.[65,66,67] A



Scheme 1. Synthesis of trifunctional DBM reagents **1**–**6**.

sequential bioconjugation protocol, in which reduction of the disulfide bond was followed by the addition of bridging reagents without intermediate purification, was applied. As a model for functional click-conjugation and to allow convenient analysis, photostable and water-soluble fluorophores Azide-Fluor 488 (AF488), Sulfo-Cyanine5.5-azide (Cy5.5-azide) and 5-Carboxyfluorescein-PEG$_3$-BCN (BCN-Fluor) were chosen, along with biotin, for prospective diverse applications utilising its affinity to streptavidin.[68,69]

Upon reduction, trastuzumab Fab was bridged with trifunctional DBMs **3**, **4**, **5**, and **6**, efficiently yielding Fab-DBM-clickable handle conjugates **7**, **8**, **9**, and **10** (Figure 1). The alkyne-containing conjugates were then functionalised with AF488 *via* CuAAC or SPAAC reactions to give Fab-DBM-AF488 conjugates **11**, **12**, and **13**. In the case of bis-alkyne, two fluorophores were attached (conjugate **11**), representing a viable approach to change the stoichiometry of attachments to two per bridged disulfide. Conjugate **12** was converted to dual-modality conjugate **15** by CuAAC; notably the copper in the CuAAC leads to less well resolved LC–MS spectra (SI Figure S13), which is presumed to be due to imperfect copper removal in the ultrafiltration. The strained alkyne was also found to be sensitive to a small amount (approximated 8%, SI Figure S10) of deactivation (presumed to be oxidation) over the course of the overnight hydrolysis step, and thus we would recommend the SPAAC being carried out prior to the hydrolysis. Precluding the copper issues, Fab-DBM-azide-tetrazine conjugate **10** was functionalised with a *trans*-cyclooctene (TCO)-biotin,[60] followed by BCN-Fluor to yield dual-modality conjugate **16** (Figure 1b for LC–MS at each stage). SDS-PAGE further provided supporting

