Stable pyrrole-linked bioconjugates through tetrazine-triggered azanorbornadiene fragmentation

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Abstract: We have developed an azanorbornadiene bromovinyl sulfone reagent that allows cysteine-selective bioconjugation. Subsequent reaction with dipyridyl tetrazine led to bond-cleavage and formation of a pyrrole-linked conjugate. The latter involves ligation of the tetrazine to the azanorbornadiene-tagged protein through inverse electron demand Diels–Alder cycloaddition with subsequent double *retro*-Diels–Alder reactions to form a stable pyrrole linkage. The sequence of site-selective bioconjugation followed by bioorthogonal bond-cleavage was efficiently employed for the labelling of three different proteins. This method benefits from easy preparation of these reagents, selectivity for cysteine, and stability after reaction with a commercial tetrazine, which lends it to the routine preparation of protein conjugates for chemical biology studies.

The installation of synthetic modifications, such as fluorescent/affinity probes or cytotoxic drugs into a pre-determined site on a protein, can help us to understand uptake and intracellular trafficking pathways[1] or enable the specific delivery of payloads to a cancer tissue in the form of antibody-drug conjugates (ADCs).[2] The chemical introduction of these modifications onto a protein may be achieved through 1) direct chemical modification of proteinogenic amino acids or 2) genetic encoding of a non-canonical amino acid in the protein sequence followed by chemical ligation.[3] Of all proteinogenic amino acids, the sulfhydryl side-chain of cysteine remains the primary choice to achieve site-selectivity because of its enhanced nucleophilicity and low abundance in its reduced form.[4] Although many cysteine-bioconjugation strategies have been developed,[5] the thio-Michael addition reaction to a maleimide acceptor is the most popular for reactions directed at cysteine[6] because it is fast and quantitative at near neutral pH, and because the reagents used are commercially available with a maleimide handle for conjugation.[6] The use of maleimides is best illustrated in the generation of clinically used ADCs, such as brentuximab vedotin[7] and ado-trastuzumab emtansine.[8] However, thiosuccinimide-cysteine-linked bioconjugates quickly degrade *in vivo* by means of a *retro*-Michael reaction, which leads to thiol-exchanged products (e.g., glutathione or the cysteine at position 34 in albumin).[9] The inherent instability of the thiosuccinimide linkage is a cause for premature drug-loss and thus potential side-toxicity and decreased therapeutic efficacy.[10] However, through either hydrolysis[11] or application of stretching force[12] stable maleimide–thiol adducts can be obtained.

Reactions that allow formation of new bonds under bioorthogonal conditions are of great interest in chemical biology.[13] Among these, the inverse electron demand Diels–Alder cycloaddition (iEDDA) between tetrazines and olefin dienophiles stands out for its selectivity, fast kinetics and biocompatibility.[14] More recently, an inverse strategy (in which a specific bond is cleaved instead of formed) has been proposed and applied for the controlled activation of proteins and prodrugs in cells and animals.[15] The reaction between aza- and oxa-benzonorbornadienes with tetrazines to afford isoindoles and isobenzofurans, respectively, (**Scheme 1**) which was reported by Warrener in the 1970's,[16] was further developed as a bioorthogonal bond-cleavage reaction, and used for drug activation[17] and signal amplification in nucleic acid templated detection of microRNA.[18] This transformation proceeds through a three-step cascade reaction that is initiated by ligation between the strained alkene of the heterobenzonorbornadiene system **A** and a tetrazine by means of an iEDDA reaction (**Scheme 1**). Upon spontaneous double *retro*-Diels–Alder (*r*DA), which leads to simultaneous extrusion of N_2 and of an aromatic pyridazine, the corresponding isoindole or isobenzofuran is formed quantitatively. However, these products are highly unstable due to their high dienic character.

