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Enabling the synthesis of multi-payload thio-antibody conjugates through the use of pyridazinediones, *p*-anisidine derivatives and various click chemistries

Clíona McMahon,[a] Christophe J. Queval,[b] Ioanna A. Thanasi,[a] Dawn H. W. Lau,[c] Michael Howell,[b] Ning Wang,[a] Maximillian T. W. Lee,[c] Josephine S. Gaynord,*[c] James R. Baker*[a] and Vijay Chudasama*[a]

In recent years, antibody–drug conjugates (ADCs) have emerged as a very promising class of targeted therapeutics, but ADC candidates still face issues such as dose-limiting toxicity and drug resistance. It has become increasingly clear that whilst there are general principles for what constitutes an effective ADC, individual ADCs require bespoke optimisation. The optimal drug-to-antibody ratio (DAR) may differ for each drug, antibody and target combination. Most recently, the use of multi-drug bearing ADCs to off-set drug resistance has aroused interest. In view of this, the modular construction of antibody conjugates with different DARs/drug classes is key to enabling the next generation of ADCs. One of the leading antibody scaffolds for making ADCs is antibodies with engineered cysteine residues (thio-antibodies). Typically, it is only the engineered site that is reacted when making ADCs from thio-antibodies, inherently limiting the potential of such conjugates. This work focuses on the development of a platform for the modular and site-selective synthesis of thio-trastuzumab mutant conjugates with defined payload-to-antibody ratios (PARs) of 1, 2, 3, 4, 5, 6, 7 and 8 by modifying engineered and native cysteines (note: in this manuscript the term payload refers to fluorophores or other functional small-molecule entities attached to an antibody). The framework to achieve this is based on using only three key molecules: a diBr-pyridazinedione (PD) bearing a strained alkyne, an azide-functionalised bisPD and a functionalised azido-aniline entity. This approach, combined with the use of clickable payloads, enables the synthesis of the full repertoire of PARs 1–8, and the ability to attach up to three different payloads, in different ratios, to thio-trastuzumab mutants. The distinct PARs and ratios of payloads can be tuned by changing the reagents used and/or the order of reagents used in combination with various clickable payload reagents. Finally, various tri-payload loaded thio-antibody conjugates were appraised *in vitro*, and were shown to bind and internalise selectively to $HER2^+$ (BT-474) over $HER2^-$ (MCF-7) cells with all payloads successfully delivered into target cells.

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Introduction

Over the past few decades, antibody–drug conjugates (ADCs) have emerged as a leading class of targeted therapeutics, with 15 ADCs receiving FDA approval over the past 15 years.[1,2] Despite considerable success, clinical trials have a high failure rate, with ADC candidates being hampered by issues such as off target-toxicity[3] and drug resistance.[4] Whilst several new toxins/antibodies/linkers have been introduced in the clinic, conjugation methods lag behind – all FDA approved ADCs use either classical lysine/cysteine conjugation (using NHS esters or maleimides, respectively).[5–7] These methods are frequently associated with heterogeneity as they indiscriminately target free lysine/cysteine residues.[8] Homogeneous ADCs, and antibody conjugates more generally, have been widely accepted to display superior properties to their heterogeneous analogues, in terms of efficacy and/or safety.[9] Considerable research has gone into developing site-selective conjugation methods over the past few years.[10] Furthermore, it is becoming increasingly clear that whilst there are general principles for what constitutes an effective ADC, individual ADCs require bespoke

[a] *Department of Chemistry, University College London, 20 Gordon Street, London, WC1H 0AJ, UK*
[b] *Screening and Automated Science Laboratory, The Francis Crick Institute, London, NW1 1AT, UK*
[c] *MSD (UK) Limited, 120 Moorgate, London, EC2M 6UR, UK.*
E-mail: v.chudasama@ucl.ac.uk

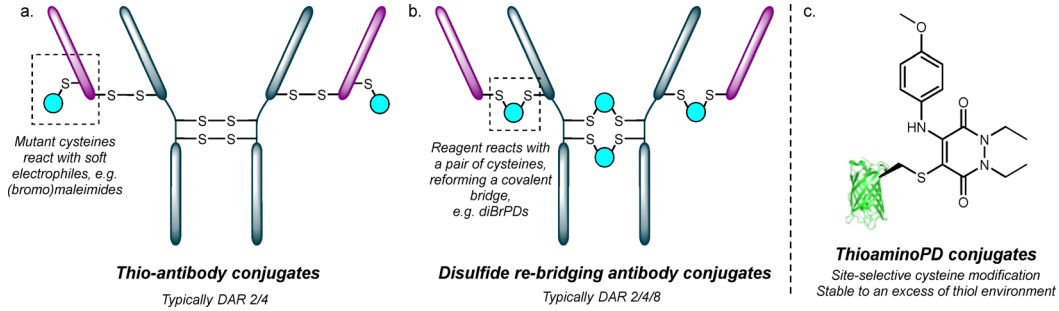

Fig. 1 Site-selective antibody conjugates can be synthesised using (a) engineered thio-antibodies or on (b) native antibodies using disulfide re-bridging (amongst other methods); (c) thioaminoPD conjugates (in this case derived from site-selective cysteine modification of GFP S147C and *para*-anisidine) are stable to an excess of thiol environment.[45]

optimisation. The optimal drug-to-antibody ratio (DAR) may differ for each drug, antibody and target combination. More potent, hydrophobic drugs may benefit from low DARs, whilst less potent drugs may be better suited to high DARs.[11,12] Most recently, the use of multi-drug bearing ADCs to off-set drug resistance has aroused interest.[13] In view of this, the modular construction of ADCs with different DARs/drug classes is highly desirable. A stepping stone to achieving this ambitious endeavour would be the plug-and-play construction of antibody-conjugates with distinct payload classes (note: in this manuscript the term payload refers to small-molecule entities such as fluorophores or other functional modules).

Strategies for site-selective antibody modification tend to be devised for use in either native or engineered antibodies.[8] That being said, there is no reason why compatible strategies that are used in native antibodies should not also work on engineered antibodies. This includes strategies based on chemoenzymatic ligation of native antibodies (*e.g.* through the use of (engineered) microbial transglutaminase (mTG)[14,15] and glycan remodelling[16]). Engineered antibodies typically have a unique reactive handle/sequence inserted into the protein which enables site-specific chemical conjugation.[8] Examples include aldehyde tags[17] (*e.g.* SMARTag[18]), SNAP-tags,[19] π-Clamp,[20] sortase-based methods,[21,22] GALaXy,[23] and the use of unnatural amino acids.[24] Most relevant to this work are antibodies that have been engineered to include reactive cysteines (*e.g.*, THIOMABs™ and other engineered cysteine-containing antibodies, hereafter referred to as thio-antibodies).[25] Thio-antibodies were originally designed to react selectively with maleimides to form DAR 2 conjugates (Fig. 1a).[25] Thio-antibody–drug conjugates have been shown to be safer and more efficacious than their non-site specifically conjugated analogues and there are now thio-antibody–drug conjugates in clinical/pre-clinical development.[26–28] Whilst thio-antibodies offer highly homogenous conjugates, odd-numbered drug loadings are often unobtainable due to the symmetry of antibodies – IgGs are composed of two identical dimers; thus it is not possible to engineer an antibody such that it contains a mutation on only one polypeptide chain. Furthermore, it is difficult to make multi-drug loaded thio-antibodies without further engineering/employing highly complex linkers.[25,29,30] Site-specific conjugation methods that are compatible with native antibodies include disulfide re-bridging (Fig. 1b),[10] glycan remodelling,[31,32] and traceless affinity peptide labelling.[33,34] Disulfide re-bridging attempts to alleviate issues associated with conventional cysteine modification of native antibodies (heterogeneity, retro-Michael deconjugation with commonly used maleimides, breaking of disulfide linkages *etc.*).[10] Reagents developed for disulfide re-bridging include dibromomaleimides,[35] bissulfone reagents (ThioBridge™),[10,36] and divinylpyrimidines.[37] One of the most widely explored class of reagents for this purpose in recent years is dibromopyridazinediones (diBrPDs),[38,39] with diBrPDs and/or bis(diBr)PDs being used to form (near) homogenous DAR 2/4/8 ADCs.[40–42] Other DARs, using disulfide re-bridging, have been achieved through other strategies, for example, Dannheim *et al.* used tetra-divinylpyrimidine linkers to make DAR 1 conjugates from a native antibody scaffold.[43] We also note that DAR 1 conjugates derived from the chemoenzymatic GlycoConnect modification of a native antibody have also been furnished and shown to be highly effective in tumour spheroids.[44]

