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Peptide Modification

Peptide-Hypervalent Iodine Reagent Chimeras: Enabling Peptide Functionalization and Macrocyclization**

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Abstract: Herein, we report a novel strategy for the modification of peptides based on the introduction of highly reactive hypervalent iodine reagents—ethynylbenziodoxolones (EBXs)—onto peptides. These peptide-EBXs can be readily accessed, by both solution- and solid-phase peptide synthesis (SPPS). They can be used to couple the peptide to other peptides or a protein through reaction with Cys, leading to thioalkynes in organic solvents and hypervalent iodine adducts in water buffer. Furthermore, a photocatalytic decarboxylative coupling to the C-terminus of peptides was developed using an organic dye and was also successful in an intramolecular fashion, leading to macrocyclic peptides with unprecedented crosslinking. A rigid linear aryl alkyne linker was essential to achieve high affinity for Keap1 at the Nrf2 binding site with potential protein-protein interaction inhibition.

Introduction

Over the past two decades, the importance of peptides in drug discovery has continuously grown, with over 60 peptide drugs approved until 2018.[1] As compared with small-molecule drugs and larger biological compounds, peptides occupy a unique intermediate position in chemical space, leading to different therapeutic applications. Nevertheless, unmodified natural peptides are poor drug candidates owing to their low bioavailability and fast degradation by proteolytic enzymes, which put them at a disadvantage when compared to small organic molecules or larger biologicals. To improve their bioactivity and stability, N and/or C-terminal protection, macrocyclization[2] and other selective modifications[3] introducing non-natural motifs into the targeted peptides have been developed (Scheme 1a). In this regard, hypervalent iodine reagents (HIRs) have been recognized as valuable and versatile reagents for peptide functionalization owing to their low toxicity, high reactivity and good functional-group selectivity.[4] They enable the transfer of various electrophiles onto different amino acid residues in peptides or proteins. Among these HIRs, ethynylbenziodoxolones (EBXs) are of particular interest.[5] Nucleophilic amino acid residues or radical species generated under photoredox conditions can be trapped by EBXs to form alkynylation products (Scheme 1a).[6] The introduction of an alkyne can not only provide an extra reactive site for further modifications,[7] but also constitutes a unique rigid linker with very low steric demand.[8] Despite these elegant advances, each new transformation required the design of specific reagents and conditions, leading to tedious and time-consuming optimization. Many reagents also have insufficient solubility in water, or display diminished reactivity when modified with water solubilizing groups. In addition, whereas progress has been made in the selective functionalization of one amino acid over others, chemical functionalization methods usually do not allow selectivity among different sites containing the same residue.

To enable complete site selectivity, another strategy is usually followed: the installation of non-natural functionalities into peptides and proteins, enabling "bioorthogonal" reactions that proceed exclusively at the modified site.[9] The use of cycloaddition reactions, in particular between azides and alkynes, has been most often exploited in the past, but new types of bioorthogonal reactions proceeding with high rates under mild conditions, especially in the absence of metal catalysts, are still urgently needed. Owing to their unique reactivity, the introduction of HIRs into peptides and proteins appears highly attractive in this context (Scheme 1b). Nevertheless, the modification of hypervalent iodine reagents without touching the highly reactive iodine center is difficult and has been achieved only on small organic molecules so far.[10] The only successful approach was reported by our group in 2019 and 2022.[11] Vinylbenziodoxolone (VBX) products were formed through the addition of Cys/Tyr-containing peptides/proteins to EBXs. The VBXs could undergo bioorthogonal Suzuki coupling reactions to introduce aryl substituents onto peptides and proteins. However, high Pd catalyst loading and tedious optimization are necessary for this cross-coupling, which therefore fails to meet the requirements for a "click

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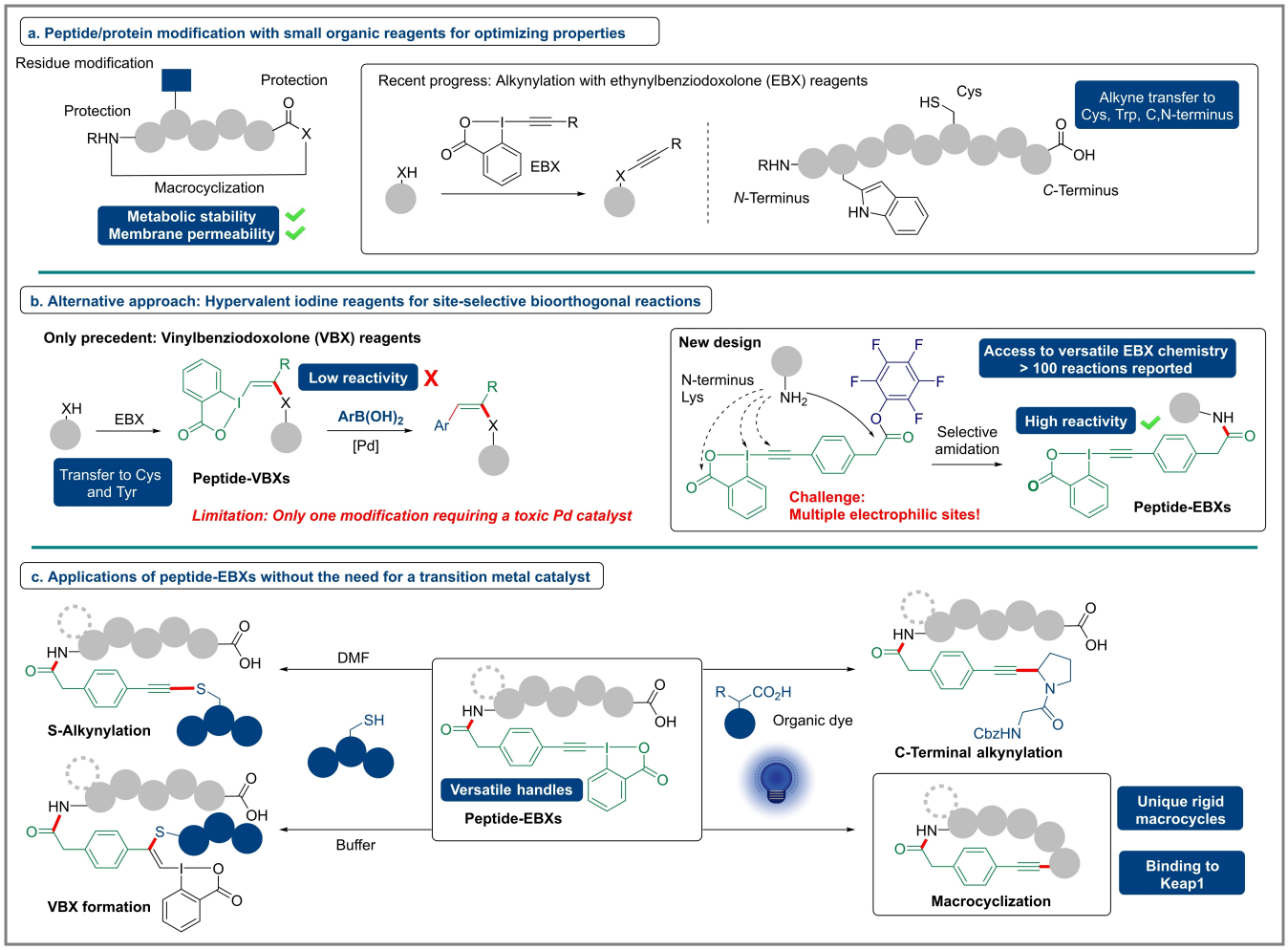


Scheme 1. Peptide modifications enabled by hypervalent iodine reagents (HIRs). a) Chemical modifications of bioactive peptides: Recent progress using alkynylation with ethynylbenziodoxolones (EBXs). b) Using hypervalent iodine reagents for bioorthogonal reactions on peptides: Limited single precedence and new reagent design. c) Metal-free bioorthogonal transformations with the new Peptide-EBX reagents.

reaction".[12] In fact, VBXs reagents are far less reactive than EBXs, and only a few transformations are possible, whereas hundreds of reactions under mild conditions are known for EBXs.[5] If a method could be found to install EBXs onto peptides/proteins, this would therefore open the door to a broad range of new bioorthogonal transformations.

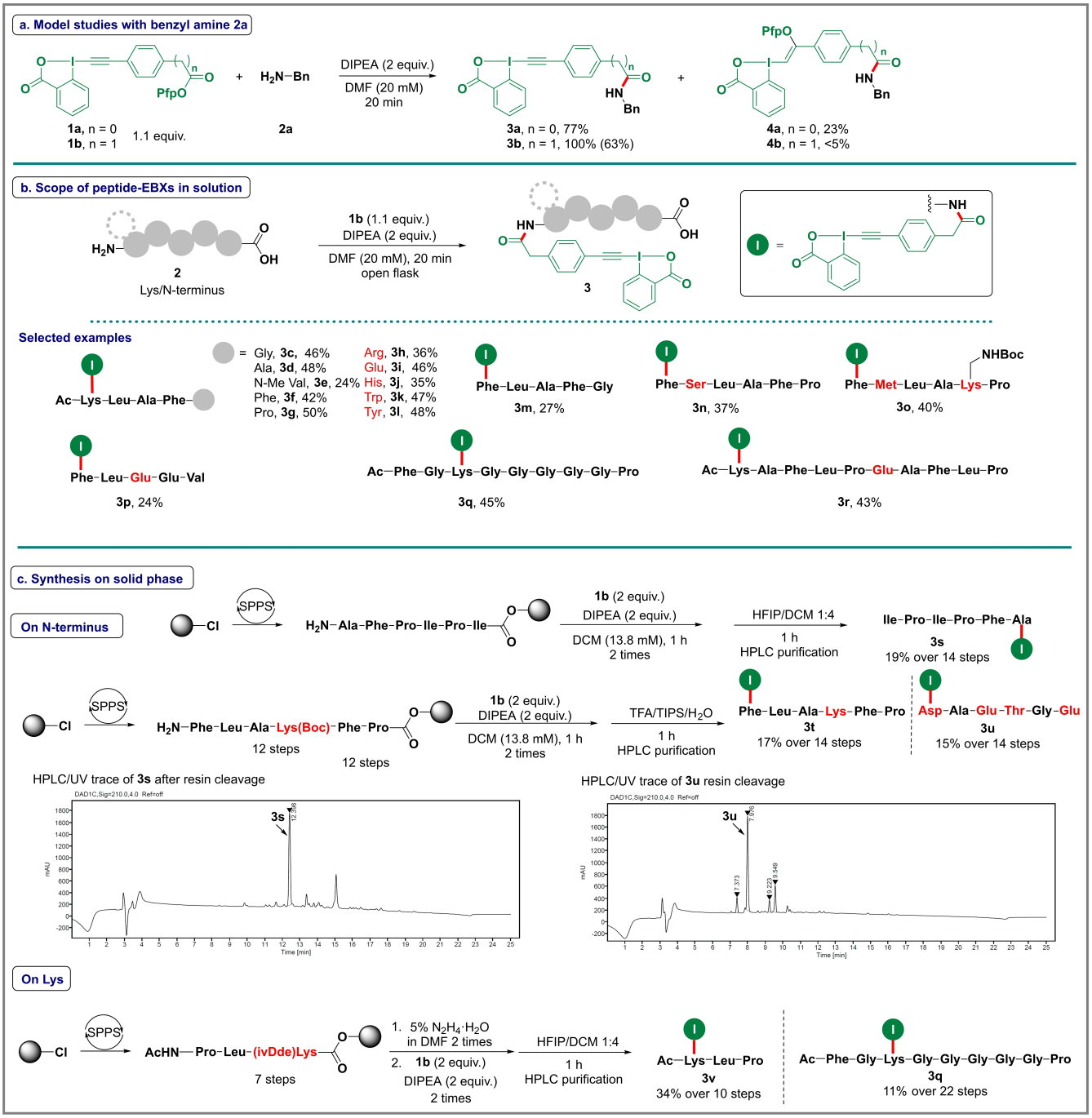
Herein, we show that a bifunctional EBX reagent containing two electrophilic sites: the alkynyl benziodoxolone (BX) and an activated pentafluorophenyl (PFP) ester, can undergo selective amide bond formation at the N-terminus or a Lys side chain with the PFP ester, forming peptide-EBX reagents (Scheme 1b). This transformation was possible both in solution and on solid phase, the latter allowing straightforward purification. Using these reagents, two transformations proceeding in the absence of a transition-metal catalyst were investigated. First, fast and selective S-alkynylation and -alkenylation with Cys-containing peptides and proteins were developed, a simple solvent switch allowing the outcome of the reaction to be changed (Scheme 1c). These transformations could also be accomplished in one pot, without purification of the EBX reagents. Second, a visible-light-mediated C-terminal decarboxylative alkynylation of peptides catalyzed by organic dyes was implemented. In the absence of a reaction partner, if the peptide bearing the EBX reagent had a free C-terminus, a new type of macrocyclization occurred, resulting in unprecedented macrocycles incorporating the rigid alkyne unit. The method was used to develop new potential inhibitors of the Keap1-Nrf2 protein-protein interaction. The rigidity of the aryl alkyne linker was important to achieve efficient binding to Keap1, as reduction of the triple bond led to a 5.5-fold decrease in binding affinity.

Results and Discussion

Synthesis of Peptide-EBXs

Recently, our group developed a novel bifunctional EBX reagent **1a** for Lys-Cys peptide stapling.[13] In this case, thiol attack onto EBXs occurs first, followed by proximity-driven amide bond formation on the pentafluorophenyl (PFP)

ester. Considering the low abundance of Cys in biomolecules, we wondered if a direct reaction between Lys or the N-terminus could be developed in the absence of free thiols to introduce the highly reactive EBX core into biomolecules. As a model reaction, we first examined selective amide formation of bifunctional EBX **1a** with benzyl amine (**2a**; Scheme 2a). We were pleased to see that the desired product **3a** was formed, albeit with a small amount of undesired O-VBX product **4a** generated through the addition of PFP to the triple bond.[14] To diminish the electrophilicity of **1a**, we synthesized modified bifunctional reagent **1b** containing one CH_2 between the EBX core and the activated ester. Indeed, no O-VBX product **4b** was formed, but amide bond formation was still efficient. We then examined the reaction on pentapeptides, focusing first on the more accessible Lys side chain in the presence of the protected N-terminus (Scheme 2b). Peptide-EBXs **3c–g** bearing only aliphatic residues were isolated in 24–50 % yield. The reactions were



Scheme 2. Synthesis of peptide-EBXs. a) Model studies. b) Synthesis of peptide-EBXs in solution. c) Synthesis of peptide-EBXs on solid phase. Yields of isolated products are given. Full experimental details are provided in the Supporting Information. HPLC and MS data can be found in Figures S1–S66 and Tables S1–S30.

nearly quantitative after only 20 min in open flasks, and mass loss occurred owing to the instability of EBX cores on reverse-phase HPLC. Importantly, the reaction was selective for Lys in the presence of nucleophilic amide (**3h** and **3i**) or aromatic (**3j–l**) residues. We then examined the potentially more challenging functionalization of the N-terminus. Gratifyingly, peptide-EBXs **3n–p** with a free N-terminal Phe group residue were obtained in comparable yield, even in the presence of unprotected Ser (**3n**) or protected Lys (**3o**). The Lys side chain could also be functionalized on larger peptides, leading to **3q** and **3r** with 9 and 10 amino acids, respectively. Although the synthesis of peptide-EBXs in solution was successful, a disadvantage was the significant mass loss upon HPLC purification. This was mostly due to partial degradation of the compound on HPLC. For example, when 10 mg pure compound **3g** were submitted to HPLC, only 6.8 mg could be recovered. Therefore, we investigated if the reagent could be synthesized directly on a solid phase (Scheme 2c). In fact, peptide-EBX **3s** functionalized on the N-terminus could be obtained on solid phase with high purity (HPLC trace of **3s** and **3u** in Scheme 2c) in 19 % over 14 steps from commercial building blocks. Moreover, we could introduce the EBX core selectively on the N-terminus (**3t**) in the presence of Boc-protected Lys during solid-phase synthesis, and the acidic stability of the EBX core allowed us to cleave the resin and Lys protecting group using TFA. The protecting group on other side chains, such as Glu, Thr and Asp, can be also removed efficiently without influencing the overall yield (**3u**). By using a well-established orthogonal protecting group on Lys,[15] peptide-EBXs **3v** and **3q** were accessed in 34 % over 10 steps and 11 % over 22 steps, respectively.

Intermolecular Reactions with Peptide-EBXs and Product Modification

After establishing the synthesis of peptide-EBXs, we explored their application in different reactions. Among the most robust reactions for EBX reagents are the alkynylation[16] and alkenylation[11b] of thiols (Scheme 3a). In an organic solvent, protected Cys was incorporated efficiently (HPLC/UV trace of **5a** after reaction in Scheme 3a) to give thioalkynes **5a–d**. Alternatively, **5a** could also be obtained in 34 % yield in a one-pot procedure from peptide **2c** without isolation of the peptide-EBX. One-pot amidation with **1b** followed by reaction with protected Cys gave **5a** in 34 % yield. Nucleophilic aromatic amino acids, such as His, Tyr and Trp, were well tolerated (**5b–d**). Glutathione could also be incorporated efficiently, either on Lys (peptide **5e**) or on the N terminus (peptide **5f**). In aqueous media, alkenylation of peptide-EBXs occurred by simple addition of the thiol to generate S-VBX products **6b** (HPLC/UV trace of **6a** after reaction in Scheme 3a). These physiological conditions can also be used for protein functionalization. As proof of concept, His_6-Cys-Ubiquitin was examined as a substrate. A quantitative reaction was observed, although in this case a 1.4:1 mixture of VBX **6b** and thioalkyne **5g** was observed.

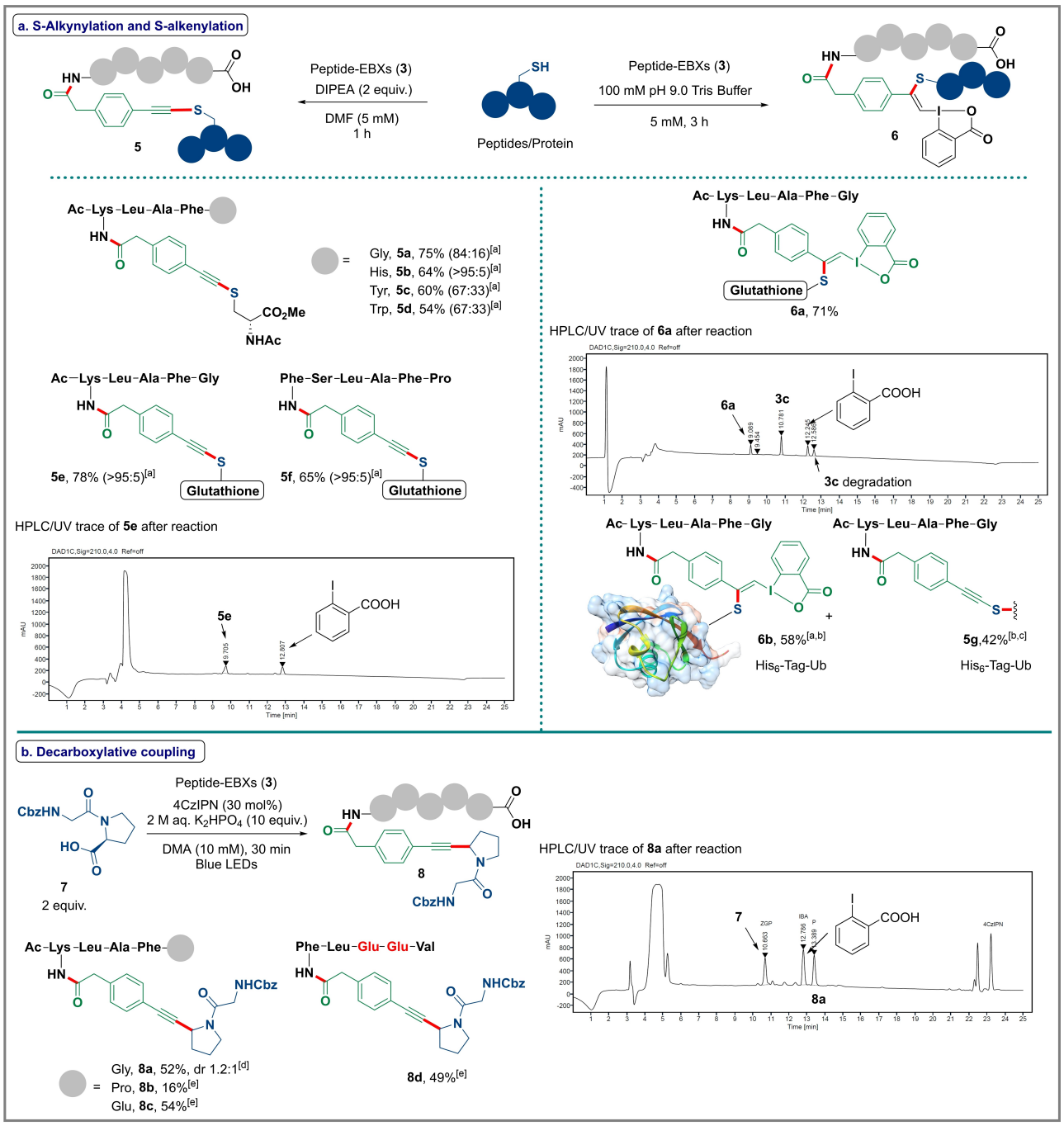
As a second transformation, we investigated C-terminal decarboxylative alkynylation using photoredox catalysis with visible light and the organic dye 4CzIPN (Scheme 3b).[6d] We selected dipeptide Cbz-GP-OH (**7**) as the coupling partner, as the decarboxylation is faster on proline as compared to other C-terminal amino acids. Decarboxylative alkynylation occurred on Lys-modified peptide-EBXs **3** to give products **8a–c** bearing a Gly (HPLC/UV trace of **8a** after reaction in Scheme 3b), a Pro and a Glu C-terminal amino acid respectively (Scheme 3b). As expected, the yield was lower for **8b** due to the competition with the C-terminal proline of **3g**. In addition, decarboxylative alkynylation was also successful in N-terminal modified EBX reagent **3p** to give alkyne **8d** in 49 % yield.

To enhance the practicability of the functionalization reactions, we then performed them directly on solid phase (Scheme 4a). To our delight, the resin-bound peptide-EBXs reacted efficiently with protected Cys to give S-alkynylation products **5h** and **5i** (HPLC/UV trace in Scheme 4a) in excellent overall yields. Interestingly, for peptide sequences FLEEV and FLAFF, by switching the resin cleavage conditions from HFIP/DCM to more acidic TFA/TIPS/H_2O, we could at the same time remove the protecting groups on the side chains and hydrate the triple bond of the thioalkyne to generate the corresponding thioester product **9a** (HPLC/UV trace in Scheme 4a)/**9b** in 22 %/19 % yield over 13 steps. Furthermore, we could extend our previous method for Cys-Lys stapling[13] on solid phase by introducing an orthogonal protecting group on Cys. The stapling reaction, followed by acidic hydration of the thioalkyne, afforded the N-terminus-Cys cyclization product **10**. Unfortunately, the photomediated decarboxylative alkynylation was not successful on solid phase so far.

Thiol and carboxylic acid alkynylation can be performed in tandem to access more functionalized products (Scheme 4c). For example, addition of a protected Cys residue to peptide-EBX **3g**, followed in one-pot by C-terminal decarboxylative alkynylation with PhEBX under photoredox conditions, gave dual functionalized product **11** in 42 % yield. In addition, the S-alkynylation product can undergo Ru^{II}-catalyzed azide-thioalkyne cycloaddition (RuAtAC)[17] to give triazole **12a**. This reaction was used to introduce a fluorescent dye (6-FAM) on thioalkyne **5a**, leading to **12b** in excellent yield and regioselectivity (Scheme 4d).

