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

Ilias Koutsopetras[+],[a] Abhaya Kumar Mishra[+],[b] Rania Benazza,[c, d] Oscar Hernandez-Alba,[c, d] Sarah Cianférani,[c, d] Guilhem Chaubet,*[a] Stefano Nicolai,*[b] and Jérôme Waser*[b]

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 biomolecules 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.

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

[a] I. Koutsopetras,[+] Dr. G. Chaubet
UMR 7199 CNRS-UdS
Chime Bio-Fonctionnelle
Faculté de Pharmacie
74 route du Rhin
67401Illkirch cedex (France)
E-mail: chaubet@unistra.fr

[b] Dr. A. K. Mishra,[+] Dr. S. Nicolai, Prof. Dr. J. Waser
Laboratory of Catalysis and Organic Synthesis
Institute of Chemical Sciences and Engineering
Ecole Polytechnique Fédéralede de Lausanne
1015 Lausanne (Switzerland)
E-mail: stefano.nicolai@epfl.ch
jerome.waser@epfl.ch

[c] R. Benazza, O. Hernandez-Alba, S. Cianférani
Laboratoire de Spectrométrie de Masse BioOrganique, IPHC UMR 7178
Université de Strasbourg
CNRS, 67087 Strasbourg (France)

[d] R. Benazza, O. Hernandez-Alba, S. Cianférani
Infrastructure Nationale de Protéomique ProFI-FR2048
67087 Strasbourg (France)

[+] These authors contributed equally to this work.

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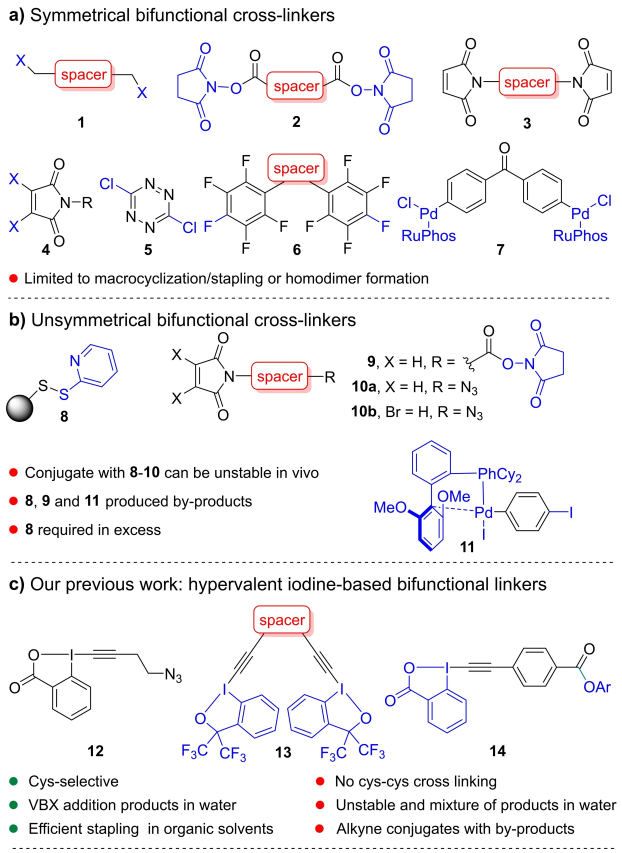


Figure 1. Bis-electrophilic linkers for bioconjugation, a) homo-bifunctional, b) hetero-bifunctional, and c) our previous work: hypervalent iodine based linkers.

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 a 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.

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.

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

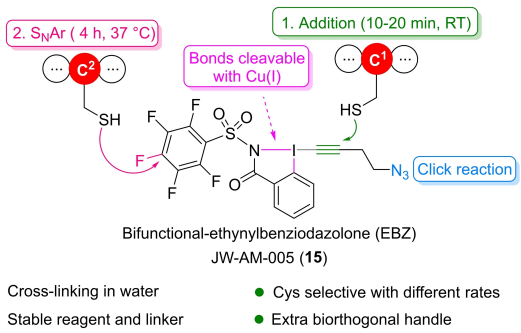
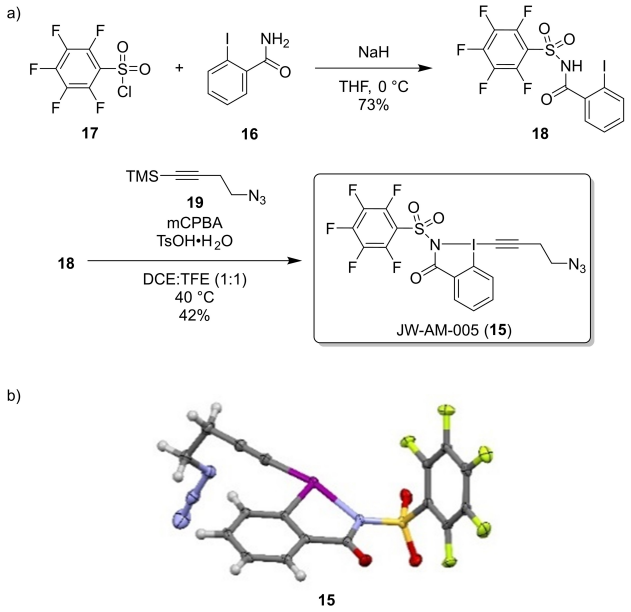


Figure 2. This work: JW-AM-005 (15) – a bifunctional EBZ reagent with high rate difference for Cys-Cys cross-linking.



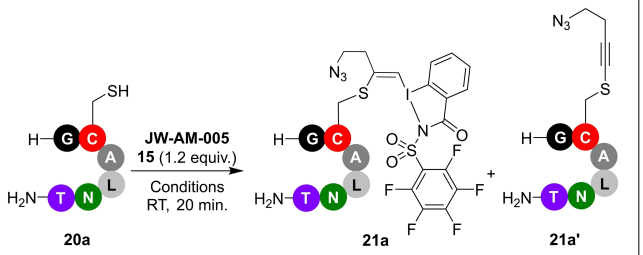
Scheme 1. a) Synthesis of reagent JW-AM-005 (**15**). b) X-Ray crystal structure of **15**.

the desirable compound **15** in 42% yield, whose structure was also confirmed by X-ray single crystal analysis (Scheme 1b).[34]

Unprotected hexapeptide amide **20a** bearing a cysteine, a threonine and a free *N*-terminus was then synthesized using standard Fmoc-solid phase synthesis (See Supporting Information: 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 Supporting Information: 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.

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

Table 1. Optimization of the first Cys bioconjugation.



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

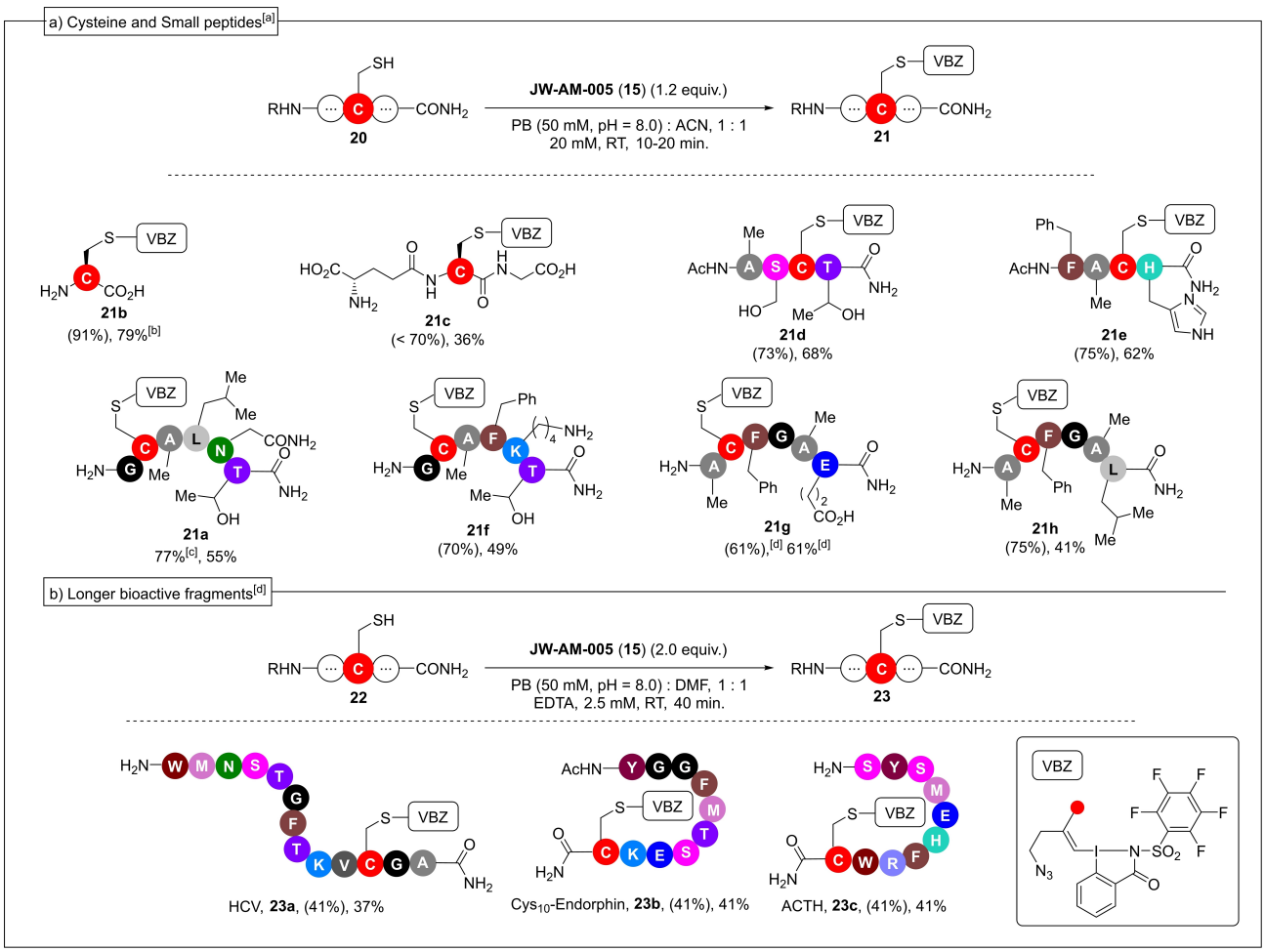
Reaction conditions: 0.0010 mmol of **20a**, 0.0012 mmol of **15**, shaken in the indicated 1:1 mixture of buffer and organic solvent for 20 minutes. The overall concentration was 10 mM with respect to **20a**, unless specified otherwise. [a] Yields of **21a** were determined by HPLC-MS and are calibrated; 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.

DMF (75% yield of **21a**; entry 5) and it 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 (reaction in water-ACN, entry 8). The reaction 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.

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 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,

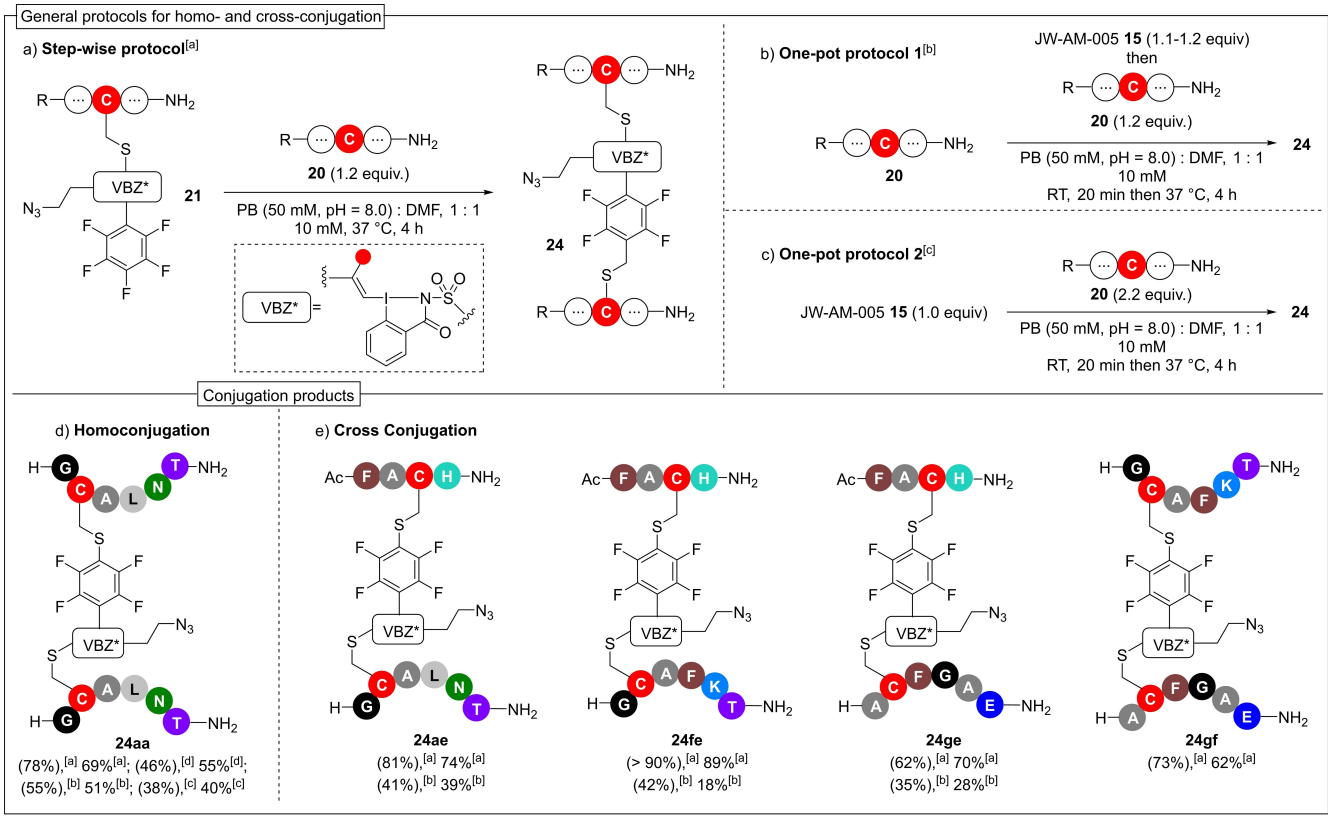


Scheme 2. [a] 0.020 mmol **20**, 0.022-0.024 mmol **15**, PB (50 mM, pH = 8.0) : ACN, 1 : 1, 20 mM with respect to **20**, at room temperature; (indicative HPLC-MS-based yields are reported in parentheses), isolated yields. [b] Reaction performed starting from 0.040 mmol L-cysteine. [c] Reaction performed 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.

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 Supporting Information 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.

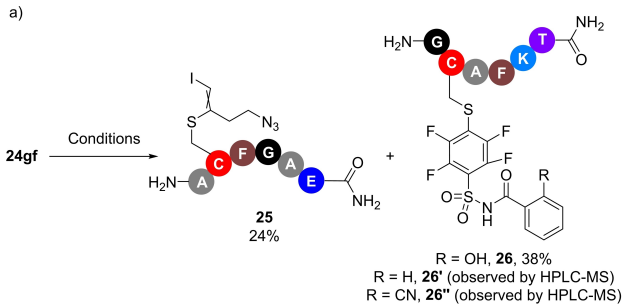
At this point, we wondered whether our stepwise and one-pot methods might be applied to the cross-conjugation of two different thiol-containing molecules (Scheme 3e). The use of the stepwise protocol was successful in all the examined examples, with heterodimers **24ae**, **24fe**, **24ge** and **24gf** 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



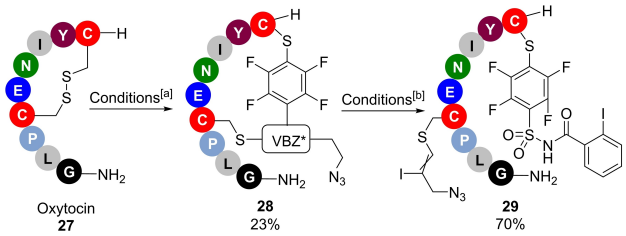
Scheme 3. [a] 0.0040 mmol **21**, 0.0044-0.0048 mmol **15**, PB (50 mM, pH = 8.0):DMF, 1:1, 10 mM with respect to **21**, at 37 °C; (indicative HPLC-MS-based yields are reported in parentheses), isolated yields. [b] 0.0040 mmol **20**, 0.0048 mmol **15**, PB (50 mM, pH = 8.0):DMF, 1:1, 10 mM with respect 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.

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 **24 ge** 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 **24 ae** 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 Supporting Information: 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 **24 gf** 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.



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



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.

Having shown the utility JW-AM-005 (**15**) for the cross-linking 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]

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 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.

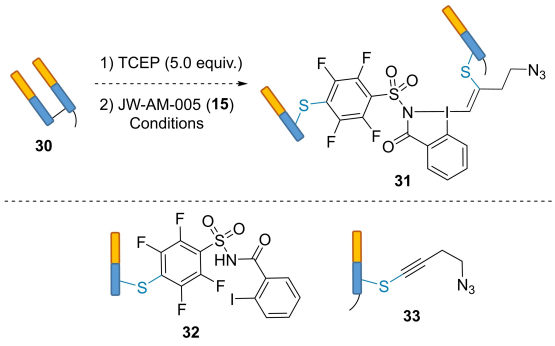
In an effort to evaluate the potential of JW-AM-005 (**15**) as disulfide crosslinking reagent on more complex substrates, we investigated the rebridging of fragment antigen-binding (Fab) species. Fabs are ~50 kDa proteins classically produced by the enzymatic digestion of a parent monoclonal antibody (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 Supporting Information, 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.

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 Supporting Information, 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 Supporting Information 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 Supporting Information, 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.

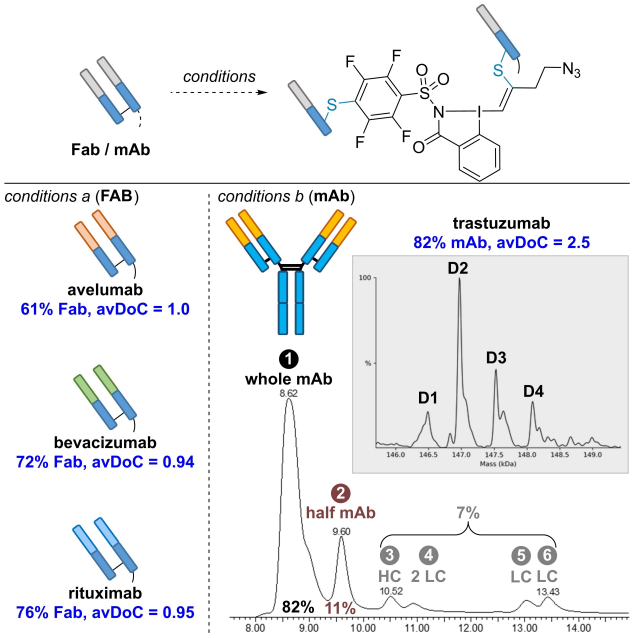
Table 2. Optimisation of rebridging of Fab-trastuzumab **30**.



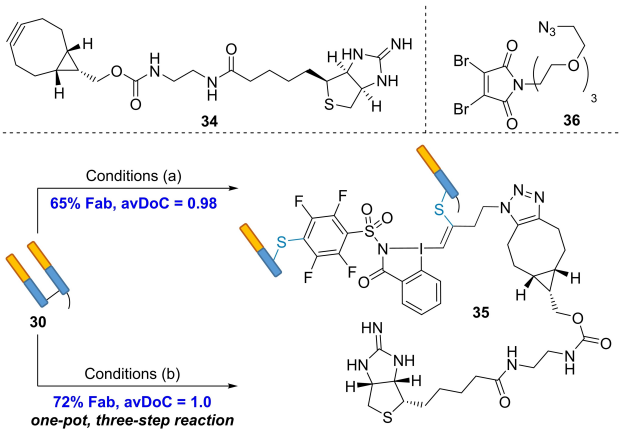
Entry	Reaction conditions	Fab [%][a]	avDoC
1	**15** (5 equiv. in DMSO), **BBS**, 2 mM EDTA, pH 8, 37 °C, 16 h	70	0.80
2	**15** (5 equiv. in DMSO), BBS, 2 mM EDTA, pH 8, 37 °C, **6 h**	79	0.41
3[b]	**15** (5 equiv. in DMSO), BBS, 2 mM EDTA, pH 8, 37 °C, 6 h	70	1.0
4[b]	**15** (5 equiv. in DMSO), **PBS 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 Supporting Information, page S4 for more details); [b]: Reduction and rebridging conducted in one pot.

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



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.



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.

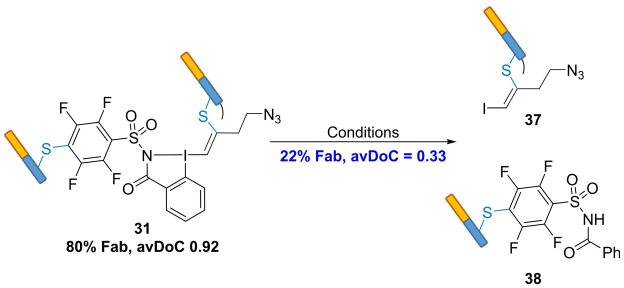
be applied to whole mAbs: under slightly tweaked conditions, trastuzumab led to 82% of rebridged mAb (avDoC = 2.5), with only 11% of half mAb and 7% of LC and HC subspecies, demonstrating the broader applicability of our strategy. For the rebridged mAb, the incorporation of two VBZs was most frequent (**D2**), but insertion of 1, 3 and even 4 VBZs was also observed (**D1**, **D3** and **D4**).

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]

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).

Conclusions

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



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.

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]

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

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

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

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Table of Contents

1. Experimental Procedures

1.1 Experimental procedures implemented in the laboratories @EPFL (sections 2, 3, 4.1)

Materials and Methods

All reactions using anhydrous conditions were performed with oven-dried glassware, under an atmosphere of nitrogen, unless stated otherwise. All reactions related to peptide conjugation were set up on the benchtop and carried out in 1.5 mL vial without oxygen exclusion. Buffers were not degassed and prepared with milliQ water. Anhydrous acetonitrile was purchased from Sigma-Aldrich. All the Fmoc-protected amino acids, Rink Amide MBHA resin and 2-chlorotrityl chloride resin were purchased from GL Biochem. O-Benzotriazole-N,N,N',N'-etramethyluronium-hexafluoro-phosphate (HBTU, GL Biotech), N,N-di*iso*propylethylamine (DIPEA, Iris Biotech GmbH) and hydroxybenzotriazole (HOBt, GL Biotech) were used as received. All the other reagents were purchased from ABCR, Acros, Aldrich, AlfaAesar, Apollo Scientific, Fluorochem, Fluka, Roth and TCI and were used without additional purification.

Spectroscopy and spectrometry

NMR spectra were recorded on a Brucker DPX-400 400 MHz spectrometer in $CDCl_3$, DMSO-d_6 or D_2O. All signals are reported in ppm with the internal $CHCl_3$ signal at 7.26 ppm, the internal DMSO signal at 2.50 ppm or the internal H_2O signal at 4.79 ppm, MeOD at 4.35 as standard. The data is being reported as: s= singlet, d = doublet, t = triplet, q = quadruplet, qi = quintet, m = multiplet or unresolved, br = broad signal, app = apparent, coupling constant(s) in Hz, integration, interpretation.^{13}C-NMR spectra were recorded with ^{1}H-decoupling on a Brucker DPX-400 100 MHz spectrometer in $CDCl_3$, DMSO-d_6 or D_2O. All signals are reported in ppm with the internal $CHCl_3$ signal at 77.0 ppm or the internal DMSO signal at 39.5 ppm as standard.
Melting points were measured on a Büchi B-540 melting point apparatus using open glass capillaries. The data is uncorrected.

Low resolution HPLC-MS measurements were performed using either of instruments described here following. Instrument (a) was utilized as the default one unless otherwise indicated. High-resolution MS measurements were performed by the mass spectrometry service of ISIC at the EPFL using a MICROMASS (ESI) Q-TOF Ultima API.
Instrument (a): Agilent 1290 Infinity HPLC system with a 1290 Autosampler, a 1290 Bin Pump and a 1290 DAD detector, connected to a 6130 Quadrupole LC/MS, coupled with a Waters XBridge C18 column (250 x 4.6 mm, 5 µm). Water:acetonitrile 95:5 + 0.1% formic acid (solvent A), water:acetonitrile 5:95 + 0.1% formic acid (solvent B) were used as the mobile phase, at a flow rate of 0.6 mL/min. The column temperature was set up to 25 °C. The HPLC-MS base line profile in the absence of any eluate was obtained by running a blank injection of a 1:5 mixture DMF:water (Figure S1).

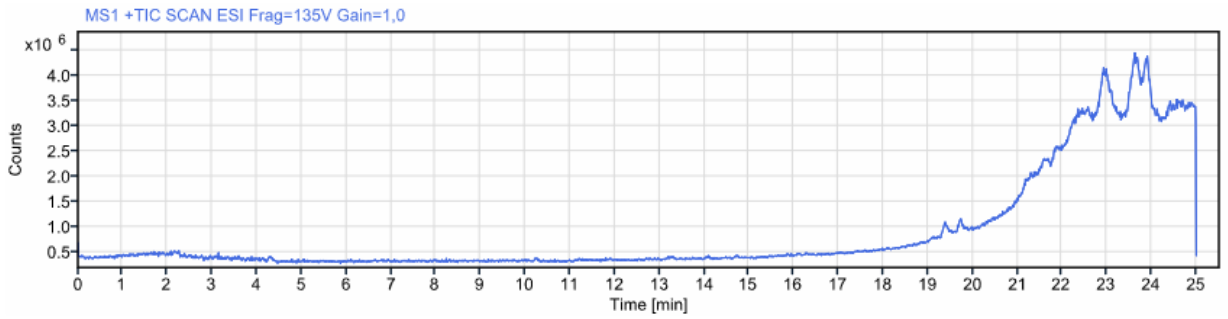


Figure S1: HPLC-MS base line profile with instrument (a).

Instrument (b) Agilent 1290 Infinity II HPLC system with a vial sampler, a 1290 high speed pump and a 1260 MWD detector, connected to a LC/MS iQ mass system, coupled with a Agilent poroshell 120 EC-C18 column (2.1 x 50 mm, 2.7 µm). Water:acetonitrile 95:5 + 0.1% formic acid (solvent A), water:acetonitrile 5:95 + 0.1% formic acid (solvent B) were used as the mobile phase, at a flow rate of 0.6 mL/min. The column temperature was set up to 25 °C. The HPLC-MS base line profile in the absence of any eluate was obtained by running a blank injection of a 1:5 mixture DMF:water (Figure S2).

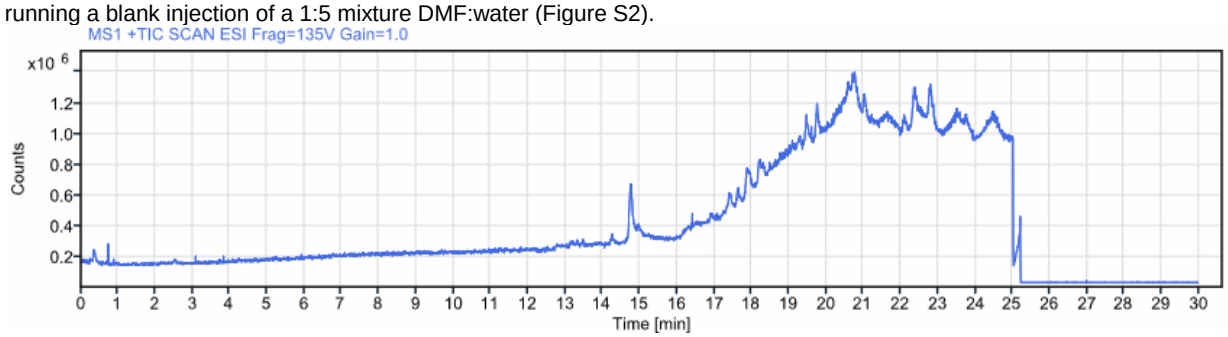


Figure S2: HPLC-MS base line profile with instrument (b).

Methods used for the HPLC-MS and HPLC-UV/VIS analyses described in this report.
- Method 1: 100% A to 100% B 0-20 minutes, then 100% B 20- 25 minutes.
- Method 2: 100% A for 5 minutes isocratic, 100% A to 100% B 0-25 minutes, then 100% B 25-35 minutes.

Preparative RP-HPLC experiments were performed on an Agilent 1260 HPLC system with a G2260A 1260 Prep ALS Autosampler, a G1361A 1260 Prep Pump, a G1365C 1260 MWD detector and a G1364B 1260 FC-PS collector, coupled with a Waters XBridge semi-preparative C18 column (19 x 150 mm, 5 µm). Water + 0.1% TFA (solvent C), water:acetonitrile 5:95 + 0.1% TFA (solvent B) were used as the mobile phase at a flow rate of 20 $mL.min^{-1}$. The following methods were used.

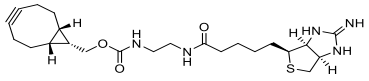
Methods used for the preparative HPLC purifications described in this report.
- Method 3: 100% C to 100% B in 0-20 minutes, then 100% B 20-25 minutes.
- Method 4: 100% C to 70% B in 0-20 minutes; 70% to 100% B 20-25 minutes; then then 100% B 25-30 minutes.
- Method 5: 100% C to 60% B in 0-20; 60% to 100% B 20-25 minutes; then 100% B 20-25 minutes.
- Method 6: 100% C to 50% B in 0-20; 50% to 100% B 20-25 minutes; then 100% B 20-25 minutes.

1.2 Experimental procedures implemented in the laboratories @CNRS-UdS (section 4.2)

Materials and Methods: Synthetic chemistry

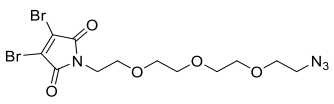
All reagents and solvents were obtained from commercial sources and used without any further purifications. Dry solvents were obtained from Sigma-Aldrich. Column chromatography was carried out as “Flash Chromatography” using silica gel G-25 (40-63 µm) from Macherey-Nagel. Thin layer chromatography (TLC) was performed using plates cut in aluminum sheets (ALUGRAM Xtra SIL G/UV254) purchased from Macherey-Nagel. Visualization was achieved under a 254 or 365 nm UV light and by using an appropriate TLC stain.

(Bicyclo[6.1.0]non-4-yn-9-yl)methyl (2-(5-((3a*S*,4*S*,6a*R*)-2-iminohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamido)ethyl)carbamate “BCN-iminobiotin” 34



Compound **34** was synthesized according to a previously reported procedure.[1]

1-(2-(2-(2-(2-Azidoethoxy)ethoxy)ethoxy)ethyl)-3,4-dibromo-1*H*-pyrrole-2,5-dione 36



Compound **36** was synthesized according to a previously reported procedure.[2]

Materials and Methods: Biomolecules

All reagents, proteins and solvents were obtained from commercial sources – i.e., Sigma Aldrich France, Fischer Scientific France, VWR France – and used without further purifications. Antibody concentration was determined by UV absorbance using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Illkirch, France). Fragment antigen-binding (Fab) conjugates were purified by gel filtration chromatography on Zeba™ Spin Desalting Columns, 7K MWCO, 0.5 mL (Thermo Fisher Scientific, Pierce Biotechnology, USA). Vivaspin micro-concentrators (10 kDa cutoff, 500 µL, Sartorius, Gottingen, Germany) were used for buffer exchange.

Reducing glycine-SDS-PAGE was performed on 4–15% Mini-PROTEAN® TGX™ Gel (Bio-Rad, ref. 4561084) following standard procedures. Samples (10 µL at ~7 µM in total Fab) were mixed with 3 µL of loading buffer (1:19 2-mercaptoethanol / 4x Laemmli Sample Buffer; Bio-Rad, ref. 1610747) and heated at 90 °C for 5 min. The gel was run at constant voltage (200 V) for 35 min using TRIS 0.25 M - Glycine 1.92 M - SDS 1% as a running buffer. Fluorescence was visualized on ImageQuant LAS 4000 series (GE Healthcare Life Sciences).
Purification by steric exclusion chromatography was performed on an ÄKTA Pure system (GE Healthcare) with a Superdex column (S200 10/300 GL), using DPBS 1X + 5 mM EDTA (pH 7.4) as eluent at a flow rate of 0.5 mL/min.

Spectroscopy and spectrometry

^{1}H and ^{13}C NMR spectra were recorded at 23 °C on Bruker Avance III - 400 MHz / 500 MHz spectrometers. Recorded shifts are reported in parts per million (δ) and calibrated using residual non-deuterated solvent. Data are represented as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad), coupling constant (*J*, Hz), integration and assignment in case of ^{1}H NMR data.

Analytical LC-MS analyses were carried out on Waters 2695 separations module equipped with Waters 2487 UV detector, Waters Acquity QDa mass detector and CORTECS, 2.7 µm, C18, 50 x 4.6 mm column. The flow rate was 1 mL/min and the solvent system was composed as follows: solvent A: 0.05% HCOOH in water; solvent B: acetonitrile. The gradient run was: 0 to 5 min. – 5% to 95% B; 5 to 6 min. – 95% B; 6 to 7.8 min. – 5% B. Mass detector was operated in positive MS Scan mode with 600 °C probe temperature, 1.5 kV capillary voltage and 10 V cone voltage.

Denaturing SEC-MS (dSEC-MS) experiments were performed on a BioAccord LC-MS system (Waters, Milford, United States). It comprises an Acquity UPLC M-Class system; including a binary solvent manager, a sample manager at 4°C, a column oven at room temperature for SEC separation and at 80°C for rpLC and a UV detector operating at 214 nm and 280 nm, coupled to an RDa detector (a high-resolution time-of-flight (ToF) mass spectrometer). The mass spectrometer was calibrated in the 400-7000 m/z range in the positive mode using a solution containing, 50 ng/µL of sodium iodide in isopropanol/water (80/20 v/v) and 0.5 ng/µL of rubidium iodide in isopropanol/water (80/20 v/v). A LockMass solution containing 3.75 ng/µL of leucine encephalin, 12.5 ng/µL of caffeine and 2.5 ng/µL of 1-pentanesulfonic acid in ACN/water (80/20 v/v) was injected automatically prior to each analysis. SEC analysis was performed using the ACQUITY UPLC BEH SEC (4.6 mm x 150 mm, 2.5 µm, 450 Å) column (Waters, Milford, United States) kept at room temperature. The separation was carried out using an isocratic gradient of mobile phase (20% ACN+0.1% FA) at a flowrate of 0.1 mL/min for 15 minutes. One to 3 µg of protein samples were injected. The BioAccord was operated with a capillary voltage of +1.5 kV. Desolvation temperature was set to 300 °C, the cone voltage to 60V and the source pressure was fixed at 2 mbar. Acquisitions were performed on the m/z range 400-7000 with a 1 s scan time.

All MS data interpretations were performed using UNIFI v1.913.9 (Waters, Manchester, UK) and MassLynx V4.1 (Waters, Manchester, UK). The avDoC values were calculated based on the relative peak intensities measured from the raw mass spectra (four charge states) using the equation below:

$$\boldsymbol{avDoC} = \frac{\sum_{k=0}^{n} \boldsymbol{k} \times \boldsymbol{I_k}}{\sum_{k=0}^{n} \boldsymbol{I_k}}$$

Where *k* is the number of drugs and I_k is the relative peak intensity of DoC_k.

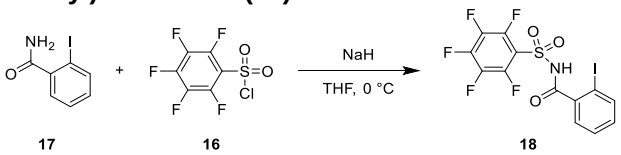
To determine the rebridging efficiency, we quantified the amount of covalently rebriged Fab by integration of the peak areas from UV chromatograms, using the following equation:

$$\boldsymbol{Amount\ of\ rebriged\ Fab}\ (\%) = \frac{\sum \boldsymbol{Peak\ area}_{\boldsymbol{covalently\ rebriged\ Fab}}}{\sum \boldsymbol{Peak\ area}_{\boldsymbol{all\ species}}} \times \mathbf{100}$$

The sum of all species include the covalently rebriged Fab and the by-products corresponding to unrebriged species; i.e., the light chain (LC) and the Fd subunit.

2. Synthesis of JW-AM-005

2-Iodo-*N*-((perfluorophenyl)sulfonyl)benzamide (18)

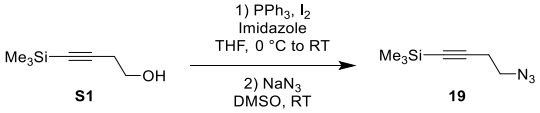


17 16 18

To a solution of 2-iodobenzamide (**17**) (0.486 g, 1.97 mmol, 1.05 equiv.) in THF (9.4 mL) was added NaH (60% dispersion in mineral oil; 0.113 g, 2.83 mmol, 1.50 equiv.) at 0 °C (ice – water bath). The resulting suspension was stirred at 0 °C for 1 hour. 2,3,4,5,6-pentaflurobenzenesulfonyl chloride (**16**) (0.28 mL, 1.90 mmol, 1.00 equiv.) was added slowly. The reaction mixture was then stirred at 0 °C for another 3 hours. After completion of the reaction as determined by TLC analysis, THF was evaporated under reduced pressure, and the residue was diluted with EtOAc (50 mL). Aq. HCl (1.0 M; 5 mL) was added to the resulting mixture. The organic layer was separated, and the aqueous layer was extracted with EtOAc (3 x 50 mL). The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The resulting crude solid was submitted to column chromatography (Biotage Flash chromatographer; SiO_2; 10 to 30% ethyl acetate in DCM) to afford 2-iodo-*N*-((perfluorophenyl)sulfonyl)benzamide (**18**) (0.65 g, 1.4 mmol, 73% yield) as a white solid.

R_f = 0.4 (ethyl acetate).
Melting Point: 85-87 °C
^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.94 (s, 1H, N*H*), 7.84 (d, *J* = 8.8 Hz, 1H, Ar*H*), 7.53 – 7.28 (m, 2H, Ar*H*), 7.23 – 7.08 (m, 1H, Ar*H*).
^{13}C NMR (101 MHz, Chloroform-*d*) δ 166.0, 146.8 (m), 144.2 (m), 140.8, 139.2 (m), 137.2, 136.6 (m), 133.3, 129.0, 128.6, 91.7.
HRMS (ESI/QTOF) m/z: $[M + Na]^+$ Calcd for $C_{13}H_5F_5INNaO_3S^+$ 499.8847; Found 499.8860.

(4-Azidobut-1-yn-1-yl)trimethylsilane (19).



S1 19

Following a reported procedure,[1] triphenylphosphine (6.14 g, 23.4 mmol, 1.3 equiv.) was added to a solution of 4-(trimethylsilyl)but-3-yn-1-ol (**S1**) (3.0 mL, 18 mmol, 1.0 equiv.) in THF (68 mL), preliminarily cooled to 0 °C (ice - water bath). Under stirring at the same temperature, imidazole (1.60 g, 23.0 mmol, 1.3 equiv.) was added, followed by iodine (5.70 g, 22.0 mmol, 1.25 equiv.). The resulting red solution (showing a whitish solid in suspension) was stirred at room temperature for 8 hours. The reaction was then quenched by addition of sat. aq. $Na_2S_2O_3$ (30 mL) and water (30 mL). The aqueous layer was extracted with ether (3 x 50 mL). The combined organic layers were washed with brine, dried over $MgSO_4$, filtered, and concentrated under vacuum. The resulting solid was triturated with pentane. The solids were then filtered off, and washed with pentane. The filtrate was concentrated under reduced pressure, and the so-obtained pale yellow, crude oil was submitted to column chromatography (Biotage Flash chromatographer; SiO_2; elution with pure pentane) to provide pure (4-iodobut-1-yn-1-yl)trimethylsilane (4.50 g, 18.0 mmol, quantitative yield) as a colorless oil.

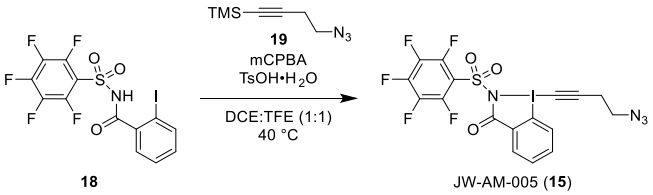
^{1}H NMR (400 MHz, Chloroform-*d*) δ 3.22 (t, *J* = 7.5 Hz, 2H, CH_2), 2.79 (t, *J* = 7.5 Hz, 2H, CH_2), 0.16 (s, 9H, Si(CH_3)$_3$). NMR signals corresponded to the data reported in the literature.[1]

Following a reported procedure,[1] sodium azide (0.722 g, 11.1 mmol, 1.4 equiv.) was partially dissolved in DMSO (16 mL). At room temperature, (4-iodobut-1-yn-1-yl)trimethylsilane (2.0 g, 7.90 mmol, 1.0 equiv.) was added to the resulting mixture. Upon stirring, the latter became slightly turbid, with apparent complete dissolution of the salt. The mixture was stirred at room temperature for 24 hours, becoming pale yellow over this time. It was then poured onto a mixture of ice and water. The aqueous layer was extracted with ether (4 x 30 mL). The combined organic layers were washed with water (2 x 30 mL), brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The resulting pale yellow crude oil was submitted to column chromatography (Biotage Flash chromatographer; SiO_2; elution with pure pentane) to provide (4-azidobut-1-yn-1-yl)trimethylsilane (**19**) (1.02 g, 6.10 mmol, 77% yield) as a colorless liquid.

^{1}H NMR (400 MHz, Chloroform-*d*) δ 3.38 (t, *J* = 6.9 Hz, 2H, CH_2), 2.52 (t, *J* = 6.9 Hz, 2H, CH_2), 0.16 (s, 9H, Si(CH_3)$_3$). NMR signals corresponded to the data reported in the literature.[1]

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

JW-AM-005 (15)



In a 25 mL, two-necked, round-bottomed flask, equipped with a Findenser© (air reflux condenser) 2-iodo-*N*-(pentafluorosulfonyl)benzamide (**18**) (0.757 g, 1.59 mmol, 1.0 equiv.) was suspended in a mixture of DCM (2.3 mL) and TFE (2.3 mL). *p*-TsOH hydrate (0.360 g, 1.90 mmol, 1.5 equiv.) and mCPBA (0.530 g, 2.40 mmol, 1.5 equiv.) were added and the mixture was stirred at 40 °C for 1 hour. (4-Azidobut-1-yn-1-yl)trimethylsilane (**19**) (0.40 g, 2.40 mmol, 1.5 equiv.) was then added, after which the mixture turned from pale yellow to yellow. It was stirred at 40 °C overnight, further darkening to brown: at this point, the mixture looked like the suspension of a whitish solid in a dark brown liquid. The latter was concentrated under reduced pressure. The solid residue was dissolved in DCM (50 mL), followed by the addition of sat. aq. $NaHCO_3$ (5 mL). This mixture was vigorously stirred at room temperature for 45 minutes. The aquoeus layer was then diluted with water, separated, and extracted with DCM (3 x 50 mL). The combined organic layers were washed with brine, dried over $MgSO_4$, filtered, and concentrated under vacuum. The resulting orange-pink crude solid was submitted to column chromatography (Biotage flash chromatographer, SiO_2; EtOAc in DCM, 2 to 5) to provide JW-AM-005 (**15**) (0.376 g, 0.659 mmol, 42% yield) as an off-white solid.

Rf = 0.6 (9:1, DCM: ethyl acetate).
Melting Point: 138-140 °C.
^{1}H NMR (400 MHz, $CDCl_3$) δ 8.31 (ddd, *J* = 17.4, 7.8, 1.6 Hz, 1H, Ar*H*), 7.94 – 7.66 (m, 1H, Ar*H*), 3.60 (t, *J* = 6.4 Hz, 2H, CH_2), 2.91 (t, *J* = 6.4 Hz, 2H, CH_2).
^{19}F NMR (376 MHz, Chloroform-*d*) δ -135.8 (dt, *J* = 21.0, 5.3 Hz), -146.7 (m), -159.6 (m).
^{13}C NMR (101 MHz, $CDCl_3$; the multiplets corresponding to fluorinated carbons were not completely resolved) δ 162.2, 146.8-143.0 (m), 139.0-137.0 (m), 136.2, 133.4, 132.2, 132.0, 127.6, 114.5, 106.5, 49.2, 45.4, 21.5.
HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{17}H_9F_5IN_4O_3S^+$ 570.9355; Found 570.9366.

X-Ray diffraction results for JW-AM-005 (15)

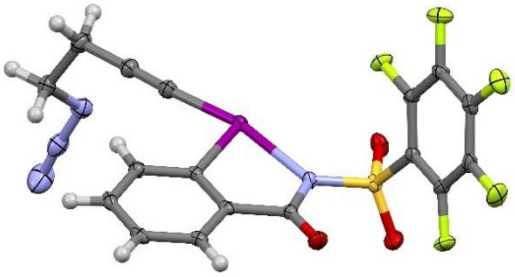


Figure S3. X-ray crystal structure of JW-AM-005 (**15**).

Crystallization Procedure. A 5 mL vial was charged with 5.0 mg **15** in 5 mL DCM:EtOAc (1:1). Subsequently, the vial was kept for slow evaporation. After slow evaporation for 2 days, transparent crystals were obtained.

Experimental. Single clear pale colourless prism crystals of am-5-r38 were used as supplied. A suitable crystal with dimensions 0.79 × 0.29 × 0.20 mm3 was selected and mounted on a SuperNova, Dual, Cu at home/near, AtlasS2 diffractometer. The crystal was kept at a steady T = 140.00(10) K during data collection. The structure was solved with the ShelXT (Sheldrick, 2015) solution program using dual methods and by using Olex2 (Dolomanov et al., 2009) as the graphical interface. The model was refined with ShelXL 2018/3 (Sheldrick, 2015) using full matrix least squares minimisation on F2.

Crystal Data. $C_{17}H_8F_5IN_4O_3S$, M_r = 570.23, triclinic, *P*-1 (No. 2), a = 7.3999(3) Å, b = 9.6743(3) Å, c = 13.2499(4) ⊕, $\alpha = 91.178(2)^\circ$, $\beta = 92.608(3)^\circ$, $\gamma = 97.972(3)^\circ$, $V = 938.05(5)$ ⊕^{3}, $T = 140.00(10)$ K, $Z = 2$, $Z' = 1$, μ(Mo K_α) = 1.897, 22977 reflections measured, 6415 unique (R_{int} = 0.0336) which were used in all calculations. The final wR_2 was 0.0524 (all data) and R_1 was 0.0235 (I≥2 σ(I)).

Compound	**JW-05-005**
Formula	$C_{17}H_8F_5IN_4O_3S$
$D_{calc.}$/ g cm^{-3}	2.019
μ/mm^{-1}	1.897
Formula Weight	570.23
Colour	clear pale colourless
Shape	prism
Size/mm^3	0.79×0.29×0.20
T/K	140.00(10)
Crystal System	triclinic
Space Group	P-1
a/Å	7.3999(3)
b/Å	9.6743(3)
c/Å	13.2499(4)
α/°	91.178(2)
β/°	92.608(3)
γ/°	97.972(3)
V/Å^3	938.05(5)
Z	2
Z'	1
Wavelength/Å	0.71073
Radiation type	Mo K□
Θ_{min}/°	2.659
Θ_{max}/°	32.889
Measured Refl's.	22977
Indep't Refl's	6415
Refl's I≥2 σ(I)	5933
R_{int}	0.0336
Parameters	281
Restraints	231
Largest Peak	0.564
Deepest Hole	-0.539
GooF	1.062
wR_2 (all data)	0.0524
wR_2	0.0510
R_1 (all data)	0.0268
R_1	0.0235

Structure Quality Indicators

Reflections:	d min (Mo) CIF 0.65	I/σ(I) CIF 29.5	Rint CIF 3.36%	complete 100% (IUCr) 100%
Refinement:	Shift CIF 0.002	Max Peak CIF 0.6	Min Peak CIF -0.5	GooF CIF 1.062

A clear pale colourless prism-shaped crystal with dimensions 0.79 × 0.29 × 0.20 mm^3 was mounted. Data were collected using a SuperNova, Dual, Cu at home/near, AtlasS2 diffractometer operating at T = 140.00(10) K.

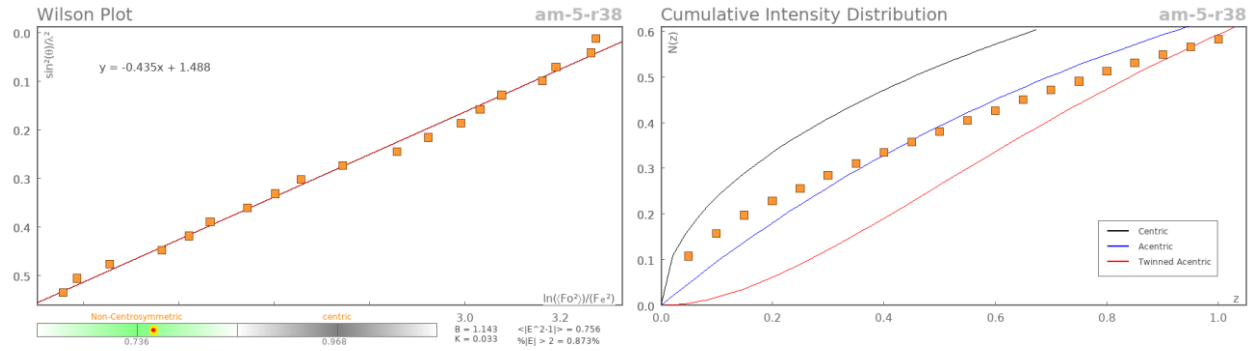
Data were measured using ω scans using Mo K_α radiation. The diffraction pattern was indexed and the total number of runs and images was based on the strategy calculation from the program CrysAlisPro (Rigaku, V1.171.40.84a, 2020). The maximum resolution that was achieved was Θ = 32.889° (0.83 Å).
The diffraction pattern was indexed and the total number of runs and images was based on the strategy calculation from the program CrysAlisPro (Rigaku, V1.171.40.84a, 2020). The unit cell was refined using CrysAlisPro (Rigaku, V1.171.40.84a, 2020) on 13851 reflections, 60% of the observed reflections.

Data reduction, scaling and absorption corrections were performed using CrysAlisPro (Rigaku, V1.171.40.84a, 2020). The final completeness is 99.90 % out to 32.889° in Θ. A gaussian absorption correction was performed using CrysAlisPro 1.171.40.84a (Rigaku Oxford Diffraction, 2020) Numerical absorption correction based on gaussian integration over a multifaceted crystal model Empirical absorption correction using spherical harmonics, implemented in SCALE3 ABSPACK scaling algorithm. The absorption coefficient μ of this material is 1.897 mm^{-1} at this wavelength (λ = 0.71073Å) and the minimum and maximum transmissions are 0.242 and 1.000.

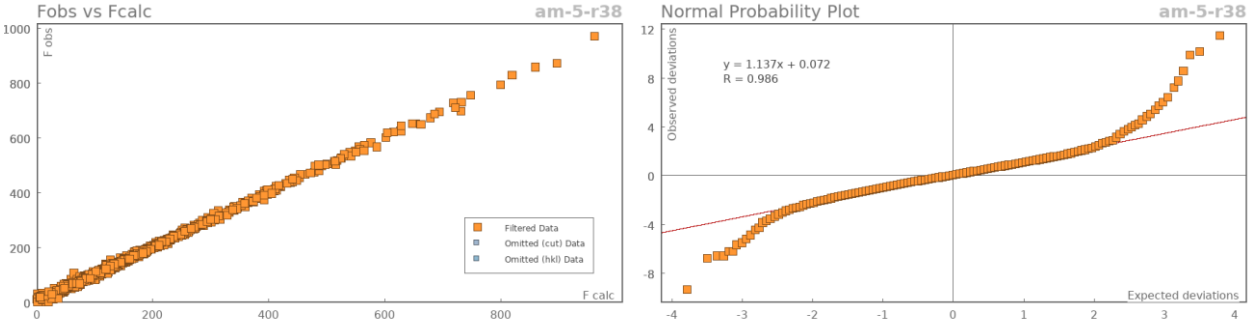
The structure was solved and the space group *P*-1 (# 2) determined by the ShelXT (Sheldrick, 2015) structure solution program using using dual methods and refined by full matrix least squares minimisation on F^2 using version 2018/3 of ShelXL 2018/3 (Sheldrick, 2015). All non-hydrogen atoms were refined anisotropically. Hydrogen atom positions were calculated geometrically and refined using the riding model. Hydrogen atom positions were calculated geometrically and refined using the riding model.

_exptl_absorpt_process_details: CrysAlisPro 1.171.40.84a (Rigaku Oxford Diffraction, 2020)Numerical absorption correction based on gaussian integration overa multifaceted crystal modelEmpirical absorption correction using spherical harmonics,implemented in SCALE3 ABSPACK scaling algorithm.
There is a single molecule in the asymmetric unit, which is represented by the reported sum formula. In other words: Z is 2 and Z' is 1.

Data Plots: Diffraction Data



Data Plots: Refinement and Data



Reflection Statistics

Total reflections (after filtering)	22977	Unique reflections	6415
Completeness	0.914	Mean I/σ	23.06
hkl_{max} collected	(10, 13, 19)	hkl_{min} collected	(-10, -14, -20)
hkl_{max} used	(10, 14, 20)	hkl_{min} used	(-10, -14, 0)
Lim d_{max} collected	100.0	Lim d_{min} collected	0.36
d_{max} used	7.66	d_{min} used	0.65
Friedel pairs	6032	Friedel pairs merged	1
Inconsistent equivalents	0	R_{int}	0.0336
R_{sigma}	0.0339	Intensity transformed	0
Omitted reflections	0	Omitted by user (OMIT hkl)	0
Multiplicity	(5690, 4382, 1478, 552, 239, 77, 13, 11, 5)	Maximum multiplicity	14
Removed systematic absences	0	Filtered off (Shel/OMIT)	0

Table S1: Fractional Atomic Coordinates (×10^4) and Equivalent Isotropic Displacement Parameters (Å^2×10^3) for JW-AM-005 (**15**). U_{eq} is defined as 1/3 of the trace of the orthogonalised U_{ij}.

Atom	x	y	z	U_{eq}
I1	4271.4(2)	7533.1(2)	4443.5(2)	14.52(4)
S1	4467.1(6)	8365.6(4)	7077.9(3)	17.16(8)
F1	2509.9(18)	7647.1(12)	9070.9(8)	29.1(3)
F2	-490.5(18)	8543.5(12)	9676.1(8)	31.2(3)
F3	-2092.5(16)	10435.4(12)	8605.1(9)	28.9(3)
F4	-788.4(16)	11257.2(11)	6791.8(9)	26.7(2)
F5	2103.1(16)	10268.6(11)	6091.0(7)	23.0(2)
O1	2061(2)	5688.1(13)	7254.4(9)	23.0(3)
O2	5400.0(19)	7773.6(14)	7891.4(10)	25.5(3)
O3	5442.7(19)	9509.2(13)	6563.9(10)	23.8(3)
N1	3661(2)	7250.2(14)	6206.2(10)	16.2(3)
N2	8650(2)	6737.4(17)	1360.2(12)	26.8(3)
N3	9860(3)	5987(2)	1278.7(12)	30.1(4)
N4	11065(3)	5387(3)	1253.7(16)	46.7(5)
C1	2913(2)	5467.2(16)	4568.3(12)	14.5(3)
C2	2597(2)	4593.4(17)	3720.1(12)	17.6(3)
C3	1623(2)	3269.7(18)	3828.7(13)	19.8(3)
C4	991(2)	2843.1(17)	4760.7(14)	19.4(3)
C5	1339(2)	3741.8(17)	5601.1(13)	17.6(3)
C6	2300(2)	5066.8(16)	5516.9(12)	14.5(3)
C7	2651(2)	6018.2(17)	6434.4(12)	16.1(3)
C8	4645(3)	7261.8(18)	2943.1(13)	21.6(3)
C9	5072(3)	7242.8(19)	2081.1(14)	23.1(4)
C10	5536(3)	7169(2)	1017.0(14)	28.4(4)

Atom	x	y	z	U_{eq}
C11	6895(3)	6161(2)	833.0(14)	26.2(4)
C12	2491(2)	8963.4(17)	7565.1(12)	16.0(3)
C13	1746(3)	8519.3(18)	8474.9(12)	19.5(3)
C14	198(3)	8992.3(18)	8805.8(12)	20.9(3)
C15	-655(2)	9932.7(18)	8256.3(13)	19.8(3)
C16	28(2)	10359.5(17)	7344.0(13)	17.9(3)
C17	1553(2)	9862.7(17)	7006.2(12)	16.4(3)

Table S2: Anisotropic Displacement Parameters ($\times 10^4$) for JW-AM-005 (**XX**). The anisotropic displacement factor exponent takes the form: $-2\pi^2[h^2a^{*2} \times U_{11} + ... + 2hka^* \times b^* \times U_{12}]$

Atom	U_{11}	U_{22}	U_{33}	U_{23}	U_{13}	U_{12}
I1	14.04(6)	13.41(6)	16.08(6)	0.32(3)	2.90(3)	1.12(4)
S1	16.7(2)	16.19(18)	18.06(19)	-2.95(14)	0.23(14)	1.13(15)
F1	43.4(8)	29.6(6)	17.4(5)	8.5(4)	0.8(5)	15.3(5)
F2	42.8(8)	34.6(7)	16.8(5)	3.7(4)	12.8(5)	3.0(6)
F3	21.7(6)	31.2(6)	34.9(6)	-5.8(5)	8.2(5)	6.3(5)
F4	21.9(6)	23.3(5)	35.4(6)	11.1(4)	-2.8(4)	5.1(4)
F5	24.7(6)	26.5(6)	17.3(5)	9.0(4)	3.3(4)	-0.2(4)
O1	30.5(8)	21.0(6)	17.1(6)	4.0(5)	3.2(5)	0.4(5)
O2	23.2(7)	30.1(7)	23.7(6)	-4.2(5)	-6.4(5)	8.7(6)
O3	22.3(7)	17.7(6)	29.9(7)	-3.8(5)	6.7(5)	-3.5(5)
N1	19.4(7)	13.3(6)	15.5(6)	-2.0(5)	2.7(5)	1.0(5)
N2	28.7(9)	25.7(8)	25.3(8)	3.0(6)	2.6(6)	0.5(7)
N3	27.3(9)	41.4(10)	20.8(8)	3.0(7)	5.0(6)	0.2(8)
N4	32.2(11)	73.6(16)	37.0(11)	-5.5(10)	2.6(8)	17.6(11)
C1	13.0(7)	11.5(7)	19.2(7)	0.6(5)	-0.3(5)	2.5(5)

Atom	U_{11}	U_{22}	U_{33}	U_{23}	U_{13}	U_{12}
C2	17.3(8)	17.8(8)	18.0(8)	-1.7(6)	0.1(6)	3.8(6)
C3	17.0(8)	17.4(8)	24.7(8)	-6.1(6)	-3.4(6)	4.2(6)
C4	15.1(8)	12.9(7)	29.8(9)	-0.1(6)	-2.4(6)	1.8(6)
C5	16.7(8)	14.2(7)	22.5(8)	3.3(6)	0.5(6)	3.5(6)
C6	12.2(7)	13.5(7)	18.3(7)	0.8(5)	-0.3(5)	3.7(6)
C7	16.7(8)	14.2(7)	17.8(7)	1.5(5)	0.0(6)	3.7(6)
C8	24.4(9)	19.1(8)	21.3(8)	0.8(6)	1.6(7)	2.8(7)
C9	25.7(10)	22.5(9)	21.7(8)	1.3(6)	3.0(7)	4.2(7)
C10	33.9(11)	35.3(11)	17.5(8)	4.0(7)	3.2(7)	9.4(9)
C11	27.5(10)	32.0(10)	19.0(8)	-3.0(7)	2.6(7)	3.2(8)
C12	18.5(8)	14.9(7)	14.4(7)	-1.4(5)	1.9(6)	1.9(6)
C13	28.5(9)	16.7(8)	13.6(7)	1.7(6)	-0.3(6)	4.5(7)
C14	29.8(10)	18.7(8)	13.8(7)	-0.9(6)	5.8(6)	0.7(7)
C15	19.0(8)	18.1(8)	21.9(8)	-4.5(6)	4.5(6)	1.0(6)
C16	18.2(8)	13.5(7)	21.6(8)	1.6(6)	-1.0(6)	1.0(6)
C17	19.6(8)	15.0(7)	13.5(7)	1.6(5)	1.6(6)	-1.7(6)

Table S3: Bond Lengths in Å for **JW-AM-005** (**15**).