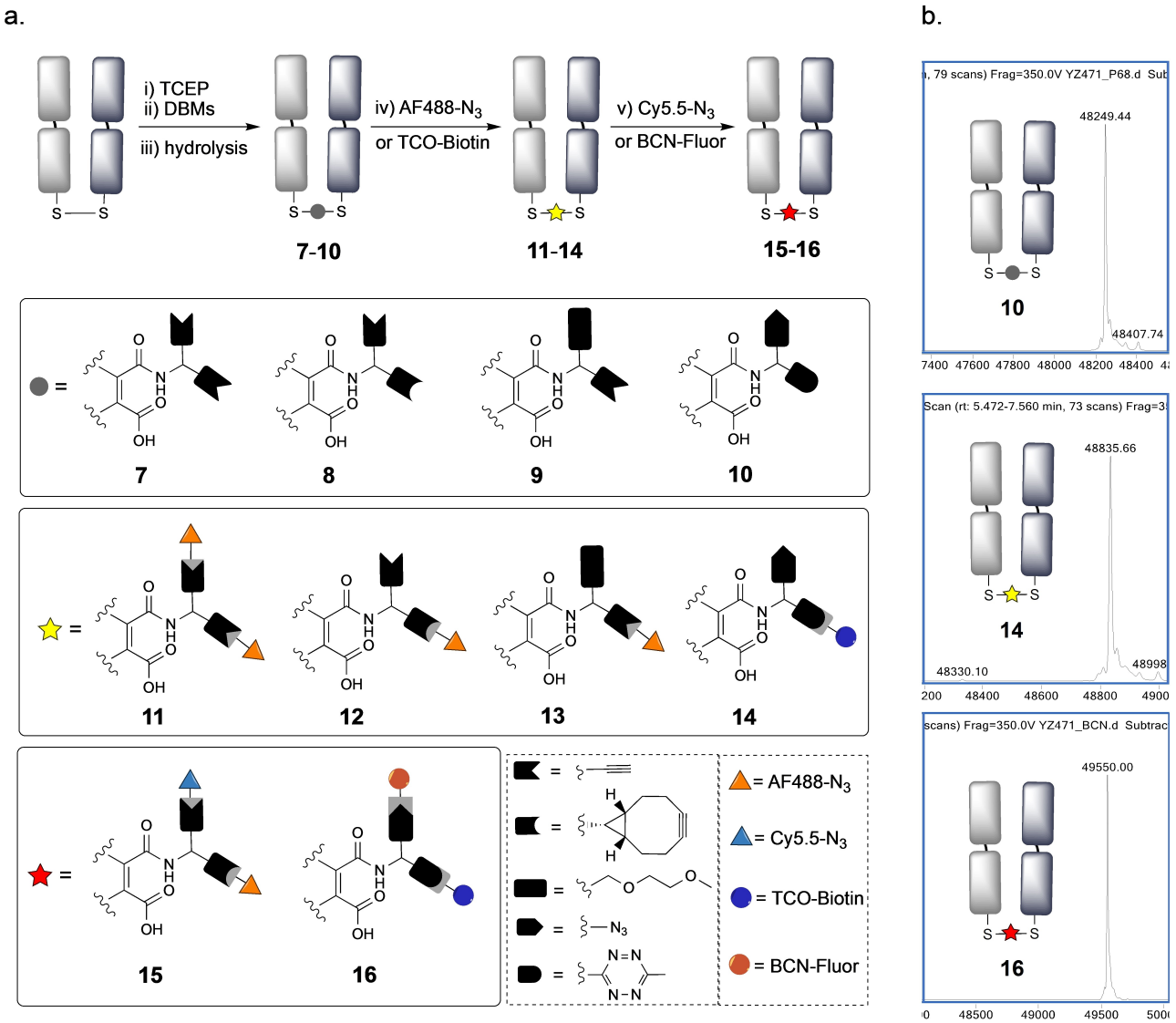


Figure 1. Dual-modality functionalisation of trastuzumab Fab: a. i) 3 eq. TCEP, 37 °C, 1.5 h, pH 8.5; ii) 5 eq. DBMs **3**, **4**, **5** or **6**, 22 °C, 1 h, pH 8.5; iii) ultrafiltration, then 37 °C, 16 h, pH 8.5; iv) 60 eq. THPTA, 12 eq. $CuSO_4$, 10 eq. AF488, 10 mM sodium ascorbate, 22 °C, 4 h, pH 7.0 (Conjugates **11** and **13**) or 10 eq. AF488, 22 °C, 4 h, pH 7.0 (Conjugate **12**) or 10 eq. TCO-Biotin 22 °C, 4 h, pH 7.0 (Conjugate **14**); v) 60 eq. THPTA, 12 eq. $CuSO_4$, 10 eq. Cy5.5-azide, 10 mM sodium ascorbate, 22 °C, 16 h, pH 7.0 (Conjugate **15**) or 10 eq. BCN-Fluor, 22 °C, 4 h, pH 7.0 (Conjugate **16**). b. LC–MS of Fab conjugate **10**: HL expected 48250 Da, HL observed 48249 Da; LC–MS of Fab conjugate **14**: HL expected 48837 Da, HL observed 48836 Da; LC–MS of Fab conjugate **16**: HL expected 49563 Da, HL observed 49550 Da.

evidence for the construction of all these conjugates (SI Figures S26-S27).

Functionalisation of trastuzumab antibody

Full IgG conjugates are the major focus of clinical efforts towards ADCs, and hence focus next shifted to native trastuzumab conjugation. Bridging with trifunctional DBMs **3**–**6** successfully generated clickable conjugates **17**–**20**, and subsequent click conjugations afforded the desired targeted array of mono- and dual-modality conjugates **21**–**26** (Figure 2a). Again the DBM-azide-tetrazine reagent **6** avoided issues with copper use, affording the most well resolved LC–MS analysis (Figure 2b), with the expected mixture of full antibody with 4+4 attachments and the half-antibody with 2+2 attachments (observed due to well-known hinge disulfide scrambling, and only detectable due to harsh denaturing conditions of LC–MS[70]). Densitometry analysis of the SDS-PAGE (SI Figures S28–S29) indicated the ratio of full antibody to half-antibody was approximately 7:3. Currently, no studies have suggested that