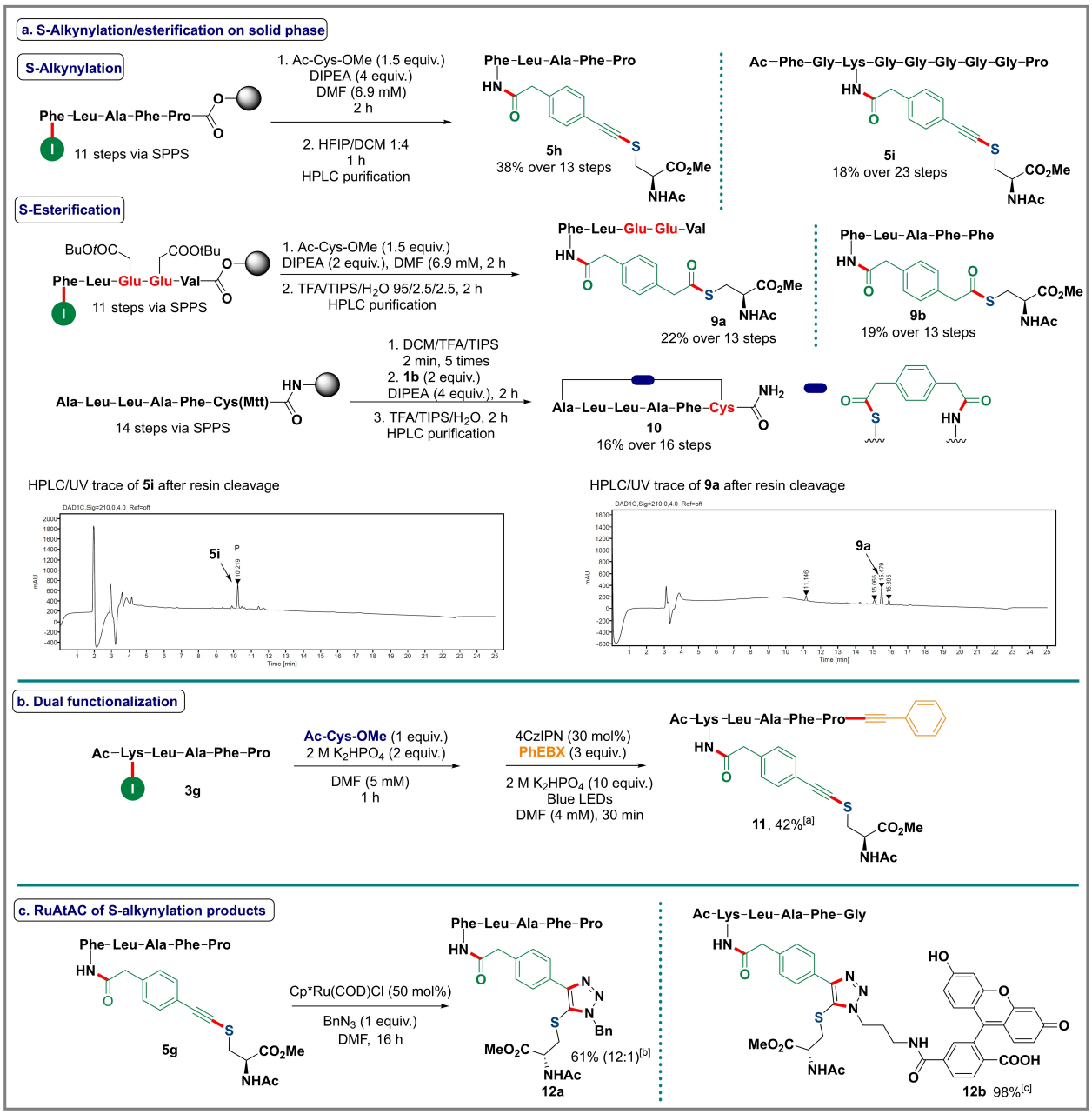
Decarboxylative Macrocyclization via Peptide-EBXs

We then wondered if the peptide-EBX reagents could be used to develop a new method for macrocyclization. Cyclic peptides are privileged scaffolds in drug discovery. As compared with their linear counterparts, they exhibit greater metabolic stability and enhanced pharmacokinetic properties.[18] Based on our group's previous work[6d] and good results from the decarboxylative cross-coupling with peptide-EBXs, we envisioned that the C-terminal radical generated from decarboxylation under photoredox conditions could be trapped by EBXs in an intramolecular



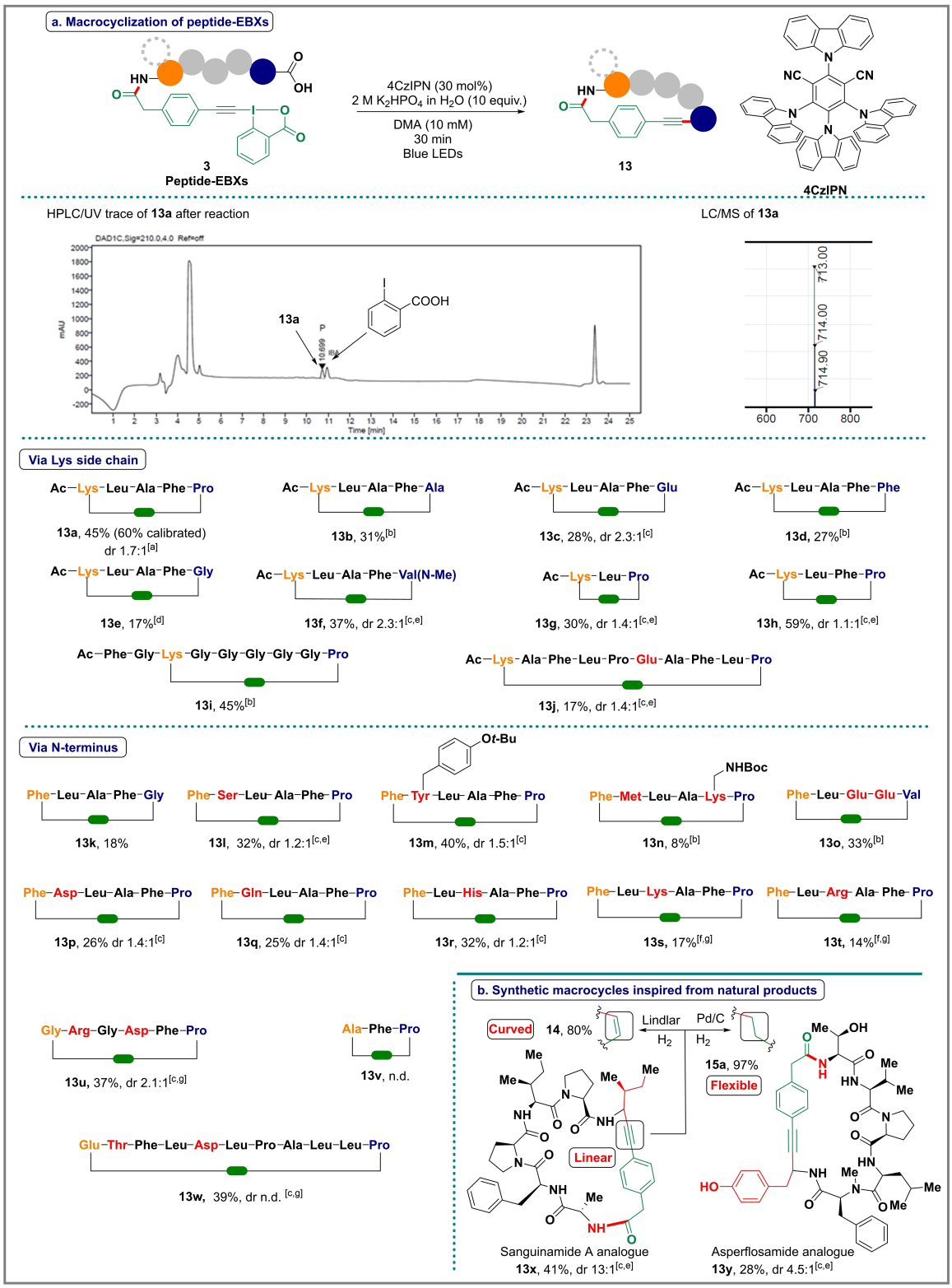
Scheme 3. Reactions with peptide-EBXs. Yields of isolated products are given. a) S-alkynylation/alkenylation of peptide-EBXs. b) Decarboxylative alkynylation of peptide-EBXs. Full experimental details are provided in the Supporting Information. HPLC and MS data for **5**, **6** and **8** can be found in Figures S67–S81 and Tables S31–S43. [a] Only the S-alkynylation product was isolated, the HPLC-UV ratio of S-alkynylation **5** and S-alkenylation product **6** is indicated in parenthesis. [b] The yield was determined based on LC–MS. [c] Ratio of S-VBX : S-alkynylation = 1.4 : 1. [d] The dr value was determined by ^{13}C NMR spectroscopy of the isolated product. [e] The dr value was not determined.

fashion, which would lead to macrocycles with a unique rigid and lipophilic phenylacetylenyl backbone (Scheme 5). Visible-light photocatalysis is very attractive for biomolecule functionalization,[19a] and it has also been applied to peptide macrocyclization.[19b] Concerning decarboxylative approaches, only a Giese-type addition of radical formed from the C-terminus has been reported by MacMillan and co-workers.[20] The flexible linkers obtained using this method are very different from the rigid and linear aryl alkynes accessible through the macrocyclization of peptide-EBXs. After optimization of the reaction conditions (see Figure S88 for details, HPLC/UV trace of **13a** in Scheme 5a), we obtained macrocycle **13a**, resulting from cyclization of the EBX on the Lys side chain with the C-terminus, in 45 %



Scheme 4. Reactions on solid phase and further product modification. a) S-alkynylation/esterification on solid phase. b) Dual functionalization using thiol and carboxylic acid alkynylation. c) RuAtAC of S-alkynylation products. Yields of isolated products are given. Full experimental details are provided in the Supporting Information. HPLC and MS data for the products can be found in Figures S82–S90 and Tables S44–S50. [a] The dr value was not determined. [b] The ratio of regioisomers is given. [c] The yield was determined based on the HPLC-UV ratio.

isolated yield (60 % calibrated yield) using the organic dye 4CzIPN as the photocatalyst (Scheme 5a). Simple commercially available blue LEDs could be used as the light source, and the reaction was finished in 30 min at room temperature. Only minor side product formation resulting from intermolecular reactions was observed. We then first tested the feasibility of the head-to-side chain cyclization with different C-terminal amino acids. To our delight, peptides with Ala, Glu and Phe as C-terminal amino acids were smoothly transformed into the desired products **13b–d**, demonstrating that the reaction is not limited to easier-to-decarboxylate Pro. Notably, the carboxylate on the Glu side chain remained untouched. Even in the case of a challenging Gly residue leading to a primary radical, macrocycle **13e** was still isolated in 17 % yield. When a lower yield was observed for the macrocyclization, iodoalkynes and solvent adducts were observed as additional side products (see the Supporting Information for details). Considering the importance of N-methylated residues for improving membrane permeability and hydrophobicity in drug discovery,[21] peptide-EBX **3e** containing N-methyl Val was also examined,



Scheme 5. Scope of decarboxylative macrocyclization. a) Macrocyclization of peptide-EBXs. b) Synthetic macrocycles inspired from natural products. The reactions were performed using peptide-EBX (0.01 mmol, 1 equiv.), 30 mol% 4CzIPN and 10 equiv. of K_2HPO_4 (2 M in milliQ water) in DMA (10 mM) at room temperature under blue LEDs for 30 min. Yields of isolated products are given. HPLC and MS data for the products can be found in Figures S92–S115. [a] The dr value was determined based on ^{1}H NMR spectroscopy of the isolated product. [b] The dr value was not determined. [c] The dr value was determined from the HPLC-UV ratio (210 nm) of the reaction crude mixture. [d] 4Cl-CzIPN (30 mol%) was used as the photocatalyst. [e] Diastereoisomers separable by RP-HPLC. [f] The yield was reported based the HPLC-UV ratio of cyclic peptides and by-products. [g] DMSO was used as the reaction solvent.

and it afforded cyclic peptide **13f** in 37 % yield as two separable diastereoisomers.

We then assessed the range of ring sizes accessible through our methodology. Macrocycles **13g–j** containing 3, 4, 9 and 10 amino acid residues, respectively, were obtained in 17–59 % yield. Besides head-to-side-chain macrocyclization, head-to-tail cyclization starting from EBX on the N-terminus was also possible (products **13k–t**). Ser (**13l**), Met (**13n**), Asp (**13p**), Gln (**13q**), His (**13r**) were well-tolerated under the reaction conditions. The commercially available peptide FLEEV, a synthetic substrate of the enzyme γ-glutamyl carboxylase (GGCX),[22] also underwent efficient cyclization to give **13o**. Free Tyr and Trp, which can be readily oxidized under photoredox conditions, are not compatible with the cyclization. Peptide sequences containing free Lys and Arg were also tested under our conditions. The corresponding cyclic peptides **13s** and **13t** were formed in lower yield, accompanied with more by-products. A RGD motif[23] containing sequence cyclized efficiently to give macrocycle **13u**. Unfortunately, the head-to-tail cyclization of the tripeptide AFP did not lead to the desired product **13v**, probably due to the strain of incorporating an alkyne in the small-ring macrocycle. The cyclization of a larger 11-mer peptide was also examined, and the corresponding cyclic product **13w** was formed in 39 % yield. Up to now, we had demonstrated the potential of our method for the synthesis of new macrocycles by diversifying both the incorporated amino acids and the ring size. As a next step, we wondered if the method could also be applied to the synthesis of synthetic analogues of known macrocyclic natural products. As a first target, we selected Sanguinamide A, a natural product extracted from *Hexabranchus sanguineus* that has been shown to have high membrane permeability and oral absorption.[24] We targeted the thioazole moiety to be replaced by the phenylacetylenyl motif, as this led to an easy-to-synthesize natural amino acid sequence. The macrocyclization step gave **13x** in 41 % yield and 13:1 diastereoselectivity. An advantage of the alkyne group is that the shape of the macrocycle can be readily changed by reducing the triple bond to either alkene **14** or alkane **15a**, leading to completely different conformations.[25] As a second target, we selected asperflosamide, recently isolated from a marine sponge-derived fungus.[26] In this case, we replaced the potentially labile ester group in the macrocycle by the rigid and stable phenylalkyne linker. The desired macrocycle **13y** was obtained in 28 % isolated yield and 4.5:1 dr.

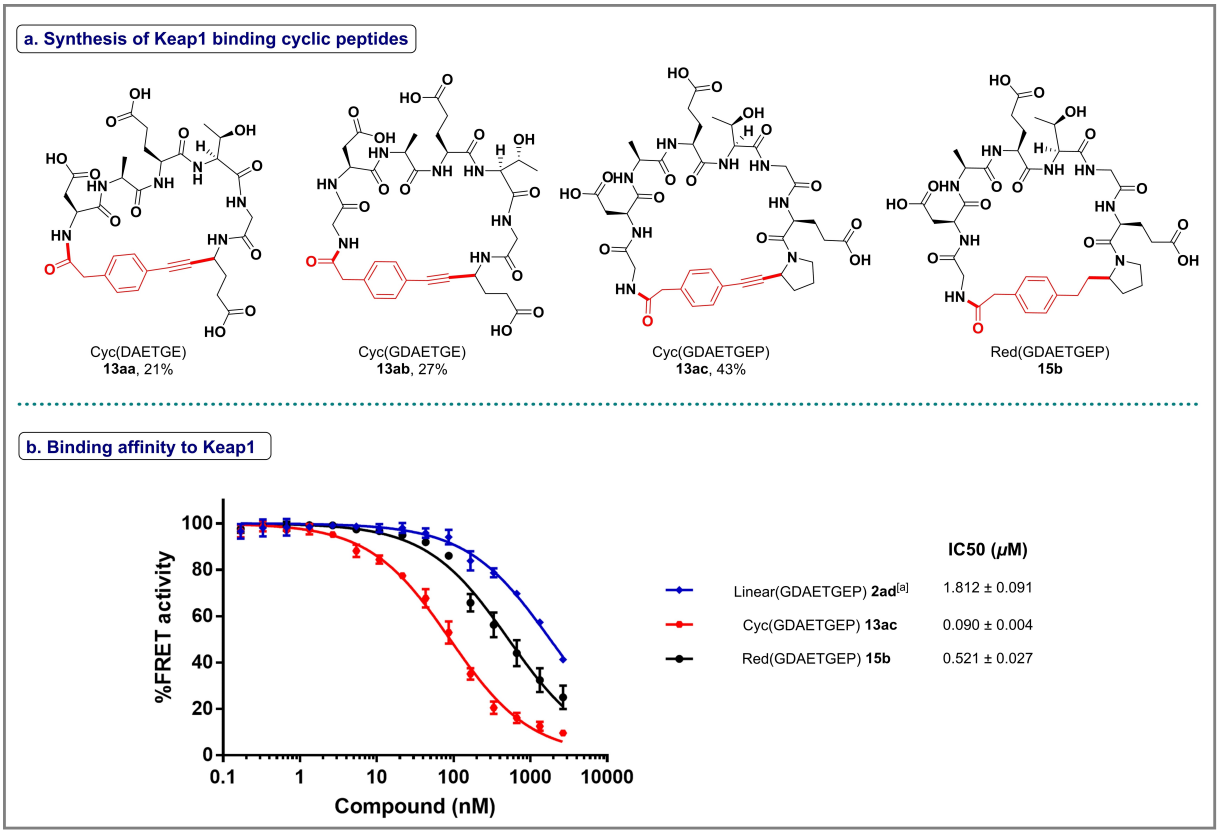


Figure 1. Binding affinity of peptidic macrocycles to the Keap1-Nrf2 PPI site a) Synthesis of Keap1 binding cyclic peptides, DMSO was used as the reaction solvent. HPLC and MS spectra for the products can be found in Figures S116–S121. b) Binding affinity was measured in TR-FRET competition assays. Average values of three independent measurements are shown. Details are provided in Figure S122 in the Supporting Information. [a] The approximate IC_{50} value of linear peptides was estimated based on the trend curves due to the low binding affinity to Keap1.

Application towards Keap1-Nrf2 Protein-Protein Interaction Inhibition

Finally, we were wondering if these macrocyclic peptides with rigid triple bonds could be potential inhibitors of protein-protein interactions (PPIs). Keap1-Nrf2 was chosen as our target due to its importance in biological pathways related to inflammation and neurodegenerative diseases.[27] Peptides containing a DxETGE motif showed good binding affinities to this target.[28] Therefore, three peptide sequences, with a DAETGE motif and varying size, from a 6-mer to an 8-mer, were synthesized. Based on our method, we could successfully cyclize the three linear peptides **13aa**–**13ac** in moderate yield (Figure 1a). The binding of cyclic peptides and their corresponding linear precursors to Keap1 was tested using a TR-FRET assay, in which the replacement of a linear Sulfo-Cy5-N_3-labeled peptide inhibitor (sequence: Ac-Propargylglycine-Peg2-LDEETGEFL-NH_2) was measured. As compared with linear peptides, enhanced binding affinity was observed for all three cyclic peptides (Figure S118). Notably, the 8-mer cyclic peptide **13ac** binds to KEAP1 with 20-fold higher affinity (IC50 = 90 nM) when compared to the linear precursor **2ad** (IC50 = 1.8 μM; Figure 1b). These results indicated that our cyclization strategy displayed potential for enhancing the binding affinity for the Keap1-Nrf2 PPI site. Intriguingly, a significant 5.5-fold drop in binding affinity was observed when we reduced the triple bond of **13ac** to a single bond in **15b**, highlighting the importance of the rigid alkyne for high binding affinity.[29]

Conclusion

To conclude, we have described the first efficient synthesis of peptide-EBX reagents both in solution and on solid phase. The unique reactivity of EBXs allowed efficient cross-coupling with Cys or C-terminal radicals generated under mild photoredox conditions, without the need for any metal catalyst or strong activating reagents, in contrast to previously existing peptide-based hypervalent iodine reagents. The decarboxylative alkynylation method could then be applied intramolecularly to the synthesis of macrocycles incorporating a rigid and linear phenylalkyne unit. This methodology allowed rapid access to cyclic peptides acting as potential inhibitors of Keap1-Nrf2 protein-protein interactions. The importance of the alkyne moiety is further supported by the lower binding affinity observed after reduction to the alkane. Considering that EBXs can be used in numerous other transformations under mild conditions, we anticipate that the discovery and application of peptide-EBXs will greatly expand the toolbox of peptide/protein modifications. Further studies on introducing the EBX group in more biocompatible ways, as well as exploring new transformations on peptide-EBXs, are ongoing in our laboratory.[30]

Supporting Information

The authors have cited additional references within the Supporting Information.[31] Experimental procedures and analytical data for all new compounds. Details of TR-FRET competition assay. HPLC, MS, ^{1}H NMR and ^{13}C NMR spectra. Raw data for NMR, IR, MS and HPLC are freely available on the platform zenodo: https://doi.org/10.5281/zenodo.8020620.

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Conflict of Interest

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: Hypervalent Iodine Reagents · Peptide/Protein Modification · Peptide Macrocyclization · Keap1-Nrf2 Protein-Protein Interaction Inhibitors.

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Supporting Information

Peptide-EBXs: Enabling Peptide Functionalization and Macrocyclization

Xing-Yu Liu, Xinjian Ji, Christian Heinis, Jerome Waser*

Table of Contents

1. General procedures

All reactions using anhydrous conditions were performed with oven-dried glassware, under an atmosphere of nitrogen, unless stated otherwise. Tetrahydrofuran, acetonitrile, diethyl ether and dichloromethane (DCM) were dried by passage over activated alumina, under nitrogen atmosphere, on an Innovative Technology Solvent Delivery System (water content < 10 ppm, Karl-Fischer titration). Dichloroethane and ethanol were purchased from Acros and trifluoroethanol was purchased from Fluorochem. DMSO was purchased from Sigma-Aldrich. All the Fmoc-protected amino acids and Rink Amide MBHA resin were purchased from GL Biochem or Bachem. 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU, Bachem) and N,N-diisopropylethylamine (DIPEA, Iris Biotech GmbH) were used as received. All the other reagents were purchased from ABCR, Acros, AlfaAesar, Apollo Scientific, Fluorochem, Fluka, Roth, Sigma-Aldrich and TCI and were used as such. For flash chromatography, distilled technical grade solvents were used. Chromatographic purification was performed as flash chromatography using Macherey-Nagel silica 40-63, 60 Å, using the solvents indicated as eluent with 0.1 – 0.5 bar pressure. TLC was performed on Merck silica gel 60 F254 TLC aluminum or glass plates and visualized with UV light or permanganate stain. Melting points were measured on a Büchi B-540 melting point apparatus using open glass capillaries. ^{1}H-NMR spectra were recorded on a Brucker DPX-400 400 MHz spectrometer in $CDCl_3$, DMSO-d_6, CD_3OD, 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 and CD_3OD as 3.35 ppm 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 1H-decoupling on a Brucker DPX-400 100 MHz spectrometer in $CDCl_3$, DMSO-d_6 or CD_3OD. All signals are reported in ppm with the internal $CHCl_3$ signal at 77.16 ppm or the internal DMSO signal at 39.52 ppm as standard. Spectra were fully assigned using COSY, HSQC, HMBC and ROESY. Infrared spectra were recorded on a JASCO FT-IR B4100 spectrophotometer with an ATR PRO410-S and a ZnSe prisma and are reported as cm^{-1} (w = weak, m = medium, s = strong, br = broad). High-resolution mass spectrometric measurements were performed by the mass spectrometry service of ISIC at the EPFL on LTQ Orbitrap ELITE ETD (Thermo fisher), Xevo G2-S QTOF (Waters), or LTQ Orbitrap ELITE ETD (Thermo fisher).

2. HPLC-MS and preparative HPLC information

HPLC-MS analysis

HPLC-MS measurements were performed on an Agilent 1290 Infinity HPLC system with a G4226a 1290 Autosampler, a G4220A 1290 Bin Pump and a G4212A 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 (solvent A) and water:acetonitrile 5:95 (solvent B), each containing 0.1% formic acid, were used as the mobile phase, at a flow rate of 0.6 $mL.min^{-1}$. The gradient was programmed as follows:

Method 1. 100% A to 100% B in 20 minutes then isocratic for 5 minutes.

Method 2: 100% A to 100% B in 30 minutes then isocratic for 5 minutes.

Method 3: 100% A to 40% B in 8 min then isocratic for 5 min, followed by 40% B to 100% B in 7 min then isocratic for 5 min.

Method 4: 100% A for 2 minutes, then 100% A to 30% A for 2-32 minutes, then 30% A to 100% B for 32-38 minutes.