We have recently developed a method for residue-specific dual protein labelling that consists of cysteine-selective thio-Michael addition to 7-azanorbornadiene system **B** followed by bioorthogonal iEDDA labelling of the resulting 7-azanorbornene conjugate with fluorogenic tetrazines (**Scheme 2**).[19] The ability to perform the iEDDA reaction enabled the incorporation of a second label and also avoided collateral *r*DA of 7-azanorbornene **C**. Here we describe the use of [2.2.1]bicyclic 2-bromovinyl sulfone of type **D** as a reagent for site-selective protein bioconjugation purposes. We hypothesized that the strained

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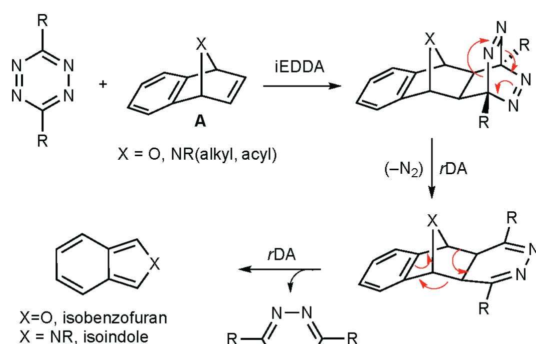
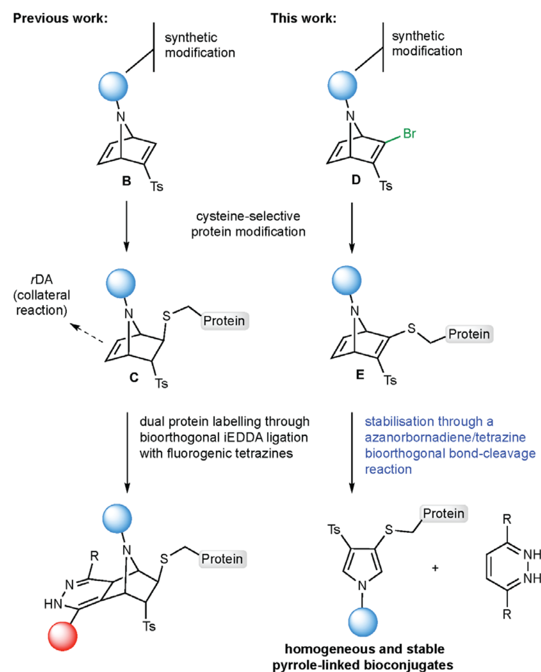
bromovinyl sulfone could react with the sulfhydryl side-chain of cysteine in a selective and fast manner to afford **E**. However, and contrary to the 7-azanorbornene analogue **C**, the subsequent reaction of 7-azanorbornadiene **E** with tetrazines would proceed by means of a tetrazine ligation/*r*DA cascade as Warrener's azabenzonorbornadienes. This new ligation would produce an unprecedented pyrrole-linked protein conjugate. Although Warrener's chemistry has been broadly exploited for the preparation of isobenzofurans and isoindoles from 7-oxa- and 7-azabenzonorbornadienes, respectively,[20] to the best of our knowledge no examples have been reported for the tetrazine-mediated preparation of pyrrole derivatives from 7-azanorbornadiene.



Scheme 1. Warrener's synthesis of isobenzofurans and isoindoles.

The synthesis of racemic [2.2.1]bicyclic bromovinyl sulfone **1** was achieved through Diels–Alder reaction between bromoethynyl *p*-tolyl sulfone and commercial *N*-*tert*-butyloxycarbonyl (Boc)-pyrrole according to a reported procedure (**Scheme S1**).[21] Bromovinyl sulfone **1** was then reacted simultaneously with both *N*-Boc-cysteine methyl ester (*N*-Boc-Cys-OMe) and *N*-Boc-lysine methyl ester (*N*-Boc-Lys-OMe) in a competition experiment in buffer (NaP_i, 50 mM, pH 8.0) with dimethylformamide (DMF) as co-solvent. As hypothesized, cysteine reacted rapidly with **1** and the corresponding [2.2.1]bicyclic thiovinyl sulfone **2** was obtained as the sole product in quantitative yield after 15 min (**Scheme 3**). This reaction was calculated to follow a concerted nucleophilic vinylic substitution (S_NV_σ) mechanism with an activation barrier of $\Delta G^\ddagger \approx 12$ kcal mol^{-1} for *N*-Moc-[2.2.1]azabicyclic bromovinyl sulfone and methyl thiolate abbreviated models (**Figure 1a** and SI for calculations with an *N*-Ac analogue, **Figures S11 and S12**).