Atom	Atom	Length/Å
I1	N1	2.4128(14)
I1	C1	2.1223(16)
I1	C8	2.0363(18)
S1	O2	1.4261(14)
S1	O3	1.4363(14)
S1	N1	1.6006(14)

Atom	Atom	Length/Å
S1	C12	1.7877(17)
F1	C13	1.332(2)
F2	C14	1.3380(19)
F3	C15	1.327(2)
F4	C16	1.3365(19)
F5	C17	1.3457(18)
O1	C7	1.222(2)
N1	C7	1.363(2)
N2	N3	1.235(3)
N2	C11	1.479(3)
N3	N4	1.131(3)
C1	C2	1.384(2)
C1	C6	1.399(2)
C2	C3	1.393(2)
C3	C4	1.391(3)
C4	C5	1.391(2)
C5	C6	1.386(2)
C6	C7	1.500(2)
C8	C9	1.199(3)
C9	C10	1.469(3)
C10	C11	1.519(3)
C12	C13	1.399(2)
C12	C17	1.391(2)
C13	C14	1.378(3)
C14	C15	1.379(3)

Atom	Atom	Length/Å
C15	C16	1.380(2)
C16	C17	1.375(2)

Table S4: Bond Angles in ° for JW-AM-005 (**15**)

Atom	Atom	Atom	Angle/°
C1	I1	N1	73.77(5)
C8	I1	N1	166.19(6)
C8	I1	C1	92.87(7)
O2	S1	O3	118.09(9)
O2	S1	N1	113.42(8)
O2	S1	C12	107.59(8)
O3	S1	N1	105.33(8)
O3	S1	C12	107.27(8)
N1	S1	C12	104.14(8)
S1	N1	I1	123.61(8)
C7	N1	I1	115.41(10)
C7	N1	S1	120.87(11)
N3	N2	C11	113.68(16)
N4	N3	N2	174.0(2)
C2	C1	I1	119.88(12)
C2	C1	C6	122.56(15)
C6	C1	I1	117.51(11)
C1	C2	C3	117.85(15)
C4	C3	C2	120.94(15)
C5	C4	C3	119.89(16)

Atom	Atom	Atom	Angle/°
C6	C5	C4	120.53(16)
C1	C6	C7	122.29(14)
C5	C6	C1	118.22(14)
C5	C6	C7	119.49(15)
O1	C7	N1	126.99(15)
O1	C7	C6	122.30(15)
N1	C7	C6	110.71(14)
C9	C8	I1	170.88(17)
C8	C9	C10	177.6(2)
C9	C10	C11	112.51(16)
N2	C11	C10	107.88(16)
C13	C12	S1	123.49(13)
C17	C12	S1	119.94(13)
C17	C12	C13	116.48(16)
F1	C13	C12	122.06(16)
F1	C13	C14	116.80(15)
C14	C13	C12	121.12(16)
F2	C14	C13	119.84(16)
F2	C14	C15	119.17(17)
C13	C14	C15	121.00(16)
F3	C15	C14	120.58(16)
F3	C15	C16	120.51(16)
C14	C15	C16	118.91(16)
F4	C16	C15	120.00(16)
F4	C16	C17	120.10(15)

Atom	Atom	Atom	Angle/°
C17	C16	C15	119.90(16)
F5	C17	C12	120.45(15)
F5	C17	C16	117.04(15)
C16	C17	C12	122.50(15)

Table S5: Torsion Angles in ° for JW-AM-005 (**15**)

Atom	Atom	Atom	Atom	Angle/°
I1	N1	C7	O1	-174.05(15)
I1	N1	C7	C6	5.06(17)
I1	C1	C2	C3	-176.96(12)
I1	C1	C6	C5	177.18(12)
I1	C1	C6	C7	-2.9(2)
S1	N1	C7	O1	9.6(3)
S1	N1	C7	C6	-171.28(12)
S1	C12	C13	F1	2.7(2)
S1	C12	C13	C14	-178.67(14)
S1	C12	C17	F5	1.2(2)
S1	C12	C17	C16	-179.79(13)
F1	C13	C14	F2	-1.7(3)
F1	C13	C14	C15	177.90(16)
F2	C14	C15	F3	2.9(3)
F2	C14	C15	C16	-178.05(15)
F3	C15	C16	F4	-1.8(2)
F3	C15	C16	C17	178.02(15)
F4	C16	C17	F5	-3.1(2)

Atom	Atom	Atom	Atom	Angle/°
F4	C16	C17	C12	177.85(15)
O2	S1	N1	I1	-126.87(9)
O2	S1	N1	C7	49.16(16)
O2	S1	C12	C13	-18.03(17)
O2	S1	C12	C17	165.43(13)
O3	S1	N1	I1	3.73(12)
O3	S1	N1	C7	179.76(13)
O3	S1	C12	C13	-146.04(14)
O3	S1	C12	C17	37.42(16)
N1	S1	C12	C13	102.62(15)
N1	S1	C12	C17	-73.92(15)
N3	N2	C11	C10	178.72(16)
C1	C2	C3	C4	-0.1(3)
C1	C6	C7	O1	177.30(16)
C1	C6	C7	N1	-1.9(2)
C2	C1	C6	C5	-0.1(2)
C2	C1	C6	C7	179.83(15)
C2	C3	C4	C5	-0.3(3)
C3	C4	C5	C6	0.5(3)
C4	C5	C6	C1	-0.3(2)
C4	C5	C6	C7	179.79(15)
C5	C6	C7	O1	-2.7(3)
C5	C6	C7	N1	178.09(15)
C6	C1	C2	C3	0.3(3)
C9	C10	C11	N2	-66.6(2)

Atom	Atom	Atom	Atom	Angle/°
C12	S1	N1	I1	116.47(9)
C12	S1	N1	C7	-67.50(15)
C12	C13	C14	F2	179.60(15)
C12	C13	C14	C15	-0.8(3)
C13	C12	C17	F5	-175.61(14)
C13	C12	C17	C16	3.4(2)
C13	C14	C15	F3	-176.65(16)
C13	C14	C15	C16	2.4(3)
C14	C15	C16	F4	179.17(15)
C14	C15	C16	C17	-1.0(3)
C15	C16	C17	F5	177.10(15)
C15	C16	C17	C12	-2.0(3)
C17	C12	C13	F1	179.33(15)
C17	C12	C13	C14	-2.0(2)

TableS6: Hydrogen Fractional Atomic Coordinates (×10⁴) and Equivalent Isotropic Displacement Parameters (Å²×10³) for JW-AM-005 (**15**). U_{eq} is defined as 1/3 of the trace of the orthogonalised U_{ij}.

Atom	x	y	z	U_{eq}
H2	3031.17	4886.47	3084.27	21
H3	1387.16	2649.78	3257.93	24
H4	323.66	1939.23	4823.24	23
H5	913.48	3445.32	6238	21
H10A	6057.54	8111.8	806.97	34
H10B	4406.31	6871.41	593.72	34
H11A	6444.05	5233.11	1096.7	31
H11B	7059.39	6053.4	99.52	31

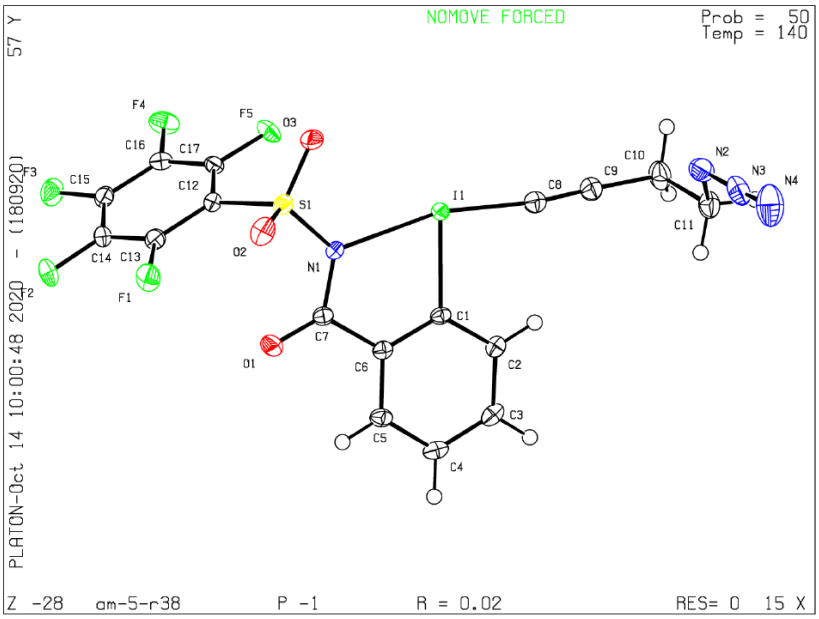


Figure S4. Crystal structure of **15**.

3. Sequential homo- and cross-linking of peptides

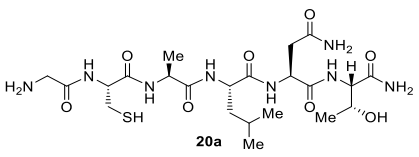
3.1 Synthesis of the starting peptides

Solid-Phase Peptide Synthesis (SPPS): Peptides were synthesized on an Advanced ChemTech 348-Ω parallel peptide synthesizer (AAPPTec) using standard Fmoc SPPS-chemistry and Rink Amide MBHA resin (0.26 mmol/g resin, 0.05 mmol scale) for C-terminal amide. The coupling was carried out by shaking the resin with a Fmoc-protected monomer (4.0 equiv.), 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU, 4.0 equiv.), 4-methylmorpholine (NMM, 6.0 equiv.), in DMF (1.3 mL), at 400 rpm, over 30 minutes. Each coupling step was performed twice. Capping was run at the end of each coupling using mixture of Ac_2O : 2,6-lutidine : DMF (5:6:89), followed by washing with dimethylformamide (4 x 3 mL). Fmoc groups were then removed by shaking the resin with a solution of piperidine in DMF (20% v/v) at 400 rpm, over 5 minutes. This step was carried out twice. Next, washing steps were achieved with DMF (5 x 3 mL). Finally, resin was washed with DCM (5 x 3 mL) and dried under vacuum.

Peptide cleavage and deprotection: Peptides were deprotected and cleaved from the resin under reducing conditions, by treatment with a mixture of water (2.5% v/v) and tri*iso*propylsilane (TIPS; 2.5% v/v) in trifluoroacetic acid (5 mL). The resulting mixture was shaken for 2 hours at 400 rpm, at rt. The resin was removed by filtration and peptides were precipitated in cold diethyl ether (20 mL), followed by a 2 hours incubation at -20 °C. Peptides were pelleted by centrifugation at 4000 rpm for 5 minutes. Finally, the mother liquors were carefully removed. The crude peptides were dissolved in a minimal amount of water and ACN and the resulting solutions were submitted to lyophilization

Peptide purification and analyses: Upon lyophilization, the crude peptides were dissolved in a 1 : 1 mixture of water and an organic co-solvent (ACN or DMF, up to a volume of 1.8 mL) and submitted to preparative RP-HPLC. The fractions containing the desired peptides were subsequently lyophilized.
All the peptide were isolated as their corresponding trifluoroacetate salts.

H-Gly-Cys-Ala-Leu-Asn-Thr-NH_2 (20a)



20a

Isolated by preparative HPLC, using method 4, as a mixture of diastereoisomers (as shown by ^{1}H-NMR analysis; d.r.: 80 : 20);[2] Retention time: 11.5-12.5 minutes.

^{1}H NMR (400 MHz, Methanol-d_4; the resolved signals corresponding the minor diastereoisomer are underlined and reported with no indication of the relative integrals) δ 4.73 (t, *J* = 6.5 Hz, 1H, C*H*), 4.62 (t, *J* = 6.0 Hz, 1H, C*H*), 4.41 (t, *J* = 6.9 Hz, 1H, C*H*), 4.39 – 4.31 (m, 4H, C*H*), 4.27 (d, *J* = 3.1 Hz, 1H), 3.84 (d, *J* = 16.0 Hz, H_2NCH_2CO), 3.77 (s, 2H, H_2NCH_2CO), 2.95 (dd, *J* = 14.0, 5.6 Hz, 1H, $CHCH_2$), 2.88 (dd, *J* = 15.2, 6.7 Hz, 1H, $CHCH_2$), 2.83 (m, 1H, $CHCH_2$), 2.78 (m, 1H, $CHCH_2$), 2.68 (dd, *J* = 15.5, 6.5 Hz, $CHCH_2$), 1.73 (dd, *J* = 12.8, 6.5 Hz, 1H, CH_2CHCH_3), 1.68 – 1.60 (m, 2H, CH_2CHCH_3), 1.46 (m, CH_3), 1.43 (d, *J* = 7.2 Hz, 3H, CH_3), 1.20 (d, *J* = 6.4 Hz, 3H, CH_3), 1.18 (m, 1H, CH_3), 0.99 (s, CH_3), 0.97 (d, *J* = 6.5 Hz, 3H, CH_2CHCH_3), 0.94 (d, *J* = 6.4 Hz, 3H, CH_2CHCH_3).

HPLC-MS: Retention time (Method 1) = 5.5 minutes.
HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{22}H_{41}N_8O_8S^+$ 577.2763; Found 577.2773.

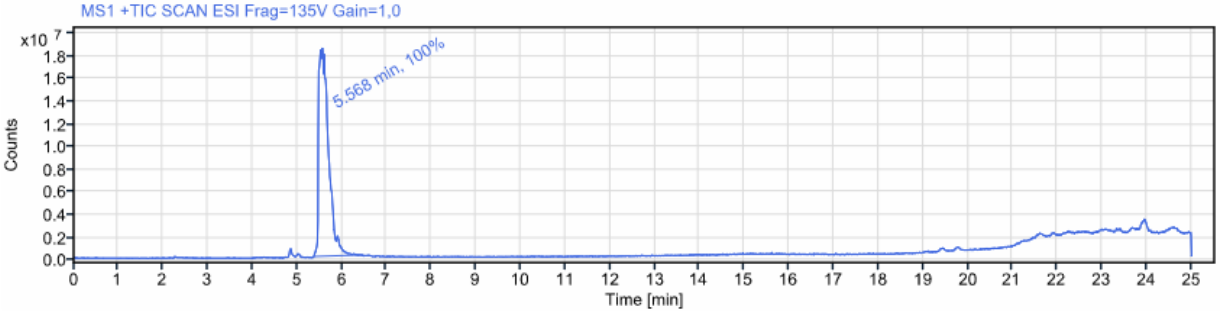
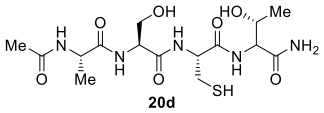


Figure S5. HPLC-MS chromatogram of **20a** upon purification.

Ac-Ala-Ser-Cys-Thr-NH_2 (20d)



20d

Isolated by preparative HPLC, method 3, as a mixture of diastereoisomers (d.r.: 10 : 1).

HPLC-MS: Retention time (Method 1) = 4.7 minutes.
LRMS (ESI) m/z: $[M + H]^+$ Calcd for $C_{15}H_{28}N_5O_7S^+$ 422.17; Found 422.20.

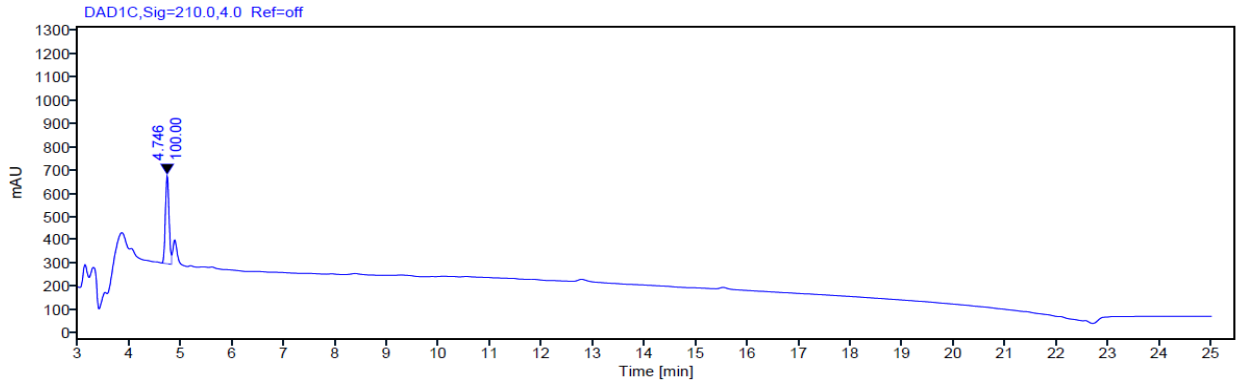
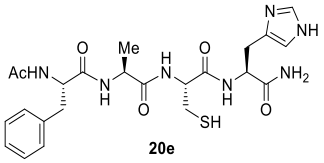


Figure S6. HPLC-UV (at 210 nm)chromatogram of **20d** upon purification.

Ac-Phe-Ala-Cys-His-NH_2 (20e)



20e

Isolated by preparative HPLC, using method 3, as a mixture of diastereoisomers (as shown by ^{1}H-NMR analysis; d.r.: 85 : 15);[2] Retention time: 7.0-7.7 minutes.

^{1}H NMR (400 MHz, DMSO-d_6; the resolved signals corresponding the minor diastereoisomer are underlined and reported with no indication of the relative integrals; the signal corresponding to SH was not resolved) δ 8.95 (s, 1H, NC*H*=N), 8.25 (d, *J* = 8.1 Hz, CON*H*); 8.20 (d, *J* = 6.6 Hz, 1H, CON*H*), 8.16 (d, *J* = 8.4 Hz, 1H, CON*H*), 8.06 (d, *J* = 8.2 Hz, 1H, CON*H*), 7.99 (d, *J* = 7.2 Hz, 1H, CON*H*), 7.32 (s, 1H, C=C*H*N), 7.29 (br s, 1H, N*H*), 7.27 – 7.23 (m, 5H, Ar*H* and N*H*), 7.18 (ddd, *J* = 6.6, 4.8, 3.2 Hz, 2H, Ar*H*), 4.53 – 4.46 (m, 2H, 2 x C*H*), 4.33 (m, 1H, C*H*), 4.26 (q, *J* = 6.6, 6.2 Hz, 1H, C*H*), 3.21 (dd, *J* = 4.2 Hz, CH_2); 3.15 (dd, *J* = 15.4, 5.1 Hz, 1H, CH_2), 3.01 (dd, *J*

[2] The two diastereoisomers were eluted with the same retention time. Nonetheless, they could be discriminated by ^{1}H-NMR analysis.

= 13.8, 3.8 Hz, 1H, CH_2), 2.94 (m, 1H, CH_2), 2.83 – 2.65 (m, 3H, CH_2), 2.35 (t, J = 8.5 Hz, 1H, CH_2), 1.76 (s, 3H, COCH_3), 1.75 (s, COCH_3), 1.26 (d, J = 7.1 Hz, CHCH_3), 1.22 (d, J = 7.1 Hz, 3H, CHCH_3).
^{13}C NMR (101 MHz, DMSO-d_6; only the signals corresponding to the major diastereoisomer were resolved) δ 172.4, 171.52, 171.48, 169.6, 169.4, 138.0, 133.7, 129.4, 129.1, 128.0, 126.2, 116.8, 55.3, 54.0, 51.6, 48.4, 37.3, 26.7, 25.7, 22.4, 17.6.
HPLC-MS: Retention time (Method 1) = 4.7 minutes.
HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{23}H_{32}N_7O_5S^+$ 518.2180; Found 518.2187.

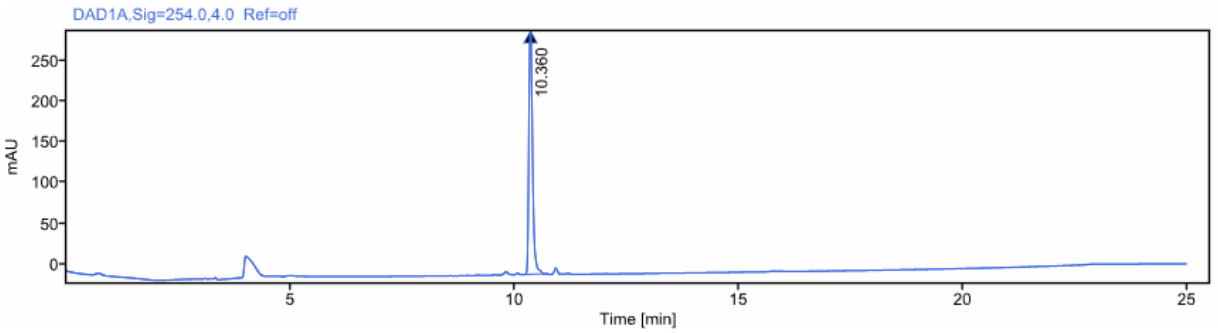
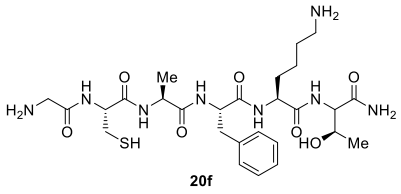


Figure S7. HPLC-UV (at 254 nm) chromatogram of **20e** upon purification.

H-Gly-Cys-Ala-Phe-Lys-Thr-NH_2 (20f)



Isolated by preparative HPLC, using method 3, as a mixture of diastereoisomers (as shown by ^{1}H-NMR analysis; d.r.: 90 : 10);[2] Retention time: 4.0-5.4 minutes.

^{1}H NMR (400 MHz, DMSO-d_6; the resolved signals corresponding the minor diastereoisomer are underlined and reported with no indication of the relative integrals; X = N, O or S) δ 8.61 (d, *J* = 8.1 Hz, 1H, CONH), 8.32 (d, *J* = 7.4 Hz, 1H, CONH), 8.25 (d, *J* = 7.8 Hz, 1H, CONH), 8.08 (d, *J* = 8.3 Hz, CONH), 8.04-7.87 (br s, 2H, XH), 7.95 (d, *J* = 8.0 Hz, 4H, XH and $CONH_2$), 7.80-7.61 (br s, 2H, XH). 7.65 (d, *J* = 8.6 Hz, 1H, CONH), 7.24 (d, *J* = 4.4 Hz, 3H, ArH), 7.22 – 7.15 (m, 2H, ArH), 7.12 (s, 1H, XH), 4.95 (d, *J* = 5.0 Hz, 1H, CH), 4.58 – 4.46 (m, 2H, CH), 4.32 (m, 1H, CH), 4.24 (q, *J* = 7.2 Hz, 1H, CH), 4.14 (dd, *J* = 8.6, 3.3 Hz, 1H, CH), 4.04 (m, 1H, CH), 3.62 (s, 2H, H_2NCH_2CO), 3.05 (dd, *J* = 14.0, 4.1 Hz, 1H, CH_2), 2.87 – 2.61 (m, 4H, CH_2), 2.68 (d, *J* = 1.8 Hz, 1H), 1.72 (m, 1H, CH_2), 1.65 – 1.46 (m, 3H, CH_2), 1.33 (s, 2H, CH_2), 1.18 (partially resolved d, CH_3), 1.16 (d, *J* = 7.1 Hz, 3H, CH_3), 1.04 (d, *J* = 6.3 Hz, 3H, CH_3).
HPLC-MS: Retention time (Instrument b, method 1) = 2.0 minutes.
LRMS (ESI) m/z: $[M + H]^+$ Calcd for $C_{27}H_{45}N_8O_7S^+$ 625.31; Found 625.40.

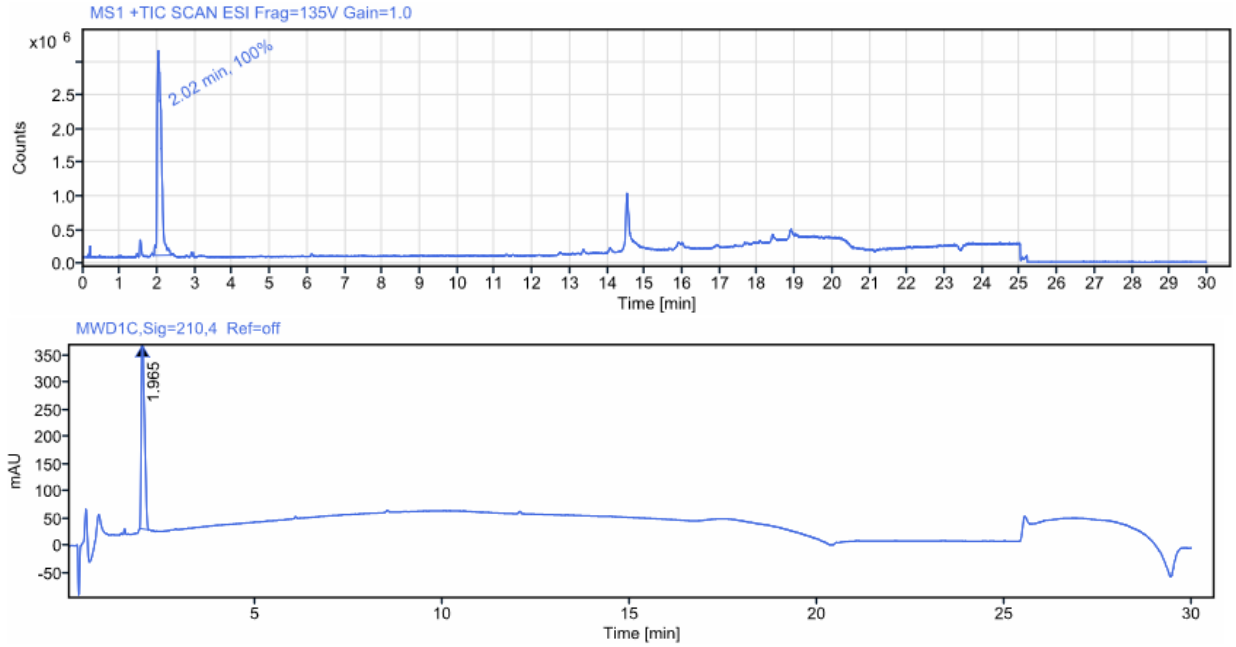
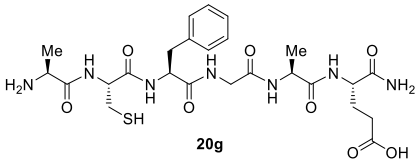


Figure S8. HPLC-MS and HPLC-UV (at 210 nm) chromatogram of **20a** upon purification.

H-Ala-Cys-Phe-Gly-Ala-Glu-NH_2 (20g)



Isolated by preparative HPLC, using method 5. Retention time: 5-7 minutes.

HPLC-MS: Retention time (Instrument b; Method 1) = 2.5 minutes.
HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{25}H_{38}N_7O_8S^+$ 596.2497; Found 596.2495.

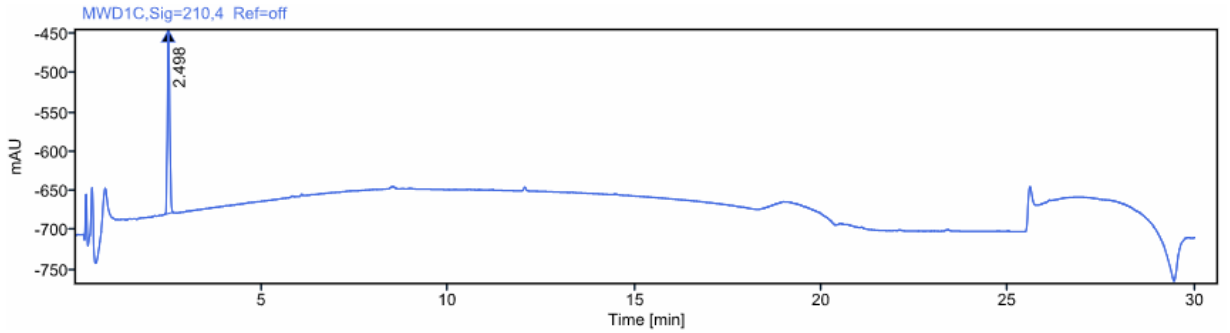
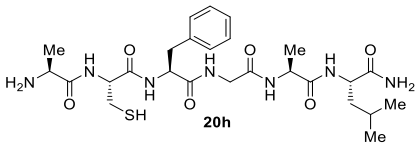


Figure S9. HPLC-UV (at 210 nm) chromatogram of **20g** upon purification.

H-Ala-Cys-Phe-Gly-Ala-Leu-NH_2 (20h)



Isolated by preparative HPLC, using method 6; Retention time: 11.5-11.9 minutes.

HPLC-MS: Retention time (Method 1) = 8.0 minutes.
HRMS (ESI/QTOF) m/z: $[M + Na]^+$ Calcd for $C_{26}H_{41}N_7NaO_6S^+$ 602.2731; Found 602.2724.

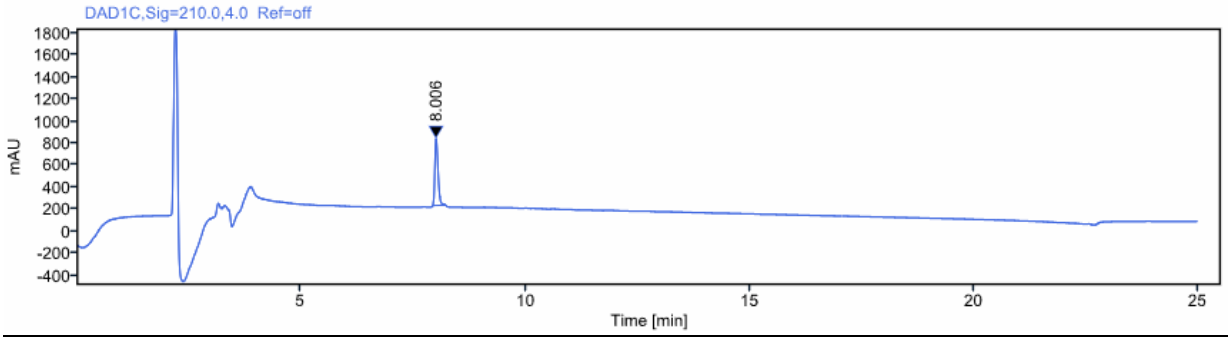
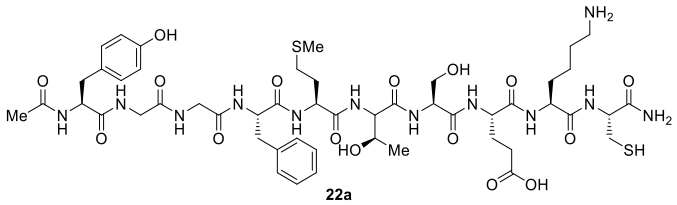


Figure S10. HPLC-UV (at 210 nm) chromatogram of **20h** upon purification.

Ac-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Cys-NH_2 (Endorphin-C_{10}, 22a)



Isolated by preparative HPLC, using method 3; Retention time: 9.0-9.5 minutes.

HPLC-MS: Retention time (Method 1) = 8.0 minutes.
LRMS (ESI) m/z: $[M + H]^+$ Calcd for $C_{50}H_{75}N_{12}O_{16}S_2^+$; 1163.47, Found 1163.70.

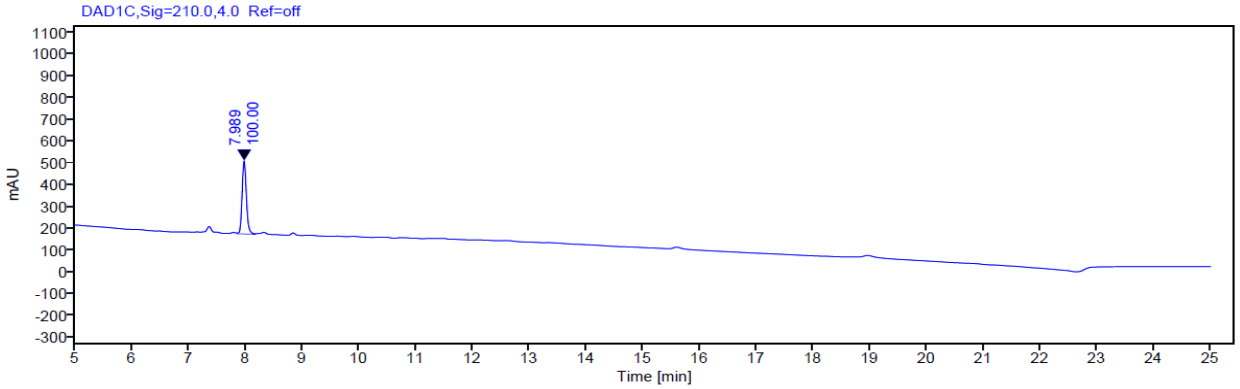
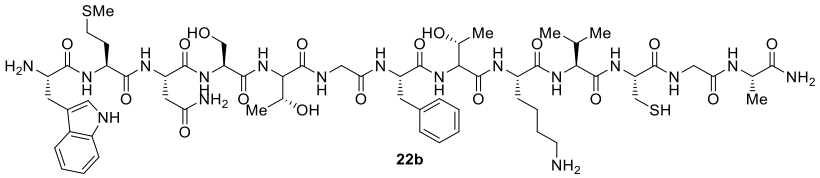


Figure S11. HPLC-UV (at 210 nm) chromatogram of **22a** upon purification.

H-Trp-Met-Asn-Ser-Thr-Gly-Phe-Thr-Lys-Val-Cys-Gly-Ala-NH_2 (Fragment of HCV, 22b)



Isolated by preparative HPLC, using method 6; Retention time: 13.4-14.0 minutes.

HPLC-MS: Retention time (Method 2) = 7.5 minutes.
HRMS (ESI/QTOF) m/z: $[M + 2H]^{+2}$ Calcd for $C_{61}H_{95}N_{17}O_{17}S_2^{+2}$ 700.8261; Found 700.8262.

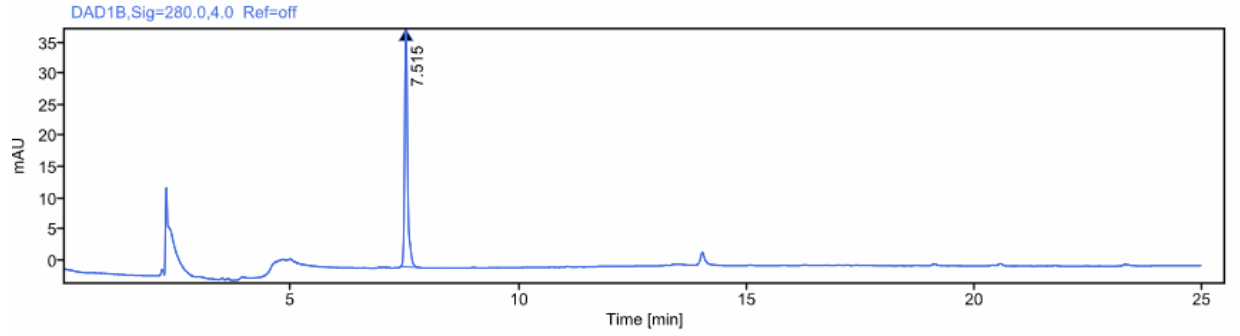
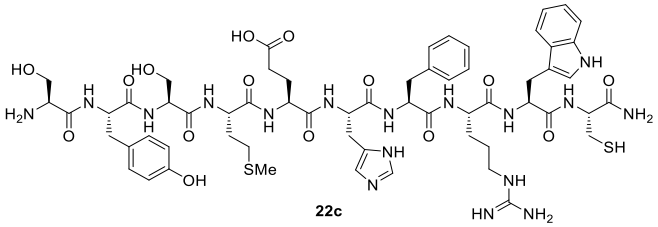


Figure S12. HPLC-UV (at 280 nm) chromatogram of **22b** upon purification.

H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Cys-NH_2 (ACTH, 22c)



Isolated by preparative HPLC, using method 5; Retention time: 11.3-11.6 minutes.

HPLC-MS: Retention time (Method 1) = 7.4 minutes.
HRMS (ESI/QTOF) m/z: $[M + 2H]^{+2}$ Calcd for $C_{60}H_{83}N_{17}O_{15}S_2^{+2}$ 672.7843; Found 672.7820.

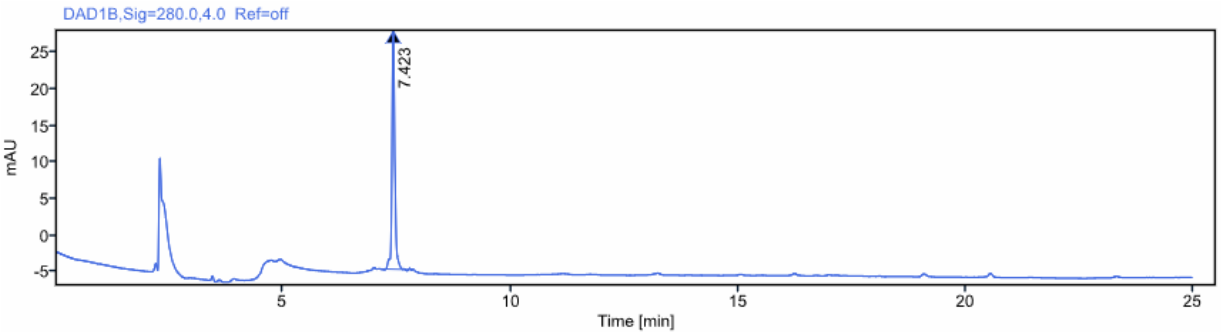
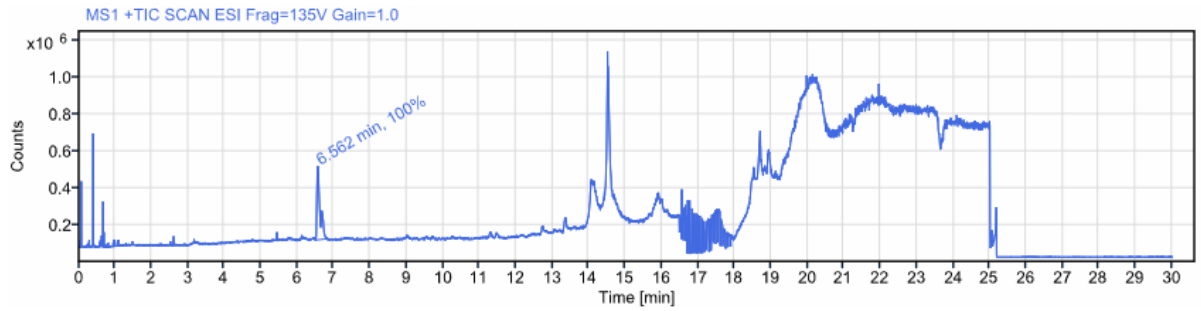


Figure S13. HPLC-UV (at 280 nm) chromatogram of **22c** upon purification.

3.2 Cys-Coupling of peptides with JW-AM-005: Optimization

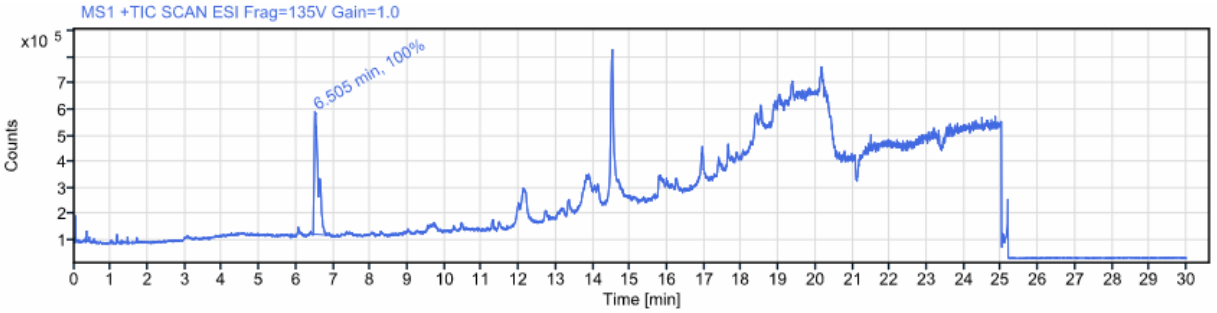
Calibration line for the quantification of 21a
Each of the following amounts of peptide derivative **21a** (trifluoroacetate salt; mixture of not resolved diastereoisomers) were dissolved in 0.40 mL solvent (0.20 mL milliQ water + 0.20 mL ACN). Samples 1-4 were then prepared by diluting 0.010 mL (10 µL) of each solution in with 90 µL of milliQ water. The resulting solutions were then submitted to HPLC-MS analysis (instrument b, method 2). N.B.: Theoretical yields refer to reactions performed with 0.0010 mmol of starting peptide **21a** (Figure S14)**.**

Sample 1 - 1.20 mg → 4 x 0.000261 mmol (26% yield)
Sample 2 - 2.40 mg → 4 x 0.000523 mmol (52% yield)
Sample 3 - 3.49 mg → 4 x 0.000761 mmol (76% yield)
Sample 4 - 4.55 mg → 4 x 0.000992 mmol (99% yield)



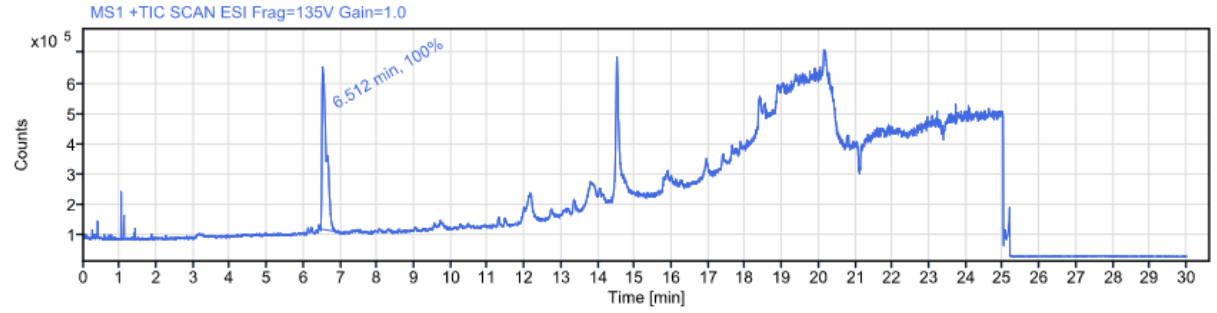
Peak Results (Area Percent at least 1%)

RT (min)	Signal Description	Width (min)	Area	Height	Area%
6.562	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.312	2484346.5	397913.4	100.00



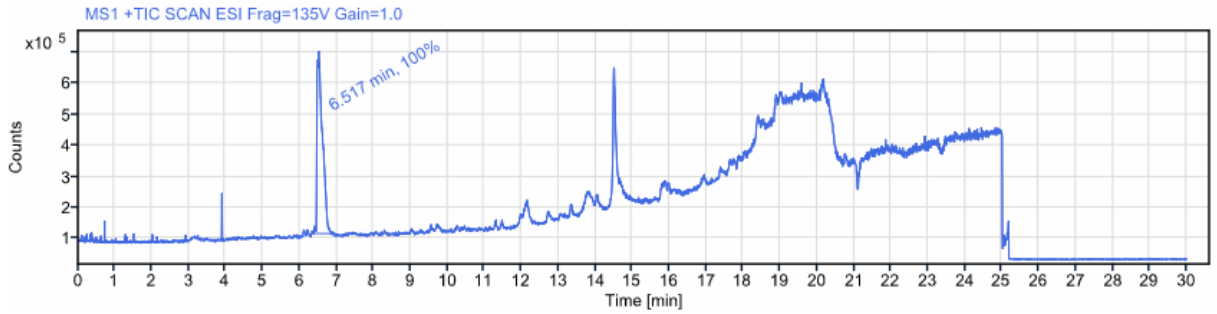
Peak Results (Area Percent at least 1%)

RT (min)	Signal Description	Width (min)	Area	Height	Area%
6.505	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.374	3765439.6	472324.6	100.00



Peak Results (Area Percent at least 1%)

RT (min)	Signal Description	Width (min)	Area	Height	Area%
6.512	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.441	4677584.7	534337.0	100.00



Peak Results (Area Percent at least 1%)

RT (min)	Signal Description	Width (min)	Area	Height	Area%
6.517	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.579	5912289.5	586105.8	100.00

Figure S14. HPLC-MS chromatograms corresponding to samples 1-4 of product **21a** and used to establish a calibration line.

The calibration line (Figure S15) was subsequently established by considering the integrals of the peaks in the MS-based chromatograms.

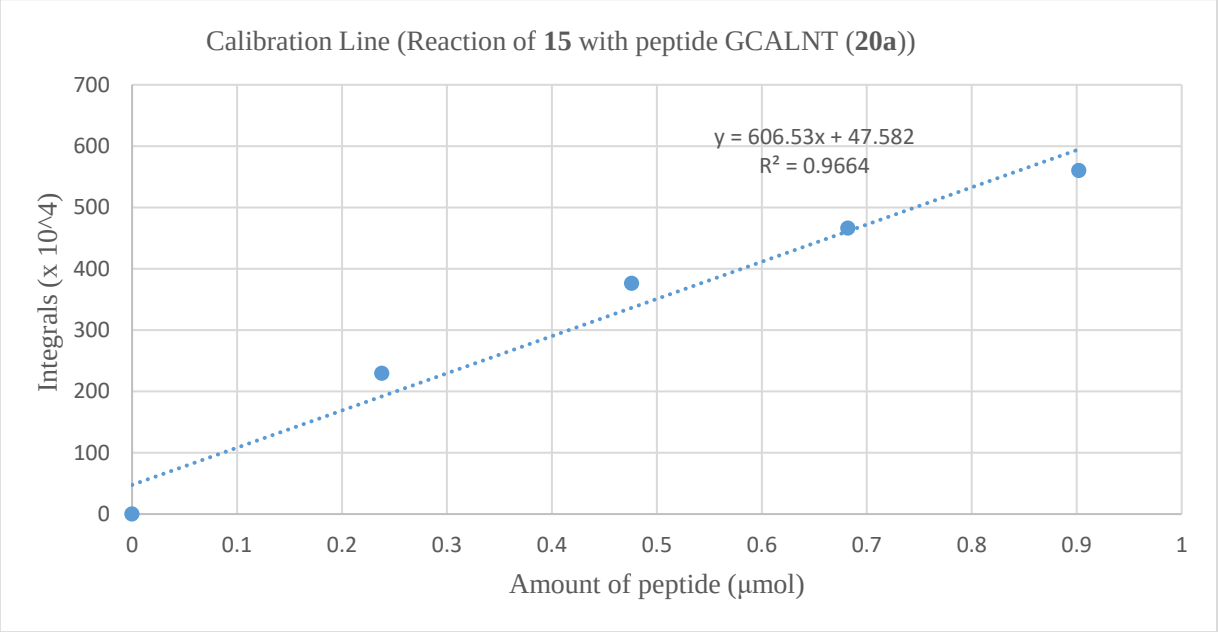


Figure S15: Calibration line relative to the quantification of **17a** obtained from the reaction of **15** with **20a**.

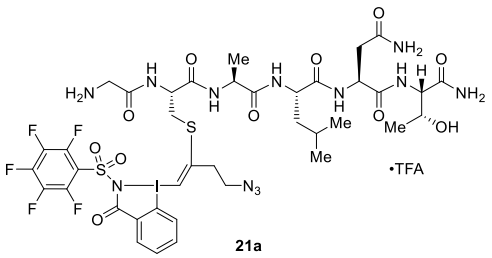
Stock solution of peptide (A): Prepared by dissolving 6.91 mg of peptide **20a** (as trifluoroacetate salt) (0.010 mmol) in a mixture of 0.250 mL of the selected organic solvent and 0.100 mL of milliQ water.

Stock solution of reagent (B): Prepared by dissolving 6.84 mg of **15** (0.012 mmol) in 0.250 mL of the selected organic solvent.

Experimental procedure for the optimization: A 0.5 mL Eppendorf vial was charged with 0.040 mL of the buffer solution. 0.035 mL of a solution A (0.0010 mmol, 1.0 equiv.) and 0.025 mL solution B (0.0012 mmol, 1.2 equiv.) were added. The mixture was shaken vigorously for 20 minutes. After this time, a sample of 10 μL was taken, diluted with 90 μL milliQ water, and submitted to HPLC-MS analysis (Method 1, instrument b). The amount of **20a** formed after 20 minutes was determined based on the calibration line showed in Figure S5. ***High dilution experiments (2 mM):*** were performed by diluting the aforementioned mixture with further volumes of buffer (0.2 mL) and of the organic solvent (0.2 mL). The reaction mixture was shaken for 80 minutes. Sampling for HPLC-MS analysis was done by diluting 50 μL of the reaction mixture with 50 μL milliQ water. The results are reported in Table S7.

Scale-up of the reaction under the best conditions and determination of the isolated yield: A 4.0 mL Eppendorf vial was charged with peptide **20a** (5.6 mg,. 0.0080 mmol, 1.0 equiv.) and the selected buffer (0.40 or 0.20 mL) (solution A). In a second 4.0 mL Eppendorf vial, the reagent **15** (5.5 mg, 0.0096 mmol, 1.2 equiv.) was dissolved in the organic solvent (0.40 or 0.20 mL) (solution B). Solution B was then added to solution A. The resulting mixture was then shaken vigorously for 20 minutes. After this time, a sample of the mixture (10 μL) was taken, diluted with milliQ water (0.20 mL) and submitted to HPLC-MS analysis. The crude mixture was then diluted with milliQ water (up to a total volume of 1.8 mL) and submitted to preparative HPLC (elution according to Method 4). The collected fractions were then lyophilized to provide the derivative **21a** as a fluffy, white solid. Derivative **21a'** was not isolated.

HPLC-MS: Retention time of **21a**: 6.5 min. (Instrument b, method 1)
HRMS (ESI/QTOF) m/z of **21a**: $[M + 2H]^{+2}$ Calcd for $C_{39}H_{50}F_5IN_{12}O_{11}S_2^{+2}$ 574.1059; Found 574.1070.
HPLC-MS: Retention time of **21a'**: 4.0 min. (Instrument b, method 1)
HRMS (ESI/QTOF) m/z of **21a'**: $[M + Na]^+$ Calcd for $C_{26}H_{43}N_{11}NaO_8S^+$ 692.2909; Found 692.2918.



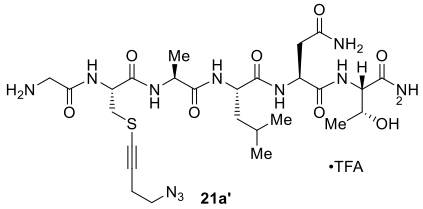


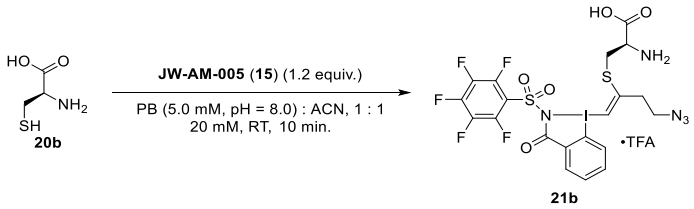
Table S7: Optimization of the model reaction for the conjugation of peptide **20a** with JW-AM-005 (**15**)

Entry	Conditions[a]	Yield of 21a	21a:21a' (yield of 21a')[c]
1	TRIS, 50 mM, pH = 8.0; ACN; 10 mM	76%	93 : 7 (~5%)
2	TRIS, 50 mM, pH = 8.0; DMSO; 10 mM	68%	82 : 18 (~12%)
3	TRIS, 50 mM, pH = 8.0; DMF; 10 mM	65%	88 : 12 (~8%)
4	HEPES, 50 mM, pH = 8.0; ACN; 10 mM	84%	92 : 8 (~7%)
5	HEPES, 50 mM, pH = 8.0; DMSO; 10 mM	79%	80 : 20 (~16%)
6	HEPES, 50 mM, pH = 8.0; DMF; 10 mM	77%	85 : 15 (~11%)
7	PB, 50 mM, pH = 8.0; ACN; 10 mM	75%	92 : 8 (~6%)
8	PB, 50 mM, pH = 8.0; DMSO; 10 mM	47%	70 : 30 (~14%)
9	PB, 50 mM, pH = 8.0; DMF; 10 mM	74%	82 : 18 (~13%)
10[b]	PB, 50 mM, pH = 8.0; ACN; 2 mM	76%	95 : 5 (~4%)
11[b]	PB, 50 mM, pH = 8.0; DMF; 2 mM	47%	77 : 23 (~11%)
12	PB, 50 mM, pH = 7.4; ACN; 10 mM	73%	90 : 10 (~7%)
13	PB, 50 mM, pH = 7.4; DMF; 10 mM	75%	18 : 82 (~13%)
14	PB, 50 mM, pH = 6.0; ACN; 10 mM	79%	90 : 10 (~8%)
15	PB, 50 mM, pH = 6.0; DMSO; 10 mM	71%	78 : 22 (~16%)
16	PB, 50 mM, pH = 6.0; DMF; 10 mM	64%	85 : 15 (~10%)
17	Water; ACN; 10 mM	75%	90 : 10 (~7%)
18	Water; DMSO; 10 mM	25%	70 : 30 (~7%)
19	Water; DMF; 10 mM	65%	90 : 10 (~6%)
20[d]	HEPES, 50 mM, pH = 8.0; ACN; 10 mM	80%[e]	90 : 10 (~8%)
21[d]	PB, 50 mM, pH = 8.0; ACN; 10 mM	82%	91 : 9 (~7%)
22[d]	PB, 50 mM, pH = 8.0; ACN; 20 mM	77%	92 : 8 (~6%)

[a] ***General 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. [b] Shaken for 80 minutes. [c] Estimated based on the ratio of integrals of **21a**:**21a'** in HPLC-MS chromatograms. [d] ***Reaction conditions:*** 0.0080 mmol of **20a**, 0.0096 mmol of **15**, shaken in the indicated 1 : 1 mixture of buffer and organic solvent for 20 minutes. [e] Average over two reiterations of the experiment.

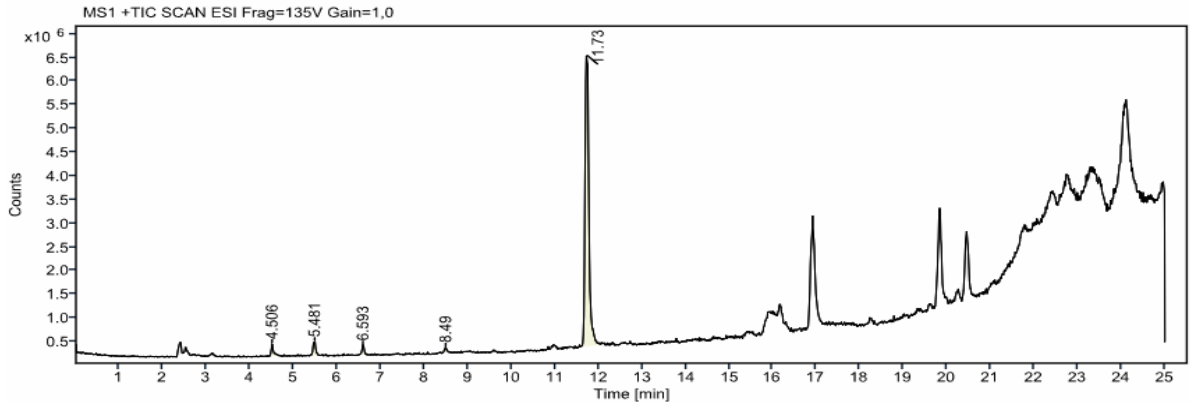
3.2 Cys-Coupling of peptides with JW-AM-005: Scope

(*Z*)-1-(4-Azido-2-mercaptobut-1-en-1-yl)-2-((perfluorophenyl)sulfonyl)-1,2-dihydro-3*H*-1λ3-benzo[*d*][1,2]iodazol-3-one (Cysteine S-VBZ) (21b)



A 2.0 mL vial was charged with L-cysteine (**20b**) (4.8 mg, 0.040 mmol, 1.0 equiv.) and JW-AM-005 (**15**) (27.4 mg, 0.048 mmol, 1.2 equiv.). PB (50 mM, pH 8.0; 1.0 mL) and ACN (1.0 mL) were added, and the resulting pale yellow suspension was stirred very vigorously at room temperature for 10 minutes, turning into a homogenous, off-white solution. HPLC-MS analysis (method 1) revealed, at this point, that no residual starting material was left (Figure S16; indicative HPLC-MS-based yield: ca. 91%).

The mixture was directly submitted to preparative HPLC (method 3; rentetion time 10.0-10.5 minutes). Upon lyophilization, (Z)-1-(4-azido-2-mercaptobut-1-en-1-yl)-2-((perfluorophenyl)sulfonyl)-1,2-dihydro-3*H*-1λ3-benzo[*d*][1,2]iodazol-3-one (trifluoroacetic acid salt) (**21b**) (25.4 mg, 0.315 mmol, 79% yield) was collected as an amorphous, white solid.



RT [min]	Signal Description	Symmetry	Resolution	Height [count]	Area [count*min]	Rel. Area [%]
4.506	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.51056		266408	1099089	2.6
5.481	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	1.03648		299083	1348665	3.2
6.593	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.95128		209573	822197	1.9
8.490	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.86517		109866	335152	0.8
11.730	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.75812		6155224	38672444	91.5

Filtered on peak height > 100000 counts

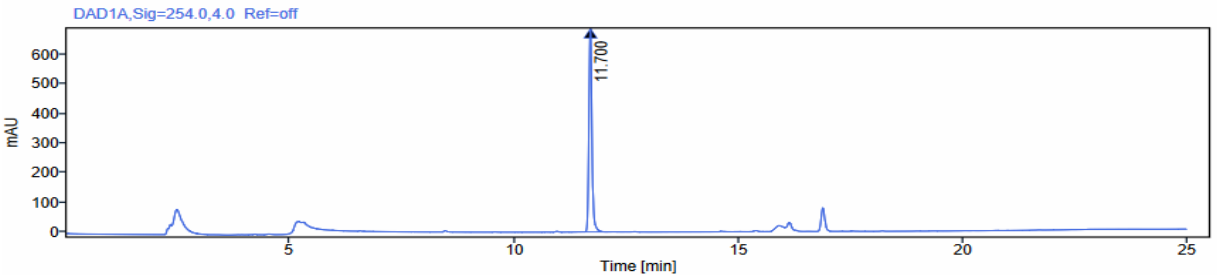


Figure S16: HPLC-MS and HPLC-UV (at 254 nm) chromatograms of the reaction of cysteine with **15** after 10 minutes. The compound eluted at ca. 17.0 minutes is the residual unreacted **15**.

^{1}H NMR (400 MHz, DMF-d_7) δ 8.37 (m, 1H, Ar*H*), 7.86 (m, 1H, Ar*H*), 7.81 – 7.73 (m, 2H, Ar*H*), 7.44 (s, 1H, C=C*H*), 4.40 (t, *J* = 5.6 Hz, 1H, H_2NC*H*), 3.88 (t, *J* = 6.4 Hz, 2H, CH_2), 3.70 (d, *J* = 4.9 Hz, 1H, CH_2), 3.68 (d, *J* = 6.4 Hz, 1H, CH_2), 3.31 (m, 1H, CH_2), 3.25 (m, 1H CH_2).
^{13}C NMR (101 MHz, DMSO-d_6) δ 168.9, 167.3, 158.2 (d, *J* = 32.3 Hz), 156.5, 145.2 (m), 142.8 (m), 135.8 (m), 135.0, 133.5, 132.1, 130.8, 128.8, 118.4, 115.4, 112.7, 104.7, 51.8, 49.1, 35.2, 31.4.

^{19}F NMR (376 MHz, DMSO-d_6) δ -73.9, -135.9 – -141.1 (m), -150.0 (t, J = 21.7 Hz), -157.8 – -165.2 (m).
HPLC-MS: Retention time (Method 1) = 11.7 minutes.
HRMS (ESI/QTOF) m/z: [M + Na]$^+$ Calcd for $C_{20}H_{15}F_5IN_5NaO_5S_2^+$ 713.9372; Found 713.9390.