The column temperature was set up to 25 °C. Low-resolution mass spectrometric measurements were acquired using the following parameters: positive electrospray ionization (ESI), temperature of drying gas = 350 °C, flow rate of drying gas = 12 $L. min^{-1}$, pressure of nebulizer gas = 60 psi, capillary voltage = 2500 V and fragmentor voltage = 70 V.

Preparative HPLC

Preparative RP-HPLC 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 (solvent A) and water:acetonitrile 5:95 (solvent B), each containing 0.1% formic acid, were used as the mobile phase at a flow rate of 20 $mL.min^{-1}$.

Method 5: 100% A for 5 minutes and then a gradient to 100% B in 20 minutes, then isocratic for 5 minutes.

Method 6: 100% A to 100% B in 30 minutes then isocratic for 5 minutes.

Method 7: 100% A to 70% B in 10 minutes then isocratic for 5 minutes, then to 100% B in 10 min.

Solid-Phase Peptide Synthesis (SPPS):

Peptides were synthesized on an MultiPep RSi parallel peptide synthesizer (Intavis) using standard Fmoc SPPS-chemistry and 2-chlorotrityl chloride resin (1.38 mmol/g, 100-200 mesh). The first amino acid was loaded on the resin by incubation of the Fmoc-protected monomer (3 equiv of the number of active sites on the resin), DIPEA (4 equiv) in dichloromethane for 2 h. Each coupling cycle was initiated by Fmoc deprotection achieved by shaking the resin with 800 µL of 20% v/v

piperidine in dimethylformamide (DMF) at 400 rpm, over 5 minutes twice. Then the resin was washed with DMF (6000 µL x7). The coupling was carried out by shaking 2-chlorotrityl chloride resin with a Fmoc-protected monomer (4.0 equiv.), HATU (4.0 equiv.), *N*-Methylmorpholine (6.0 equiv.), in DMF (1.3 mL), at 400 rpm, over 30 minutes twice. Capping using Cap Mixture (5% v/v Ac_2O and 6% v/v 2,6-lutidine in DMF) was carried out at the end of each cycle, followed by a DMF wash (6000 µL x7). The synthesis was finished by deprotection of Fmoc using 20% v/v piperidine in dimethylformamide at 400 rpm, over 5 minutes two times. The N-terminus was either left unprotected or was acylated. Acetylation of the N-terminal was achieved by incubating the resin with Cap Mixture three times. Next, washing steps were performed with dimethylformamide (5 x 3 mL). Finally, resin was dried with dichloromethane (5 x 3 mL).

Peptide cleavage and deprotection:

Peptides without protecting groups

Peptides were deprotected and cleaved from the resin by treatment with 2.5% v/v water and 2.5% v/v Triisopropyl silane in neat trifluoroacetic acid (5 mL). The resulting mixture was shaken for 2 hours, at room temperature. The resin was removed by filtration and peptides were precipitated in cold diethyl ether (50 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.

Peptides with protecting groups

Peptides were deprotected and cleaved from the resin by treatment with a 20% solution of HFIP in DCM. The resulting mixture was shaken for 1 hour at room temperature. The resin was removed by filtration and peptides were precipitated in cold diethyl ether (50 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 precipitations were further dissolved in water and acetonitrile, shell freeze and lyophilize to yield the desired crude peptides. If necessary, preparative HPLC purification was carried out.

Peptide analysis:

MS/MS fragmentation:

The regioselectivity of the introduction of EBX onto peptides was confirmed using MS/MS analysis. The spectra were obtained by the mass spectrometry service of ISIC at the EPFL using Thermo Orbitrap Elite instrument. The desired ion was selected using mass filters and submitted to fragmentations. The obtained data was analyzed using fragment generation program on eln.epfl.ch.[1] For the calculations peak threshold for intensity was set to 0.5% and 0.03% for quantity, precision was set to 5 ppm and minimal similarity: 70%. The peaks were compared to

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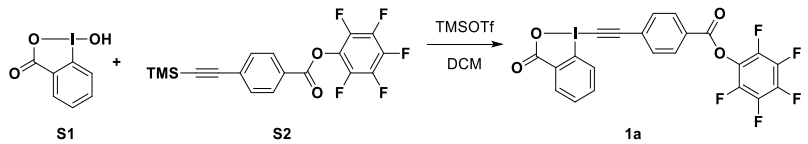
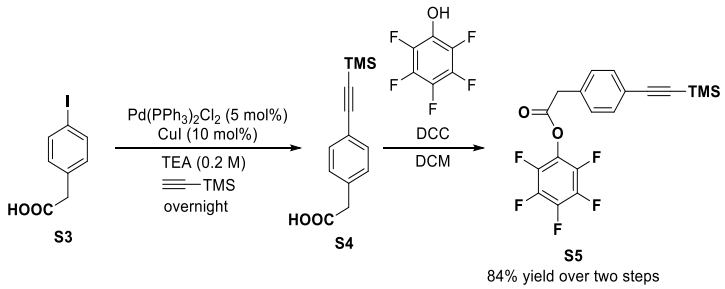
theoretical peaks. The theoretical peak width was calculated from the mass of the ion by the formula provided in the script. The zone was set to -0.5 to 3.5 ppm. y and b fragments with and without linker were selected and reported. In the cases were fragmentation was low, c and z fragments and/or fragments arising from neutral losses were included.

3. Synthesis of bifunctional alkynylation reagents



General procedure:

Following a reported procedure,[2] trimethylsilyl triflate (0.12 mL, 0.66 mmol, 1.1 equiv) was added to a suspension of 2- iodosylbenzoic acid **S1** (158 mg, 0.600 mmol, 1.00 equiv) in DCM (2 mL) at RT. The resulting suspension was stirred for 1 h, followed by the drop wise addition of **S2** (498 mg, 1.25 mmol, 1.1 equiv), which was dissolved in DCM (1 mL). The resulting suspension was stirred for 4 h at RT. A saturated solution of $NaHCO_3$ (5 mL) was then added and the mixture was stirred vigorously for 10 minutes, the two layers were separated and the organic layer was washed with sat. $NaHCO_3$ (20 mL), dried over Na_2SO_4, filtered and evaporated under reduced pressure. The mixture was purified by column chromatography with pure ethyl acetate to afford **1a** (212 mg, 0.380 mmol, 63%) as a colorless solid.
^{1}H NMR (400 MHz, $CDCl_3$) δ 8.45 (dd, *J* = 6.7, 2.5 Hz, 1H, ArH), 8.30 – 8.23 (m, 3H, ArH), 7.82 (ddd, *J* = 6.5, 4.4, 1.7 Hz, 2H, ArH), 7.79 (d, *J* = 8.4 Hz, 2H, ArH).
Spectroscopic data was consistent with the values reported in literature.[2]



General procedure:[3]

To a solution of 2-(4-iodophenyl)acetic acid **S3** (2.5 g, 10 mmol, 1 equiv.), $PdCl_2(PPh_3)_2$ (0.35 g, 0.50 mmol, 5 mol%), CuI (0.19 mg, 1.0 mmol, 10 mol%) in Et_3N (40 mL) under argon, trimethylsilylacetylene (1.7 g, 2.4 mL, 17 mmol, 1.7 equiv.) was added. The mixture was stirred overnight at room temperature, then filtered through a pad of celite®. The residue was washed with EtOAc (100 mL) and the organic layer was successively washed with aqueous 1 M HCl solution (3 x 30 mL), water (50 mL) and brine (2 x 30 mL). After drying (Na_2SO_4), filtration and concentration under reduced pressure, the desired product **S4** was obtained without further purification.
The crude product **S4** and DCC (2.1 g, 20 mmol, 2 equiv.) were added in the 50 mL vial and dissolved in DCM (20 mL) and stirred for 10 min, at which time, pentaflurophenol (2.8 g, 15 mmol, 1.5 equiv.) was added and the reaction was stirred overnight. After the reaction, the mixture was filtered through a pad of celite. The solution was concentrated under vaccum, and the residue

[2] Ceballos, J.; Grinhagena, E.; Sangouard, G.; Heinis, C.; Waser, J., Cys–Cys and Cys–Lys Stapling of Unprotected Peptides Enabled by Hypervalent Iodine Reagents. *Angew. Chem., Int. Ed.* **2021,** *60* (16), 9022-9031.
[3] Pantin, M.; Bodinier, F.; Saillour, J.; Youssouf, Y. M.; Boeda, F.; Pearson-Long, M. S. M.; Bertus, P., Convenient and easy access to 2-hydroxycyclopent-2-enones from acylcyanohydrins. *Tetrahedron* **2019,** *75* (33), 4657-4662.

was purified by column chromatography (Pentane/EA 10:1) to afford the desired product **S5** as colorless solid (3.4 g, 8.4 mmol, 84% yield,).

R_f (Pentane/EA 10:1) = 0.25. M.P. 65.6-68.9 ºC.

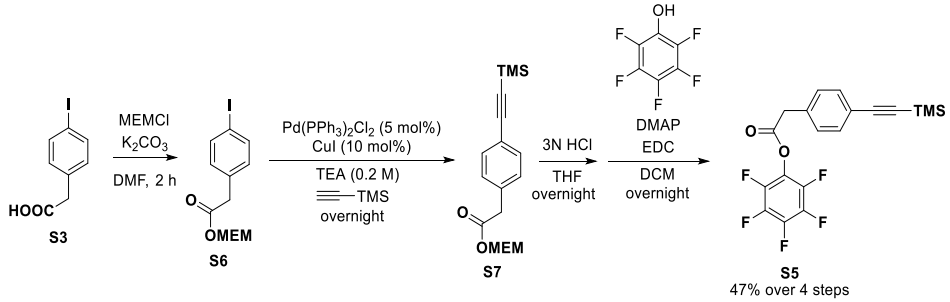
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.48 (d, *J* = 8.4 Hz, 2H, C_{Ar}-H), 7.29 (d, *J* = 8.5 Hz, 2H, C_{Ar}-H), 3.95 (s, 2H, CH_2), 0.25 (s, 9H, $SiCH_3$).

^{13}C NMR (101 MHz, $CDCl_3$) δ 167.1, 141.2 (dm, *J* = 239.7 Hz, C_{Ar}-F), 139.6 (dm, *J* = 253.5 Hz, C_{Ar}-F), 137.9 (dm, *J* = 248.7 Hz, C_{Ar}-F), 132.5 (C_{Ar}), 132.3 (C_{Ar}), 125.1 (m, C_{Ar}-O), 122.9 (C_{Ar}), 104.5 (C_{Ar}), 95.0 (C*C*-C_{Ar}), 77.3 (*C*C-C_{Ar}), 40.1 (*C*H_2), 0.00 (Si*C*H_3).

IR (cm^{-1}): 2962, 2160, 1790, 1520, 1250, 1219, 1092.

HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{19}H_{16}F_5O_2Si^+$ 399.0834; Found 399.0842.

Alternative pathway:



Sometimes the work-up of Sonogashira coupling with the free acid didn't work properly, which resulted in brown or yellow solid for the product. One alternative pathway was developed. Following a reported procedure:[4]

MEM protection:

K_2CO_3 (1.6 g, 12 mmol, 1.2 equiv.) and 2-methoxyethoxymethyl chloride (MEMCl) (1.5 mL, 13 mmol, 1.3 equiv.) were added to a reaction vessel containing a solution of 2-(4-iodophenyl)acetic acid **S3** (2.6 g, 10 mmol, 1 equiv.) in DMF (20 mL). The mixture was stirred at room temperature for 2 h, poured onto 5% LiCl solution (20 mL), and then extracted with EtOAc (3 x 10 mL). The combined layer was washed with brine, dried over Na_2SO_4 and concentrated. The desired product **S6** was obtained as brown oil (3.2 g, 9.5 mmol, 95% yield) without further purification.

Sonogashira coupling:

To a solution of crude compound **S6** (3.2 g, 9.5 mmol. 1 equiv.), $PdCl_2(PPh_3)_2$ (167 mg, 0.238 mmol, 2.5 mol%), CuI (90 mg, 0.48 mmol, 5 mol%) in Et_3N (30 mL) under argon, trimethylsilylacetylene (1.4 g, 2.0 mL, 14 mmol, 1.5 equiv.) was added. The mixture was stirred overnight at room temperature, then filtered through a pad of celite®. The residue was concentrated under reduced pressure, the desired product **S7** was purified by column chromatography with (Pentane/EA 4:1) as brown oil (2.8 g, 8.7 mmol, 92% yield).

R_f (Pentane/EA 4:1) = 0.3.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.42 (d, *J* = 8.3 Hz, 2H, C_{Ar}-H), 7.22 (d, *J* = 8.3 Hz, 2H, C_{Ar}-H), 5.32 (s, 2H, OCH_2O), 3.74 – 3.67 (m, 2H, OCH_2), 3.64 (s, 2H, CH_2), 3.53 – 3.46 (m, 2H, OCH_2), 3.36 (s, 3H, OCH_3), 0.24 (s, 9H, $Si(Me)_3$).

^{13}C NMR (126 MHz, $CDCl_3$) δ 170.8, 134.2, 132.3, 129.3, 122.2, 104.9, 94.5, 89.9, 71.5, 69.7, 59.2, 41.5, 0.1.

[4] Shen, J.; Woodward, R.; Kedenburg, J. P.; Liu, X.; Chen, M.; Fang, L.; Sun, D.; Wang, P. G., Histone Deacetylase Inhibitors through Click Chemistry. *J. Med. Chem.* **2008,** *51* (23), 7417-7427.

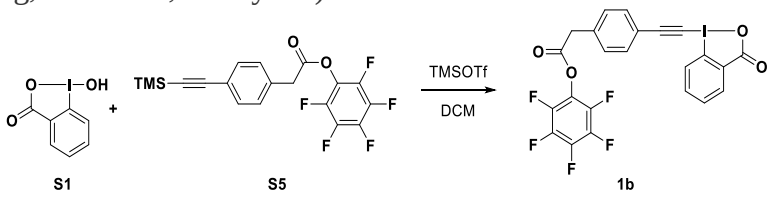
HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + Na]^+$ Calcd for $C_{17}H_{24}NaO_4Si^+$ 343.1336; Found 343.1336.

MEM deprotection:

3N HCl (12 mL) and MEM-protected **S6** (2.8 g, 8.7 mmol, 1 equiv.) were added into in THF (30 mL). The reaction mixture was stirred overnight and was then concentrated under vacuum. The residue was extracted 3 times with EA, dried over Na_2SO_4 and concentrated to give the desired product **S4** as yellow solid (1.8 g, 7.9 mmol, 83% yield) without further purification.

Condensation:

The product **S4** (1.8 g, 7.9 mmol), pentaflurophenol (2.2 g, 12 mmol, 1.5 equiv.), DMAP (0.096 g, 0.79 mmol ,0.1 equiv.) and EDC • HCl (2.2 g, 12 mmol, 1.5 equiv.) were added in the 50 mL vial and dissolved in DCM (30 mL) at room temperature and stirred overnight. After the reaction, the mixture was quenched with 20 mL saturated $NaHCO_3$, and extracted with DCM (3 x 10 mL). The combined solution was dried with Na_2SO_4 and concentrated under vacuum. The residue was purified by column chromatography (Pentane/EA 10:1) to afford the desired product **S5** as colorless solid (1.9 g, 4.7 mmol, 60% yield).



S1 S5 1b

General procedure:

Trimethylsilyl triflate (0.23 mL, 1.3 mmol, 1.1 equiv) was added to a suspension of 2-iodosylbenzoic acid **S1** (300 mg, 1.13 mmol, 1.0 equiv) in DCM (2 mL) at RT. The resulting suspension was stirred for 1 h, followed by the drop wise addition of **S5** (498 mg, 1.25 mmol, 1.1 equiv), which was dissolved in DCM (1 mL). The resulting suspension was stirred for 4 h at RT. A saturated solution of $NaHCO_3$ (5 mL) was then added and the mixture was stirred vigorously for 10 minutes, the two layers were separated and the organic layer was washed with sat. $NaHCO_3$ (20 mL), dried over Na_2SO_4, filtered and evaporated under reduced pressure. The mixture was purified by column chromatography with pure ethyl acetate to afford **1b** (190 mg, 0.332 mmol, 29%) as a colorless fluffy solid.

R_f (EA) = 0.2. ^{1}H NMR (800 MHz, $CDCl_3$) δ 8.42 (dd, *J* = 7.1, 2.0 Hz, 1H, C_{Ar}-H), 8.24 (dd, *J* = 7.8, 1.3 Hz, 1H, C_{Ar}-H), 7.81 – 7.75 (m, 2H, C_{Ar}-H), 7.62 (d, *J* = 8.2 Hz, 2H, C_{Ar}-H), 7.44 (d, *J* = 7.9 Hz, 2H, C_{Ar}-H), 4.04 (s, 2H, CH_2).

^{13}C NMR (201 MHz, $CDCl_3$) δ 166.9, 166.7, 141.3 (dm, *J* = 254.1 Hz, *C*$_{Ar}$-F), 139.8 (dm, *J* = 253.7 Hz, *C*$_{Ar}$-F), 138.0 (dm, *J* = 251.9 Hz, *C*$_{Ar}$-F), 135.1, 135.0, 133.5, 132.7, 131.8, 131.4, 129.9, 126.4, 125.0 (m, *C*$_{Ar}$-O), 120.3, 116.3, 106.0 (C*C*-*C*$_{Ar}$), 51.2(C*C*-*C*$_{Ar}$), 40.2.

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{23}H_{11}F_5IO_4^+$ 572.9617; Found 572.9617.

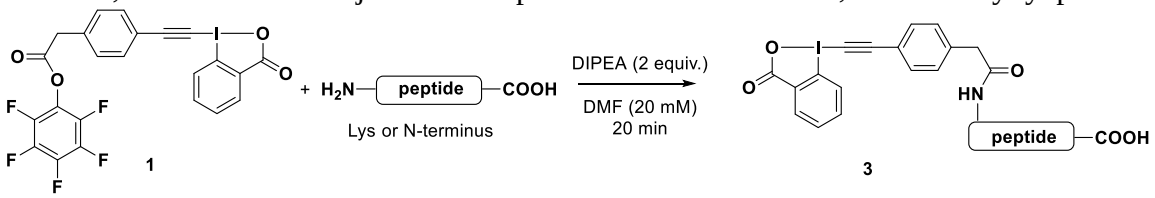
IR (cm^{-1}): 3070, 2962, 2148, 1790, 1620, 1520, 1338, 1092, 999.

M.P. 91.5-92.7 ºC.

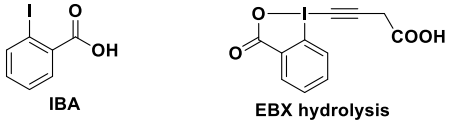
4. General procedure for synthesis of peptide-EBXs

To a solution of peptide (0.03 mmol), bifunctional EBX reagent **1b** (0.033 mmol, 1.1 equiv.) in DMF (1.5 mL), DIPEA (10 μL, 0.060 mmol, 2 equiv.) was added into the solution (concentration:

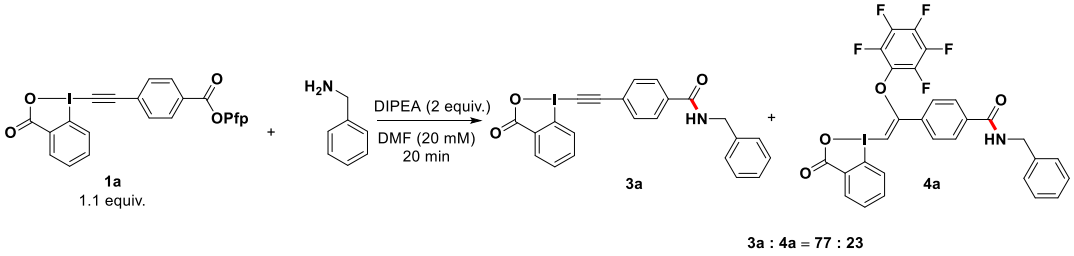
20 mM) and the mixture was stirred for 20 min without protection of atmosphere or light. For the isolation, the crude was subjected to Prep-HPLC without dilution, followed by lyophilization.



Common side-products that can be observed in HPLC:



4.1 Test on the selective amide formation



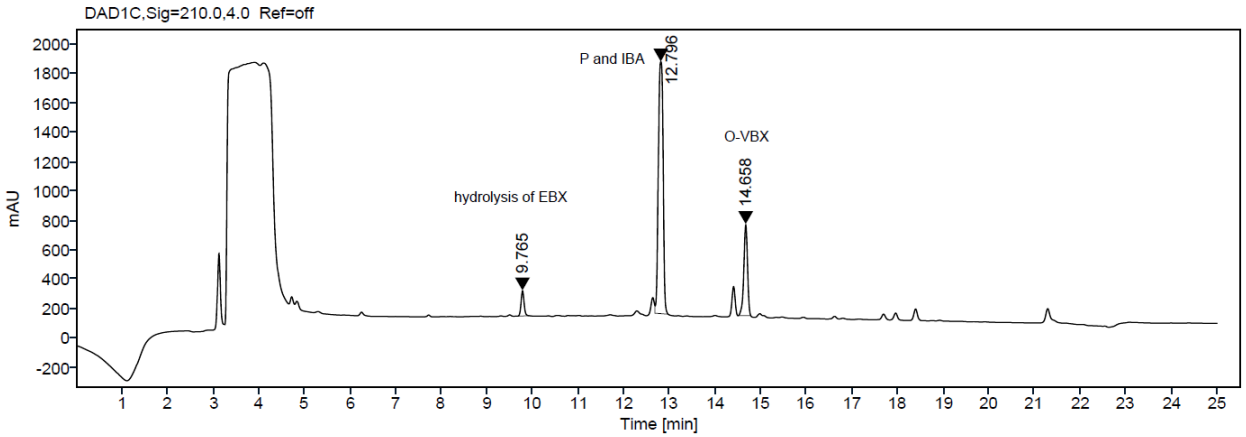
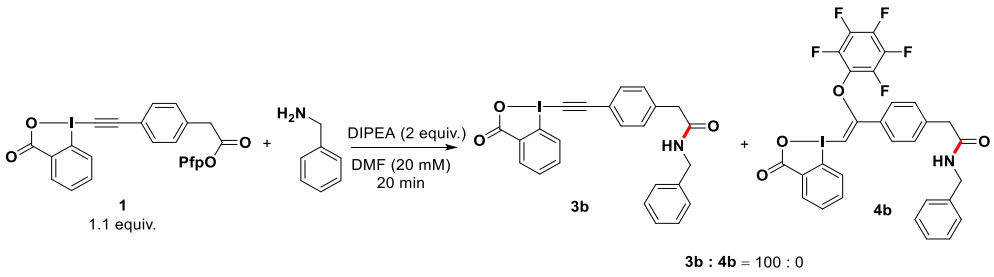


Figure S1: HPLC-UV chromatogram (210 nm) of the crude **3a** by **Method 1**. The ratio was determined based on the UV absorption (210 nm) of **3a** and **4a**.



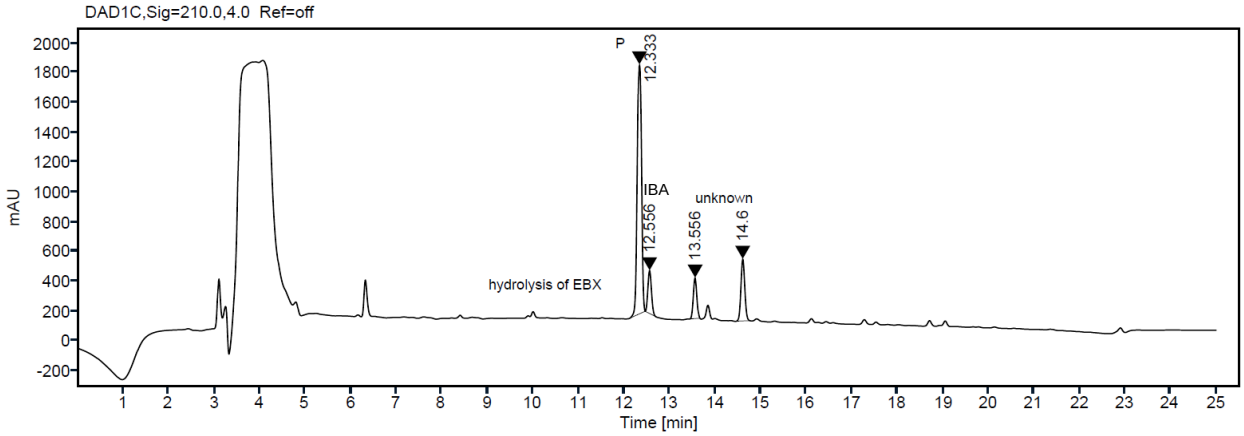
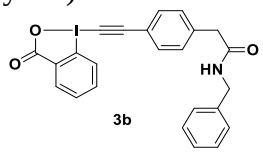


Figure S2: HPLC-UV chromatogram (210 nm) of the crude by **Method 1.** The ratio was determined based on the UV absorption (210 nm) of **3b** and **4b**.