Whilst work has been carried out to make ADCs with various DARs and/or multi-drug loaded ADCs,[29,46–48] as well as the complementary work of Journeaux *et al.* on forming quadruple-functionalised antibodies albeit not in the form of ADCs,[29] very limited work has been carried on enabling such features for the leading class of engineered antibodies for ADCs, *i.e.* thio-antibodies. In almost all cases, it is only the engineered site that is reacted when making ADCs from thio-antibodies, inherently limiting the potential of such conjugates. We theorised that diBrPDs should react with thio-antibodies as (i) they have been shown to react with single cysteine mutants[45] and (ii) they are structurally similar to bromomaleimides that have been shown to form stable, near-homogenous thio-antibody ADCs.[49] Moreover, post-conjugation, if the remaining bromine on the pyridazinedione ring could be substituted with an aniline-like molecule, the thioaminoPD small-molecule should be deactivated and stable to TCEP as they have been shown to be stable in a reducing environment (Fig. 1c).[45] This would enable the native interchain disulfide bonds to be subsequently reduced and then undergo disulfide re-bridging without affecting the previously conjugated mutant cysteines. Hence, by combining these distinct technologies (engineered cysteine modification,

native disulfide re-bridging and aniline conjugate addition) the formation of engineered cysteine containing antibody-conjugates with discrete, controlled (multi-)payload loadings in a site-selective, modular manner would be feasible.

In this manuscript, we demonstrate that by using three click handle-containing molecules – a diBrPD bearing a strained alkyne (BCN PD **1**), a bisPD containing an azide (ArN_3 bisPD **2**) and an azide functionalised aniline (N_3 aniline **3**) – in combination with clickable payloads, access to a wide range of payload loadings (payload-to-antibody ratio (PAR) from 1 through to 8) is realised. Moreover, by changing the order in which the reactions are carried out/which combination of molecules are used, we show that up to three different entities can be attached site-selectively and in a modular manner. We demonstrate by ELISA that thio-antibody conjugates retained binding affinity for their biological target HER2 (relevant in breast/stomach cancers). Finally, we show that the tri-functional conjugates bind and internalise selectively to $HER2^+$ over $HER2^-$ cell lines with all three payloads delivered into target cells only. It is acknowledged that the conjugation of toxin payloads can be different compared to the model payloads in this manuscript (*e.g.* biotin and fluorophores), but as we are using well established ''click'' chemistries to append the payloads, we would anticipate efficient translation from fluorophores/biotin to small-molecule toxins.

Results and discussion

Our study began with the synthesis of the three aforementioned molecules **1**–**3** (Fig. 2). Firstly, a bicyclo[6.1.0]non-4-yne (BCN)-containing PD (BCN PD) **1** was synthesised by adapting a protocol described in our previous work (see SI Fig. S1 for full details).[50] Next, aryl–azide/methyl bisPD (ArN_3 bisPD) **2** was synthesised by coupling a diBrPD methyl/acid and diBrPD aryl–azide/acid together *via* a bis-amine-PEG_3 entity (see SI Fig. S2 for full details). For the functionalised aniline, 4-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethoxy)aniline (N_3 aniline **3**), a procedure reported in our previous work was adapted for its synthesis (see SI Fig. S3 for full details).[45] An unfunctionalised diBrPD, dibromo diethyl pyridazinedione (diEt PD) **4**, and bisPD, dibromo dimethyl bis-pyridazinedione (diMe bisPD) **5** were also synthesised (in an analogous manner to BCN PD **1**/ArN_3 bisPD **2**, respectively, see SI Fig. S4 and S5 for full details), to act as model PDs where necessary (Fig. 2).

With these molecules in hand, we set about obtaining suitable thio-antibody mutants to appraise our methodology. We obtained two different thio-trastuzumab mutants (from MSD UK): cysteine-capped LC S168C **6**, and HC S378C **7**. The mutations were on different parts of the antibody as we were especially interested in elucidating any differing reactivity between the two types of mutant. Whilst the engineered cysteine on HC S378C **7** was available for reaction, the LC S168C mutant cysteines were capped with cysteine (calculated as the mass difference between the capped and uncapped form was ~240 Da); this is common with thio-antibodies as a result of their synthesis.[25] As such, before the LC S168C **8** mutant could be used, LC S168C **6** had to be ''uncapped'' whilst retaining the native interstrand disulfides in two-step reduction–oxidation procedure to afford the conjugate (Fig. 3, see SI Tables S6–S8 and Fig. S8–S31 for details on optimisation). The ability to successfully conjugate the engineered

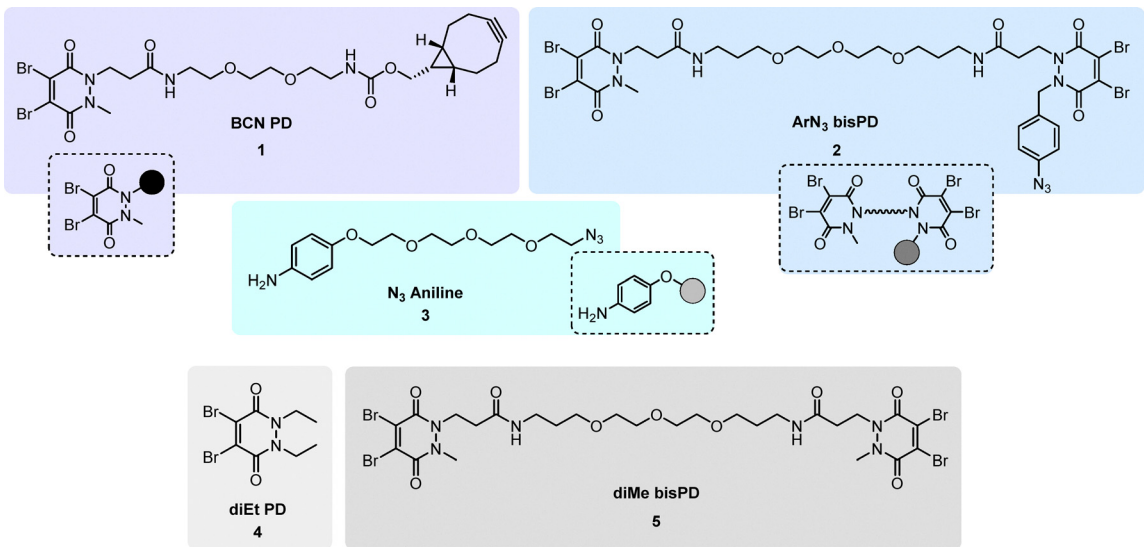

Fig. 2 Click-handle containing BCN PD **1** (5 step synthesis from 1-boc-1-methylhydrazine; this compound is also available commercially from various suppliers (*e.g.* Merck and Precise PEG)), ArN3 bisPD **2** (8 step synthesis from 1,2-diboc-hydrazine (di-*tert*-butyl hydrazodiformate); that said, many intermediates in the synthesis are commercially available) and N_3 aniline **3** (5 step synthesis from *para*-aminophenol and tetraethylene glycol), and model PDs diEt PD **4** (2 step synthesis from 1,2-diboc-hydrazine (di-*tert*-butyl hydrazodiformate)) and diMe bisPD **5** (4 step synthesis from 1-boc-1-methylhydrazine; this compound is also available commercially from Precise PEG). For full synthetic schemes, see SI Fig. S1–S5. Note: many synthetic steps are common in the synthesis of the various PDs.