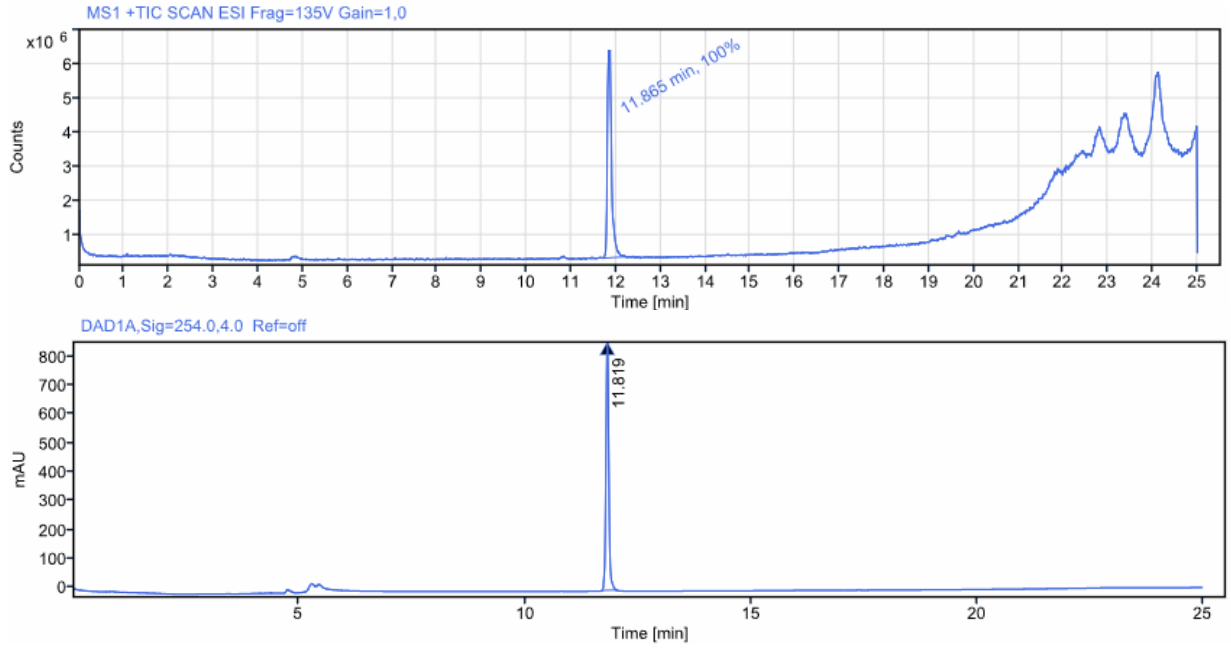
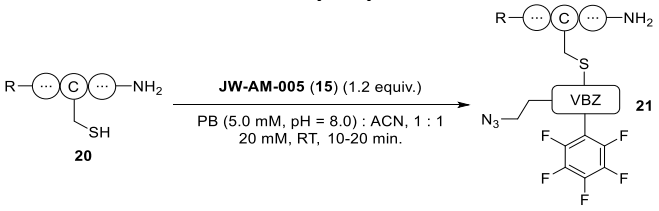


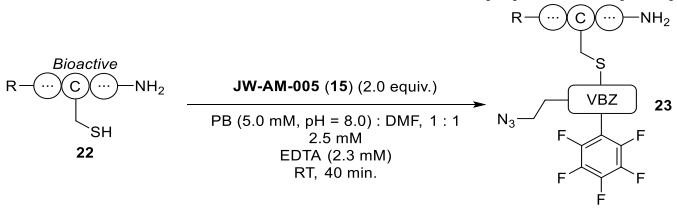
Figure S17. HPLC-MS and HPLC-UV (at 254 nm) chromatograms of pure **21b**.

General procedure for the synthesis of S-VBZ derivatives 21 (GP1)



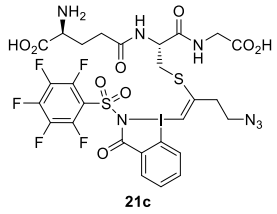
A 2.0 mL Eppendorf vial was charged with the peptide substrate **20** (ca. 0.020 mmol; 1.0 equiv.) and JW-AM-005 (**15**) (ca. 0.024 mmol, 1.2 equiv.). Equal volumes of PB (50 mM, pH = 8.0) and ACN were added to give an overall concentration of 20 mM. The resulting mixture was the stirred vigorously at room temperature for 20 minutes. The full conversion of the starting material was assessed by HPLC-MS (method 1). Unless otherwise indicated, the final product **21** was isolated by column chromatography (preparative HPLC).

General procedure for the synthesis of S-VBZ derivatives 23 from bioactive peptides 22 (GP2)



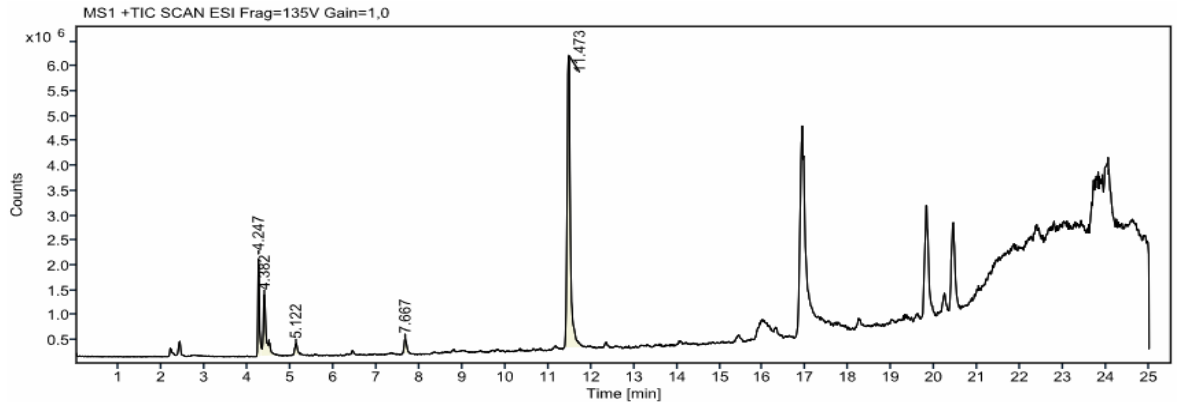
A 2.0 mL Eppendorf vial was charged with the peptide substrate **22** (0.0040; 1.0 equiv.) and JW-AM-005 (**15**) (4.5 mg, 0.0080 mmol, 2.0 equiv.). PB (Solution A: 50 mM, pH = 8.0; 0.53 mL), EDTA (Solution B: 6.7 mM in solution A; 0.27 mL) and DMF (0.80 mL) were added. A second 2.0 mL Eppendorf vial was charged with JW-AM-005 (**15**) (1.1-1.2 equiv.) and ACN (overall concentration: 20 mM). The resulting mixture was vigorously stirred at room temperature for 40 minutes. The full conversion of the staring material was then assessed by HPLC-MS (method 1). Unless otherwise indicated, the final product **23** was isolated by column chromatography (preparative HPLC).

Glutathione S-VBZ (21c)



The title compound was prepared following the **GP1** using reduced glutathione (6.1 mg, 0.020 mmol, 1.0 equiv.), **15** (13.7 mg, 0.0240 mmol, 1.2 equiv.), 0.50 mL PB (50 mM, pH 8.0) and 0.50 mL ACN. The reaction was monitored by HPLC-MS analysis, which showed that it stopped progressing within 40 minutes, with unreacted residual glutathione in both its reduced and oxidized forms (method 1, retention times: 4.2 minutes and 4.4 minutes, respectively. Indicative HPLC-MS based yield: ca. 70%. Figure S18).

The reaction mixture was diluted with milliQ water (up to 1.8 mL) and directly submitted to preparative HPLC (Method 3; retention time 10.5-12.0 min.). Upon lyophilization of the collected fractions, compound **21c** (6.3 mg, 0.072 mmol, 36% yield) was isolated as an amorphous, white solid.



RT [min]	Signal Description	Symmetry	Resolution	Height [count]	Area [count*min]	Rel. Area [%]
4.247	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.70732		2040739	4937616	10.7
4.382	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.48644		1239189	6504409	14.1
5.122	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.74527		232246	1055983	2.3
7.667	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.61907		305337	1187232	2.6
11.473	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.66433		5882797	32369910	70.3

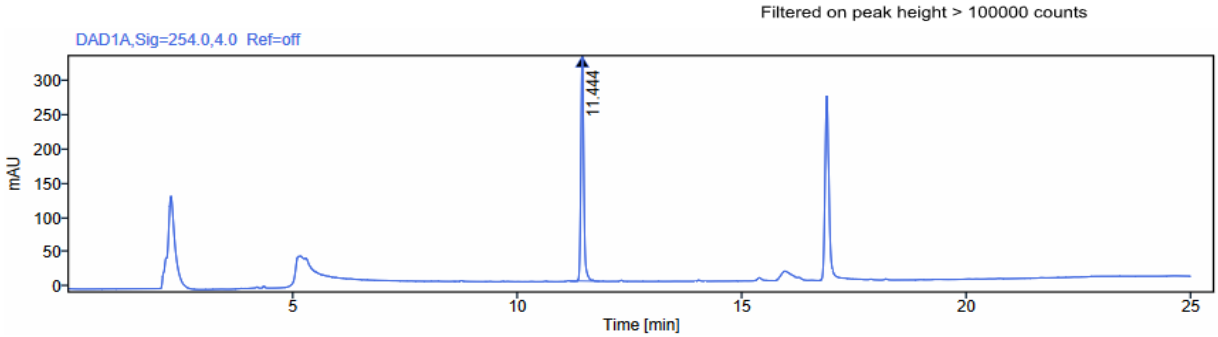


Figure S18: HPLC-MS and HPLC-UV (at 254 nm) chromatograms of the reaction of glutathione with **15** after 40 minutes. The compounds eluted at ca. 4.2 and 4.4 minutes are the residual st. material and the corresponding oxidized derivative (S-S disulfide dimer). The compound eluted at ca. 17.0 minutes is the residual unreacted **15**.

^{1}H NMR (600 MHz, DMSO-*d*6) δ 13.89 (br s, 1H, CO_2H), 12.66 (br s, 1H, CO_2H), 8.47 (t, *J* = 5.7 Hz, 1H, CONH), 8.43 (d, *J* = 8.2 Hz, 1H, CONH), 8.25 (m, 1H, ArH), 8.21 (br s, 2H, NH_2), 7.70 (dd, *J* = 5.9, 3.4 Hz, 2H, ArH), 7.64 (dd, *J* = 6.0, 3.3 Hz, 1H, ArH), 7.16 (s, 1H, C=CH), 4.47 (dd, *J* = 8.2 Hz, 1H, CHNHCO), 3.90 (br s, 1H, $CHNH_2$), 3.81 – 3.70 (m, 3H, CH_2CO_2H and CH_2), 3.64 (dd, *J* = 17.6, 5.5 Hz, 1H), 3.20 (dd, *J* = 13.7, 5.2 Hz, 1H, CH_2), 3.15 – 3.01 (m, 3H, CH_2), 2.27 (t, *J* = 7.8 Hz, 2H, CH_2), 1.95 (tq, *J* = 14.3, 6.9, 6.4 Hz, 2H, CH_2).
^{13}C NMR (151 MHz, DMSO-d_6; the signals corresponding to two aromatic Cs were not resolved) δ 171.6, 171.3, 170.0, 158.7, 158.3, 158.1, 145.2 (m), 143.6 (m), 135.4, 133.9, 132.6, 131.2, 129.1, 116.6, 112.7, 52.9, 52.0, 49.6, 41.1, 35.6, 34.1, 30.8, 26.1.
HPLC-MS: Retention time (Method 1) = 11.4-11.6 minutes.
HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{27}H_{26}F_5IN_7O_9S_2^+$ 878.0193; Found 878.0203.

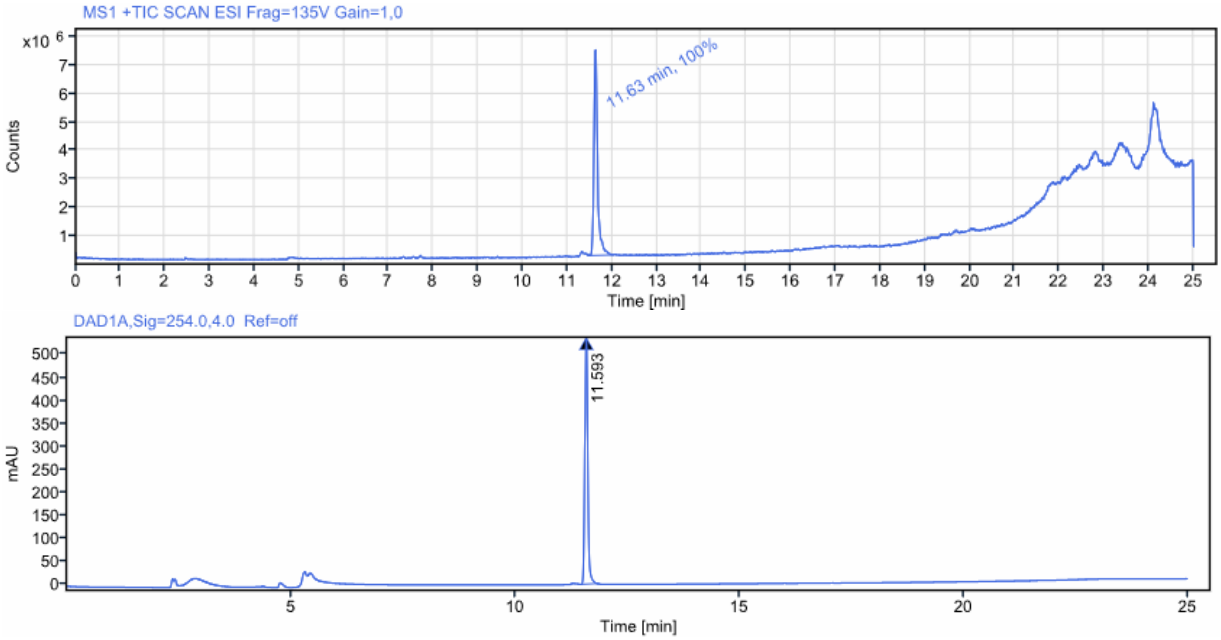
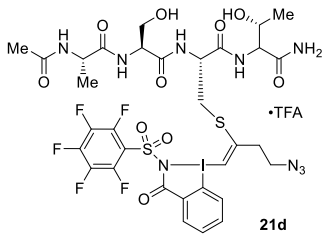


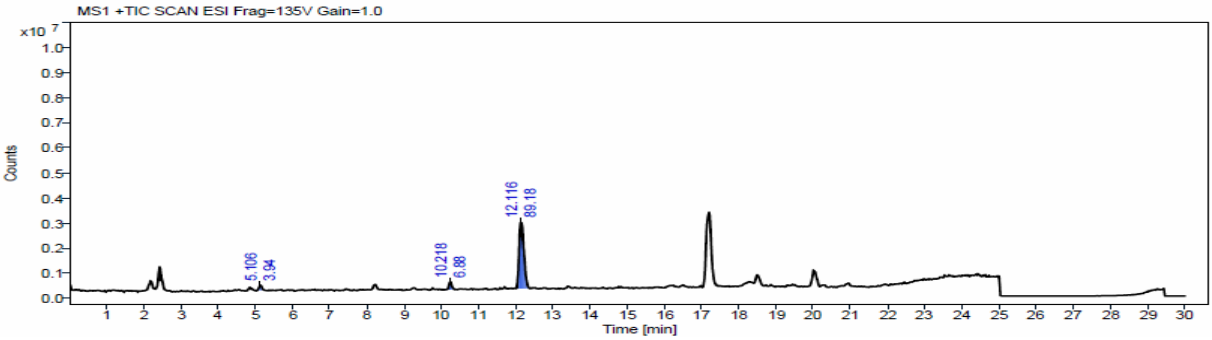
Figure S19. HPLC-MS and HPLC-UV (at 254 nm) chromatograms of pure **21c**.

Ac-Ala-Ser-Cys(S-VBZ)-Thr-NH_2 21d



The title compound was prepared following the **GP1** using peptide **20d** (10 mg, 0.024 mmol, 1.0 equiv.), **15** (14 mg, 0.026 mmol, 1.1 equiv.), 0.060 mL PB (50 mM, pH 8.0) and 0.060 mL ACN. After vigorous stirring for 10 minutes, full conversion was observed based on HPLC-MS analysis (10 μL sample, method 1; indicative HPLC-MS based yield: ca. 73%. Figure S20).

The reaction mixture was diluted with milliQ water (up to 1.8 mL) and directly submitted to preparative HPLC (Method 3; retention time 10.0-11.0 min.). Upon lyophilization of the collected fractions, compound **21d** (16 mg, 0.016 mmol, 68% yield; mixture of diastereoisomers, d.r.: 10 : 1) was isolated as a fluffy, white solid.



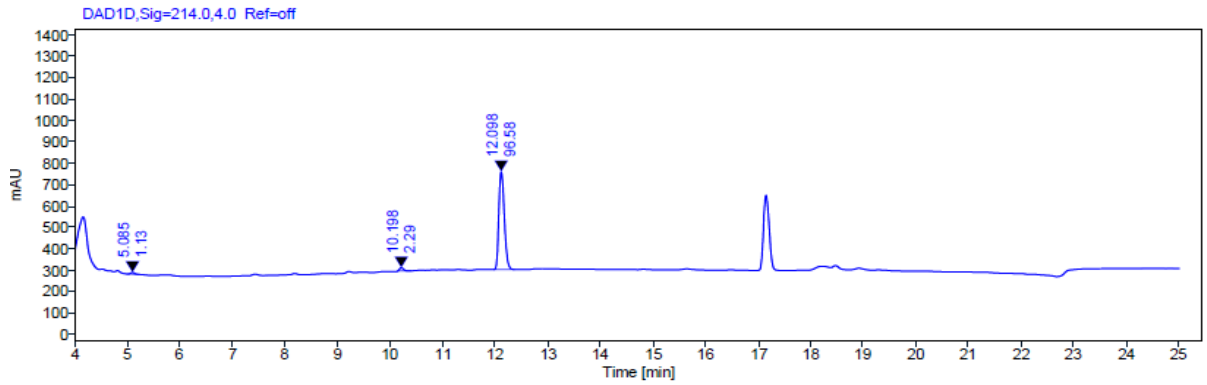


Figure S20: HPLC-MS and HPLC-UV (at 214 nm) chromatograms of the reaction of peptide **20d** with **15**. The compound eluted at ca. 17.0 minutes is the residual unreacted **15**.

^{1}H NMR (400 MHz, MeOD:D_2O (3:1); only the signals corresponding to the major diastereoisomers are reported; the signal corresponding to 1H at 3.30 ppm is partially overlapped with the signal of the solvent) δ 8.38 (m, 1H, Ar*H*), 7.78 – 7.66 (m, 3H, Ar*H*), 7.06 (s, 1H, IC*H*=C), 4.59 (dd, *J* = 8.1, 5.5 Hz, 1H, *CH* or CH_2), 4.36 (t, *J* = 5.3 Hz, 1H, *CH*), 4.32 – 4.24 (m, 2H, *CH* or CH_2), 4.16 (dd, *J* = 6.4, 4.1 Hz, 1H, *CH* or CH_2), 3.87 (dd, *J* = 11.2, 5.3 Hz, 1H, CH_2), 3.81 – 3.76 (m, 3H, 2 x CH_2), 3.46 (dd, *J* = 14.4, 5.5 Hz, 1H, CH_2), 3.30 (m, 1H, CH_2), 3.14 (t, 2H, *J* = 5.6 Hz, CH_2), 1.99 (s, 3H, COCH_3), 1.37 (d, *J* = 7.2 Hz, 3H, CHCH_3), 1.17 (d, *J* = 6.5 Hz, 3H, CHCH_3).
^{19}F NMR (376 MHz, MeOD) δ -138.7 (d, *J* = 31.0 Hz, 2F)), -150.97, (t, J = 36.4 Hz, 1F)), -162.79, (t, J = 22.5 Hz, 2F).
^{13}C NMR (101 MHz, methanol-*d4*; the signals corresponding to fluorinated aromatic carbons were not resolved) δ 174.6, 173.4, 173.1, 171.6, 170.2, 160.2, 138.7, 135.3, 132.9, 132.5, 130.9, 128.3, 111.3, 102.3, 67.1, 61.1, 58.7, 55.5, 54.12, 49.9, 49.6, 35.4, 32.4, 21.3, 18.7, 16.3. Carbon (F-C) could not be resolved).
HRMS (ESI/QTOF) m/z: $[M + Na]^+$ Calcd for $C_{32}H_{35}F_5IN_9NaO_{10}S_2^+$ 1014.0805; Found 1014.0798.
HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{32}H_{36}F_5IN_9O_{10}S_2^+$ 992.0986; Found 992.1014. MS-MS analysis shown in Figure S9.

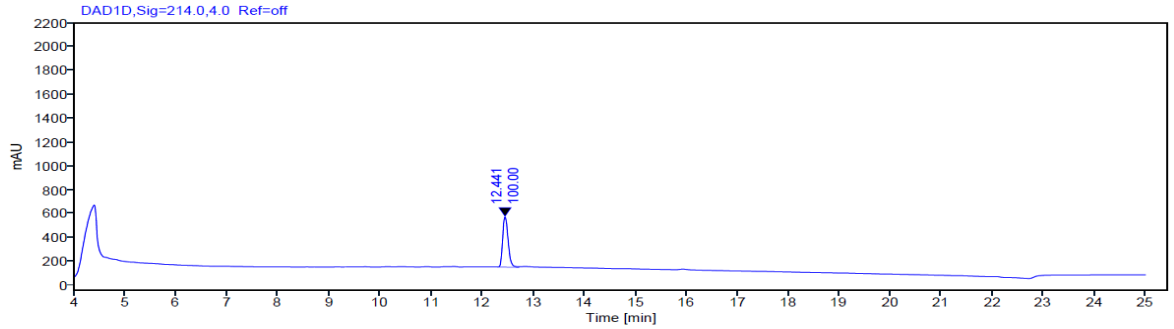


Figure S21. HPLC-MS and HPLC-UV (at 254 nm) chromatograms of pure **21d**.

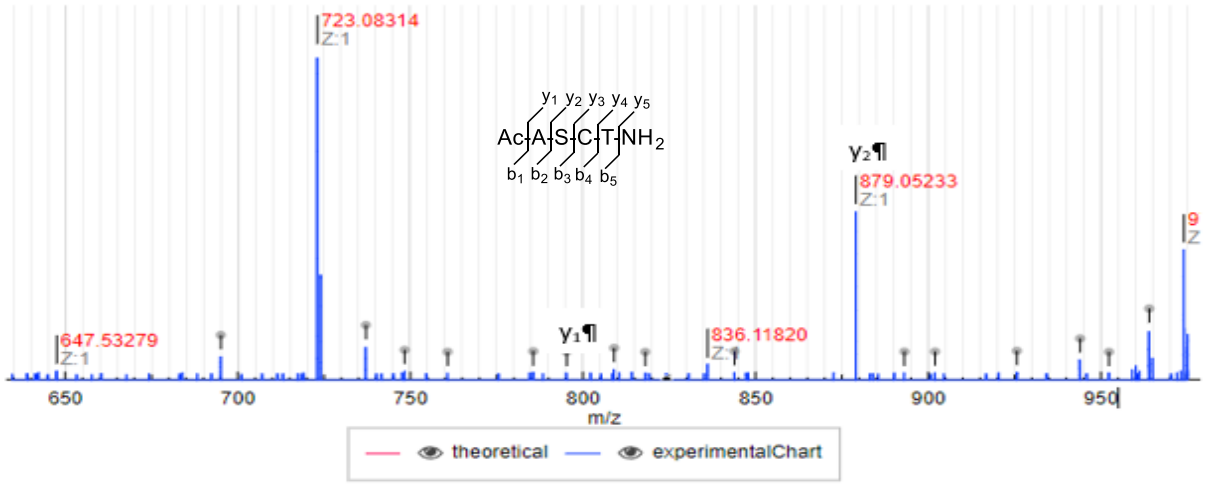
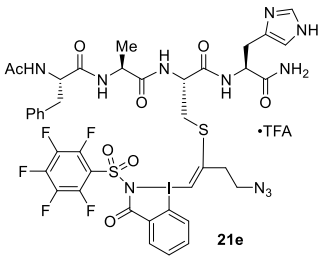


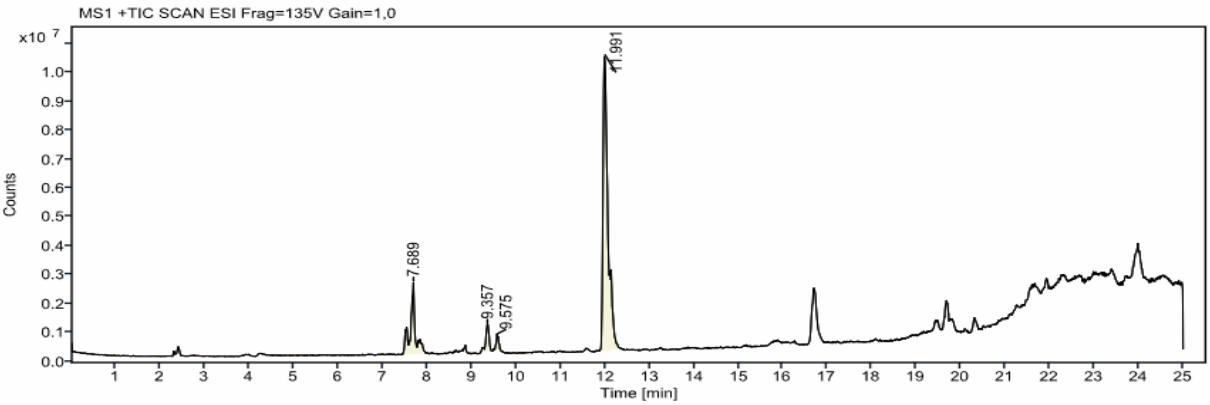
Figure S22. LC-MS/MS of **21d**

Ac-Phe-Ala-Cys(S-VBZ)-His-NH_2 21e



The title compound was prepared following the **GP1** using peptide **20e** (mixture of diastereoisomers, d.r. 85 : 15; 12.3 mg, 0.020 mmol, 1.0 equiv.), **15** (12.5 mg, 0.022 mmol, 1.1 equiv.), 0.50 mL PB (50 mM, pH 8.0) and 0.50 mL ACN. After vigorous stirring for 20 minutes, full conversion was observed based on HPLC-MS analysis (method 1) (Indicative HPLC-MS based yield: ca. 75%. Figure S23).

The reaction mixture was diluted with milliQ water (up to 1.8 mL) and directly submitted to preparative HPLC (Method 3; retention time 10.8-12.6 min.). Upon lyophilization of the collected fractions, compound **21e** (mixture of diastereoisomers, d.r. 85 : 15; 15.6 mg, 0.0129 mmol, 62% yield) was isolated as a fluffy, solid.



RT [min]	Signal Description	Symmetry	Resolution	Height [count]	Area [count*min]	Rel. Area [%]
7.689	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	1.35348		2522899	18930716	18.2
9.357	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	1.30321		976996	4703195	4.5
9.575	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	1.03042		593630	2022493	1.9
11.991	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.40658		10247332	78268579	75.3

Filtered on peak height > 100000 counts

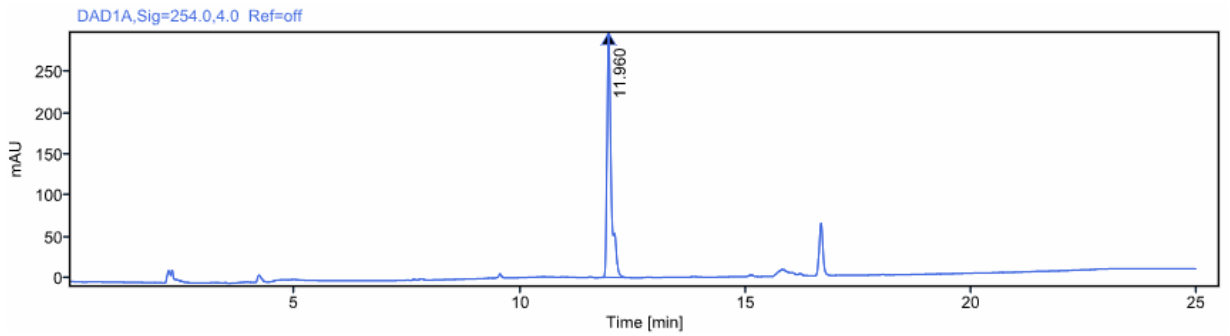


Figure S23: HPLC-MS and HPLC-UV (at 254 nm) chromatograms of the reaction of peptide **20e** with **15**. The compound eluted at ca. 7.7 minutes is the oxidized derivative of the st. material (S-S disulfide dimer). The compound eluted at ca. 17.0 minutes is the residual unreacted **15**.

^{1}H NMR (400 MHz, DMSO-d_6; acquired at 90 °C;[3] the resolved signals corresponding the minor diastereoisomer are underlined and reported with no indication of the relative integrals) δ 8.84 (s, 1H, NC*H*=N), 8.28 (m, 1H, Ar*H*), 8.05 (d, *J* = 8.1 Hz, 1H, CON*H*), 8.01 (d, *J* = 7.7 Hz, 1H, CON*H*), 7.87 – 7.77 (m, 2H, CON*H*), 7.72 – 7.65 (m, 2H, Ar*H*), 7.65 – 7.60 (m, 1H, Ar*H*), 7.27 (m, 1H, C=C*H*N), 7.26 – 7.19 (m, 4H, Ar*H* and N*H*), 7.19 – 7.14 (m, 2H, Ar*H*), 7.13 (s, 1H, IC*H*=C), 7.06 (s, 2H, CONH_2), 4.54 – 4.42 (m, 2H, 2 x C*H*), 4.39 (td, *J* = 7.9, 5.4 Hz, 1H, C*H*), 4.18 (m, 1H, C*H*CH_3), 3.73 (t, *J* = 6.5 Hz, 2H, $CH_2CH_2N_3$), 3.30 (dd, *J* = 13.7, 5.3 Hz, 2H, CH_2), 3.18 (m, 1H, CH_2), 3.13 (dd, *J* = 9.3, 4.4 Hz, 1H, CH_2), 3.08 (t, *J* = 7.3 Hz, 2H, $CH_2CH_2N_3$), 2.97 (dd, *J* = 15.3, 8.1 Hz, 1H, C$H_{2(His)}$), 2.79 (dd, *J* = 14.1, 9.4 Hz, 1H, C$H_{2(His)}$), 1.79 (s, 3H, COCH_3), 1.18 (d, *J* = 7.1 Hz, 3H, CHCH_3).
^{13}C NMR (101 MHz, DMSO-d_6; acquired at 25 °C; the multiplets corresponding to the fluorinated aromatic carbons were not resolved) δ 173.1, 172.0, 171.8, 170.0, 169.5, 158.8, 158.4, 138.4, 135.4, 134.2, 133.8, 132.6, 131.3, 129.7, 129.5, 129.0, 128.5, 126.7, 117.3, 112.5, 54.4, 53.6, 52.1, 49.7, 48.9, 37.7, 35.7, 33.4, 27.2, 22.9, 18.2.
^{19}F NMR (376 MHz, DMSO-d_6) δ -74.1, -138.1 (d, *J* = 23.7 Hz), -150.1, -161.4 (d, *J* = 18.2 Hz).
HPLC-MS: Retention time (Method 1) = 11.4-11.6 minutes.
HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{40}H_{40}F_5IN_{11}O_8S_2^+$ 1088.1462; Found 1088.1441**.**

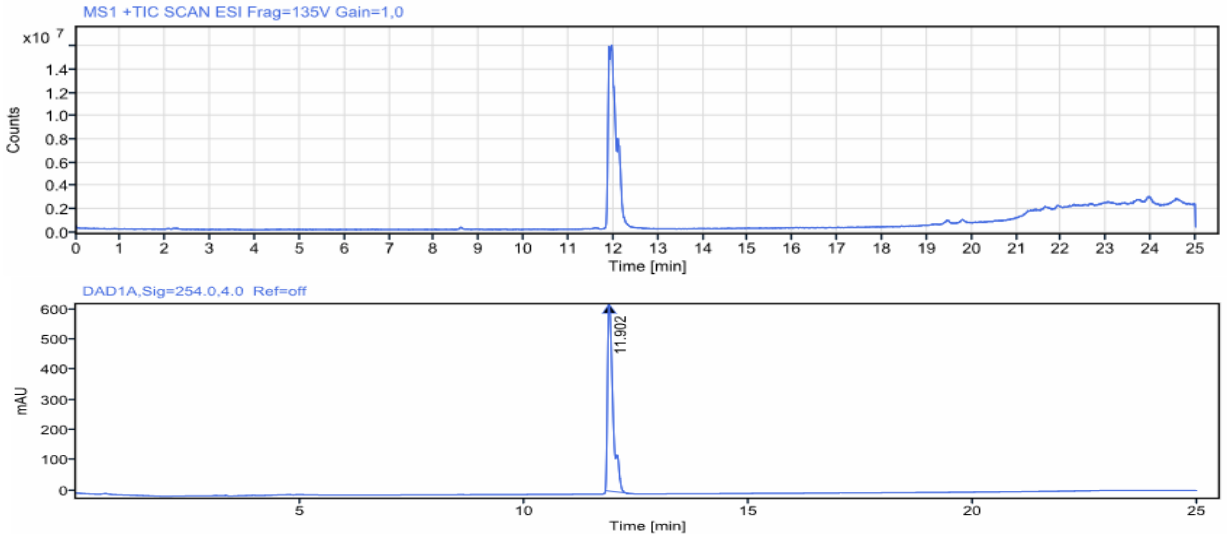
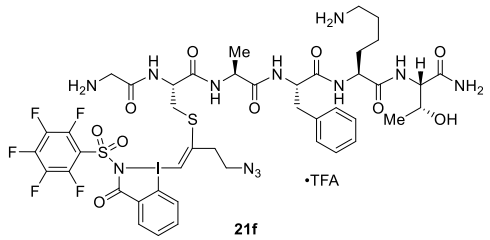


Figure S24. HPLC-MS and HPLC-UV (at 254 nm) chromatograms of pure **21e**.

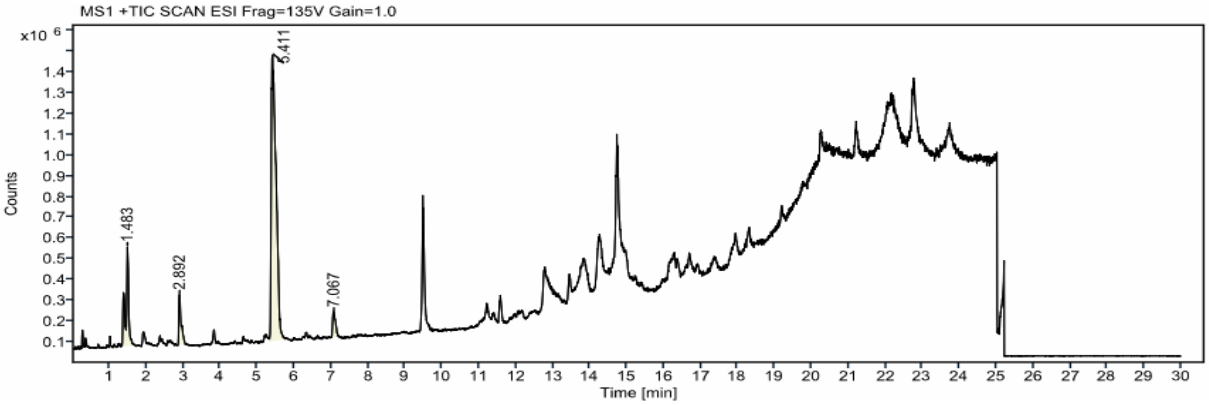
[3] Acquisition at 90 °C resulted in better resolution than at room temperature. Signal assignment was done based on the 1D and 2D spectra acquired at room temperature.

H-Gly-Cys(S-VBZ)-Ala-Phe-Lys-Thr-NH_2 21f



The title compound was prepared following the **GP1** using peptide **20f** (mixture of diastereoisomers, d.r.: 10:1; 14.8 mg, 0.020 mmol, 1.0 equiv.), **15** (14 mg, 0.026 mmol, 1.1 equiv.), 0.50 mL PB (50 mM, pH 8.0) and 0.50 mL ACN. After vigorous stirring for 20 minutes, full conversion was observed based on HPLC-MS analysis (instrument b; method 1) (Indicative HPLC-MS-based yield: ca. 70%. Figure S25).

The reaction mixture was diluted with milliQ water (up to 1.8 mL) and directly submitted to preparative HPLC (Method 3; retention time 10.0-11.0 min.). Upon lyophilization of the collected fractions, compound **21f** (mixture of atropoisomers, d.r. 10: 1; 12.9 mg, 0.00981 mmol, 49% yield) was isolated as a fluffy, white solid.



RT [min]	Signal Description	Symmetry	Resolution	Height [count]	Area [count*min]	Rel. Area [%]
1.483	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	1.32757		480666	2606625	14.3
2.892	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.37382		243694	1142070	6.3
5.411	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.40487		1387467	12832988	70.6
7.067	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.56274		124730	730260	4.0

Filtered on peak height > 100000 counts

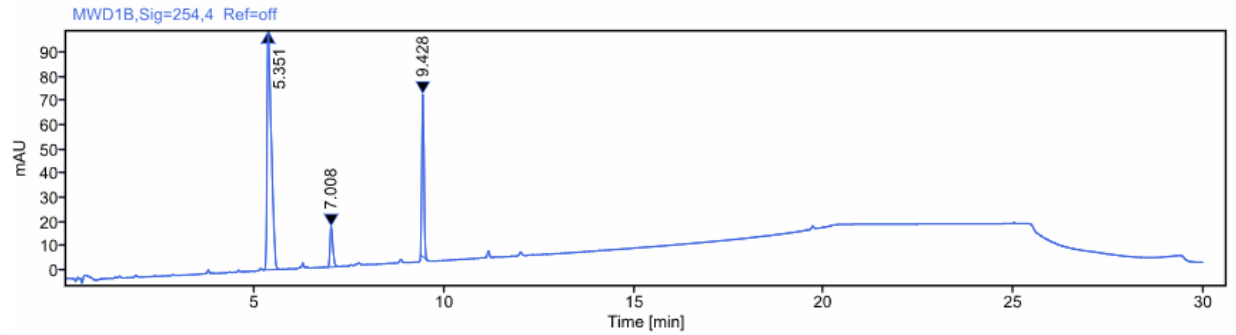
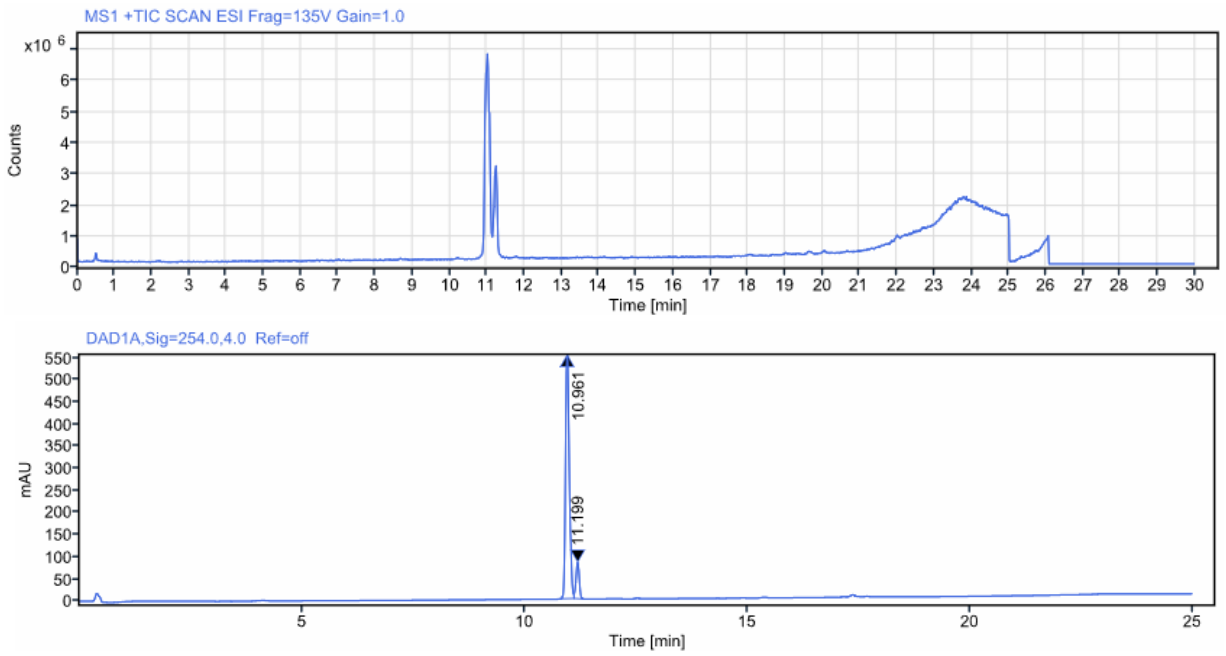


Figure S25: HPLC-MS and HPLC-UV (at 254 nm) chromatograms (instrument b) of the reaction of peptide **20f** with **15**. The compound eluted at ca. 9.5 minutes is the residual unreacted **15**.

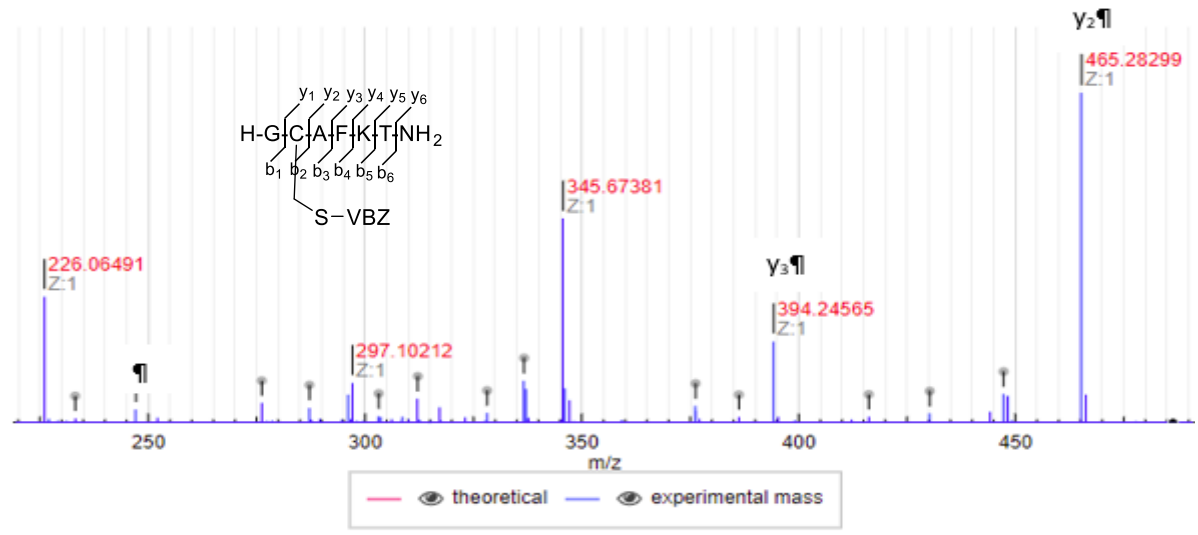
HPLC-MS: Retention time (Method 1) = 11.4-11.6 minutes.
HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{44}H_{53}F_5IN_{12}O_{10}S_2^+$ 1195.2408; Found 1195.2438. MS-MS analysis shown in Figure S12.



Signal: DAD1A,Sig=254.0,4.0 Ref=off

RT [min]	Type	Width [min]	Area	Height	Area%	Name
10.961	MM m	0.2893	3191.8317	552.6301	88.3459	
11.199	MM m	0.2459	421.0481	82.2334	11.6541	

Figure S25. HPLC-MS and HPLC-UV (at 254 nm) chromatograms of pure **21f**.



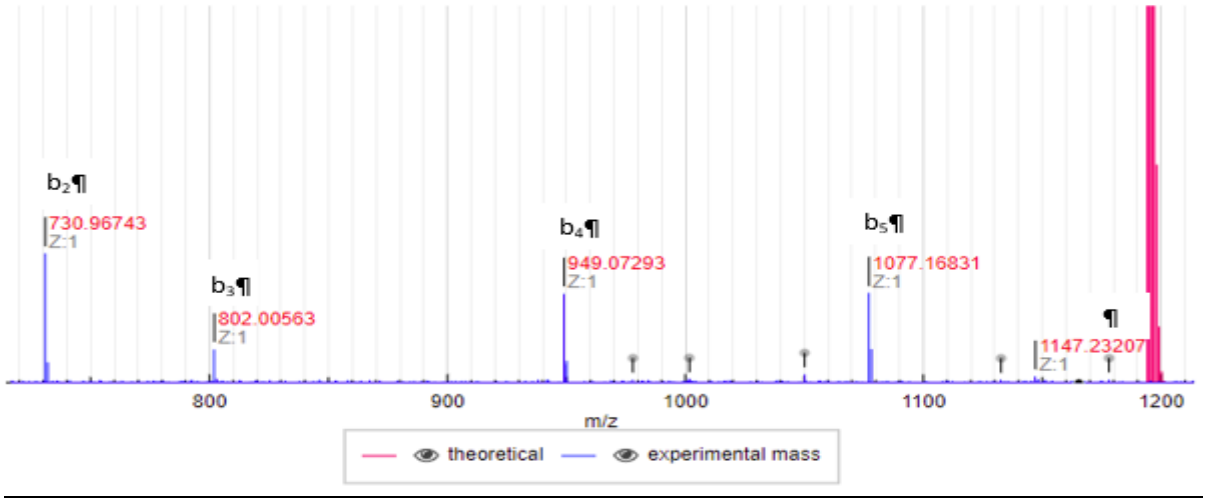
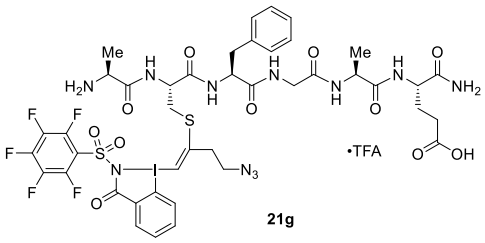


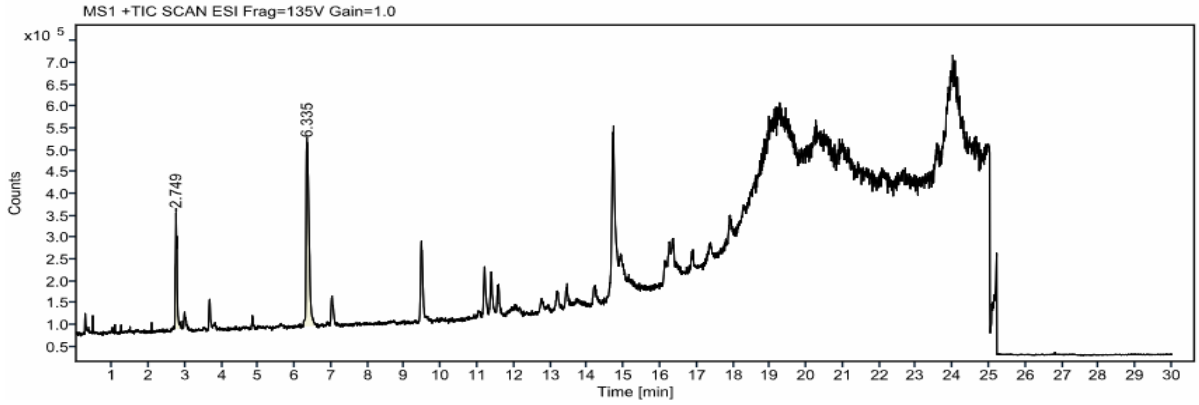
Figure S26. LC-MS/MS of **21f**.

H-Ala-Cys(S-VBZ)-Phe-Gly-Ala-Glu-NH_2 21g



The title compound was prepared following the **GP1** using peptide **20g** (14.2 mg, 0.020 mmol, 1.0 equiv.), **15** (14 mg, 0.026 mmol, 1.1 equiv.), 0.50 mL PB (50 mM, pH 8.0) and 0.50 mL ACN. After vigorous stirring for 20 minutes, full conversion was observed based on HPLC-MS analysis (instrument b; method 1). (Indicative HPLC-MS-based yield: ca. 61%. Figure S27).

The reaction mixture was diluted with milliQ water (up to 1.8 mL) and directly submitted to preparative HPLC (Method 3; retention time 12.0-12.5 min.). Upon lyophilization of the collected fractions, compound **21g** (15.6 mg, 0.0122 mmol, 61% yield) was isolated as a fluffy, white solid.



RT [min]	Signal Description	Symmetry	Resolution	Height [count]	Area [count*min]	Rel. Area [%]
2.749	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.54891		266909	827692	20.4
6.335	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.55006		423476	2463908	60.8

Filtered on peak height > 100000 counts

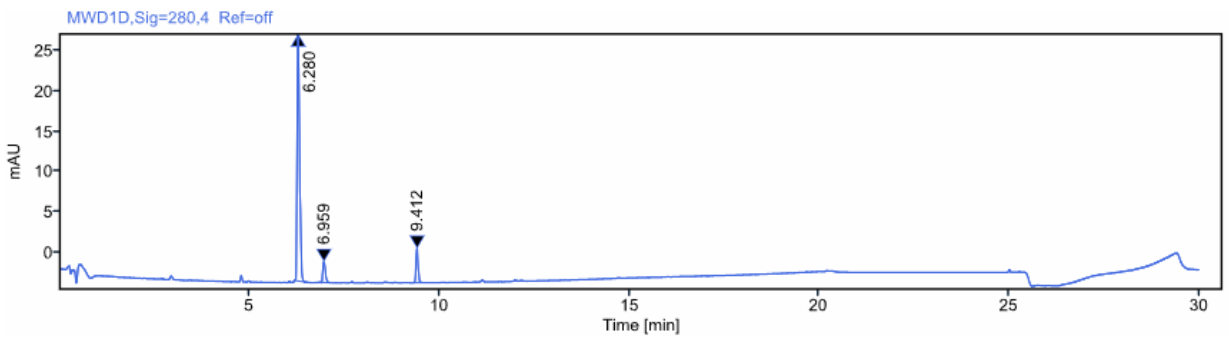


Figure S27: HPLC-MS and HPLC-UV (at 280 nm) chromatograms of the reaction of peptide **20g** with **15**. The compound eluted at 2.7 minutes is the oxidized derivative of the st. material (S-S disulfide dimer). The compound eluted at ca. 7.0 minutes is non-identified compound, likely of peptidic origin. The compound eluted at ca. 9.5 minutes is the residual unreacted **15**.

HPLC-MS: Retention time (Instrument b; method 1) = 6.3 minutes.
HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{42}H_{46}F_5IN_{11}O_{11}S_2^+$ 1166.1779; Found 1166.1814.

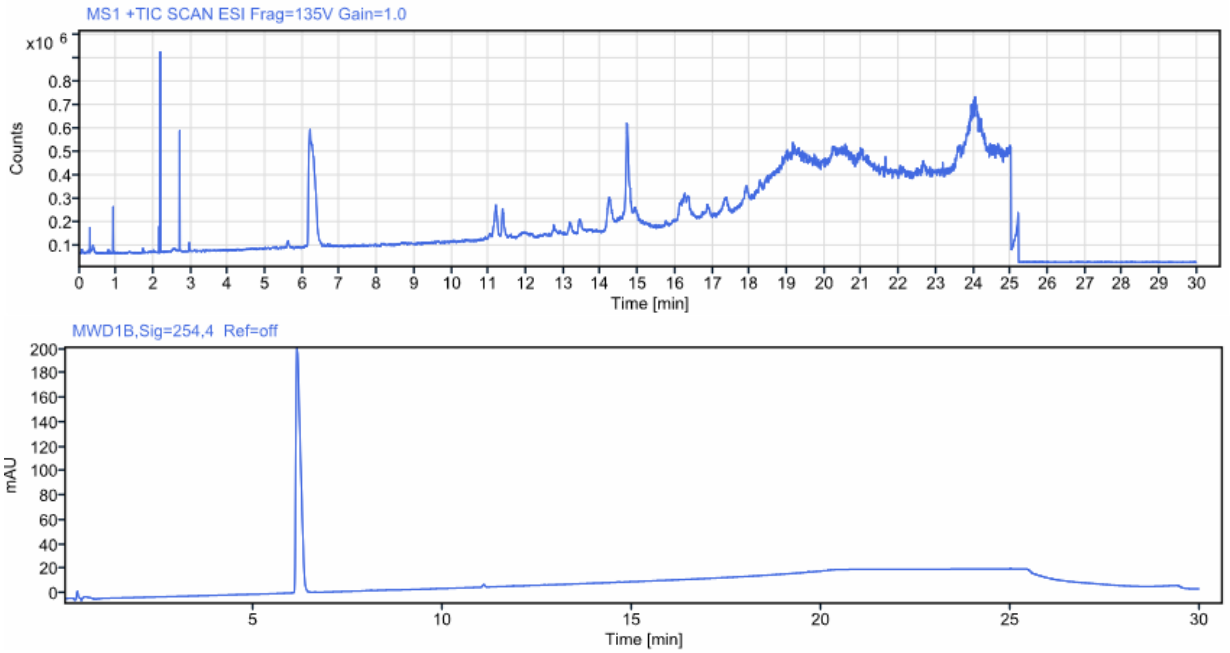
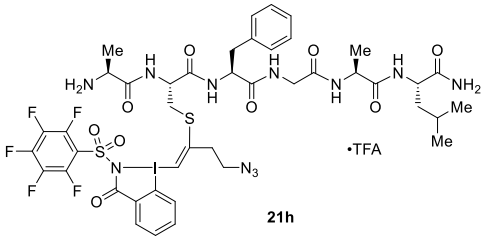


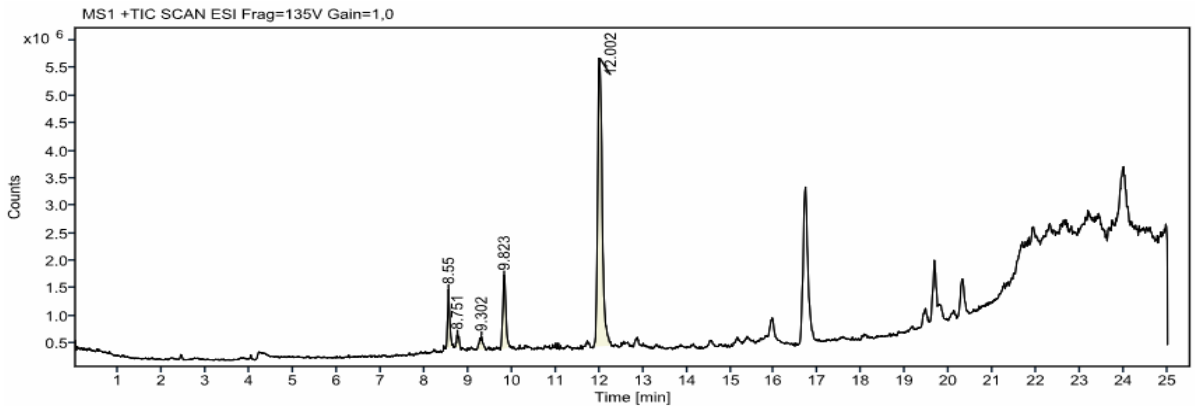
Figure S28. HPLC-MS and HPLC-UV (at 254 nm) chromatograms of pure **21g**.

H-Ala-Cys(S-VBZ)-Phe-Gly-Ala-Leu-H_2 (21h)



The title compound was prepared following the **GP1** using peptide **20h** (12.5 mg, 0.018 mmol, 1.0 equiv.), **15** (12.6 mg, 0.022 mmol, 1.2 equiv.), 0.50 mL PB (50 mM, pH 8.0) and 0.50 mL ACN. After vigorous stirring for 20 minutes, full conversion was observed based on HPLC-MS analysis (method 1). (Indicative HPLC-MS-based yield: ca. 75%. Figure S29).

The reaction mixture was diluted with milliQ water (up to 1.8 mL) and directly submitted to preparative HPLC (Method 4; retention time 14.5-15.2 min.). Upon lyophilization of the collected fractions, compound **21h** (9.4 mg, 0.0074 mmol, 41% yield) was isolated as a fluffy solid.



RT [min]	Signal Description	Symmetry	Resolution	Height [count]	Area [count*min]	Rel. Area [%]
8.550	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.74542		1071483	3486125	7.1
8.751	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.51087		238977	870881	1.8
9.302	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	1.24847		253353	1498819	3.1
9.823	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.81537		1340164	6375976	13.0
12.002	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.58905		5247272	36740741	75.0

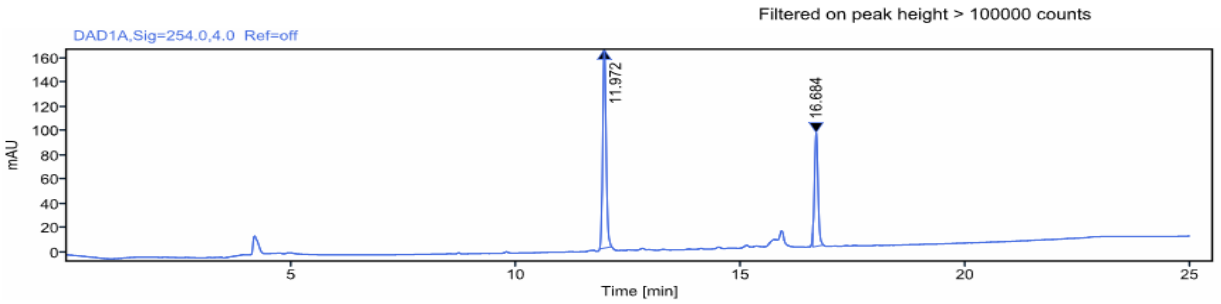


Figure S29: HPLC-MS and HPLC-UV (at 254 nm) chromatograms of the reaction of peptide **20h** with **15**. The compound eluted at ca. 8.5 minutes is the oxidized derivative of the st. material (S-S disulfide dimer). The compound eluted at ca. 9.8 minutes is a non-identified peptide derivative. The compound eluted at ca. 16.7 minutes is the residual unreacted **15**.

HPLC-MS: Retention time (Method 1) = 11.9 minutes.
HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{43}H_{50}F_5IN_{11}O_9S_2^+$ 1150.2194; Found 1150.2220.