***N*-benzyl-2-(4-((3-oxo-1l3-benzo[d][1,2]iodaoxol-1(3*H*)-yl)ethynyl)phenyl)acetamide (3b)**

Following the general procedure, the reaction was conducted in 0.048 mmol scale. The desired product **3b** (12 mg, 0.025 mmol, 63% yield) was isolated by **Method 6**.



3b

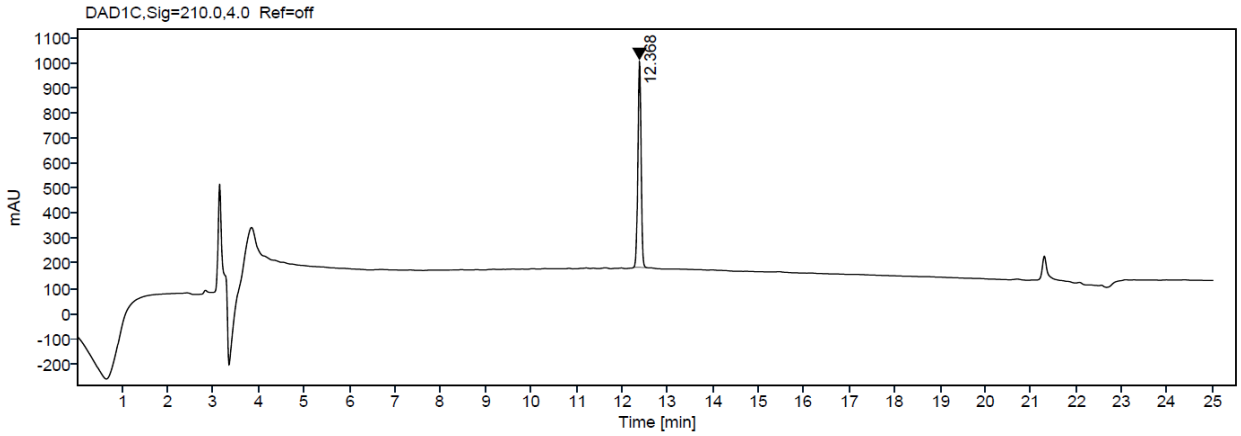


Figure S3: HPLC-UV chromatogram (210 nm) of **3b** by **Method 1**.

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.62 (t, *J* = 5.9 Hz, 1H, NH), 8.31 (d, *J* = 8.1 Hz, 1H, C_{Ar}-H), 8.14 (dd, *J* = 7.4, 1.7 Hz, 1H, C_{Ar}-H), 7.91 (ddd, *J* = 8.4, 7.2, 1.7 Hz, 1H, C_{Ar}-H), 7.81 (t, *J* = 7.3 Hz, 1H, C_{Ar}-H), 7.70 – 7.62 (m, 2H, C_{Ar}-H), 7.44 – 7.38 (m, 2H, C_{Ar}-H), 7.36 – 7.28 (m, 2H, C_{Ar}-H), 7.27 – 7.22 (m, 3H, C_{Ar}-H), 4.28 (d, *J* = 5.9 Hz, 2H, NCH_2), 3.58 (s, 2H, CH_2).

^{13}C NMR (151 MHz, DMSO-d_6) δ 169.5, 166.3, 139.4, 139.3, 135.1, 132.5, 132.1, 131.3, 131.3, 129.8, 128.4, 127.5, 127.3, 126.9, 118.5, 116.4, 104.4 (C*C*-*C*$_{Ar}$), 51.8 (C*C*-*C*$_{Ar}$), 42.3, 42.2.

HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{24}H_{19}INO_3^+$ 496.0404; Found 496.0408.

4.2 Scope of peptide-EBXs

Peptide EBX of Ac-KLAFG (3c)

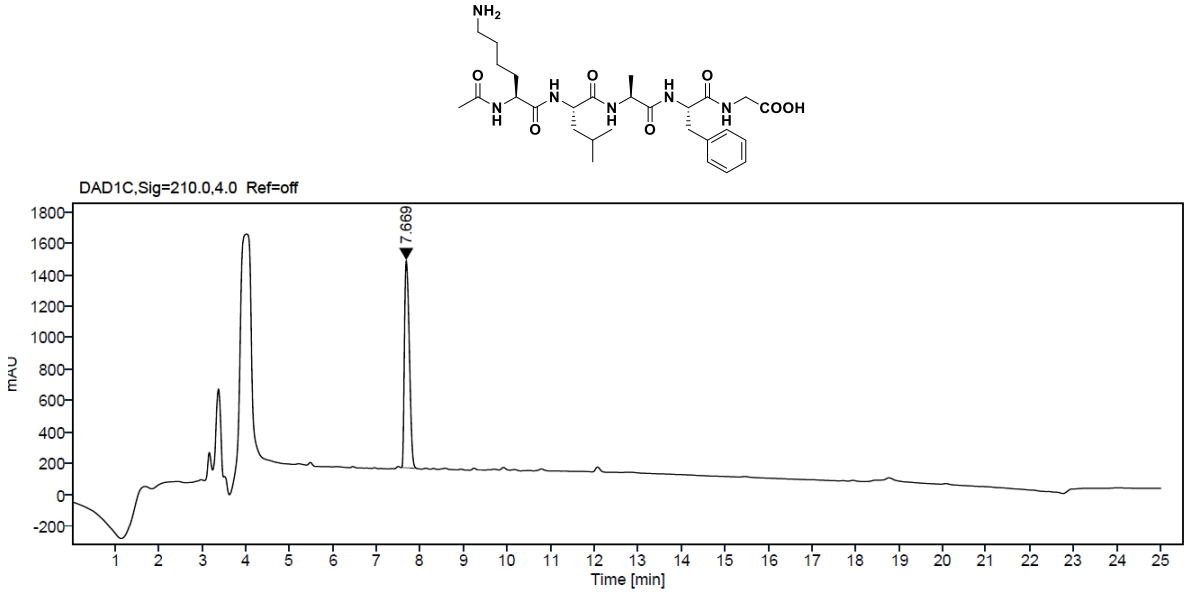
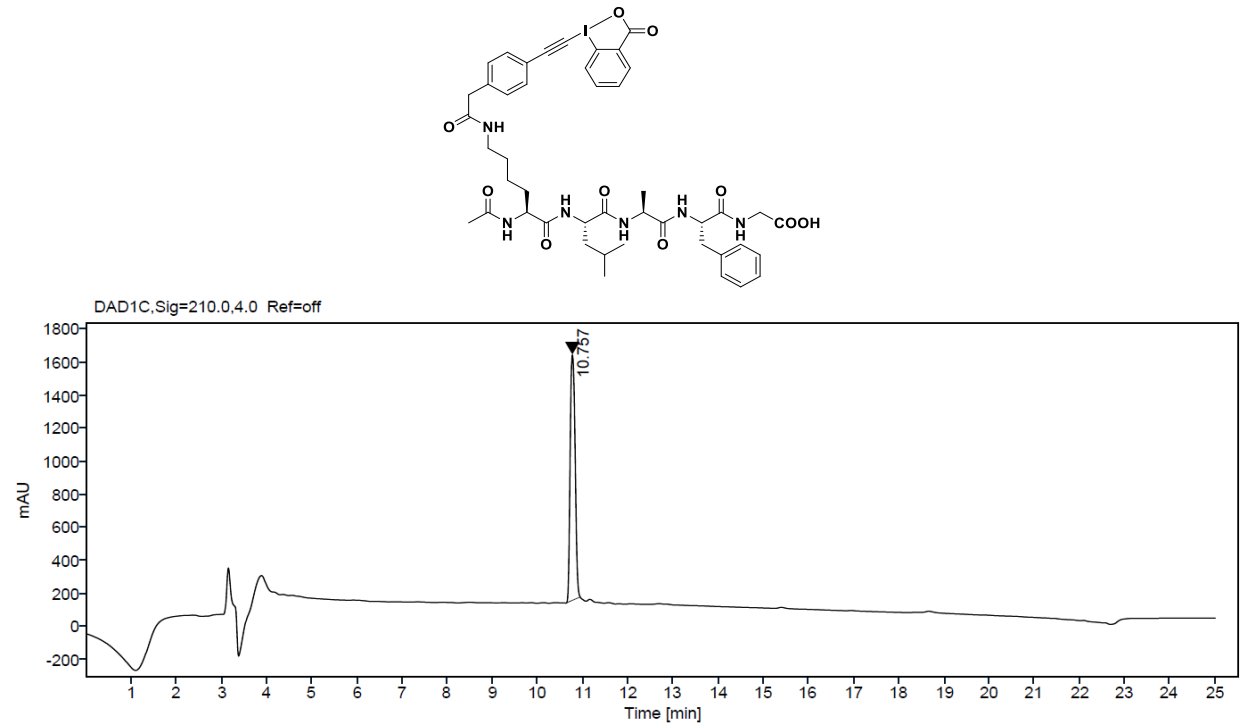


Figure S4: HPLC-UV chromatogram (210 nm) of Ac-KLAFG by **Method 1.**

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{28}H_{45}N_6O_7^+$ 577.3344; Found 577.3359.
Following the general procedure, the reaction was conducted on a 0.03 mmol scale. The desired product **3c** (13 mg, 0.013 mmol, 46% yield) was isolated by **Method 5**.



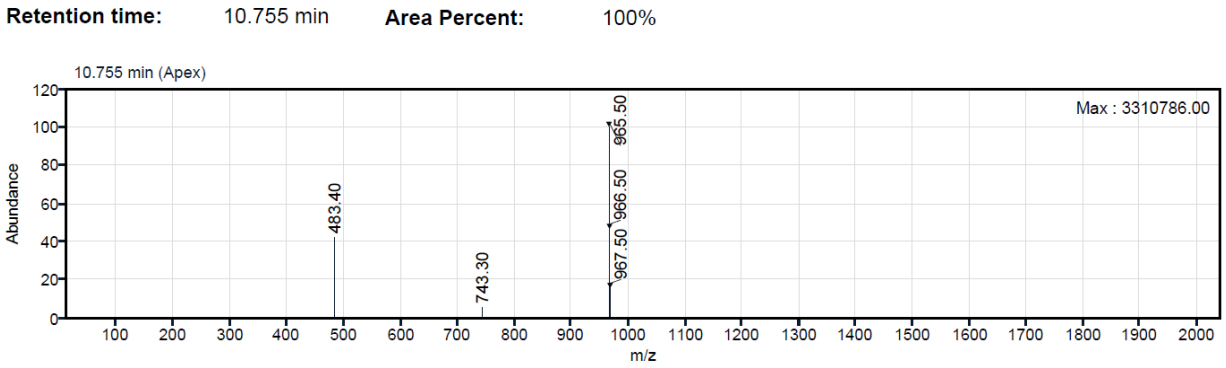


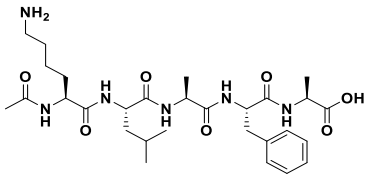
Figure S5: HPLC-UV chromatogram (210 nm) and MS(ESI) of **3c** by **Method 1**.

HRMS (LTQ-Orbitrap) m/z: $[M + H_2]^{+2}$ Calcd for $C_{45}H_{55}IN_6O_{10}^{+2}$ 483.1507; Found 483.1517.
Table S1: MS/MS fragmentation of **3c**:

κ = Lys(C17H9IO3)
Nter = C2H3O

Sequence	Type	MF	MF Mass	m/z	Intensity	Similarity
FG	y2	C11H15N2O3(+1)	223.1083	223.1077	17.5	94.64%
AFG	y3	C14H20N3O4(+1)	294.1454	294.1448	1.92	93.53%
LAFG	y4	C20H31N4O5(+1)	407.2294	407.2289	4.24	92.76%
KLAF	b4	C43H49IN5O8(+1)	890.2626	445.6347	102.79	91.61%
KLAF	a4	C42H49IN5O7(+1)	862.2677	431.6372	0.65	91.59%
K	b1	C25H24IN2O5(+1)	559.073	559.0724	4.54	91.53%
KL	b2	C31H35IN3O6(+1)	672.1571	672.1565	3.02	90.91%
KLA	b3	C34H40IN4O7(+1)	743.1942	743.1936	27.32	89.77%
KLA	b3	C34H40IN4O7(+1)	743.1942	372.1004	7.86	87.26%

Peptide EBX of Ac-KLAFA (3d)



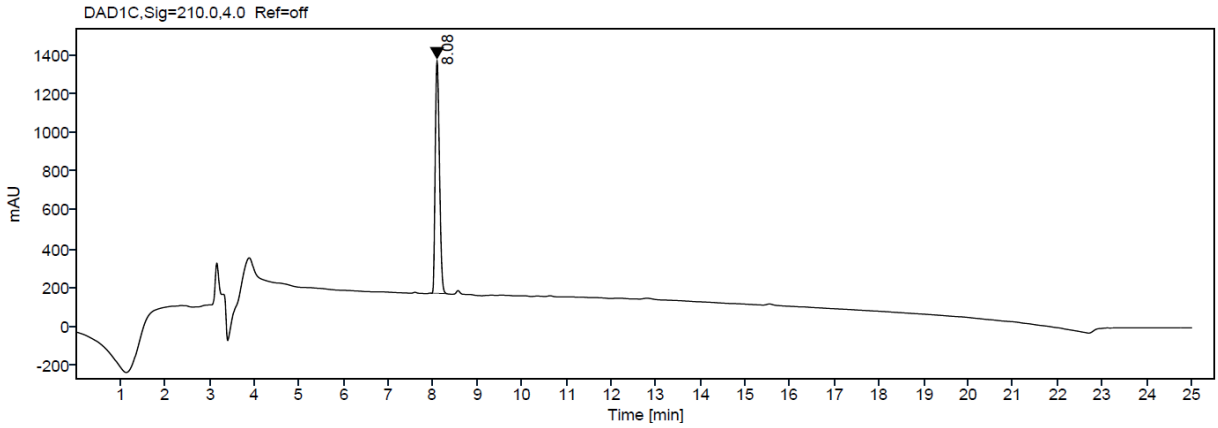
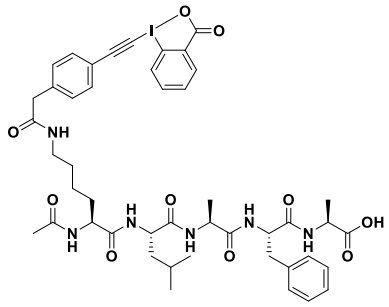
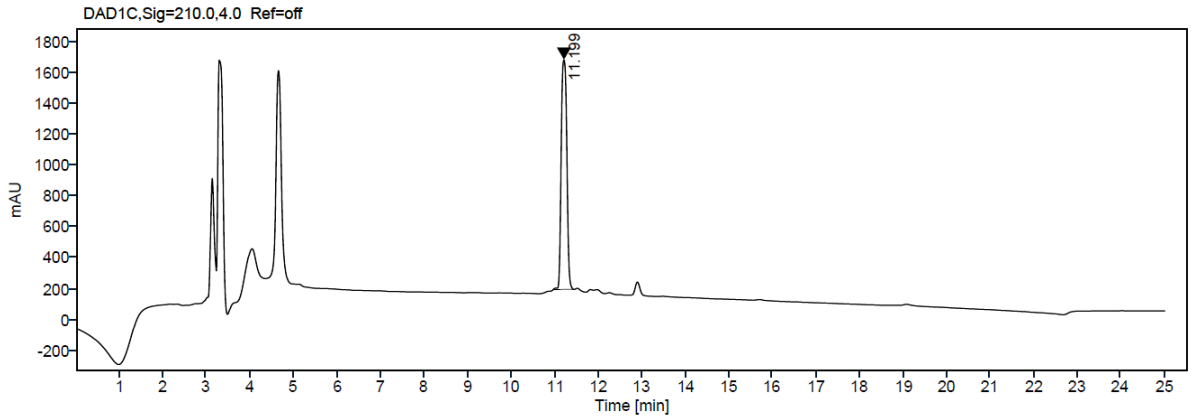


Figure S6: HPLC-UV chromatogram (210 nm) of Ac-KLAFA by **Method 1.**
HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{29}H_{47}N_6O_7^+$ 591.3501; Found 591.3516.
Following the general procedure, the reaction was conducted on a 0.02 mmol scale. The desired product **3d** (9.4 mg, 0.096 mmol, 48% yield) was isolated by **Method 5**.





Retention time: 11.167 min **Area Percent:** 100%

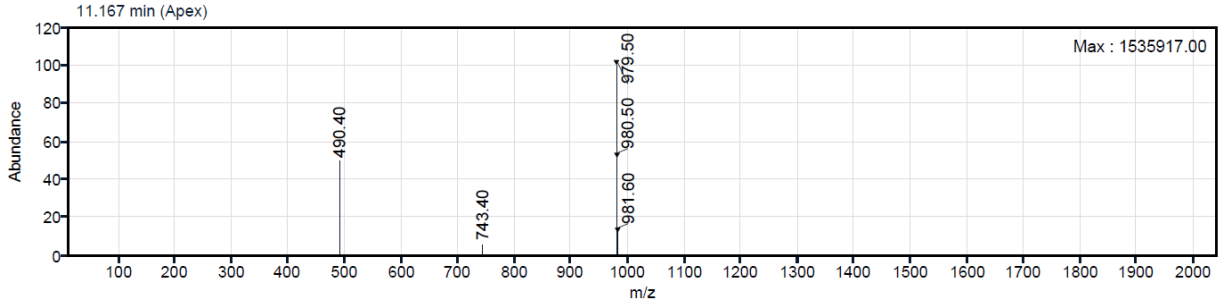
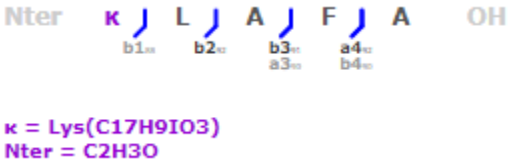


Figure S6: HPLC-UV chromatogram (210 nm) and MS(ESI) of **3d** by **Method 1**.
HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{46}H_{56}IN_6O_{10}^+$ 979.3097; Found 979.3103.
Table S2: MS/MS fragmentation of **3d**:



Sequence	Type	MF	MF Mass	m/z	Intensity	Similarity
KL	b2	C31H35IN3O6(+1)	672.1571	672.1565	9.68	92.16%
KLAF	a4	C42H49IN5O7(+1)	862.2677	862.2671	76.88	91.57%
KLA	b3	C34H40IN4O7(+1)	743.1942	743.1936	33.46	90.84%
KLAF	b4	C43H49IN5O8(+1)	890.2626	890.262	102.26	89.81%
KLA	a3	C33H40IN4O6(+1)	715.1993	715.1987	2.58	89.56%
K	b1	C25H24IN2O5(+1)	559.073	559.0724	13.02	87.89%

Peptide EBX of Ac-KLAF(N-Me)V (3e)

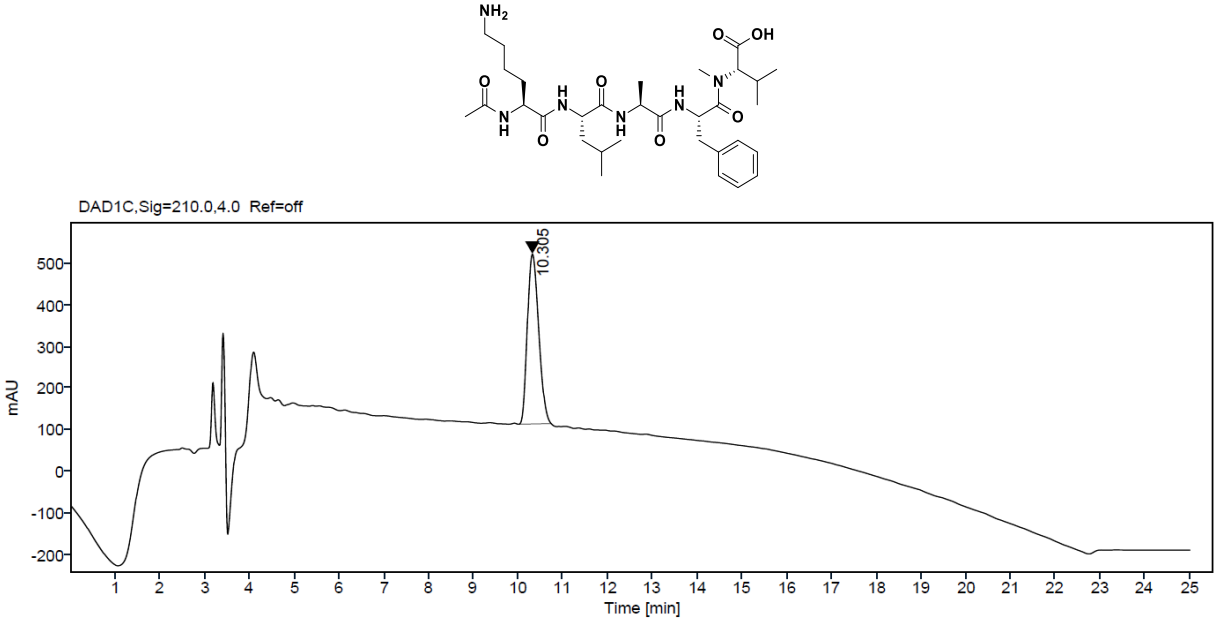


Figure S7: HPLC-UV chromatogram (210 nm) of Ac-KLAF(N-Me)V by **Method 1.**

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{32}H_{53}N_6O_7^+$ 633.3970; Found 633.3982.
Following the general procedure, the reaction was conducted on a 0.03 mmol scale. The desired product **3e** (7.4 mg, 0.013 mmol, 24% yield) was isolated by **Method 5**.

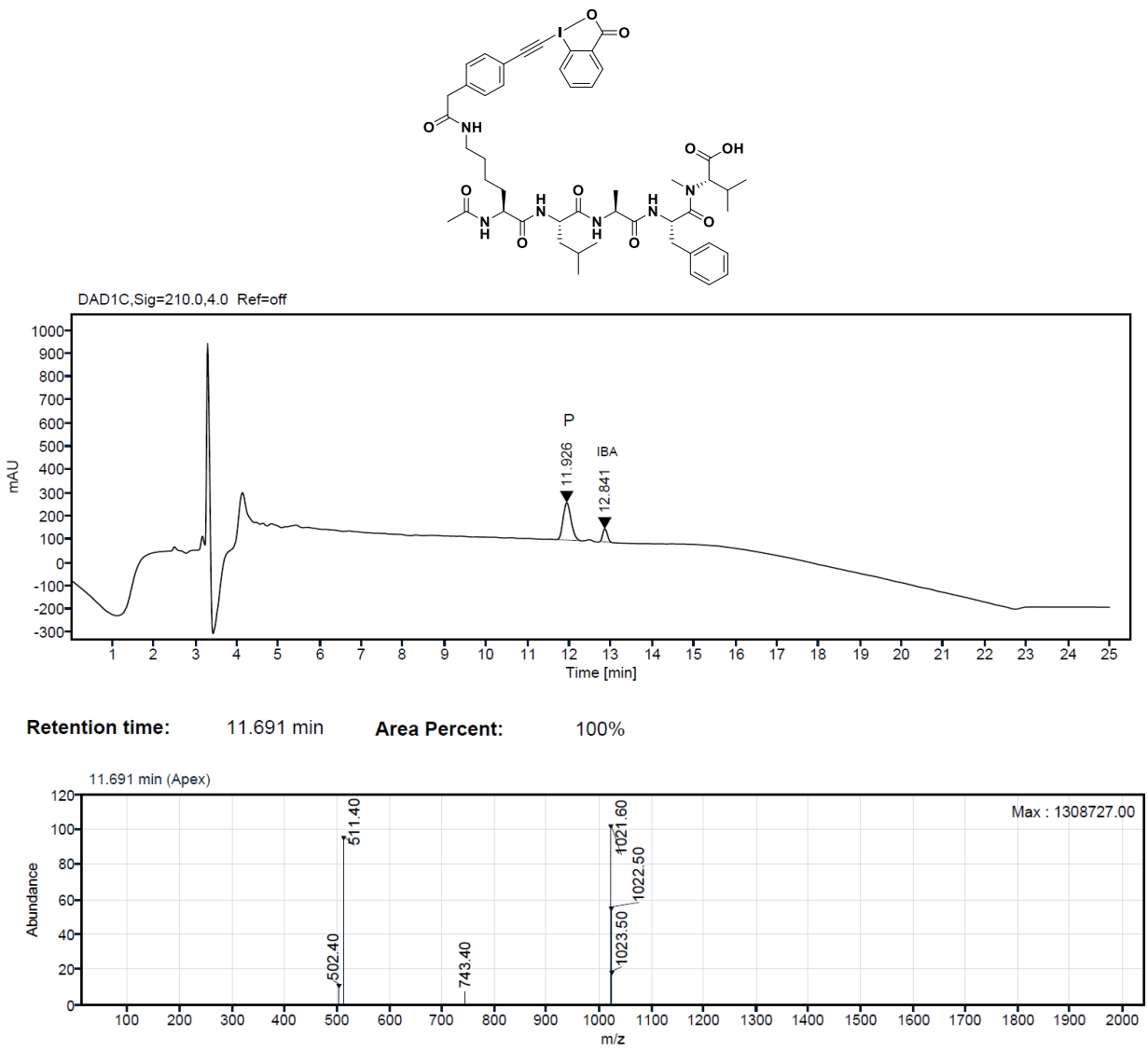
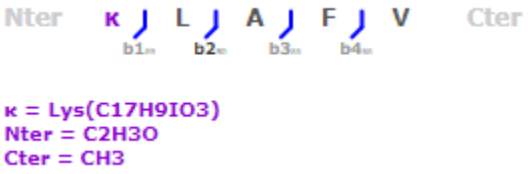


Figure S8: HPLC-UV chromatogram (210 nm) and MS(ESI) of **3e** by **Method 1**.
HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{49}H_{62}IN_6O_{10}^+$ 1021.3567; Found 1021.3561.