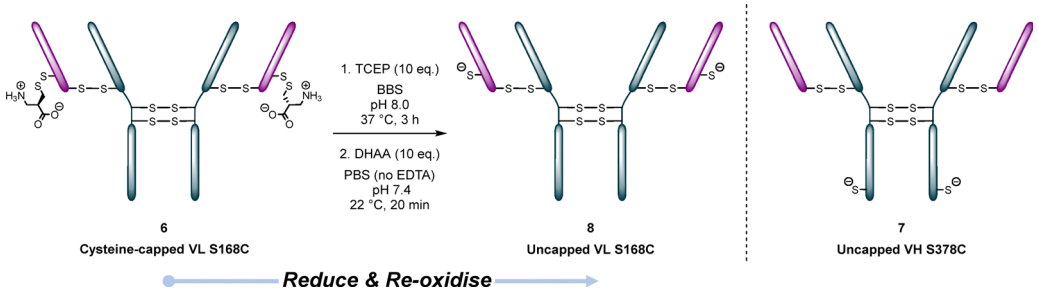

Fig. 3 Uncapping of cysteine-capped LC S168C **6** to afford LC S168C **8**, and HC S378C **7**.

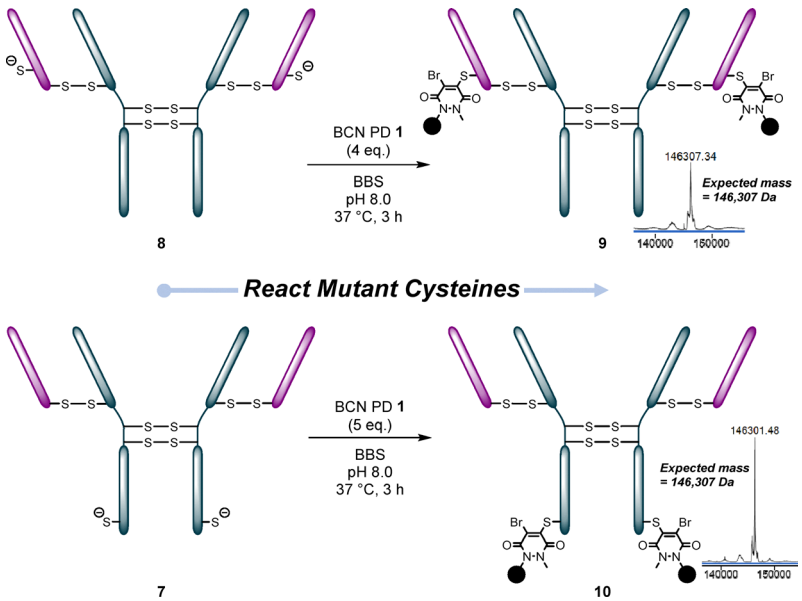

Fig. 4 Reaction of the thio-trastzumab mutants LC S168C **8** and HC S378C **7** with BCN PD **1**.

cysteines of uncapped LC S168C **8** and HC S378C **7** was confirmed by reaction with *N*-methylmaleimide (see SI conjugates S14 and S15 (respectively) for details).

PARs 2, 4 and 2 + 2

Uncapped thio-trastuzumab mutants LC S168C **8** and HC S378C **7** were then reacted with BCN PD **1** (Fig. 4). After some minor optimisation for reactions with HC S378C **7** (see SI Table S9 and Fig. S34–S38 for details), conjugates **9** and **10** were formed in excellent conversion yields. However, the main peak on LC-MS for the product was split in two, with the secondary peak −35 Da off the expected mass. We observed this splitting intermittently on both mutants. We believed it to be an artefact related to the LC-MS conditions, as the splitting always disappeared following displacement of the bromines with an aniline-like molecule (details below, Fig. 5).

Both conjugates **9** and **10** were clicked with Azide-Fluor 488 to give HC S378C-Azide Fluor 488 **11** and LC S168C-Azide Fluor 488 **12** (respectively). We then showed that the free bromines could be displaced by N_3 aniline **3**, affording conjugates **13** and **14**, respectively. These conjugates could subsequently be clicked again using strained alkyne bearing payloads, to give either PAR 4 (if the same payload was clicked on) or 2 + 2 (if a different payload was clicked on) conjugates. This was exemplified using BP-Fluor 568 for the LC S168C mutant, giving conjugate **15** (Fig. 5).

PARs 1, 3 and 1 + 2

As BCN PD **1** reacted well with the thio-trastuzumab mutants, it was theorised that bisDiBrPDs could react across the engineered cysteines if the linker between the PDs was long enough/the two mutant cysteines were sufficiently close in space. If successful, this would be a simple method of synthesising thio-antibody conjugates with the unusual DARs of 1 and 3 if the bisDiBrPD had one click handle and anilines bearing a click handle could react with the thioBrPDs.

The distance between the reactive sites on ArN_3 bisPD **2** was measured to be *ca.* 22.5 Å by MolView. The distance between the HC mutant cysteines on HC S378C **7** was measured to be *ca.* 22.5 Å by PyMol (using PDB: 1HZH (human IgG1 b12) as there is no available crystal structure for full trastuzumab, see SI Fig. S50 for details), making the HC S378C mutant an excellent candidate for this reaction. On the other hand, the distance between the LC mutant cysteines was measured to be