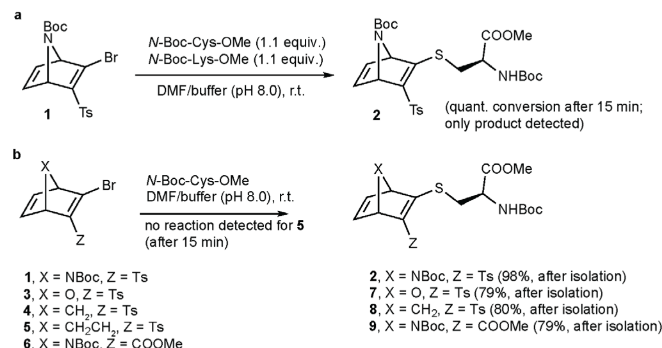


Scheme 2. Different behaviour of azanorbornadienes type **B** and **D** and their reactions with tetrazines for protein bioconjugation.

The cysteine derivative *N*-Boc-Cys-OMe only required 5 min for quantitative conversion to **2**. Conversely, the lysine derivative *N*-Boc-Lys-OMe only resulted in 32% conversion to the corresponding product even after 2 h of reaction time (**Figure S5**). Accordingly, the reaction between the same *N*-Moc-azabicycle and methylamine as a lysine model was calculated to be stepwise (i.e. addition then elimination) and thousands of times slower with an activation barrier of $\Delta G^\ddagger \approx 16$ kcal mol^{-1} (**Figure S11** and **12**). Other amino acid nucleophilic side-chains (serine, arginine, methionine or threonine) showed no reactivity towards **1** while histidine showed a similar reactivity profile to lysine (**Figures S1–S6**). Altogether this data demonstrates the superior kinetics of the reaction between [2.2.1]bicyclic bromovinyl sulfone reagents and cysteine.

7-Azanorbornadiene **2** showed high stability and no *r*DA reaction was observed after 1 week at 37 °C in $CDCl_3$, in agreement with the high activation barrier of $\Delta G^\ddagger \approx 28$ kcal mol^{-1} calculated for an *N*-Ac-azabicycle analogue in chloroform (**Figure S13**). This result contrasts with the 7-azanorbornene analogue of type C, which we found to be more prone to undergo slow *r*DA breakdown under the same conditions (calculated activation barrier of $\Delta G^\ddagger \approx 25$ kcal mol^{-1} in chloroform).[19] The exceptional reactivity of the bromovinyl sulfone moiety embedded in a [2.2.1]bicyclic skeleton over a less-strained bicyclic system was confirmed through reaction of *N*-Boc-Cys-OMe with differently bridged bicyclic derivatives **3**–**5**. Compounds **3** and **4**, which feature a [2.2.1]bicyclic skeleton, reacted rapidly with *N*-Boc-Cys-OMe (calculated activation barrier with MeS^- of $\Delta G^\ddagger = 11$ and 14 kcal mol^{-1}, respectively, **Figure S12**), whereas less-strained [2.2.2]bicyclic derivative **5** (calculated activation barrier with MeS^-

of $\Delta G^{\ddagger} \approx 16$ kcal mol^{-1}, **Figure S12**) did not react under the same conditions. The calculated activation barrier for the reaction of MeS^- with *N*-Moc abbreviated model of [2.2.1]azabicyclic bromovinyl ester **6** ($\Delta G^{\ddagger} \approx 13.8$ kcal mol^{-1}, **Figure S16**) is slightly higher than for the bromovinyl sulfone analogue **1**.



