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Cysteine-Cysteine Cross-Conjugation of both Peptides and Proteins with a Bifunctional Hypervalent Iodine-Electrophilic Reagent

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Abstract: Peptide and protein bioconjugation sees ever-growing applications in the pharmaceutical sector. Novel strategies and reagents that can address the chemo- and regioselectivity issues inherent to these biomolecules, while delivering stable and functionalizable conjugates, are therefore needed. Herein, we introduce the crosslinking ethynylbenziodazolone (EBZ) reagent JW-AM-005 for the conjugation of peptides and proteins through the selective linkage of cysteine residues. This easily accessed compound gives access to peptide dimers or stapled peptides under mild and tuneable conditions. Applied to the antibody fragment of antigen binding (Fab) species, JW-AM-005 delivered rebridged proteins in a one-pot three-reaction process with high regioselectivity, outperforming the standard reagents commonly used for this transformation.

Introduction

The bioconjugation of peptides and proteins with natural or synthetic molecules stands at the forefront or modern drug discovery and biomaterial sciences.[1-5] Chemical modifications allow optimizing the pharmacological properties and bioactivity of peptides, proteins and oligonucleotides.[6-8] The development of new bioconjugation techniques is essential for continuing progress in this area, yet the multiple functional groups present in biomolecule constitute a formidable challenge for selective transformations.[1-10] The most successful approaches to perform bioconjugation are based on either the installation and modification of non-natural amino acids with unique reactivity, or the functionalization of more reactive natural amino acids present in low abundance.[6-12] The latter has the advantage of not requiring time-consuming and technically difficult modifications of peptides and proteins with non-natural amino acids, but represents a challenge of selectivity, as most reactive natural amino acids are inherently nucleophilic.[13-16] Cysteine bioconjugation has been most successful due to the unique nucleophilicity of the sulfur atom combined with its low abundance.[16]

When considering the preponderance of nucleophilic functionalities in biomolecules, the use of bis-electrophilic linkers appears especially attractive for bioconjugation (Figure 1a). Linkers based on alkylation (**1**),[8,16] acylation (**2**),[17] Michael addition (**3**,**4**),[18] nucleophilic aromatic substitution (**6**),[19] and metal mediated coupling (**7**)[20] have been therefore developed for cysteine-cysteine and, to a lesser measure, lysine-lysine cross-conjugation. The use of these linkers with two identical electrophilic groups limits potential applications to either intramolecular processes (macrocyclization, stapling, disulfide re-bridging) or the formation of homodimers.[1,9,19] However, the intermolecular coupling of two different biomolecules is highly desirable for the formation of peptide-peptide, protein-peptide, peptide-drug,[21,22] protein-drug, antibody-drugs conjugates (ADC)[23] and antibody-oligo conjugates (AOC),[24] all of which are extensively used in vaccines, immunotherapy, drug delivery and cancer therapy.

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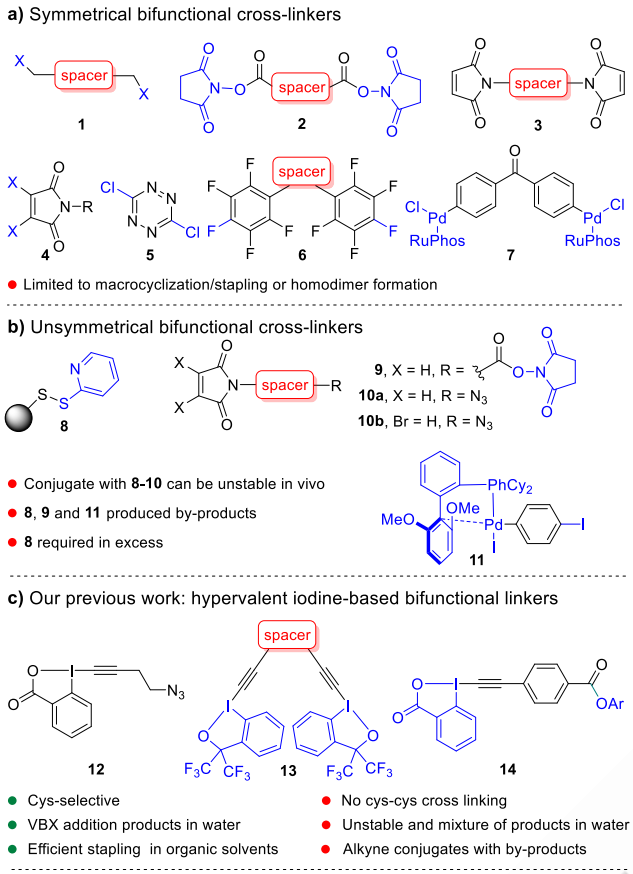

Figure 1. Bis-electrophilic linkers for bioconjugation, a) homo-bifunctional, b) hetero-bifunctional, and c) our previous work: hypervalent iodine based linkers.

In order to realize such selective cross-conjugation, the use of non-symmetrical linkers with sufficient difference in reaction rates between the two electrophilic groups is needed. Maleimide-succinimidyl esters **9** are currently among the most popular hetero-bifunctional cross linkers used in bioconjugation.[1] However, maleimide conjugates can also present issues of stability in biological systems and the activated ester of **9** get rapidly hydrolyzed in alkaline aqueous media and reacts with both thiols and amines.[26] The issue of the ester reactivity can be solved by replacing it with an azide (**10a**) to perform a biorthogonal reaction, but this does not allow any longer the use of natural amino acids as conjugation partners.[27] Next generation maleimide (NGM) dibromide cross-linkers (**10b**) have also been recently found particularly attractive for their high cysteine selectivity as well as for their convenient use in the generation of ADCs through disulfide rebridging.[28-29] Nevetheless, there is still an urgent need for crosslinkers that are stable under physiological conditions and easily further functionalized after rebridging. In a recent work, Pentelute, Buchwald and co-workers reported the successful use of palladium complex **11** for protein-protein cysteine bioconjugation leading to stable products with high selectivity.[30] However, this complex requires arduous and costly synthesis to be accessed, thus limiting its application.

In the Waser group, we have introduced ethynylbenziodoxolones (EBXs) hypervalent iodine reagents for cysteine bioconjugation. These compounds present higher thiol selectivity than alkylation reagents, with reaction rates comparable to Michael addition to maleimides, and lead to the formation of stable thioalkynes or vinylbenziodoxolones (VBXs) as conjugates.[31,32] The use of EBXs such as **12** was first reported, which could be used in water for the generation of VBX adducts without by-product formation, and enable further conjugation via biorthogonal azide cycloaddition reactions.[31] More recently, we developed reagents with two electrophilic reactive sites, such as **13** – bearing two EBX groups – or **14**, which has one EBX and one activated ester.[32] These reagents could be respectively used for cysteine-cysteine or cysteine-lysine conjugation, albeit exclusively in organic solvents, as the use of water often led to a mixture of products and degradation.

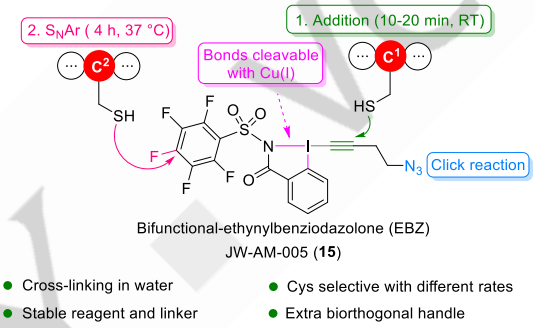

Figure 2. This work: JW-AM-005 (15) – a bifunctional EBZ reagent with high rate difference for Cys-Cys cross-linking.