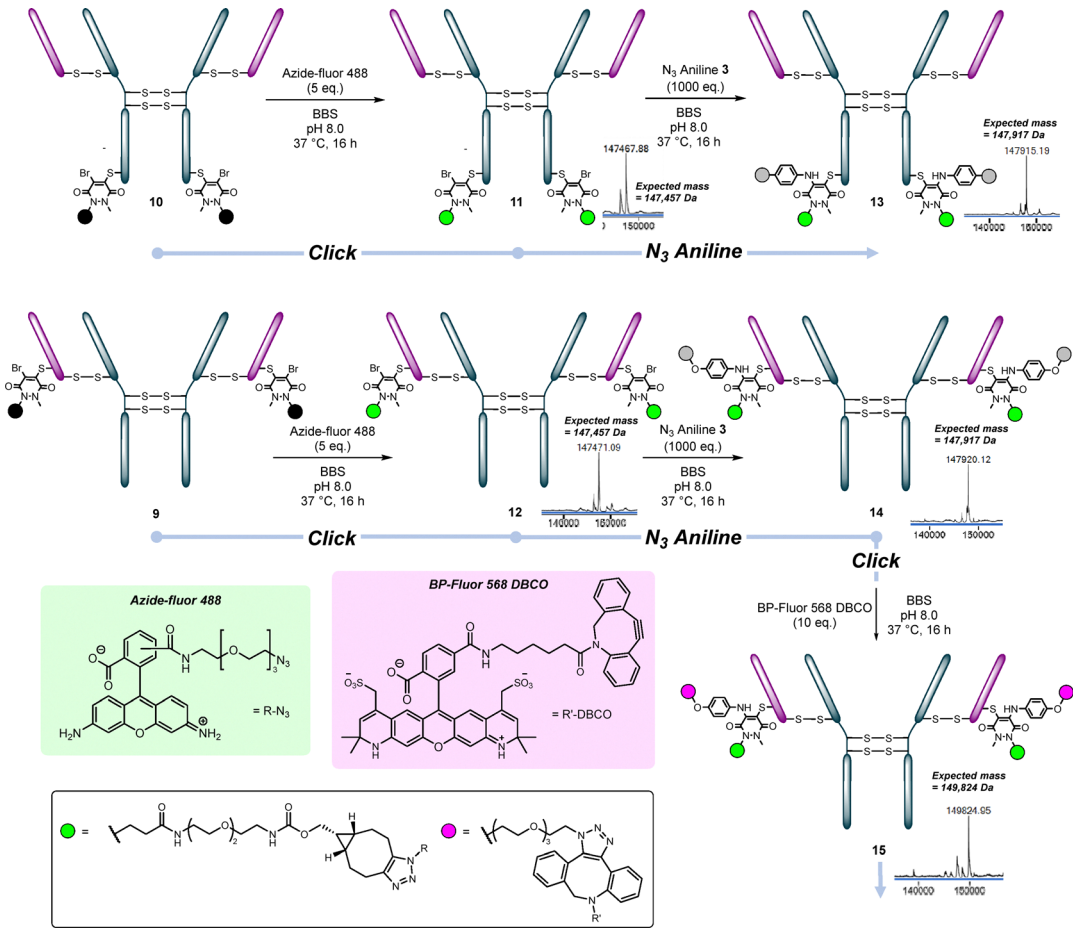

Fig. 5 The reactions of conjugates **9** (LC S168C conjugated to BCN PD **1**) and **10** (HC S378C conjugated to BCN PD **1**) with Azide-fluor 488 and subsequent reactions with N_3 aniline **3**. Deconvoluted, zoomed-in mass spectra are shown beside conjugates.

ca. 87.1 Å by PyMol (using PDB: 1HZH (human IgG1 b12) as there is no available crystal structure for full trastuzumab, see SI Fig. S50 for details), so we judged that it was unlikely that the desired PAR 1 product would be obtained when reacting ArN_3 bisPD **2** with LC S168C **8**. That all being said, it was appreciated that antibodies are flexible and the distances in space may in fact be different when dynamic antibodies are in solution.

Reaction of model diMe bisPD **5** with LC S168C **8** and HC S378C **7** gave results that were somewhat consistent with expectations. Reaction of LC S168C **8** with diMe bisPD **5** resulted in the formation of an appreciable amount of one bis PD per mutant cysteine – likely formed due to a lack of favourability of the bridging the bisPD across the mutant cysteines (see SI Fig. S64 and S65 for details). We hypothesise that by using a bisPD with a longer PEG chain connecting the two PDs, re-bridging across the mutant cysteine could be attainable, but this is outside the scope of this study.

In contrast to the light chain mutant, reaction of diMe bisPD **5** (5 eq.) with HC S378C **7** at 37 °C for 3 h in BBS (pH 8) afforded the desired conjugate S16 (see SI Fig. S49 for details) in excellent conversion. Moreover, the subsequent reaction of this conjugate **7** with *p*-anisidine (1000 eq.) at 37 °C for 16 h in BBS (pH 8) also worked well in excellent conversion (see SI Fig. S51 and S52 for details). Pleasingly, ArN_3 bisPD **2** reacted with HC S378C mutant **7** in a similar manner to diMe bisPD **5** under analogous conditions, giving conjugate **16** in excellent conversion. Subsequently clicking the azide of conjugate **16** with BP-Fluor 568 DBCO afforded desired homogenous PAR 1 conjugate **17**. Displacement of the bromines of the thioBrPDs with N_3 aniline **3** (conjugate **18**) and subsequent clicking of the two azides with BP Fluor 647 DBCO (to form conjugate **19**) provided access to a PAR 1 + 2 (or 3) conjugate (Fig. 6). Alternatively, conjugate **16** could be clicked with DBCO biotin, then the bromines displaced with N_3 aniline **3**, and the two azides subsequently clicked to BP Fluor 568 DBCO to form conjugate **20** (Fig. 11). As the two heavy chain mutant cysteines are relatively close in space, and potentially quite sterically hindered (especially as they had already been clicked), a large excess of BP Fluor 647 DBCO (to form conjugate **19**) and BP Fluor 568 DBCO (to form conjugate **20**) were used to ensure full conversion.

Disulfide re-bridging of trastuzumab

As many of the further proposed thio-antibody conjugates rely on the ability of PDs to successfully re-bridge the native disulfide bonds of trastuzumab, we wanted to ensure we had a robust protocol for re-bridging trastuzumab with both BCN PD

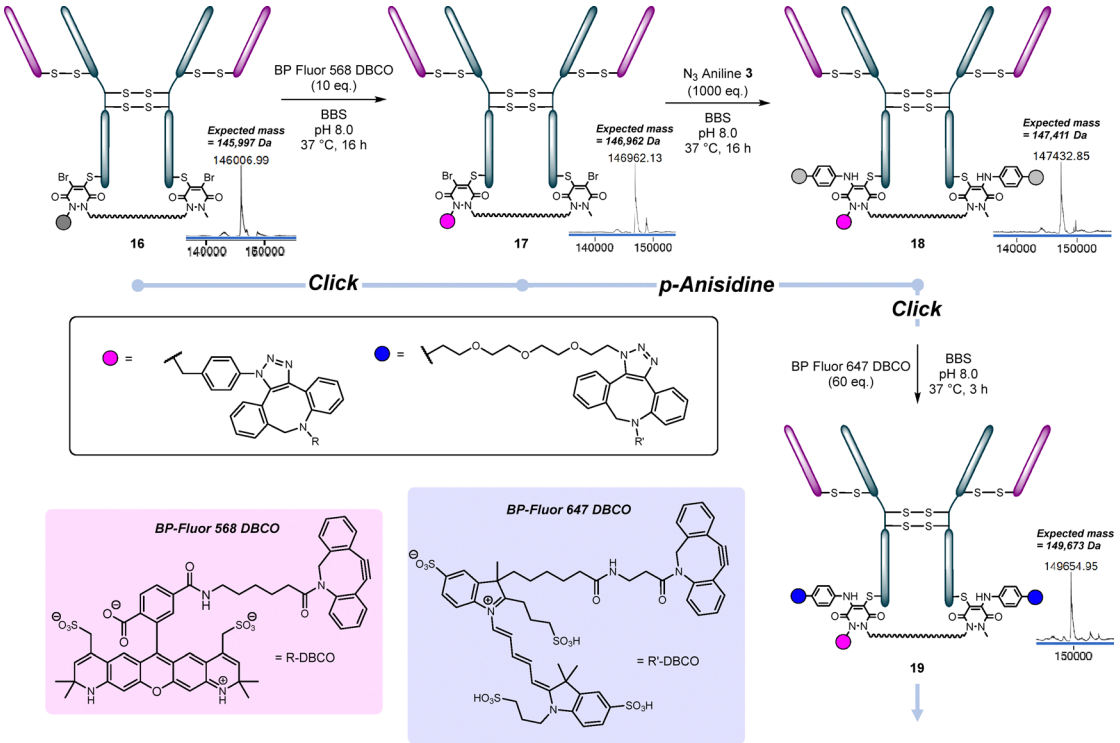

Fig. 6 Reactions of HC S378C **7** with ArN_3 bisPD **2** followed by reaction with various DBCO fluorophores and N_3 aniline **3**.