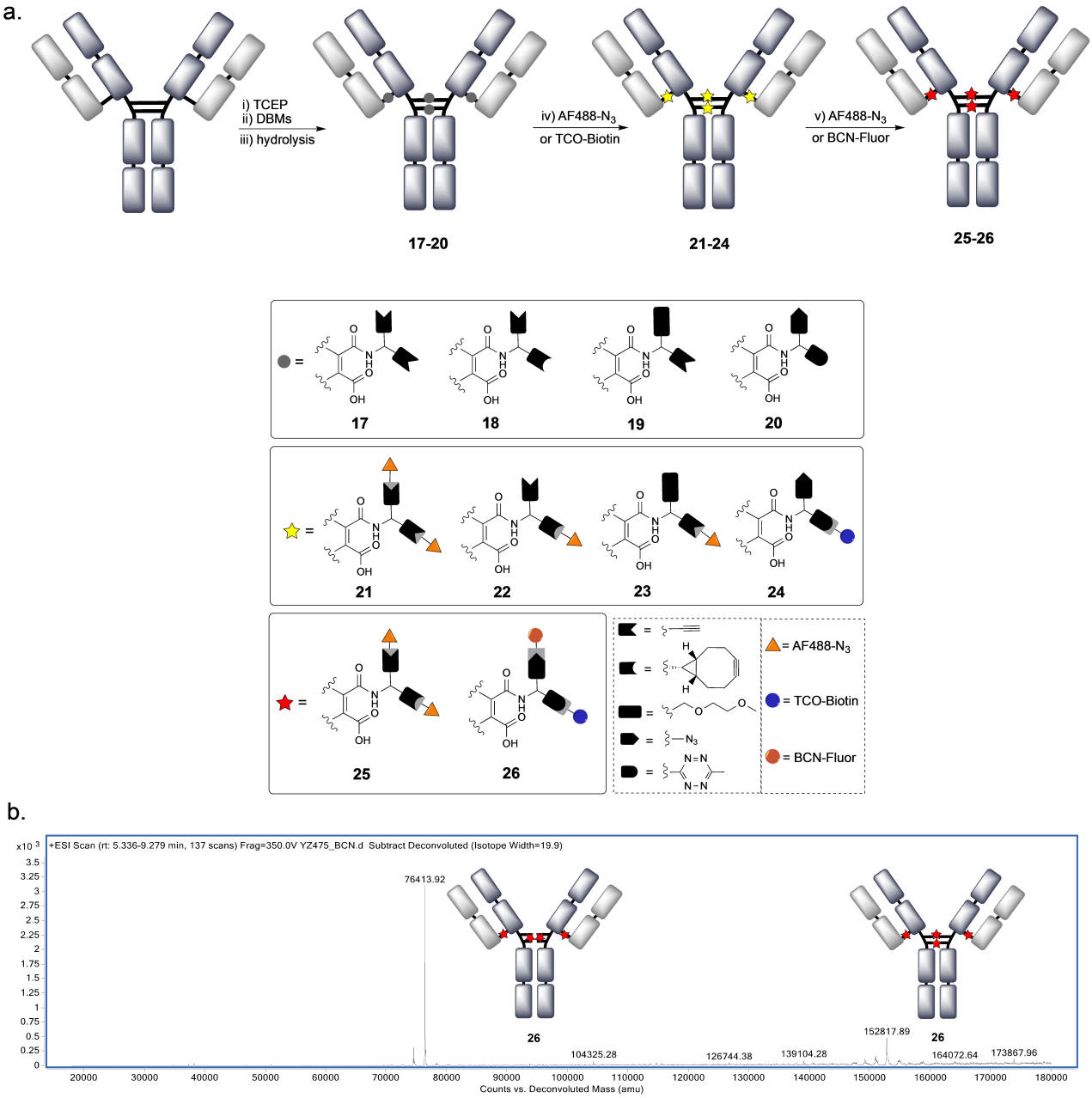


Figure 2. Dual modality functionalisation of trastuzumab antibody: a. i) 8 eq. TCEP, 37 °C, 2 h, pH 8.5; ii) 15 eq. DBMs **3**, **4**, **5** or **6**, 22 °C, 1 h, pH 8.5; iii) ultrafiltration, then 37 °C, 16 h, pH 8.5; iv) 180 eq. THPTA, 36 eq. $CuSO_4$, 20 eq. AF488, 10 mM sodium ascorbate, 22 °C, 4 h, pH 7.0 (Conjugates **21** and **23**) or 20 eq. AF488, 22 °C, 4 h, pH 7.0 (Conjugate **22**) or 20 eq. TCO-Biotin, 22 °C, 4 h, pH 7.0 (Conjugate **24**); v) 180 eq. THPTA, 36 eq. $CuSO_4$, 20 eq. AF488, 10 mM sodium ascorbate, 22 °C, 16 h, pH 7.0 (Conjugate **25**) or 20 eq. BCN-Fluor, 22 °C, 16 h, pH 7.0 (Conjugate **26**). b. LC–MS of Ab conjugate **26**: HL expected 76441 Da, HL observed 76414 Da, HHLL expected 152879 Da, HHLL observed 152818 Da.

half-antibody species would negatively affect the *in vivo* profile of such an ADC.[7]

As a final challenge of these trifunctional reagents, we set out to challenge how rapidly the bioconjugation sequence could be achieved. The first step involves the reduction of the full antibody, which requires TCEP (8 eq.) for 2 h at 37 °C. To accelerate this step, aryl selenols attracted our attention, as we had previously identified that benzeneselenol can deliver reduction of disulfides in antibodies.[71,72] However, the lack of water solubility and pungent odour of this reagent discouraged its widespread use in such applications. Therefore, we explored a range of aryl selenols with potentially improved properties and tuned reactivity (Figure 3a). As aryl selenols are prone to rapid oxidation, the corresponding aryl