Herein, we report the design and application of the new crosslinking hypervalent iodine reagent JW-AM-005 (**15**), a nitrogen analogue of EBXs coined ethynylbenziodazolone (EBZ) (Figure 2). In the Waser group, we have used EBZs as electrophilic alkynylation reagents for the enantioselective copper-catalyzed oxyalkynylation of diazo compounds,[33] but they had never been used for bioconjugation so far. Compared to EBXs, they have the unique advantage of possessing a nitrogen substituent poised for further diversification. Inspired by the work of the Pentelute group, who have extensively studied the reactivity of perfluoro aryl groups in bioconjugation via S_NAr reactions,[19] we designed water compatible JW-AM-005 (**15**), which is easily accessible in two steps. Similarly to EBX reagent **12**, JW-AM-005 (**15**) undergoes fast addition to thiols (< 10 minutes at RT) at low concentrations (5-20 mM range) to give the corresponding S-VBZ derivatives. The electron-poor perfluorinated aryl group on the sulfonamide can then be reacted in a second step with another thiol-containing molecule. This second reaction is orders of magnitude slower (3-5 h at 37 °C) than the first one, allowing smooth and selective crosslinking of two different thiols without the use of a large excess of reagent or reaction partner. Added advantages of this new EBZ crosslinking reagent is that only fluoride is generated as a by-product and the hypervalent bond can be easily cleaved using a copper(I) salt, offering promising perspectives for the controlled release of the conjugated thiols.

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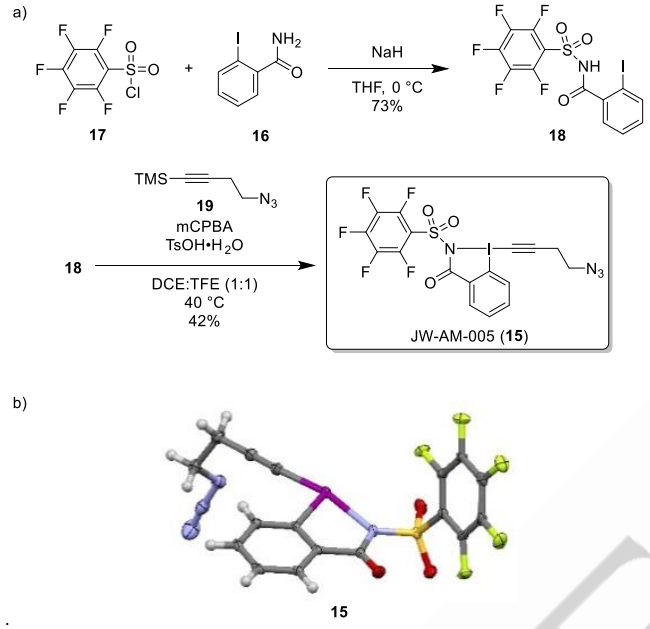
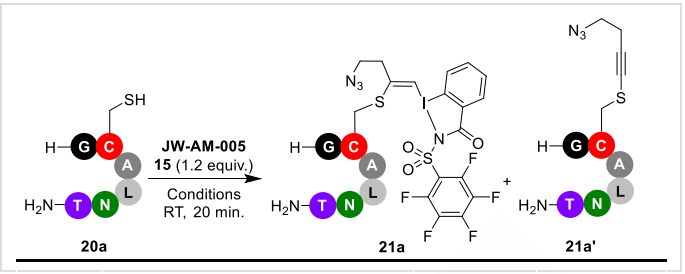
Results and Discussion

The synthesis of reagent JW-AM-005 (**15**) was carried out in just two steps starting from the commercially available 2-iodobenzamide (**16**) (Scheme 1a). The latter was first treated with sulfonyl chloride **17** in the presence of sodium hydride to give sulfonamide **18** in 73% yield. Subsequently, oxidation of **18** with *m*CPBA followed by reaction with silyl alkyne **19**, furnished the desirable compound **15** in 42% yield, whose structure was also confirmed by X-ray single crystal analysis (Scheme 1b).[34]



Scheme 1. [a] Synthesis of reagent JW-AM-005 (**15**) [b] X-Ray crystal structure of **15**.

Unprotected hexapeptide amide **20a** bearing a cysteine, a threonine and a free *N*-terminus was then synthesized using standard Fmoc-solid phase synthesis (See ESI: page S20; the syntheses of the peptides used in this work are reported in pages S20-25) (Table 1). This peptide was selected to optimize the reaction for the first Cys bioconjugation, as it would allow assessing selective sulfur functionalization over oxygen or nitrogen nucleophiles. When **20a** was treated with only 1.2 equivalents of **15** in a mixture of 50 mM Tris buffer pH 8.0: DMF (1:1), vinylbenziodazolone (VBZ) product **21a** was obtained in 65% HPLC calibrated yield (for the calibration, see ESI: pages S25-26) (entry 1). The reaction profile was very clean, with complete conversion of the starting peptide **20a**. Only two by-products could be observed: a small variable amount of disulfide dimer (2-14%), and alkyne **21a'**, which was formed in 8% yield. Thioalkynes are the major products obtained, when the reaction with EBX or EBZ reagents is carried out in organic solvents.[35-36] Neither reaction on threonine or the *N*-terminus nor degradation of product **21a'** occurred.



Entry	Reaction conditions	21a (%)[a]	21a' (%)[a]
1	50 mM Tris:DMF, pH 8.0	65	8
2	50 mM HEPES:DMF, pH 8.0	77	11
3	50 mM PB:DMF, pH 8.0	74	13
4	50 mM PB:DMSO, pH 8.0	47	14
5	50 mM PB:ACN, pH 8.0	75	6
6	50 mM PB:ACN, pH 7.4	73	7
7	50 mM PB:ACN, pH 6.0	79	8
8	H_2O:ACN	75	7
9[b]	50 mM PB:ACN, pH 8.0	76	4
10[c]	50 mM PB, pH 8.0;DMF 2%v/v	53%	
11[d]	50 mM PB:ACN, pH 8.0	82[f]	7
12[d][e]	50 mM PB:ACN, pH 8.0	77[f]	6

Reaction conditions: 0.0010 mmol of **20a**, 0.0012 mmol of **15**, shaken in the indicated 1 : 1 mixture of buffer and organic solvent for 20 minutes. The overall concentration was 10 mM with respect to **20a**, unless specified otherwise. [a] Yields of **21a** were determined by HPLC-MS and are calibrated; yields of **21a'**, were estimated without calibration. [b] The reaction was performed at a 2 mM concentration, for 80 minutes. [c] The reaction was performed at a 2 mM concentration in 2% DMF/50 mM PB pH 8.0 (*v*/*v*), for 40 min. The yield was estimated by uncalibrated HPLC-MS. [d] The reaction was performed using 0.0080 mmol of **20a**, 0.0096 mmol of **15**. [e] The reaction was performed at 20 mM concentration. [f] Isolated yield upon preparative RP-HPLC. ACN: acetonitrile, PB: phosphate buffer.

Using a HEPES buffer resulted in a higher 77% yield of **21a**, with no significant relative increase in the formation of alkyne **21a'** (entry 2). A very similar result was obtained in phosphate buffer (PB; 50 mM, pH = 8.0) (entry 3), and prompted us to further investigate the reaction in this more physiologically compatible buffer. Other organic co-solvents were tested at first. While isopropanol had to be excluded because of the limited solubility of the reacting species, the transformation worked in DMSO, although only in 47% yield, and with a less favorable 3 : 1 ratio between **21a** and **21a'** (entry 4). The reaction in acetonitrile (ACN) provided comparable results to DMF (75% yield of **21a**; entry 5) and was therefore adopted as the optimal co-solvent for the following experiments. Interestingly, lowering the pH from 8.0 to 7.4 or even 6 had no impact on the yield (entries 6 and 7), which remained unaffected even when no buffer at all was used

Table 1. Optimization of the first Cys bioconjugation.

