

Stable Pyrrole-Linked Bioconjugates through Tetrazine-Triggered Azanorbornadiene Fragmentation

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Abstract: An azanorbornadiene bromovinyl sulfone reagent for cysteine-selective bioconjugation has been developed. Subsequent reaction with dipyrindyl tetrazine leads 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 has potential for the routine preparation of protein conjugates for chemical biology studies.

The installation of synthetic modifications, such as fluorescent/affinity probes or cytotoxic drugs, at a pre-determined site on a protein can either 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, thio-succinimide-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 does not reach the intended tissue, having and decreased therapeutic efficacy.^[10] However, through either hydrolysis^[11] or application of a 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 alternate strategy (in which a specific bond is cleaved instead of formed) has been proposed and used 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 a spontaneous double retro-Diels–Alder (rDA) reaction, which leads to simultaneous extrusion of N₂ and of an aromatic pyridazine, the corresponding isoindole or isobenzofuran is formed



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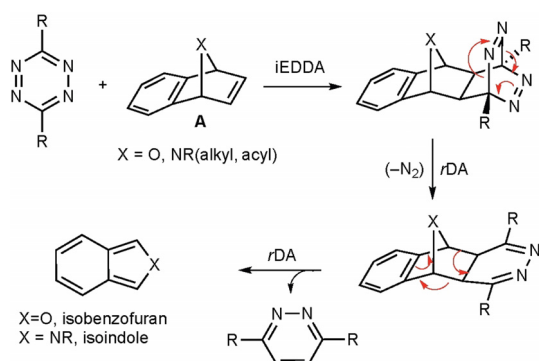
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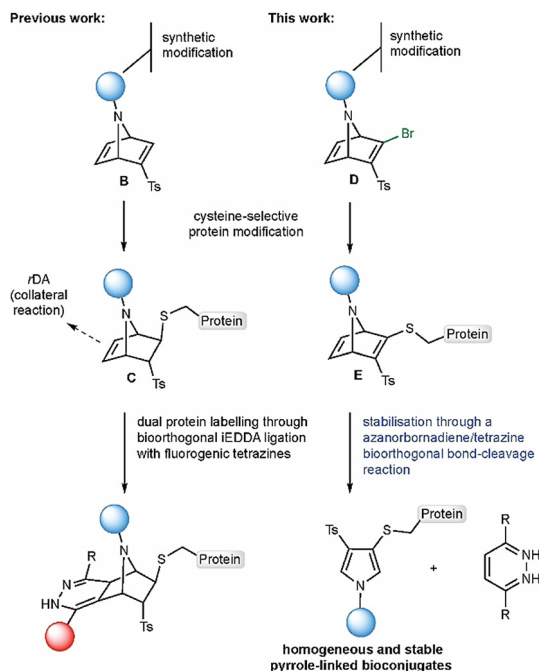
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Scheme 1. Warrener's synthesis of isobenzofurans and isoindoles.

quantitatively. However, these products are highly unstable because of their high dienic character.

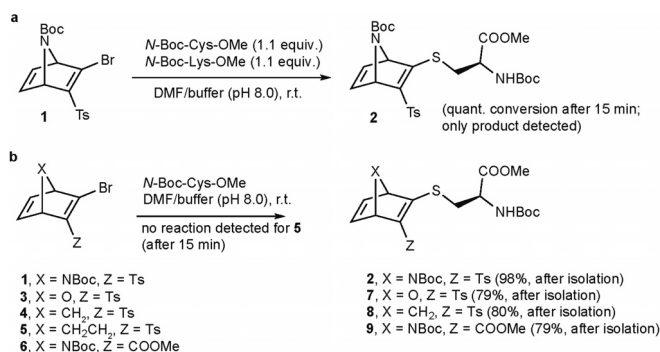
We have recently developed a method for residue-specific dual protein labelling that consists of cysteine-selective thio-Michael addition to the 7-azanorbornadiene system **B** followed by bioorthogonal iEDDA labelling of the resulting 7-azanorbornene conjugate with fluorogenic tetrazines (Scheme 2).^[19] The ability to perform the iEDDA reaction enabled the incorporation of a second label and also avoided collateral rDA of the 7-azanorbornene **C**. Here we describe the use of a [2.2.1]bicyclic 2-bromovinyl sulfone of type **D** as a reagent for site-selective protein bioconjugation purposes. We hypothesized that the strained bromovinyl sulfone could react with the sulfhydryl side-chain of cysteine in a selective and fast manner to afford **E**. However, and contrary to **C**, the subsequent reaction of **E** with tetrazines would proceed by means of a tetrazine ligation/rDA cascade as Warrener's