1 and ArN_3 bisPD **2** before attempting further work on the thio-antibodies. We trialled a variety of different conditions on native trastuzumab (Ontruzant®), see SI Table S10 and Fig. S66–S86, S89 and S90. Our optimised conditions for re-bridging with both BCN PD **1** or the ArN_3 bisPD **2** were as follows: the antibody (20 μM in BBS, pH 8.0) was reduced with TCEP (10 eq., 37 °C) for 1.5 h. After this, TCEP was removed and the reduced antibody reacted with (bis)PD (10 eq., 37 °C) for 3 h. This protocol consistently gave us a near homogenous conjugate with a PD-to-antibody ratio (PDAR 4) for BCN PD; this could be then clicked with Azide-fluor 488 with full conversion (fluorophore-to-antibody ratio (FAR) = 4.0). The same protocol resulted in a clean PDAR 2 when using the ArN_3 bisPD **2**. This conjugate could be clicked too (exemplified with DBCO-biotin). In both cases, the corresponding half antibody conjugate species were also present as the minor product; this does not affect the PDARs. We also note that the formation of analogous half antibody conjugate species, owing to some non-native PD rebridging in the hinge region when reacting the reduced native disulfide bonds of thio-antibodies (or conjugates) thereof with BCN PD **1** and/or ArN_3 bisPD **2** (in all relevant experiments described below), is observed – this will be denoted by a dagger symbol (†) for each relevant figure.

Concurrent reaction of the disulfides and mutant cysteines of thio-antibodies: PARs 6, 8 and 6 + 2

In general, when uncapping thio-antibodies, reducing the inter-chain disulfides is generally unavoidable, so a re-oxidation step is needed (see Fig. 3). This extra step is usually undesirable, and we hypothesised that if a diBrPD was added straight after reduction of a thio-antibody (capped or uncapped) that disulfide re-bridging as well as conjugation to the mutant cysteines would occur (Fig. 7). For this to occur, the mutant cysteines would have to be sufficiently far from the interchain disulfides so that one PD could not react with a mutant cysteine and one liberated from a reduced interchain disulfide bond.

This was first attempted on the HC S378C mutant **7**. Although already uncapped, this would show us whether it was possible for the PDs to differentially react with the mutant cysteines and the disulfides. The mutant was reduced using 10 eq. TCEP, the excess TCEP then removed by ultrafiltration and the reduced antibody (conjugate **21**) then subsequently reacted with 25 eq. BCN PD **1** to afford the desired PDAR 6 conjugate **22**. This conjugate could then be clicked with Azide-fluor 488 and then reacted with *p*-anisidine to create conjugates **23** and **24**, providing proof of concept for PAR 8 (6 + 2) (Fig. 8).

Next, the one-pot reaction was performed using cysteine-capped LC S168C mutant **6**. This time, the mutant was uncapped and reduced using 40 eq. TCEP to ensure that the antibody was both uncapped and fully reduced. The reduced antibody **25** was subsequently reacted with 20 eq. BCN PD **1** after removal of the excess TCEP by ultrafiltration. This reaction worked well, giving **26** (PDAR 6) as the main product. A very small amount of PDAR 5 (one mutant cysteine and all four disulfides reacted) was also visible on LC-MS. This conjugate could then be clicked and reacted with N_3 aniline **3**, creating conjugates **27** and **28**. N_3 aniline conjugate **28** was clicked with DBCO-biotin to form PAR 8 conjugate **29**. This gave us our proof-of-concept for PAR 6, 8 and 6 + 2 conjugates (Fig. 8).

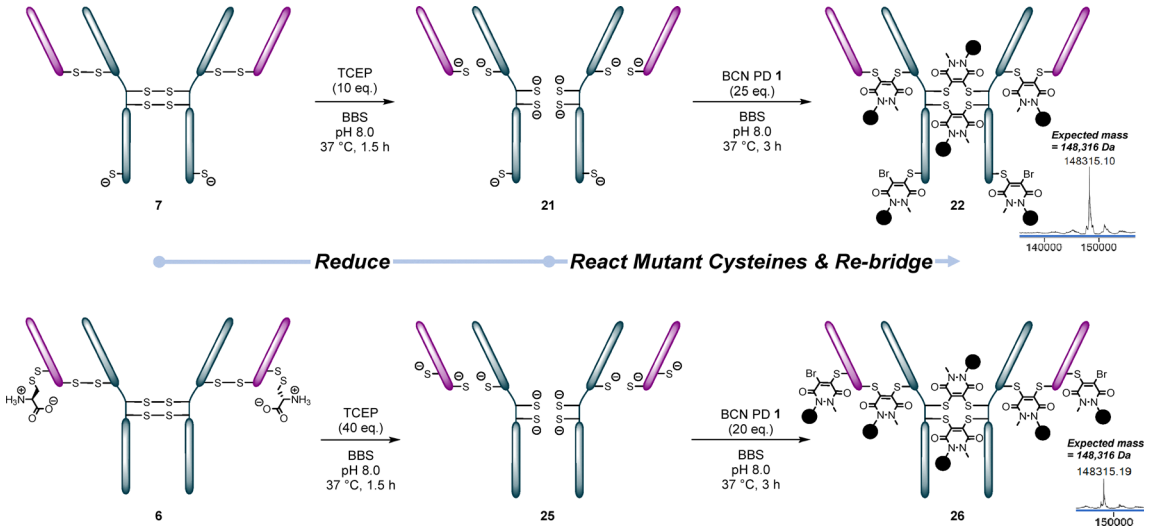

Fig. 7 One-pot reaction of reduced thio-trastuzumab mutants **7** and **6** with BCN PD **1** to afford conjugates **22** and **26**.†