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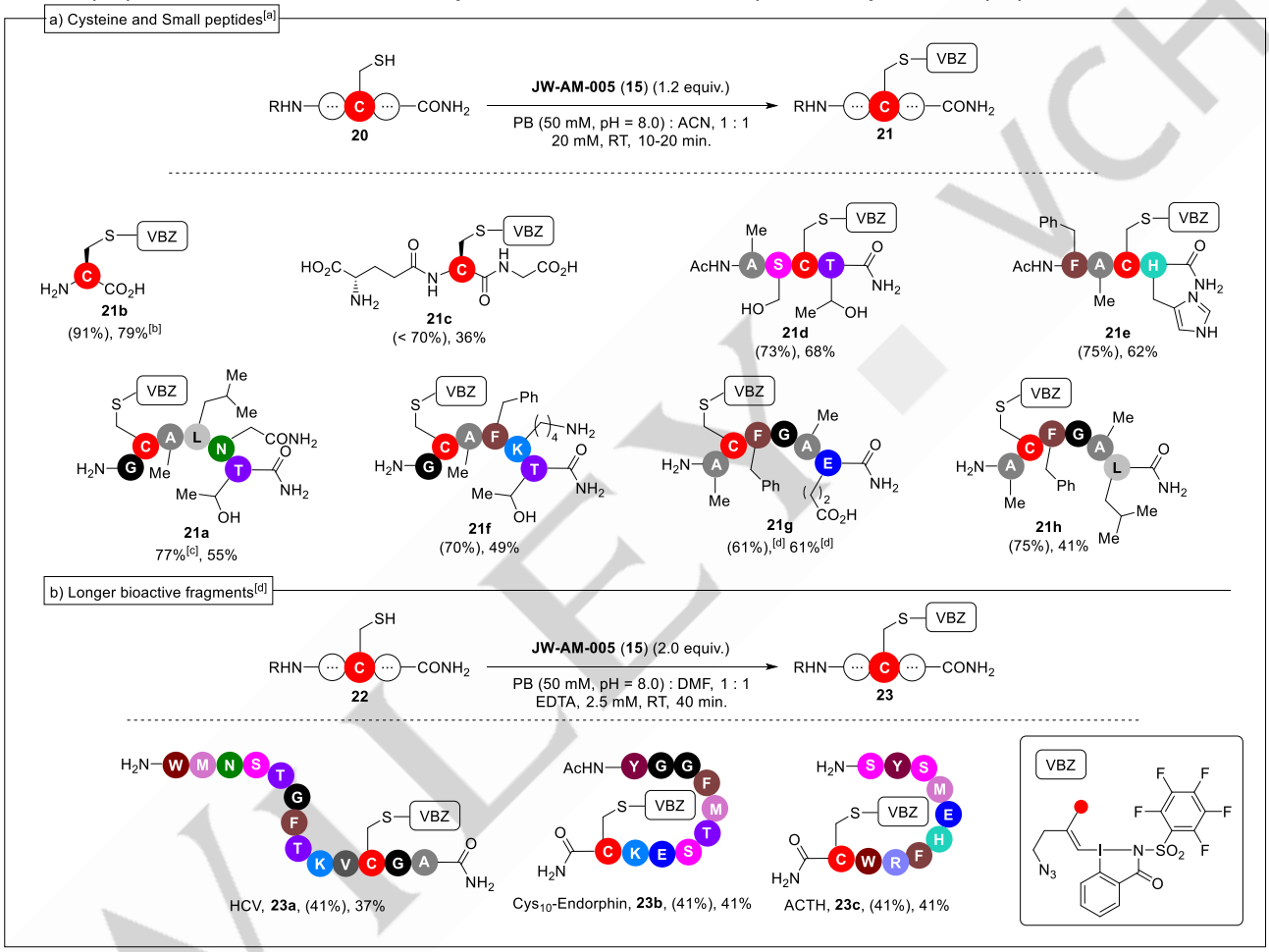
(reaction in water-ACN, entry 8). The reaction also worked as well under much more diluted conditions (2 mM), providing **21a** in 76% yield after 80 minutes (entry 9) with minimal amounts of alkyne by-product. These evidences highlight that the Cys-conjugation of **15** is very robust and highly tolerant to variations in buffer composition, solvent, pH and concentration. In order to be closer to physiological conditions, we then run the transformation in the presence of smaller amounts of organic co-solvent. At 2 mM and with only 2% of DMF in a 50 mM PB buffer, **21a** was still obtained in 53% yield after 40 min, alongside with 5% of **21a'** and disulfide (entry 10). Under these conditions, precipitation of **15** was observed, explaining the lower yield. Selecting the 50 mM PB pH 8.0 / ACN (1:1) mixture as our model solvent system, we were also pleased to observe that the reaction could be effectively scaled up (81% yield; entry 11) and successfully carried out at higher concentration (20 mM) with no significant diminution of yield (77% yield; entry 12).

Interested in how broadly applicable our method was (Scheme 2), we tested it first on unprotected L-cysteine, which was efficiently converted into the corresponding VBZ **21b** in 79% yield. With glutathione, no complete conversion was obtained even after a longer reaction time (40 minutes). The reason for the lower reactivity shown by this substrate remains unclear. Moreover, while the yield of product **21c** was estimated around 70% based on HPLC-MS analysis, only 36% of it could be isolated. This could indicate a poor stability of **21c** on preparative HPLC.



Scheme 2. [a] 0.020 mmol **20**, 0.022-0.024 mmol **15**, PB (50 mM, pH = 8.0) : ACN, 1 : 1, 20 mM with respect to **20**, at room temperature; (indicative HPLC-MS-based yields are reported in parentheses), isolated yields. [b] Reaction performed starting from 0.040 mmol L-cysteine. [c] Reaction performed at a 10 mM overall concentration. [d] Reaction performed starting from 0.0040 mmol **22**, 0.0080 mmol **15**, 0.53 mL PB (5.0 mM, pH = 8.0), 0.27 mL EDTA 6.7 mM in PB (50 mM, pH = 8.0), 0.80 mL DMF, at room temperature; (indicative HPLC-MS-based yields are reported in parentheses), isolated yields.

Better results were obtained with the Cys-conjugation of *N*-acylated tetrapeptides **21d** and **21e**. The former, derived from SARS-COV 2 (Ala_{211}-Thr_{214}) protein and containing both serine and threonine residues gave the corresponding VBZ **21d** in 68% yield, with full conversion within 20 minutes. Similarly, **21e** was isolated in 62% yield starting from histidine-containing **20e**. As shown with model substrate **20a**, peptides with unprotected *N*-termini also worked effectively, with complete cysteine selectivity observed in all cases. In particular, the reaction tolerated the presence of nucleophilic side chains, with both basic (product **21f**, isolated yield: 49%) and acidic character (product **21g**, isolated yield 61%). Hexapeptide **20h**, mostly containing lipophilic residues, provided VBZ derivative **21h** in 41% yield. We then turned our attention to larger peptides, and in particular to fragments with biological significance. With these more complex substrates, issues with solubility of the peptides and competing oxidative homodimerization, through disulfide bond formation, became more pronounced. An adjustment of the reaction

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conditions was necessary to address these problems: the Cys-conjugation was carried out at higher dilution (2.5 mM), with replacement of ACN with DMF, while the addition of EDTA helped to limit oxidative dimerization. Under such re-optimized conditions, we could isolate VBZ **23a** in 36% yield starting from fragment **22a**, derived from hepatitis C virus envelope glycoprotein E2.[37] Endorphine, one of the most important natural peptide hormone, was modified on its *C*-terminus to add a cysteine residue,[38] resulting in modified peptide **22b** that gave its corresponding VBZ conjugate **23b** in 41% yield. Finally, we synthesized peptide **22c** containing the $H_6F_7R_8W_9$ sequence, which is present in human adrenocorticotropic hormone (ACTH) and acting as binding site to the melanocortin receptor 2 (MC2R).[39] The corresponding product **23c** was also isolated in 41% yield. Overall, in all these larger peptides, the presence of potentially reactive amino acids such as histidine, tyrosine, arginine and methionine was tolerated, although the corresponding VBZ derivatives **24** were obtained in lower yields and the formation of several (unidentified) by-products was observed by HPLC-MS analysis.