diselenides **27**–**32** were synthesised instead and reduced with TCEP immediately before application to trastuzumab Fab (SI Figure S30 and Table S3). Reduction with bis(4-aminophenyl)diselenide **27**[73,74,75] was found to be most effective, being complete in just 2 minutes. It was also confirmed to be a much more practicable analogue, non-pungent and with improved aqueous solubility. Interestingly, ultrafiltration was required straight after complete Fab reduction (2 minutes), as otherwise Fab re-oxidation would start to occur (e.g. complete re-oxidation observed in 30 minutes, SI Table S3). The continued presence of TCEP in these reactions is thus crucial as it maintains the presence of selenols - once the TCEP is consumed, the diselenide is presumed to reform and promote the re-oxidation of the Fab disulfide. The success in the use of the bis(4-aminophenyl)diselenide **27**, and lack of any reduction observed for pyridyl diselenide **32** (SI Table S3) is consistent with a trend towards increased pKa of the formed aryl selenol being an important design feature. Finally, it should be noted that sub-stoichiometric amounts of the diselenide **27** (0.5 eq.) could be employed with TCEP (2 eq.) for complete Fab reduction, and as such these reagents may serve as useful catalytic additives more widely to accelerate protein disulfide reductions.

On the full antibody, an optimised procedure of 4-minute reduction with bis(4-aminophenyl)diselenide **27** (4 eq.) and TCEP (30 eq.) delivered full antibody reduction. Finally, the dual-modality full antibody conjugate was accessed in 20 min total