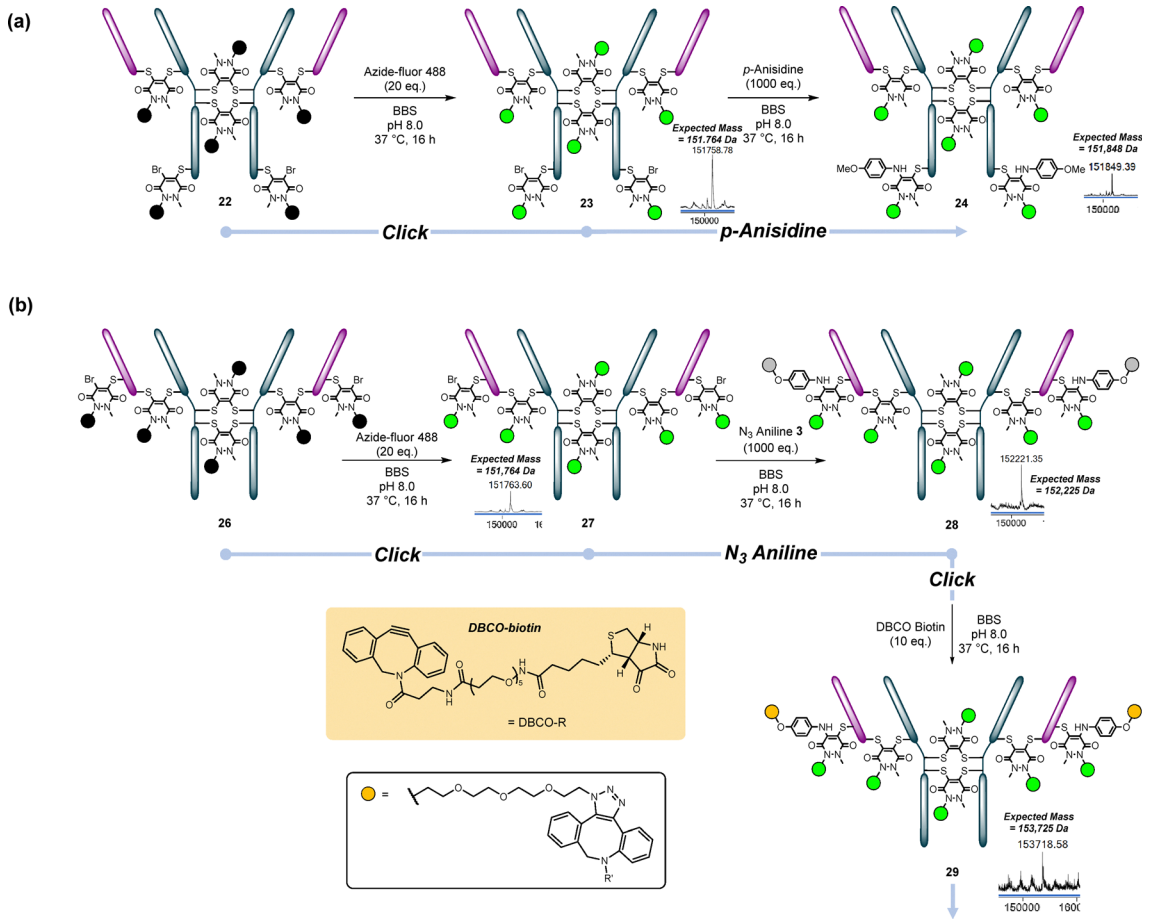

Fig. 8 (a) Reactions of conjugate **22** (HC S378C reacted/re-bridged with BCN PD **1**) with *p*-anisidine/N_3 aniline **3** and Azide-fluor 488. (b) Reactions of conjugate **26** (LC S168C reacted/re-bridged with BCN PD **1**) with *p*-anisidine/N_3 aniline **3** and Azide-fluor 488.†

Disulfide re-bridging of mutant cysteine modified thio-antibody conjugates

In order to synthesise trifunctional thio-trastuzumab-PD conjugates, and to access PARs 5 and 7, the PD-conjugated mutant cysteines would need to be stable to TCEP reducing conditions, at least under the conditions required for full reduction of the interchain disulfides (10 eq. TCEP, 1.5 h, 37 °C). To test this, the HC S378C thio-trastuzumab mutant was reacted with a

model PD, diEt PD **4**, followed by reaction with *p*-anisidine. This thioaminoPD conjugate was then incubated with TCEP under disulfide reduction conditions. After 1.5 h, excess reagents were removed and the conjugate analysed by LC-MS. To our delight, no apparent deconjugation of the thioaminoPD linkage was observed. We also independently corroborated this reactivity profile on model protein GFP S147C (see SI Fig. S114, S116 and S118 for details of these stability tests). These results suggested that thioaminoPD-conjugates of thio-trastuzumab antibodies would be stable to TCEP reduction conditions required to reduce the native interstrand disulfide bonds of the thio-antibody. With this in mind, we moved towards attempting the proposed antibody disulfide re-bridging reactions where the interstrand disulfide bonds were to be reduced in the presence of a thioaminoPD linkage.

Proof-of-concept for PAR 6: 2 + 4

We began by synthesising LC S168C-BCN PD-*p*-anisidine–Azide Fluor 488 conjugate **30**. The native disulfides of this conjugate were then reduced and re-bridged with BCN PD **1**, using our optimised disulfide re-bridging protocol, to give re-bridged conjugate **31**. Pleasingly, this worked well and the subsequent click reaction with 10 eq. Azide-fluor 488 gave a clean conjugate, **32**, providing proof-of-concept of these two-step reactions, as well as for PARs 6 and 2 + 4 (Fig. 9).

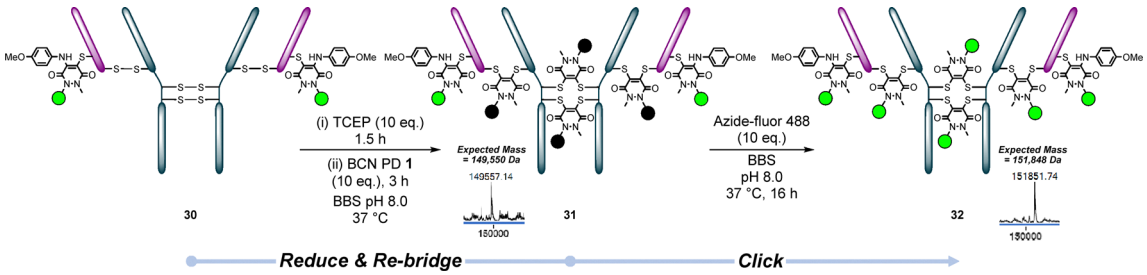

Fig. 9 Conjugate **30** (LC S168C reacted with BCN PD **1** clicked with Azide-fluor 488 and *p*-anisidine) was re-bridged with BCN PD **1** to form conjugate **31**, then clicked with Azide-fluor 488 to form conjugate **32**.†

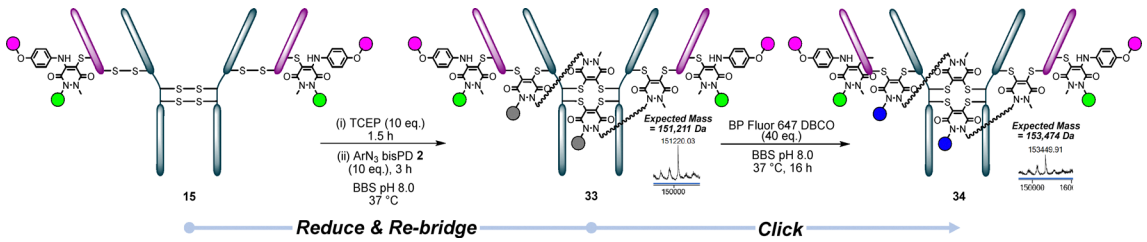

Fig. 10 Conjugate **15** (LC S168C reacted with BCN PD clicked with Azide-fluor 488, reacted with N_3 aniline **3** which was clicked with BP Fluor 568) was re-bridged with BCN PD **1** to form conjugate **33**, which was then clicked with BP Fluor 647 to form trifunctional conjugate **34**.†

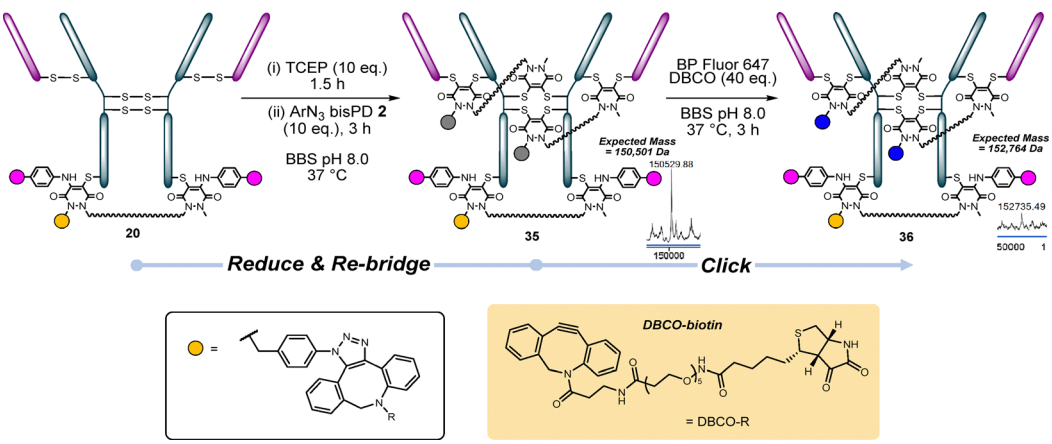

Fig. 11 Conjugate **20** (HC S378C reacted with ArN_3 PD **2** clicked with DBCO biotin and N_3 aniline **3** which was clicked with BP Fluor 568) was re-bridged with ArN_3 bis PD **2** to form conjugate **35**, which was then clicked with BP Fluor 647 to form conjugate **36**.†

PAR 6: 2 + 2 + 2

Next, we attempted to disulfide re-bridge conjugate **15** (BCN PD-Azide-fluor 488-N_3 aniline-BP Fluor 589-LC S168C) with ArN_3 bisPD **2** across pairs of native disulfide bonds. Once again, this reaction proceeded smoothly, forming conjugate **33** and, following a click with BP Fluor 647, affording conjugate **34**, providing access to 2 + 2 + 2 (PAR 6) (Fig. 10).