Having developed an efficient method for the monoconjugation of reagent **15** with cysteine-containing substrates, we then investigated the possibility to add a second thiol peptide to the so obtained conjugates **21** via a S_NAr reaction on the polyfluorinated aromatic group. As a proof of concept, we started our study on the homoconjugation of our model hexapeptide **21a**. A stepwise protocol was considered at first, consisting in the treatment of the preformed VBZ-derivative **21a** with a small excess (1.5 equivalents) of **20a**. After a short reoptimization of the reaction conditions (see ESI for details: pages S47-50), we found that an effective procedure relied on the reaction of the two species in a 1:1 mixture of PB buffer (50 mM, pH 8.0) and DMF at 37 °C for 4 hours (Scheme 3a). While the transformation also occurred at room temperature within the same reaction time, mild heating led to higher yield and cleaner HPLC-MS profiles. Under these adjusted conditions, homodimer **24aa** was isolated by preparative RP-HPLC in 69% yield (Scheme 3d). In order to avoid the isolation and purification of VBZ intermediate **21**, we then developed a one-pot protocol (one-pot protocol 1; Scheme 3b). Accordingly, upon reacting **20a** with 1.2 equiv. **15** at room temperature for 30 minutes, adding an excess of **20a** to the untreated reaction mixture resulted in the formation of **24aa** in 51% yield after 4 hours at 37 °C. An even more straightforward approach implied the direct treatment of **15** with 2.2 equivalents of the starting peptide (one-pot protocol 2; Scheme 3c). In this case, homodimer **24aa** was obtained in 41% yield.

At this point, we wondered whether our stepwise and one-pot methods might be applied to the cross-conjugation of two different thiol-containing molecules (Scheme 3e). The use of the stepwise protocol was successful in all the examined examples, with heterodimers **24ae**, **24fe**, **24ge** and **24gf** isolated in good to excellent yields (62-89%). The application of the one-pot procedure, while viable, proved less effective. Much lower yields were obtained, and the formation of the homoconjugation products corresponding to both reacting peptides was also observed. In particular, under such conditions **24ae** and **24fe** could be only isolated in 39% and 18% yields respectively. While this homodimerization is due to an S_NAr process occurring on the cross conjugates, it remains unclear why it was not observed in the stepwise experiments. The specific reactivity of the used second peptide must certainly play a significant role, as suggested by the fact that no homoconjugate was generated together with **24ge** when the one-pot protocol was utilized.

Importantly, all obtained VBZ cross-linked conjugates were shown to be stable under the conditions classically used for their synthesis or purification. In addition, treating conjugate **24ae** with an excess of glutathione in a 10 mM DMF : PB 1 : 1 mixture for 24 hours did not lead to any significant degradation (see ESI: pages S56-58). Nevertheless, the weak hypervalent iodine bond should be still cleavable using stronger reductants. This has the potential to make our method even more attractive, as it could be used to release selectively the two individual biomolecules, each labelled with a fragment of the initial EBZ reagent. In a preliminary approach, a modified version of the L-proline-promoted Rosenmund-von Braun reaction first reported by Yoshikai and co-workers on VBX compounds[40] was successfully applied to cross-conjugate **24gf** to generate two main fragments **25** and **26** in 24% and 38% yield, respectively (Scheme 4). Interestingly, two other analogues of **26** were also detected by HPLC-MS, the reduced arene **26'** and nitrile derivative **26''**, presumably resulting from the reductive elimination of a Cu(III)CN intermediate. While these results are promising, they also indicate that further investigations will be needed to obtain a clean and selective linker cleavage.

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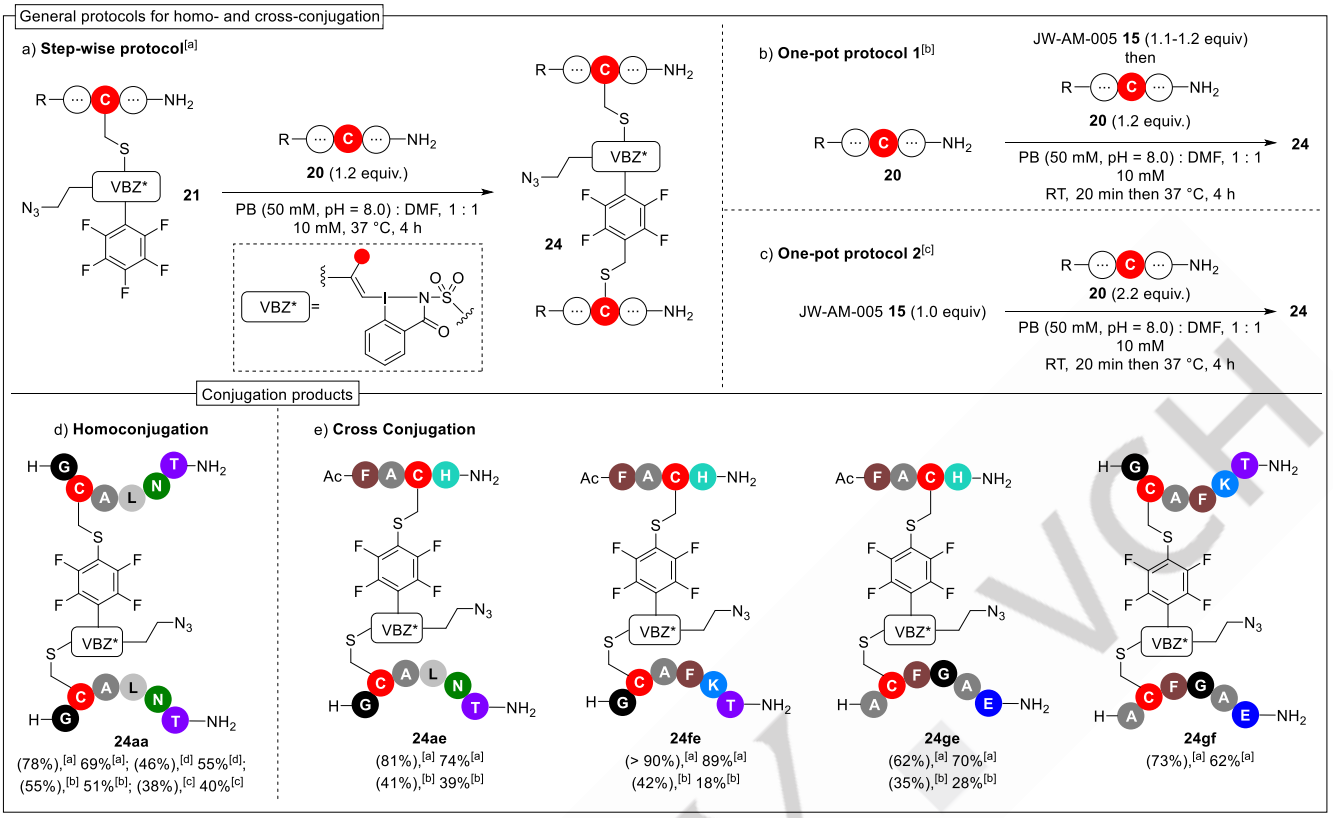
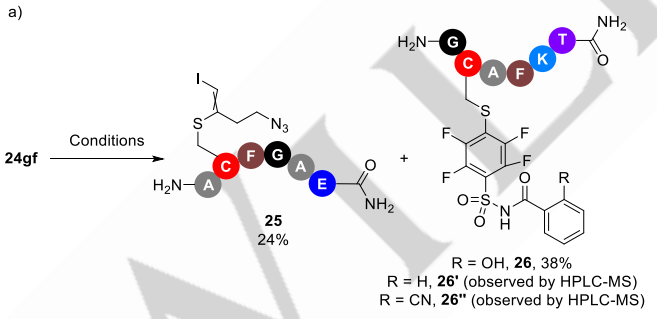
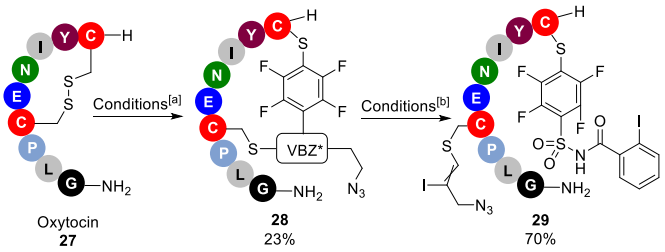