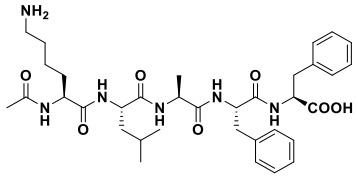
Table S3: MS/MS fragmentation of **3e**:



Sequence	Type	MF	MF Mass	m/z	Intensity	Similarity
KL	b2	C31H35IN3O6(+1)	672.1571	672.1565	0.56	90.37%
KLAF	b4	C43H49IN5O8(+1)	890.2626	890.262	10.41	89.87%
K	b1	C25H24IN2O5(+1)	559.073	559.0724	0.83	89.26%
KLAF	b4	C43H49IN5O8(+1)	890.2626	445.6347	29.36	87.00%

KLA	b3	C34H40IN4O7(+1)	743.1942	743.1936	7.01	84.81%

Peptide EBX of Ac-KLAFF (3f)



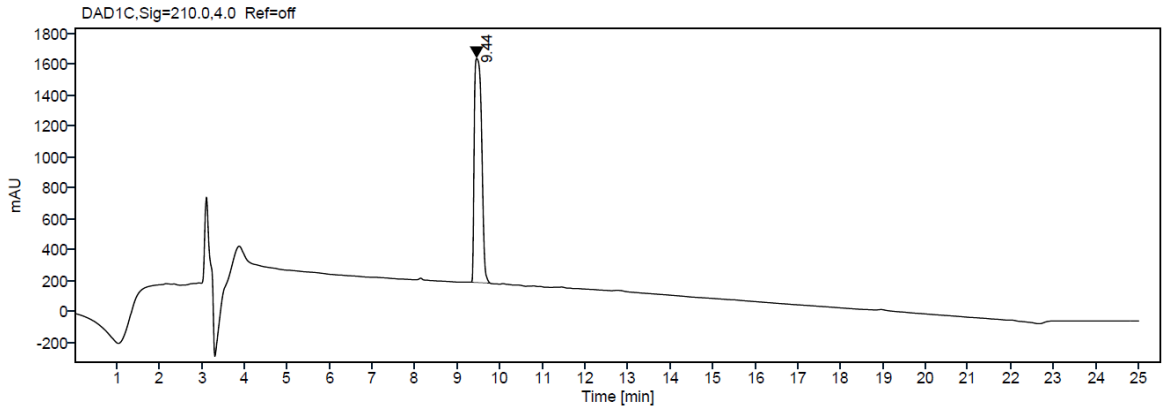
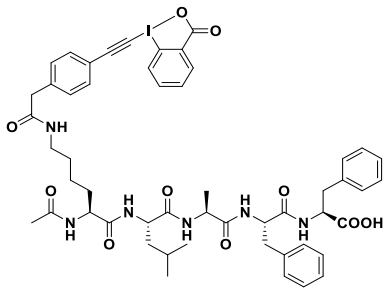
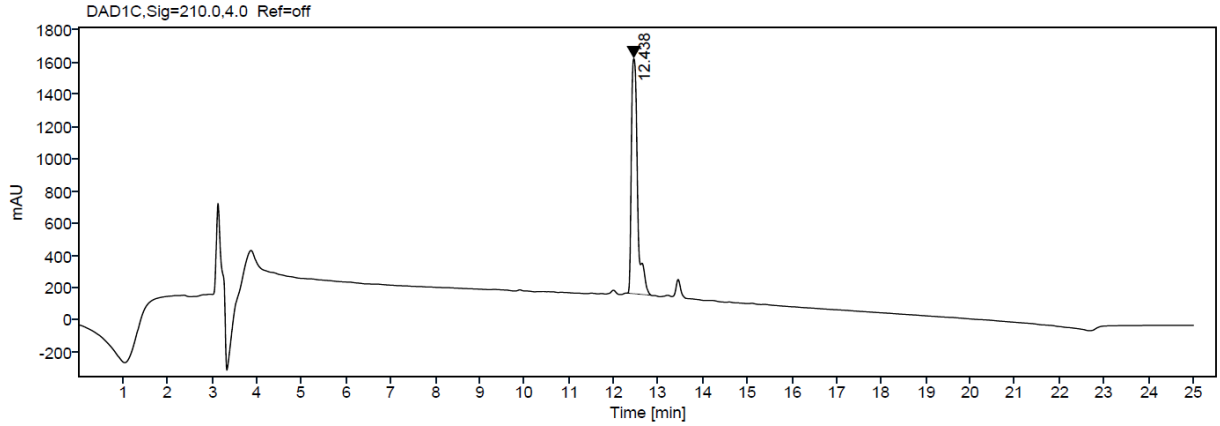


Figure S9: HPLC-UV chromatogram (210 nm) of Ac-KLAFF by **Method 1.**
HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{35}H_{51}N_6O_7^+$ 667.3814; Found 667.3803.



Following the general procedure, the reaction was conducted on a 0.045 mmol scale. The desired product **3f** (20 mg, 0.019 mmol, 42% yield) was isolated by **Method 5**.



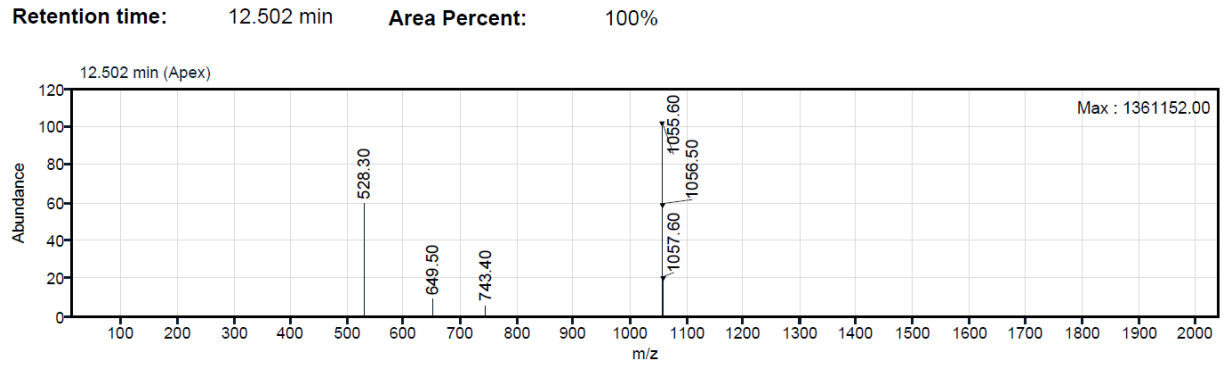


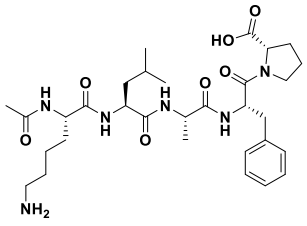
Figure S10: HPLC-UV chromatogram (210 nm) of **3f** and MS(ESI) by **Method 1.**
HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{52}H_{60}IN_6O_{10}^+$ 1055.3410; Found 1055.3397.

Table S4: MS/MS fragmentation of **3f**:

κ = Lys(C17H9IO3)
Nter = C2H3O

Sequence	Type	MF	MF Mass	m/z	Intensity	Similarity
KLA	a3	C33H40IN4O6(+1)	715.1993	715.1987	5.3	91.51%
FF	y2	C18H21N2O3(+1)	313.1552	313.1547	16.18	91.46%
KL	b2	C31H35IN3O6(+1)	672.1571	672.1565	12.94	90.77%
K	b1	C25H24IN2O5(+1)	559.073	559.0724	11.46	90.38%
LAFF	y4	C27H37N4O5(+1)	497.2764	497.2758	48.13	86.42%
KLA	b3	C34H40IN4O7(+1)	743.1942	743.1936	38.08	86.05%
KLAF	a4	C42H49IN5O7(+1)	862.2677	862.2671	100.39	84.39%
KLAF	b4	C43H49IN5O8(+1)	890.2626	890.262	101.36	83.60%

Peptide EBX of Ac-KLAFP (3g)



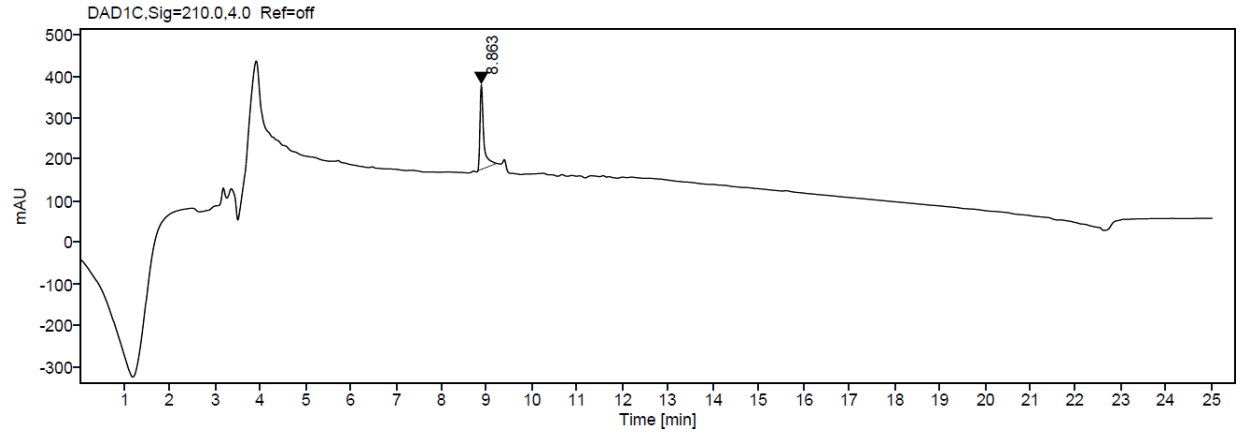
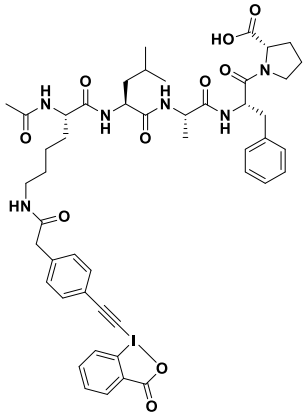
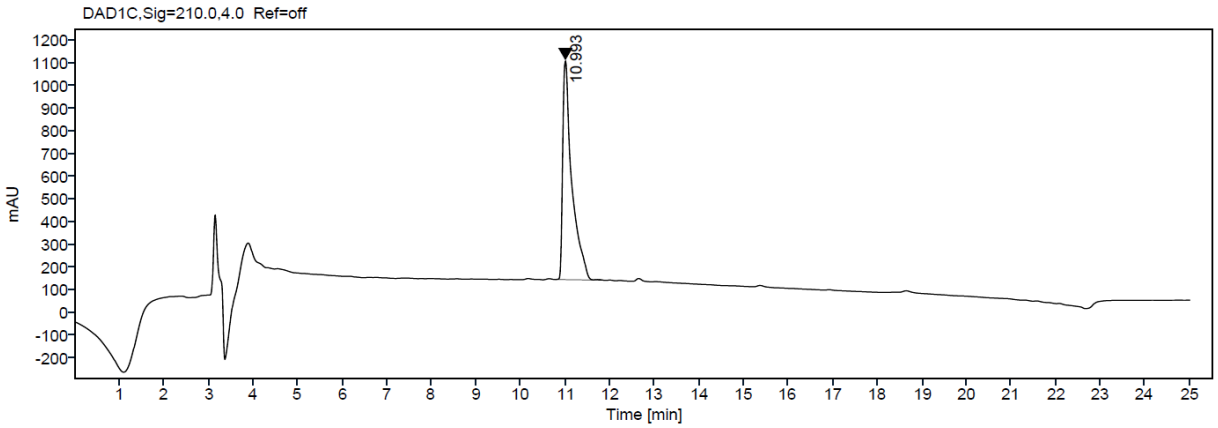


Figure S11: HPLC-UV chromatogram (210 nm) of Ac-KLAFP by **Method 1**.
HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{28}H_{45}N_6O_7^+$ 577.3344; Found 577.3359.



Following the general procedure, the reaction was conducted on a 0.03 mmol scale. The desired product **3g** (15 mg, 0.015 mmol, 50% yield) was isolated by **Method 5**.



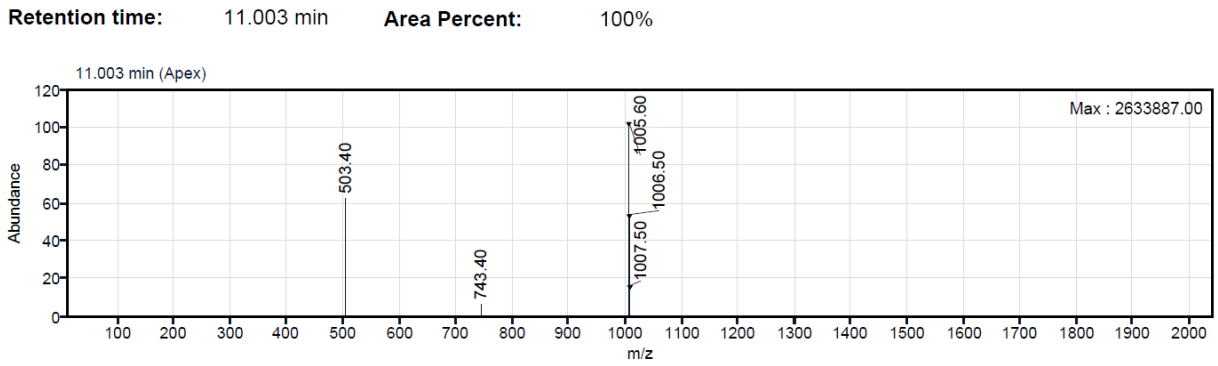


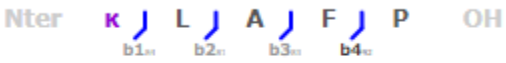
Figure S12: HPLC-UV chromatogram (210 nm) and MS(ESI) of **3g** by **Method 1.**

^{1}H NMR (800 MHz, DMSO-d_6) δ 8.31 (d, *J* = 8.2 Hz, 1H), 8.14 (d, *J* = 7.4 Hz, 1H), 7.91 (d, *J* = 7.9 Hz, 1H), 7.80 (d, *J* = 7.2 Hz, 1H), 7.64 (d, *J* = 7.7 Hz, 2H), 7.38 (d, *J* = 7.8 Hz, 2H), 7.28 – 7.13 (m, 5H), 4.63 (td, *J* = 8.0, 5.3 Hz, 1H), 4.26 – 4.22 (m, 2H), 4.19 (ddd, *J* = 13.7, 8.4, 4.6 Hz, 1H), 3.59 (dt, *J* = 9.4, 7.0 Hz, 2H), 3.02 (dq, *J* = 13.3, 6.8 Hz, 2H), 2.98 (dd, *J* = 14.1, 5.2 Hz, 1H), 2.77 (dd, *J* = 14.1, 8.2 Hz, 1H), 2.13 (dq, *J* = 12.0, 7.9 Hz, 1H), 1.90 – 1.81 (m, 6H), 1.59 (ddq, *J* = 31.2, 13.5, 6.7, 6.1 Hz, 3H), 1.42 (m, 6H), 1.31 – 1.21 (m, 3H), 1.13 (d, *J* = 7.2 Hz, 3H), 0.87 (dd, *J* = 14.9, 6.6 Hz, 3H), 0.83 (dd, *J* = 16.7, 6.5 Hz, 3H).

^{13}C NMR (201 MHz, DMSO-d_6) δ 173.2, 171.8, 171.8, 171.5, 169.4, 169.4, 169.3, 166.3, 139.6, 137.3, 135.2, 132.5, 132.1, 131.4, 131.3, 129.7, 129.4, 128.1, 127.5, 126.4, 118.4, 116.4, 104.5, 58.7, 52.6, 51.9, 51.6, 50.8, 48.0, 46.4, 42.3, 40.5, 38.7, 36.7, 31.7, 28.8, 28.7, 24.6, 24.2, 23.2, 22.9, 22.6, 21.5, 18.2.

HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{48}H_{58}IN_6O_{10}^+$ 1005.3254; Found 1005.3259.

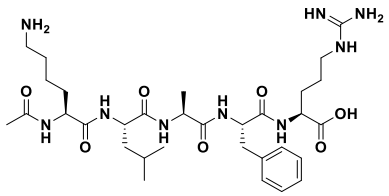
Table S5: MS/MS fragmentation of **3g**:



κ = Lys(C17H9IO3)
Nter = C2H3O

Sequence	Type	MF	MF Mass	m/z	Intensity	Similarity
KLAF	b4	C43H49IN5O8(+1)	890.2626	890.262	100.48	92.47%
K	b1	C25H24IN2O5(+1)	559.073	559.0724	1.33	84.13%
KLA	b3	C34H40IN4O7(+1)	743.1942	743.1936	9.93	82.90%
KL	b2	C31H35IN3O6(+1)	672.1571	672.1565	1.49	81.42%

Peptide EBX of Ac-KLAFR (3h)



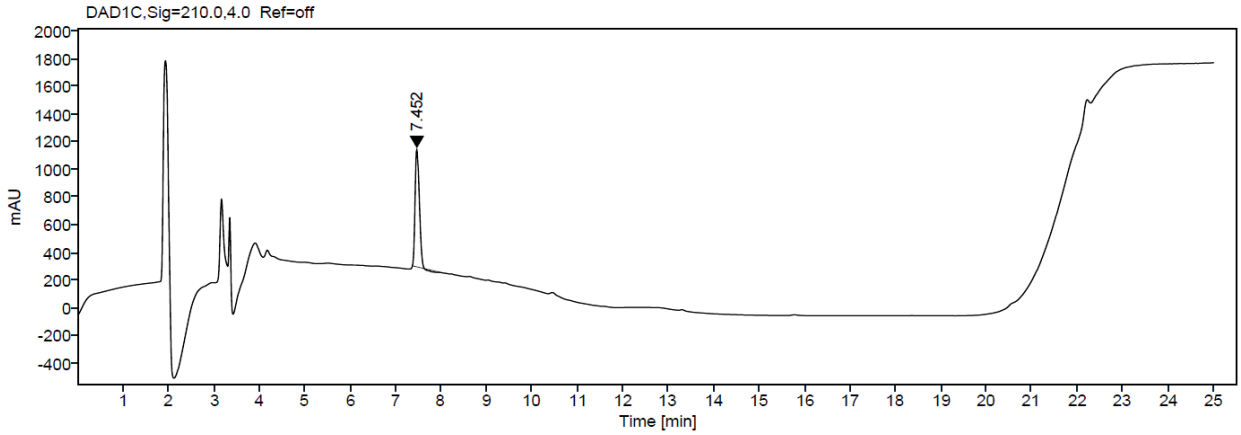
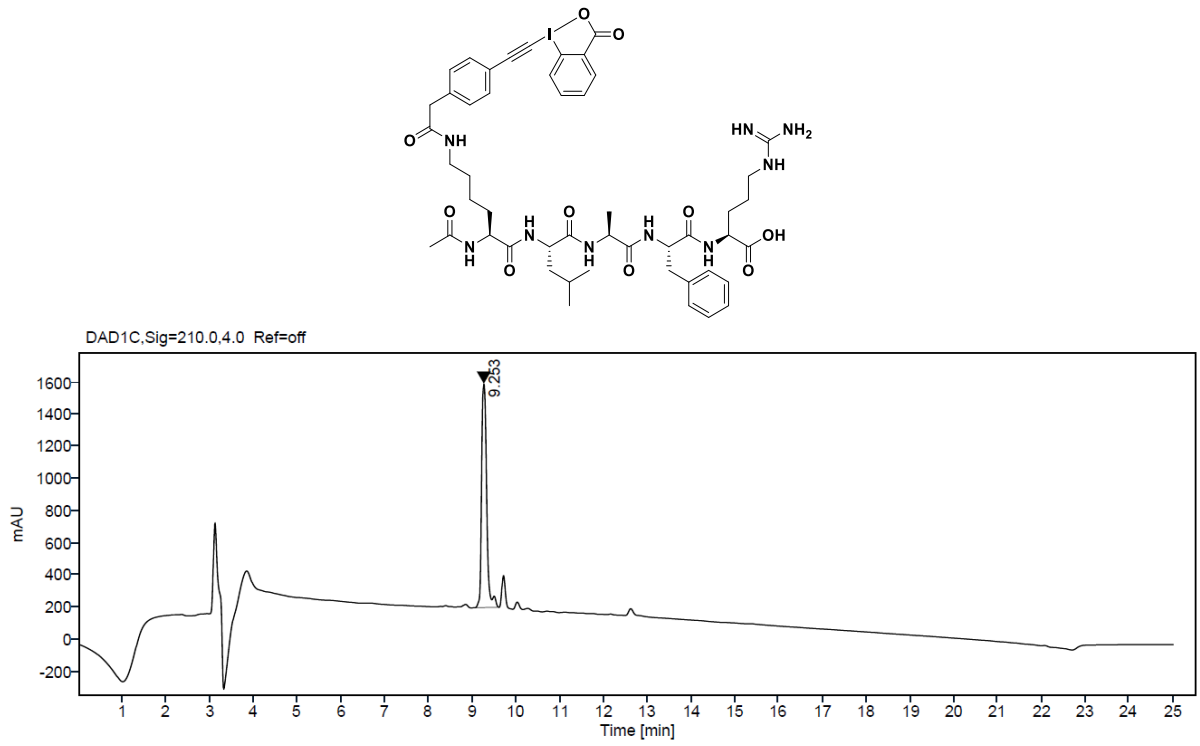


Figure S13: HPLC-UV chromatogram (210 nm) of Ac-KLAFR by **Method 1.**

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{32}H_{54}N_9O_7^+$ 676.4141; Found 676.4147.
Following the general procedure, the reaction was conducted in 0.044 mmol scale. The desired product **3h** (17 mg, 0.016 mmol, 36%) was isolated by **Method 5**.