With this result in-hand, we decided to move onto appraising the reaction of ArN_3 bisPD-HC S378C conjugates **19** and **20** with disulfide re-bridging PDs, as this would theoretically provide access to our final remaining PARs: 5 and 7.

PAR 5: 2 + 1 + 2

To form these conjugates we followed a similar protocol as for the formation of the aforementioned PAR 8 and 6 conjugates.

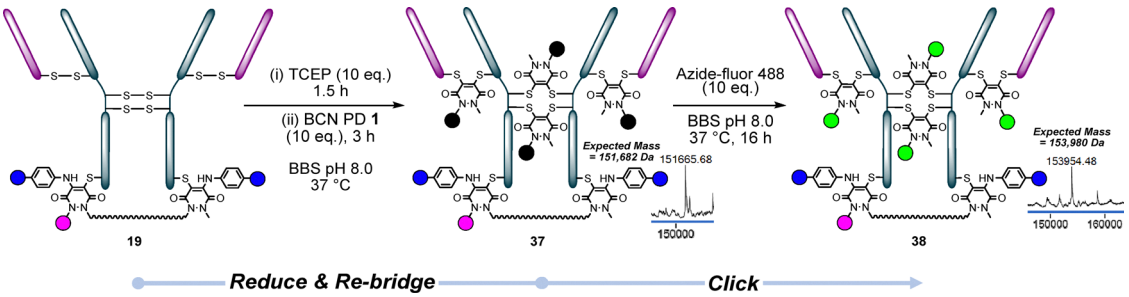

Fig. 12 Conjugate **19** (HC S378C reacted with ArN_3 PD **2** clicked with BP Fluor 568, reacted with N_3 aniline **3** which was clicked with BP Fluor 647) was re-bridged with BCN PD **1** to form conjugate **37**, which was then clicked with Azide-fluor 488 to form trifunctional conjugate **38**.

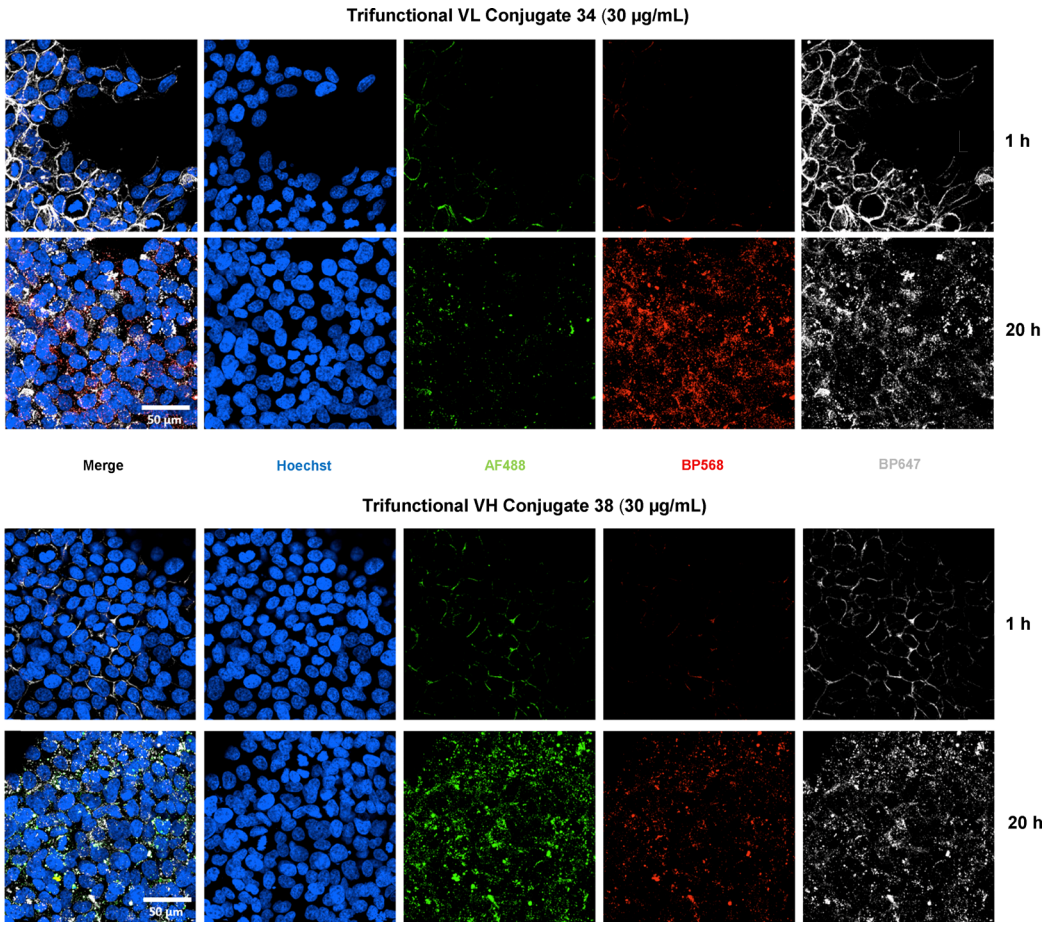

Fig. 13 High magnification (63×) images of HER2$^+$ (BT-474) cells 1 h and 20 h post treatment with LC S168C conjugate **34** and HC S378C conjugate **38** at a concentration of 30 μg mL^{-1}. The three fluorophores are visualised as follows: Dapi (nuclei) in blue; Azide-fluor 488 in green; BP Fluor 568 in red and BP Fluor 647 in white-grey.

First, we reduced conjugate **20**, then disulfide re-bridged it with ArN_3 bisPD **2** to form conjugate **35**. This was successfully clicked with BP Fluor 647 to form conjugate **36**, thus providing access to 2 + 1 + 2 (PAR 5) (Fig. 11).

PAR 7: 2 + 1 + 4

To form our final unmet PAR conjugate target, we reduced conjugate **19** (ArN_3 bisPD-BP Fluor 568-N_3 aniline-BP Fluor 647 HC S378C) and then re-bridged the reduced disulfides with BCN PD **1**, to form conjugate **37**. Finally, we clicked this with Azide-Fluor 488 to form trifunctional conjugate **38**, thus providing access to 2 + 1 + 4 (PAR 7) (Fig. 12).

ELISAs and internalisation studies

Having formed thio-antibody conjugates with PARs 1–8 in a modular and site-selective manner, we next evaluated if our conjugation strategies had compromised antibody–antigen binding. To appraise this, we performed ELISA analysis on PDAR 6 LC S168C conjugate S34 (see SI conjugate S34 for synthesis and characterisation) and HC S378C conjugate **16** using HER2 antigen with the unmodified thio-trastuzumab antibodies acting as controls. These conjugates were chosen as they were representative of the range of conjugates synthesised – one light chain conjugate and one heavy chain conjugate, one with a bisPD, one with all disulfides re-bridged and with six modules attached. Gratifyingly, we found no significant difference in HER2 binding between our conjugates and the native thio-trastuzumab controls (see ELISA section in the SI for details).

Finally, we performed internalisation studies on selected conjugates. The conjugates chosen for this were trifunctional LC S168C conjugate **34** and HC S378C conjugate **38**. Trifunctional conjugates were chosen for these assays as visualising the three different fluorophores would allow us to determine if all three modules were internalised into targeted cells treated with the conjugates. The three different fluorophores – Azide-fluor 488, BP Fluor 568 DBCO and BP Fluor 647 DBCO were chosen as they each fluoresce under a different excitation/emission wavelength and could be independently captured by fluorescence microscopy.