Scheme 3. [a] 0.0040 mmol **21**, 0.0044-0.0048 mmol **15**, PB (50 mM, pH = 8.0) : DMF, 1 : 1, 10 mM with respect to **21**, at 37 °C; (indicative HPLC-MS-based yields are reported in parentheses), isolated yields. [b] 0.0040 mmol **20**, 0.0048 mmol **15**, PB (50 mM, pH = 8.0) : DMF, 1 : 1, 10 mM with respect to **20** at room temperature for 20 minutes; then 0.0044-0.0048 mmol **20** at 37 °C; (indicative HPLC-MS-based yields are reported in parentheses), isolated yields. [c] 0.0072 mmol **20**, 0.0032 mmol **15**, PB (5.0 mM, pH = 8.0) : DMF, 1 : 1, 10 mM with respect to **20**, at 37 °C; (indicative HPLC-MS-based yields are reported in parentheses), isolated yield. [d] Same conditions as in [a] but at room temperature.



Scheme 4. Reaction conditions: 0.0025 mmol **24af**, 0.025 mmol L-proline, 0.050 mmol CuCN, ACN : PB (50 mM, pH = 8.0) = 1 : 1, 2.5 mM with respect to **24gf**, at 37 °C for 18 hours; isolated yields.

Having shown the utility JW-AM-005 (**15**) for the crosslinking of different peptides, we wondered if this compound might also be used for the cysteine-cysteine rebridging of peptidic fragments resulting from the reduction of disulfide bonds. Such an operation would provide a convenient access to VBZ conjugates easily further modified thanks to the azide group. A large number of bioactive peptides in nature are in fact characterized by one or multiple intramolecular disulfide bridges. As a notable example, oxytocin (**27**) is a hormone and neuropeptide containing a single S-S bond that plays a role in processes such as reproduction, childbirth and lactation (Scheme 5).[41]



Scheme 5: Reaction conditions [a] 0.0090 mmol Oxytocin (**27**, as acetate salt), 0.0180 mmol TCEP, 5 mM in PB (50 mM, pH = 8.0), at 37 °C for 2 hours; then EDTA in PB (50 mM, pH = 8.0), ACN (2 mM overall concentration), 0.0085 mmol **15**, at room temperature for 24 hours; isolated yield. [b] 0.0021 mmol **28**, 0.025 mmol CuI, PB (50 mM, pH = 8.0) : DMF, 1 : 1, 5 mM with respect to **28**, at 37 °C for 30 hours; isolated yield.

Native oxytocin was treated with the reductant TCEP in a PB buffer. The subsequent addition of JW-AM-005 (**15**) in the presence of EDTA (to minimize intramolecular reoxidation) and ACN as the organic co-solvent provided the desired macrocycle **28** in 23% isolated yield. Surprisingly, only one of the two possible isomers was observed. It cannot be excluded that the second one

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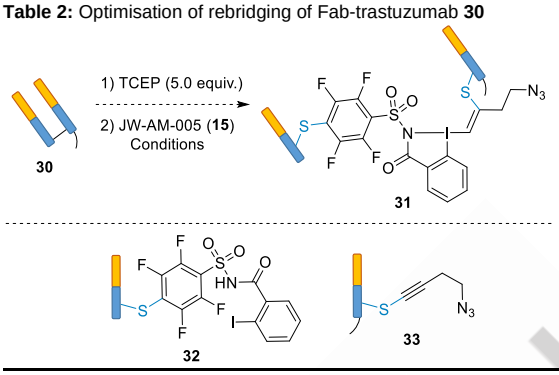
was not stable under the reaction conditions. The structure of **28** was later on confirmed by the MS-MS analysis of product **29** resulting from the reductive cleavage of the hypervalent bond in **28**, which was efficiently accomplished in 70% yield using copper(I) iodide under aqueous conditions. In this reaction, the formation of multiple coupling derivatives of the VBZ core could be avoided by using CuI instead of the aforementioned combination of CuCN and L-proline: the 2-iodobenzamide **29** was cleanly obtained in 70% yield as the sole product.

In an effort to evaluate the potential of JW-AM-005 (**15**) as disulfide crosslinking reagent on more complex substrates, we investigated the rebridging of fragment antigen-binding (Fab) species. Fabs are ~50 kDa proteins classically produced by the enzymatic digestion of a parent monoclonal antibody (mAb). With the retained targeting properties of whole mAbs but a smaller size, Fab conjugates are attractive due to their enhanced tissue penetration.[42] Structurally speaking, they consist of a light chain (LC) covalently connected through a single interchain disulfide bond to a Fd chain, the digested remnant of the mAb's heavy chain (HC). For our optimization studies, we opted for the Fab fragment **30** of trastuzumab – an FDA-approved monoclonal antibody (mAb) used against HER2$^+$ breast cancer cells –,[43] which we easily obtained after two consecutive pepsin- and papain-mediated digestion steps.[44] Reduction of the single interchain disulfide bond to free the cysteines' thiols was carried out following known procedures, using 5 equivalents of *tris*(2-carboxyethyl)phosphine (TCEP) at 37 °C, followed by gel filtration chromatography to eliminate the excess of reagent. Rebridging efficacy was determined by SEC-MS in denaturing conditions (dSEC-MS), an approach specifically developed for our study,[45] according to two parameters: the amount of Fab detected (corresponding to the percentage of both Fab species – i.e., native disulfide-bonded Fab **30** and VBZ-rebridged Fab **31** – versus that of LC and Fd sub-species, including fragmented adducts **32** and **33**) and its average degree of conjugation (avDoC), used as a direct indicator of the rebridging efficiency.

We began our investigations by applying to our reduced trastuzumab-Fab similar conditions as those previously optimized for the production of dimers **22** (see ESI, Table S8, page S73). However, to our dismay, this led to poor rebridging due to two main factors: the generation of LC and Fd side species **32** and **33**, respectively, bearing only fragments of the expected payload; and the incomplete conjugation of **30** (avDoC < 0.3). A plausible explanation behind the formation of **32** and **33** is the fragmentation of the JW-AM-005 (**15**) payload upon thiolate addition through elimination; the reasons behind the observed selectivity of this side reaction, with the alkynyl and sulfonamide fragments being only detected on the Fd and the LC, respectively, remain poorly understood.