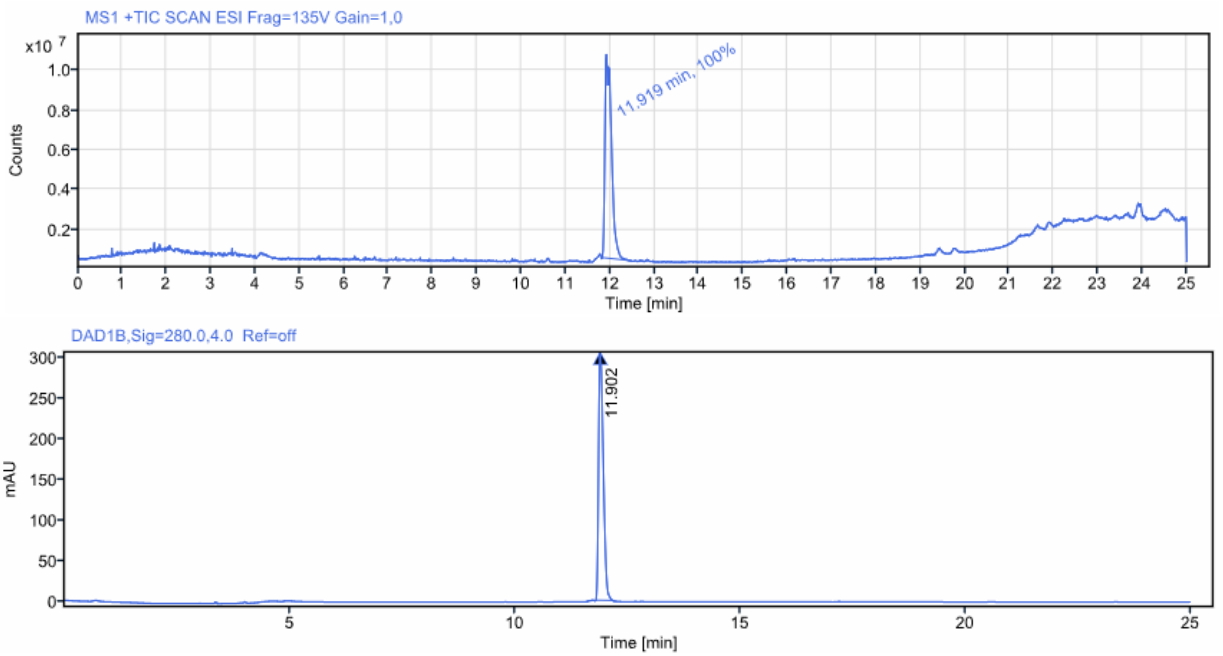
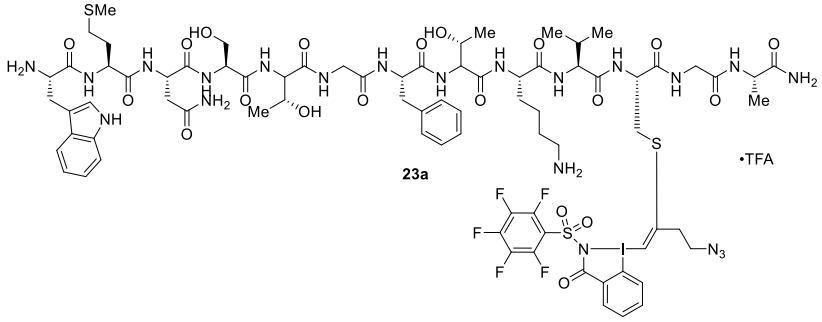


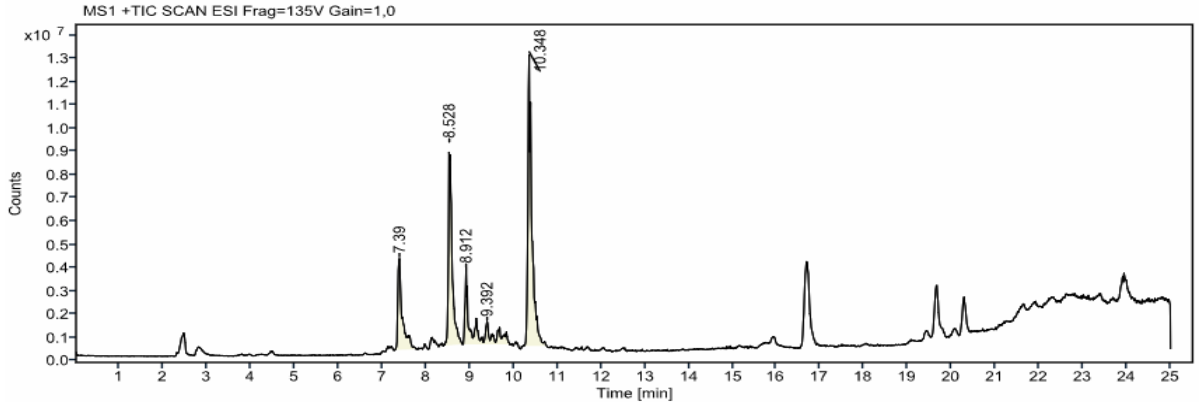
Figure S30. HPLC-MS and HPLC-UV (at 280 nm) chromatograms of pure **21h**.

Fragment of HCV: H-Trp-Met-Asn-Ser-Thr-Gly-Phe-Thr-Lys-Val-Cys(S-VBZ)-Gly-Ala-NH_2 (**23a**)



The title compound was prepared following the **GP2** using peptide **22a** (6.1 mg, 0.0040 mmol, 1.0 equiv.), **15** (4.7 mg, 0.0080 mmol, 2.0 equiv.), PB (Solution A: 50 mM, pH = 8.0; 0.53 mL), EDTA (Solution B: 6.7 mM in solution A; 0.27 mL), and 0.80 mL DMF. After vigorous stirring for 40 minutes, full conversion was observed based on HPLC-MS analysis (method 1) (Indicative HPLC-MS-based yield: ca. 41%. Figure S31).

The reaction mixture was diluted with milliQ water (up to 1.8 mL) and directly submitted to preparative HPLC (Method 3; retention time 13.3-13.8 min.). Upon lyophilization of the collected fractions, compound **23a** (3.1 mg, 0.0014 mmol, 37% yield) was isolated as a fluffy, white solid.



RT [min]	Signal Description	Symmetry	Resolution	Height [count]	Area [count*min]	Rel. Area [%]
7.390	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.40626		3984294	27717103	14.5
8.528	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.32383		8735473	50207761	26.3
8.912	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.26160		3296426	20229025	10.6
9.392	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.16032		1003878	13476387	7.1
10.348	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.31868		12706531	78918023	41.4

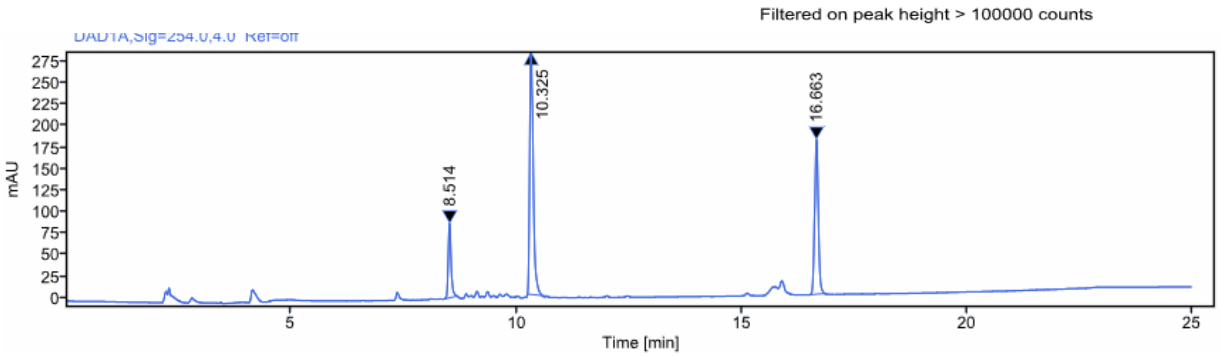


Figure S31: HPLC-MS and HPLC-UV (at 254 nm) chromatograms of the reaction of peptide **22a** with **15**. The compound eluted at ca. 7.4 minutes is the oxidized derivative of the st. material (S-S disulfide dimer). The compound eluted at ca. 8.5 minutes is a non-identified peptide derivative. The compound eluted at ca. 16.6 minutes is the residual unreacted **15**.

HPLC-MS: Retention time (Method 1) = 10.3 minutes.
HRMS (ESI/QTOF) m/z: $[M + 2H]^{+2}$ Calcd for $C_{78}H_{103}F_5IN_{21}O_{20}S_3^{+2}$ 985.7902; Found 985.7907.

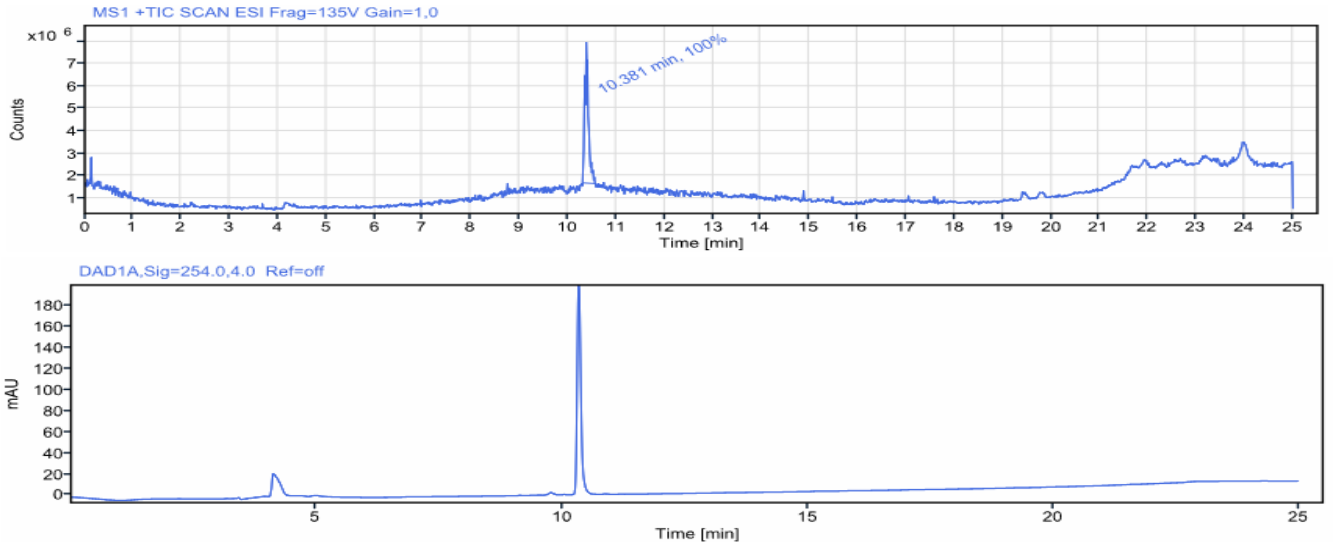
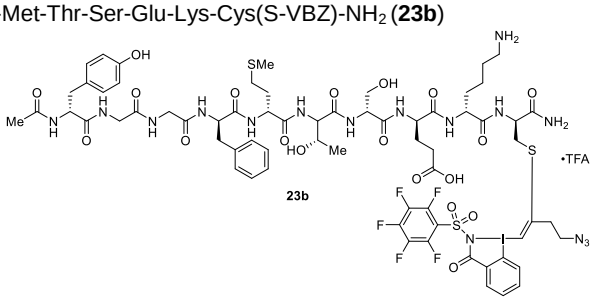


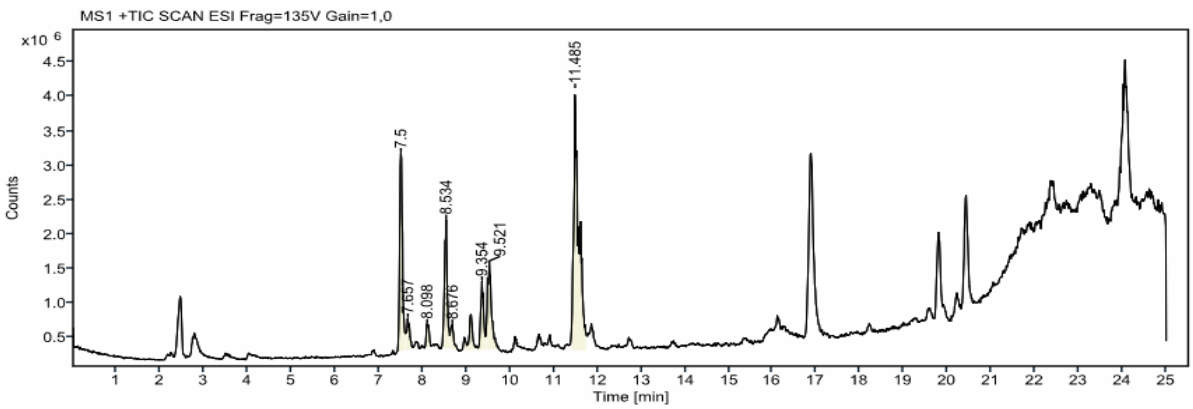
Figure S32. HPLC-MS and HPLC-UV (at 254 nm) chromatograms of pure **23a**.

Endorphin-C_{10}: Ac-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Cys(S-VBZ)-NH_2 (**23b**)



The title compound was prepared following the **GP2** using peptide **22b** (5.7 mg, 0.0040 mmol, 1.0 equiv.), **15** (4.7 mg, 0.0080 mmol, 2.0 equiv.), PB (Solution A: 50 mM, pH = 8.0; 0.53 mL), EDTA (Solution B: 6.7 mM in solution A; 0.27 mL), and 0.80 mL DMF. After vigorous stirring for 40 minutes, residual starting material was still present based on HPLC-MS analysis (method 1), but no increase of the coversion could be observed after a longer reaction time (80 minutes) (Indicative HPLC-MS-based yield: ca. 41%; Figure S33).

The reaction mixture was diluted with milliQ water (up to 1.8 mL) and directly submitted to preparative HPLC (Method 3; retention time 13.9-14.2 min.). Upon lyophilization of the collected fractions, compound **23b** (3.4 mg, 0.0016 mmol, 41% yield) was isolated as a fluffy, white solid.



RT [min]	Signal Description	Symmetry	Resolution	Height [count]	Area [count*min]	Rel. Area [%]
7.500	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.74276		2884002	13328201	17.9
7.657	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.89274		461177	2409344	3.2
8.098	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.45263		342567	1437513	1.9
8.534	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	1.16779		1878171	8214507	11.0
8.676	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	1.34355		362066	1857540	2.5
9.354	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	2.33707		1007130	8207921	11.0
9.521	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.77601		1291952	8425185	11.3
11.485	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.38757		3808974	30541252	41.0

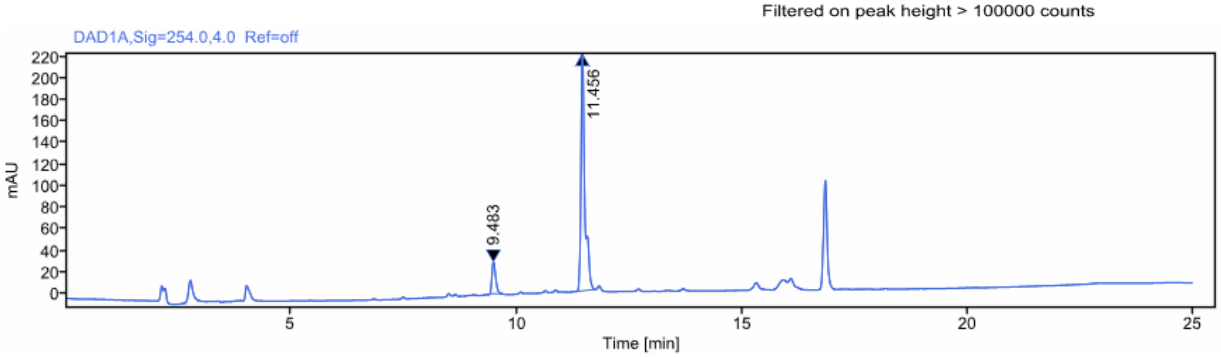


Figure S33: HPLC-MS and HPLC-UV (at 254 nm) chromatograms of the reaction of peptide **22b** with **15**. The compound eluted at ca. 7.5 minutes is the residual unreacted st. material. The compounds eluted at ca. 8.5 and 9.5 minutes are non-identified peptide derivatives. The compound eluted at ca. 17.0 minutes is the residual unreacted **15**.

HPLC-MS: Retention time (Method 1) = 11.6 minutes.
HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + 2H]^{+2}$ Calcd for $C_{67}H_{84}F_5IN_{16}O_{19}S_3^{+2}$ 867.2107; Found 867.2104.

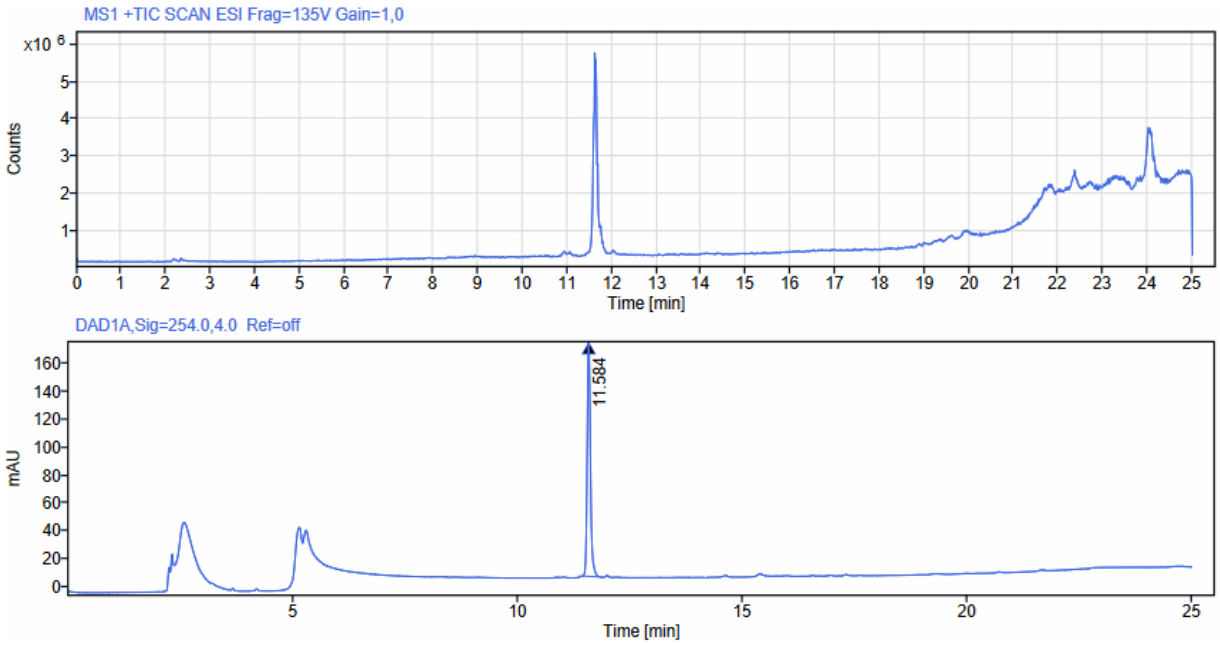
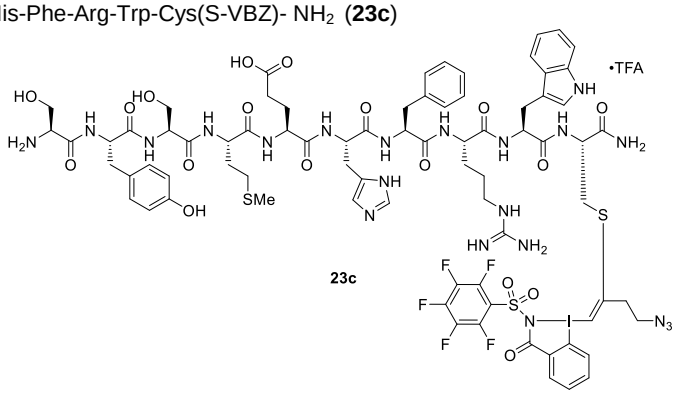


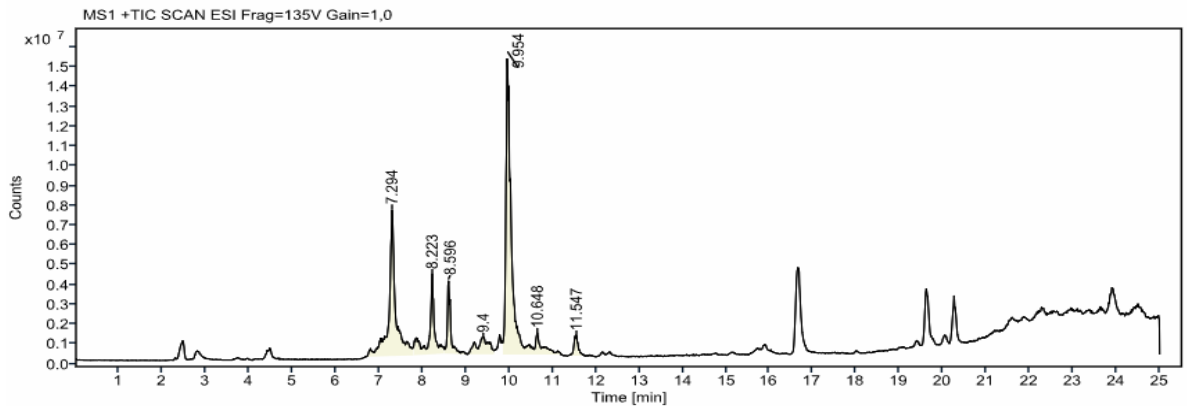
Figure S34. HPLC-MS and HPLC-UV (at 254 nm) chromatograms of pure **23b**.

ACTH: H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Cys(S-VBZ)- NH_2 (**23c**)



The title compound was prepared following the **GP2** using peptide **22c** (6.0 mg, 0.0040 mmol, 1.0 equiv.), **15** (4.7 mg, 0.0080 mmol, 2.0 equiv.), PB (Solution A: 50 mM, pH = 8.0; 0.53 mL), EDTA (Solution B: 6.7 mM in solution A; 0.27 mL), and 0.80 mL DMF. After vigorous stirring for 40 minutes, full conversion was observed based on HPLC-MS analysis (method 1) (HPLC-MS-based yield: ca. 41%. Figure S35).

The reaction mixture was diluted with milliQ water (up to 1.8 mL) and directly submitted to preparative HPLC (Method 3; retention time 13.3-13.8 min.). Upon lyophilization of the collected fractions, compound **23c** (ca. 95% pure; 3.3 mg, 0.0016 mmol, 41% yield) was isolated as a fluffy, white solid.



RT [min]	Signal Description	Symmetry	Resolution	Height [count]	Area [count*min]	Rel. Area [%]
7.294	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.84497		7444794	80740131	26.7
8.223	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	1.46074		4082032	34943445	11.6
8.596	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.45055		3753547	19821982	6.6
9.400	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	1.04879		877794	16841809	5.6
9.954	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.37226		15333997	124000438	41.0
10.648	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.76607		1144256	18346972	6.1
11.547	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	1.03714		1053267	7476611	2.5

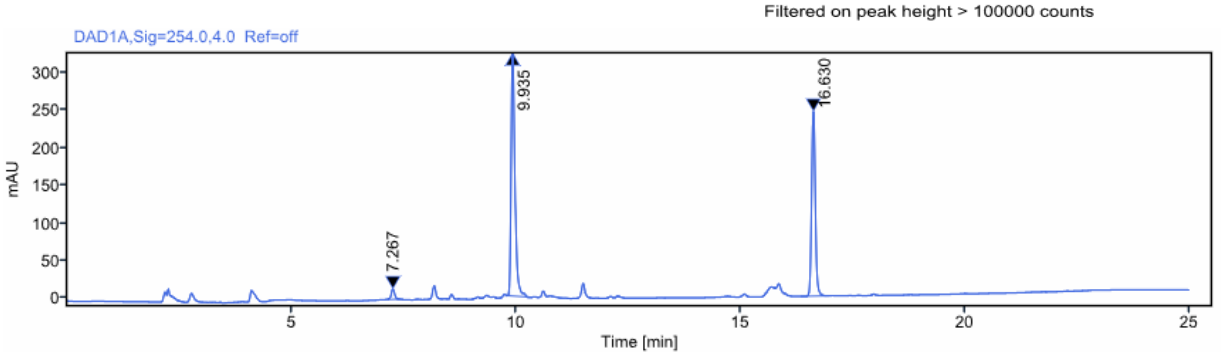


Figure S35: HPLC-MS and HPLC-UV (at 254 nm) chromatograms of the reaction of peptide **22c** with **15**. The compound eluted at ca. 8.5 minutes is the oxidized derivative of the st. material (S-S disulfide dimer). The compounds eluted between 8.0 and 9.0 minutes are non-identified peptide derivatives. The compound eluted at ca. 16.6 minutes is the residual unreacted **15**.

HPLC-MS: Retention time (Method 1) = 9.9 minutes.
HRMS (Nanochip-based ESI/LTQ-Orbitrap) m/z: $[M + 3H]^{+3}$ Calcd for $C_{77}H_{91}F_5IN_{20}O_{19}S_3^{+3}$ 639.1627; Found 638.8352.

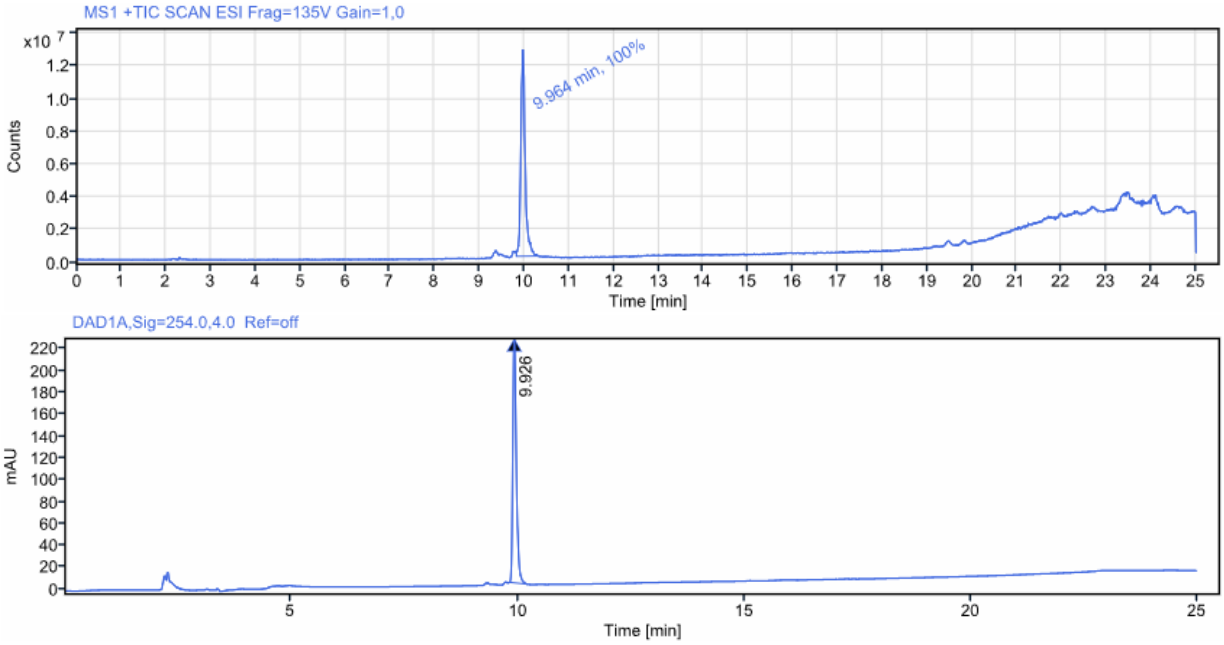
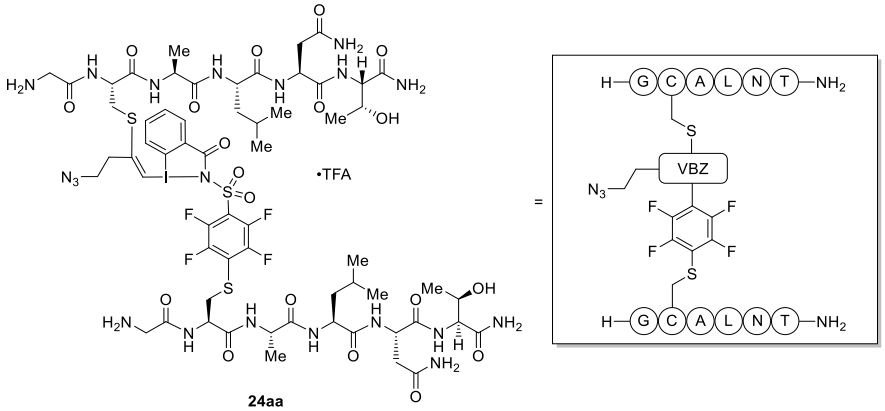


Figure S36. HPLC-MS and HPLC-UV (at 254 nm) chromatograms of pure **23c**.

3.4 Sequential Homo- and Cross-conjugation of two peptides using JW-AM-005

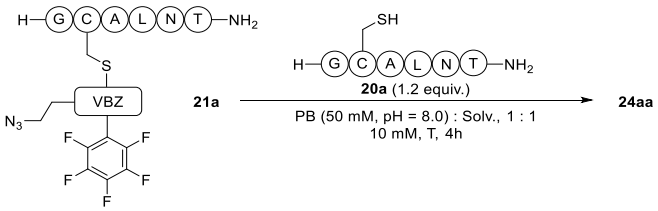
3.4.1 Homo-conjugation experiments

Homoconjugation of H-Ala-Cy-Gly-Leu-Asn-Thr-NH_2 (20a) to give 24aa



Step-wise protocol:

Premise: The homoconjugation of model peptide **20a** was investigated. Initial attempts to perform the reaction at a 20 mM overall concentration were unsuccessful using either DMF or ACN as the organic solvent, due to the low or very low solubility of **20a** and **21a** in both solvents. (Partial) dissolution of **20a** and **21a** was observed at a 10 mM overall concentration, at which the experiments were viable.



A 2.0 mL Eppendorf vial was charged with H-Ala-Cy-Gly-Leu-Asn-Thr-NH_2 S-VBZ derivative **21a** (5.1 mg, 0.0040 mmol, 1.0 equiv.) and an excess of the peptide **20a** (3.2 mg, 0.0047 mmol, 1.2 equiv.). PB (50 mM, pH = 8.0; 0.20 mL) and the organic solvent (0.20 mL) were added. The mixture was vortexed until the complete dissolution of the solids, and it was then shaken at 37 °C for the indicated time. Once no further conversion was observed according to HPLC-MS analysis, the crude mixture was then diluted with milliQ water up to a volume of 1.8 mL, and submitted to preparative HPLC (Method 3; retention time of **24aa**: 12.0-12.5 minutes). The collected fractions were then lyophilized to provide homodimeric conjugate product **24aa** as a fluffy, white solid.

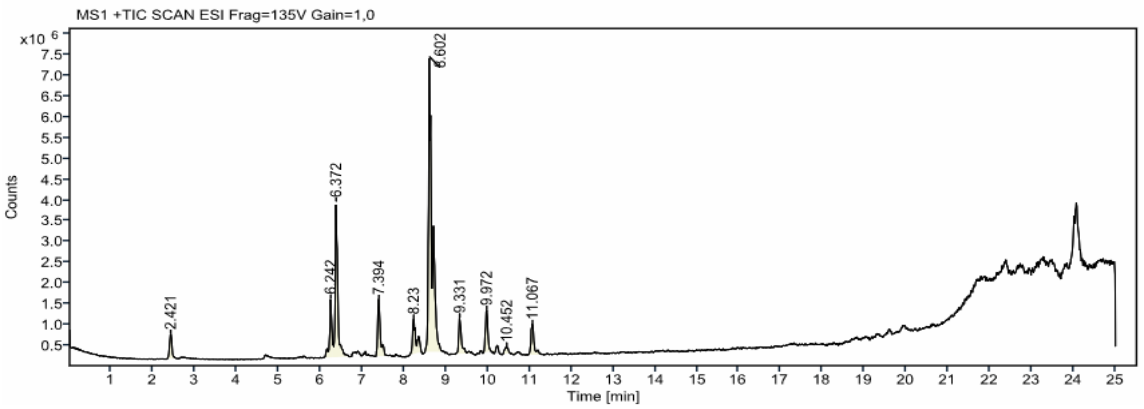
Results:

a) In DMF at room temperature (Figure S37):

After 5+16 hours: Ratio (**24aa** : st. material **21a**) > 95 : 5

The reaction mixture was shaken at room temperature for overall 5.0 hours. Indicative HPLC-MS-based yield: ca. 46%.

Upon preparative HPLC, 3.9 mg of **24aa** were isolated (0.0022 mmol, 55% yield).



RT [min]	Signal Description	Symmetry	Resolution	Height [count]	Area [count*min]	Rel. Area [%]
2.421	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.92228		592503	2417268	2.8
6.242	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.99593		1415228	5249934	6.2
6.372	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.48039		3741415	14052357	16.5
7.394	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.48513		1373363	6056910	7.1
8.230	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.40055		843718	5346595	6.3
8.602	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.28321		7114077	39487486	46.4
9.331	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.53895		874692	3986324	4.7
9.972	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	1.19452		1013424	3648233	4.3
10.452	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	2.24981		169469	719372	0.8
11.067	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.73576		743493	4123393	4.8

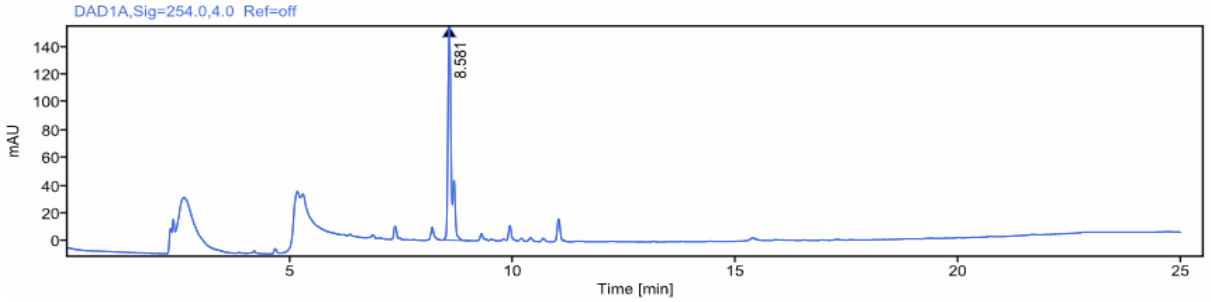


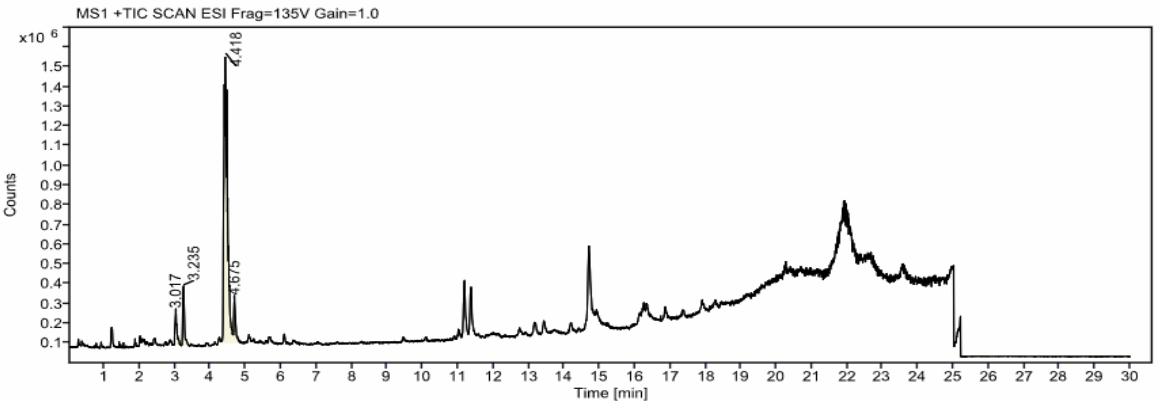
Figure S37: HPLC-MS and HPLC-UV (at 254 nm) chromatograms of the homoconjugation reaction following the step-wise protocol in PB/DMF at room temperature, after 5 hours.

b) In DMF at 37 °C (Figure S38):

After 3 hours: Ratio (**24aa** : st. material **21a**) > 95 : 5.

The reaction mixture was shaken at 37 °C for overall 3.5 hours. Indicative HPLC-MS-based yield: ca. 78%.

Upon preparative HPLC, 5.4 mg of **2aa** were isolated (0.0027 mmol, 69% yield).



RT [min]	Signal Description	Symmetry	Resolution	Height [count]	Area [count*min]	Rel. Area [%]
3.017	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.59217		162033	598051	4.8
3.235	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.68472		307206	1029309	8.3
4.418	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.54685		1474694	9757529	78.5
4.675	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.48434		222096	800727	6.4

Filtered on peak height > 100000 counts

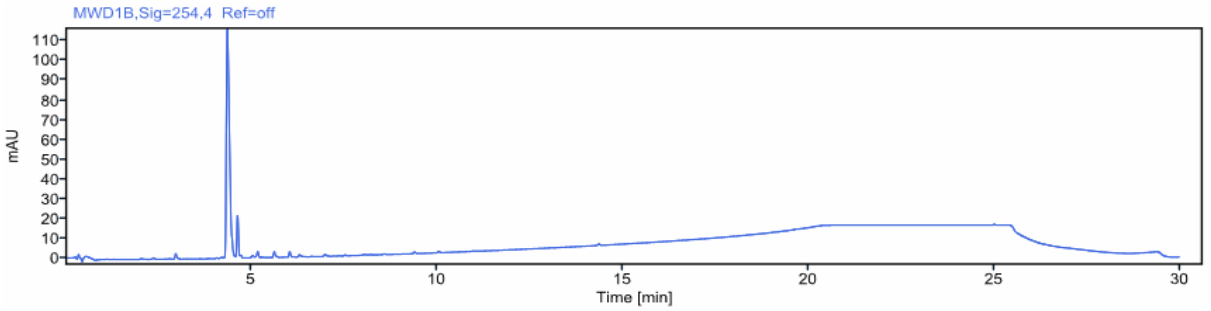
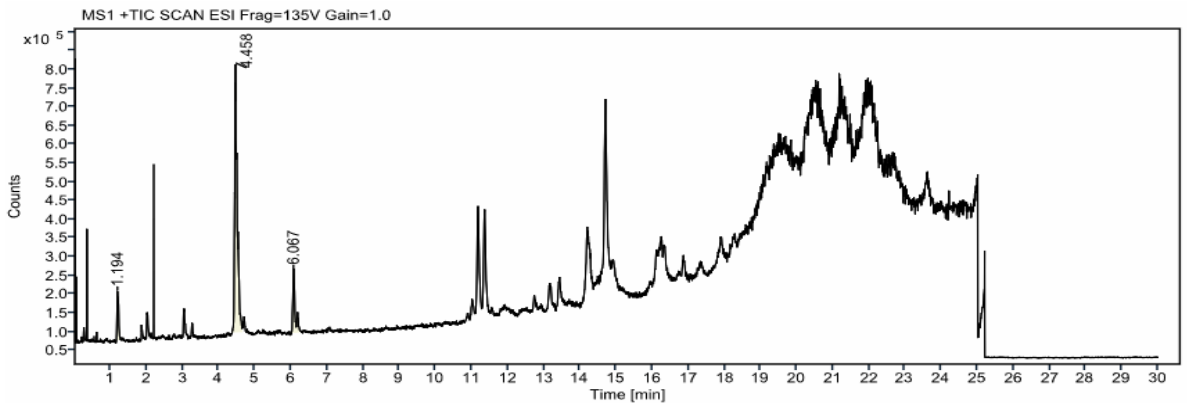


Figure S38: HPLC-MS and HPLC-UV (at 254 nm) chromatograms of the homoconjugation reaction following the step-wise protocol in PB/DMF, after 3 hours.

c) In ACN at 37 °C (Figure S39):

After 3 hours: Ratio (**24aa** : st. material **21a**) = 80 : 20
After 5 hours: Ratio (**24aa** : st. material **21a**) = 80 : 20
The reaction mixture was shaken at 37 °C for overall 5 hours. Indicative HPLC-MS-based yield: ca. 66%.

Upon preparative HPLC, 3.9 mg of **24aa** were isolated (0.0020 mmol, 50% yield).



RT [min]	Signal Description	Symmetry	Resolution	Height [count]	Area [count*min]	Rel. Area [%]
1.194	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.49222		132862	362715	6.9
4.458	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.48690		720556	3496466	66.5
6.067	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.41610		170955	746092	14.2

Filtered on peak height > 100000 counts

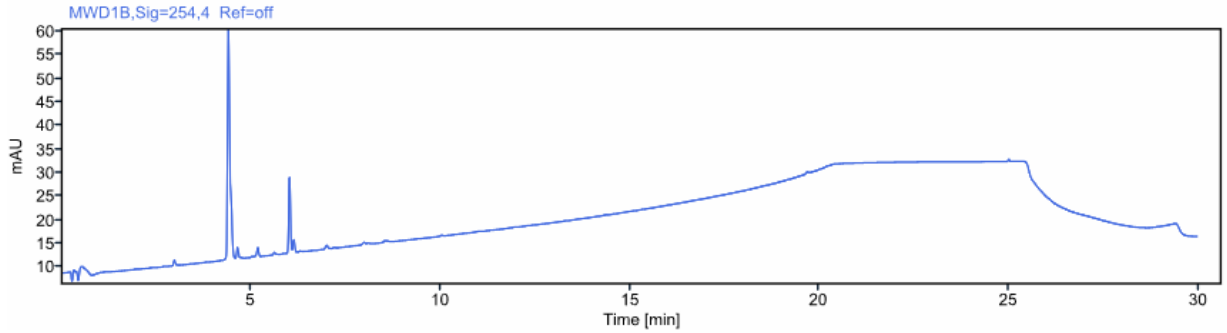
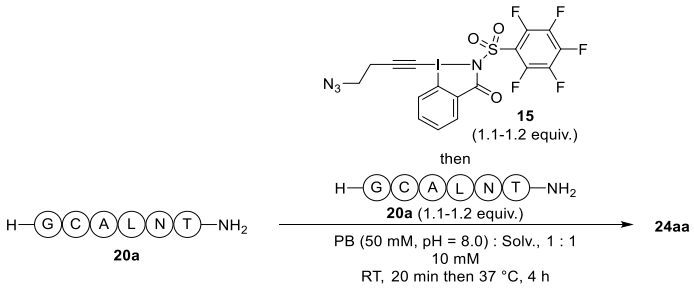


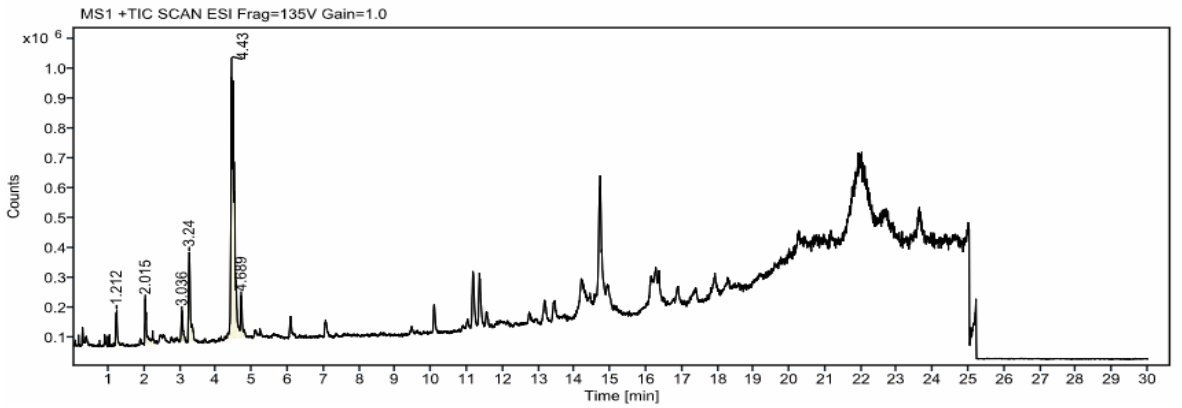
Figure S39: HPLC-MS and HPLC-UV (at 254 nm) chromatograms of the homoconjugation reaction following the step-wise protocol in PB/DMF, after 5 hours.

One-Pot Protocol 1:



A 2.0 mL Eppendorf vial was charged with peptide H-Ala-Cy-Gly-Leu-Asn-Thr-NH_2 (**20a**) (2.8 mg, 0.0040 mmol, 1.0 equiv.) and buffer PB (50 mM, pH = 8.0; 0.20 mL). To the resulting colorless solution was added a pale yellow solution of **15** (2.7 mg, 0.0048 mmol, 1.2 equiv.) in DMF (0.20 mL). The mixture was shaken at room temperature for 20 minutes. It was then transferred into a second 4.0 mL Eppendorf vial containing a second portion of peptide **20a** (3.0 mg, 0.0044 mmol, 1.1 equiv.). The resulting solution was shaken at 37 °C for 4 hours (Indicative HPLC-MS-based yield: 55%. Figure S40).

It was then diluted up to a volume of 1.8 mL by addition of water, and submitted to preparative HPLC (Method 3). Upon lyophilization of the collected fraction, homodimeric conjugate product **24aa** (3.8 mg, 0.0020 mmol, 51% yield).was collected as a fluffy, white solid



RT [min]	Signal Description	Symmetry	Resolution	Height [count]	Area [count*min]	Rel. Area [%]
1.212	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.85289		120462	350290	3.6
2.015	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.26616		155094	614839	6.4
3.036	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.55661		103604	343853	3.6
3.240	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.50150		302406	1136018	11.8
4.430	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.36715		946268	5366358	55.6
4.689	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.87831		142802	646661	6.7

Filtered on peak height > 100000 counts

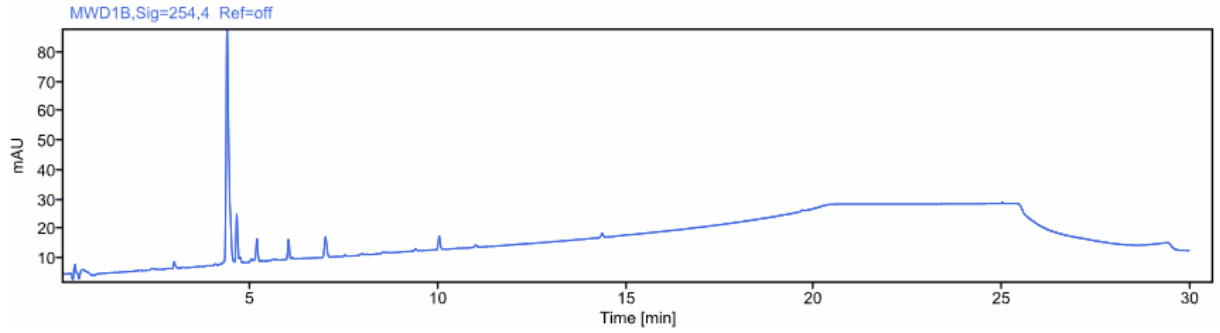
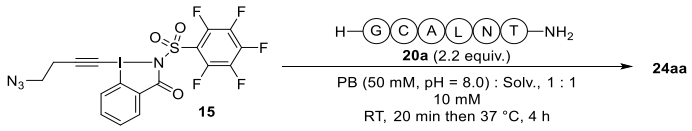


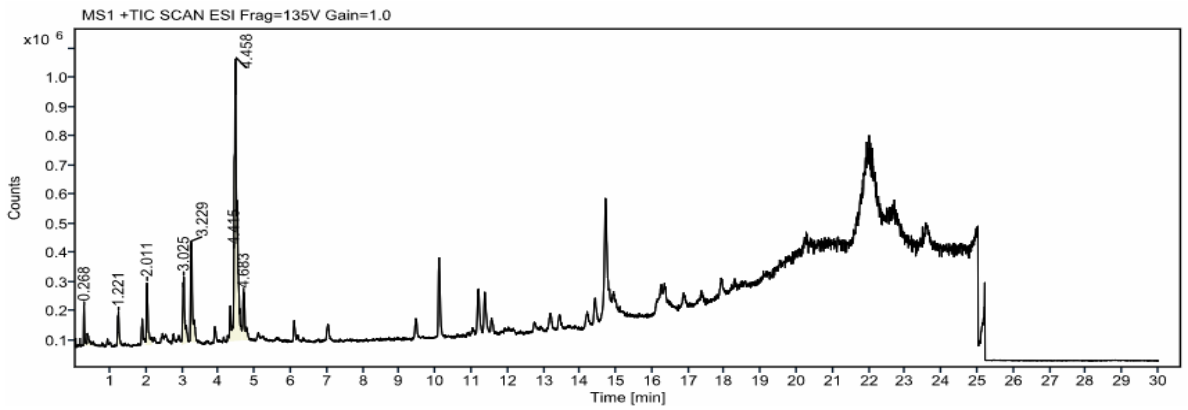
Figure S40: HPLC-MS and HPLC-UV (at 254 nm) chromatograms of the homoconjugation reaction following the one-pot protocol 1, after 4 hours.

One-Pot Protocol 2:



A 2.0 mL Eppendorf vial was charged with peptide H-Ala-Cy-Gly-Leu-Asn-Thr-NH_2 (**20a**) (5.0 mg, 0.0072 mmol, 2.2 equiv.) and buffer PB (50 mM, pH = 8.0; 0.17 mL). To the resulting colorless solution was added a pale yellow solution of **15** (1.9 mg, 0.0032 mmol, 1.0 equiv.) in DMF (0.170 mL). The mixture was shaken at room temperature for 20 minutes and then at 37 °C for 4 hours (Indicative HPLC-MS based yield: 38%. Figure S41).

It was then diluted up to a volume of 1.8 mL by addition of water and submitted to preparative HPLC purification (Method 3). Upon lyophilization of the collected fraction, homodimeric conjugate product **24aa** (2.5 mg, 0.0012 mmol, 40% yield), was collected as a fluffy, white solid.



RT [min]	Signal Description	Symmetry	Resolution	Height [count]	Area [count*min]	Rel. Area [%]
0.268	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.20574		138263	459055	3.8
1.221	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	1.21592		116492	317401	2.6
2.011	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.69296		219482	798622	6.6
3.025	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.83785		235508	906183	7.5
3.229	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.37231		346820	1413434	11.8
4.415	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	7.04340		317926	772089	6.4
4.458	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.62656		974999	4558930	37.9
4.683	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.53974		165847	771896	6.4

Filtered on peak height > 100000 counts

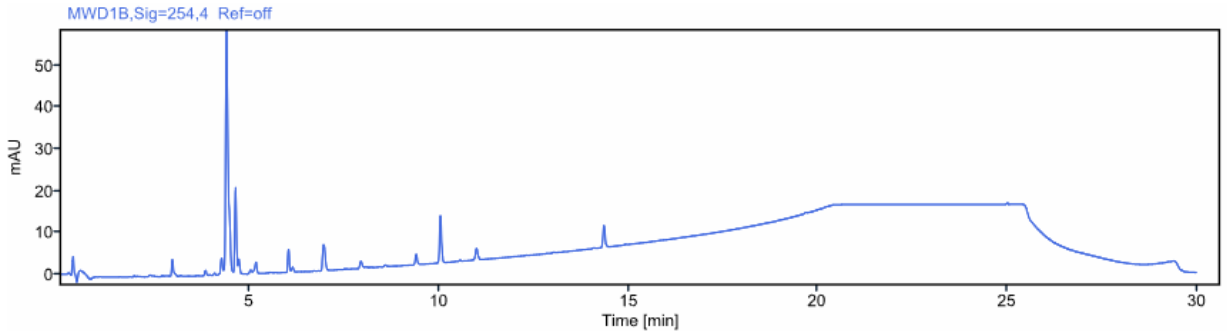


Figure S41: HPLC-MS and HPLC-UV (at 254 nm) chromatograms of the homoconjugation reaction following the one-pot protocol 2, after 4 hours.

HPLC-MS: Retention time (Instrument b, method 1) = 4.4-4.5 minutes
HRMS (ESI/QTOF) m/z: $[M]^{+2}$ Calcd for $C_{61}H_{89}F_4IN_{20}O_{19}S_3^{+2}$ 852.2372; Found 852.2365.

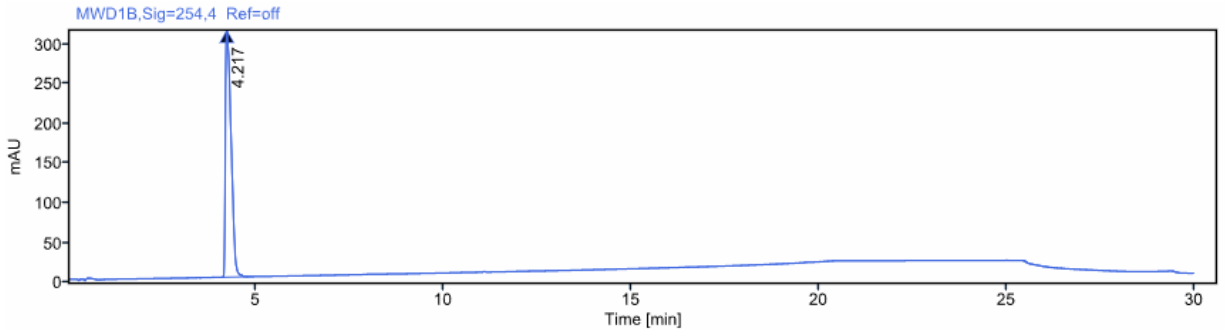
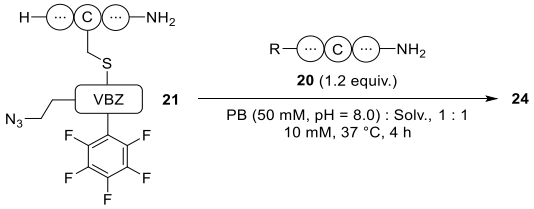


Figure S42. HPLC-MS and HPLC-UV (at 254 nm) chromatograms of pure **22aa**.

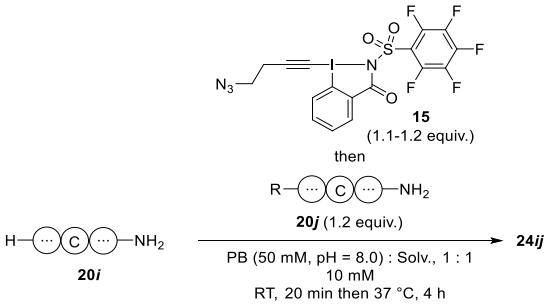
3.4.2 Cross-conjugation experiments

Step-wise protocol: General procedure (GP2)



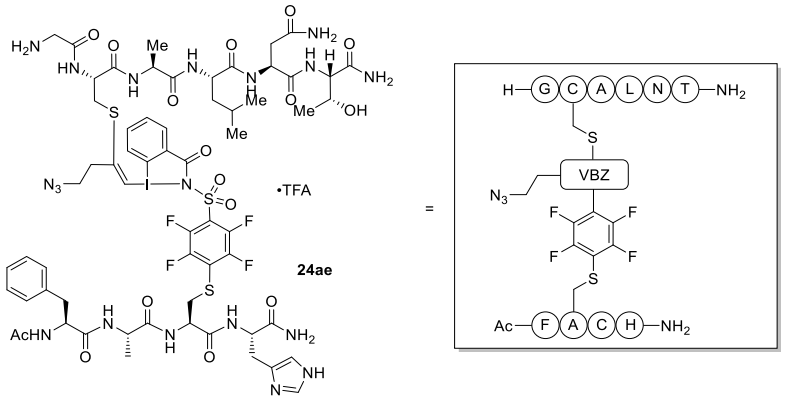
A 2.0 mL Eppendorf vial was charged with peptide S-VBZ derivative **21** (0.0040 mmol, 1.0 equiv.) and an excess of the partner peptide (ca. 0.0044 mmol, 1.1 equiv.). PB (50 mM, pH = 8.0; 0.20 mL) and DMF (0.20 mL) were then added. The mixture was vortexed until the complete dissolution of the solids, and it was then shaken at 37 °C for 4 hours. The mixture was then allowed to cool down to room temperature, diluted with water (0.7 mL) and ACN (0.7 mL) and directly submitted to preparative HPLC.

One-pot protocol: General procedure (GP3)

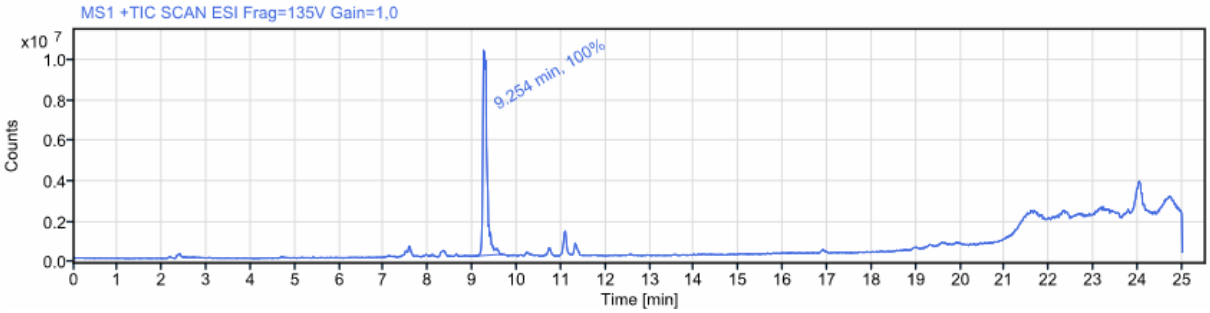


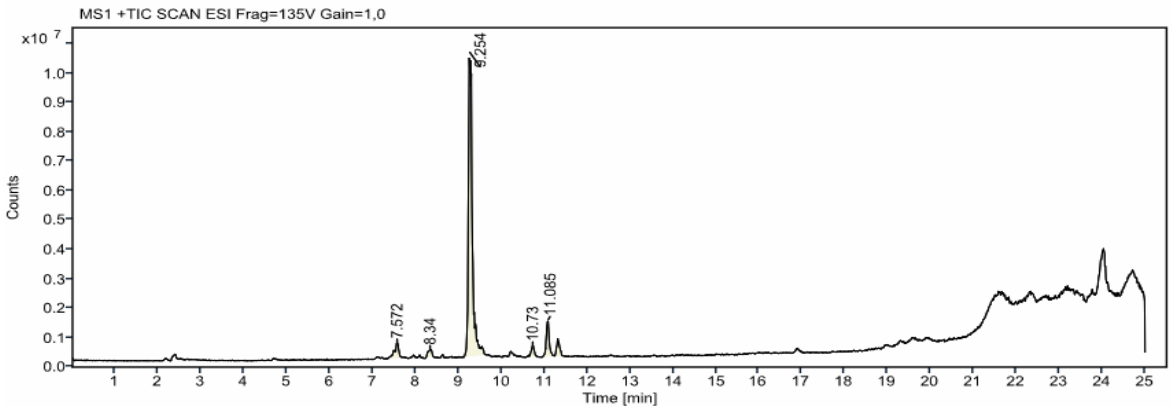
A 2.0 mL Eppendorf vial was charged with first peptide **20*i*** (0.0100 mmol, 1.0 equiv.) and buffer PB (50 mM, pH = 8.0; 0.50 mL). To the resulting colorless solution was added a pale yellow solution of **15** (0.012 mmol, 1.2 equiv.) in DMF (0.50 mL). The mixture was shaken at room temperature for 20 minutes. It was then transferred into a second 4.0 mL Eppendorf vial containing second peptide **20*j*** (0.0044~0.0048 mmol, 1.1-1.2 equiv.). The resulting solution was shaken at 37 °C for 4 hours. The mixture was then allowed to cool down to room temperature, diluted with water (0.8 mL) and directly submitted to preparative HPLC.

Cross-conjugation of H-Gly-Cys-Ala-Leu-Gln-Thr-NH_2 (20a) with Ac-Phe-Ala-Cys-His-NH_2 (20e)



Step-wise protocol: The title conjugate was prepared following the **GP2** and using H-Gly-Cys-Ala-Leu-Gln-Thr-NH_2 S-VBZ derivative (**21a**) (5.0 mg, 0.0040 mmol, 1.0 equiv.) and peptide Ac-Phe-Ala-Cys-His-NH_2 (**20e**) (2.3 mg, 0.0044 mmol, 1.1 equiv.) in a mixture of PB (50 mM, pH = 8.0; 0.20 mL) and DMF (0.20 mL). After shaking at 37 °C for 4 hours (Indicative HPLC-MS-based yield: 81%. Figure S43), preparative HPLC (Method 4; retention time of **24ae**: 11.1-11.7 minutes) afforded cross-conjugate **24ae** (5.2 mg, 0.0029 mmol, 74% yield) as a fluffy, white solid.