Scheme 3. a. Competition experiment of the reaction between [2.2.1]azabicyclic bromovinyl sulfone **1** with cysteine and lysine amino acids. **b.** Reactivity of other [2.2.1/2]bicyclic bromovinyl sulfones/ester towards a cysteine model.

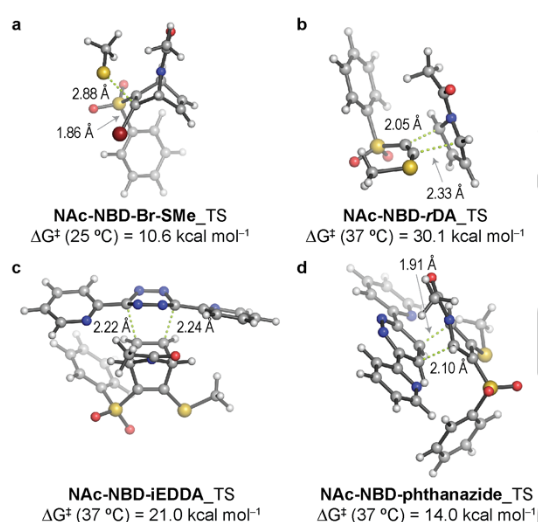
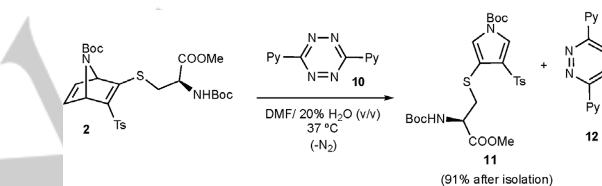


Figure 1. Lowest-energy structures for the **a**) concerted nucleophilic vinylic substitution (S_NV_σ) of the *N*-acetyl analogue of **1** (*N*-Ac-NBD-Br) with methyl thiolate, **b**) *retro*-Diels–Alder (*r*DA) of thiovinyl sulfone *N*-Ac-NBD-SMe, **c**) inverse electron demand Diels–Alder (iEDDA) cycloaddition of *N*-Ac-NBD-SMe with tetrazine DPTz **10**, and **d**) final *r*DA reaction of the azanorbornadienic sulfone model *N*-Ac-NBD calculated with PCM(H_2O)/M06-2X/6-31+G(d,p). Relative free activation energies ($\Delta G^{\ddagger}$, in kcal mol^{-1}) are calculated from the corresponding separated reactants. For the calculations on the bromovinyl methyl ester analogue **6**, please see the SI.

Next, we studied the reactivity of the cysteine-conjugated 7-azanorbornadiene **2** towards tetrazines and found that the most electron-rich double bond present in **2** undergoes rapid iEDDA ligation with the electron-deficient 3,6-di-(2-pyridyl)-*s*-tetrazine (DPTz) **10** under aqueous conditions (**Scheme 4**). The initial iEDDA was followed by two consecutive *r*DA reactions, which led to the formation of a thiol-conjugated pyrrole **11** in excellent 91% yield after isolation along with pyridazine **12** as a by-product. These observations were corroborated by the much lower activation barrier calculated for the iEDDA reaction between MeS^- conjugated 7-azanorbornadiene models and DPTz in water ($\Delta G^{\ddagger}$ = 21–22 kcal mol^{-1}, **Figure 1c**) with respect to the potentially competitive but unobserved *r*DA decomposition to give the corresponding pyrrole and alkyne products ($\Delta G^{\ddagger} \approx 30$ kcal mol^{-1}, **Figure 1b**). The *r*DA fragmentation from the tricyclic dihydropyridazine adduct was calculated as fast and thus not rate-limiting ($\Delta G^{\ddagger} \approx 14$ kcal mol^{-1}, **Figure 1d**). Accordingly, the reaction between the tetrazine and **2** was monitored through the decrease in tetrazine absorbance over time. A second-order rate constant (k) of 0.026 (±0.002) $M^{-1}s^{-1}$ at 37 ºC was determined for the triple-cascade reaction with the initial cycloaddition step as rate limiting (**Figure S10**). This value is similar to the one determined for iEDDA ligation between DPTz and azanorbornene of type **C** (**Scheme 2**).[19] Similar reaction rates were also reported for iEDDA reactions with aza/oxabenzonorbornadienes.[17] Finally, [2.2.1]bicyclic analogues **7**–**9** reacted with DPTz **10** in a similar manner to **2** (**Scheme S3**).