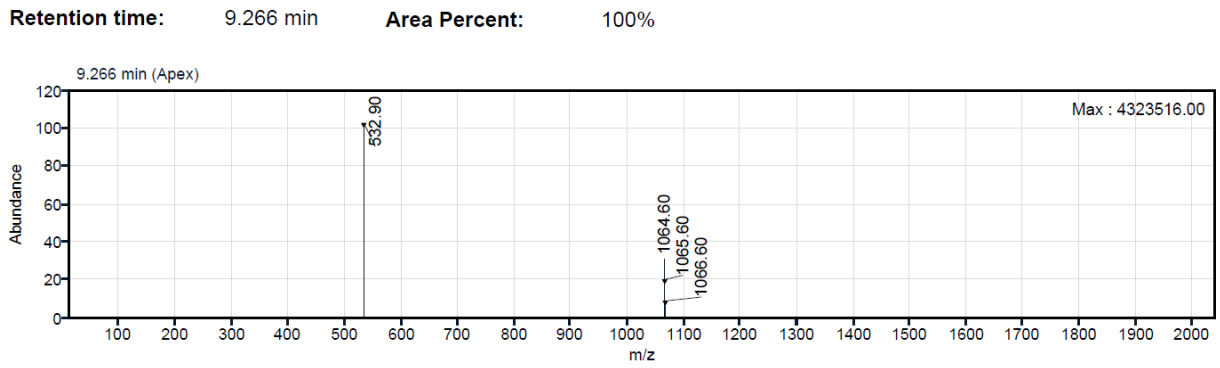
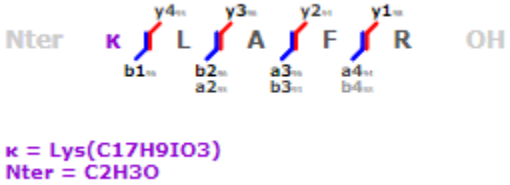


Figure S14: HPLC-UV chromatogram (210 nm) of **3h** and MS(ESI) by **Method 1.**

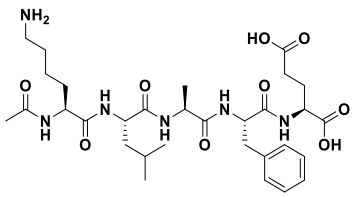
HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H_2]^{+2}$ Calcd for $C_{49}H_{64}IN_9O_{10}^{+2}$ 532.6905; Found 532.6898.

Table S6: MS/MS fragmentation of **3h**:



Sequence	Type	MF	MF Mass	m/z	Intensity	Similarity
R	y1	C6H15N4O2(+1)	175.1195	175.119	15.2	97.66%
AFR	y3	C18H29N6O4(+1)	393.225	393.2245	92.73	96.32%
K	b1	C25H24IN2O5(+1)	559.073	559.0724	83.47	96.29%
KLA	a3	C33H40IN4O6(+1)	715.1993	715.1987	5.23	96.04%
KL	b2	C31H35IN3O6(+1)	672.1571	336.5819	2.45	95.62%
KL	b2	C31H35IN3O6(+1)	672.1571	672.1565	69.96	95.55%
KLA	b3	C34H40IN4O7(+1)	743.1942	743.1936	23.17	95.20%
KL	a2	C30H35IN3O5(+1)	644.1621	644.1616	6.66	94.84%
LAFR	y4	C24H40N7O5(+1)	506.3091	506.3085	100.06	94.51%
FR	y2	C15H24N5O3(+1)	322.1879	322.1874	24.29	94.49%
KLAF	a4	C42H49IN5O7(+1)	862.2677	862.2671	8.47	94.15%
KLA	b3	C34H40IN4O7(+1)	743.1942	372.1004	4.45	91.14%
KLAF	b4	C43H49IN5O8(+1)	890.2626	890.262	2.27	87.83%

Peptide EBX of Ac-KLAFE (3i)



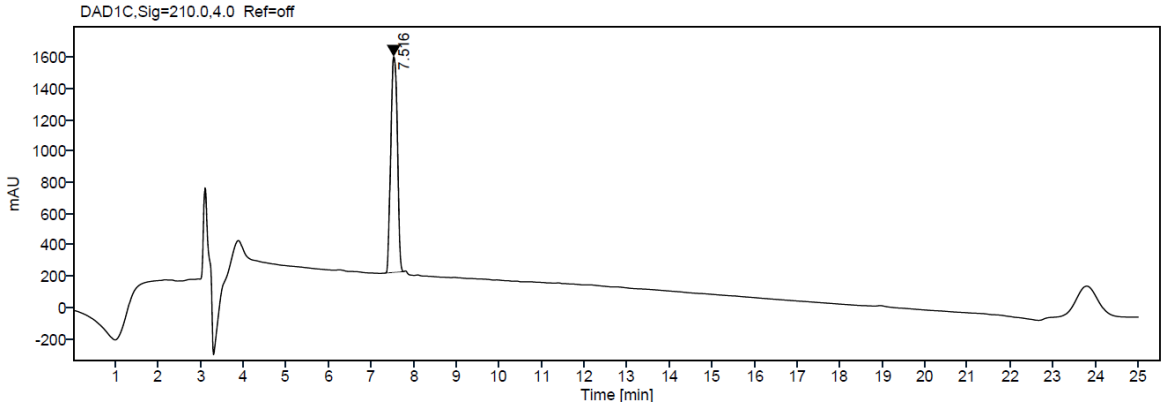
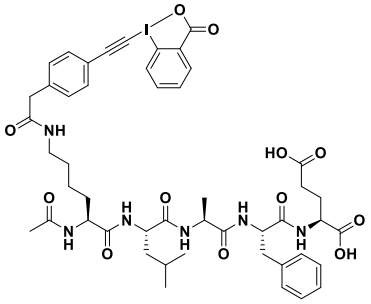
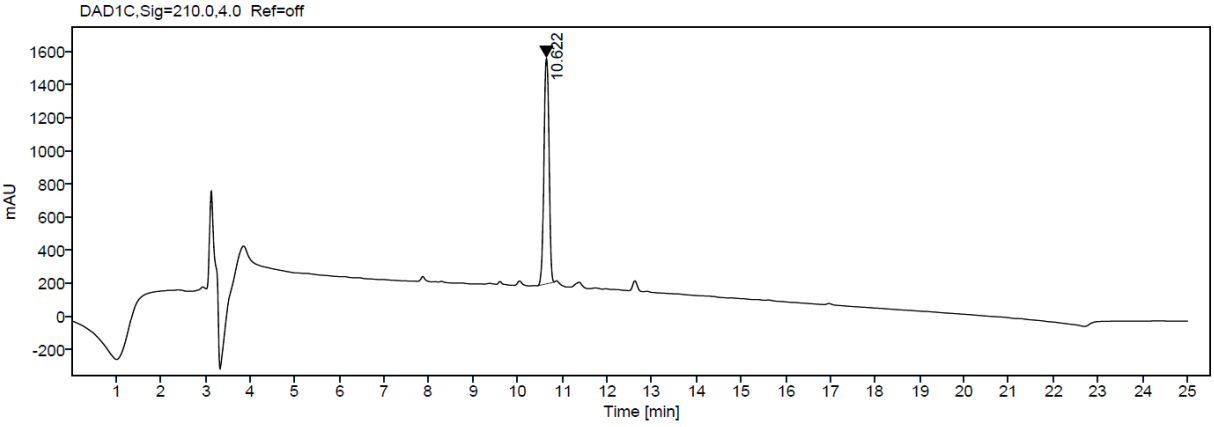


Figure S15: HPLC-UV chromatogram (210 nm) of Ac-KLAFE by **Method 1**.

HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{31}H_{49}N_6O_9^+$ 649.3556; Found 649.3545.

Following the general procedure, the reaction was conducted on a 0.046 mmol scale. The desired product **3i** (22 mg, 0.021 mmol, 46% yield) was isolated with **Method 5**.





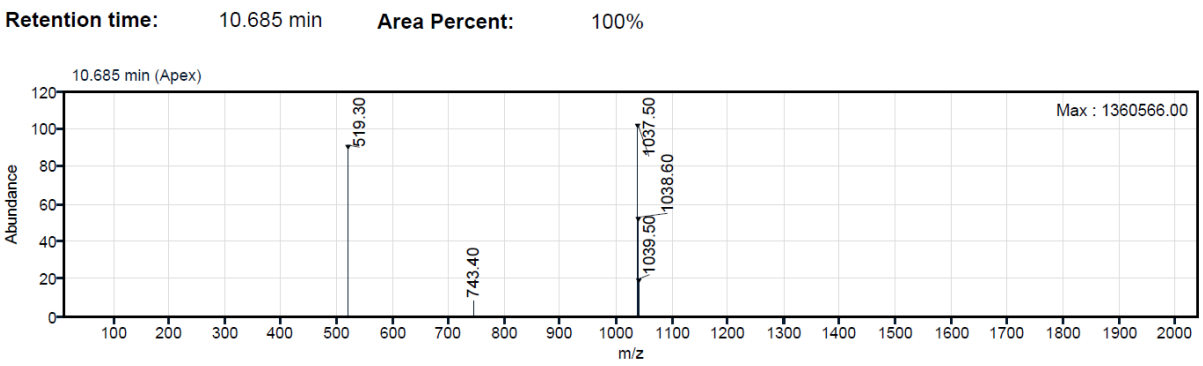
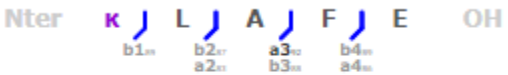


Figure S16: HPLC-UV chromatogram (210 nm) of **3i** and MS(ESI) by **Method 1**.

HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{48}H_{58}IN_6O_{12}^+$ 1037.3152; Found 1037.3157.

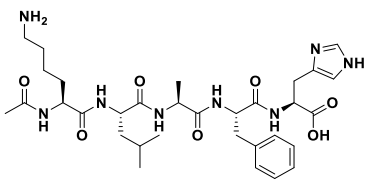
Table S7: MS/MS fragmentation of **3i**:



κ = Lys(C17H9IO3)
Nter = C2H3O

Sequence	Type	MF	MF Mass	m/z	Intensity	Similarity
KLA	a3	C33H40IN4O6(+1)	715.1993	715.1987	6.5	91.58%
K	b1	C25H24IN2O5(+1)	559.073	559.0724	16.42	89.33%
KLAF	b4	C43H49IN5O8(+1)	890.2626	890.262	91.91	88.88%
KLA	b3	C34H40IN4O7(+1)	743.1942	743.1936	45.38	87.71%
KL	b2	C31H35IN3O6(+1)	672.1571	672.1565	17.8	86.79%
KLAF	a4	C42H49IN5O7(+1)	862.2677	862.2671	100.15	85.98%
KL	a2	C30H35IN3O5(+1)	644.1621	644.1616	1.36	82.69%

Peptide EBX of Ac-KLAFH (3j)



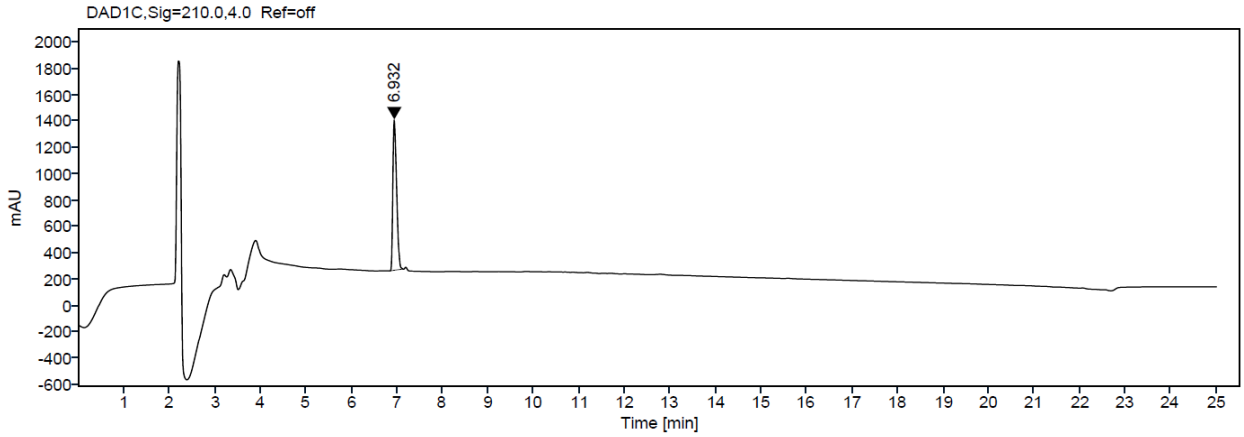
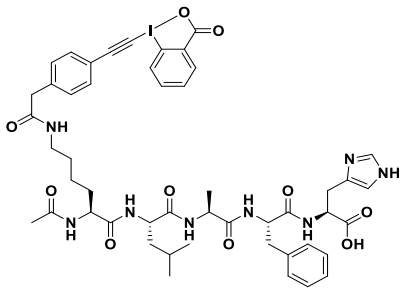
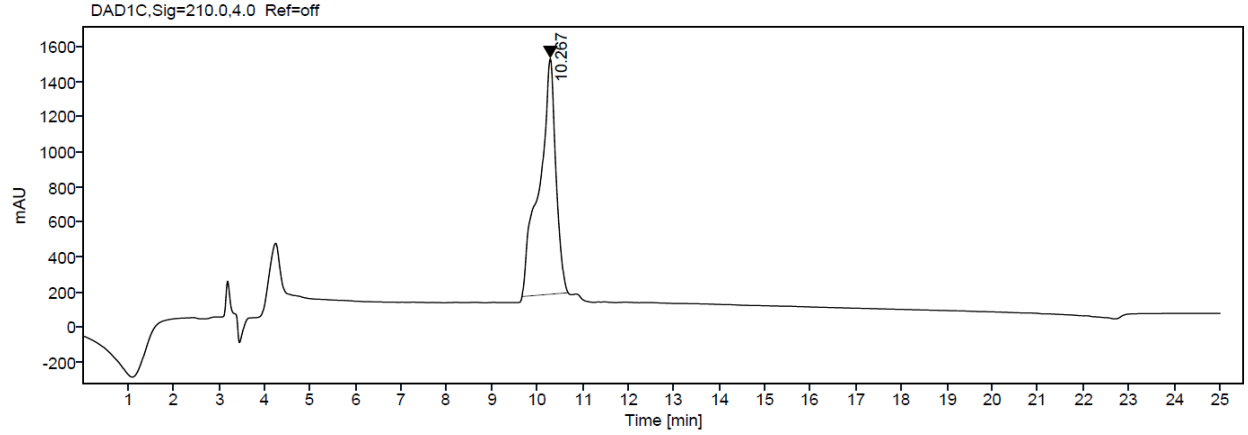


Figure S17: HPLC-UV chromatogram (210 nm) of Ac-KLAFH by **Method 1.**
HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{32}H_{49}N_8O_7^+$ 657.3719; Found 657.3724.



Following the general procedure, the reaction was conducted on a 0.037 mmol scale. The desired product **3j** (12 mg, 0.012 mmol, 35%) was isolated by **Method 5**.



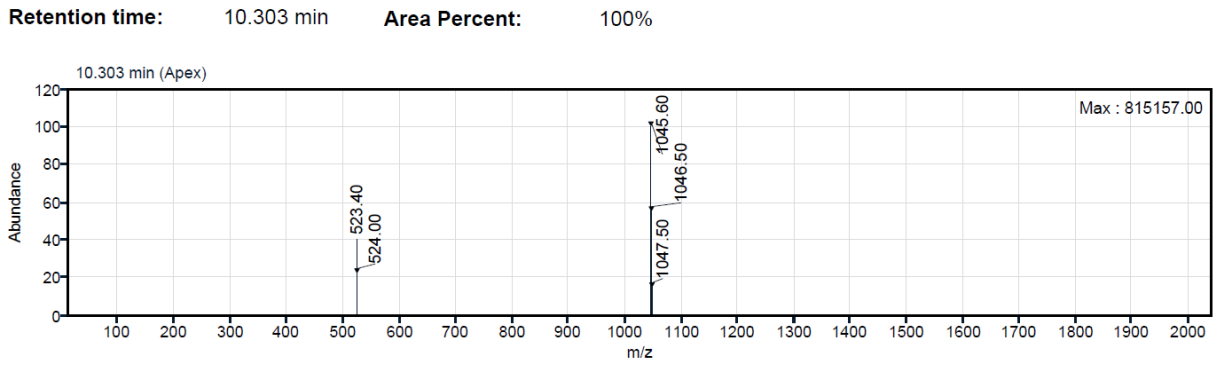
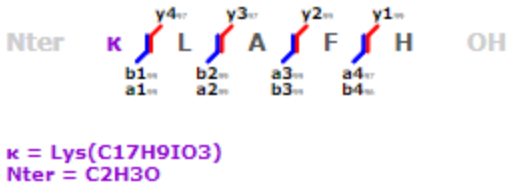


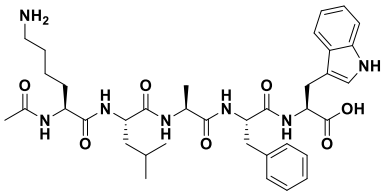
Figure S18: HPLC-UV chromatogram (210 nm) of **3j** and MS(ESI) by **Method 1.**
HRMS (Nanochip-based ESI/LTQ-Orbitrap) m/z: $[M + H_2]^{+2}$ Calcd for $C_{49}H_{59}IN_8O_{10}^{+2}$ 523.1694; Found 543.2601

Table S8: MS/MS fragmentation of **3j**:



Sequence	Type	MF	MF Mass	m/z	Intensity	Similarity
K	b1	C25H24IN2O5(+1)	559.073	559.0724	21.88	99.16%
KLA	a3	C33H40IN4O6(+1)	715.1993	715.1987	5.81	99.15%
KL	b2	C31H35IN3O6(+1)	672.1571	672.1565	25.53	99.09%
KLA	b3	C34H40IN4O7(+1)	743.1942	743.1936	13.04	98.97%
KLAFH		C49H57IN8O10	1044.324	523.1694	3.72	98.84%
H	y1	C6H10N3O2(+1)	156.0773	156.0768	101.15	98.83%
KL	a2	C30H35IN3O5(+1)	644.1621	644.1616	4.33	98.79%
FH	y2	C15H19N4O3(+1)	303.1457	303.1452	15.7	98.79%
K	a1	C24H24IN2O4(+1)	531.0781	531.0775	2.21	98.72%
KL	b2	C31H35IN3O6(+1)	672.1571	336.5819	1.42	98.11%
K	b1	C25H24IN2O5(+1)	559.073	280.0399	1.08	97.97%
KLAF	a4	C42H49IN5O7(+1)	862.2677	862.2671	3.48	97.45%
AFH	y3	C18H24N5O4(+1)	374.1828	374.1823	2.78	97.00%
LAFH	y4	C24H35N6O5(+1)	487.2669	487.2663	1	97.00%
KLAF	b4	C43H49IN5O8(+1)	890.2626	890.262	0.51	95.51%

Peptide EBX of Ac-KLAFW (3k)



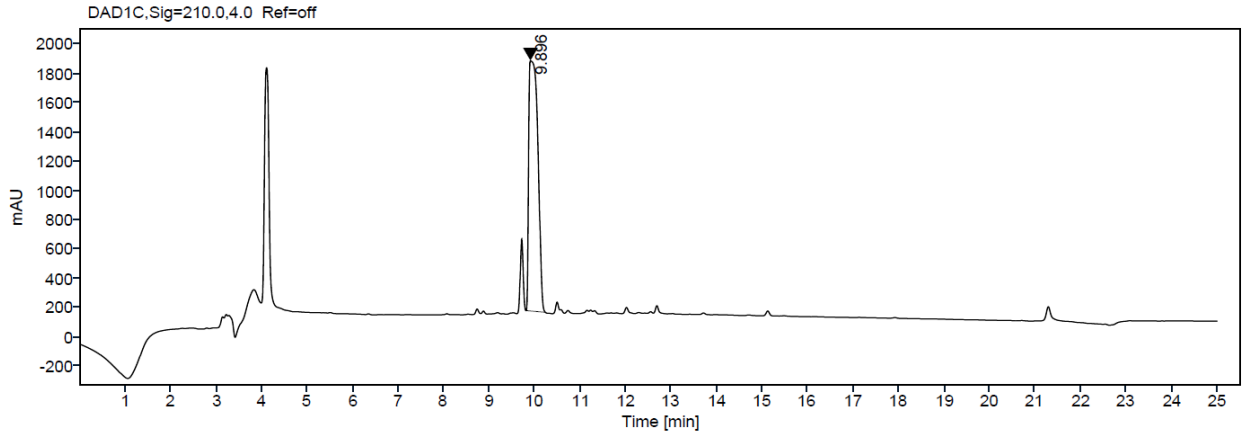
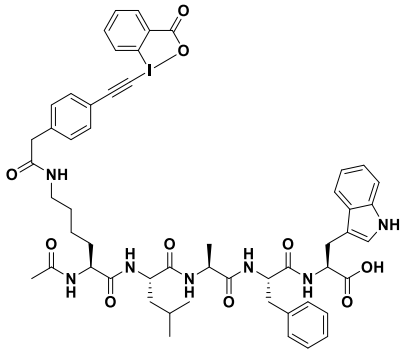
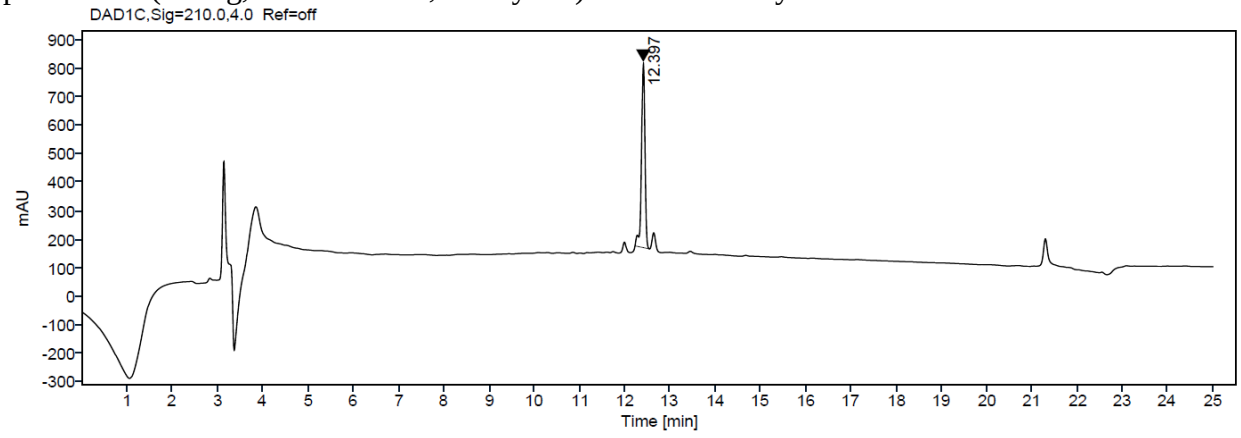


Figure S19: HPLC-UV chromatogram (210 nm) of Ac-KLAFW by **Method 1**.
HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{37}H_{52}N_7O_7^+$ 706.3923; Found 706.3927.



Following the general procedure, the reaction was conducted on a 0.03 mmol scale. The desired product **3k** (15 mg, 0.0014 mmol, 47% yield) was isolated by **Method 5**.



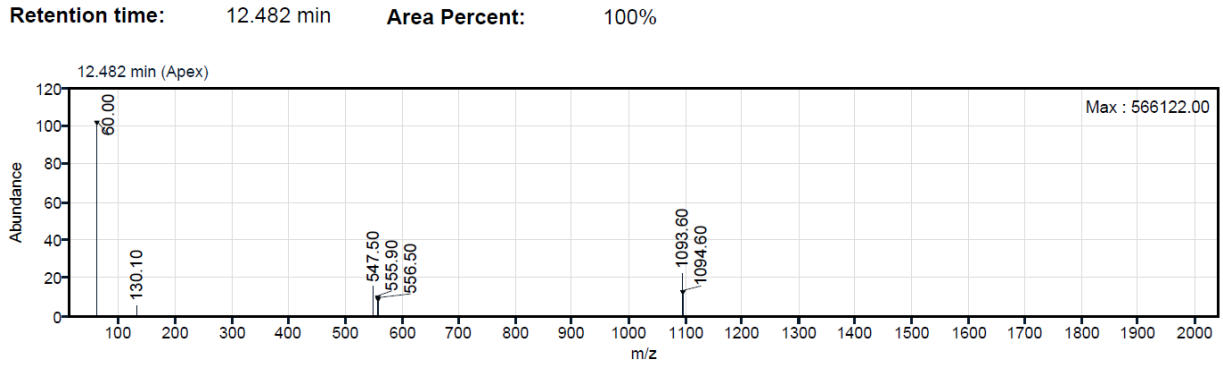
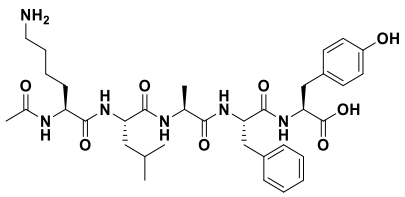


Figure S20: HPLC-UV chromatogram (210 nm) of EBX and MS(ESI) of **3k** by **Method 1**. HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{54}H_{61}IN_7O_{10}^+$ 1094.3519; Found 1094.3447.