RT [min]	Signal Description	Symmetry	Resolution	Height [count]	Area [count*min]	Rel. Area [%]
7.572	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	1.60821		519097	3752723	4.5
8.340	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.65453		250156	1084236	1.3
9.254	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.36958		10393802	67645041	81.0
10.730	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.98966		381206	1913534	2.3
11.085	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.42616		1243473	9094663	10.9

Filtered on peak height > 100000 counts

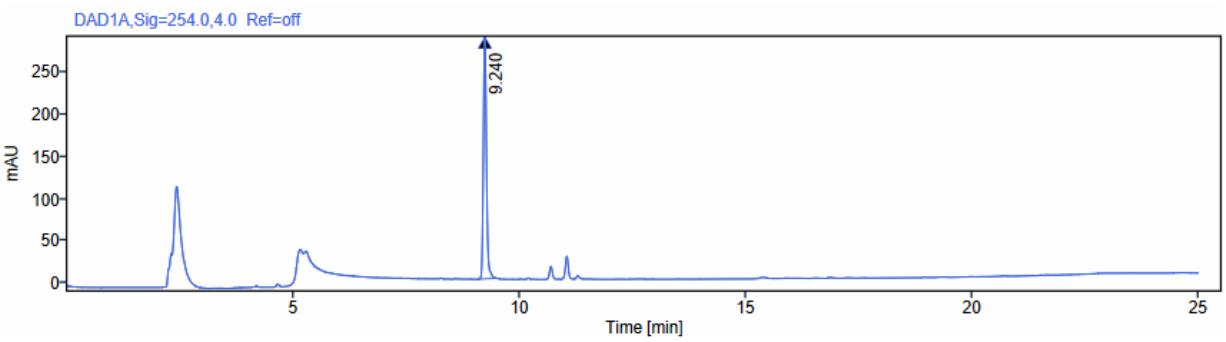


Figure S43: HPLC-MS and HPLC-UV (at 254 nm) chromatograms of the step-wise cross-conjugation reaction of peptides GCALNT (**20a**) and AcFACH (**20e**), after 4 hours. Retention time of **24ae** = 9.2 minutes.

One-one protocol: The title compound was also prepared following the **GP3** and using peptide Gly-Cys-Ala-Leu-Gln-Thr-NH_2 (**20a**) (12.6 mg, 0.0100 mmol, 1.0 equiv.), peptide Ac-Phe-Ala-Cys-His-NH_2 (**20e**) (6.2 mg, 0.012 mmol, 1.2 equiv.) and **15** (6.8 mg, 0.012 mmol, 1.2 equiv.) in a mixture of PB (50 mM, pH = 8.0; 0.50 mL) and DMF (0.50 mL). The solution of **20a** and **15** was shaken at room temperature for 20 minutes; upon addition of **20e**, shaking was continued at 37 °C for 5 hours (Indicative HPLC-MS-based yield: 41%. Figure S44). Preparative HPLC (Method 4) afforded cross-conjugate **24ae** (4.0 mg, 0.0022 mmol, 23% yield) as a fluffy, white solid. Homodimer **24aa** (7.5 mg 0.0039 mmol, 39% recovery) was collected as the major product.

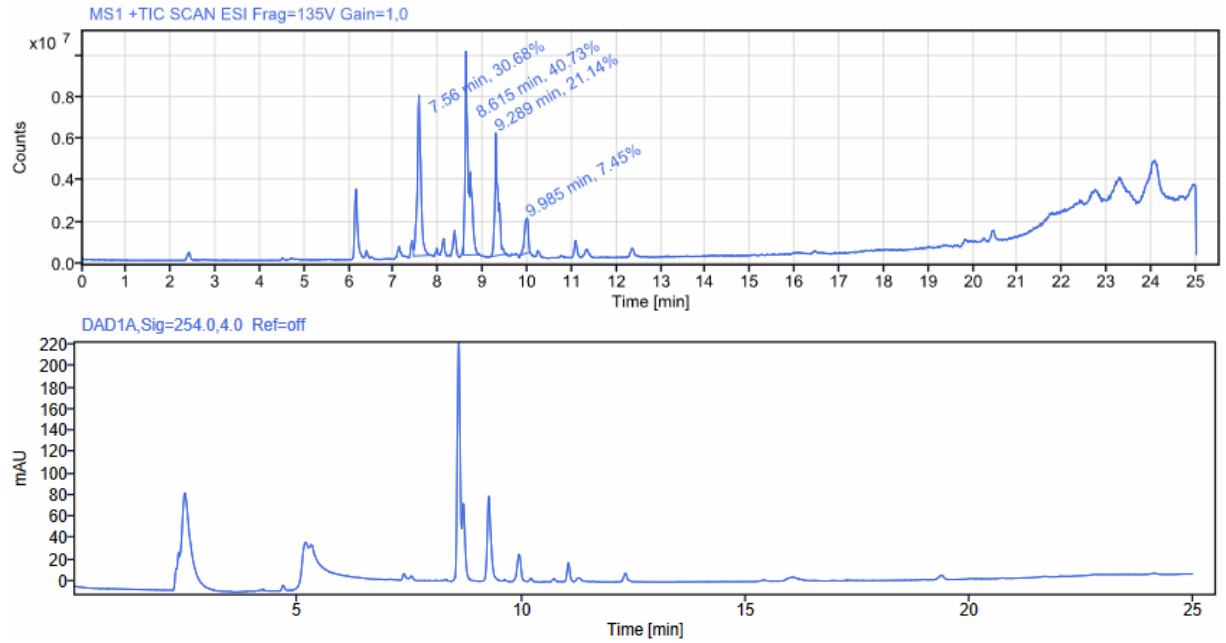
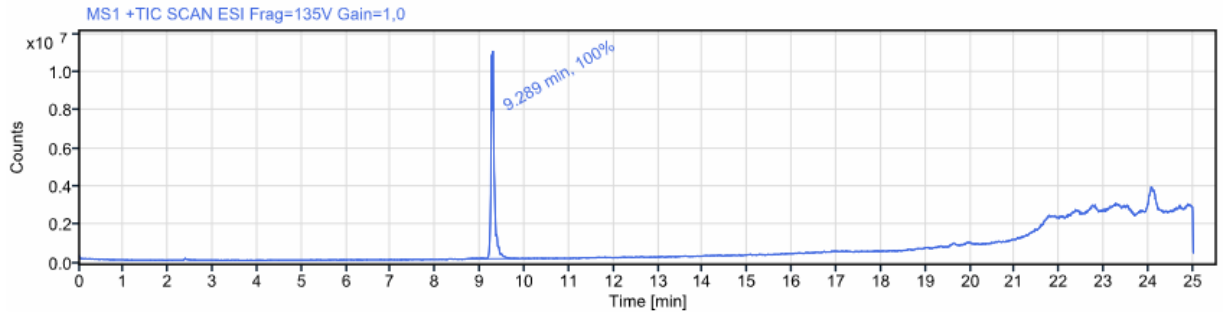


Figure S44: HPLC-MS and HPLC-UV (at 254 nm) chromatograms of the cross-conjugation reaction of peptides **20a** and **20e** following the ope-pot protocol, after 5 hours at 37 °C. Retention time of desired cross-conjugate **24ae**: 9.2 minutes. The compound eluted at ca. 8.6 minutes is the homoconjugate **24aa**. The compound eluted at ca. 10.0 minutes is the homoconjugate **24ee**. The compound eluted at ca. 11.1 minutes is the unreacted S-VBZ **21a**.

HPLC-MS: Retention time (Method 1) = 9.2 minutes.
HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + 2H]^{+2}$ Calcd for $C_{62}H_{80}F_4IN_{19}O_{16}S_3^{+2}$ 822.7081; Found 822.7096.



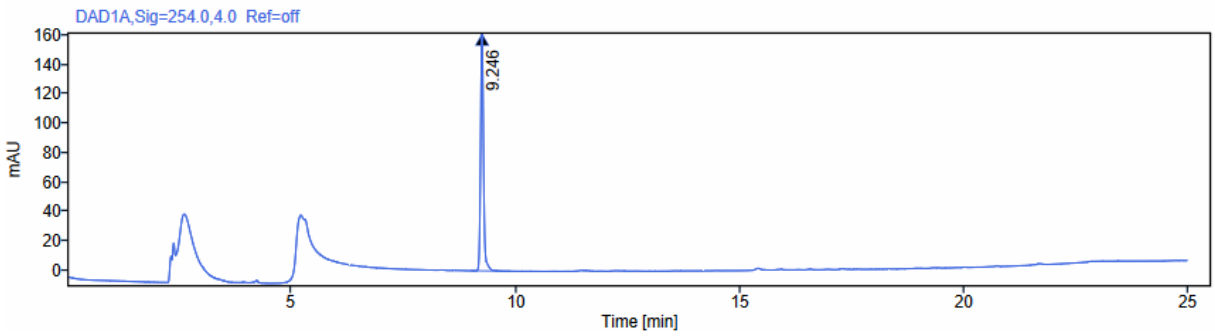
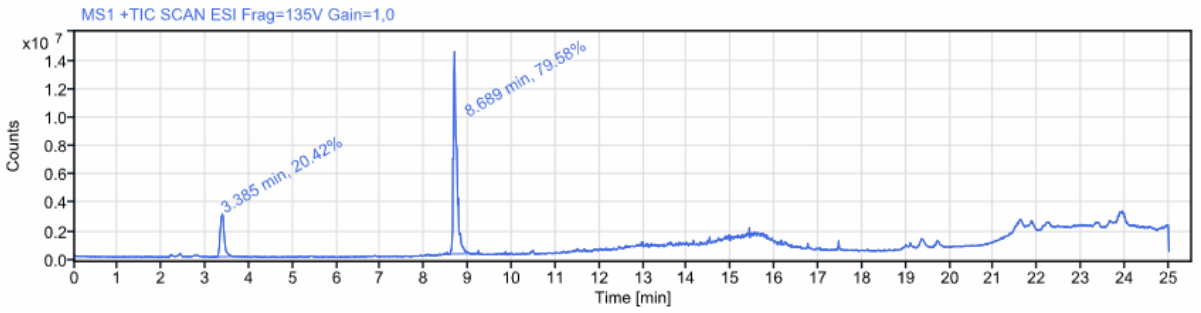


Figure S45. HPLC-MS and HPLC-UV (at 254 nm) chromatograms of pure **24ae**.

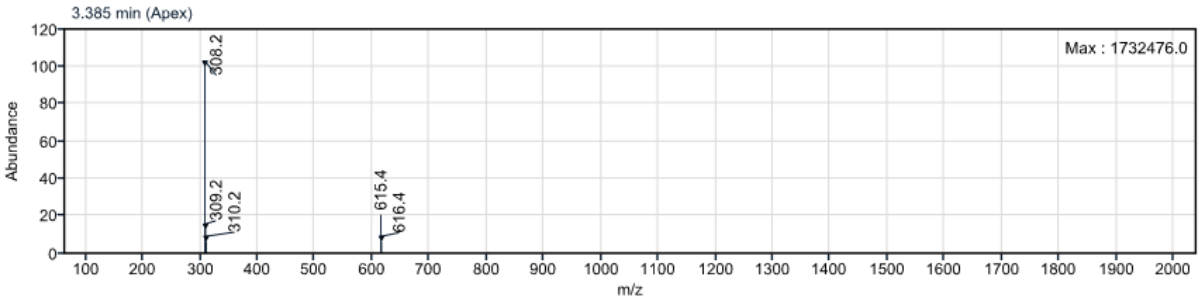
Test of stability: In a 2 mL Eppendorf vial containing a stirring bar, cross-conjugate **24ae** (3.5 mg, 0.0020 mmol, 1.0 equiv.) and reduced glutathione (2.8 mg, 0.0090 mmol, 4.5 equiv.) were dissolved in a mixture of DMF (0.10 mL), PB (solution (a); 5.0 mM, pH = 8.0; 0.066 mL) EDTA (6.7 mM in solution (a); 0.034 mL). The clear mixture was stirred at room temperature for 24 hours, becoming slightly turbid during this time. The mixture was submitted to HPLC-MS analysis after 4 hours (Figure S46) and after 24 hours (Figure S47). The composition of the mixture was shown unchanged during the aforementioned period of time. The ratio between the integrals of the peaks corresponding to the two mixed species remained practically unchanged as well.



Peak Results (Area Percent at least 1%)

RT (min)	Signal Description	Width (min)	Area	Height	Area%
3.385	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.497	18929668.2	2985300.8	20.42
8.689	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.513	73770262.1	14355744.6	79.58

Retention time: 3.385 min **Area Percent:** 20%



Retention time: 8.689 min **Area Percent:** 80%

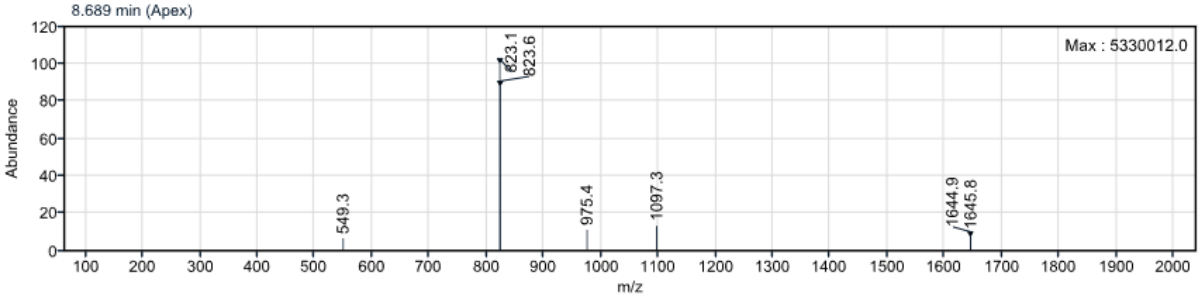
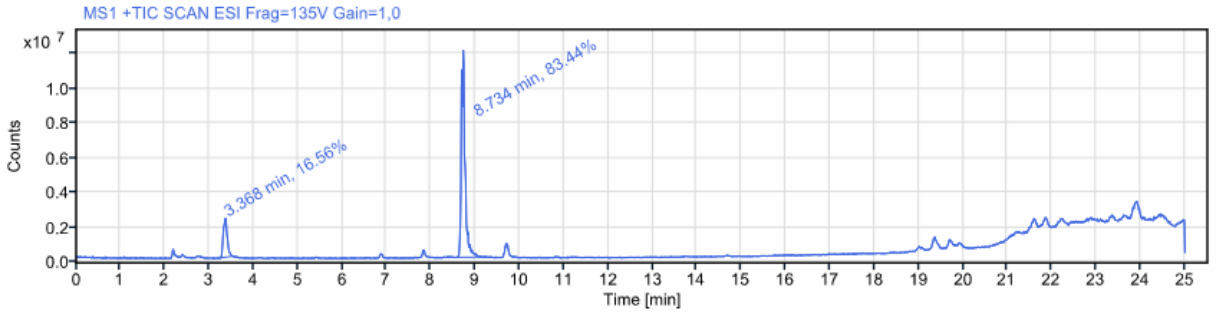


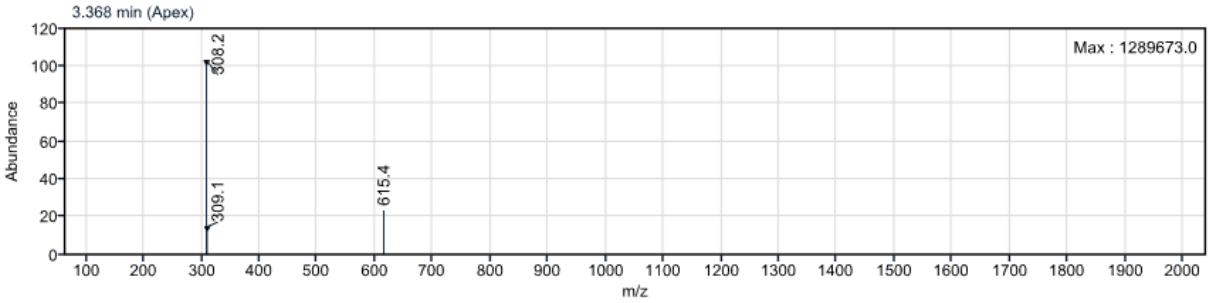
Figure S46: HPLC-MS analysis of the mixture of **24ae** with reduced glutathione after 4 hours. MS spectra are also provided to confirm the identity of the two compounds.



Peak Results (Area Percent at least 1%)

RT (min)	Signal Description	Width (min)	Area	Height	Area%
3.368	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.285	13383912.2	2258241.8	16.56
8.734	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.775	67460074.3	12087540.7	83.44

Retention time: 3.368 min **Area Percent:** 17%



Retention time: 8.734 min **Area Percent:** 83%

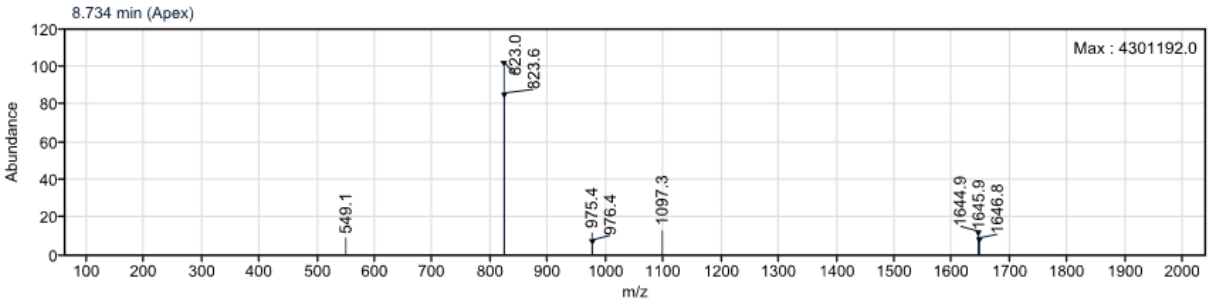
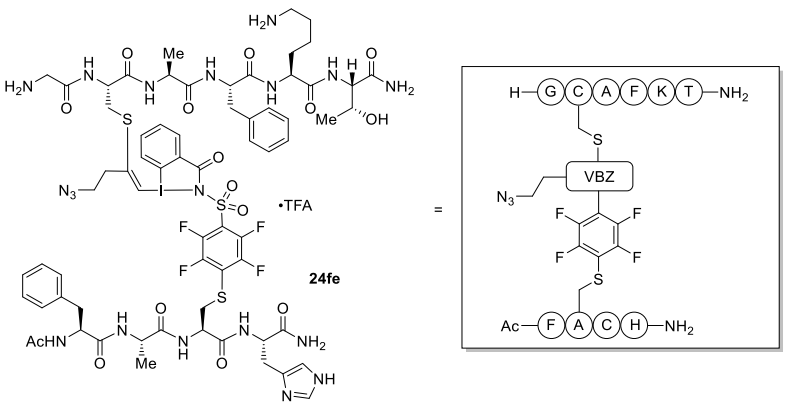


Figure S47: HPLC-MS analysis of the mixture of **24ae** with reduced glutathione after 24 hours. MS spectra are also provided to confirm the identity of the two compounds.

Cross-conjugation of H-Gly-Cys-Ala-Phe-Lys-Thr-NH_2 (20f) with Ac-Phe-Ala-Cys-His-NH_2 (20e)



Step-wise protocol: The title conjugate was prepared following the **GP2** and using Ala-Cys-Phe-Gly-Ala-Glu-NH_2 S-VBZ derivative (**21f**) (5.3 mg, 0.0040 mmol, 1.0 equiv.) and peptide Ac-Phe-Ala-Cys-His-NH_2 (**20e**) (2.3 mg, 0.0044 mmol, 1.1 equiv.) in a mixture of PB (50 mM, pH = 8.0; 0.20 mL) and DMF (0.20 mL). After shaking at 37 °C for 4 hours (Indicative HPLC-MS-based yield > 90%. Figure S48), preparative HPLC (Method 3; retention time of **24fe**: 8.3-8.8 minutes) afforded cross-conjugate **24fe** (6.6 mg, 0.0036 mmol, 89% yield) as a fluffy, white solid.

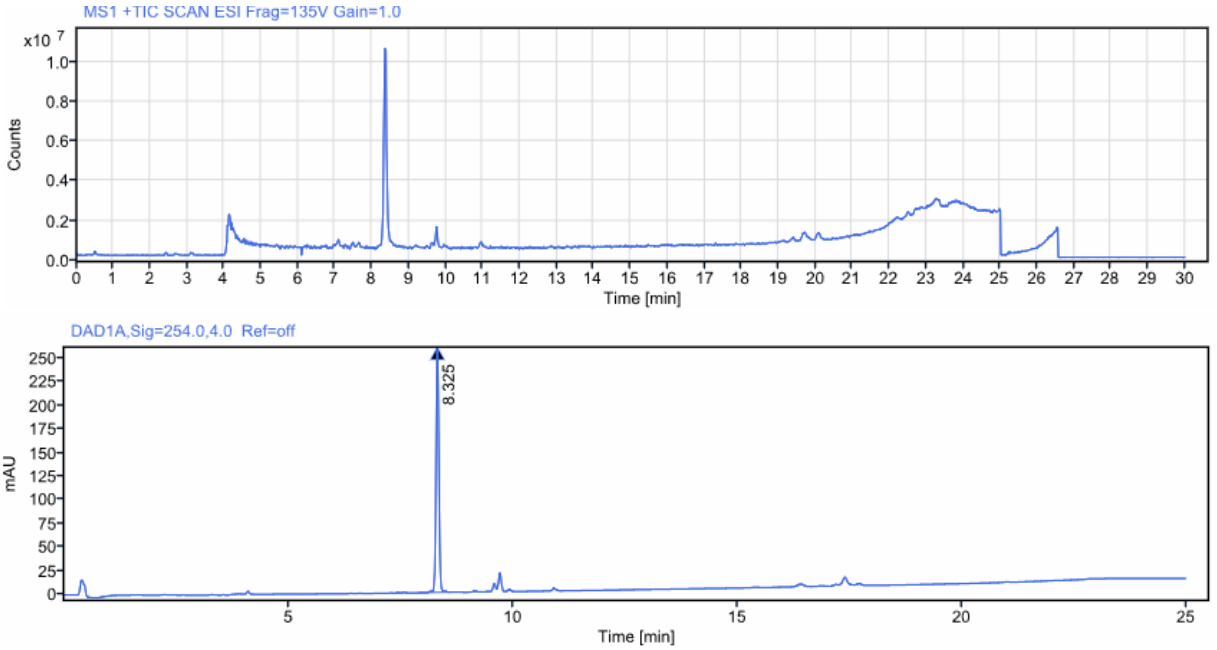
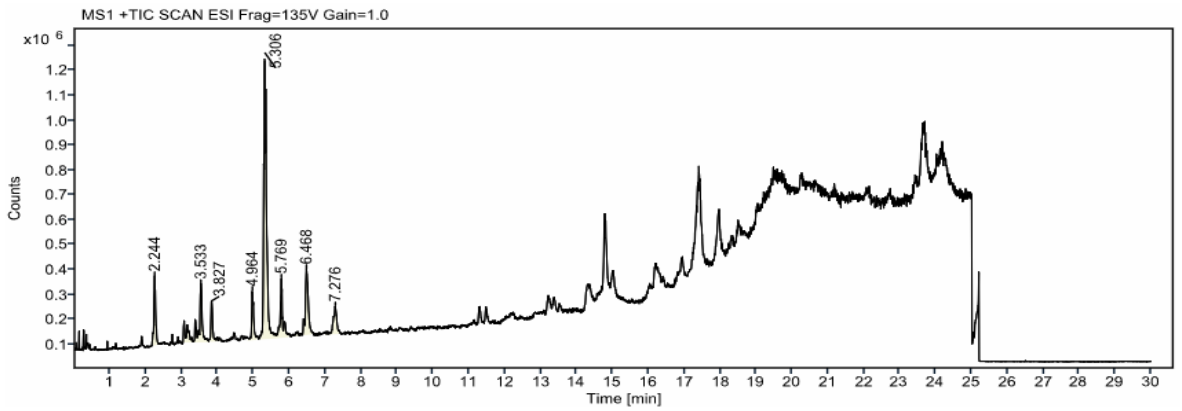


Figure S48: HPLC-MS and HPLC-UV (at 254 nm) chromatograms of the step-wise cross-conjugation reaction of peptides GCALNT (**20f**) and AcFACH (**20e**), after 4 hours. Retention time of **24fe** = 8.3 minutes.

One-one protocol: The title compound was also prepared following the **GP3** and using peptide Ala-Cys-Phe-Gly-Ala-Glu-NH_2 (**20f**) (6.2 mg, 0.0100 mmol, 1.0 equiv.), peptide Ac-Phe-Ala-Cys-His-NH_2 (**20e**) (6.2 mg, 0.012mmol, 1.2 equiv.) and **15** (6.8 mg, 0.012 mmol, 1.2 equiv.) in a mixture of PB (50 mM, pH = 8.0; 0.50 mL) and DMF (0.50 mL). The solution of **20f** and **15** was shaken at room temperature for 20 minutes; upon addition of **20e**, shaking was continued at 37 °C for 5 hours (Indicative HPLC-MS-based yield: ca. 42%. Figure S49). Preparative HPLC (Method 3) afforded cross-conjugate **24fe** (3.1 mg, 0.0018 mmol, 18% yield) as a fluffy, white solid. Both homoconjugation by-products could be additionally isolated, also as fluffy, white solids: homoconjugation of Gly-Cys-Ala-Phe-Lys-Thr-NH_2 (**24ff**) (2.0 mg, 0.0011 mmol, 11% yield) and homoconjugation of Ac-Phe-Ala-Cys-His-NH_2 (**24ee**) (2.6 mg, 0.0016 mmol, 16% yield).



RT [min]	Signal Description	Symmetry	Resolution	Height [count]	Area [count*min]	Rel. Area [%]
2.244	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.81869		279385	944820	6.8
3.533	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	2.66093		236430	1946406	14.0
3.827	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.41941		165650	676990	4.9
4.964	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.58153		200460	696744	5.0
5.306	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.49674		1153947	5867639	42.3
5.769	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.79111		240213	1177586	8.5
6.468	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.82700		266900	1769278	12.8
7.276	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	1.57151		113235	784771	5.7

Filtered on peak height > 100000 counts

Figure S49: HPLC-MS chromatogram of the one-pot cross-conjugation reaction after 4 hours. Retention time of **24fe** = 8.3 minutes. The compound eluted at ca. 7.3 minutes is the homoconjugate corresponding to peptide **24ee**. The compound eluted at ca. 9.6 minutes is the homoconjugate corresponding to peptide **24ff**. The compound eluted at ca. 12 minutes is the exceeding unreacted **20e**.

HPLC-MS: Retention time (Method 1) = 8.4 minutes.
HRMS (Nanochip-based ESI/LTQ-Orbitrap) m/z: $[M + 2H]^{+2}$ Calcd for $C_{67}H_{84}F_4IN_{19}O_{15}S_3^{+2}$ 846.7263; Found 846.7260.

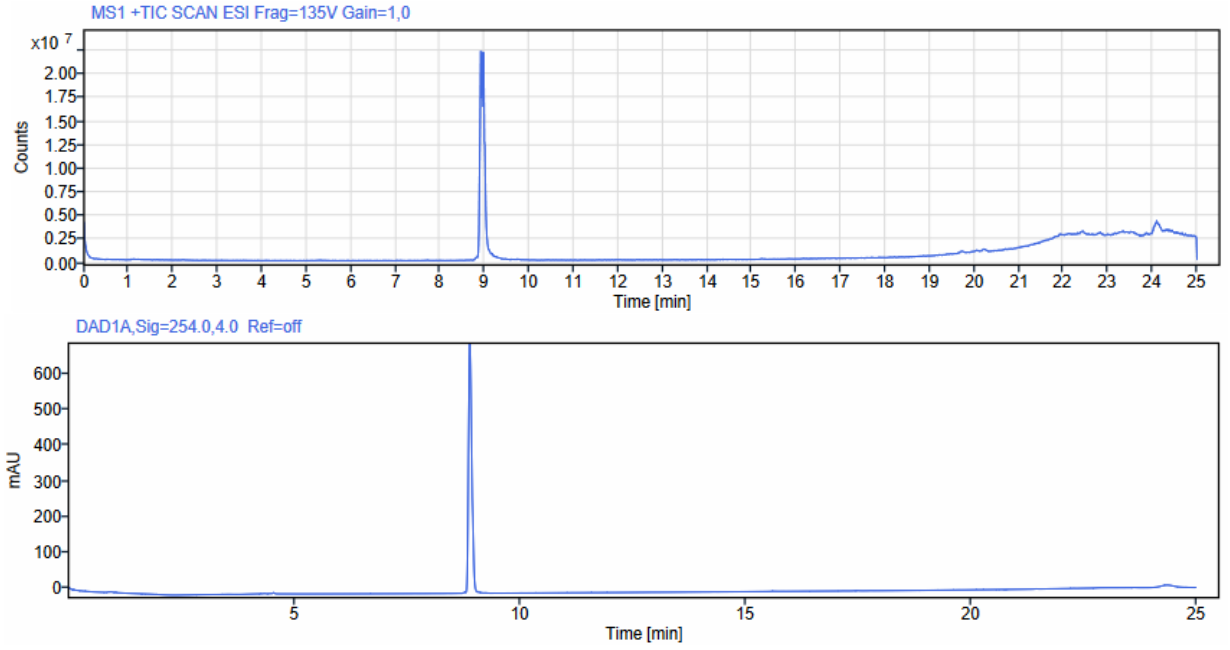
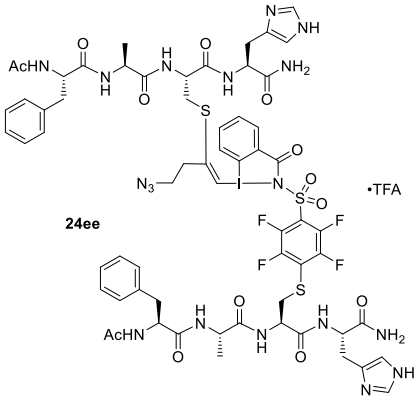


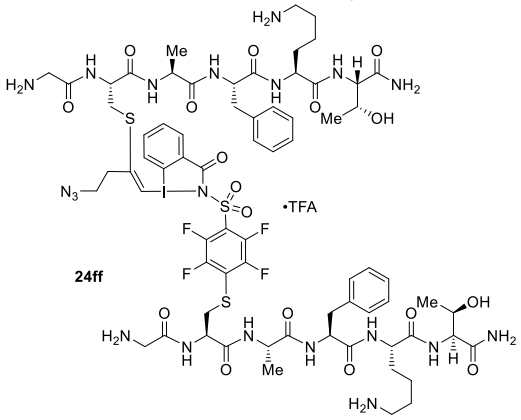
Figure S50: HPLC-MS and HPLC-UV (at 254 nm) chromatograms of pure **24fe**.

By-product 24ee:

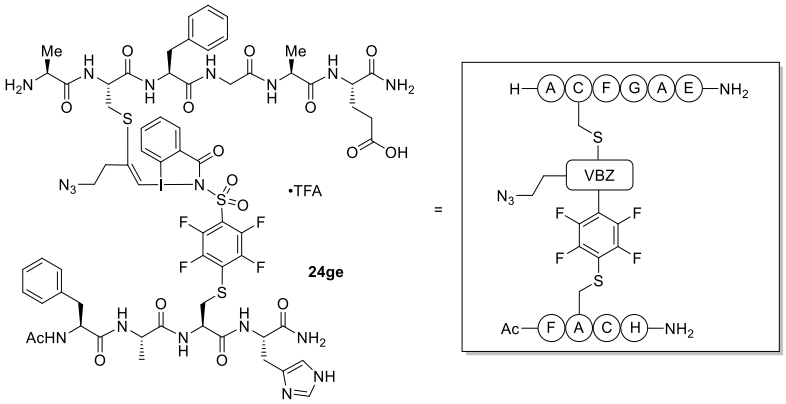
HPLC-MS: Retention time (method 1) = 7.3 minutes.
HRMS (Nanochip-based ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{36}H_{36}F_4IN_8O_8S_2^+$ 975.1073; Found 975.1077.



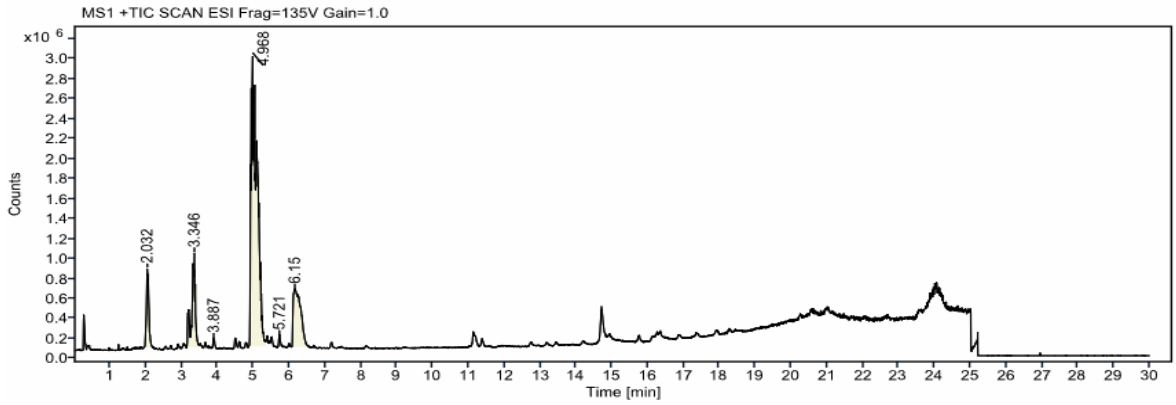
By-product 24ff:
HPLC-MS: Retention time (method 1) = 9.7 minutes.
HRMS (ESI/QTOF) m/z: $[M + 3H]^{+3}$ Calcd for $C_{71}H_{98}F_4IN_{20}O_{17}S_3^{+3}$ 600.5182; Found 600.5169.



Cross-conjugation of H-Ala-Cys-Phe-Gly-Ala-Glu-NH_2 (20g) with Ac-Phe-Ala-Cys-His-NH_2 (20e)



Step-wise protocol: The title conjugate was prepared following the **GP2** and using Ala-Cys-Phe-Gly-Ala-Glu-NH_2 S-VBZ derivative (**21g**) (5.1 mg, 0.0040 mmol, 1.0 equiv.) and peptide Ac-Phe-Ala-Cys-His-NH_2 (**20e**) (2.3 mg, 0.0044 mmol, 1.1 equiv.) in a mixture of PB (50 mM, pH = 8.0; 0.20 mL) and DMF (0.20 mL). After shaking at 37 °C for 4 hours (Indicative HPLC-MS-based yield: ca. 62%. Figure S51), preparative HPLC (Method 4; retention time of **24ge**: 11.5-12.0 minutes) afforded cross-conjugate **24ge** (5.0 mg, 0.0028 mmol, 70% yield) as a fluffy, white solid. Unreacted S-VBZ **21g** (1.3 mg 0.0011 mmol, 28% recovery) was also collected.



RT [min]	Signal Description	Symmetry	Resolution	Height [count]	Area [count*min]	Rel. Area [%]
2.032	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.56763		800458	3779212	7.1
3.346	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	2.19553		959394	6305510	11.9
3.887	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.45189		105337	208649	0.4
4.968	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.26312		2945856	32858719	62.1
5.721	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.80747		119872	280496	0.5
6.150	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.27640		590247	8659937	16.4

Filtered on peak height > 100000 counts

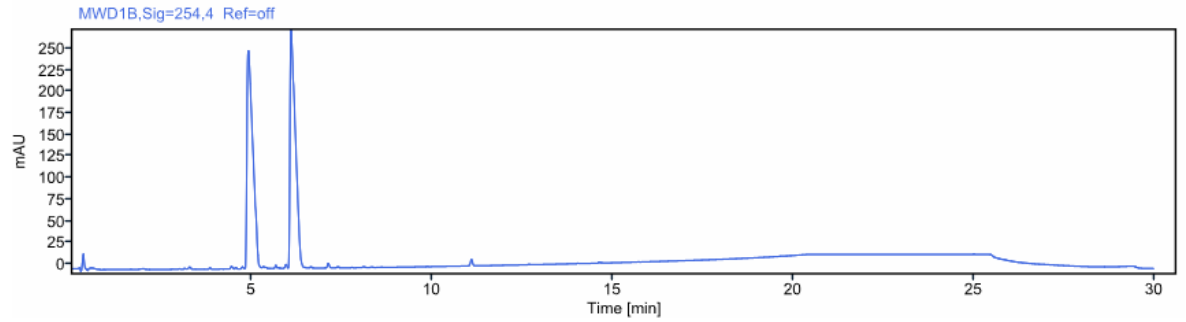
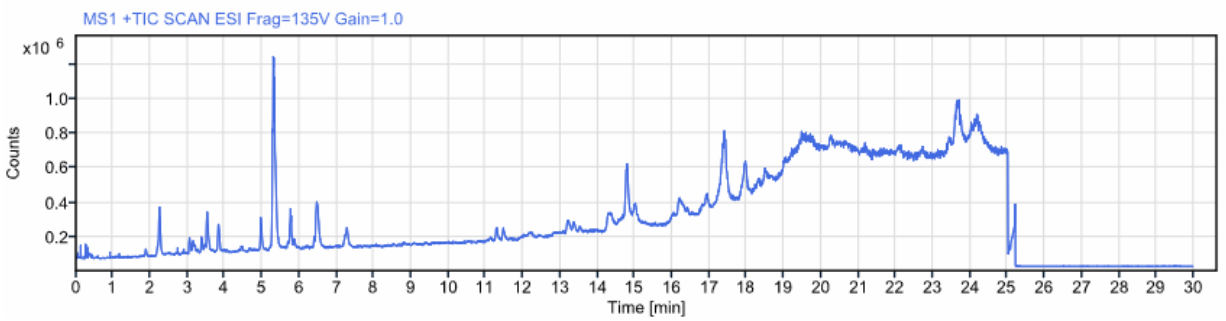


Figure S51: HPLC-MS and HPLC-UV (at 254 nm) chromatograms (instrument b) of the step-wise cross-conjugation reaction after 4 hours. Retention time of **24ge** = 5.0 minutes. The compound eluted at ca. 6.1 minutes in unreacted **21g**.

One-one protocol: The title compound was also prepared following the **GP3** and using peptide Ala-Cys-Phe-Gly-Ala-Glu-NH_2 (**20f**) (7.3 mg, 0.010 mmol, 1.0 equiv.), peptide Ac-Phe-Ala-Cys-His-NH_2 (**20e**) (5.7 mg, 0.011 mmol, 1.1 equiv.) and **15** (6.8 mg, 0.012 mmol, 1.2 equiv.) in a mixture of PB (50 mM, pH = 8.0; 0.50 mL) and DMF (0.50 mL). The solution of **20g** and **15** was shaken at room temperature for 20 minutes; upon addition of **20e**, shaking was continued at 37 °C for 5 hours (Indicative HPLC-MS-based yield: ca. 35%. Figure S52). Preparative HPLC (Method 4) afforded cross-conjugate **24ge** (5.0 mg, 0.0028 mmol, 28% yield) as a fluffy, white solid. Unreacted S-VBZ **21g** (2.0 mg 0.0017 mmol, 17% recovery) was also collected.



RT (min)	Signal Description	Width (min)	Area	Height	Area%
2.079	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.311	1707059.4	386930.8	7.61
2.884	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.365	1121041.8	139534.5	5.00
3.365	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.163	923607.9	284233.7	4.12
3.662	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.178	982433.7	282804.6	4.38
4.822	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.243	826539.6	266783.6	3.68
5.153	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.329	7822885.2	1312132.5	34.86
5.624	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.165	1239687.8	307319.1	5.52
6.298	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.292	2714352.4	389491.4	12.10
7.139	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.128	662636.7	181990.8	2.95
11.182	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.208	563691.8	122845.7	2.51
14.222	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.140	575298.1	135674.3	2.56
14.699	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.110	753754.2	346180.8	3.36
14.712	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.134	1133965.6	359341.2	5.05
24.993	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.037	576704.2	377861.7	2.57
25.192	MS1 +TIC SCAN ESI Frag=135V Gain=1.0	0.139	837936.7	243707.6	3.73

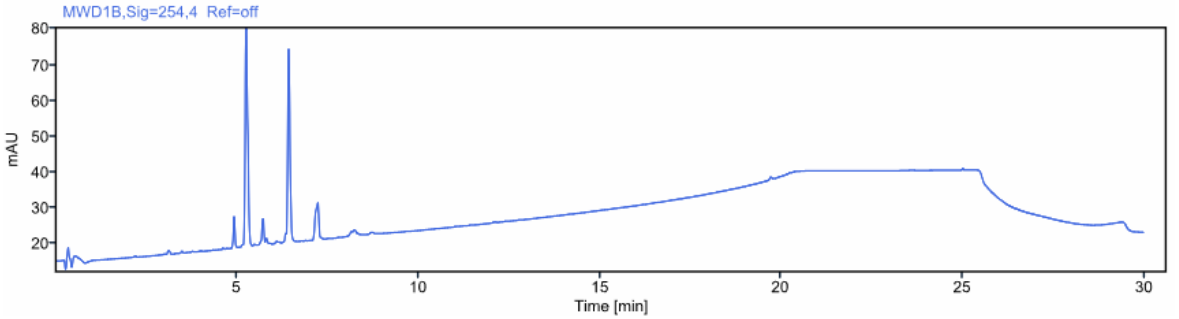
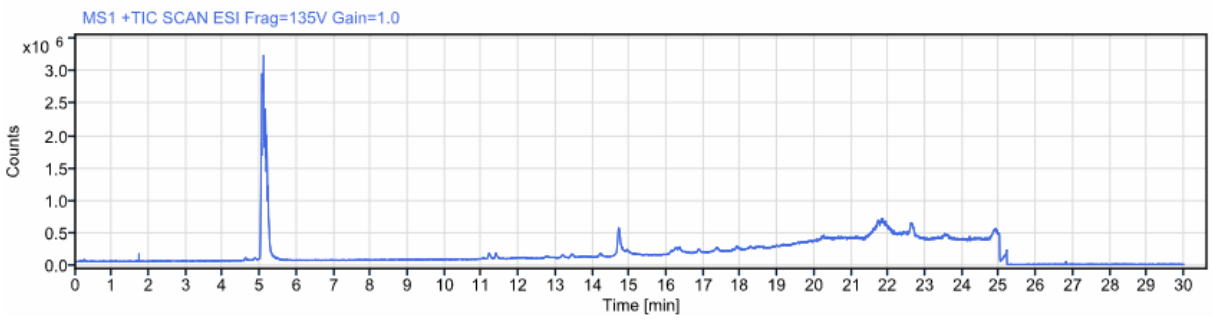


Figure S52: HPLC-MS and HPLC-UV (at 254 nm) chromatograms (instrument b) of the one-pot cross-conjugation reaction after 4 hours. Retention time of **24ge** = 5.0 minutes. The compound eluted at ca. 6.1 minutes in unreacted **21g**.

HPLC-MS: Retention time (Instrument b, method 1) = 4.9-5.0 minutes.
HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + 2H]^{+2}$ Calcd for $C_{65}H_{77}F_4IN_{18}O_{16}S_3^{+2}$ 832.1948; Found 832.1968.



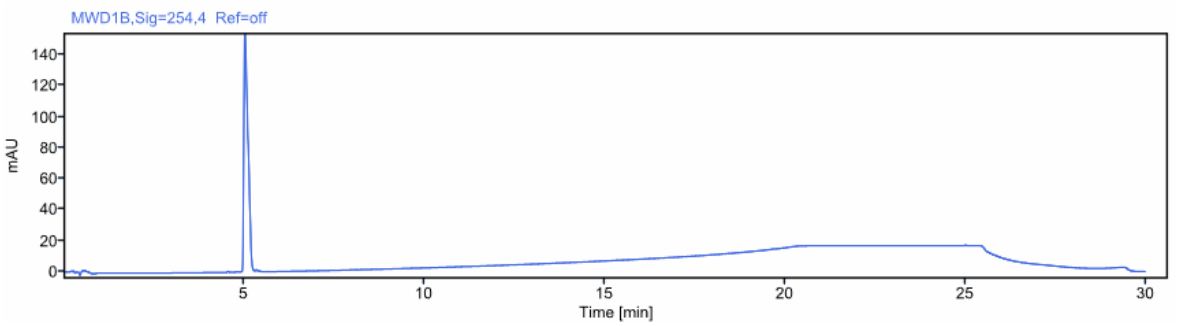
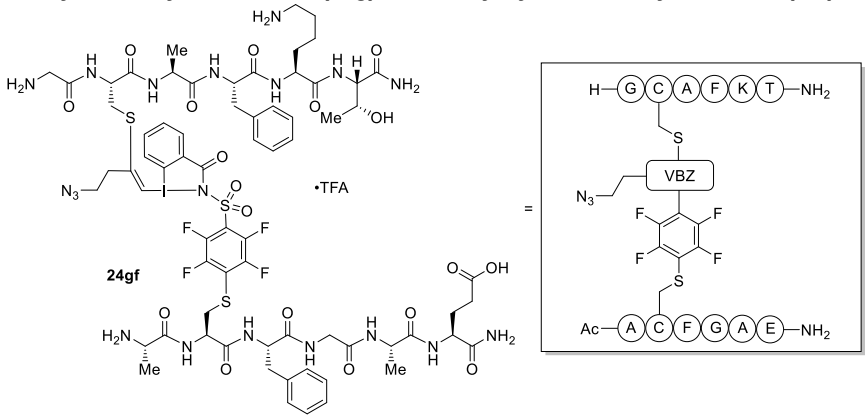
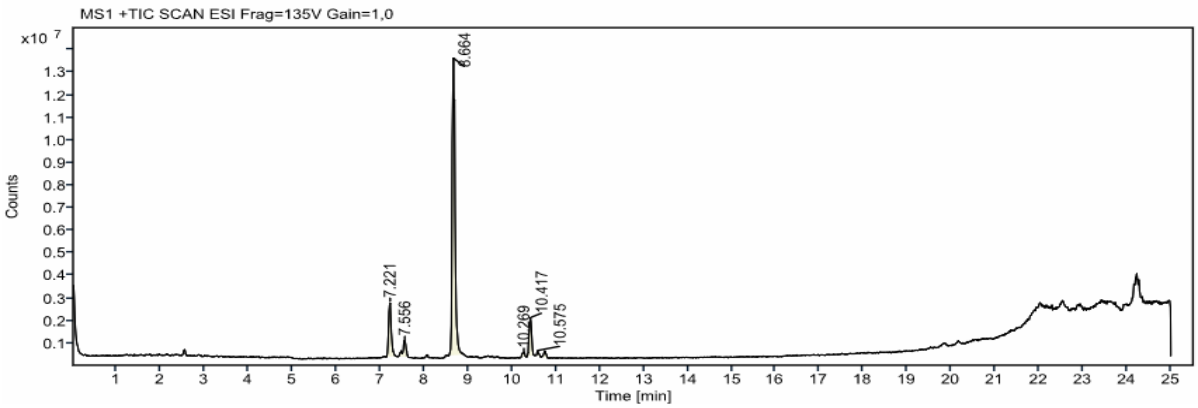


Figure S53. HPLC-MS and HPLC-UV (at 254 nm) chromatograms of pure **24ge**.

Cross-conjugation of Ala-Cys-Phe-Gly-Ala-Glu-NH_2 (20g) with H-Gly-Cys-Ala-Phe-Lys- Thr-NH_2 (20f)



Step-wise protocol: The title conjugate was prepared following the **GP2** and using Ala-Cys-Phe-Gly-Ala-Glu-NH_2 S-VBZ (**21g**) derivative (5.3 mg, 0.0040 mmol, 1.0 equiv.) and peptide Ala-Cys-Phe-Gly-Ala-Glu-NH_2 (**20f**) (3.1 mg, 0.0044 mmol, 1.1 equiv.) in a mixture of PB (50 mM, pH = 8.0; 0.20 mL) and DMF (0.20 mL). After shaking at 37 °C for 4 hours (Indicative HPLC-MS-based yield: ca. 73%. Figure S54), preparative HPLC (Method 3; retention time of **24gf**: 7.8-8.1 minutes) afforded cross-conjugate **24gf** (4.8 mg, 0.0025 mmol, 62% yield) as a fluffy, white solid.



RT [min]	Signal Description	Symmetry	Resolution	Height [count]	Area [count*min]	Rel. Area [%]
7.221	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.96311		2421587	9883692	11.9
7.556	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	1.46015		751959	3765233	4.5
8.664	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.66669		13179438	60398421	73.0
10.269	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	1.74758		198349	685280	0.8
10.417	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.90172		1699254	6382048	7.7
10.575	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.14161		231344	1655752	2.0

Filtered on peak height > 100000 counts

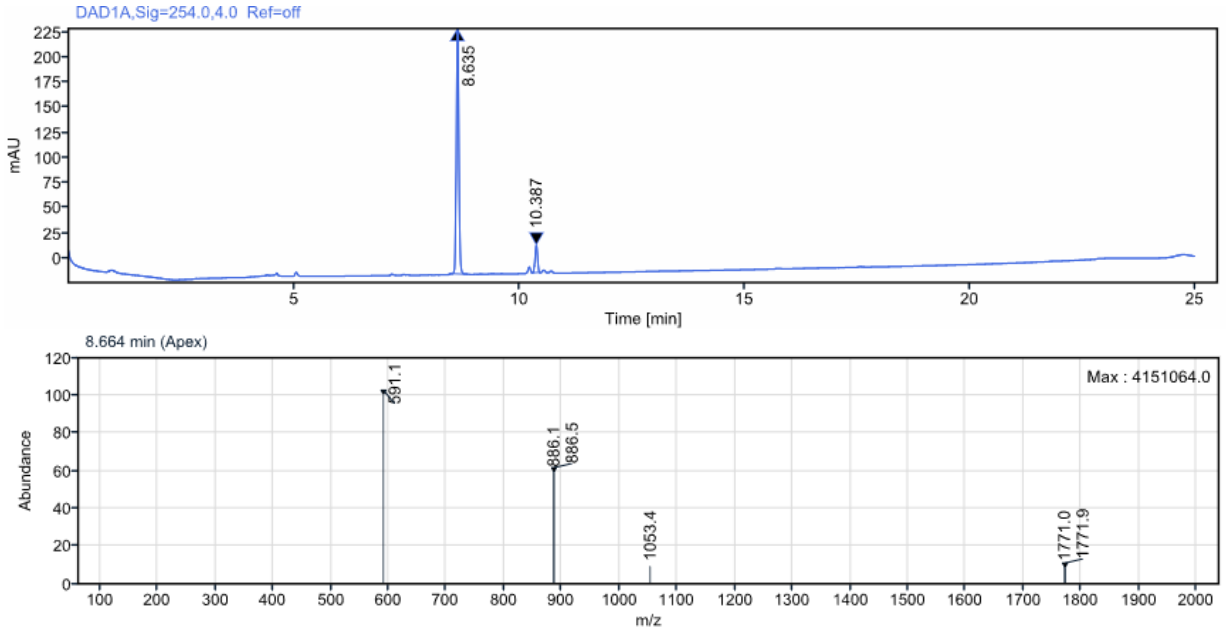


Figure S54: HPLC-MS and HPLC-UV (at 254 nm) chromatograms of the stepwise cross-conjugation reaction after 4 hours. Retention time of **24gf** = 8.7 minutes. The compound eluted at ca. 10.4 minutes in unreacted **21g**. The MS spectrum is here also shown to prove the identity of the compound, the elution time of which changed significantly upon isolation from the crude reaction mixture.

HPLC-MS: Retention time (Method 1) = 8.4 minutes.
HRMS (ESI/QTOF) m/z: $[M]^{+2}$ Calcd for $C_{69}H_{90}F_4IN_{19}O_{18}S_3^{+2}$ 885.7422; Found 885.7455.

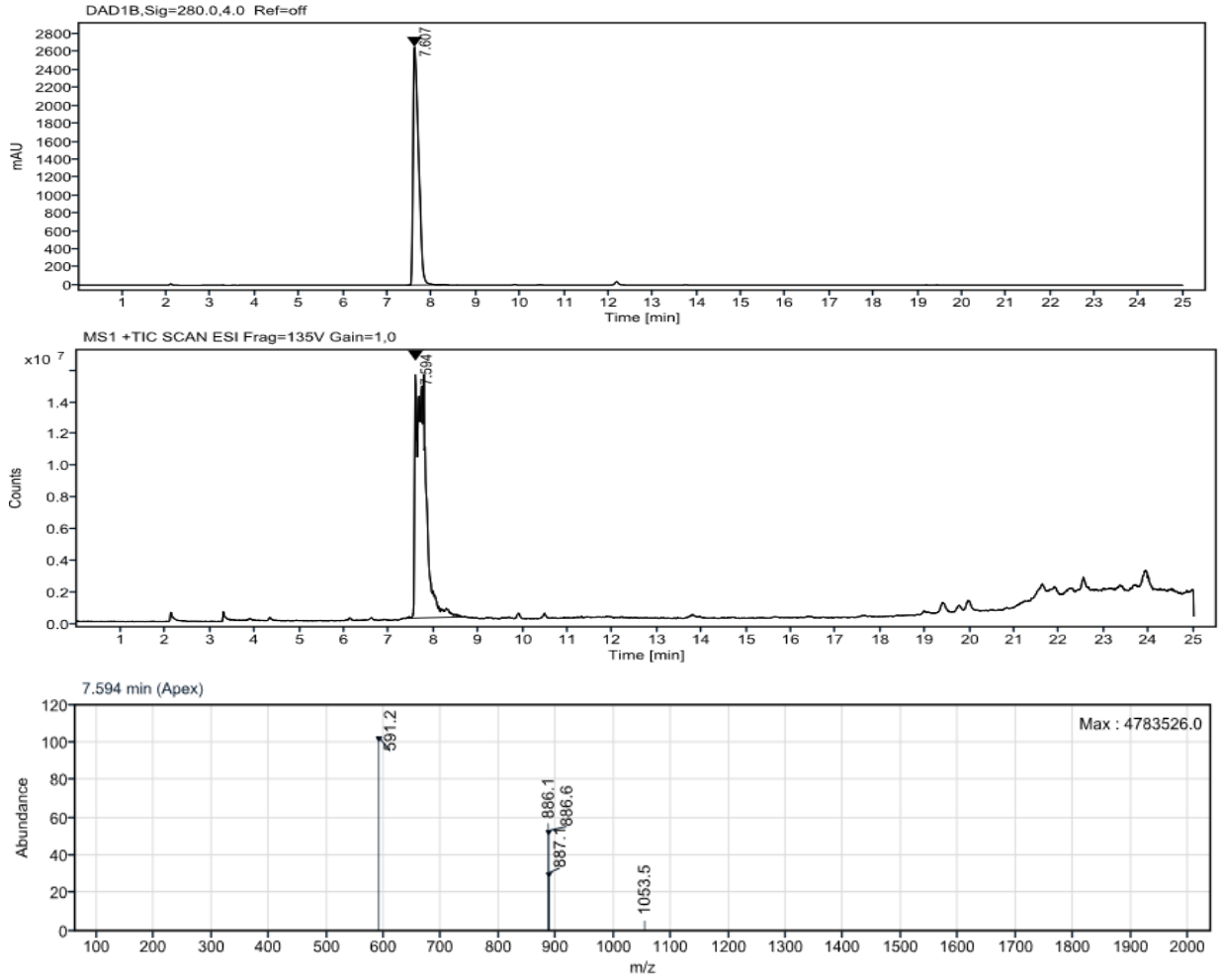
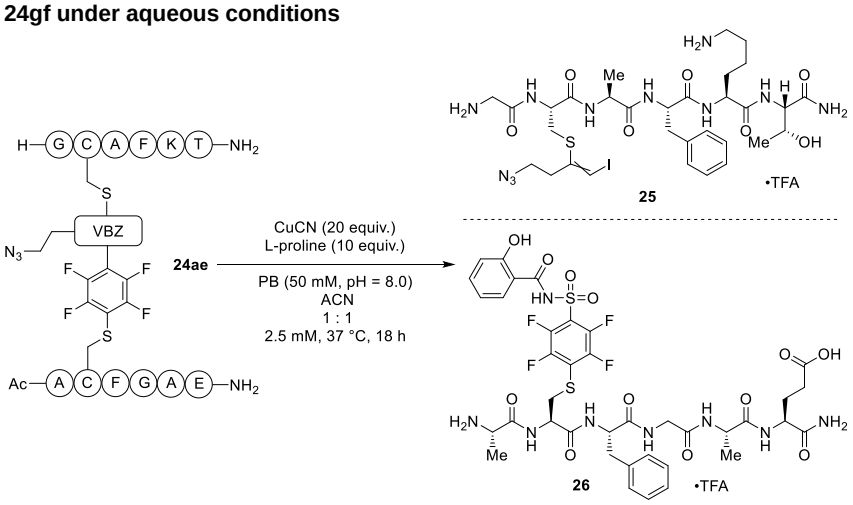


Figure S55. HPLC-MS and HPLC-UV (at 280 nm) chromatograms of pure **24gf**. The MS spectrum is here also shown to prove the identity of the compound, the elution time of which changed significantly upon isolation from the crude reaction mixture.

Cleavage of conjugate 24gf under aqueous conditions



Following a significantly modified version of a reported procedure,[3] a 5.0 mL round-bottomed vial was charged with L-proline (2.9 mg, 0.025 mmol, 10 equiv.) and CuCN (4.5 mg, 0.050 mmol, 20 equiv.). ACN (0.5 mL) was added. The resulting white suspension was stirred at room temperature for 30 minutes. A solution of the title conjugate **24gf** (4.7 mg, 0.0025 mmol, 1.0 equiv.) in a mixture of PB (50 mM, pH = 8.0; 0.50 mL) was then added. The resulting mixture was then stirred at 37 °C. A gradual change of color from white to pale blue was observed. After 18 hours at 37 °C, full conversion of the starting material was observed based HPLC-MS analysis (Figure S56). The mixture was then diluted with water (0.6 mL) and ACN (0.4 mL), the solids were filtered off, and the filtrate was submitted directly to preparative HPLC (method 3; retention time of **25** = 10.3-10.5 minutes; retention time of **26** = 12.2-12.4 minutes). Upon lyophilization of the collected fractions, two products were isolated, both as white, fluffy solids: iodoalkene **25** (0.50 mg, 0.00059 mmol, 24% yield) and 2-hydroxy benzamide **26** (0.90 mg, 0.00095 mmol, 38% yield).

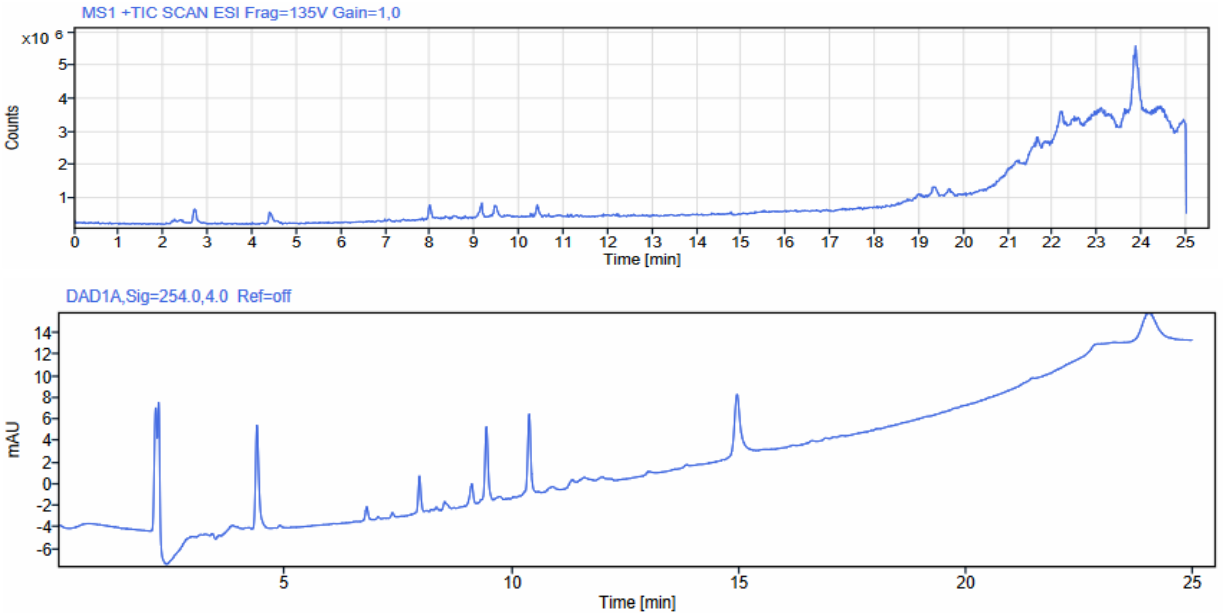


Figure S56: HPLC-MS and HPLC-UV (at 254 nm) chromatograms of the cleavage of cross-conjugate **24gf**, after 18 hours.

- 25
HPLC-MS: Retention time (Method 1) = 7.9-8.0 minutes.
HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{31}H_{49}IN_{11}O_7S^+$ 846.2576; Found 846.2587.