Scheme 2. Different behavior of azanorbornadienes of types **B** and **D** and their reactions with tetrazines for protein bioconjugation. Ts = 4-toluenesulfonyl.

azanorbornadienes. 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-azanorbornadienes, respectively,^[20] to the best of our knowledge no examples have been reported for the tetrazine-mediated preparation of pyrrole derivatives from 7-azanorbornadienes.

The synthesis of the racemic [2.2.1]bicyclic bromovinyl sulfone **1** (for structure see Scheme 3) was achieved through Diels–Alder reaction between bromoethynyl *p*-tolyl sulfone and commercially available *N*-*tert*-butyloxycarbonyl (Boc)-pyrrole according to a reported procedure (see Scheme S1 in the Supporting Information).^[21] The 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 *N,N*-dimethylformamide (DMF) as a cosolvent. 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



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. Boc = *tert*-butoxycarbonyl.

nucleophilic vinylic substitution (S_NV_o) mechanism with an activation barrier of $\Delta G^\ddagger \approx 12$ kcal mol⁻¹ for the *N*-Moc-[2.2.1]azabicyclic bromovinyl sulfone and methyl thiolate abbreviated models (Figure 1 a; Figures S11 and S12).

The cysteine derivative *N*-Boc-Cys-OMe only required 5 min for quantitative conversion into **2**. Conversely, the lysine derivative *N*-Boc-Lys-OMe only resulted in 32% conversion into the corresponding product after 2 h of reaction time (see Figure S5). Accordingly, the reaction between the same *N*-Moc-azabicyclic 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 (see Figures S11 and S12). Other amino acid nucleophilic side-chains (serine, arginine, methionine, or threonine) showed no reactivity towards **1**, whereas histidine showed a similar reactivity profile to that of lysine (see Figures S1–S6). Altogether these data demonstrate the superior kinetics of the reaction

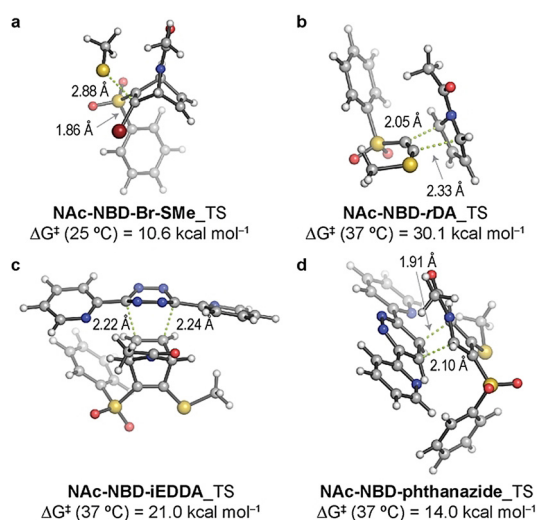
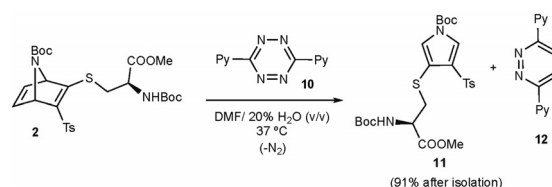


Figure 1. Lowest-energy structures for the a) concerted nucleophilic vinylic substitution (S_NV) of the *N*-acetyl analogue of **1** (*N*-Ac-NBD-Br) with methyl thiolate, b) retro-Diels–Alder (*rDA*) of thiovinyl sulfone *N*-Ac-NBD-SMe, c) inverse electron demand Diels–Alder (iEDDA) cycloaddition of *N*-Ac-NBD-SMe with tetrazine **10**, and d) final *rDA* 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 (ΔG^\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 Supporting Information.

between [2.2.1]bicyclic bromovinyl sulfone reagents and cysteine.