Scheme 4. Reactivity of thiol-conjugated azanorbornadiene **2** towards tetrazines.

Our data demonstrate that azanorbornadiene bromovinyl sulfones react selectively and rapidly with cysteine, whilst an electron-rich alkene remains unreacted, which enables a further iEDDA-*r*DA-*r*DA reaction cascade to afford a stable pyrrole-linked conjugate at cysteine. The combined advantages of directness, selectivity and further formation of a stable pyrrole linkage by means of iEDDA ligation with tetrazines could allow application of protein and antibody conjugation strategies with the formation of homogenous and stable pyrrole products based simply on these synthetical accessible [2.2.1]bicyclic 2-bromovinyl sulfone reagents. To test this hypothesis, we first decorated **1** with biotin as an affinity probe and model modification (compound **13**, **Figure 2**, **Scheme S4** for synthetic preparation). As a model protein, we chose ubiquitin, which has been engineered with a surface exposed cysteine at position 63 (Ub-K63C).[22] When we reacted Ub-K63C with 5 equiv. of biotin-functionalized bromovinyl sulfone **13** in NaP_i buffer (50 mM, pH 8.0) with DMF as co-solvent (10%) at room temperature for 30 min, a single and homogeneous product was formed as determined by LC-MS (**Figure 2a**). Further reaction of resulting bioconjugate **14A** with thio-specific Ellman's reagent confirmed that the cysteine was completely consumed during the reaction (**Figure S23**). To further confirm cysteine chemoselectivity, we first reacted Ub-K63C with a maleimide probe (**Figure S27**) and then examined reaction of the resulting conjugate with **13**. After incubation of Ub-thiosuccinimide with **13** for 1 h at 25 ºC (the conditions required for complete reaction of

13 with Ub-Cys), only < 5% of unspecific reaction, presumably at lysine, was observed (**Figure S28**). This further corroborates the chemoselectivity of the azanorbornadiene bromovinyl sulfone reagents towards cysteine at a protein level.

Once we demonstrated the chemoselectivity of azanorbornadiene bromovinyl sulfone for cysteine-selective protein bioconjugation, we decided to explore if the thio-vinyl sulfone could undergo iEDDA followed by double *r*DA when treated with a tetrazine. We found that treatment of **14A** with an excess of DPTz **10** at 37 °C led to the clean formation of pyrrole-linked bioconjugate **14B** as determined by LC-MS (**Figure 2a**). The scope of the method was expanded by using 7-azanorbornadiene **13** in the bioconjugation of other three medically and biologically relevant proteins containing an engineered cysteine residue, the C2A domain of synaptotagmin-I (C2Am)[23] and an anti-HER2 2Rb17c nanobody[24] (**Figure 2b** and **Figures S35–S38**). Identical reactivity was observed, which demonstrates the general utility of [2.2.1]bicyclic 2-bromovinyl sulfone reagents for the formation of stable β-thio-pyrrole linked bioconjugates. We further demonstrated that the probe **13** can react with multiple cysteine residues on an antibody. Upon interchain disulfide reduction, 8 accessible cysteine residues become available on trastuzumab which enabled the installation of an average of 4.3 modifications per antibody as detected by native MS (**Figures S39–S41**).[25] Finally, we performed extensive molecular dynamics simulations on conjugates **14B** and **15B** (see SI for details). According to these calculations, the 3D structure of the proteins remains unaltered upon the chemical modifications (**Figure S43**), which is pre-requisite for biological activity.