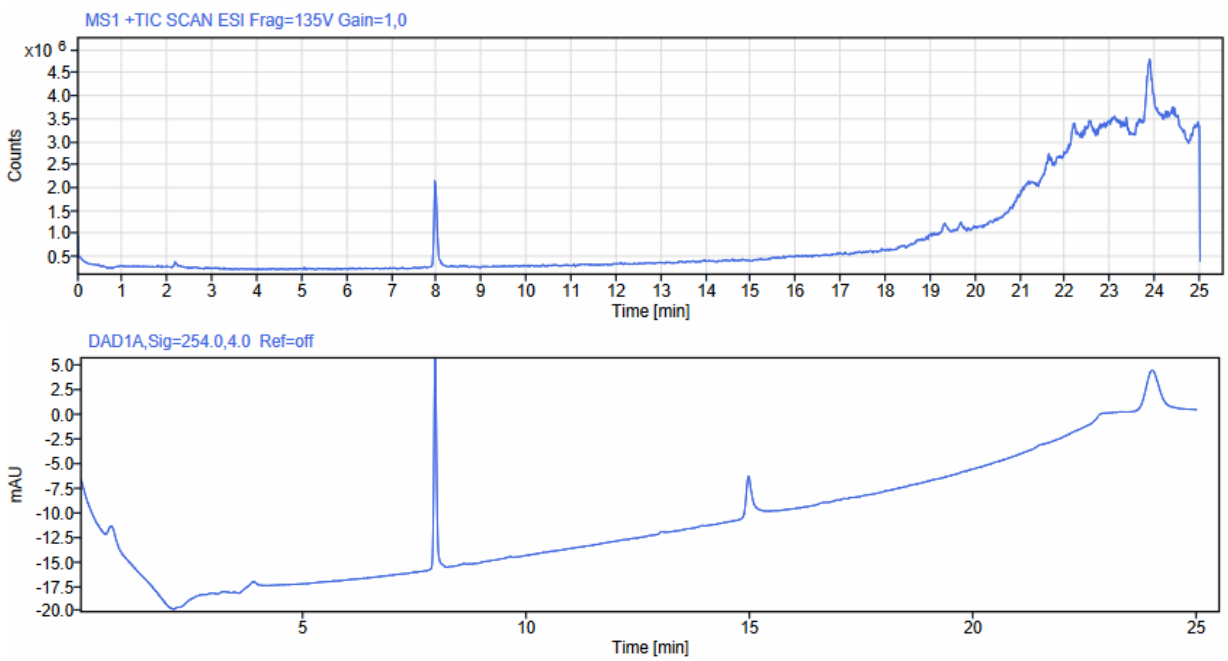


Figure S57. HPLC-MS and HPLC-UV (at 280 nm) chromatograms of pure **25**.

- 26
HPLC-MS: Retention time (Method 1) = 10.4 minutes.
HRMS (ESI/QTOF) m/z: $[M + Na]^+$ Calcd for $C_{38}H_{42}F_4N_8NaO_{12}S_2^+$ 965.2192; Found 965.2192.

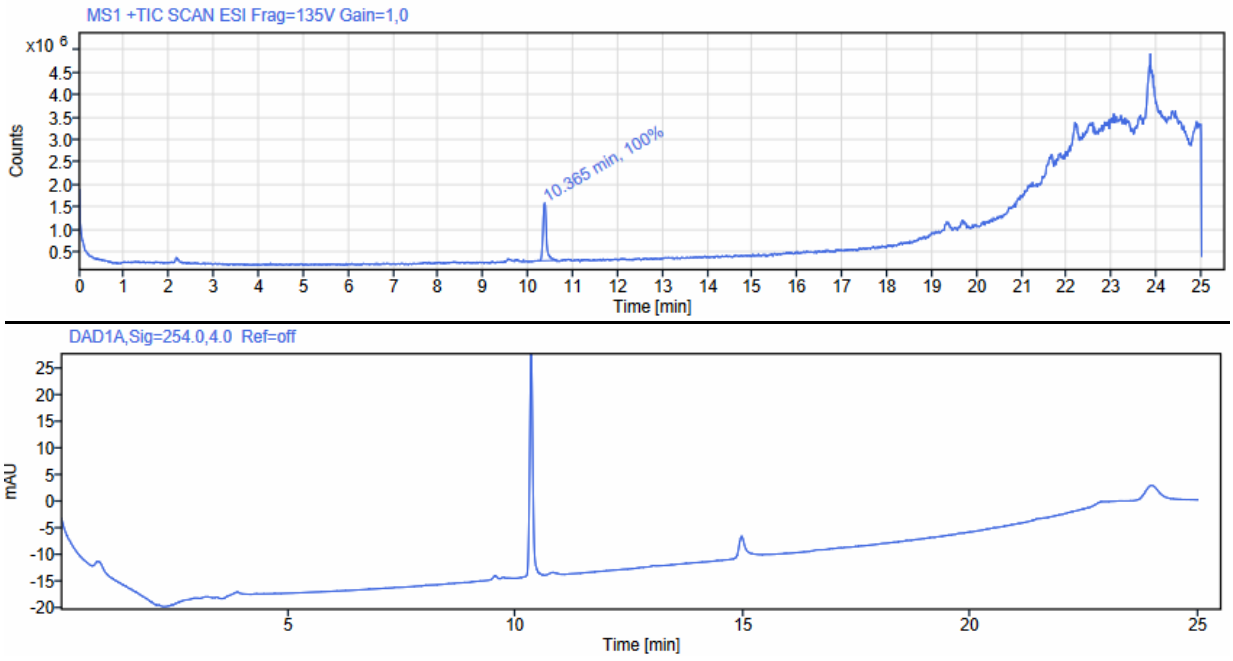
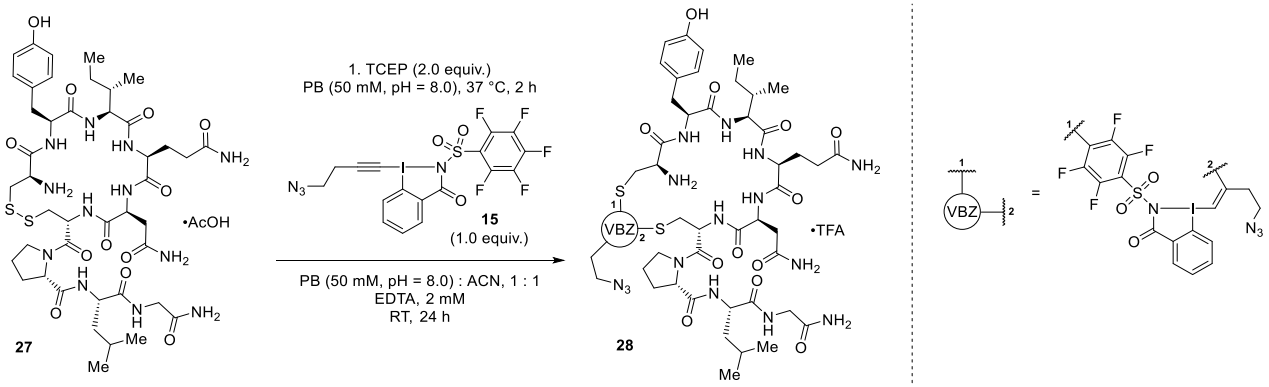


Figure S58. HPLC-MS and HPLC-UV (at 280 nm) chromatograms of pure **26**.

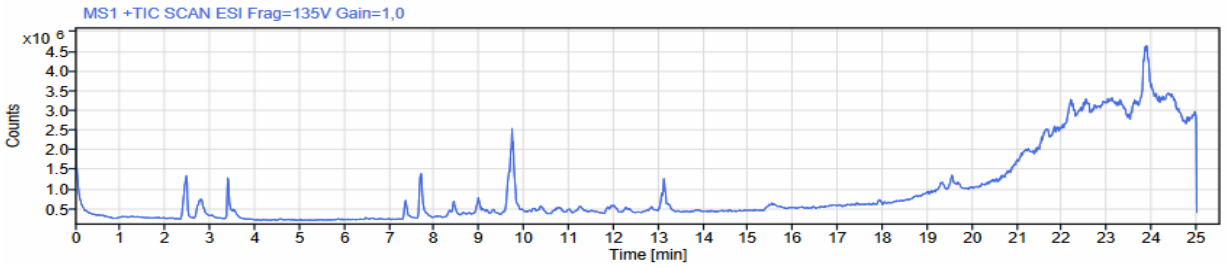
4. Rebridging of peptidic fragments upon disulfide reduction

4.1 Oxyitocin

Rebridging of oxytocin

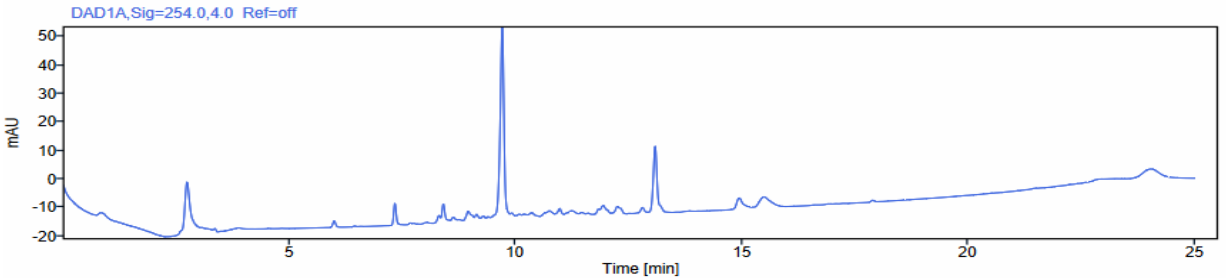


A 2.0 mL Eppendorf vial was charged with oxytocin acetate (**27**) (10 mg, 0.0090 mmol, 1.05 equiv.). A solution of TCEP (5.2 mg, 0.018 mmol, 2.0 equiv. with respect to oxytocin) in PB (50 mM, pH = 8.0; 1.8 mL) was added. The resulting clear solution was shaken at 37 °C for 2 hours, becoming a milky, slightly viscous mixture. The mixture was then diluted up to a 4.5 mL volume by adding it to a solution of EDTA in PB buffer (6.7 mmol in EDTA; 2.7 mL - final concentration in EDTA: 2.0 mM) contained in a 25 mL, round bottomed flask. Finally, a solution of **15** (4.9 mg, 0.0085 mmol, 1.0 equiv.) in ACN (4.5 mL) was added at room temperature. The now clear solution was stirred at room temperature for 24 hours (Indicative HPLC-MS based yield: ca. 41%; Figure S59). The reaction was then stopped, and the solution was lyophilized. The crude fluffy solid was diluted with water (1.0 mL) and ACN (0.8 mL). The resulting solution was submitted to preparative HPLC (method 3; retention time of **28** = 12.0-12.2 minutes). The collected fraction was lyophilized to provide bridged Oxytocin S-VBZ **28** (95% pure; 3.0 mg, 0.0020 mmol, 23% yield) as a white, fluffy solid.



Peak Results (Area Percent at least 1%)

RT (min)	Signal Description	Width (min)	Area	Height	Area%
7.353	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.346	2142931.6	469212.4	5.25
7.700	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.335	5963728.1	1150162.8	14.62
8.427	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.365	3292677.8	412857.9	8.07
8.977	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.597	3405250.0	402199.4	8.35
9.728	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.577	16706825.2	2153015.8	40.94
10.760	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.292	1370869.5	136534.4	3.36
11.979	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.379	1909592.8	170364.7	4.68
12.271	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.350	873195.1	129681.0	2.14
13.115	MS1 +TIC SCAN ESI Frag=135V Gain=1,0	0.543	5045016.3	826650.5	12.36
	Sum MS1 +TIC SCAN ESI Frag=135V Gain=1,0		40710086.4		



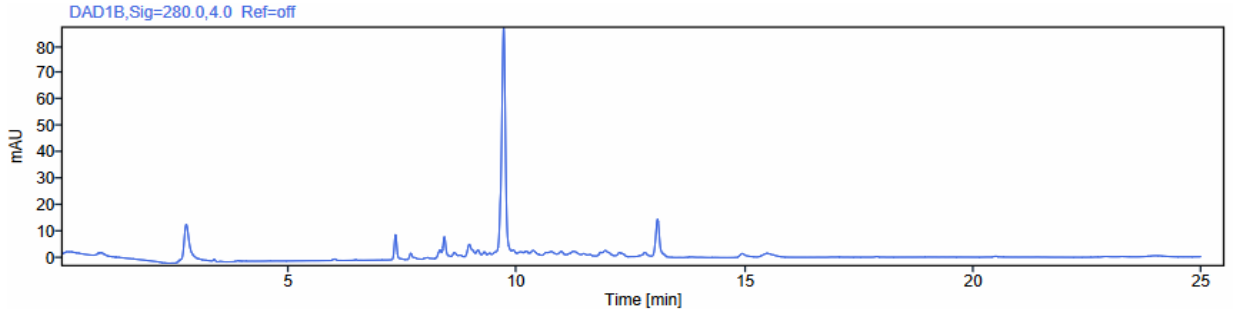


Figure S59: HPLC-MS and HPLC-UV (at 254 and 280 nm) chromatograms of the intramolecular cross-conjugation of oxytocin (**27**) with **15**, after 24 hours. The compound eluted at ca. 13.6 minutes is of non-identified origin.

HPLC-MS: Retention time (Method 1) = 10.5 minutes.
HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + 2H]^{+2}$ Calcd for $C_{60}H_{77}F_4IN_{16}O_{15}S_3^{+2}$ 780.1943; Found 780.1938.

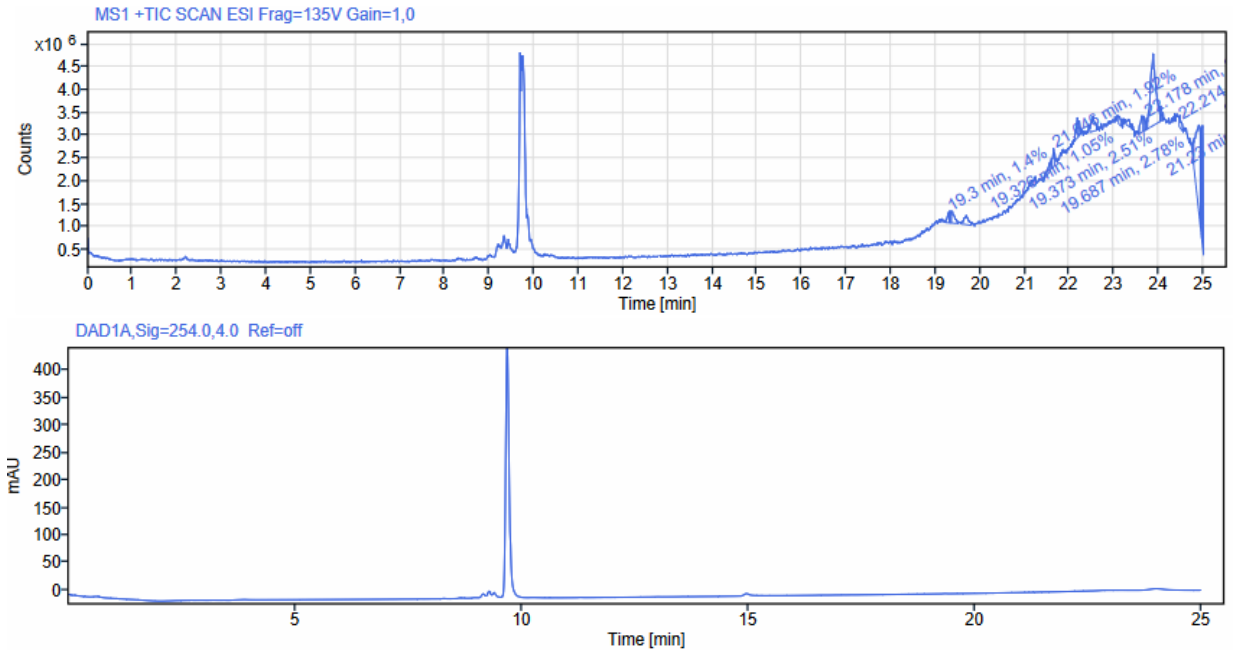
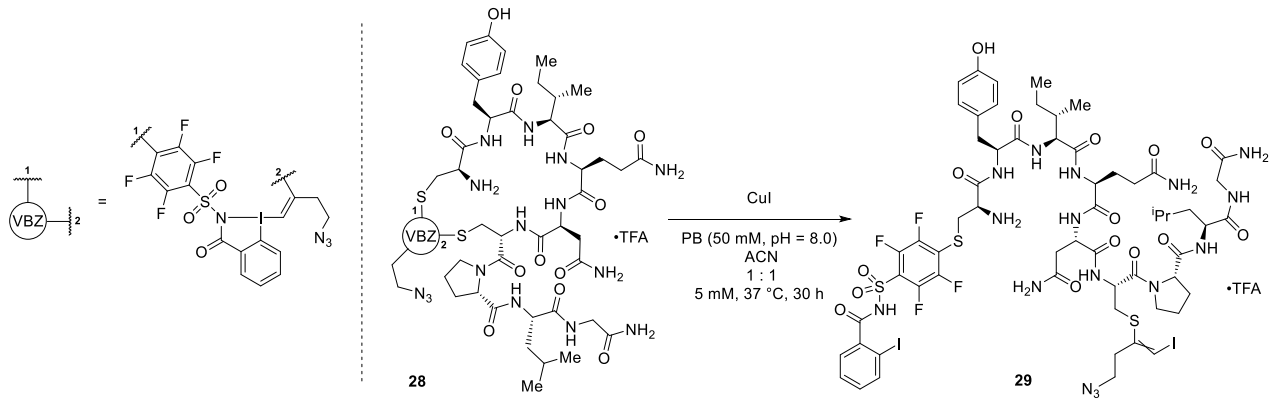


Figure S60. HPLC-MS and HPLC-UV (at 280 nm) chromatograms of pure **28**.

CuI promoted cleavage of rebridged oxytocin



In a 10 mL round-bottomed vial, oxytocin-VBZ **28** (3.3 mg, 0.0021 mmol, 1.0 equiv.) and CuI (4.8 mg, 0.025 mmol, 12 equiv.) were mixed with PB (50 mM, pH = 8.0; 0.20 mL) and DMF (0.20 mL). The resulting mixture was stirred at 37 °C, turning at first to pinkish and then becoming a homogeneous, pale green suspension. After 30 hour, HPLC-MS analysis showed the almost complete disappearance of the starting material and the presence of the cleaved product **29** (Figure S61). The reaction was therefore stopped, and the mixture was diluted with milliQ water (1.0 mL) and ACN (0.4 mL), and the solids were filtered off using a micropore filter. The filtrate was submitted to preparative HPLC (method 3; retention time of **29** = 11.3-11.5 minutes). Upon lyophilization, product **29** (90-95% purity; 2.5 mg, 0.0014 mmol, 70% yield) was collected as a white, fluffy solid.

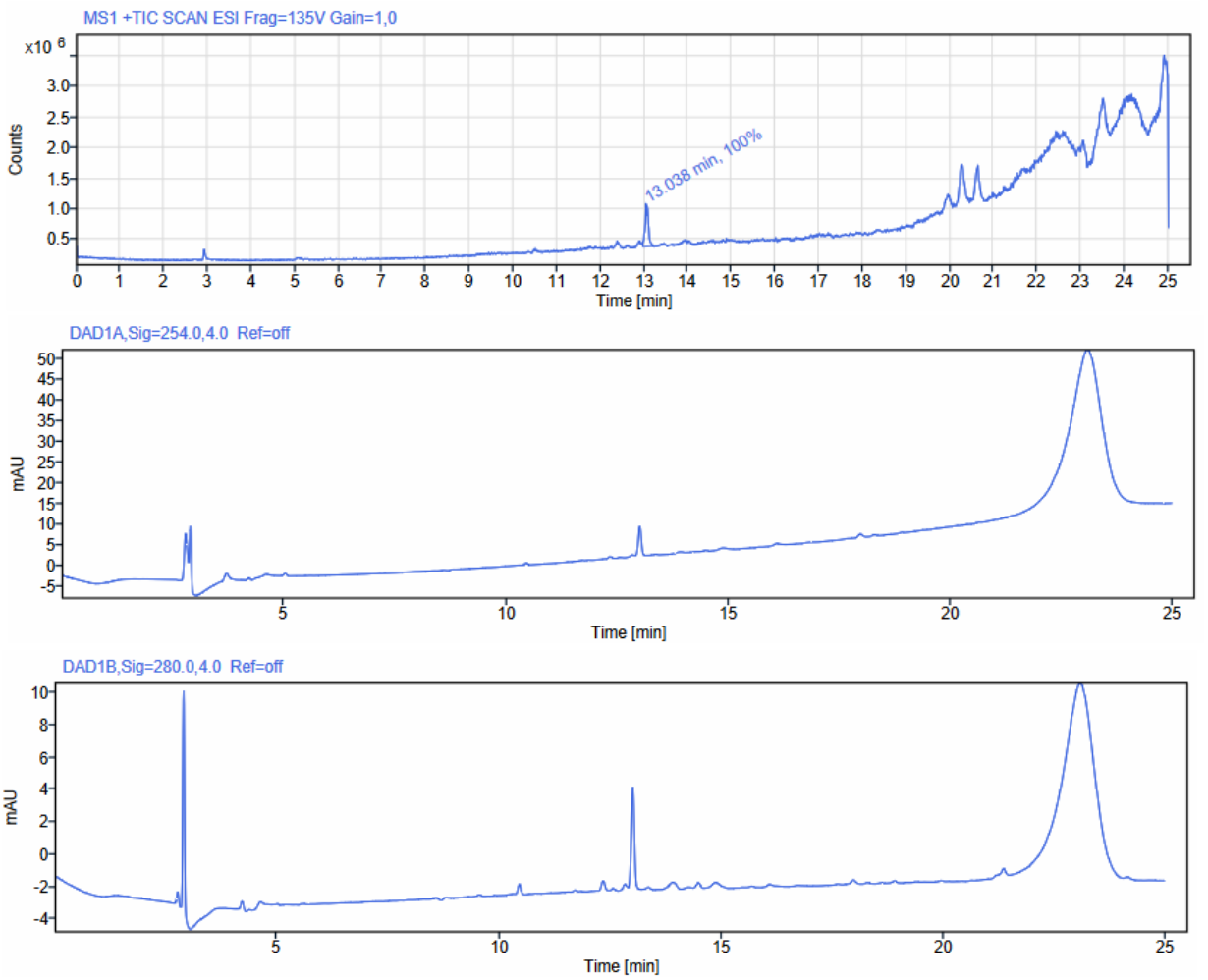


Figure S61: HPLC-MS and HPLC-UV (at 254 and 280 nm) chromatograms of the cleavage of intramolecular conjugate **28**, after 30 hours.

HPLC-MS: Retention time (Method 1) = 13.0 minutes.
HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H_2]^{+2}$ Calcd for $C_{60}H_{78}F_4I_2N_{16}O_{15}S_3^{+2}$ 844.1505; Found 844.1495.

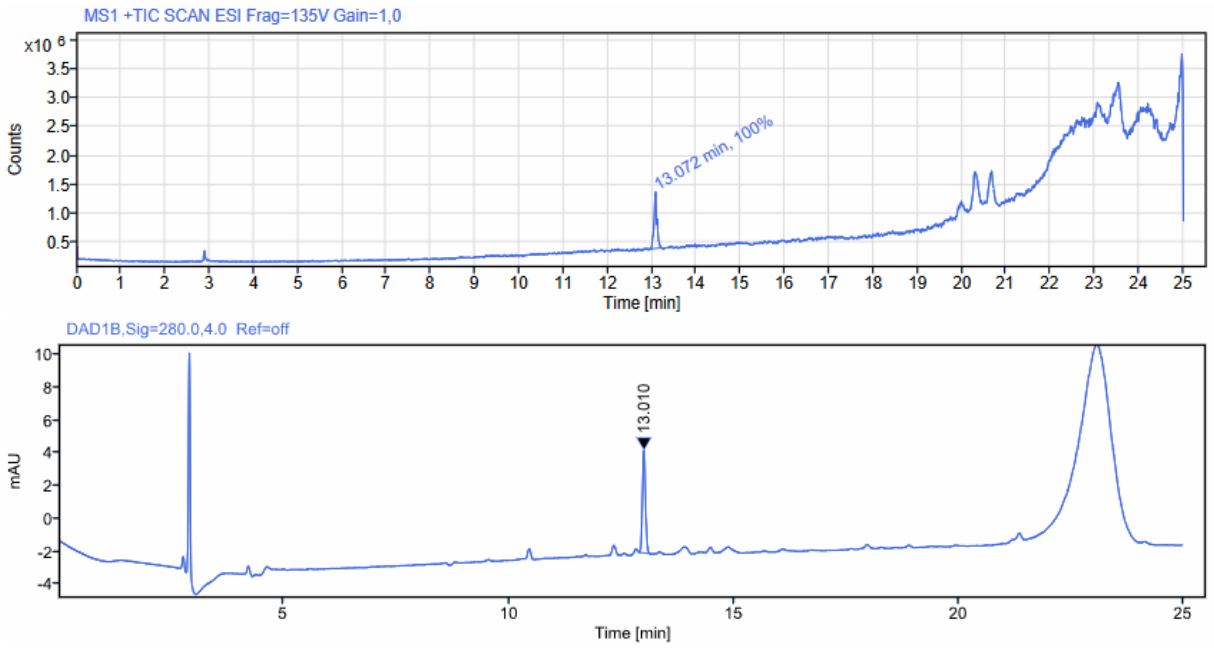


Figure S62. HPLC-MS and HPLC-UV (at 280 nm) chromatograms of pure **29**.

The structure of compound **29** was determined based on a MS-MS experiment (Figure S63)

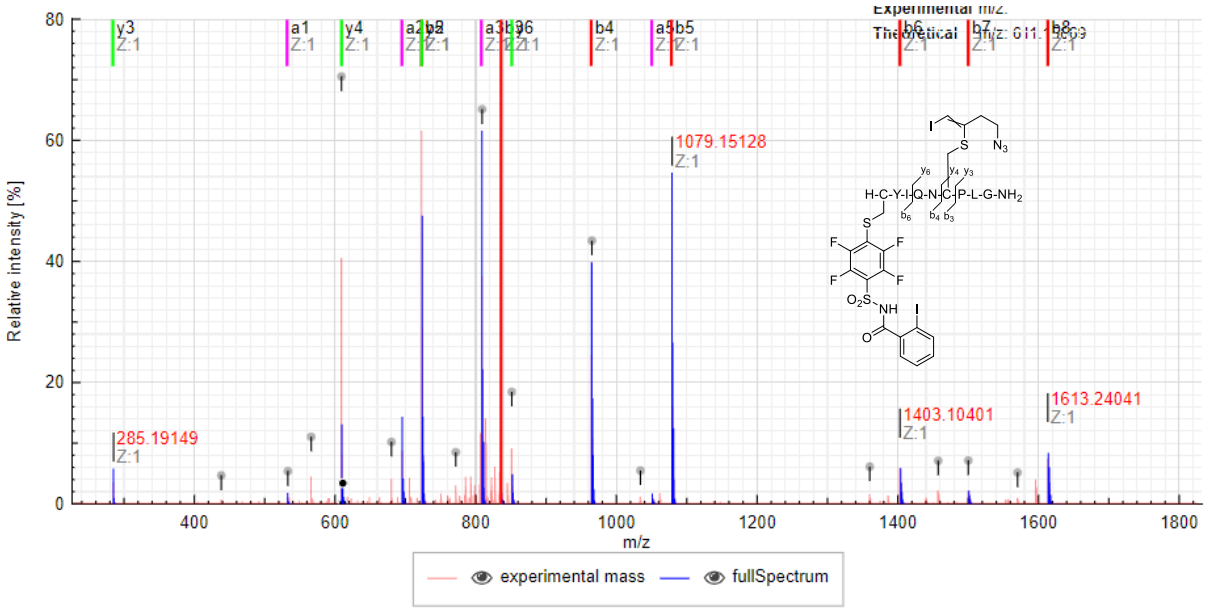


Figure S63. LC-MS/MS of **29**.

4.2 Fab Fragments

4.2.1 Preparation of Fab fragments

Buffers were prepared with double-deionized water and filter sterilized (0.20 µm). **Phosphate Buffer (PB; 1 M, pH 7.4)** contains a mixture of 1 M NaH_2PO_4 and 1 M Na_2HPO_4 in a 2:21 v/v ratio. **Borate Buffer Saline (BBS)** contains 25 mM boric acid (H_3BO_3), 25 mM sodium chloride (NaCl), and 2 mM ethylenediaminetetraacetic acid (EDTA) and pH was adjusted accordingly. **Phosphate Buffer Saline (PBS)** contains 10 mM PB, 138 mM NaCl, 2 mM EDTA, pH 8. **Tris buffer** contains 1000 mM Tris base, 2 mM EDTA, pH 8. **Sodium acetate buffer** contains 20 mM NaOAc, pH 3.1. **Digestion buffer 1** contains 50 mM PB, 150 mM NaCl, 1 mM EDTA, pH 6.8. **Digestion buffer 2** contains 50 mM PB, 150 mM NaCl, 1 mM EDTA, 10 mM DTT, pH 6.8. **Digestion buffer 3** contains sodium dihydrogen phosphate 20 mM (NaH_2PO_4), 10 mM EDTA, 80 mM cysteine·HCl, pH 7.
Incubation during digestion, reduction, and conjugation experiments took place in an Eppendorf Thermomixer Comfort equipped with a 2 mL block.

Preparation of trastuzumab (Trazimera) & avelumab Fab fragments
A solution (1 mL, 107 µM in water) of the monoclonal antibody (mAb) was buffer exchanged into **sodium acetate buffer** via ultrafiltration (10 kDa MWCO), followed by determination of concentration by NanoDrop.
Thermo Scientific™ Immobilized Pepsin (Agarose Resin) (732 µL) was split in two ways, each of which was loaded onto a Pierce™ centrifuge column and washed with sodium acetate buffer three times. Waste was discarded.
A solution of buffer-swapped mAb (0.50 mL) was then added to the activated pepsin and the mixture was incubated at 37 °C for 5 h under constant agitation (1 100 rpm). The resin was then removed from the digest using a Pierce™ centrifuge column and was washed three times with 0.5 mL of **digestion buffer 1**. The filtrates from the washes containing $F(ab')_2$ were combined, concentrated with 15 mL Falcon viva spin tubes (10 kDa MWCO) before the volume was adjusted to 0.5 mL.
At the beginning of the fourth hour of pepsin incubation, Thermo Scientific™ Immobilized Papain (Agarose Resin) (1.22 mL) was split in three ways, each of which was loaded onto a separate Pierce™ centrifuge columns, concentrated and washed three times with 0.5 mL of **digestion buffer 2**. The resulted papain in **digestion buffer 2** was then incubated at 37 °C for 90 min under constant agitation (1 100 rpm) before it was washed ten times with 0.5 mL of digestion buffer 1 (i.e., **without DTT**) using a Pierce™ centrifuge column, in order to remove DTT before adding the antibody.
The 0.5 mL of $F(ab')_2$ solution obtained before was added to the Pierce™ centrifuge column containing the activated papain and the mixture was incubated at 37 °C for 16 h under constant agitation (1 100 rpm). Effective Fab formation was confirmed by SDS-PAGE analysis. The resin was then separated and washed four times with 0.5 mL of PBS 1X pH 7.5. The filtrates were combined, concentrated with a 15 mL Falcon viva spin tube (10 kDa MWCO) and purified via steric exclusion chromatography (SEC) on an ÄKTA pure system. Aliquots of Traztuzumab/Avelumab-Fabs were stored at -20 °C for up to 6 months.

Preparation of rituximab Fab fragment
Immobilized papain (0.3 mL, 0.25 mg/mL) was loaded onto a Pierce™ centrifuge column, concentrated and washed 3 times with **digestion buffer 2**. The resin-bound papain in **digestion buffer 2** was then incubated at 37 °C under constant agitation (1 100 rpm) for 1 h before the resin was washed six times with 0.5 mL of **digestion buffer 1**. Rituximab (3 mg in 0.5 mL of digestion buffer) was then added to the resin-bound papain and the mixture was incubated for 16 h at 37 °C under constant agitation (1 100 rpm). The resin was then separated from the digested rituximab by washing the Pierce™ centrifuge column 4 times with 0.5 mL of **Pierce™ protein A binding buffer** (100 mM phosphate, 150 mM NaCl, pH 7.2). The filtrates containing digested rituximab were combined and buffer exchanged to **protein A binding buffer** – in order to get rid of **digestion buffer 1** – and the volume adjusted to 1.5 mL before it was loaded onto a NAb™ protein A column (Thermo Scientific, France) and incubated at 20 °C with end-over-end mixing for 10 min. Filtration of the NAb™ protein A column followed by three washes with protein A binding buffer delivered rituximab Fab (concentration determined by NanoDrop), whose aliquots were stored at -20 °C for up to 6 months.

Preparation of bevacizumab Fab fragment
Bevacizumab (0.5 mL, 10 mg/mL) was buffer exchanged into **digestion buffer 3**. Thermo Scientific™ Immobilized Papain (Agarose Resin) (0.5 mL, 0.25 mg/mL) was loaded onto a Pierce™ centrifuge column, concentrated and washed three times with **digestion buffer 3**. The previously made bevacizumab solution was added to the resin-bound papain and the mixture was incubated at 37 °C for 4 h under constant agitation (1 100 rpm). The resin was then separated from the digested bevacizumab by washing the Pierce™ centrifuge column four times with 0.5 mL of **Pierce™ protein A binding buffer**. The filtrates were combined and completely buffer exchanged to **Pierce™ protein A binding buffer** while the volume was adjusted to 1.5 mL. The sample was then loaded onto a NAb™ protein A column (Thermo Scientific, France) and incubated at 20 °C with end-over-end mixing for 10 min. The bevacizumab Fab was finally obtained after filtration of the protein A column followed by three washes with 0.5 mL of **Pierce™ protein A binding buffer**. The filtrates were combined, concentrated using a 15 mL Falcon viva spin tube (10 kDa MWCO) and purified by steric exclusion chromatography (SEC). Concentration in bevacizumab Fab was determined by NanoDrop and the resulting aliquots were stored at -20 °C for up to 6 months.

4.2.2 Bioconjugation experiments

Step-wise reduction-conjugation procedure
Reduction of Fab fragments. To a solution of Fab (1.5 mg/mL, 1 equiv., 50 µL in PBS 1x with 1% EDTA, pH 7.5) was added TCEP (15 mM in H_2O, 5 equiv., 0.52 µL). The reaction mixture was then incubated for 1.5 hours at 37 °C with constant agitation (650 rpm), before the excess of reagent was removed by gel filtration chromatography on Zeba™ Spin Desalting Columns, 7K MWCO, pre-equilibrated with BBS (25 mM borate, 25 mM NaCl, 2 mM EDTA, pH 8) to give a solution of reduced Fab in BBS, whose concentration was determined by NanoDrop.
Conjugation step. To a solution of reduced Fab (1.5 mg/mL, 1 equiv., 50 µL) in the desired buffer containing EDTA was added EBZ reagent **15** (10 mM solution in DMSO, MeCN or DMF). The resulting solution was then incubated at either 4 °C, 25 °C or 37 °C, with constant agitation (650 rpm), before the excess of reagent was removed by gel filtration chromatography on Zeba™ Spin Desalting Columns, 7K MWCO pre-equilibrated with PBS 1X pH 7.5 to give a solution of rebridged Fab.

One-pot reduction-conjugation procedure (Fab)
To a solution of Fab (1.5 mg/mL, 1 equiv., 50 µL in BBS, 2 mM EDTA, pH 8) was added TCEP (15 mM in water, 5 equiv., 0.52 µL) and EBZ reagent **15** (10 mM solution in DMSO, 5 equiv., 0.79 µL). The resulting solution was then incubated for 5 h at 37 °C with constant agitation (650 rpm), before the excess of reagent was removed by gel filtration chromatography on Zeba™ Spin Desalting Columns, 7K MWCO, to give a solution of rebridged Fab **31**.

One-pot reduction-conjugation procedure (mAb)
To a solution of trastuzumab (2.5 mg/mL, 1 equiv., 50 µL in BBS, 6 mM EDTA, pH 8) was added TCEP (15 mM in H_2O, 10 equiv., 0.55 µL) and EBZ reagent **15** (10 mM solution in DMSO, 10 equiv., 0.82 µL). The resulting solution was then incubated for 5 h at 37 °C with constant agitation (650 rpm), before the excess of reagent was removed by gel filtration chromatography on Zeba™ Spin Desalting Columns, 7K MWCO, to give a solution of functionalized trastuzumab.

Functionalization step (SPAAC reaction)
To a solution of rebridged Fab (1 equiv., 50 µL in PBS 1X, pH 7.5) was added BCN-Iminobiotin **34** (10 mM solution in DMSO, 30 equiv.). The resulting mixture was then incubated for 24 h at 25 °C before the excess of reagent was removed by gel filtration chromatography on Zeba™ Spin Desalting Columns, 7K MWCO.

One-pot reduction-conjugation-functionalization procedure
To a solution of Fab (1.5 mg/mL, 1 equiv., 50 µL in BBS, 2 mM EDTA, pH 8) was added TCEP (15 mM in H_2O, 5 equiv., 0.52 µL), EBZ reagent **15** (10 mM solution in DMSO, 5 equiv., 0.79 µL), and BCN-Iminobiotin **34** (10 mM solution in DMSO, 20 equiv., 3.15 µL). The resulting solution was then incubated for 5 h at 37 °C with constant agitation (650 rpm), before the excess of reagent was removed by gel filtration chromatography on Zeba™ Spin Desalting Columns, 7K MWCO, to give a solution of functionalized trastuzumab Fab **35**.

Copper(I)-mediated reduction procedure
A solution of copper(I) iodide (20 equiv., 15 mM in water), THPTA (40 equiv., 160 mM in water), aminoguanidine hydrochloride (100 equiv., 100 mM in water) and sodium ascorbate (300 equiv., 400 mM in water) was first prepared and incubated for 10 min at 25 °C without agitation, before it was added to a solution of rebridged trastuzumab Fab **31** (1 equiv., 2 mg/mL in PBS 1X, pH 7.4). The resulting reaction mixture was then incubated at 37 °C for 16 h with constant agitation (650 rpm), before the excess of reagent was removed by gel filtration chromatography on Zeba™ Spin Desalting Columns, 7K MWCO.

Optimisation of the rebridging of trastuzumab Fab 30

Table S8. Representative results for the rebridging of trastuzumab Fab **30** with EBZ **15** (highlighted in yellow are the conditions for which analytical data is provided below)

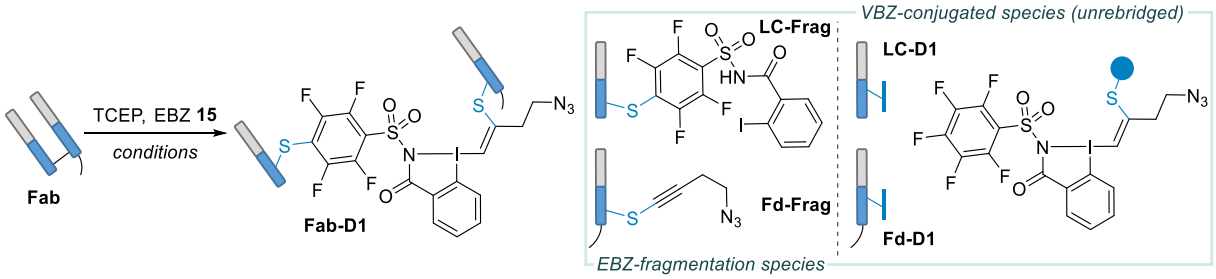
	Entry	Conditions	FAB (%)	avDoC[a]
Two-step reduction-conjugation	S1	EBZ (2 equiv. in MeCN), PBS 1x buffer, pH 8.5, 25 °C, 20 min	86	0.34
	S2	EBZ (2 equiv. in DMSO), PBS 1x buffer, pH 8.5, 25 °C, 20 min	83	0.33
	S3	EBZ (2 equiv. in DMSO), PBS 1x buffer, pH 8, 25 °C, 20 min	92	0.41
	S4	EBZ (2 equiv. in DMSO), PBS 1x buffer, pH 8, 37 °C, 20 min	82	0.57
	S5	EBZ (2 equiv. in DMSO), PBS 1x buffer, pH 8, 37 °C, 2 h	68	0.28
	S6	EBZ (2 equiv. in DMSO), PBS 1x buffer, 6 mM EDTA, pH 8, 37 °C, 2 h	77	0.25
	S7	EBZ (2 equiv. in DMSO), PBS 1x buffer, 6 mM EDTA, pH 8, 37 °C, 16 h	74	0.31
	S8	EBZ (5 equiv. in DMSO), PBS 1x buffer, 6 mM EDTA, pH 8, 37 °C, 16 h	75	0.30
	S9	EBZ (5 equiv. in DMSO), PBS 1x buffer, 2 mM EDTA, pH 8, 37 °C, 16 h	66	0.52
	S10	EBZ (5 equiv. in DMSO), BBS buffer, 2 mM EDTA, pH 8, 37 °C, 16 h	70	0.80
	S11	EBZ (5 equiv. in DMSO), BBS buffer, 2 mM EDTA, pH 8, 37 °C, 6 h	79	0.41
One-pot reduction-conjugation procedure	S12	EBZ (5 equiv. in DMSO), BBS buffer, 2 mM EDTA, pH 8, 37 °C, 6 h	70	1
	S13	EBZ (5 equiv. in DMSO), PBS 1x buffer, 2 mM EDTA, pH 8, 37 °C, 6 h	71	0.91
	S14	EBZ (5 equiv. in MeCN), PBS 1x buffer, 2 mM EDTA, pH 8, 37 °C, 6 h	69	0.91
	S15	EBZ (5 equiv. in DMF), PBS 1x buffer, 2 mM EDTA, pH 8, 37 °C, 6 h	67	0.93
	S16	EBZ (5 equiv. in DMSO), Tris buffer, 2 mM EDTA, pH 8, 37 °C, 6 h	82	0.27
	S17	EBZ (5 equiv. in MeCN), Tris buffer, 2 mM EDTA, pH 8, 37 °C, 6 h	80	0.36
	S18	EBZ (5 equiv. in MeCN), BBS buffer, 2 mM EDTA, pH 8, 37 °C, 6 h	58	0.95
	S19	EBZ (5 equiv. in DMF), BBS buffer, 2 mM EDTA, pH 8, 37 °C, 6 h	70	1
	S20	EBZ (5 equiv. in DMSO), BBS buffer, 2 mM EDTA, pH 6.5, 37 °C, 6 h	71	1
	S21	EBZ (5 equiv. in DMSO), BBS buffer, 2 mM EDTA, pH 7, 37 °C, 6 h	61	1
	S22	EBZ (5 equiv. in DMSO), BBS buffer, 2 mM EDTA, pH 7.5, 37 °C, 6 h	62	0.93
	S23	EBZ (5 equiv. in DMSO), BBS buffer, 2 mM EDTA, pH 8.5, 37 °C, 6 h	46	0.82
	S24	EBZ (5 equiv. in DMSO), BBS buffer, 2 mM EDTA, pH 8, 37 °C, 0.5 h	80	0.69
	S25	EBZ (5 equiv. in DMSO), BBS buffer, 2 mM EDTA, pH 8, 37 °C, 1 h	81	0.75
	S26	EBZ (5 equiv. in DMSO), BBS buffer, 2 mM EDTA, pH 8, 37 °C, 2 h	82	0.85
	S27	EBZ (5 equiv. in DMSO), BBS buffer, 2 mM EDTA, pH 8, 37 °C, 4 h	77	0.93
	S28	EBZ (5 equiv. in DMSO), BBS buffer, 2 mM EDTA, pH 8, 37 °C, 5 h	72	1
	S29	EBZ (5 equiv. in DMSO), BBS buffer, 2 mM EDTA, pH 8, 25 °C, 6 h	76	0.97
	S30	EBZ (5 equiv. in DMSO), BBS buffer, 2 mM EDTA, pH 8, 4 °C, 6 h	69	0.58
	S31	EBZ (7.5 equiv. in DMSO), BBS buffer, 2 mM EDTA, pH 8, 25 °C, 6 h	84	1.06[b]
	S32	EBZ (10 equiv. in DMSO), BBS buffer, 2 mM EDTA, pH 8, 25 °C, 6 h	77	1.08[b]

[a]: avDoC values concern only the FAB fragment; [b]: contains FAB-D2 species

Denaturing SEC-MS (dSEC-MS) analyses

SEC-UV chromatograms and MS spectra corresponding to the analyses of the most representative conjugation and rebridging conditions are given below.

Reduction and conjugation steps (Fab)



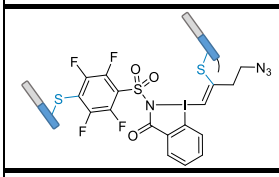
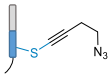
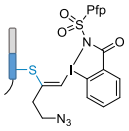
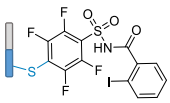
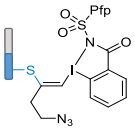
Fragment	Name (*mass increment*)	mAb	average MW (Da)	Code
	**Fab**	Trastuzumab	47 637	●
		Rituximab	47 179[a]	
		Bevacizumab	48 208	
		Avelumab	46 873	
	**Fab-D1** (*+550 Da*)	Trastuzumab	48 187	▲
		Rituximab	47 729	
		Bevacizumab	48 758	
		Avelumab	47 423	
	**Fd**	Trastuzumab	24 200	●
		Rituximab	24 145	
		Bevacizumab	24 763	
		Avelumab	24 073	
	**Fd-Frag** (*+93 Da*)	Trastuzumab	24 293	▲
		Rituximab	24 238	
		Bevacizumab	24 856	
		Avelumab	24 166	
	**Fd-D1** (*+570 Da*)	Trastuzumab	24 770	■
		Rituximab	24 715	
		Bevacizumab	25 333	
		Avelumab	24 643	
	**LC**	Trastuzumab	23 439	●
		Rituximab	23 036	
		Bevacizumab	23 447	
		Avelumab	22 802	
	**LC-Frag** (*+457 Da*)	Trastuzumab	23 896	▲
		Rituximab	23 493	
		Bevacizumab	23 904	
		Avelumab	23 259	
	**LC-D1** (*+570 Da*)	Trastuzumab	24 009	■
		Rituximab	23 606	
		Bevacizumab	24 017	
		Avelumab	23 372	

Figure S64. Overview of all species formed during Fab rebridging with their structures and molecular weights. The color code is used in the copies of mass spectra given below, to facilitate identification and understanding. [a]: the MW is that of a Fab species with two *N*-terminal pyroglutamate residues – instead of two glutamines –, as observed in various commercial samples.[4]

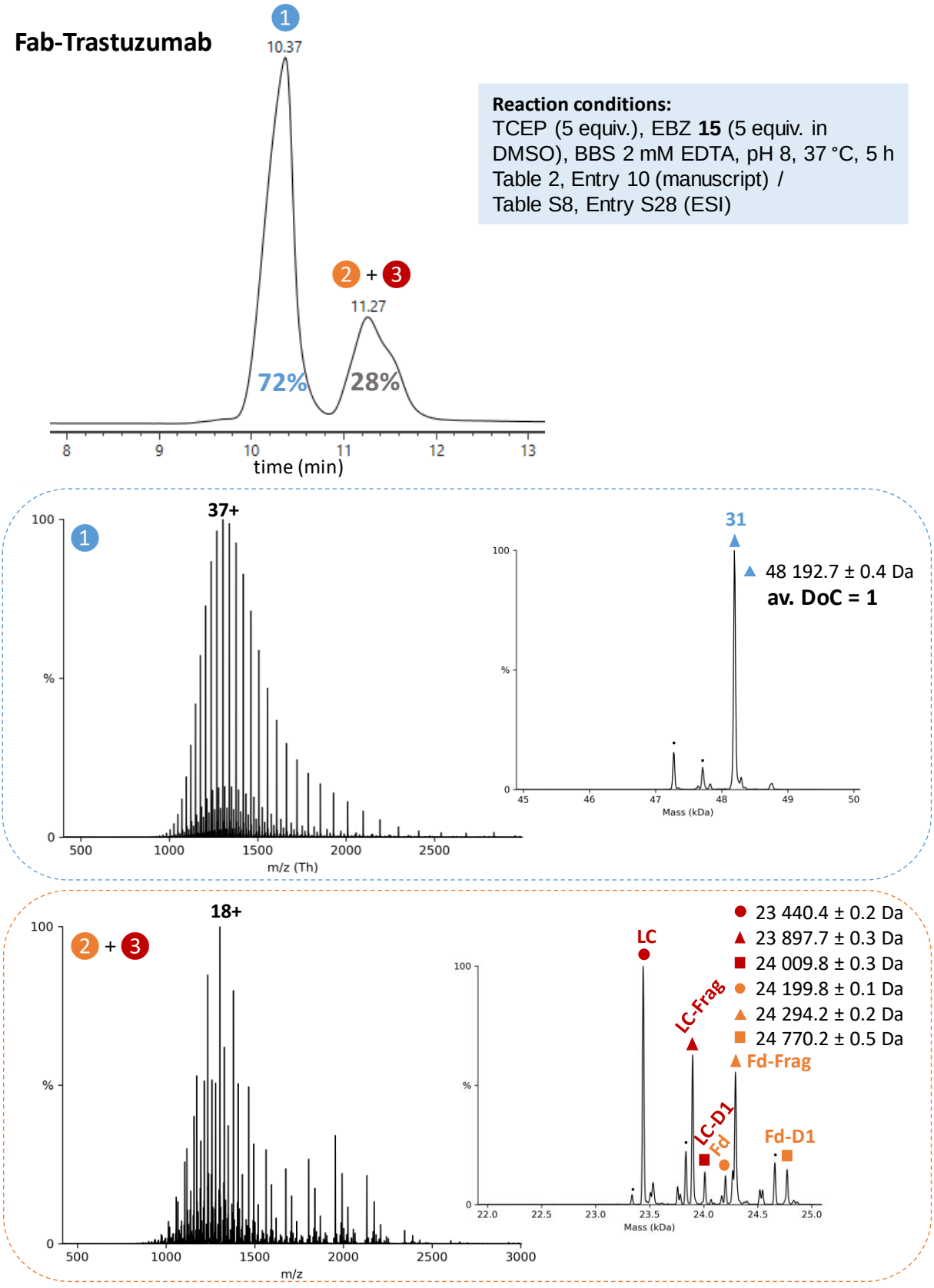


Figure S65. SEC-UV chromatogram and the corresponding MS raw and deconvoluted spectra of each peak corresponding to conjugation conditions described in Table S8, entry S28. The small black dots correspond to species resulted from enzymatic over digestion of Fab fragment and their corresponding conjugates with EBZ **15**.

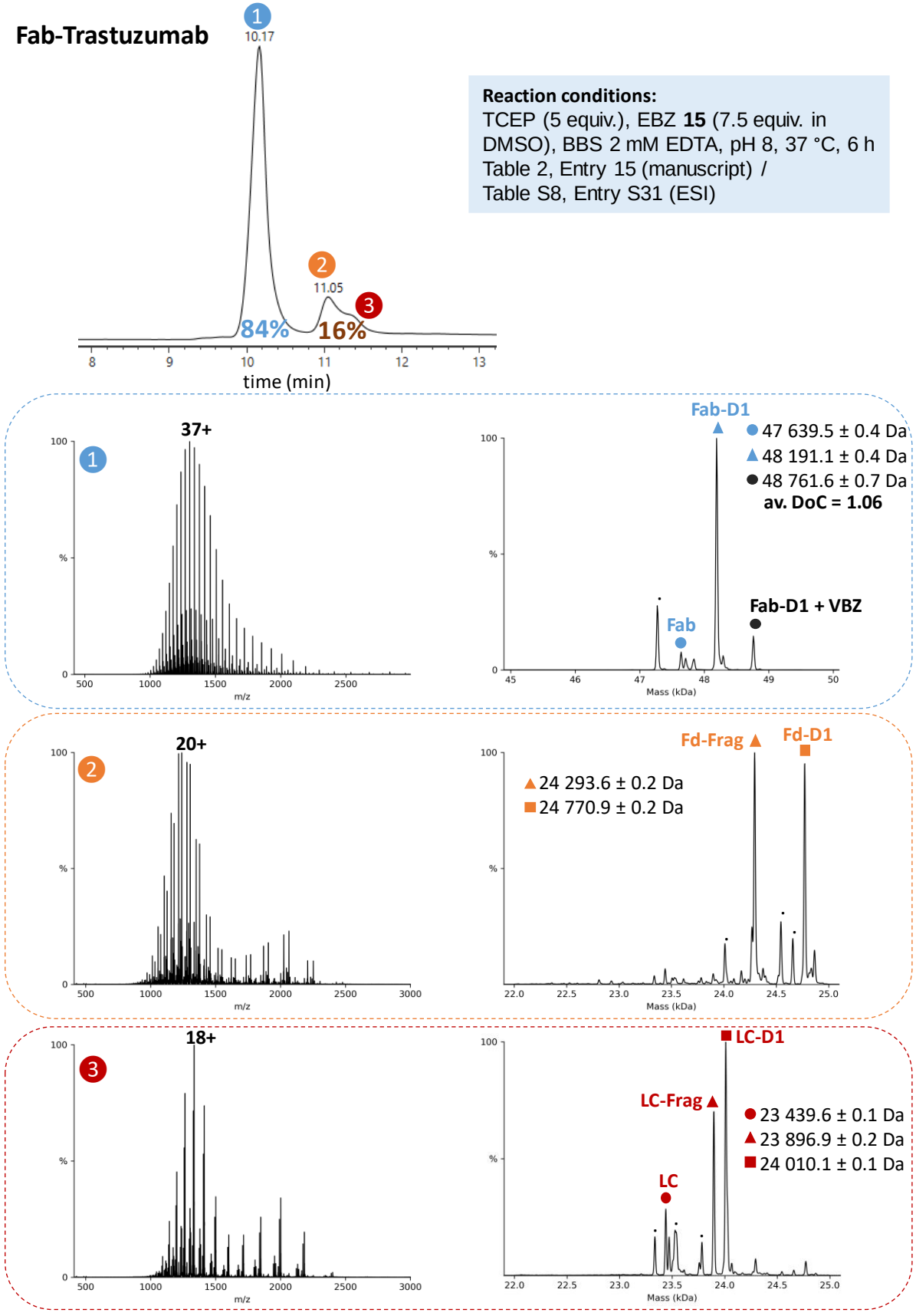


Figure S66. SEC-UV chromatogram and the corresponding raw and deconvoluted MS spectra of each peak corresponding to conjugation conditions described in Table S8, entry S31. The small black dots correspond to species resulted from enzymatic over digestion of Fab fragment and their corresponding conjugates with EBZ **15**.

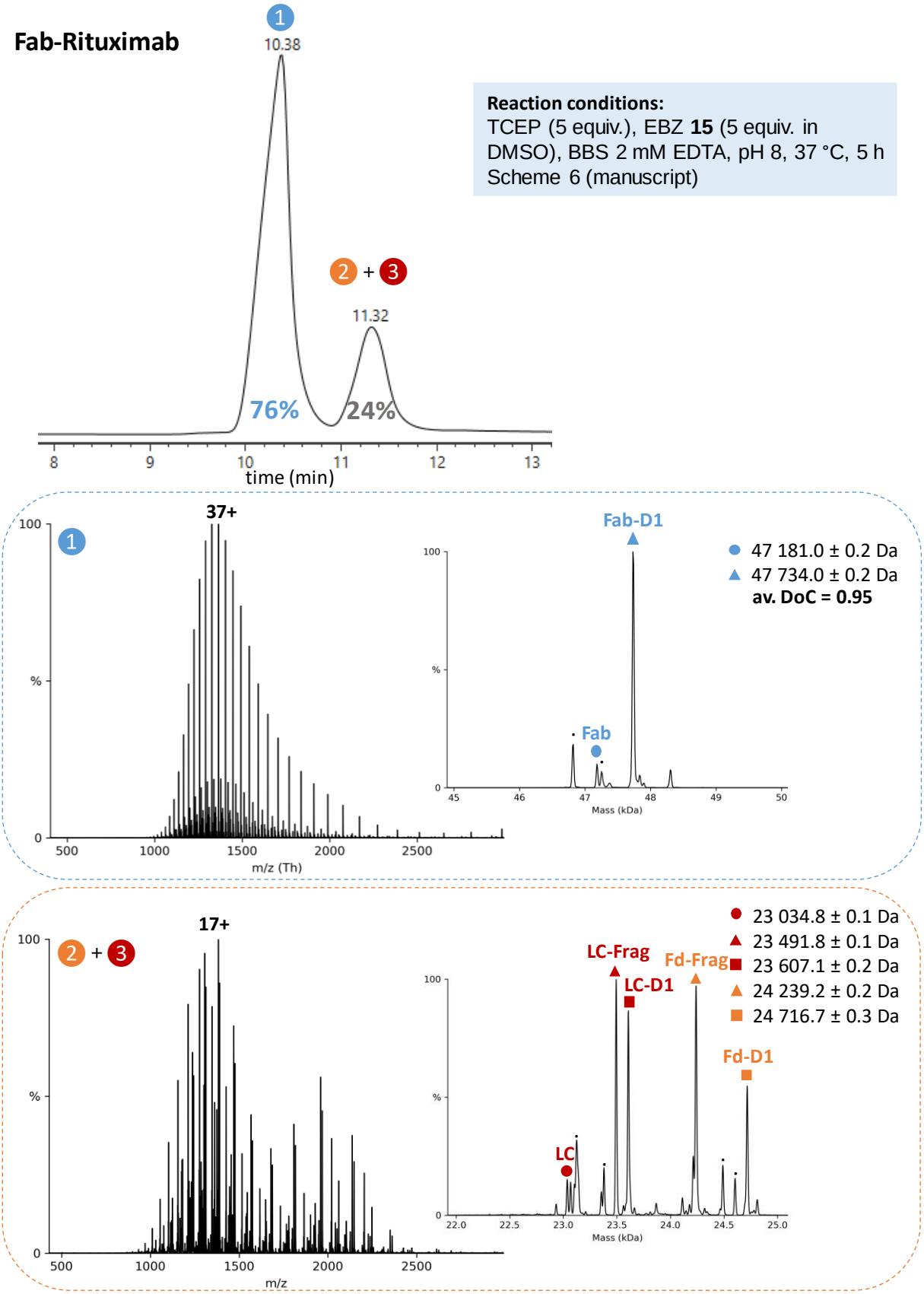


Figure S67. SEC-UV chromatogram and the corresponding raw and deconvoluted MS spectra of each peak corresponding to the conjugation of rituximab Fab under optimised conditions, *viz.* those described in Table S8, entry S28. The small black dots correspond to species resulted from enzymatic over digestion of Fab fragment and their corresponding conjugates with EBZ **15**.