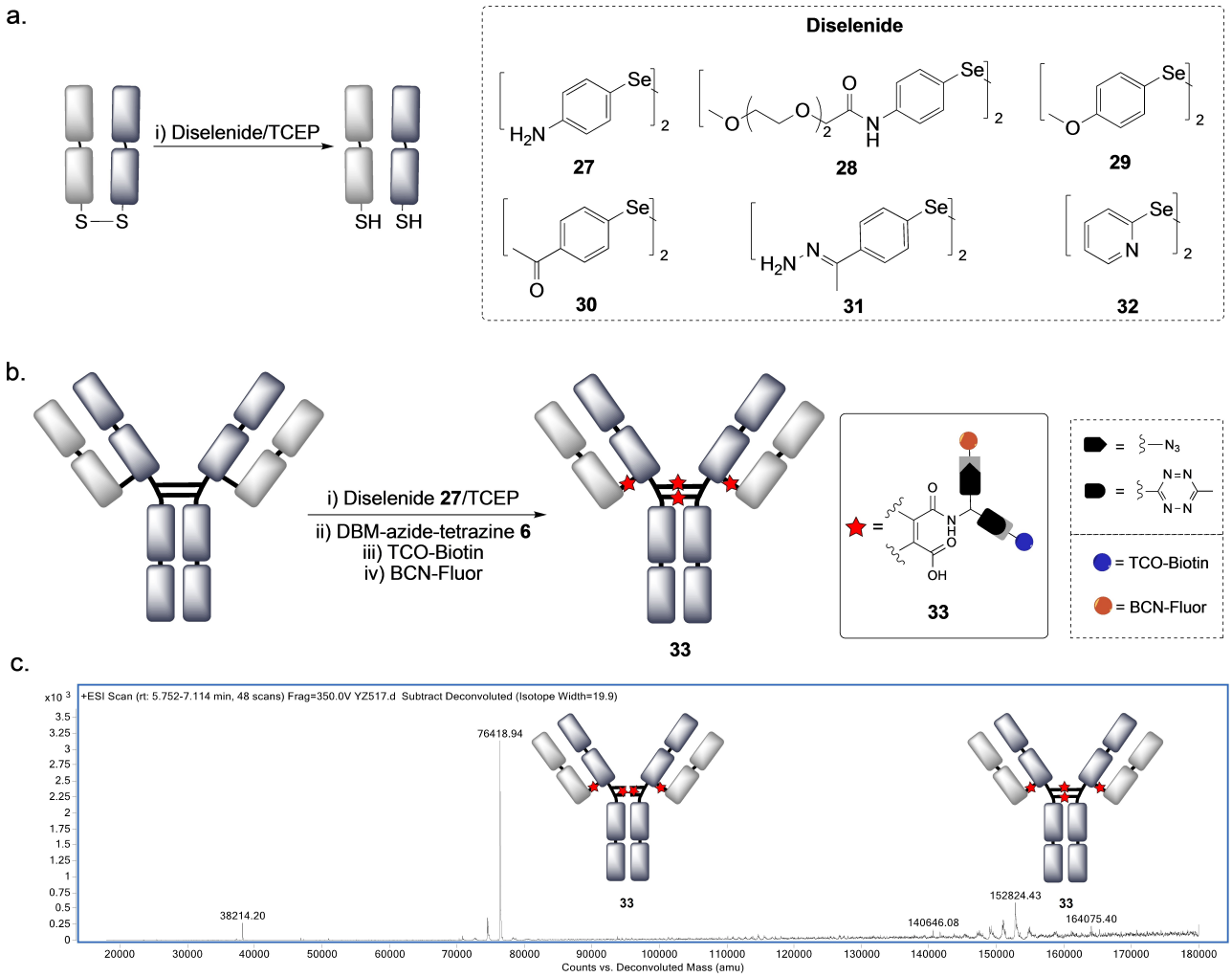


Figure 3. Selenol-catalysed reduction on trastuzumab Fab and antibody: a. Reduction on trastuzumab Fab using aryl diselenides **27**–**32**: i) 2 eq. diselenide **27**–**32**, 4 eq. TCEP, 22 °C, 2–30 min, pH 7.4. b. Reduction-bridging-IEDDA-SPAAC sequence on trastuzumab antibody using aryl diselenide **27**: i) 4 eq. diselenide **27**, 30 eq. TCEP, 22 °C, 4 min, pH 7.4; ii) 10 eq. DBM-azide-tetrazine **6**, 37 °C, 5 min, pH 7.4; iii) 15 eq. TCO-Biotin, 37 °C, 1 min, pH 7.4; iv) 20 eq. BCN-Fluor, 37 °C, 10 min, pH 7.4. c. LC–MS of Ab conjugate **33**: HL expected 76441 Da, HL observed 76419 Da, HHLL expected 152879 Da, HHLL observed 152824 Da.

time, *via* a selenol-catalysed reduction-bridging-IEDDA-SPAAC 4-step sequence to afford the trifunctional antibody conjugate **33** (Figure 3b–c and SI Figure S31). In this case, hydrolysis to create the desired serum stable maleamic acids had taken place during preparation of the samples for LC–MS. Further analogues of such diselenides can be readily envisaged, to offer further enhancements in water solubility and rates of disulfide reduction, and this work is ongoing in our laboratory.