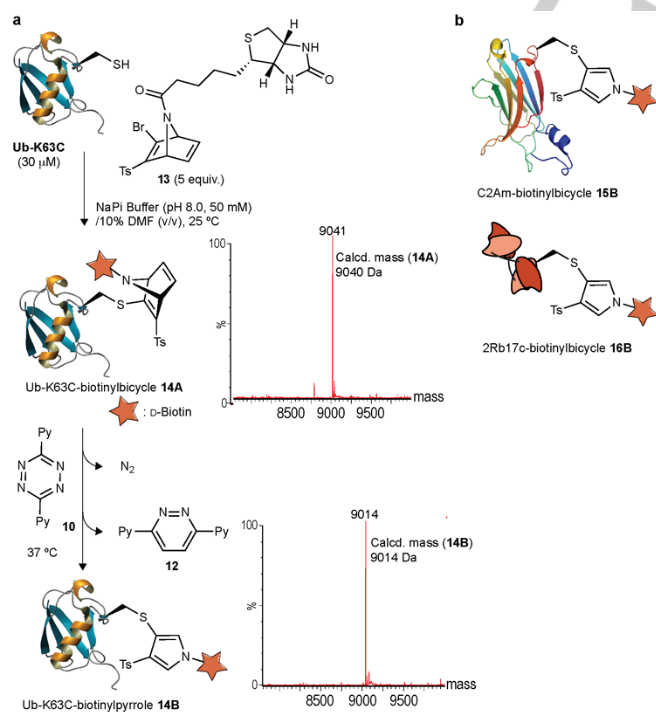


Figure 2. a. Bioconjugation of ubiquitin (Ub-K63C) with biotinyl-azanorbornadiene **13** followed by iEDDA reaction. Deconvoluted mass spectra obtained before and after the bioorthogonal reaction with DPTz **10**. **b.** Product of the reaction of C2Am and anti-HER2 2Rb17c nanobody with **13** followed by iEDDA reaction with DPTz **10**.

Next, we evaluated the stability of both ubiquitin bioconjugates **14A** and **14B** in human plasma and with an excess of glutathione. After 24 h incubation at 37 °C in plasma, the bioconjugate **14A** remained unaltered (**Figure 3a**). However, the incubation of **14A** with an excess of glutathione (100 equiv.) for 1 h converted **14A** into bioconjugate **17** as detected by LC-MS. Although the thio-vinyl sulfone functionality is not prone to suffer from thiol-exchange like typical thio-maleimide adducts, the thio-vinyl sulfone can still act as a Michael acceptor. Thus, the formation of **17** can be explained by the thio-Michael addition of glutathione to the thio-vinyl sulfone moiety, followed by spontaneous *r*DA of the resulting norbornene thioketal. In contrast, thio-pyrrole linked bioconjugate **14B** was shown to be fully stable, both in plasma and when reacted with an excess of glutathione (**Figure 3b**).