Table 2: Optimisation of rebridging of Fab-trastuzumab **30**



Entry	Reaction Conditions	Fab(%)[a]	avDoC
1	**15** (5 equiv. in DMSO), **BBS**, 2 mM EDTA, pH 8, 37 °C, 16 h	70	0.80
2	**15** (5 equiv. in DMSO), BBS, 2 mM EDTA, pH 8, 37 °C, **6 h**	79	0.41
3[b]	**15** (5 equiv. in DMSO), BBS, 2 mM EDTA, pH 8, 37 °C, 6 h	70	1.0
4[b]	**15** (5 equiv. in DMSO), **PBS 1X**, 2 mM EDTA, pH 8, 37 °C, 6 h	71	0.91
5[b]	**15** (5 equiv. in DMSO), **Tris**, 2 mM EDTA, pH 8, 37 °C, 6 h	82	0.27
6[b]	**15** (5 equiv. in **MeCN**), BBS, 2 mM EDTA, pH 8, 37 °C, 6 h	58	0.95
7[b]	**15** (5 equiv. in **DMF**), BBS, 2 mM EDTA, pH 8, 37 °C, 6 h	70	1.0
8[b]	**15** (5 equiv. in DMSO), BBS, 2 mM EDTA, **pH 8.5**, 37 °C, 6 h	46	0.82
9[b]	**15** (5 equiv. in DMSO), BBS, 2 mM EDTA, **pH 7.0**, 37 °C, 6 h	61	1.0
10[b]	**15** (5 equiv. in DMSO), BBS, 2 mM EDTA, pH 8, 37 °C, **5 h**	72	1.0
11[b]	**15** (5 equiv. in DMSO), BBS, 2 mM EDTA, pH 8, 37 °C, **2 h**	82	0.85
12[b]	**15** (5 equiv. in DMSO), BBS, 2 mM EDTA, pH 8, 37 °C, **0.5 h**	80	0.69
13[b]	**15** (5 equiv. in DMSO), BBS, 2 mM EDTA, pH 8, **25 °C**, 6 h	76	0.97
14[b]	**15** (5 equiv. in DMSO), BBS, 2 mM EDTA, pH 8, **4 °C**, 6 h	69	0.58
15[b]	**15** (**7.5 equiv.** in DMSO), BBS, 2 mM EDTA, pH 8, 37 °C, 6 h	84	1.06

[a]: Relative quantification determined by integration of peak areas from UV chromatograms – the remaining fraction of protein species are LC and Fd (see ESI, page S4 for more details); [b]: Reduction and rebridging conducted in one pot.

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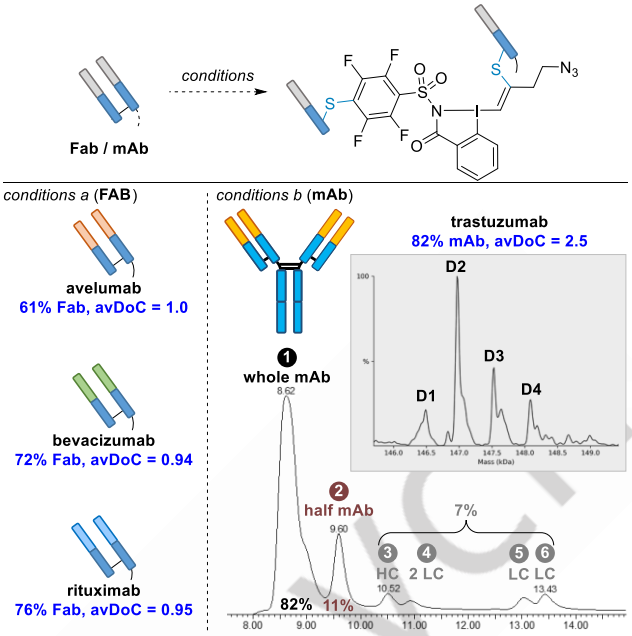
Adding EDTA in an attempt to minimize the reoxidation of cysteines and playing on both the reaction time and number of equivalents of JW-AM-005 (**15**) helped to increase rebridging, whilst maintaining the same amount of side species **32** and **33** (see ESI, Table S8, page S73).[45] Interestingly, a stark improvement was noticed when switching to borate buffered saline (BBS; entry 1; Table 2), with 70% of Fab being detected with an avDoC of 0.80, whilst decreasing the reaction time to 6 h led to improved amounts of Fab but to a less efficient rebridging, as evidenced by its halved avDoC (entry 2).

At this stage, we hypothesized that the gel purification conducted after the TCEP-mediated reduction step might favor the reoxidation of the thiols, and therefore we evaluated the possibility of conducting both the reduction and the rebridging steps in one pot (see ESI for procedure, page S72). Gratifyingly, this led to full rebridging of Fab **30** (i.e., avDoC = 1.0), the sole double-chain species detected by denaturing LC-MS (entry 3). This excellent stability of the iodine(III) bond toward these reductive conditions is remarkable, and far superior to that of more standard reagents classically used for protein rebridging (v. infra), and is further highlighted by a perfect stability of the rebridged Fab in plasma for 5 days at 37 °C (see ESI, page S93). All our subsequent efforts were thus dedicated to improving the percentage of fully rebridged Fab obtained, by minimizing the side reactions leading to partially conjugated LC **32** and Fd **33**. Varying buffer and co-solvent led systematically to a decrease in DoC, highlighting the profound influence of solvent effects on the efficacy of the conjugation (entries 4-7). Increasing pH had a detrimental impact on Fab's percentage and DoC, whilst decreasing it had little effect on both terms (entries 8-9). We also noted that similar results could be obtained after just 5 h (entry 10), but that decreasing the reaction time further led to an erosion of the DoC values and hence the rebridging efficiency (entries 11-12). Lowering the reaction temperature to 25 °C led to an improved amount of Fab with a minimal decrease in avDoC (entry 13). Interestingly, formation of side species **32** and **33** could be completely suppressed by working at 4 °C, albeit at the expense of both avDoC and amount of Fab (entry 14). Any attempt at improving the latter – notably by increasing the number of JW-AM-005 **15** equivalents (entry 15) – led to a parallel increase of the former, due to the appearance of doubly conjugated Fab species, indicating chemoselectivity issues.

Having developed optimal conditions on trastuzumab Fab, we were keen to apply our protocol to Fab from other mAb sources. Enzymatic digestion of bevacizumab, avelumab and rituximab furnished the desired Fab species in high yield and purity, which were also successfully rebridged (Scheme 6). Gratifyingly, we also demonstrated that JW-AM-005 (**15**) could be applied to whole mAbs: under slightly tweaked conditions, trastuzumab led to 82% of rebridged mAb (avDoC = 2.5), with only 11% of half mAb and 7% of LC and HC subspecies, demonstrating the broader applicability of our strategy. For the rebridged mAb, the incorporation of two VBZs was most frequent (**D2**), but insertion of 1, 3 and even 4 VBZs was also observed (**D1**, **D3** and **D4**).



Scheme 6. Reaction conditions: [a] **Fab** (1.5 mg/mL in BBS, 2 mM EDTA, pH 8), TCEP (15 mM in water, 5 equiv.), **15** (10 mM solution in DMSO, 5 equiv.) at 37 °C for 5 hours. [b] **trastuzumab** (10 mg/mL in BBS, 6 mM EDTA, pH 8), TCEP (15 mM in H_2O, 10 equiv.), **15** (10 mM solution in DMSO, 10 equiv.) at 37 °C for 5 hours. Chromatogram and mass spectrum excerpts from the dSEC-MS analysis of the rebridging of trastuzumab are provided.