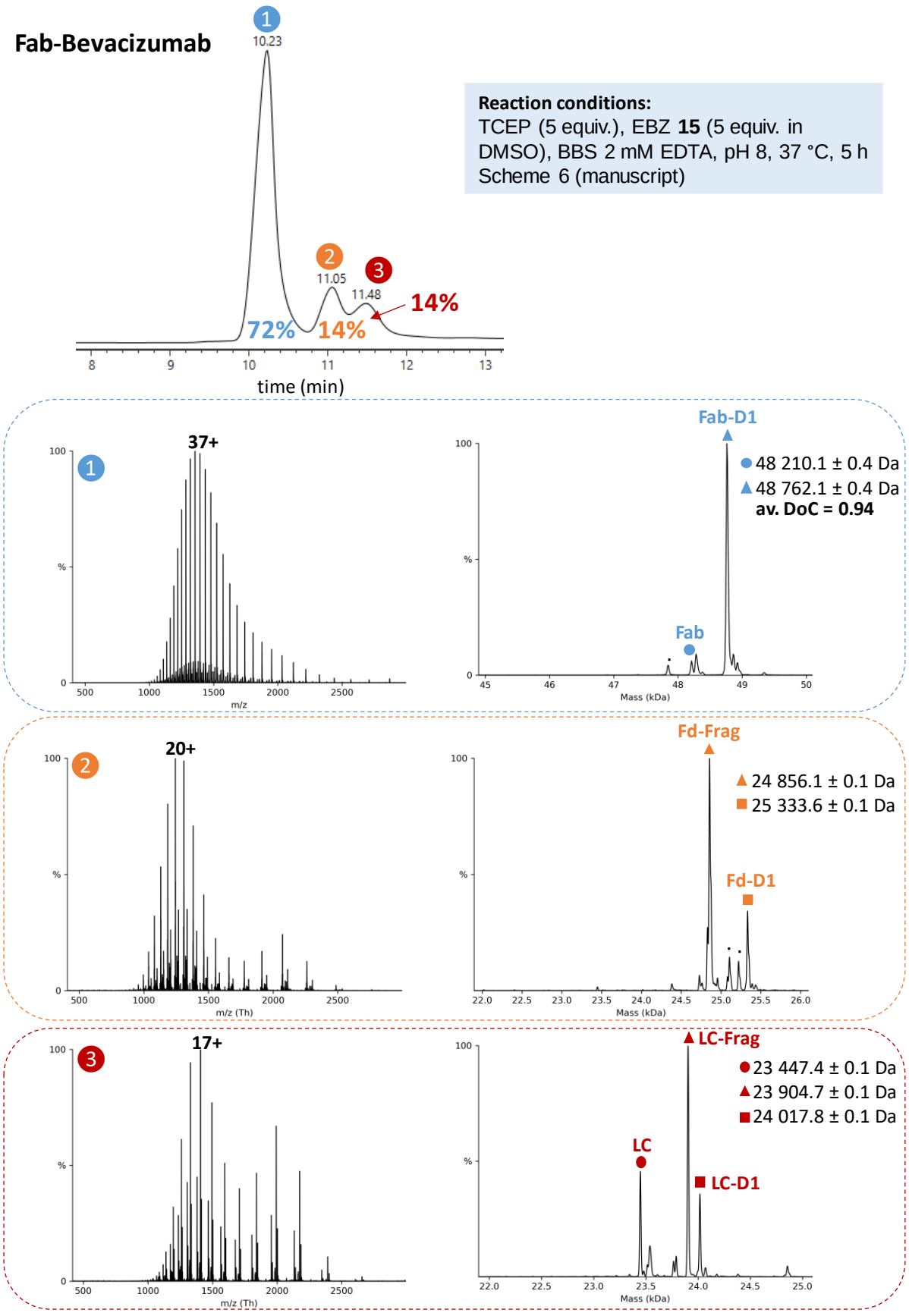


Figure S68. SEC-UV chromatogram and the corresponding raw and deconvoluted MS spectra of each peak corresponding to the conjugation of bevacizumab Fab under optimised conditions, *viz.* those described in Table S8, entry S28. The small black dots correspond to species resulted from enzymatic over digestion of Fab fragment and their corresponding conjugates with EBZ **15**.

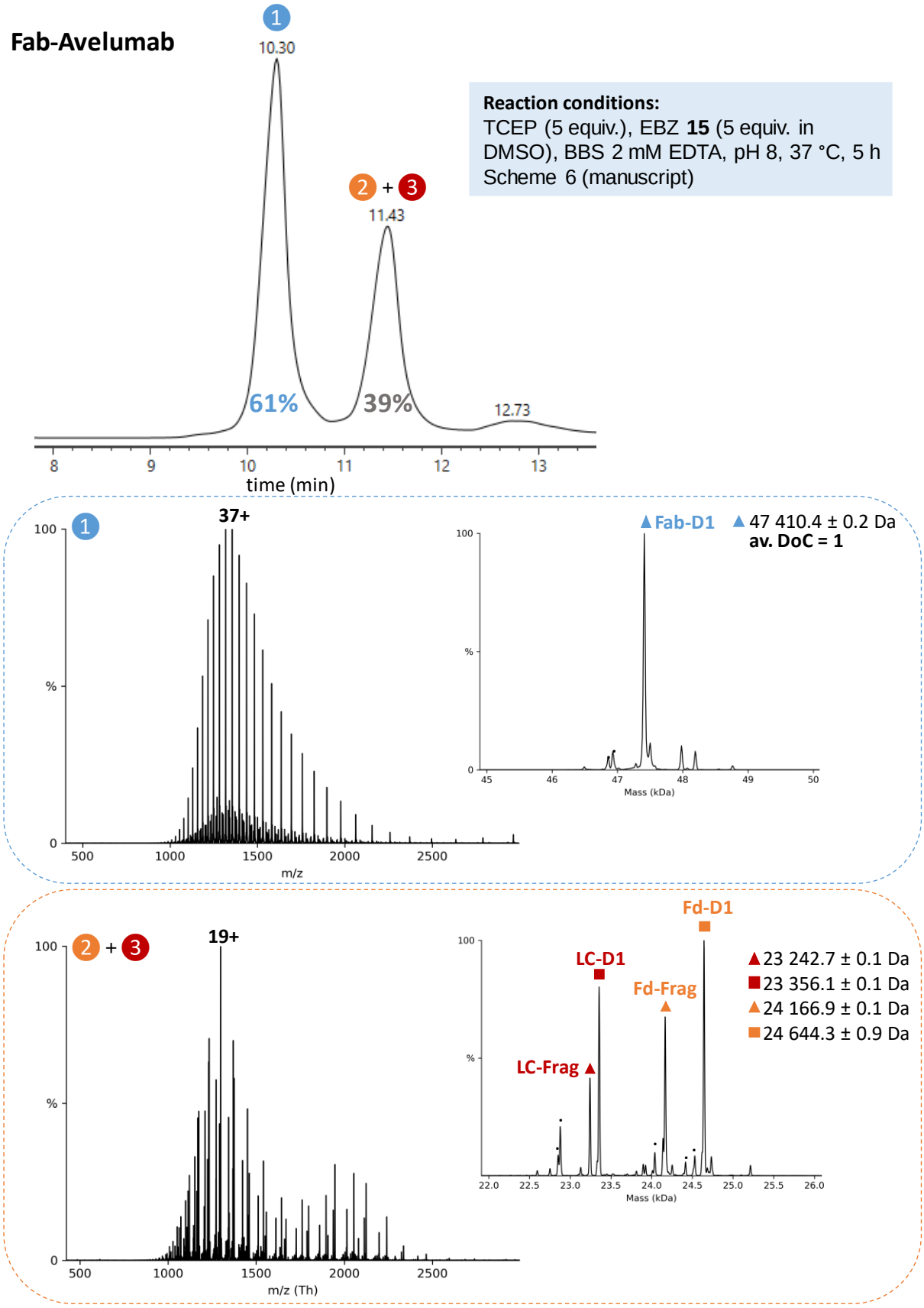
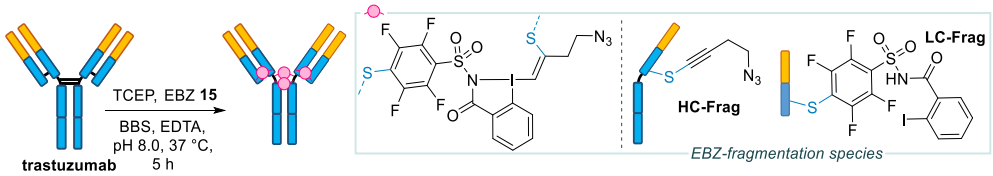


Figure S69. SEC-UV chromatogram and the corresponding raw and deconvoluted MS spectra of each peak corresponding to the conjugation of avelumab Fab under optimised conditions, *viz.* those described in Table S8, entry S28. The experimental MW indicate that the LC *N*-terminal glutamine has cyclized to a pyroglutamate. The small black dots correspond to species resulted from enzymatic over digestion of Fab fragment and their corresponding conjugates with EBZ **15**.

Reduction and conjugation steps (mAb)



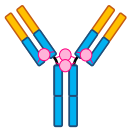
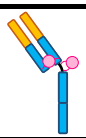
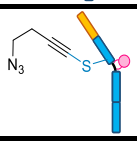
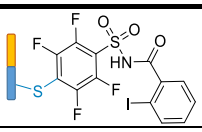
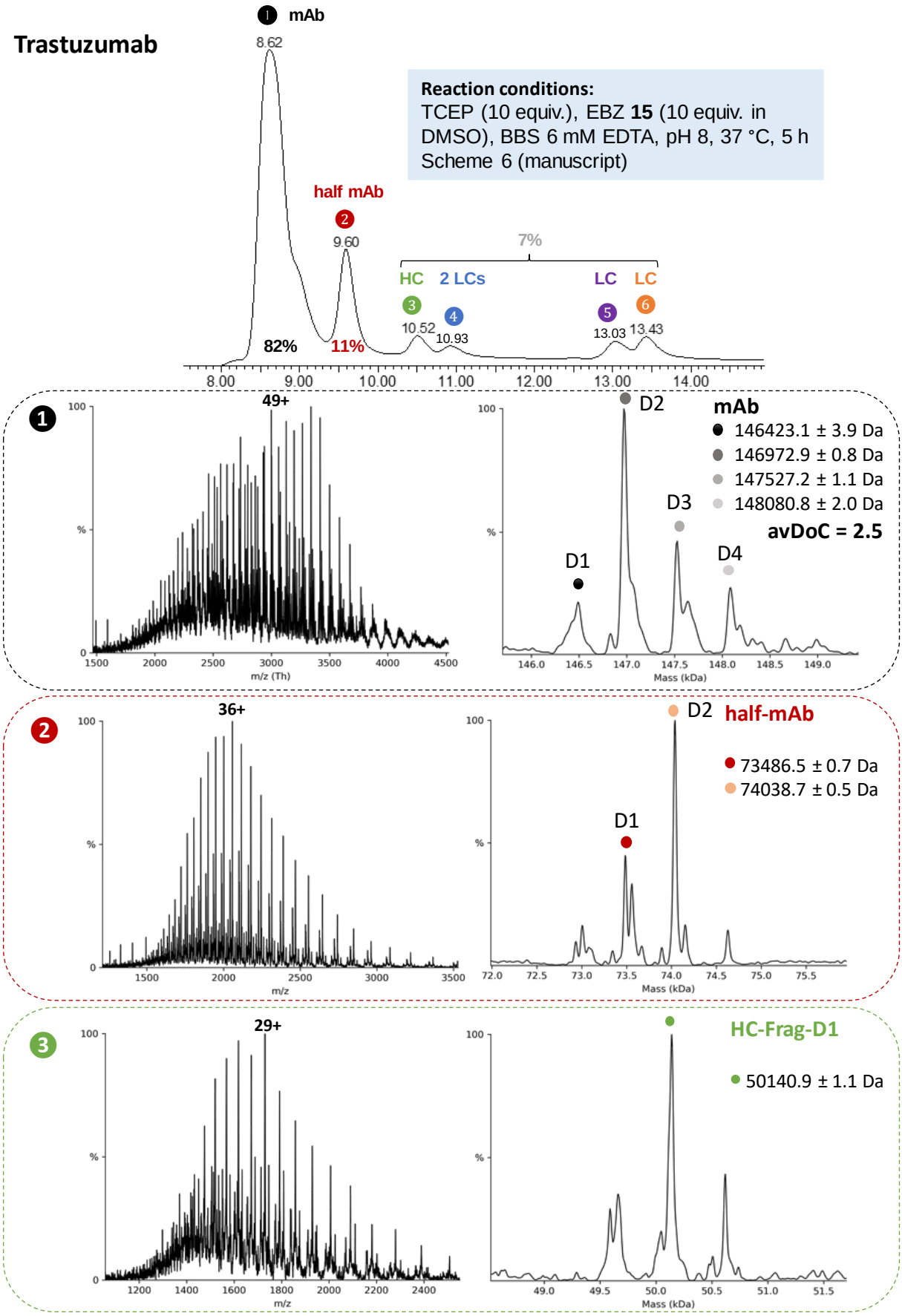
Species	Name (*mass increment*)	average MW (Da)	Code
	**mAb (D0)**	145 875	
	D1 (*+550 Da*)	146 425	
	D2 (*+1 100 Da*)	146 975	
	D3 (*+1 650 Da*)	147 525	
	D4 (*+2 200 Da*)	148 075	
	**half mAb (D0)**	72 937	
	D1 (*+550 Da*)	73 487	
	D2 (*+1 100 Da*)	74 037	
	**HC-Frag (D0)**	49 588	
	D1 (*+550 Da*)	50 138	
	**LC-dimer (D0)**	46 878	
	D1 (*+550 Da*)	47 428	
	**LC**	23 439	
	**LC-Frag** (*+457 Da*)	23 896	

Figure S70. Overview of all species formed during trastuzumab rebridging with their structures and molecular weights. The color code is used in the copies of mass spectra given below, to facilitate identification and understanding.


Trastuzumab
1 mAb
8.62
Reaction conditions:
TCEP (10 equiv.), EBZ 15 (10 equiv. in DMSO), BBS 6 mM EDTA, pH 8, 37 °C, 5 h
Scheme 6 (manuscript)
half mAb
2
9.60
7%
HC
2 LCs
LC
LC
3
4
5
6
10.52
10.93
13.03
13.43
82%
11%
8.00
9.00
10.00
11.00
12.00
13.00
14.00
1
49+
m/z (Th)
D2
D3
D1
D4
mAb
146423.1 ± 3.9 Da
146972.9 ± 0.8 Da
147527.2 ± 1.1 Da
148080.8 ± 2.0 Da
avDoC = 2.5
Mass (kDa)
2
36+
m/z
D2
half-mAb
73486.5 ± 0.7 Da
74038.7 ± 0.5 Da
D1
Mass (kDa)
3
29+
m/z
HC-Frag-D1
50140.9 ± 1.1 Da
Mass (kDa)

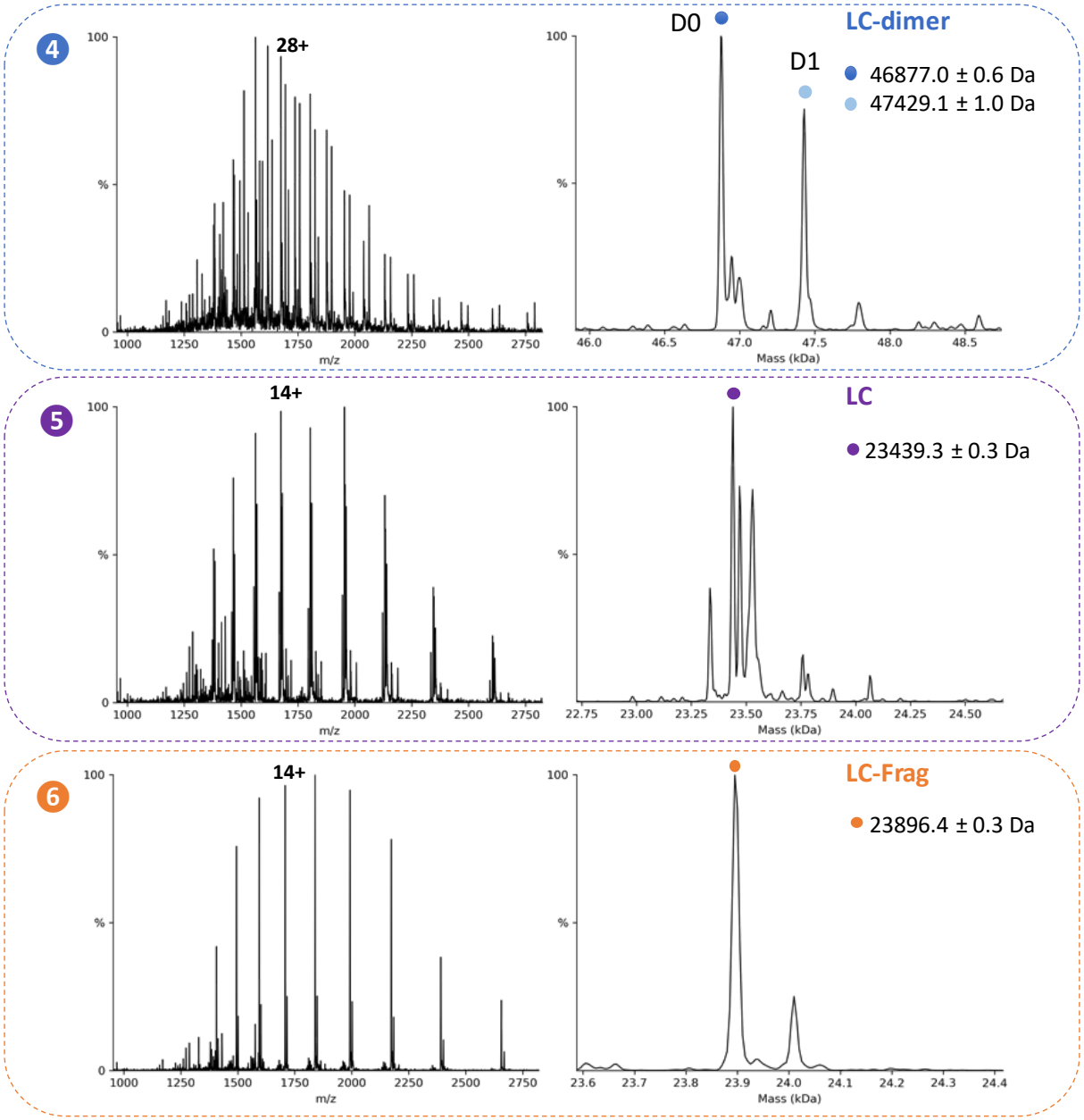
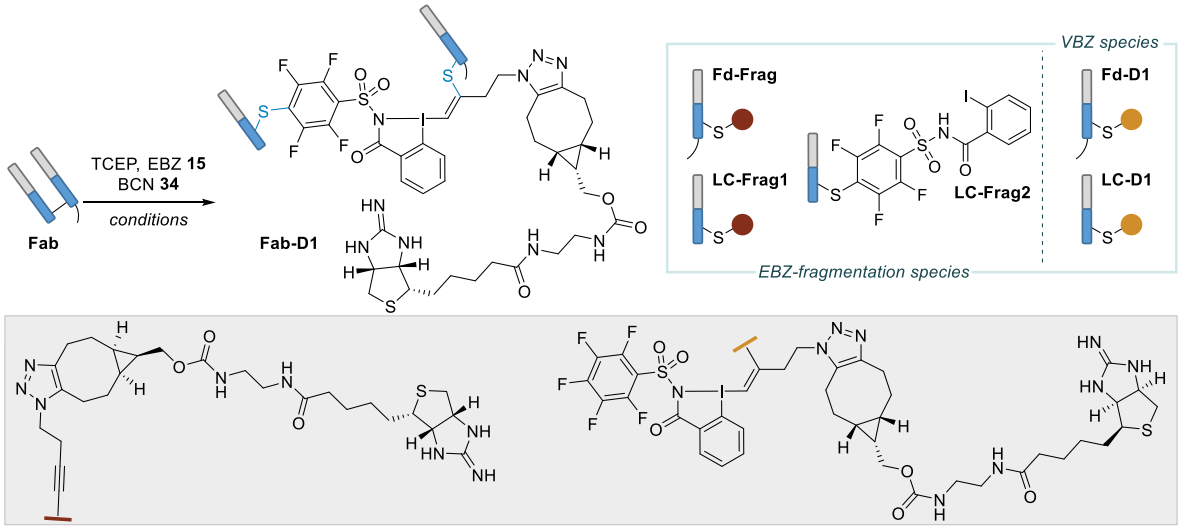


Figure S71. SEC-UV chromatogram and the corresponding MS raw and deconvoluted spectra of each peak corresponding to the one-pot reduction-conjugation of trastuzumab.

Functionalisation step



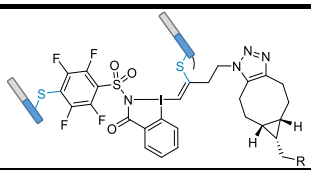
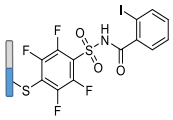
Fragment	Name (*mass increment*)	mAb	av. MW (Da)	Code
	**Fab**	Trastuzumab	47 637	
		Rituximab	47 179[a]	
		Bevacizumab	48 208	
		Avelumab	46 873	
	**Fab-D1** (*+1012 Da*)	Trastuzumab	48 649	
		Rituximab	48 191	
		Bevacizumab	49 220	
		Avelumab	47 885	
	**Fd**	Trastuzumab	24 200	
		Rituximab	24 145	
		Bevacizumab	24 763	
		Avelumab	24 073	
	**Fd-Frag1** (*+555 Da*)	Trastuzumab	24 755	
		Rituximab	24 700	
		Bevacizumab	25 318	
		Avelumab	24 628	
	**Fd-D1** (*+1032 Da*)	Trastuzumab	25 232	
		Rituximab	25 177	
		Bevacizumab	25 795	
		Avelumab	25 105	
	**LC**	Trastuzumab	23 439	
		Rituximab	23 036	
		Bevacizumab	23 447	
		Avelumab	22 802	
	**LC-Frag2** (*+457 Da*)	Trastuzumab	23 896	
		Rituximab	23 493	
		Bevacizumab	23 904	
		Avelumab	23 259	
	**LC-Frag1** (*+555 Da*)	Trastuzumab	23 994	
		Rituximab	23 591	
		Bevacizumab	24 002	
		Avelumab	23 357	
	**LC-D1** (*+1032 Da*)	Trastuzumab	24 471	
		Rituximab	24 068	
		Bevacizumab	24 479	
		Avelumab	23 834	

Figure S72. Overview of all species formed during Fab functionalization with their structures and molecular weights. The color code is used in the copies of mass spectra given below, to facilitate identification and understanding. [a]: the MW is that of a Fab species with two *N*-terminal pyroglutamate residues – instead of two glutamines –, as observed in various commercial samples.[3]

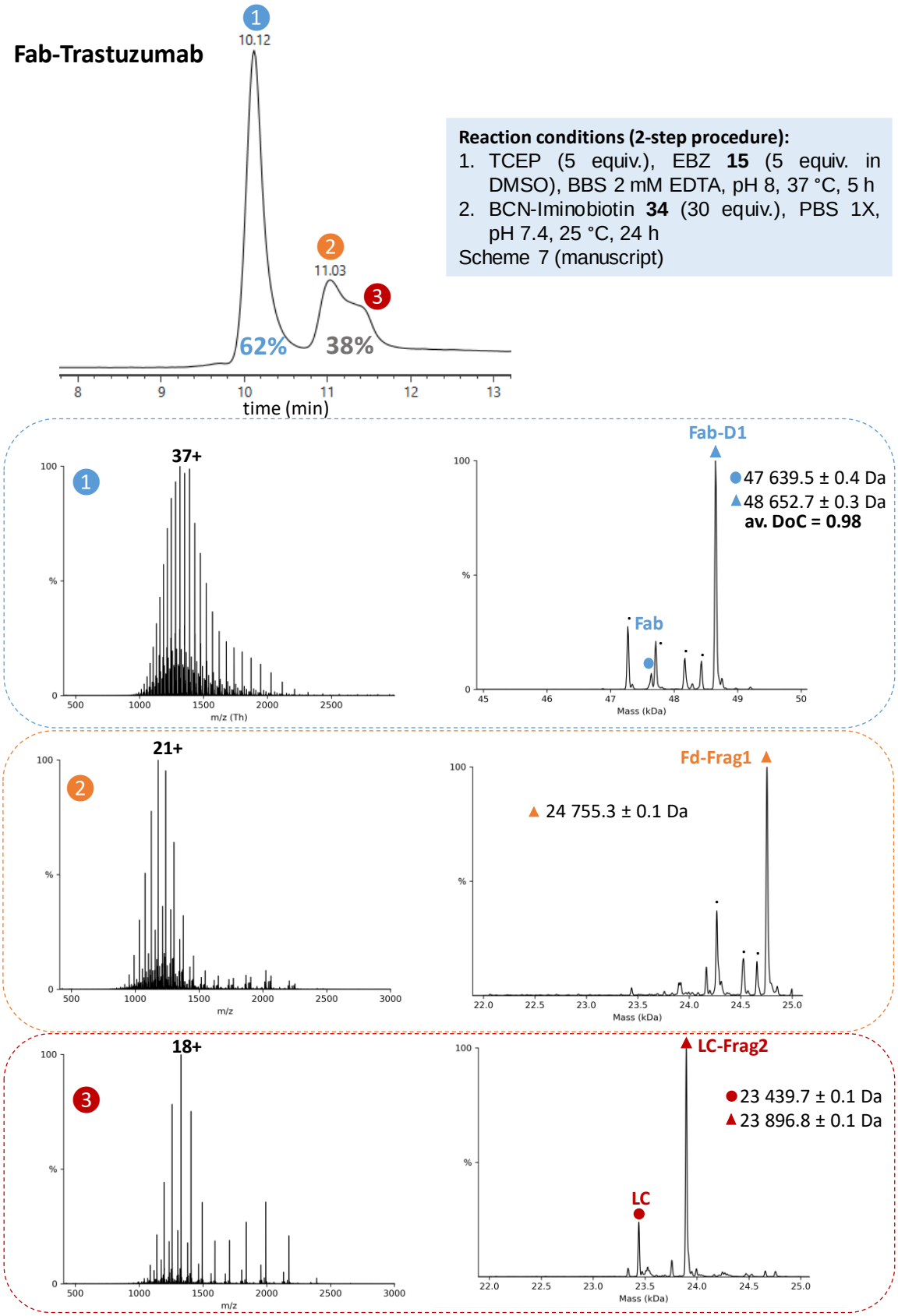


Figure S73. SEC-UV chromatogram and the corresponding raw and deconvoluted MS spectra of each peak corresponding to the two-step conjugation-functionalization of trastuzumab Fab under optimised conditions. The small black dots correspond to species resulted from enzymatic over digestion of Fab fragment and their corresponding conjugates with EBZ **15**.

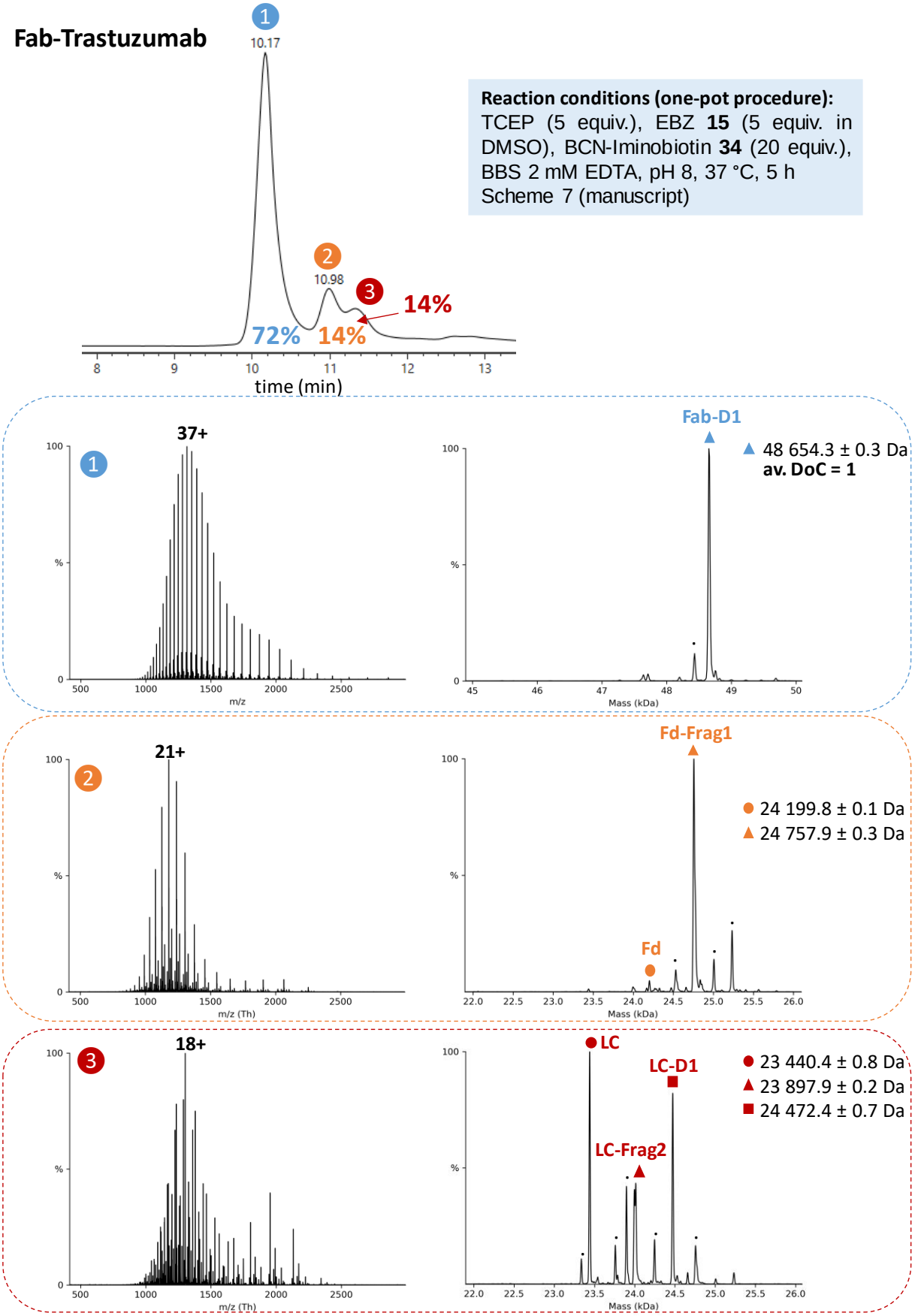


Figure S74. SEC-UV chromatogram and the corresponding raw and deconvoluted MS spectra of each peak corresponding to the one pot reduction-conjugation-functionalization of trastuzumab Fab under optimised conditions. The small black dots correspond to species resulted from enzymatic over digestion of Fab fragment and their corresponding conjugates with EBZ **15**.

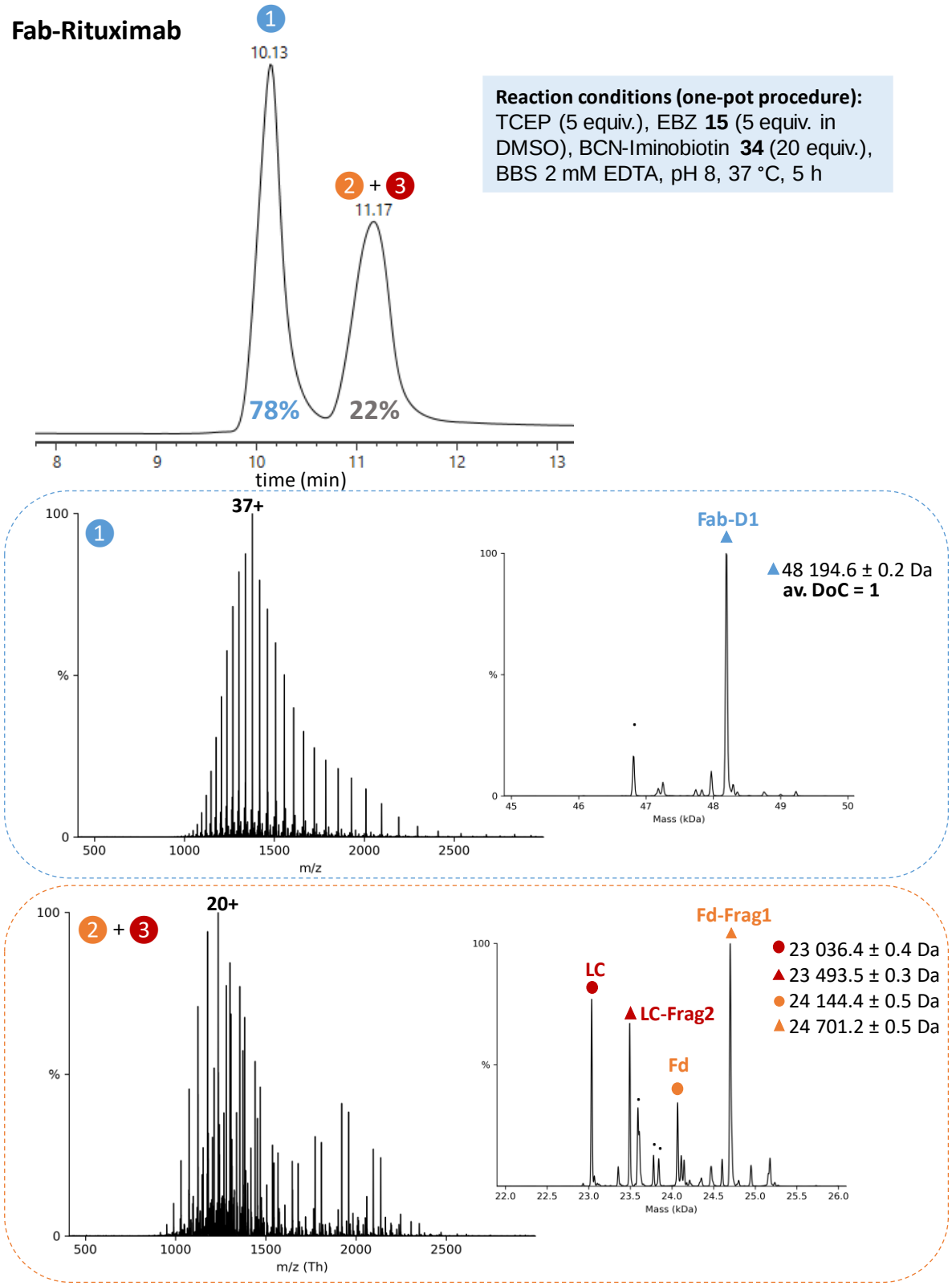


Figure S75. SEC-UV chromatogram and the corresponding raw and deconvoluted MS spectra of each peak corresponding to the one pot reduction-conjugation-functionalization of rituximab Fab under optimised conditions. The small black dots correspond to species resulted from enzymatic over digestion of Fab fragment and their corresponding conjugates with EBZ **15**.

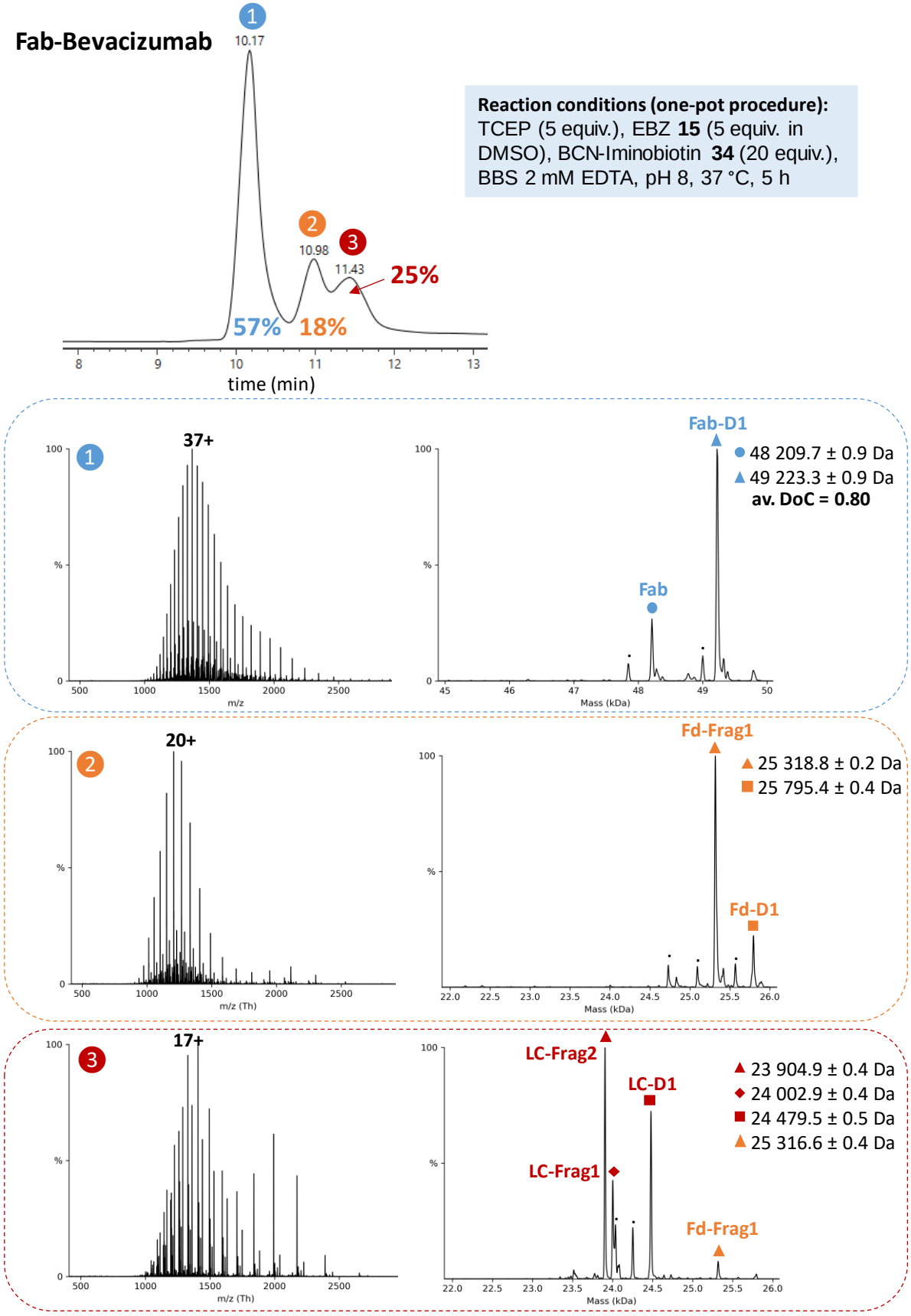


Figure S76. SEC-UV chromatogram and the corresponding raw and deconvoluted MS spectra of each peak corresponding to the one pot reduction-conjugation-functionalization of bevacizumab Fab under optimised conditions. The small black dots correspond to species resulted from enzymatic over digestion of Fab fragment and their corresponding conjugates with EBZ **15**.

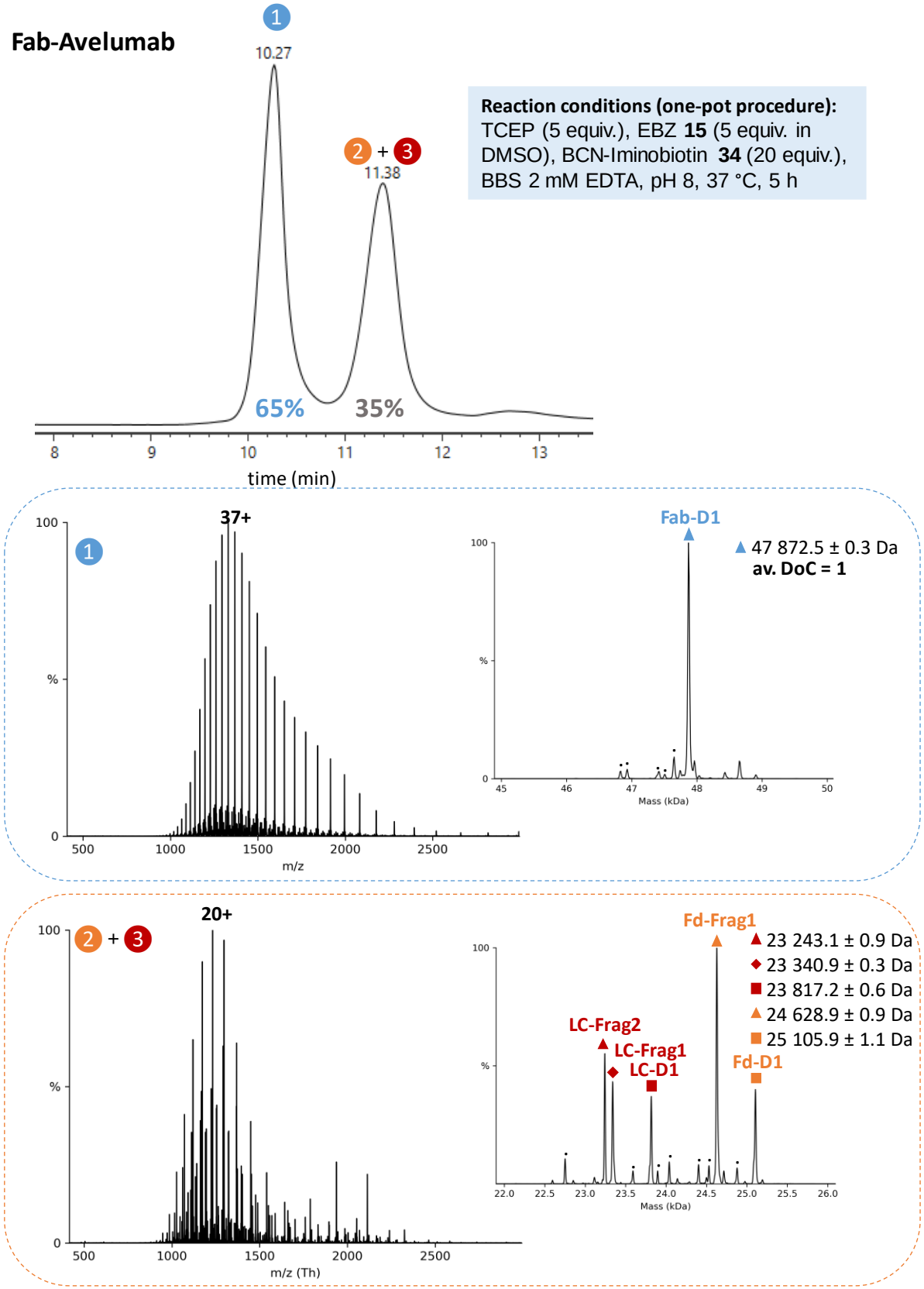
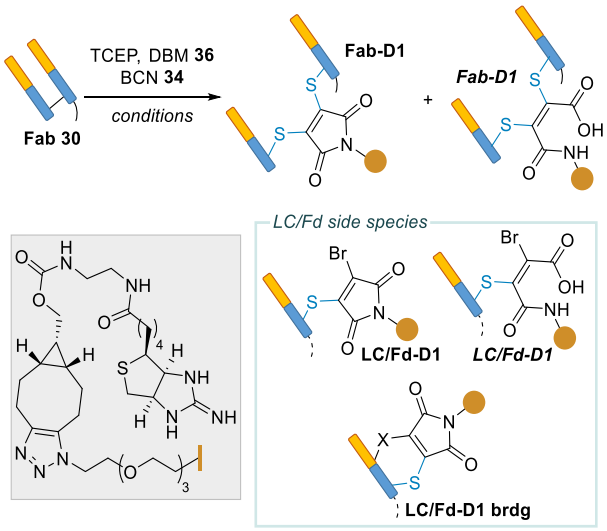


Figure S77. SEC-UV chromatogram and the corresponding raw and deconvoluted MS spectra of each peak corresponding to the one pot reduction-conjugation-functionalization of avelumab Fab under optimised conditions. The experimental MW indicate that the LC *N*-terminal glutamine has cyclized to a pyroglutamate. The small black dots correspond to species resulted from enzymatic over digestion of Fab fragment and their corresponding conjugates with EBZ **15**.



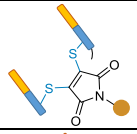
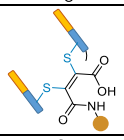
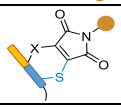
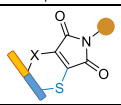
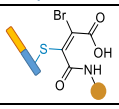
Fragment	Name (number) (*mass increment*)	average MW (Da)	Color code
	**Fab** (**30**)	47 637	
	**Fab-D1** (*+756 Da*)	48 393	
	***Fab-D1*** (*+774 Da*)	48 411	
	**Fd-D1 brdg** (*+756 Da*)	24 956	
	**LC-D1 brdg** (*+756 Da*)	24 195	
	***LC-D1*** (*+855 Da*)	24 294	

Figure S78. Overview of all species formed during trastuzumab Fab rebdriging with dibromomaleimide (DBM) reagent **36** with their structures and molecular weights. The color code is used in the copies of mass spectra given below, to facilitate identification and understanding.

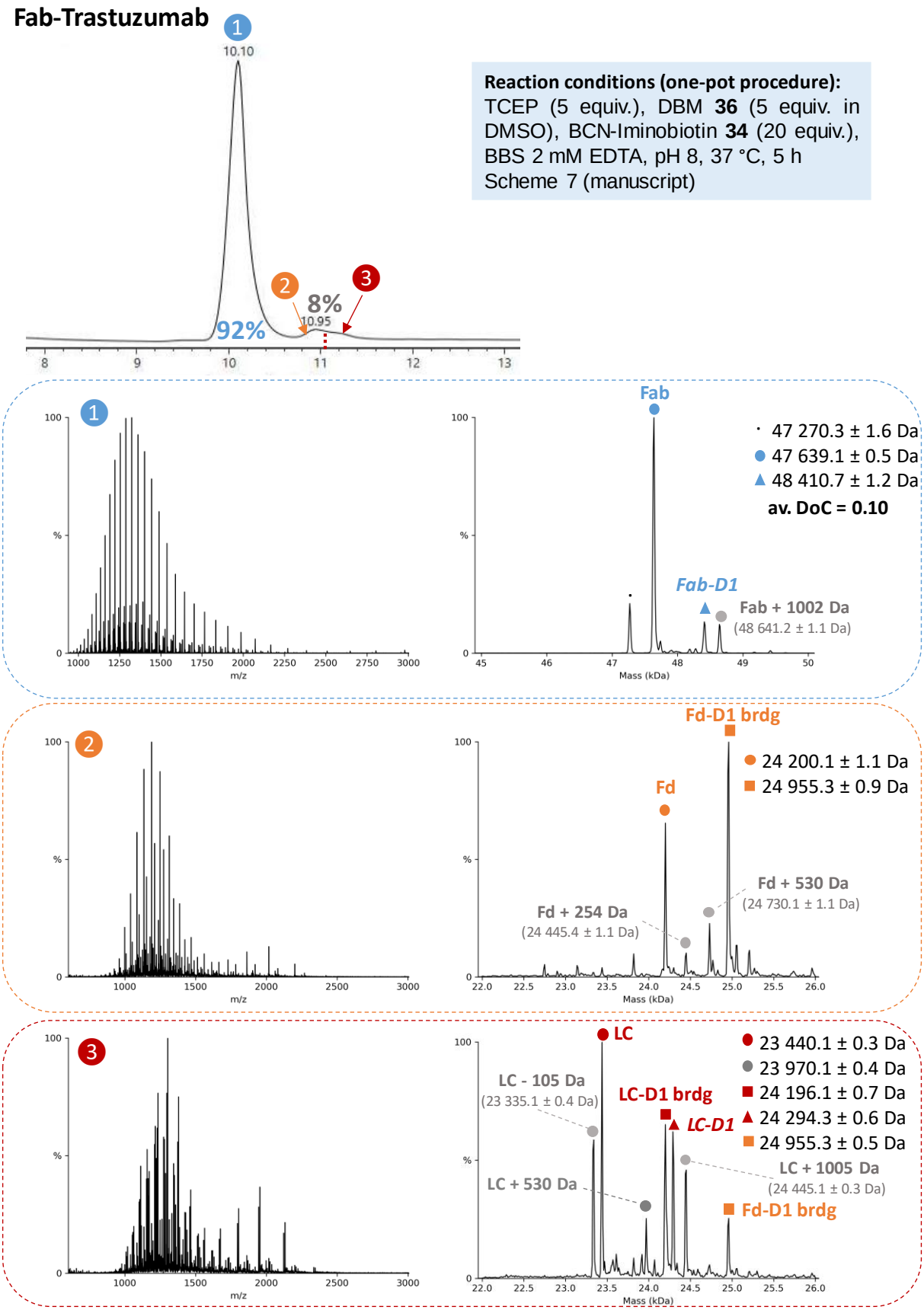
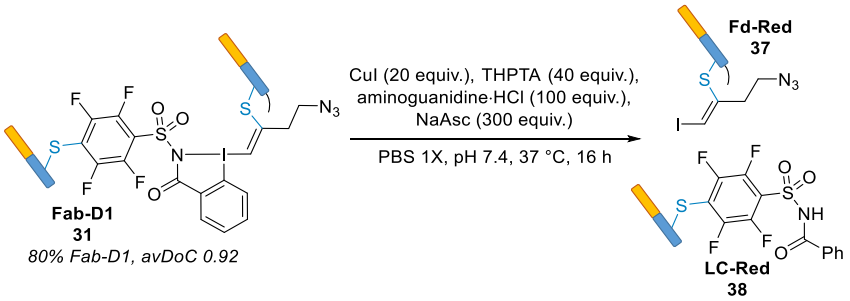


Figure S79. SEC-UV chromatogram and the corresponding raw and deconvoluted MS spectra of each peak corresponding to the attempted one pot reduction-conjugation-functionalization of trastuzumab Fab with dibromomaleimide (DBM) reagent **36** under optimised conditions. The small black dots correspond to species resulted from enzymatic over digestion of Fab fragment. The red labels indicate unidentified species, whose molecular weights are given for information.

Reductive cleavage



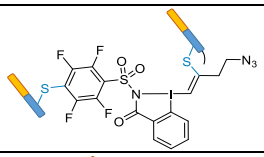
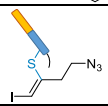
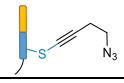
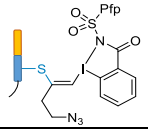
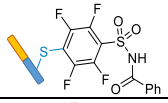
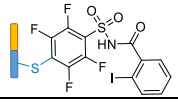
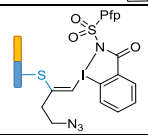
Fragment	Name (number) (*mass increment*)	average MW (Da)	Color code
	**Fab** (**30**)	47 637	
	**Fab-D1** (**31**)	48 187	
	**Fd-Red** (**37**) (*+221 Da*)	24 421	
	**Fd-Frag** (**33**) (*+93 Da*)	24 293	
	**Fd-D1** (*+570 Da*)	24 770	
	**LC-Red** (**38**) (*+331 Da*)	23 770	
	**LC-Frag** (**32**) (*+457 Da*)	23 896	
	**LC-D1** (*+570 Da*)	24 009	

Figure S80. Overview of all species formed during the reductive cleavage of trastuzumab Fab-D1 **31** with their structures and molecular weights – the species Fd-Frag, Fd-D1, LC-Frag, and LC-D1 were present in the Fab-D1 sample used for the study. The color code is used in the copies of mass spectra given below, to facilitate identification and understanding.

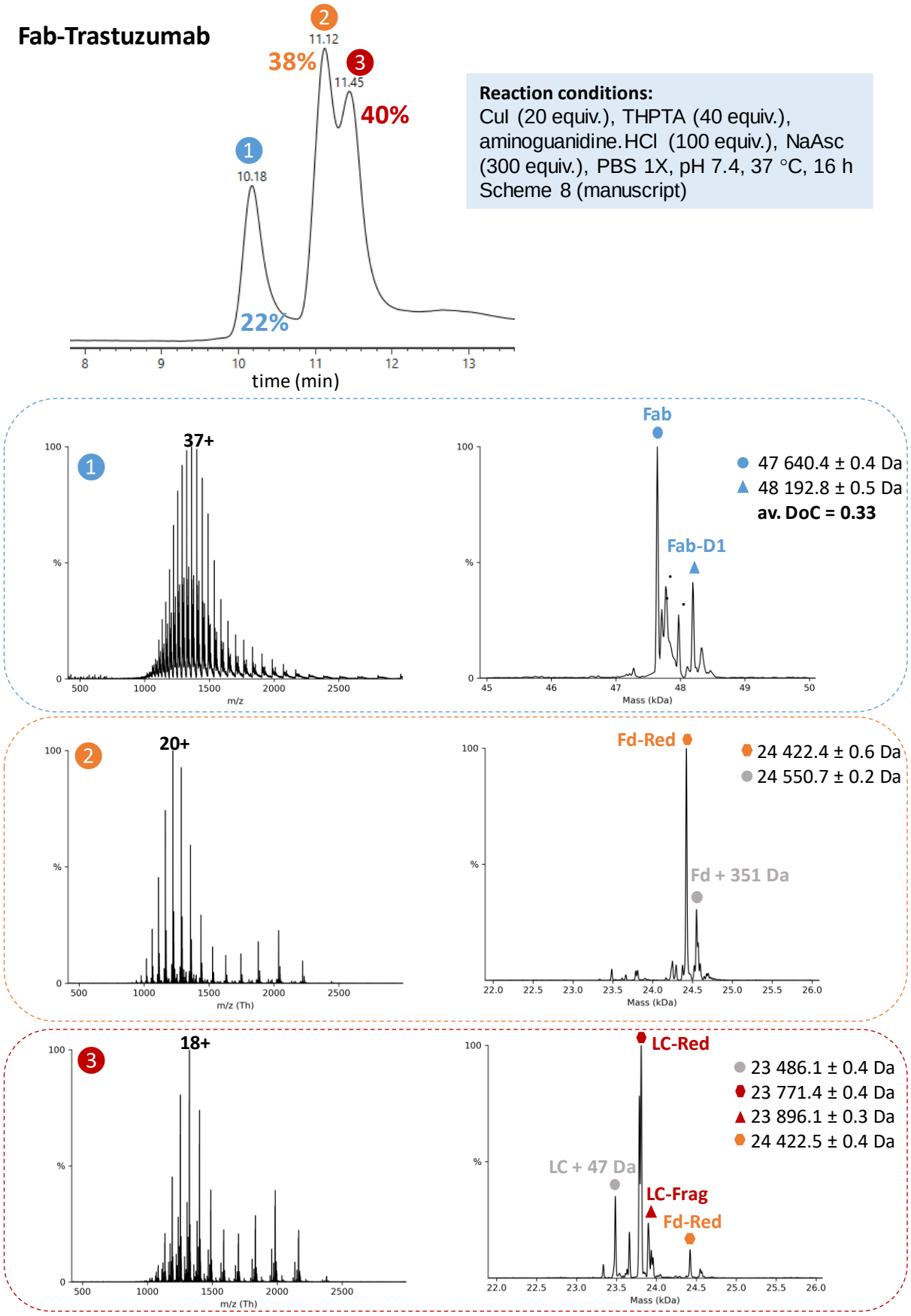


Figure S81: SEC-UV chromatogram and the corresponding raw and deconvoluted MS spectra of each peak corresponding to the reductive cleavage of trastuzumab Fab-D1 **31** under optimised conditions. The small black dots correspond to species resulted from enzymatic over digestion of Fab fragment and their corresponding conjugates with EBZ **15**. The red labels indicate unidentified species, whose molecular weights are given for information.

Stability studies

To evaluate the stability of VBZ-rebridged Fab, **Fab-TAMRA** was generated from trastuzumab Fab **30** and the commercially available **DBCO-OEG-TAMRA** (Sigma-Aldrich), following the one-pot reduction-conjugation-functionalization procedure (see Figure S74 for a representative analysis). **Fab-TAMRA** was next incubated for up to 5 days at 37 °C in human plasma and regularly analyzed by SDS-PAGE, showing the excellent stability over time compared with a Fab-TAMRA control incubated in PBS.

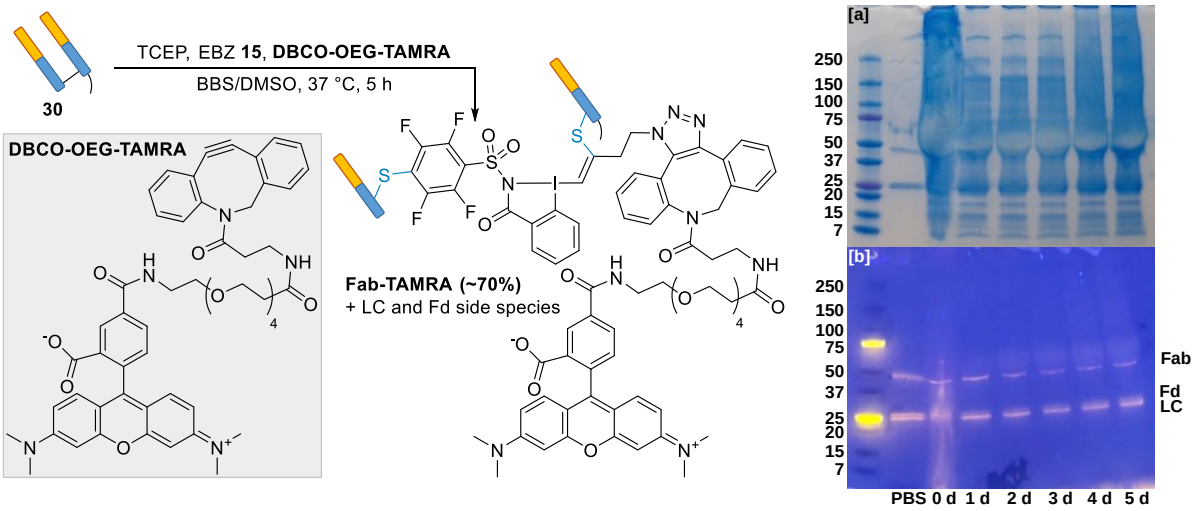


Figure S82: Stability studies of the rebridged **Fab-TAMRA** in human plasma for up to 5 days at 37 °C. [a] SDS-PAGE with Coomassie blue staining. [b] SDS-page under UV light, at 254 nm.

6. References

[1] 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.

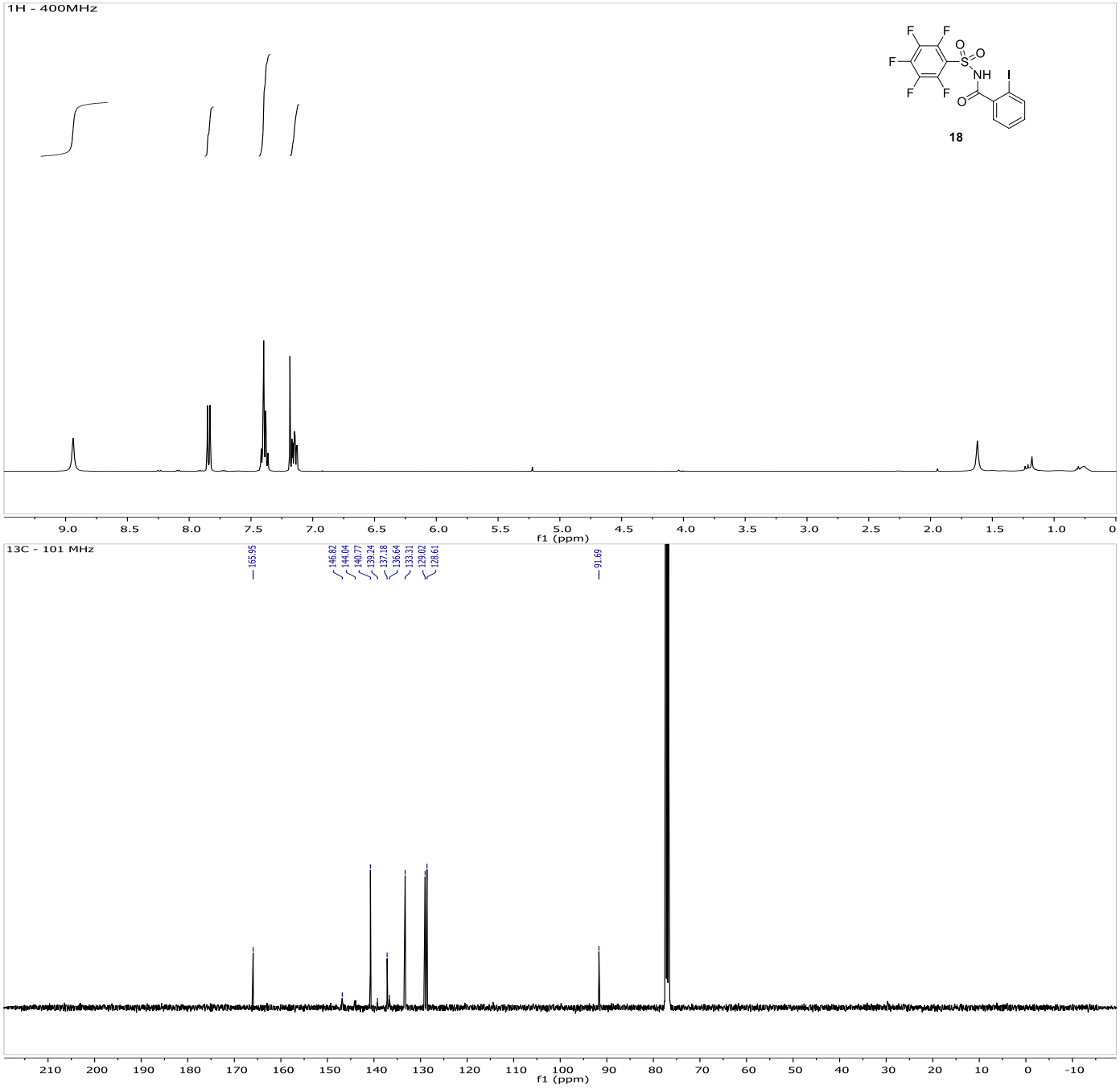
[2] L. Castañeda, A. Maruani, F. F. Schumacher, E. Miranda, V. Chudasama, K. A. Chester, J. R. Baker, M. E. B- Smith, S. Caddick, S. *Chem. Commun.* **2013**, *49*, 8187-8189.