Conclusions

In summary, we have efficiently synthesised a selection of trifunctional DBM reagents built around a lysine core, which can deliver dual-modality antibody conjugates from native antibodies by disulfide bridging and well-tolerated "click" chemistry. This strategy is highly flexible, as a range of corresponding click reagents can be readily attached to produce a diverse array of conjugates. Furthermore, we have developed a new practicable aryl diselenide additive for rapid antibody disulfide reduction. We demonstrate its use in accelerating the first step in disulfide bridging conjugation of antibodies, facilitating a rapid sequence to construct dual-modality conjugates.

Experimental Section

Chemical synthesis

Complete syntheses and characterisations of all compounds are described in the SI.

Antibody conjugation

Details of preparation, LC–MS, UV-Vis spectra and SDS-PAGE of antibody conjugates are listed in the SI. Optimised bioconjugation protocols on trastuzumab Fab and antibody using DBM-azide-tetrazine **6** are demonstrated here as an example:

Preparation of trastuzumab Fab conjugate 16 *via* TCEP reduction-DBM bridging-IEDDA-SPAAC sequence

To trastuzumab Fab (100 μL, 0.012 μmol, 120 μM, 5.72 mg/mL) in BBS buffer (pH 8.5) was added TCEP.HCl (3.6 μL, 0.036 μmol, 10 mM solution in dH_2O, 3 eq.) and the reaction mixture was incubated at 37 °C for 1.5 h. DBM-azide-tetrazine **6** (6 μL, 0.060 μmol, 10 mM solution in anhydrous DMF, 5 eq.) was then added and the reaction mixture was incubated at 22 °C for 1 h. The excess reagents were then removed *via* ultrafiltration (10 kDa MWCO) into BBS buffer (pH 8.5). The concentration was determined by UV-Vis absorbance and adjusted to 100 μM. The resultant solution was incubated at 37 °C for 16 h. The final trastuzumab Fab conjugate **10** was desalted (7 kDa MWCO) and characterised by LC–MS.

To trastuzumab Fab-DBM conjugate **10** (100 μL, 0.010 μmol,100 μM, 4.77 mg/mL) in PB (pH 7.0) was added TCO-Biotin (5 μL, 0.100 μmol, 20 mM solution in anhydrous DMF, 10 eq.). The reaction mixture was incubated at 22 °C for 4 h. The excess reagents were then removed *via* PD column and ultrafiltration (10 kDa MWCO) into EDTA conjugation buffer (pH 7.0). The final trastuzumab Fab conjugate **14** was desalted (7 kDa MWCO) and characterised by LC–MS.

To trastuzumab Fab-DBM-Biotin conjugate **14** (50 μL, 0.005 μmol,100 μM, 4.77 mg/mL) in PB (pH 7.0) was added 5-carboxyfluorescein-PEG_3-BCN (BCN-Fluor, 2.5 μL, 0.050 μmol, 20 mM solution in anhydrous DMF, 10 eq.). The reaction mixture was incubated at 22 °C for 4 h. The excess reagents were then removed *via* PD column and ultrafiltration (10 kDa MWCO) into EDTA conjugation buffer (pH 7.0). The final trastuzumab Fab conjugate **16** was desalted (7 kDa MWCO) and characterised by LC–MS.