Table S9: MS/MS fragmentation of **3k**:

Sequence	Type	MF	MF Mass	m/z	Intensity	Similarity
FW	y2	C20H22N3O3(+1)	352.1661	352.1656	19.82	95.96%
KLA	b3	C34H40IN4O7(+1)	743.1942	743.1936	22.74	95.41%
KLA	b3	C34H40IN4O7(+1)	743.1942	372.1004	11.81	94.78%
KLAF	b4	C43H49IN5O8(+1)	890.2626	445.6347	101.25	93.42%
KLAF	b4	C43H49IN5O8(+1)	890.2626	890.262	30.26	91.30%
W	y1	C11H13N2O2(+1)	205.0977	205.0972	8.71	90.97%
KL	b2	C31H35IN3O6(+1)	672.1571	336.5819	1.25	88.15%
K	b1	C25H24IN2O5(+1)	559.073	559.0724	8.23	86.91%
LAFW	y4	C29H38N5O5(+1)	536.2873	536.2867	6.97	82.87%
AFW	y3	C23H27N4O4(+1)	423.2032	423.2027	3.42	80.10%

Peptide EBX of Ac-KLAFY (3l)



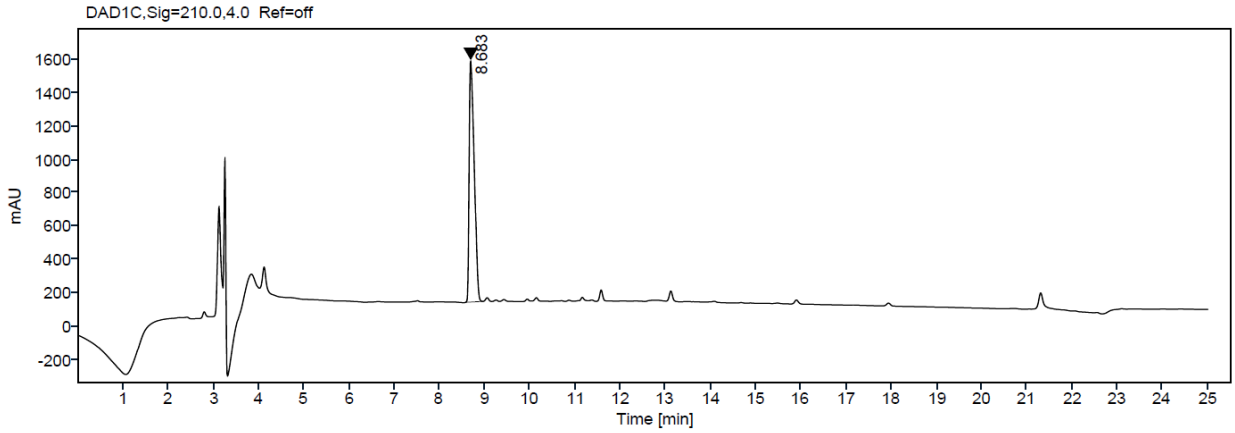
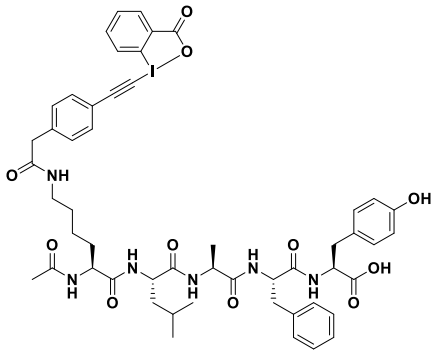
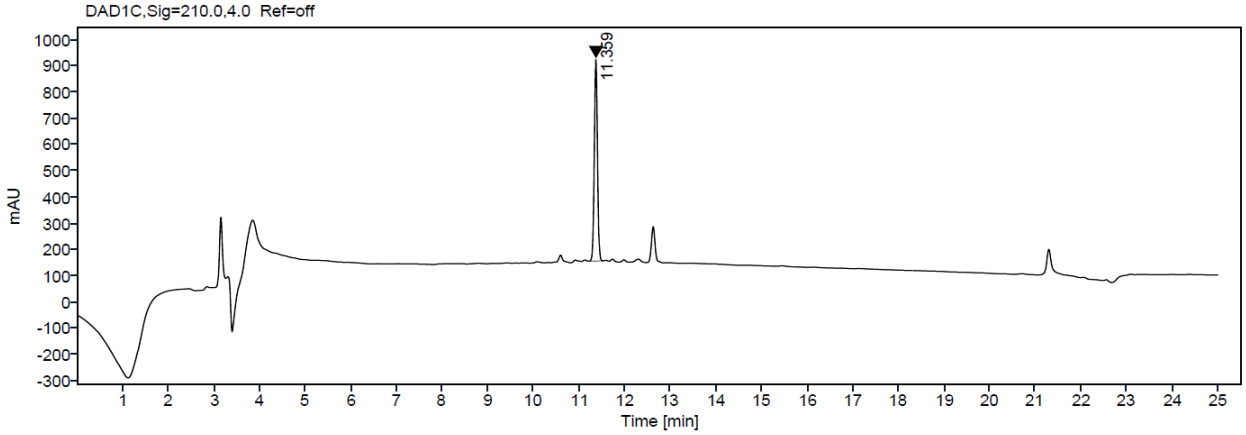


Figure S21: HPLC-UV chromatogram (210 nm) of Ac-KLAFY by **Method 1**.
HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{35}H_{51}N_6O_8^+$ 683.3763; Found 683.3765.



Following the general procedure, the reaction was conducted on a 0.03 mmol scale. The desired product **3l** (15 mg, 0.014 mmol, 48% yield) was isolated by **Method 5**.



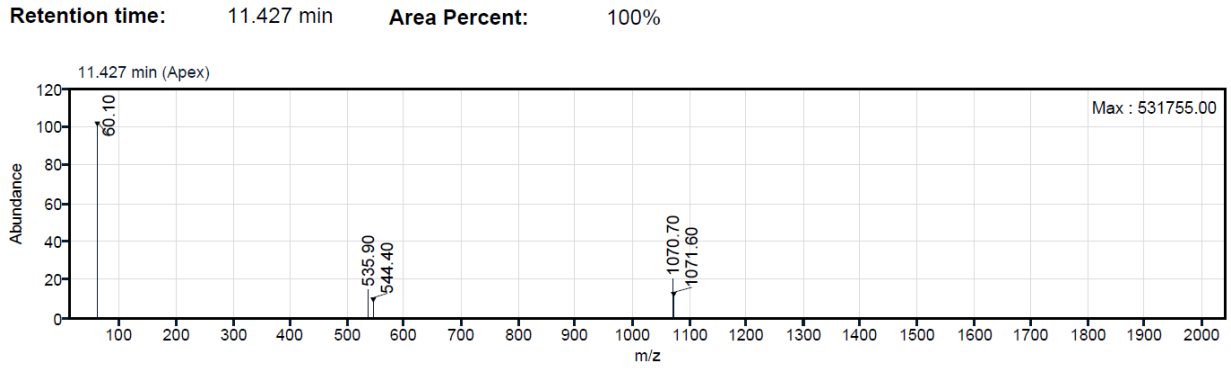


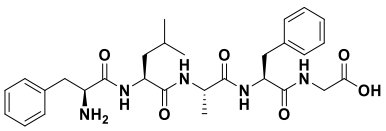
Figure S22: HPLC-UV chromatogram (210 nm) and MS(ESI) of **3l** by **Method 1**.
HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{52}H_{60}IN_6O_{11}^+$ 1071.3359; Found 1071.3344.

Table S10: MS/MS fragmentation of **3l**:

κ = Lys(C17H9IO3)
Nter = C2H3O

Sequence	Type	MF	MF Mass	m/z	Intensity	Similarity
KLAF	a4	C42H49IN5O7(+1)	862.2677	431.6372	1.07	98.96%
KLAF	b4	C43H49IN5O8(+1)	890.2626	445.6347	101.7	96.44%
FY	y2	C18H21N2O4(+1)	329.1501	329.1496	14.94	94.98%
KL	b2	C31H35IN3O6(+1)	672.1571	336.5819	1.08	94.90%
KLA	b3	C34H40IN4O7(+1)	743.1942	743.1936	27.31	93.58%
AFY	y3	C21H26N3O5(+1)	400.1872	400.1867	2.32	93.46%
K	b1	C25H24IN2O5(+1)	559.073	559.0724	6.69	92.98%
KLA	b3	C34H40IN4O7(+1)	743.1942	372.1004	23.69	92.58%
KL	b2	C31H35IN3O6(+1)	672.1571	672.1565	3.88	91.78%
LAFY	y4	C27H37N4O6(+1)	513.2713	513.2708	4.82	91.71%
KLAF	b4	C43H49IN5O8(+1)	890.2626	890.262	1.5	91.71%

Peptide EBX of FLAFG (3m)



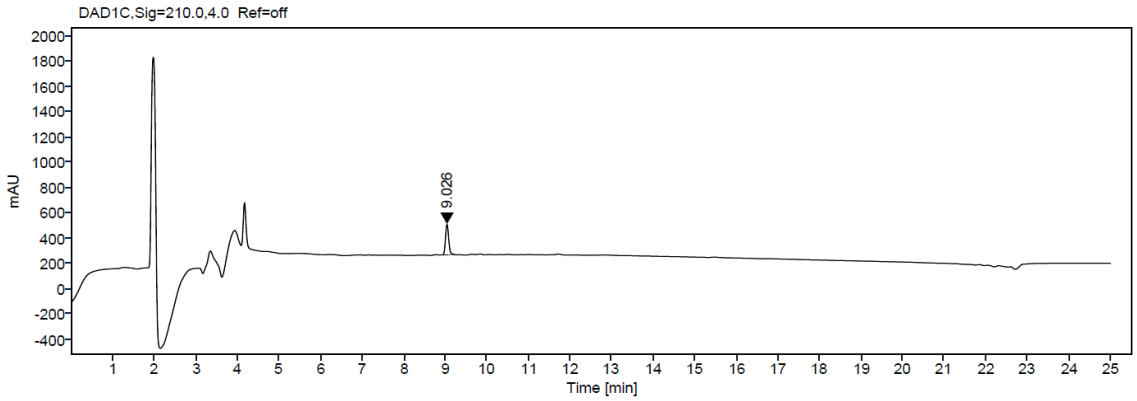
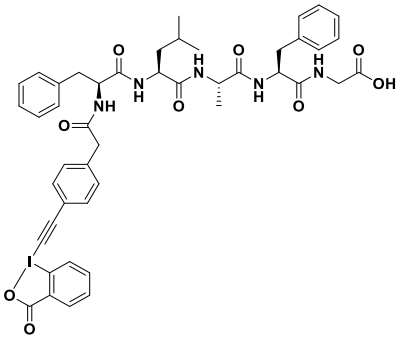
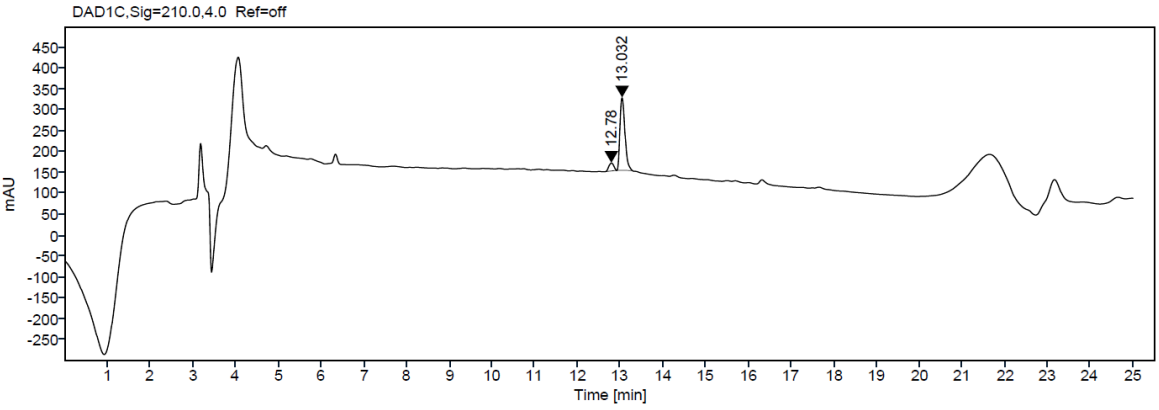


Figure S23: **HPLC-UV** chromatogram (210 nm) of FLAFG by **Method 1**.
HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{29}H_{40}N_5O_6^+$ 554.2973; Found 554.2980.



Following the general procedure, the reaction was conducted in 0.027 mmol scale. The desired product **3m** (6.9 mg, 0.0073 mmol, 27% yield) was isolated by **Method 5**.



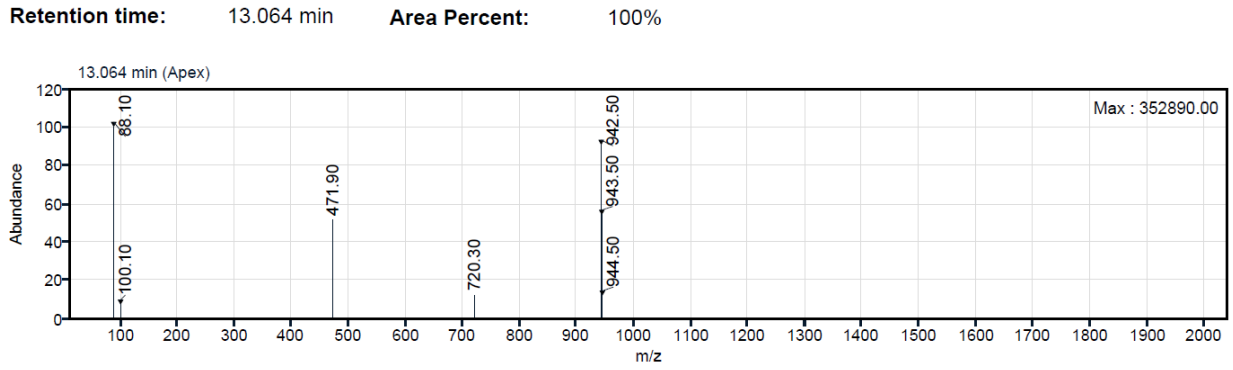
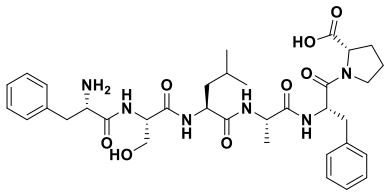


Figure S24: HPLC-UV chromatogram (210 nm) and MS(ESI) of **3m** by **Method 1**.
HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{46}H_{49}IN_5O_9^+$ 942.2570; Found 942.2560.

Table S11: MS/MS fragmentation of **3m**:

Sequence	Type	MF	MF Mass	m/z	Intensity	Similarity
FL	b2	C32H30IN2O5(+1)	649.1199	649.1194	49.9	99.09%
FLA	b3	C35H35IN3O6(+1)	720.1571	720.1565	44.13	97.27%
F	a1	C25H19INO3(+1)	508.041	508.0404	49.56	97.17%
F	b1	C26H19INO4(+1)	536.0359	536.0353	37.88	96.39%
FLAFG		C46H48IN5O9	941.2497	942.257	6.51	95.79%
FL	a2	C31H30IN2O4(+1)	621.125	621.1245	32.28	95.61%
AFG	y3	C14H20N3O4(+1)	294.1454	294.1448	6.19	95.05%
FLA	a3	C34H35IN3O5(+1)	692.1621	692.1616	11.88	94.39%
FLAF	b4	C44H44IN4O7(+1)	867.2255	867.2249	2.93	91.68%
FLAF	a4	C43H44IN4O6(+1)	839.2306	839.23	3.88	91.45%

Peptide EBX of FSLAFP (3n)



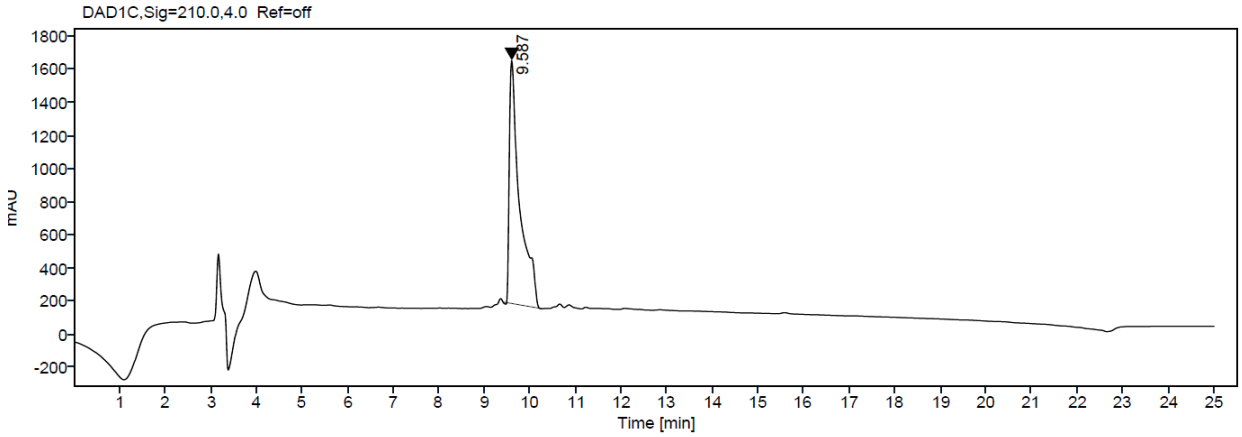
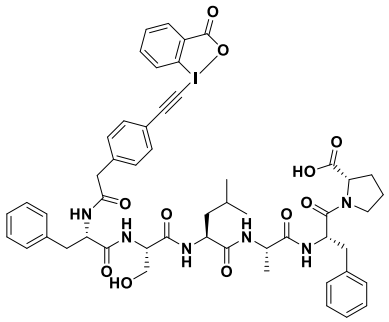
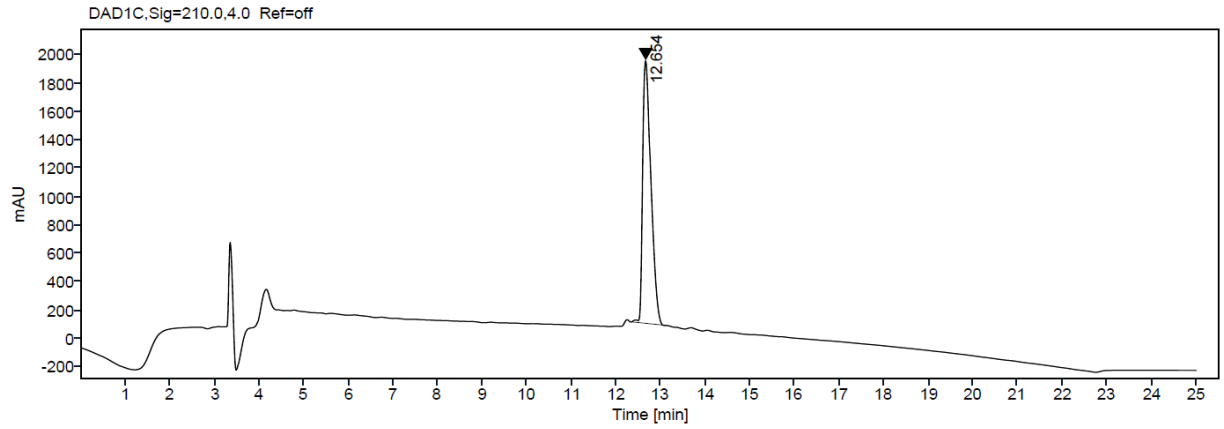


Figure S25: HPLC-UV chromatogram (210 nm) of FSLAFP by **Method 1.**

HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{35}H_{49}N_6O_8^+$ 681.3606; Found 681.3574.



Following the general procedure, the reaction was conducted on a 0.044 mmol scale. The desired product **3n** (17 mg, 0.016 mmol, 37% yield) was isolated by **Method 5**.



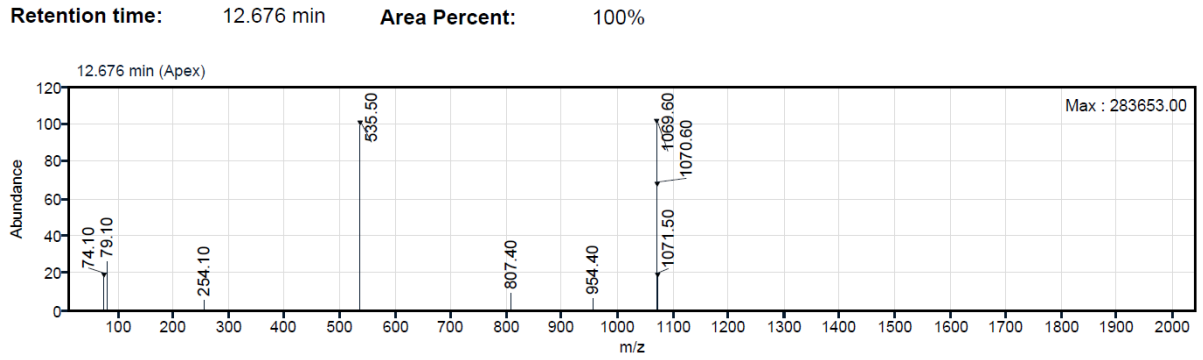
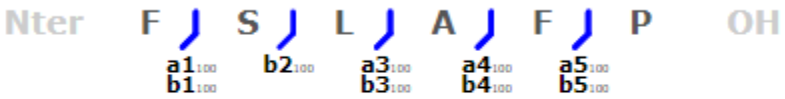


Figure S26: HPLC-UV chromatogram (210 nm) and MS(ESI) of **3n** by **Method 1**.
HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H_2]^{+2}$ Calcd for $C_{52}H_{59}IN_6O_{11}^{+2}$ 535.1638; Found 535.1612.