For these studies, BT-474 was used as the $HER2^+$ cell line, and MCF-7 as the $HER2^-$ control cell line. To test binding/internalisation, LC S168C conjugate **34** and HC S378C conjugate **38** were added and the cells incubated for 1 h at 4 °C to allow binding but preventing endocytosis. The cells were then washed with PBS to remove unbound antibody conjugates, and then incubated in fresh medium at 37 °C. As expected, conjugates **34** and **38** were bound and internalised only to the $HER2^+$ cells. Interestingly, the HC conjugate showed lower fluorescence at the same dose. After 1 h at 4 °C some cells were fixed, and this showed that the conjugates were clustered

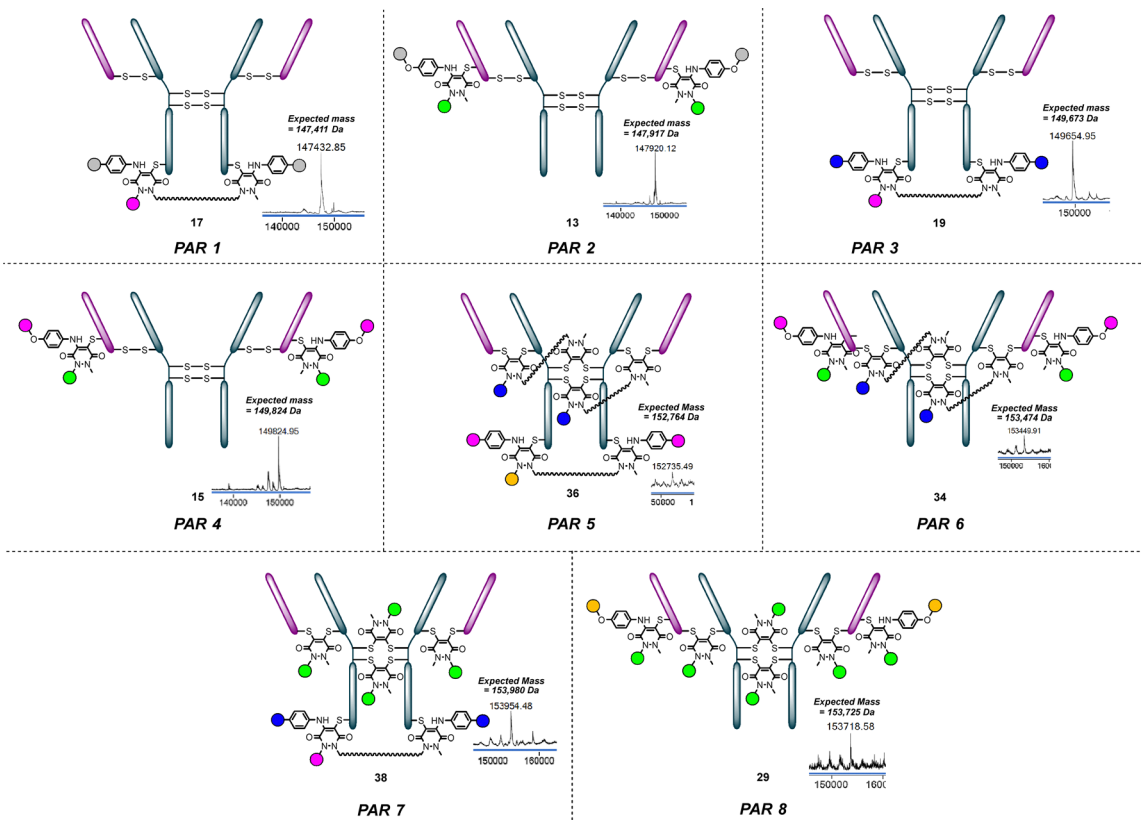

Fig. 14 PAR 1–8 conjugates were accessed by using different combinations of Mepstra PD **1**, PhN_3 bisPD **2** and N_3 aniline **3**, in combination with various clickable payloads, on thio-trastuzumab mutants.†

around the cell membrane of $HER2^+$ (BT-474) cells (Fig. 13). After switching to 37 °C, and fixing the cells at 20 h, the fluorescence appeared to be mostly concentrated in dots within the cells, suggesting internalisation into cellular compartments (Fig. 13). No binding or internalisation was observed in the negative control MCF-7 cells (see SI Fig. S143 for details).

In both cases, the BP Fluor 647 gave a much brighter signal than the Azide-fluor 488 or the BP Fluor 568. Indeed, the extinction coefficient of BP Fluor 647 is much higher than the extinction coefficient of the other two fluorophores, which could explain this difference of intensity. Despite this difference, a signal from all three fluorophores was detected. The three fluorescent probes were initially detected around the cell membranes but over time became concentrated within intracellular vacuoles. Most importantly, all three fluorophores are detectable within the cell after 20 h of treatment, and presented a similar dotted-distribution within the cytoplasm (Fig. 13).

This study clearly shows that for both trifunctional thio-antibody conjugates, the three fluorophores traffic over time from the cellular membrane to the cytosol and persist at least up to 20 h post-treatment. The co-distribution of each fluorophore present within the intracellular vacuoles after 20 h of treatment was quantified (see SI Fig. S144 for details). It was not only confirmed that the fluorescence of each fluorophore was still detectable, but also shown that some combinations of fluorophore still co-exist in close vicinity, likely sharing the same vacuolar space.

Conclusions

In this manuscript we disclose a platform for the modular and site-selective construction of thio-trastuzumab mutant conjugates with payload-to-antibody ratios (PARs) of 1, 2, 3, 4, 5, 6, 7 and 8 (Fig. 14). Using only BCN PD **1**, ArN_3 bis PD **2**, and N_3 aniline **3**, the framework to achieve all these PARs was enabled, including the ability to append up to three different payloads. The modularity of the chemistry and exploiting various chemoselective reactions enabled various ratios of different classes of payloads to be realised in a facile manner. Moreover, the use of click chemistry to attach payloads also adds a further level of modularity to the plug-and-play platform. The retention of binding of key representative thio-antibody conjugates to HER2 was demonstrated by ELISA, and tri-payload loaded thio-antibody conjugates were shown to internalise selectively to $HER2^+$ (BT-474) over $HER2^-$ (MCF-7) cells with all payloads successfully delivering into target cells only. Overall, we have demonstrated how our platform can enable the facile synthesis (multi-)payload bearing thio-antibody conjugates with PARs of 1 through to 8 with characterisation *via* LC-MS, SDS-PAGE, ELISA and on relevant cells *in vitro*.

Author contributions

C. M. synthesised the small-molecules and carried out the bioconjugation reactions and ELISAs. I. A. T. synthesised the diMe bisPD. N. W. provided advice on bioconjugation reactions and the ELISAs. C. J. Q., M. H. and D. H. W. L. designed the imaging experiments. C. J. Q. carried out the imaging experiments. J. S. G. and M. T. W. L. helped to support C. M. at MSD and during their PhD. C. M., M. T. W. L., J. R. B. and J. S. G. and V. C. conceived and designed the project/experiments. C. M. and V. C. co-wrote the manuscript. All authors read and approved the final manuscript.

Conflicts of interest

There are no conflicts to declare.

Data availability

Synthetic chemistry experimental details, including synthetic procedures and compound characterisation studies, *i.e.*, NMR, IR and MS spectra, chemical biology experimental details, including bioconjugation procedures, LC-MS methodology, full LC-MS spectra including TIC and raw data, and full details of the *in vitro* studies have been included in the supplementary information (SI). See DOI: https://doi.org/10.1039/d6cb00018e.

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