Preparation of trastuzumab antibody conjugate 26 *via* TCEP reduction-DBM bridging-IEDDA-SPAAC sequence

To trastuzumab Ab (100 μL, 0.002 μmol, 24 μM, 3.48 mg/mL) in BBS buffer (pH 8.5) was added TCEP.HCl (1.9 μL, 0.019 μmol, 10 mM solution in dH_2O, 8 eq.) and the reaction mixture was incubated at 37 °C for 2 h. DBM-azide-tetrazine **6** (3.6 μL, 0.036 μmol, 10 mM solution in anhydrous DMF, 15 eq.) was then added and the reaction mixture was incubated at 22 °C for 1 h. The excess reagents were then removed *via* ultrafiltration (10 kDa MWCO) into BBS buffer (pH 8.5). The concentration was determined by UV-Vis absorbance and adjusted to 20.0 μM. The resultant solution was incubated at 37 °C for 16 h. After this time, the sample was buffer exchanged into ammonium acetate (pH 6.9) *via* ultrafiltration (10 kDa MWCO). The final trastuzumab conjugate **20** was deglycosylated and characterised by LC–MS.

To trastuzumab Ab-DBM conjugate **20** (100 μL, 0.002 μmol, 20 μM, 2.90 mg/mL) in PB (pH 7.0) was added TCO-Biotin (2.0 μL, 0.040 μmol, 20 mM solution in anhydrous DMF, 20 eq.). The reaction mixture was incubated at 22 °C for 4 h. The excess reagents were then removed *via* PD column and ultrafiltration (10 kDa MWCO) into EDTA conjugation buffer (pH 7.0). After this time, the sample was buffer exchanged into ammonium acetate (pH 6.9) *via* ultrafiltration (10 kDa MWCO). The final trastuzumab conjugate **24** was deglycosylated and characterised by LC–MS.

To trastuzumab Ab-DBM-Biotin conjugate **24** (60 μL, 0.001 μmol, 20 μM, 2.90 mg/mL) in PB (pH 7.0) was added BCN-Fluor (1.2 μL, 0.024 μmol, 20 mM solution in anhydrous DMF, 20 eq.). The reaction mixture was incubated at 22 °C for 16 h. The excess reagents were then removed *via* PD column and ultrafiltration (10 kDa MWCO) into EDTA conjugation buffer (pH 7.0). After this time, the sample was buffer exchanged into ammonium acetate (pH 6.9) *via* ultrafiltration (10 kDa MWCO). The final trastuzumab conjugate **26** was deglycosylated and characterised by LC–MS.

Preparation of trastuzumab antibody conjugate 33 *via* selenol-catalysed reduction-DBM bridging-IEDDA-SPAAC sequence

To trastuzumab Ab (50 μL, 0.002 μmol, 50 μM, 7.25 mg/mL) in BBS (pH 7.4) was added premixed Bis(4-aminophenyl)diselenide **27** (1.0 μL, 0.010 μmol, 10 mM solution in anhydrous MeOH, 4 eq.) and TCEP.HCl (7.5 μL, 0.075 μmol, 10 mM solution in dH_2O, 30 eq.). The reaction mixture was incubated at 22 °C for 4 min. The excess reagents were then removed *via* ultrafiltration (10 kDa MWCO) into BBS buffer (pH 7.4). To the reaction mixture, DBM-azide-tetrazine **6** (2.5 μL, 0.025 μmol, 10 mM solution in anhydrous DMF, 10 eq.) was added and then incubated at 37 °C for 5 min. To the reaction mixture, TCO-Biotin (1.9 μL, 0.038 μmol, 20 mM solution in anhydrous DMF, 15 eq.) was added and incubated at 37 °C for 1 min. BCN-Fluor (2.5 μL, 0.050 μmol, 20 mM solution in anhydrous DMF, 20 eq.) was then added and incubated at 37 °C for 10 min. The

excess reagents were then removed *via* PD column and ultrafiltration (10 kDa MWCO) into EDTA conjugation buffer (pH 7.0). After this time, the sample was buffer exchanged into ammonium acetate (pH 6.9) *via* ultrafiltration (10 kDa MWCO). The final trastuzumab conjugate **33** was deglycosylated and characterised by LC–MS.

Supporting Information

Additional references cited within the SI.[76,77,78,79,80]

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Conflict of Interests

J. R. B. and V.C. are directors of UCL spin-out ThioLogics.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: Antibodies · antibody conjugation · bioorganic chemistry · click chemistry

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