The sulfone **2** showed high stability and no *rDA* 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-azanobicyclic analogue in chloroform (see Figure S13). This result contrasts with the 7-azanorbornene analogue of type **C**, which we found to be more prone to undergoing slow *rDA* breakdown under the same reaction 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**; Scheme 3). The 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; see Figure S12), whereas the 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 reaction conditions. The calculated activation barrier for the reaction of MeS^- with the *N*-Moc abbreviated model of the [2.2.1]azabicyclic bromovinyl ester **6** ($\Delta G^\ddagger \approx 13.8$ kcal mol⁻¹; see Figure S16) is slightly higher than for **1**.

Next, we studied the reactivity of **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 *rDA* reactions, which led to the formation of



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

a thiol-conjugated pyrrole (**11**) in an excellent 91 % yield after isolation, along with the 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 *rDA* decomposition of 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 $0.026 (\pm 0.002) \text{ M}^{-1} \text{ s}^{-1}$ at 37 °C was determined for the triple-cascade reaction with the initial cycloaddition step as rate limiting (see 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 **10** in a similar manner to **2** (see Scheme S3).

Our data demonstrate that azanorbornadiene bromovinyl sulfones react selectively and rapidly with cysteine, whilst an electron-rich alkene remains unreacted, thus enabling a further iEDDA-*rDA*-*rDA* 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 homogeneous and stable pyrrole products based simply on these synthetically 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; see Scheme S4 for synthetic preparation). As a model protein, we chose ubiquitin, which was engineered with a surface exposed cysteine at position 63 (Ub-K63C).^[22] When we reacted Ub-K63C with 5 equivalents **13** in NaP_i buffer (50 mM, pH 8.0) with DMF as a cosolvent (10 %) at room temperature for 30 min, a single and homogeneous product was formed as determined by LC-MS (Figure 2a). Further reaction of the resulting bioconjugate **14A** with thio-specific Ellman's reagent confirmed that the cysteine was completely consumed during the reaction (see Figure S23). To further confirm cysteine chemoselectivity, we first reacted Ub-K63C with a maleimide probe (see Figure S27) and then examined reaction of the resulting conjugate with **13**. After incubation of Ub-thiosuccinimide with **13** for 1 h at 25 °C (the