Having validated the rebridging step, we next focused on the functionalization of rebridged Fab **31** through bioorthogonal reaction, taking advantage of the azide group on the VBZ payload. Performing this stepwise rebridging/functionalization process on Fab trastuzumab with strained alkyne **34** led to fully rebdriged iminobiotin-containing Fab species **35**, albeit in a lower proportion than before (Scheme 7). Suspecting that this was caused by the two successive purification steps, we also evaluated the concomitant one-pot reduction/rebridging/functionalization sequence by mixing trastuzumab Fab with a mixture of TCEP, JW-AM-005 (**15**) and BCN **34** under our optimized conjugation conditions at 37 °C. Pleasingly, this led to an increase in rebridged Fab proportion (72%), the only double-chain species detected. To the best of our knowledge, this intricate chemoselective ballet between four reactive species has never been reported before and is key in improving both the efficacy and the efficiency of our conjugation sequence. More importantly, any attempt at performing the same one pot three-step reaction with a classical rebridging dibromomaleimide reagent **36** led to mediocre rebridging (i.e., 92% Fab, avDoC = 0.10), presumably because of TCEP-mediated decomposition of the maleimide motif.[28,44,47-49]

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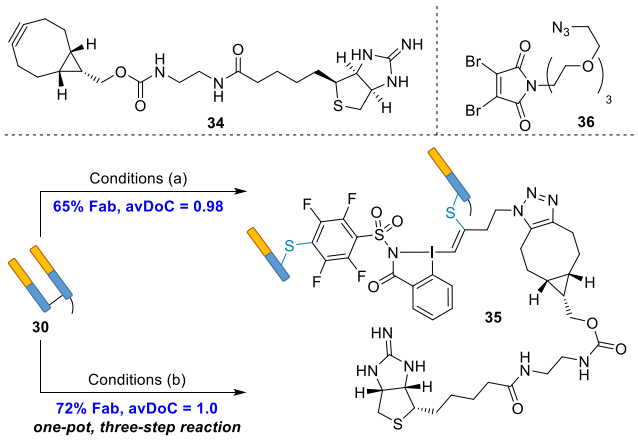
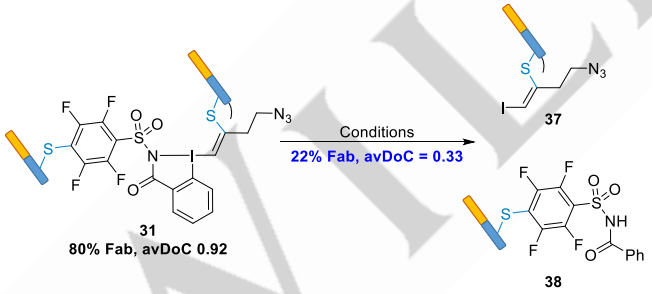


Scheme 7. Reactions conditions: [a] 1. Fab **30** (1.5 mg/mL in BBS, 2 mM EDTA, pH 8), TCEP (15 mM in water, 5 equiv.) **15** (10 mM solution in DMSO, 5 equiv.) at 37°C for 5 hours; 2. **34** (10 mM in DMSO, 30 equiv.) at 25 °C for 24 hours. [b] Fab **30** (1.5 mg/mL in BBS, 2 mM EDTA, pH 8), TCEP (15 mM in H_2O, 5 equiv.), **15** (10 mM solution in DMSO, 5 equiv.), **34** (10 mM solution in DMSO, 20 equiv.) at 37 °C for 5 hours.

Finally, we wanted to investigate whether the controlled reductive cleavage of the hypervalent iodine bond could also be applied to rebridged Fab **31**. Using a mixture of copper(I) iodide and *tris*(3-hydroxypropyltriazolylmethyl)amine (THPTA) in the presence of sodium ascorbate and aminoguanidine at 37 °C for 16 h, nearly quantitative cleavage of Fab **31** was observed, leading to fragmented species **37** – the sole Fd fragmentation species detected, which tends to suggest a regioselective rebridging – and **38** – the main LC fragmentation species detected –, as determined by denaturing LC-MS (Scheme 8).



Scheme 8. Reaction conditions: **31** (1.5 mg/mL in PBS 1X, pH 7.4), 20 equiv. CuI, 40 equiv. THPTA, 100 equiv., aminoguanidine●HCl, 300 equiv. NaAsc,at 37 °C for 16 hours.

Conclusion

In conclusion, we reported the synthesis of a novel bifunctional cross-linker incorporating EBZ and pentafluorophenyl motifs. This platform was applied to the single or double conjugation of cysteine-containing peptides and proteins, offering access to stapled peptides, homo- and hetero-dimers. We showed that the method was tolerant to a wide range of functionalities and that excellent chemo- and regioselectivity could be attained. In particular, the rebridging of the Fab fragment of several antibodies could be achieved with high efficiency and an incorporated azide group enabled further diversification of the payload. A further advantage of incorporating a hypervalent iodine bond into the cross linker is high stability under physiological conditions, even in plasma, but still inherent lability towards copper(I) species, allowing a controlled cleavage of bioconjugates. These properties further highlight the advantages of hypervalent iodine(III) reagents for the selective conjugation of cysteines and open promising perspectives in the development of new hetero-crosslinking reagents.

Supporting Information

The authors have cited one additional reference within the Supporting Information.[50]

Acknowledgements

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Keywords: antibodies • bioconjugation • cysteine • hypervalent iodine • rebridging

[1] O. Koniev, A. Wagner, *Chem. Soc. Rev.* **2015**, 44, 5495-5551.

[2] W. R. Algar, P. E. Dawson, I. I. Medintz, *Chemoselective and Bioorthogonal Ligation Reactions Concepts and Applications*, *Vol. 1*, WILEY-VCH Verlag GmbH & Co. KGaA, **2017**, https://onlinelibrary.wiley.com/doi/pdf/10.1002/9783527683451.

[3] P. Elzahhar, A. S. F. Belal, F. Elamrawy, N. A. Helal, M. I. Nounou, M. I. *Bioconjugation Drug Delivery: Practical Perspectives and Future Perceptions.* in *Pharmaceutical Nanotechnology. Methods in Molecular Biology*, *Vol 2000*, Humana, New York, **2019**, p 125.

[4] M. P. Deonarayan, G. Yahioglu, G., *Expert Opin. Drug Discov.* **2021**, *16*, 613-624.

[5] J. A. Shadish, C. A. DeForest, *Matter* **2020**, *2*, 50-77.

[6] V. G. Muir, J. A. Burdick, *Chem. Rev.* **2021**, *121*, 10908-10949.

[7] S. Benizri, A. Gissot, A. Martin, B. Vialet, M. W. Grinstaff, P. Barthelém, P. *Bioconjug. Chem.* **2019**, *30*, 366-383.

[8] O. Boutureira, G. J. L. Bernardes, *Chem. Rev.* **2015**, *115*, 2174-2195.

[9] J. N. deGruyter, L. R. Malins, P. S. Baran, *Biochemistry* **2017**, *56*, 3863-3873.

[10] N. C. Reddy, M. Kumar, R. Molla, V. Rai, *Org. Biomol. Chem*. **2020**, *18*, 4669-4691.

[11] C. Bottecchia, T. Noel, T. *Chem. Eur. J.* **2019**, *25*, 26-42.

[12] C. Sornay, S. Hessmann, S. Erb, I. Dovgan, A. Ehkirch, T. Botzanowski, S. Cianférani, A. Wagner, G. Chaubet, *Chem. Eur. J.* **2020**, *26*, 13797-13805.

[13] D. R. W. Hodgson, J. M. Sanderson, *Chem. Soc. Rev*. **2004**, *3*, 422-430.

[14] L. Gentilucci, R. De Marco, *Curr. Pharm. Des.* **2010**, *16*, 3185-3203.

[15] P. Ochtrop, C. P. R. Hackenberger, C. P. R., *Curr Opin Chem Biol.* **2020**, *58*, 28-36.

[16] R. Derba, M. R. Jafari, *Protein Pept. Lett.* **2018**, *25*, 1051-1075.