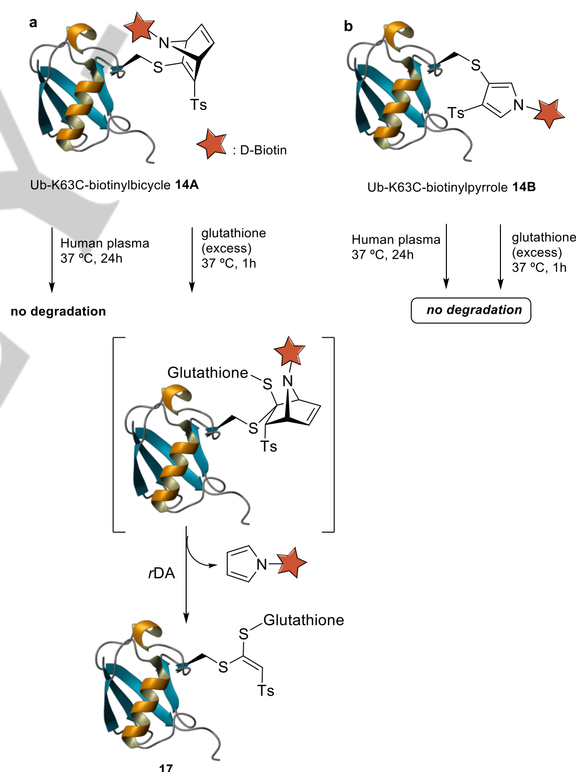


Figure 3. Stability studies of Ub-K63C bioconjugates **a) 14A** and **b) 14B** both in plasma and with an excess of glutathione, respectively.

Finally, we show that the azanorbornadiene bromovinyl sulfone reagent is capable of reacting with the cysteine proteome in HeLa cell lysates. Importantly, protein labeling increased with increasing concentration of **13** (**Figure S42**). These data serve as proof of principle for further studies and we are currently commencing on a dedicated proteomics study to annotate these hits and evaluate them against the known cysteine proteome.[26]

In summary, we report the use of azanorbornadiene bromovinyl sulfones for cysteine-selective bioconjugation. This reagent can accommodate most synthetic modifications and shows high reactivity and chemoselectivity towards cysteine residues on proteins. The corresponding conjugates, which feature an azanorbornadiene thio-vinyl sulfone linker, may be subsequently reacted with tetrazines to form pyrrole-linked protein conjugates that are stable in both human plasma and in the presence of high amounts of glutathione. The use of azanorbornadiene bromovinyl sulfones for cysteine-selective protein modification combined with azanorbornadiene/tetrazine bond-cleavage reaction, constitutes a new and robust method for the preparation of stable and chemically-defined bioconjugates.

Acknowledgements

We thank the Spanish Ministry of Education, Culture and Sport under the FPU program (E.G.M.), the Spanish Ministry of Economy and Competitiveness (CTQ2016-77270-R to A.J.M.-V., E.G.M. and I.R, RTI2018-099592-B-C22 and RYC-2013-14706 to G.J.-O. and RTI2018-099592-B-C21 to F.C.), the EU (Marie-Sklodowska Curie Actions to E.J.M., GA No. 701473) and FCT Portugal (FCT Investigator to G.J.L.B., IF/00624/2015) for funding. We thank Dr André Neves and Prof. Kevin Brindle (CRUK Cambridge Institute, UK) for providing the C2Am protein; Dr S. Massa and Prof N. Devoogdt (Vrije Universiteit Brussel, Brussels) for the generous gift of the HER2-targeting nanobody 2Rb17c, and Dr Vikki Cantrill for her help with the preparation and editing of this manuscript. We also thank CITIUS for NMR and mass spectrometry assistance and BERONIA (Universidad de La Rioja) for computer support. G.J.L.B. is a Royal Society University Research Fellow (URF\R\180019) recipient of an European Research Council Starting Grant (*TagIt*, GA. 676832).

Keywords: azanorbornadiene • bioorthogonal chemistry • tetrazine • cleavage reactions • inverse electron-demand Diels-Alder

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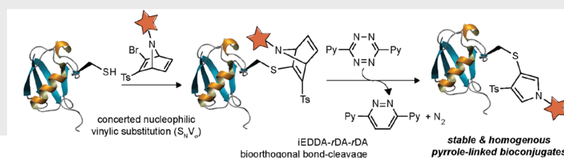

Click to stabilize: An azanorbornadiene bromovinyl sulfone reagent allowed site-selective protein modification and, in combination with a tetrazine partner, enabled the preparation of stable pyrrole-linked protein conjugates through a new bond-cleavage reaction. The latter proceeds through a three-step cascade reaction that involves iEDDA between tetrazine and the azanorbornadiene-tagged protein with subsequent double *retro*-Diels–Alder reaction conjugate.

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Stable pyrrole-linked bioconjugates through tetrazine-triggered azanorbornadiene fragmentation

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