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

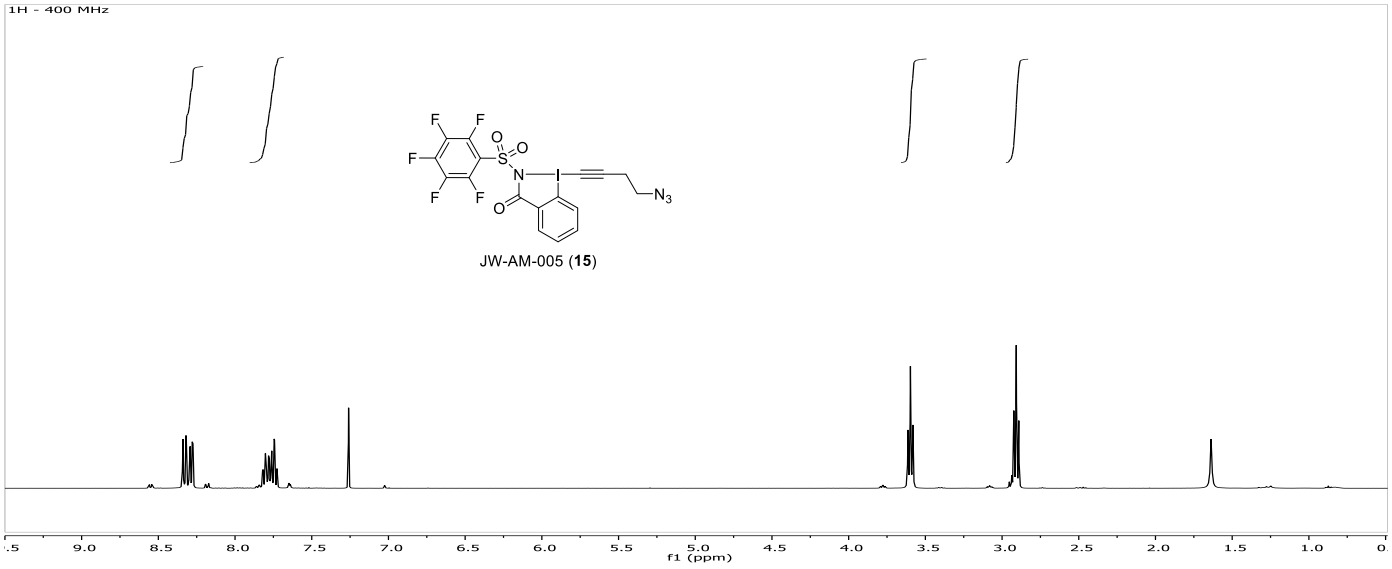
[4] B. Wang, A. C. Gucinski, D. A. Keire, L. F. Buhse, M. T. B. II *Analyst* **2013**, *138*, 3058-3065.

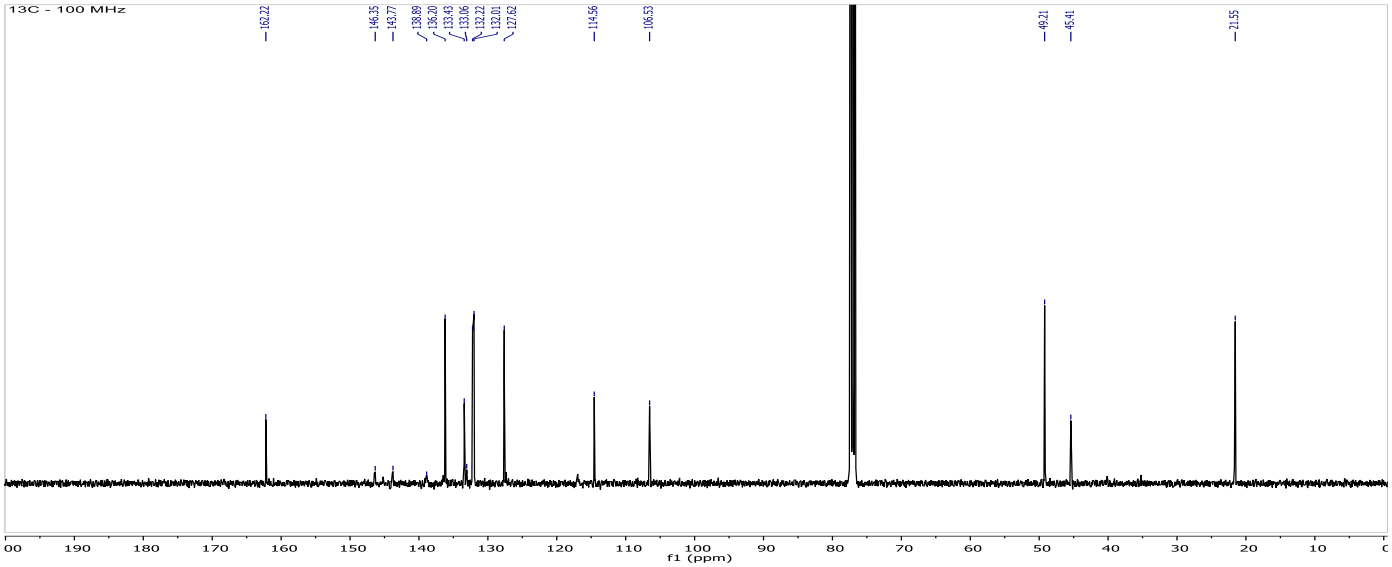
7. NMR Spectra of previously unreported compounds

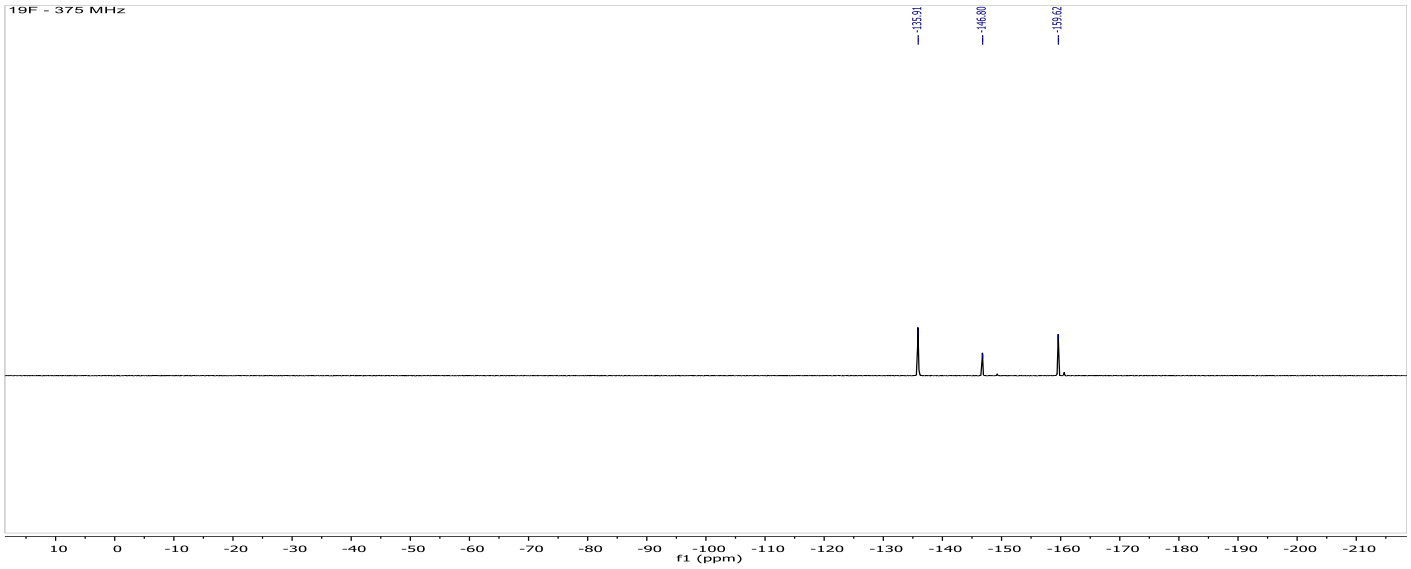
2-Iodo-N-((perfluorophenyl)sulfonyl)benzamide (18)



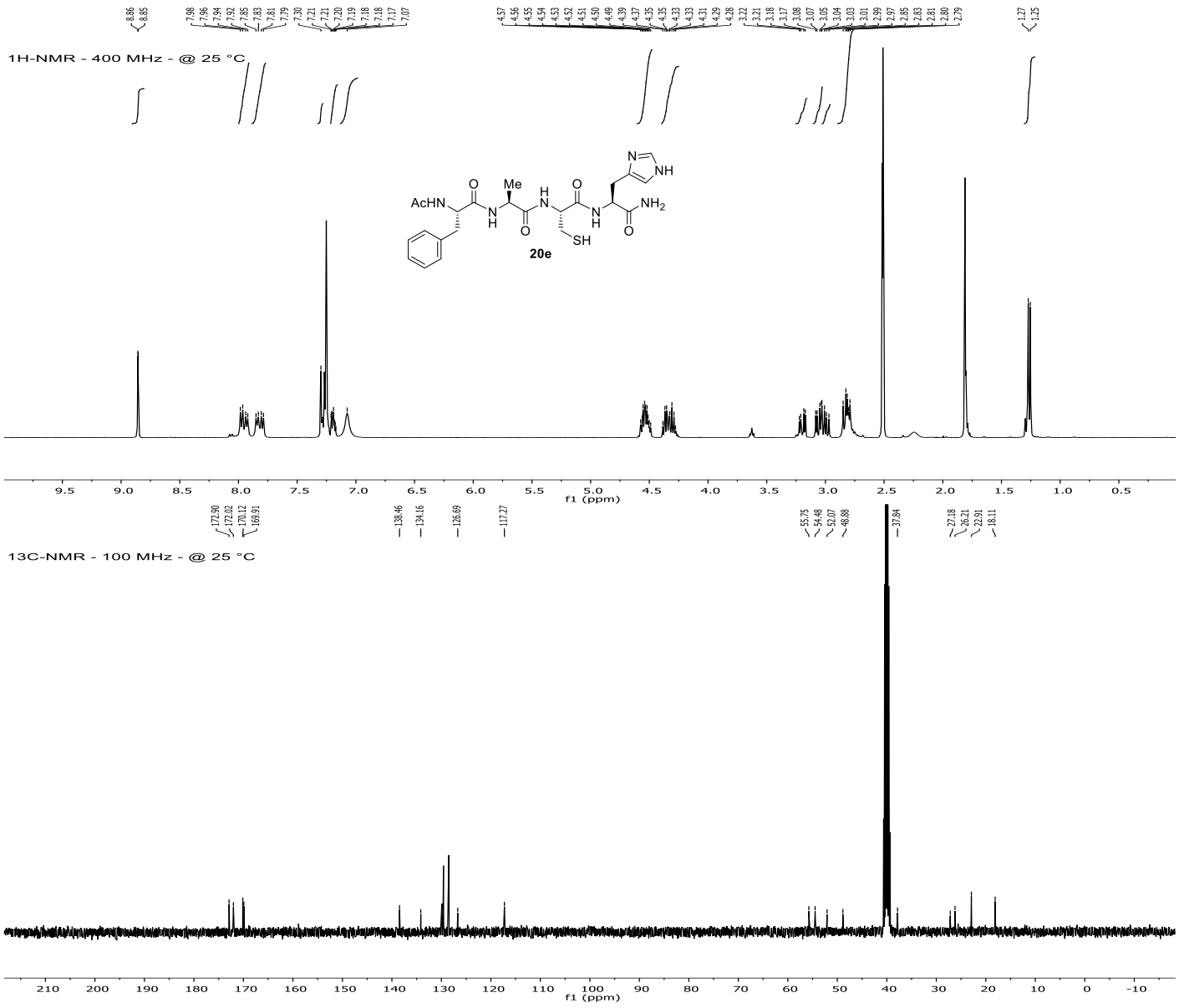
JW-AM-005 (15)


1H - 400 MHz
JW-AM-005 (15)
f1 (ppm)

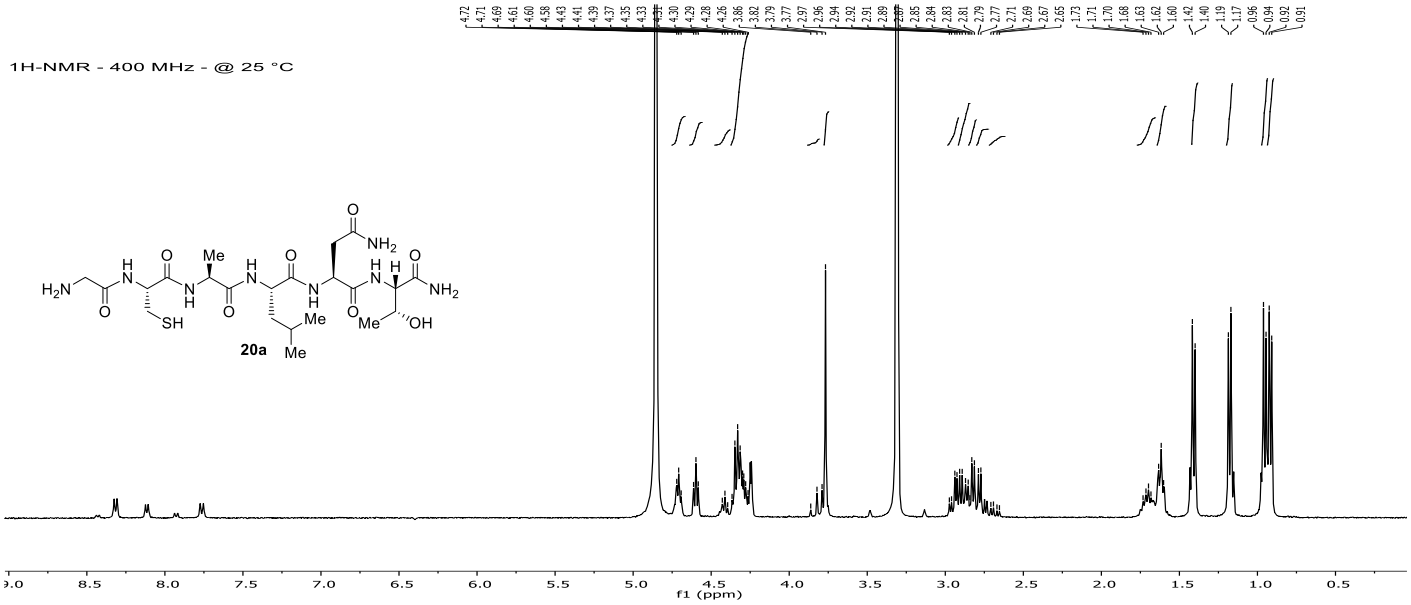

13C - 100 MHz
162.22
146.55
143.77
138.89
136.20
133.45
133.06
132.22
132.01
127.62
114.56
106.53
49.21
45.41
21.55
f1 (ppm)


19F - 375 MHz
-135.91
-146.80
-159.62
f1 (ppm)

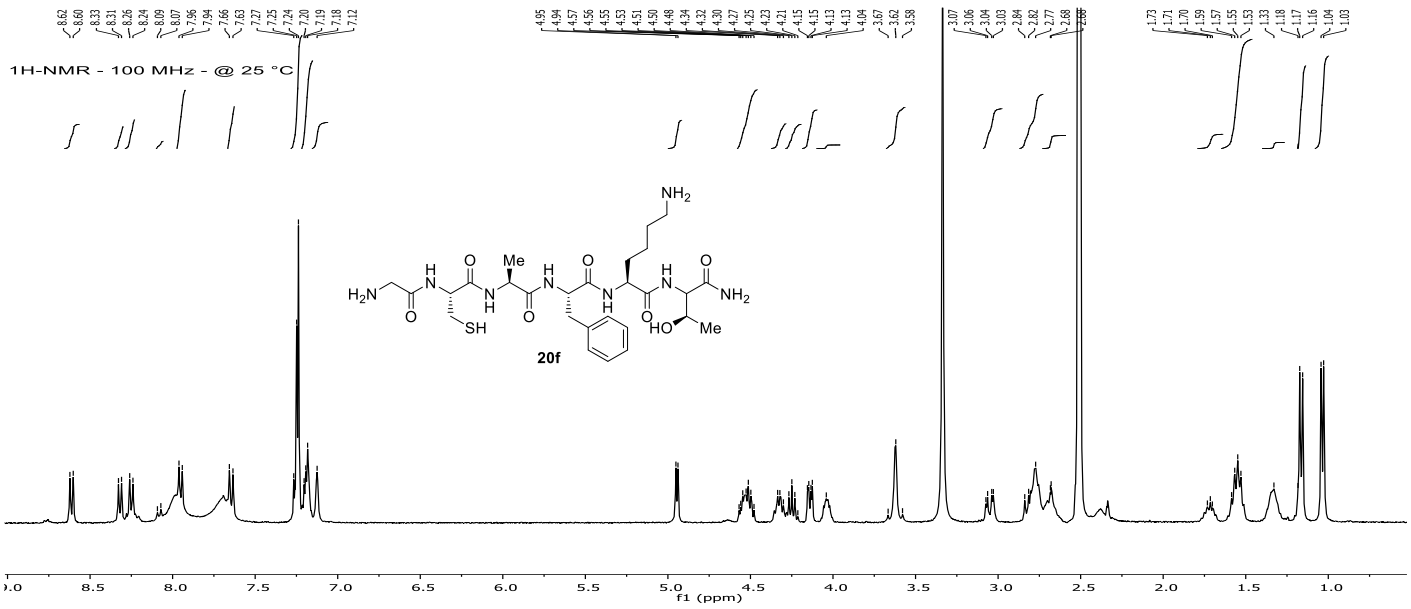
Ac-Phe-Ala-Cys-His-NH_2 (20e)



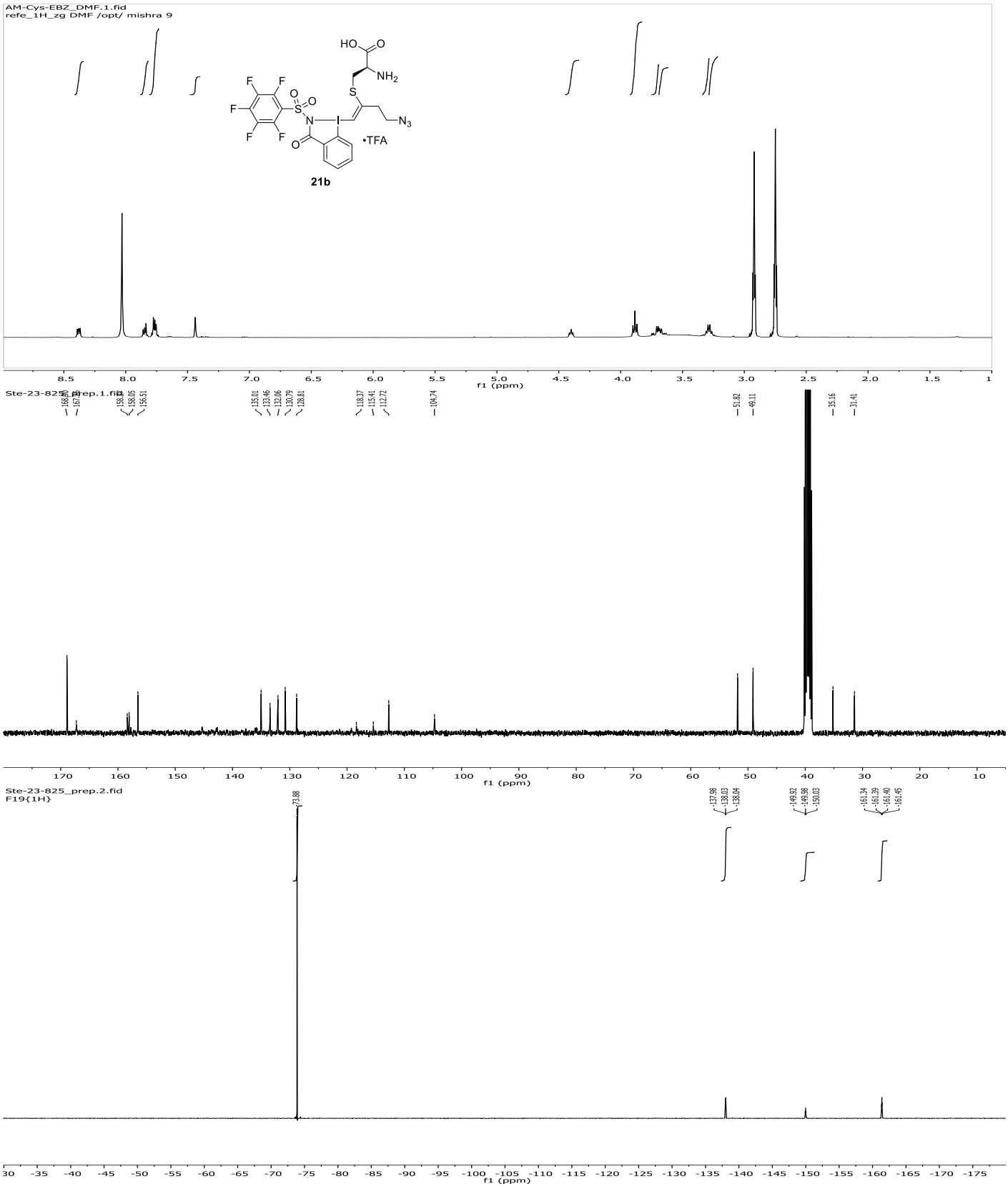
H-Gly-Cys-Ala-Leu-Asn-Thr-NH_2 (20a)



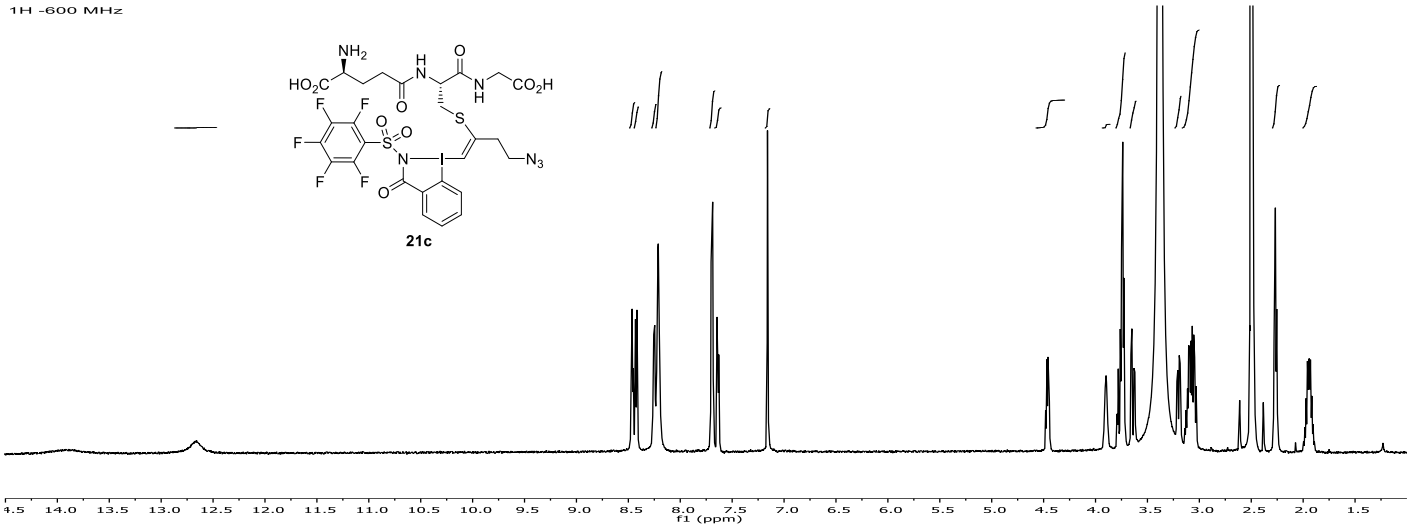
H-Gly-Cys-Ala-Phe-Lys-Thr-NH_2 (20f)

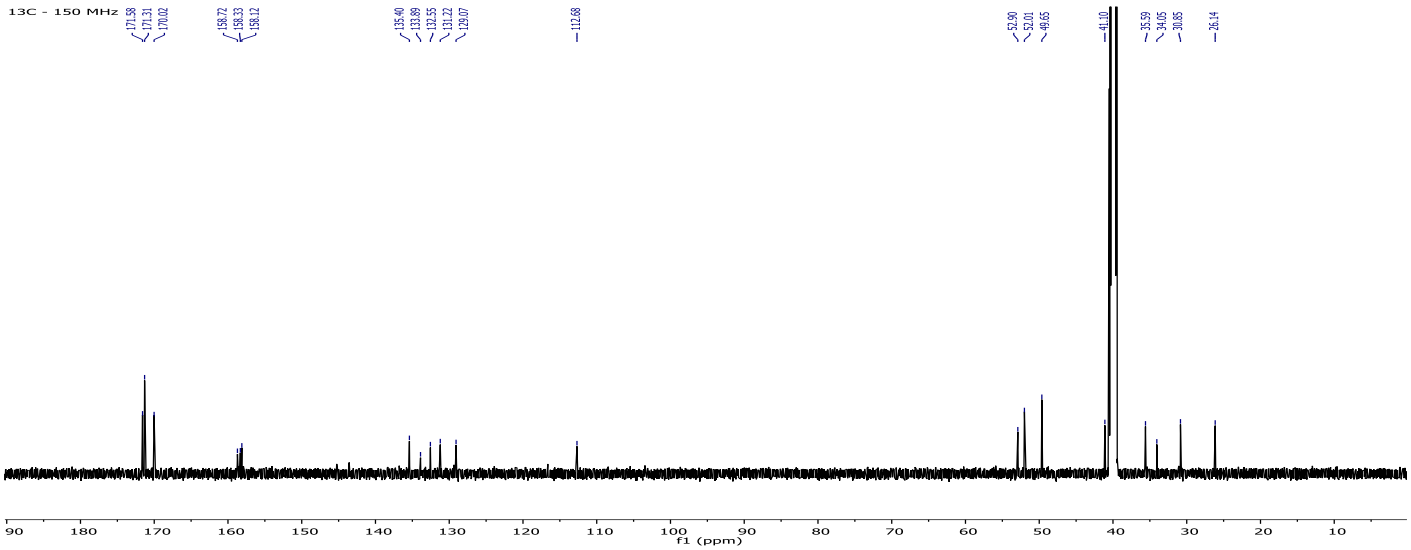


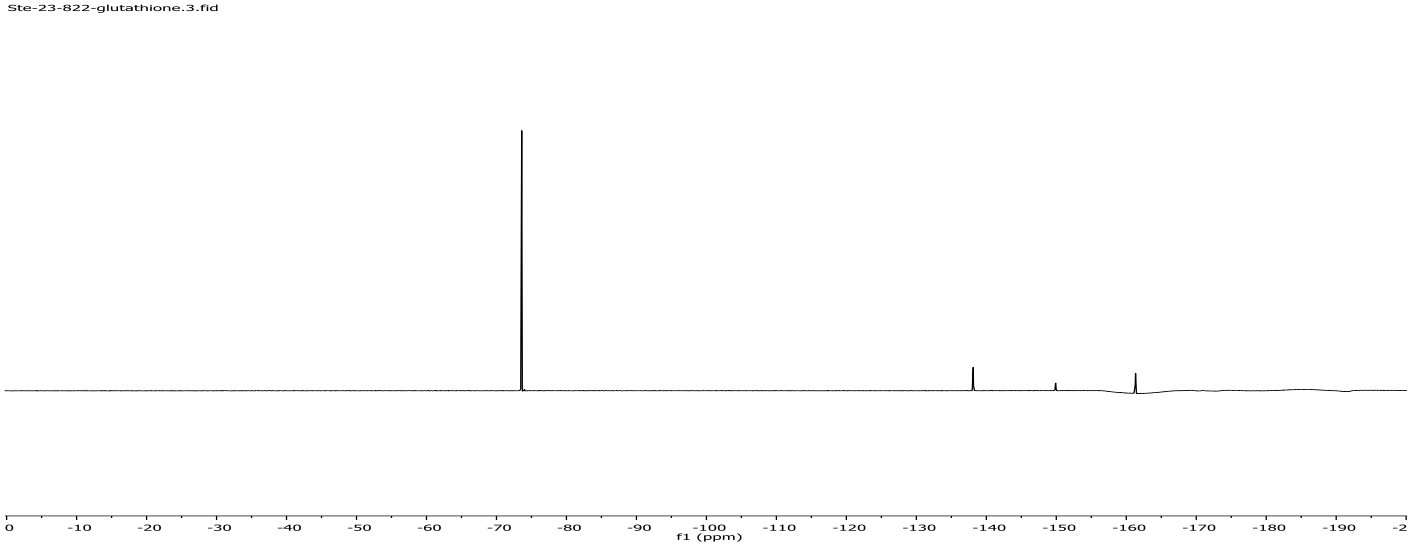
(Z)-1-(4-Azido-2-mercaptobut-1-en-1-yl)-2-((perfluorophenyl)sulfonyl)-1,2-dihydro-3*H*-1λ^3-benzo[d][1,2]iodazol-3-one (Cysteine S-VBZ) (21b)



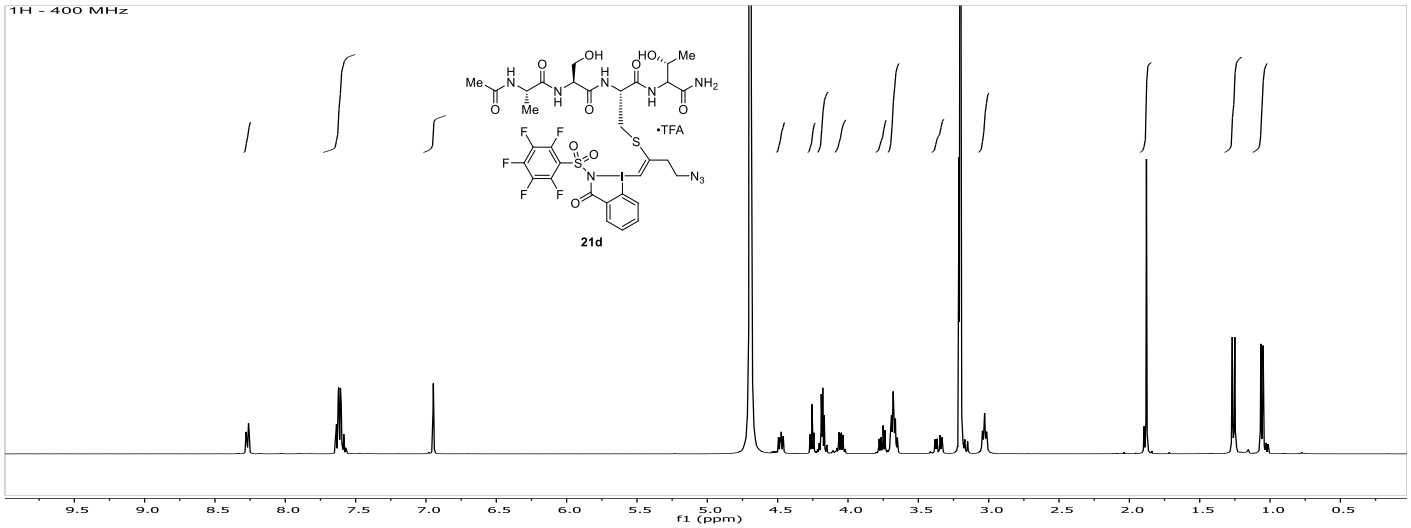
Glutathione S-VBZ (21c)

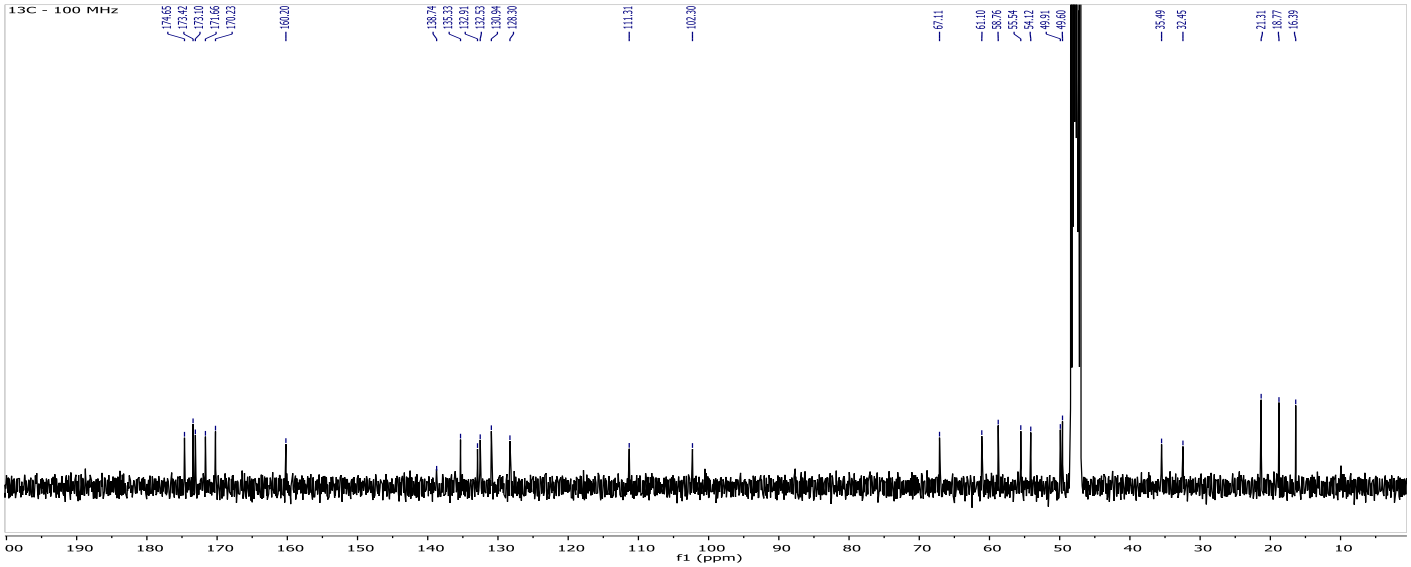


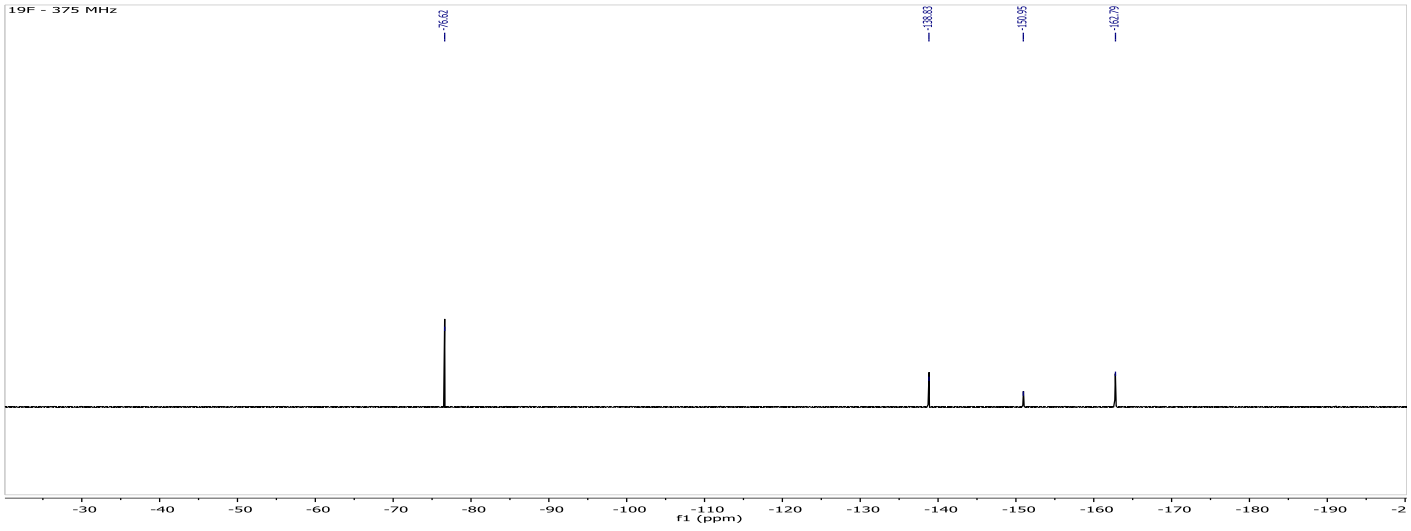


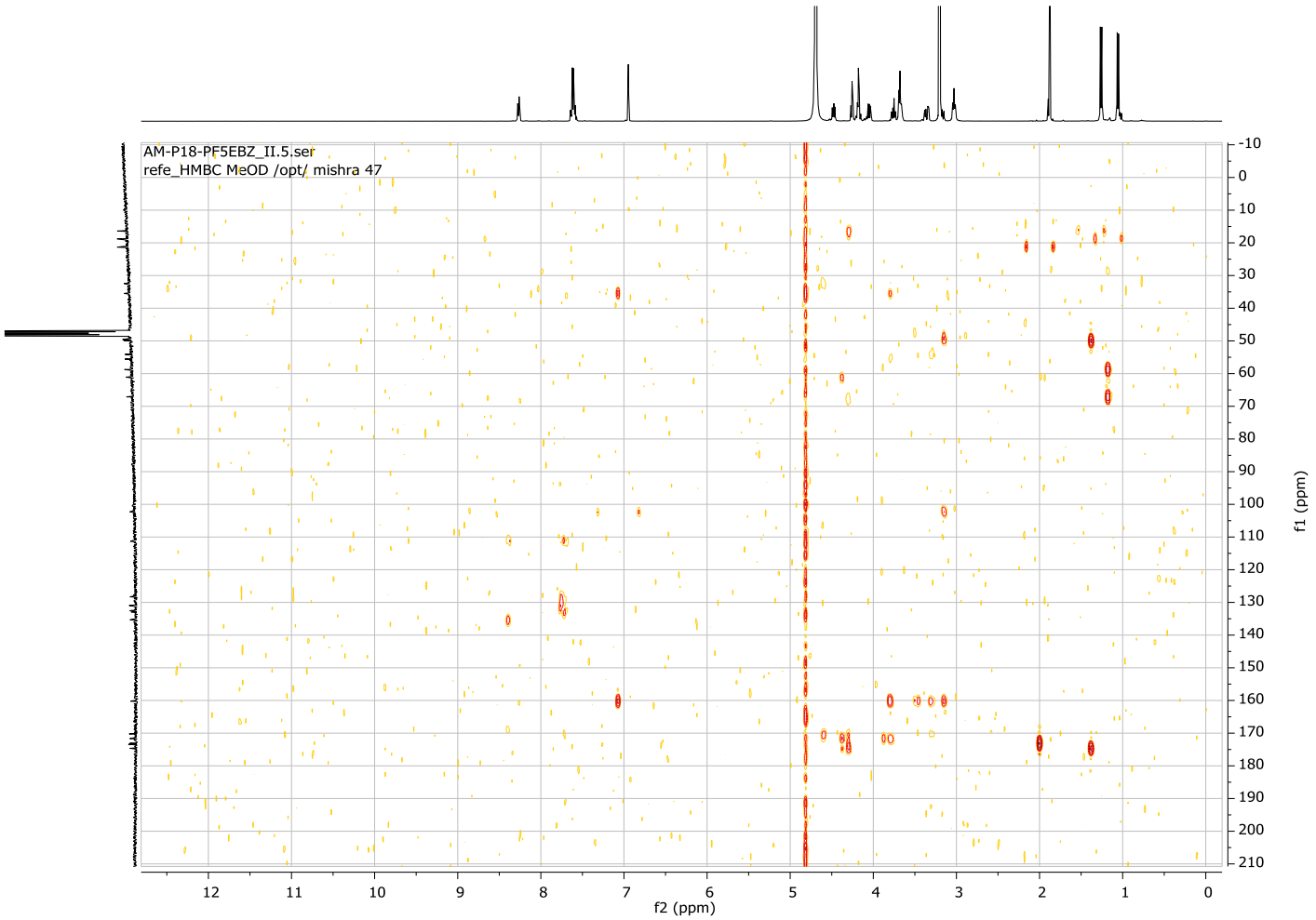


Ac-Ala-Ser-Cys(S-VBZ)-Thr-NH_2 21d



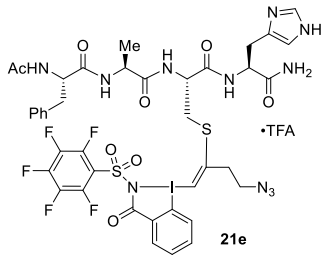




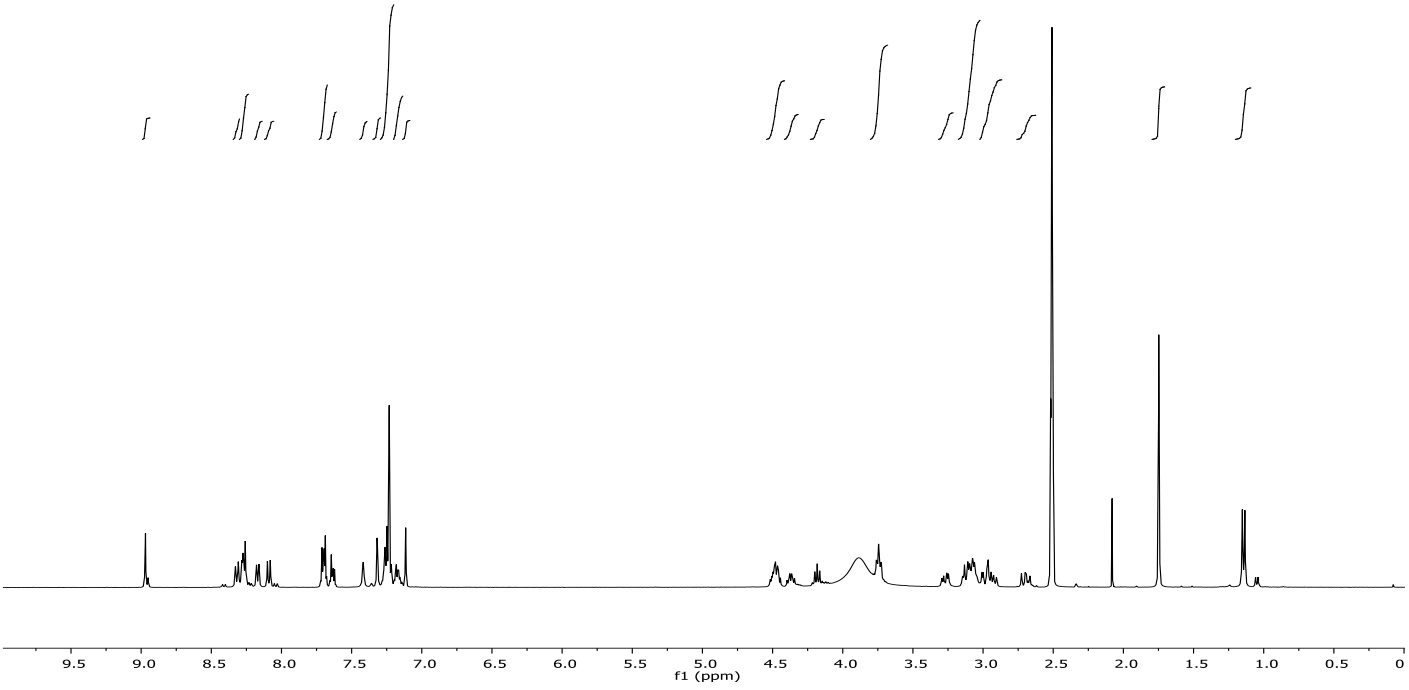

AM-P18-PF5EBZ_II.5.ser
refe_HMBC MeOD /opt/ mishra 47
f2 (ppm)
f1 (ppm)

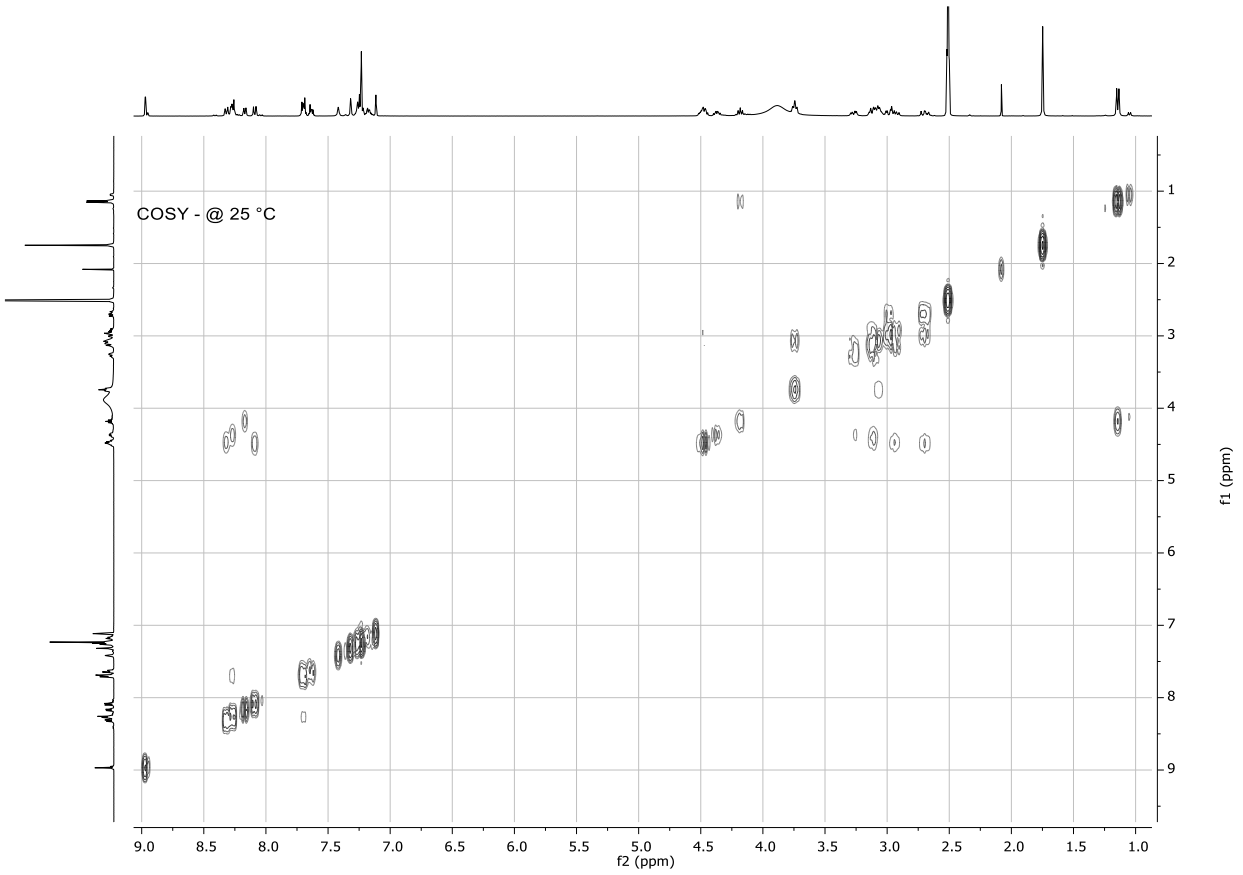
Ac-Phe-Ala-Cys(S-VBZ)-His-NH_2 21e

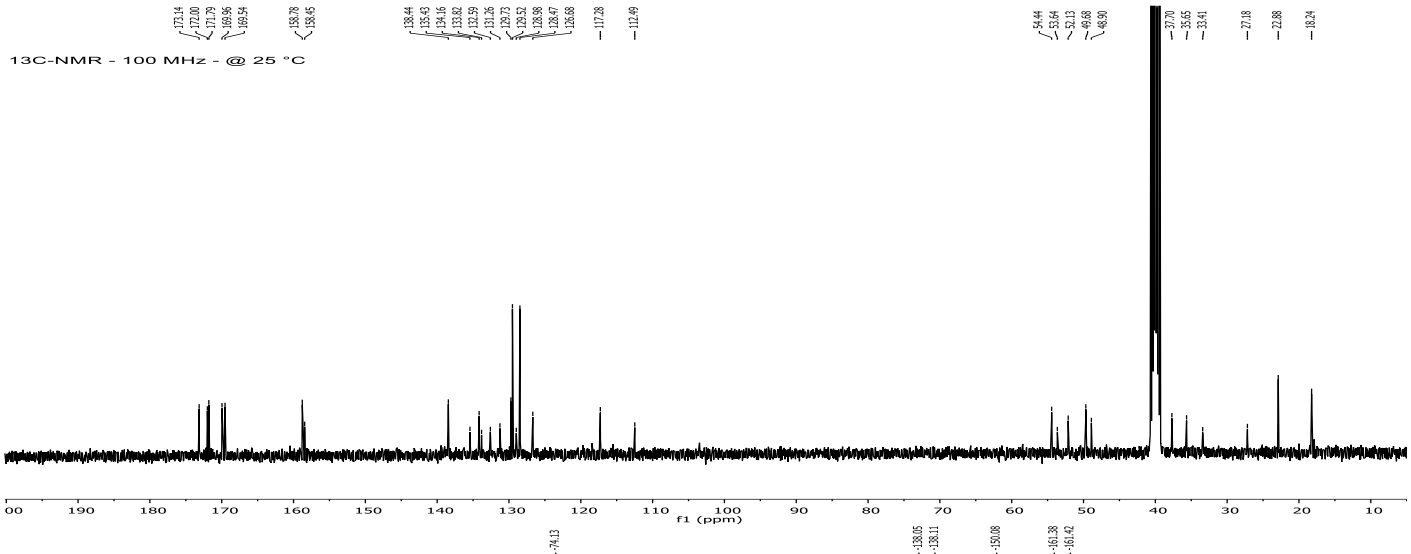
1H-NMR - 400 MHz - @ 90 °C

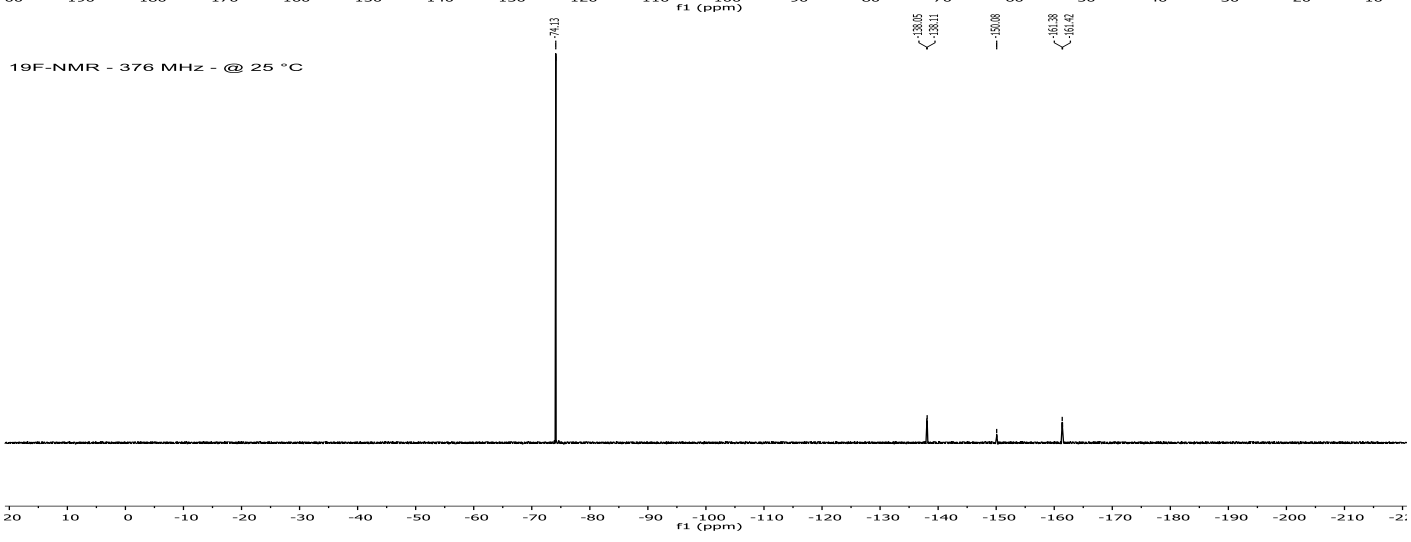


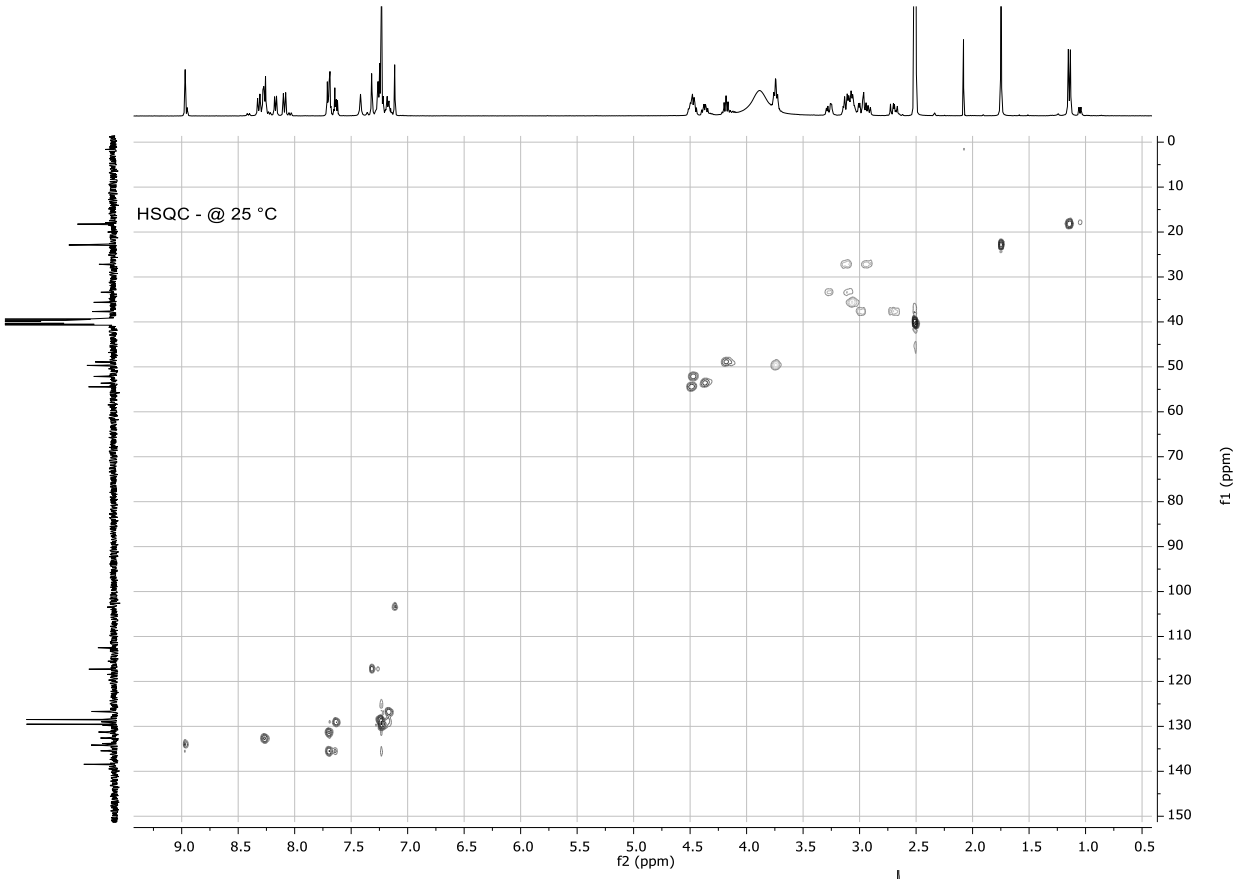
1H-NMR - 400 MHz - @ 25 °C

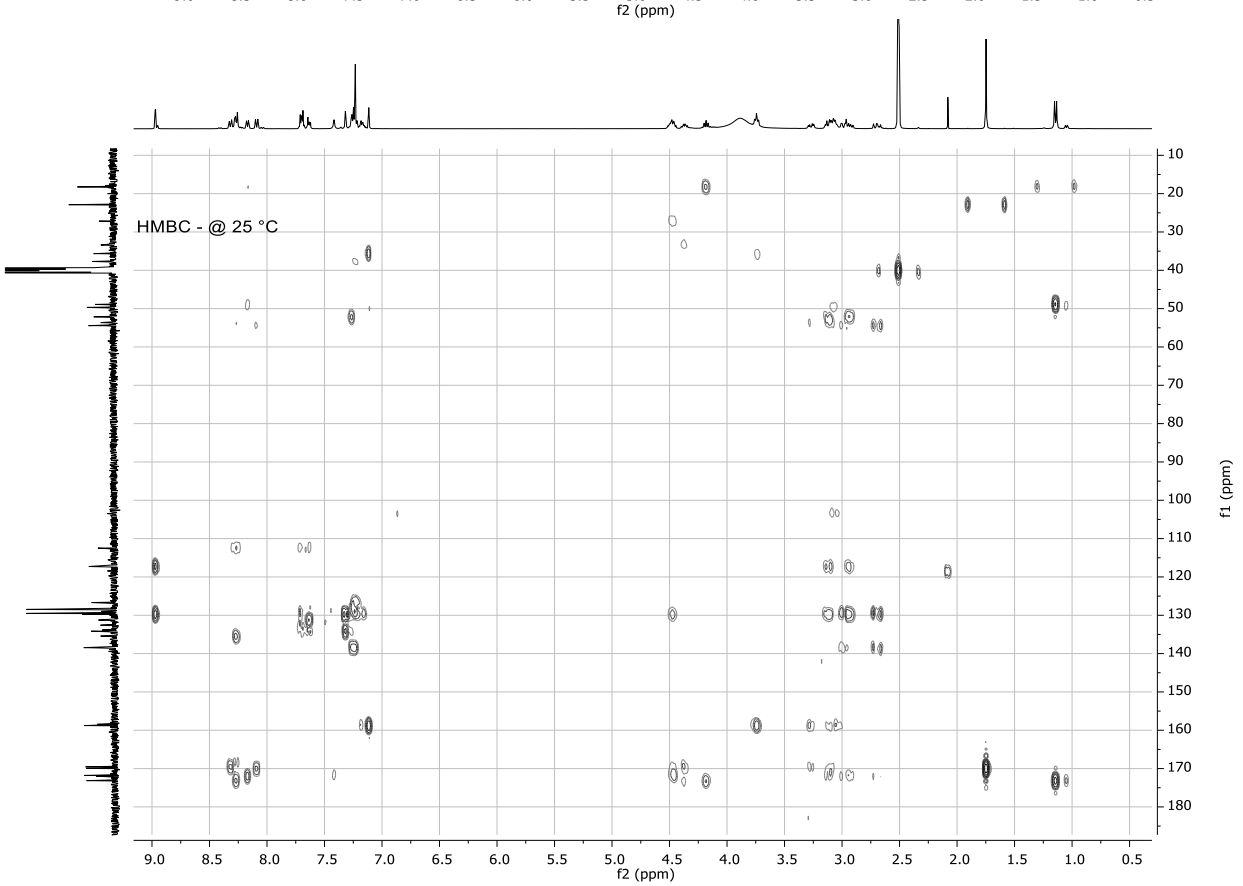



COSY - @ 25 °C
f2 (ppm)
f1 (ppm)


13C-NMR - 100 MHz - @ 25 °C
f1 (ppm)


19F-NMR - 376 MHz - @ 25 °C
f1 (ppm)


HSQC - @ 25 °C
f2 (ppm)
f1 (ppm)


HMBC - @ 25 °C
f2 (ppm)
f1 (ppm)