[17] A. R. Stasińska, P. Putajb, M. K. Chmielewski, *Bioorg. Chem*. **2019**, *92*, 103223-103239.

[18] J. M. Ravasco, H. Faustino, A. Trindade, P. M. P. Gois, *Chem. Eur. J.* **2019**, *25*, 43-59.

[19] C. Zhang, E. V. Vinogradova, A. M. Spokoyny, S. L. Buchwald, *Angew. Chem. Int. Ed.* **2019**, *58*, 4810-4839.

[20] E. V. Vinogradova, C. Zhang, A. M. Spokoyny, B. L. Pentelute, s. L. Buchwald, *Nature* **2015**, *526*, 687-691.

[21] B. M. Cooper, J. Iegre, D. H. O'Donovan, M. O. Halvarssonc, D. R. Spring, *Chem. Soc. Rev*. **2021**, *50*, 1480-1494.

[22] E. I. Vrettos, G. Mező, A. G. Tzakos, *Beilstein J. Org. Chem.* **2018**, *14*, 930-954.

[23] S. J. Walsh, J. D. Bargh, F. M. Dannheim, A. R. Hanby, H. Seki, A. J. Counsell, X. Ou, E. Fowler, N. Ashman, Y. Takada, A. Isidro-Llobet, J. S. Parker, J. S. Carroll, D. R. Spring, *Chem. Soc. Rev*. **2021**, *50*, 1305-1353.

[24] K. Klabenkova, A. Fokina, D. Stetsenko, D. *Molecules* **2021**, *26*, 5420.

[25] M. Geven, H. Luo, D. Koo, G. Panambur, R. Donno, A. Gennari, R. Marotta, B. Grimaldi, N. Tirelli, *ACS Appl. Mater. Interfaces* **2019**, *11*, 26607-26618.

[26] P. Akkapeddi, S.-A. Azizi, A. M. Freedy, P. M. S. D. Cal, P. M. P. Goisc, G. J. L- Bernardes, *Chem. Sci.* **2016**, *7*, 2954-2963.

[27] K. C. Nicolaou, S. Rigol, *Angew. Chem. Int. Ed*. **2019**, *58*, 11206-11241.

[28] J. P. M. Nunes, M. Morais, V. Vassileva, E. Robinson, V. S. Rajkumar, M. E. B. Smith, R. B. Pedley, S. Caddick, J. R. Baker, V. Chudasama *Chem. Commun.* **2015**, *51*, 10624-10627.

[29] M. Morais, J. P. M. Nunes, K. Karu, N. Forte, I. Benni, M. E. B. Smith, S. Caddick, V. Chudasama, J. R. Baker, *Org. Biomol. Chem.* **2017**, *15*, 2947-2952.

[30] H. H. Dhanjee, A. Saebi, I. Buslov, A. R. Loftis, S. L. Buchwald, B. L. Pentelute, *J. Am. Chem. Soc*. **2020**, *142*, 9124-9129.

[31] R. Tessier, J. Ceballos, N. Guidotti, R. Simonet-Davin, B. Fierz, J. Waser, *Chem* **2019**, *5*, 2243-2263.

[32] J. Ceballos, E. Grinhagena, G. Sangouard, C. Heinis, J. Waser, *Angew. Chem. Int. Ed.* **2021**, *60*, 9022-9031.

[33] D. P. Hari, L. Schouwey, V. Barber, R. Scopelliti, F. Fadaei-Tirani, J. Waser, *Chem. Eur. J.* **2019**, *25*, 9522-9528.

[34] Deposition Number 2269564 (for **15**) contains the supplementary crystallographic data for this paper. These data are provided free of charge by the joint Cambridge Crystalloraphic Data Centre and Fachinformationszentrum Karlsruhe Access Structures service.

[35] R. Frei, J. Waser, *J. Am. Chem. Soc.* **2013**, *135*, 9620-9623.

[36] R. Frei, M. D. Wodrich, D. P. Hari, P.-A. Borin, C. Chauvier, J. Waser, *J. Am. Chem. Soc.* **2014**, *136*, 16563-16573.

[37] J. Fraser, I. Boo, P. Poumbourios, H. E. Drummer, *J. Biol. Chem.* **2011**, *286*, 31984-31992.

[38] C. H. Li, T. B. Ng, D. Yamashiro, D. Chung, R. G. Hammonds Jr, L. F. Tseng, *Int. J. Pept. Protein Res.* **1981**, *18*, 242-248.

[39] C. Ghaddhab, J.-M. Vuissoz, J. Deladoëy, *Front. Endocrinol.* **2017**, *8*, 17, doi: 10.3389/fendo.2017.00017.

[40] W. Ding, J. Chai, C. Wang, J. Wu, N. Yoshikai, *J. Am. Chem. Soc.* **2020**, *142*, 8619-8624.

[41] G. Leng, R. I. Leng, *J. Neuroendocrinol.* **2021**, *33*, e13014.

[42] Y. Zhao, L. Gutshall, H. Jiang, A. Baker, E. Beil, G. Obmolova, J. Carton, S. Taudte, B. Amegadzie, *Protein Expr. Purif.* **2009**, *67,* 182-189.

[43] R. Nahta, F. J. Esteva, *Oncogene* **2007**, *26*, 3637-3643.

[44] L. Castañeda, A. Maruani, F. Schumacher, E. Miranda, V. Chudasama, K. A. Chester, J. R. Baker, M. E. B. Smith, S. Caddick *Chem. Commun.* **2013**, *49*, 89.

[45] R. Benazza, I. Koutsopetras I, V. Vaur, G. Chaubet, O. Hernandez-Alba, S. Cianferani *ChemRxiv preprint* **2023**, DOI: 10.26434/chemrxiv-2023-jmp5n

[46] G. T. Hermanson, *Bioconjugate Techniques.* Elsevier Science, Amsterdam, **2010**.

[47] F. F. Schumacher, V. A. Sanchania, B. Tolner, Z. V. F. Wright, C. P. Ryan, M. E. B. Smith, J. M. Ward, S. Caddick, C. W. M. Kay, G. Aeppli, K. A. Chester, J. R. Baker. *Sci. Rep.* **2013**, *3*, 1525.

[48] M. E. B Smith, F. F. Schumacher, C. P. Ryan, L. M. T. Danai Papaioannou, G. Waksman, S. Caddick, J. R. Baker. *J. Am. Chem. Soc.* **2010**, *132*, 1960-1965.

[49] F. Bryden, A. Maruani, H. Savoie, V. Chudasama, M. E. B. Smith, S. Caddick, R. W. Boyle *Bioconjug. Chem.* **2014**, *25*, 611-617.

[50] B. Wang, A. C. Gucinski, D. A. Keire, L. F. Buhse, M. T. B. II *Analyst* **2013**, *138*, 3058-3065.

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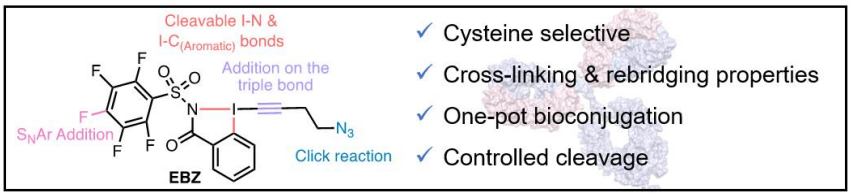
Entry for the Table of Contents



Easily accessible crosslinking ethynylbenziodazolone (EBZ) JW-AM-005 enables the conjugation of peptides and proteins through the selective linkage of cysteine residues. Peptide dimers or stapled peptides were obtained under mild and tuneable conditions. The rebridging of antibody fragments was also performed in a one-pot three-reaction process with high regioselectivity, outperforming the standard reagents commonly used for this transformation.

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