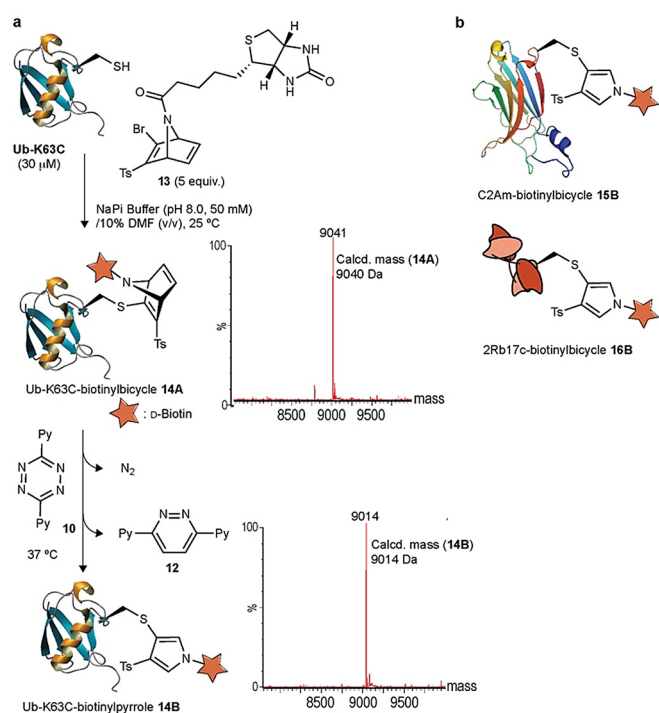


Figure 2. a) Bioconjugation of ubiquitin (Ub-K63C) with biotinyl-azanorbornadiene **13** followed by iEDDA reaction. Deconvoluted mass spectra obtained before and after the bioorthogonal reaction with **10**. b) Product of the reaction of C2Am and anti-HER2 2Rb17c nanobody with **13** followed by iEDDA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

reaction conditions required for complete reaction of **13** with Ub-Cys), only less than 5% of unspecific reaction, presumably at lysine, was observed (see Figure S28). This result 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 whether the thio-vinyl sulfone could undergo iEDDA followed by double *rDA* when treated with a tetrazine. We found that treatment of **14A** with an excess of **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 **13** in the bioconjugation of two other 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; see 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 **13** can react with multiple cysteine residues on an antibody. Upon interchain disulfide reduction, eight accessible cysteine residues become available on trastuzumab and enabled the installation of an average of 4.3 modifications per antibody as detected by native MS (see Figures S39–S41).^[25] Finally, we performed extensive molecular dynamics simulations on **14B** and **15B** (see the Supporting Information for details). According to

these calculations, the 3D structure of the proteins remains unaltered upon the chemical modification (see Figure S43), which is a pre-requisite for biological activity.

Next, we evaluated the stability of both **14A** and **14B** in human plasma and with an excess of glutathione. After 24 h of incubation at 37 °C in plasma, **14A** remained unaltered (Figure 3a). However, the incubation of **14A** with an excess

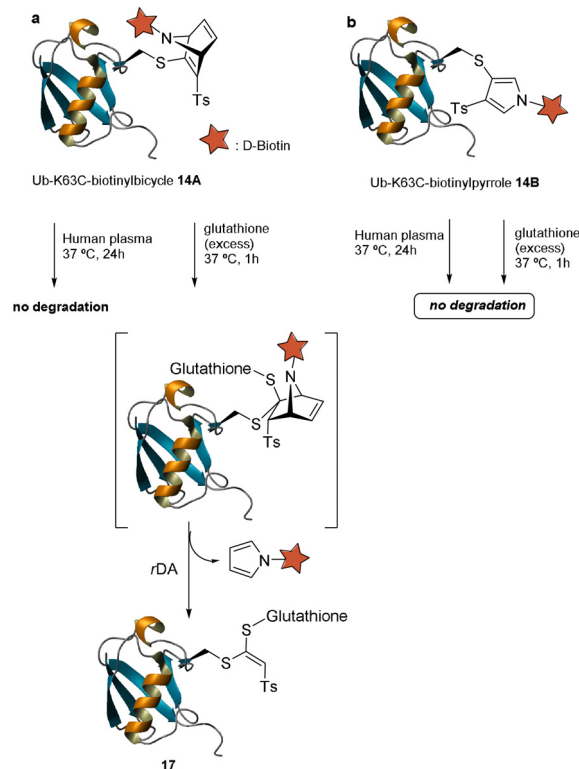


Figure 3. Stability studies of Ub-K63C bioconjugates **14A** (a) and **14B** (b) both in plasma and with an excess of glutathione, respectively.

of glutathione (100 equiv) for 1 h converted **14A** into the bioconjugate **17** as detected by LC-MS. Although the thio-vinyl sulfone functionality is not prone to 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 *rDA* of the resulting norbornene thioketal. In contrast, **14B** was shown to be fully stable, both in plasma and when reacted with excess glutathione (Figure 3b).

Finally, we show that the azanorbornadiene bromovinyl sulfone reagent reacts with the cysteine proteome in HeLa cell lysates. Importantly, protein labelling improved with increasing concentration of **13** (see 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.

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Conflict of interest

The authors declare no conflict of interest.

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