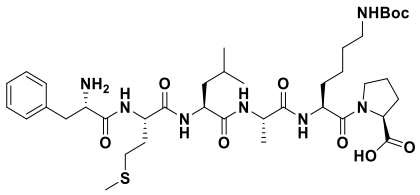
Table S12: MS/MS fragmentation of **3n**:



Nter = C17H10IO3

Sequence	Type	MF	MF Mass	m/z	Intensity	Similarity
FSLAF	b5	C47H49IN5O9(+1)	954.2575	954.257	100.92	94.56%
FSLA	b4	C38H40IN4O8(+1)	807.1891	807.1885	27.37	93.18%
FS	b2	C29H24IN2O6(+1)	623.0679	623.0674	2.28	93.10%
FSL	b3	C35H35IN3O7(+1)	736.152	736.1514	5.72	92.87%
FSL	a3	C34H35IN3O6(+1)	708.1571	708.1565	1.18	91.91%
FSLAF	a5	C46H49IN5O8(+1)	926.2626	926.262	60.96	91.68%
F	b1	C26H19INO4(+1)	536.0359	536.0353	1.22	90.41%
FSLA	a4	C37H40IN4O7(+1)	779.1942	779.1936	0.89	89.71%
F	a1	C25H19INO3(+1)	508.041	508.0404	0.76	80.14%

Peptide EBX of FMLAKP (3o)



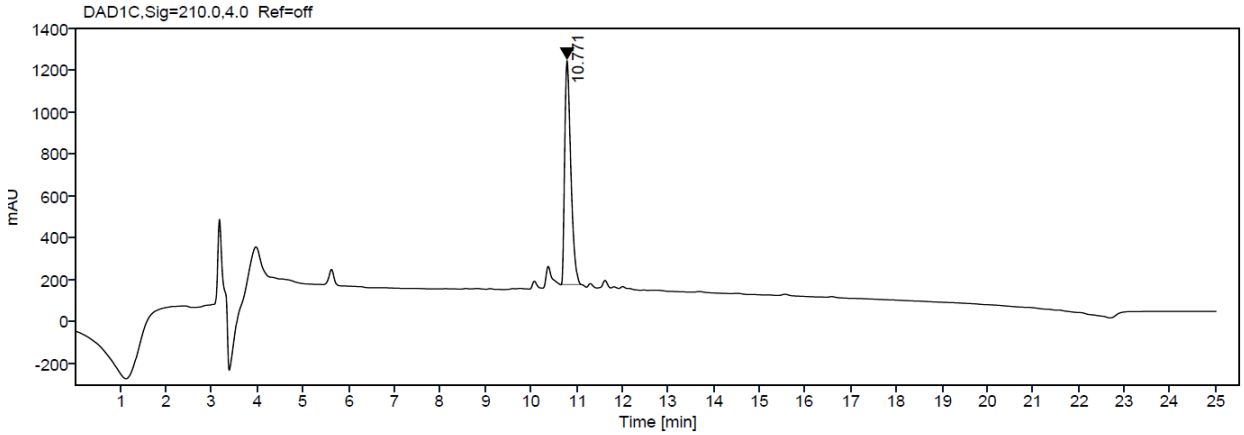
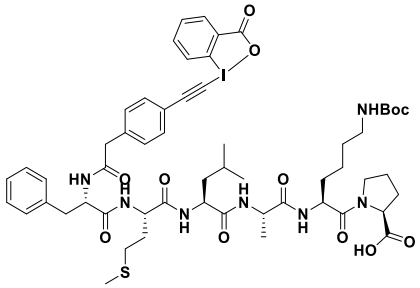
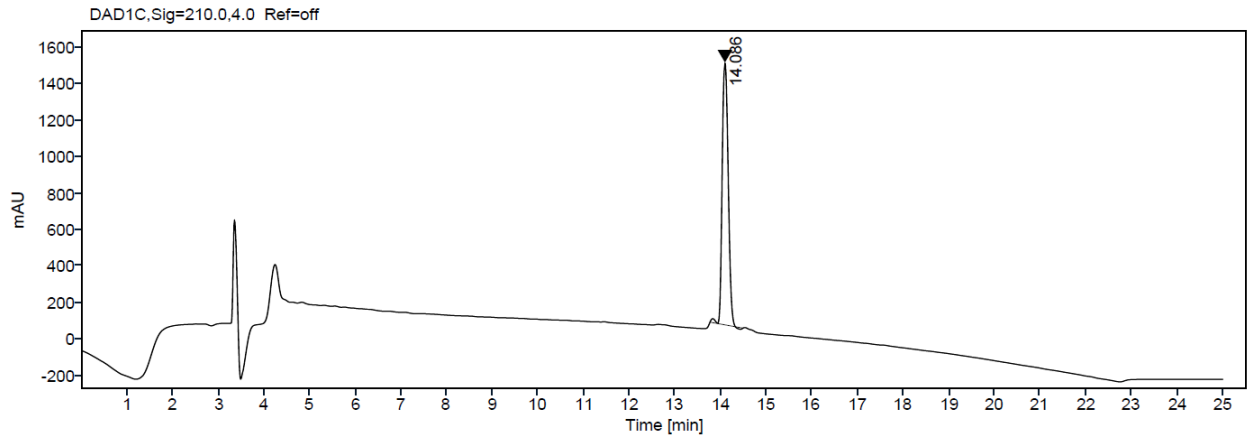


Figure S27: HPLC-UV chromatogram (210 nm) of FMLAKP by **Method 1.**
HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{39}H_{64}N_7O_9S^+$ 806.4481; Found 806.4448.



Following the general procedure, the reaction was conducted on a 0.035 mmol scale. The desired product **3o** (17 mg, 0.014 mmol, 40% yield) was isolated by **Method 5**.



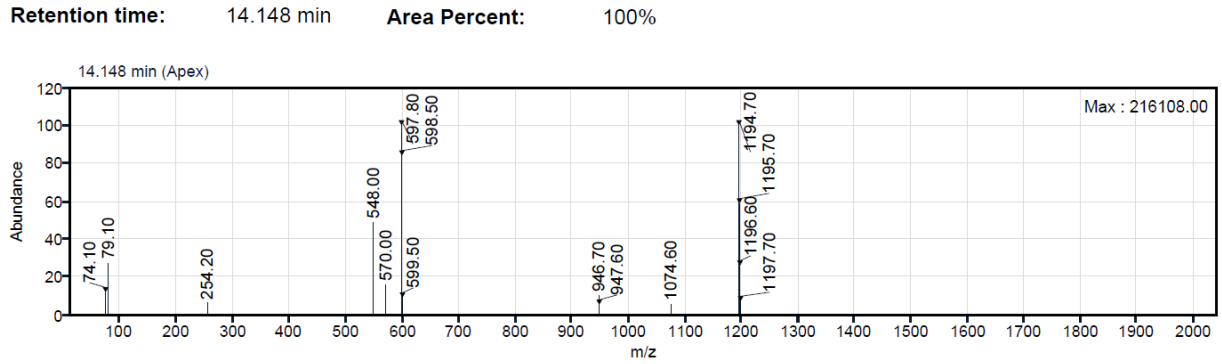
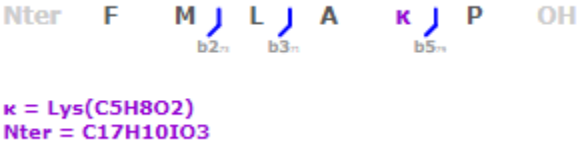


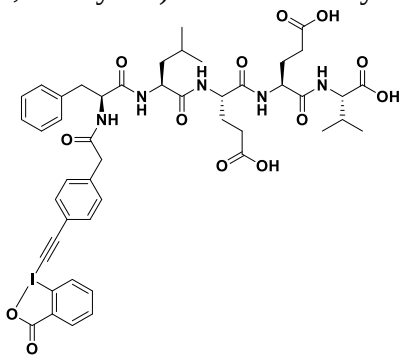
Figure S28: HPLC-UV chromatogram (210 nm) and MS(ESI) of **3o** by **Method 1**.
HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{56}H_{73}IN_7O_{12}S^+$ 1194.4077; Found 1194.4053.
Table S13: MS/MS fragmentation of **3o**:

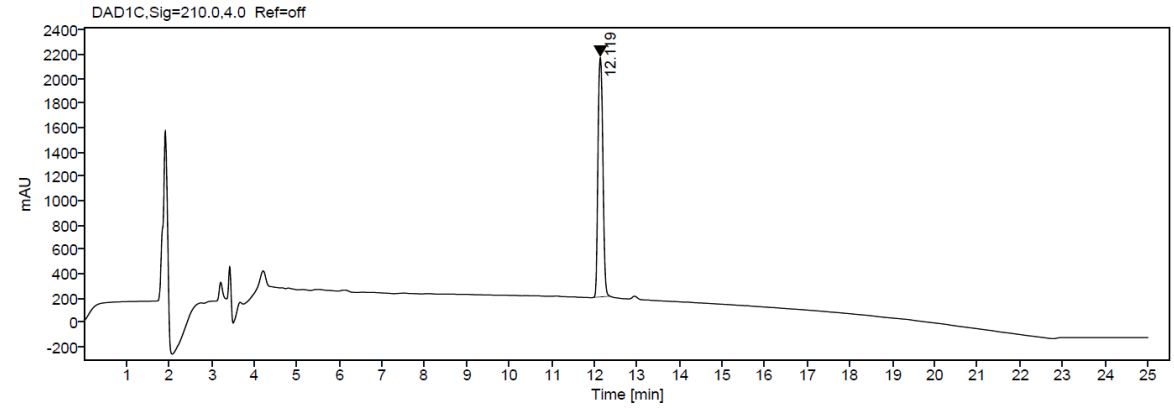


Sequence	Type	MF	MF Mass	m/z	Intensity	Similarity
FMLAK	b5	C51H64IN6O10S(+1)	1079.345	1079.344	1.88	78.97%
FM	b2	C31H28IN2O5S(+1)	667.0764	667.0758	0.51	73.26%
FML	b3	C37H39IN3O6S(+1)	780.1604	780.1599	1.28	70.72%

Peptide EBX of FLEEV 3p (commercially available peptide)

Following the general procedure, the reaction was conducted on a 0.031 mmol scale. The desired product **3p** (7.8 mg, 0.0077 mmol, 24% yield) was isolated by **Method 5**.





Retention time: 12.136 min **Area Percent:** 100%

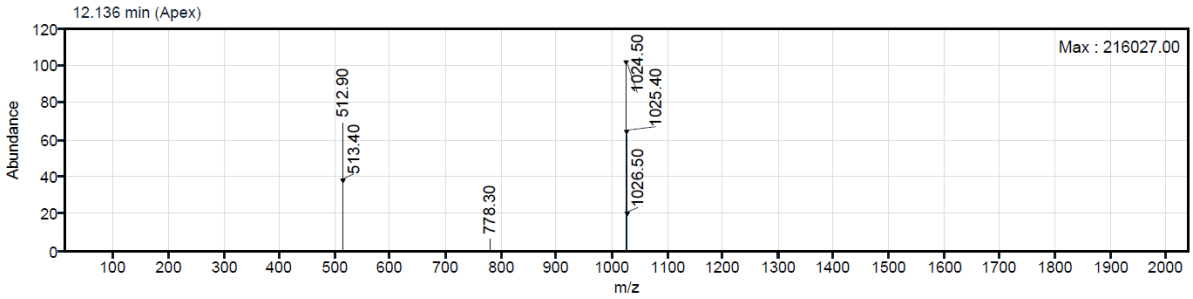
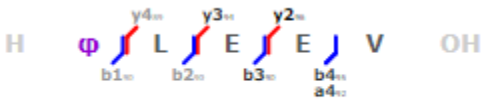


Figure S29: HPLC-UV chromatogram (210 nm) and MS(ESI) of **3p** by **Method 1**.
HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H_2]^{+2}$ Calcd for $C_{47}H_{56}IN_5O_{13}^{+2}$ 512.6454; Found 512.6449.

Table S14: MS/MS fragmentation of **3p**:



φ = Phe(C17H9IO3)

Sequence	Type	MF	MF Mass	m/z	Intensity	Similarity
EV	y2	C10H19N2O5(+1)	247.1294	247.1288	6.2	95.74%
FLEE	b4	C42H44IN4O11(+1)	907.2051	907.2046	17.25	95.04%
FLEE	b4	C42H44IN4O11(+1)	907.2051	454.1059	100.17	94.57%
EEV	y3	C15H26N3O8(+1)	376.172	376.1714	1.88	93.93%
FLEE	a4	C41H44IN4O10(+1)	879.2102	440.1085	0.94	92.20%
FLE	b3	C37H37IN3O8(+1)	778.1625	778.162	28.86	91.75%
FL	b2	C32H30IN2O5(+1)	649.1199	325.0633	0.67	90.50%
F	b1	C26H19INO4(+1)	536.0359	536.0353	2.95	89.54%
LEEV	y4	C21H37N4O9(+1)	489.2561	489.2555	0.55	89.40%
FL	b2	C32H30IN2O5(+1)	649.1199	649.1194	8.39	89.16%
FLE	b3	C37H37IN3O8(+1)	778.1625	389.5846	10.97	88.44%

Peptide EBX of Ac-FGKGGGGGP (3q)

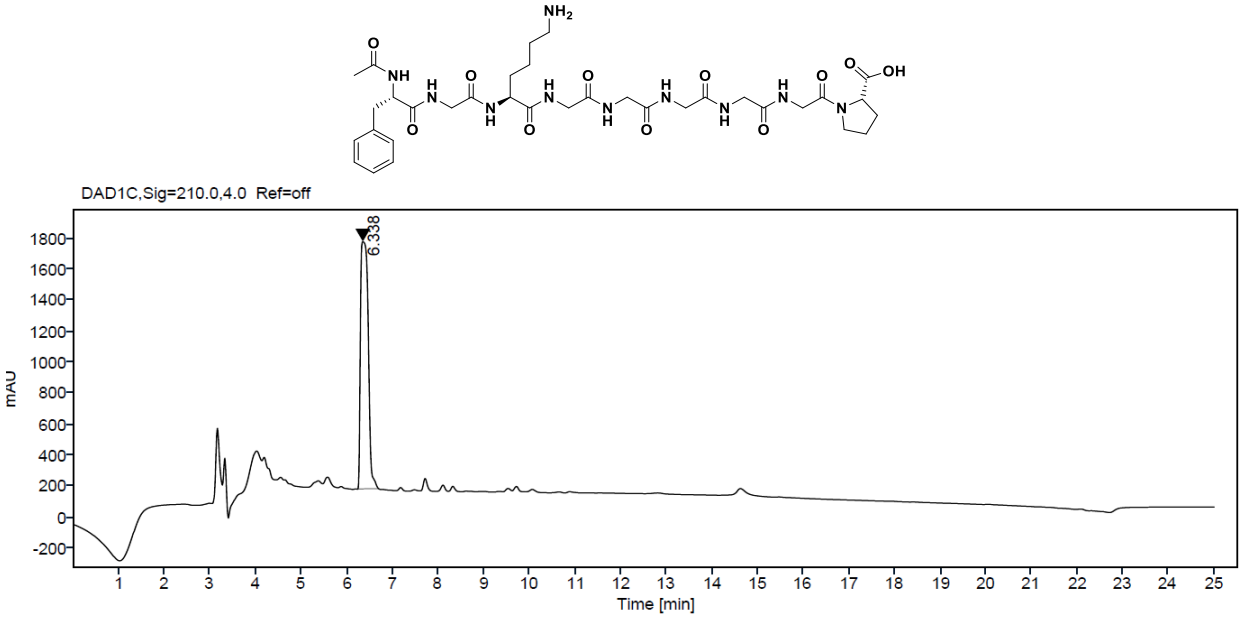
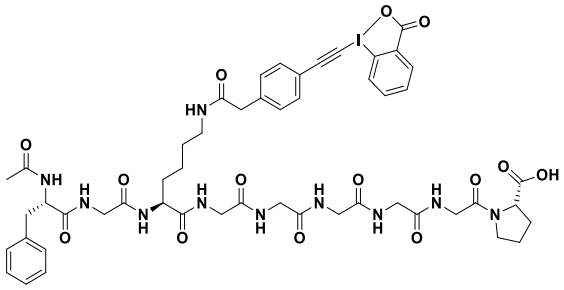
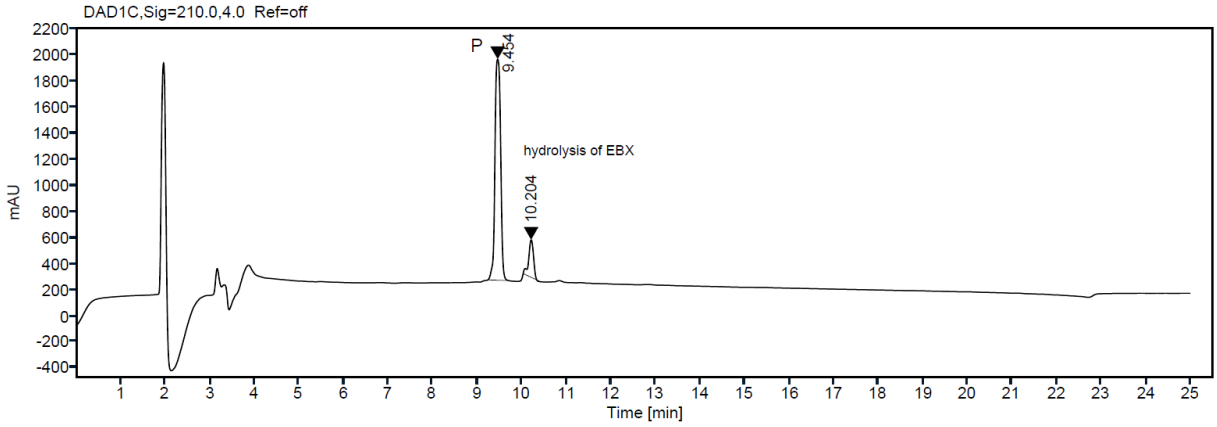


Figure S30: HPLC-UV chromatogram (210 nm) of Ac-FGKGGGGGP by **Method 1.**

HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{34}H_{51}N_{10}O_{11}^+$ 775.3733; Found 775.3719.



Following the general procedure, the reaction was conducted on a 0.045 mmol scale. The desired product **3q** (24 mg, 0.021 mmol, 45% yield) was isolated by **Method 6** (Pure compound can be obtained by SPPS, See Section 5).



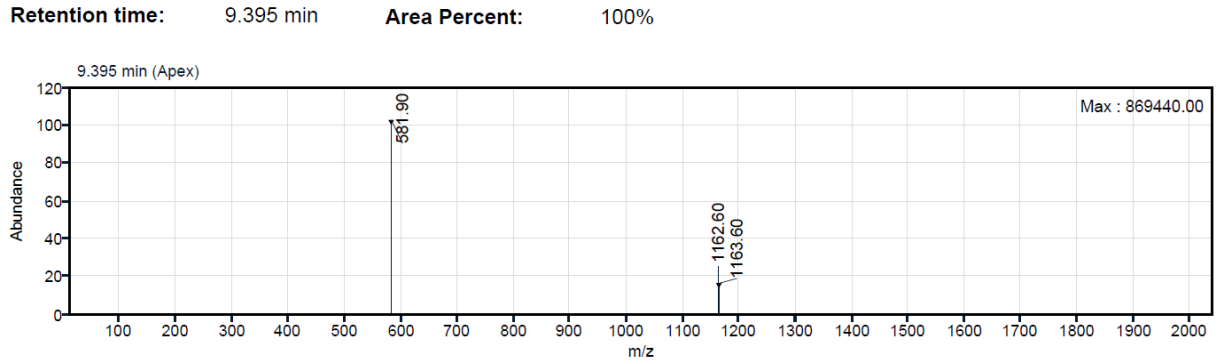


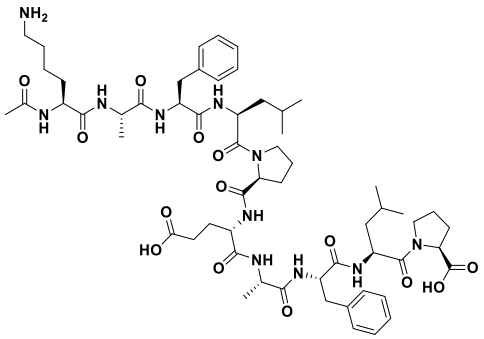
Figure S31: HPLC-UV chromatogram (210 nm) and MS(ESI) of **3q** by **Method 1.**
HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{51}H_{60}IN_{10}O_{14}^+$ 1163.3330; Found 1163.3311.

Table S15: MS/MS fragmentation of **3q**:

κ = Lys(C17H9IO3)
Nter = C2H3O

Sequence	Type	MF	MF Mass	m/z	Intensity	Similarity
FGKGG	b5	C40H42IN6O9(+1)	877.2058	877.2053	2.43	92.23%
FGKGGGG	a7	C43H48IN8O10(+1)	963.2538	963.2533	0.81	91.92%
GKGGGGGP	y8	C40H49IN9O12(+1)	974.2545	974.254	3.97	90.77%
FGKGGGGG	a8	C45H51IN9O11(+1)	1020.275	1020.275	4.33	90.00%
FGKGGGG	b7	C44H48IN8O11(+1)	991.2487	991.2482	23.81	89.99%
FGKGGGGG	b8	C46H51IN9O12(+1)	1048.27	1048.27	100.37	89.32%
FGKGGG	b6	C42H45IN7O10(+1)	934.2273	934.2267	6.84	89.10%
FGKG	b4	C38H39IN5O8(+1)	820.1843	820.1838	1.18	88.44%
FGK	b3	C36H36IN4O7(+1)	763.1629	763.1623	2.12	87.92%

Peptide EBX of Ac-KAFLPEAFLP (3r)



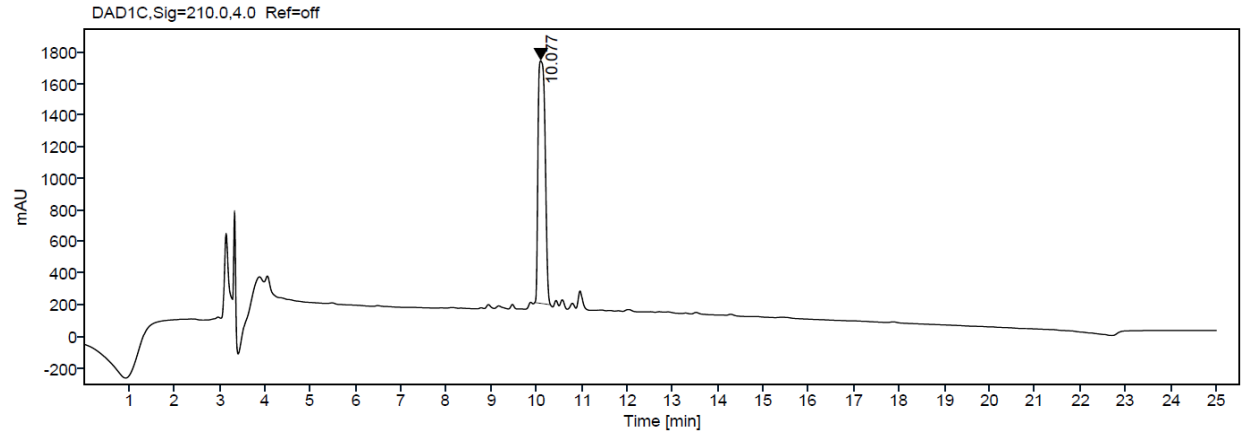
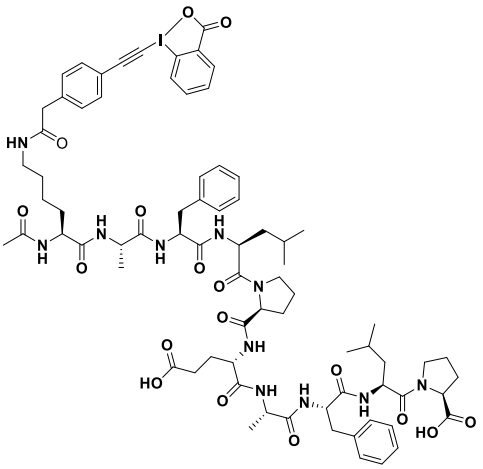
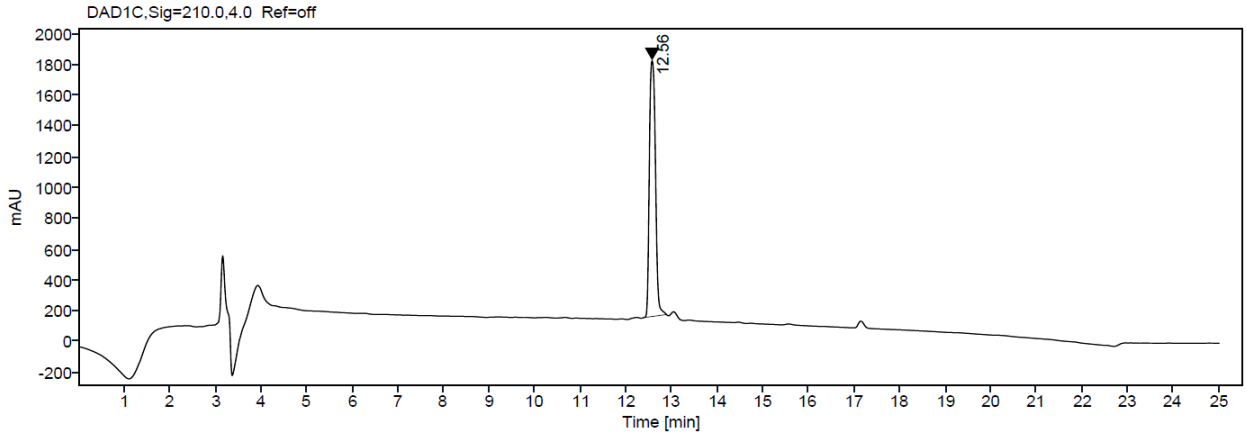


Figure S32: HPLC-UV chromatogram (210 nm) of Ac-KAFLPEAFLP by **Method 1.**

HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H_2]^{+2}$ Calcd for $C_{68}H_{93}N_{11}O_{13}^{+2}$ 635.8472; Found 635.8460.

Following the general procedure, the reaction was conducted on a 0.026 mmol scale. The desired product **3r** (17 mg, 0.011 mmol, 43% yield) was isolated by **Method 5**.





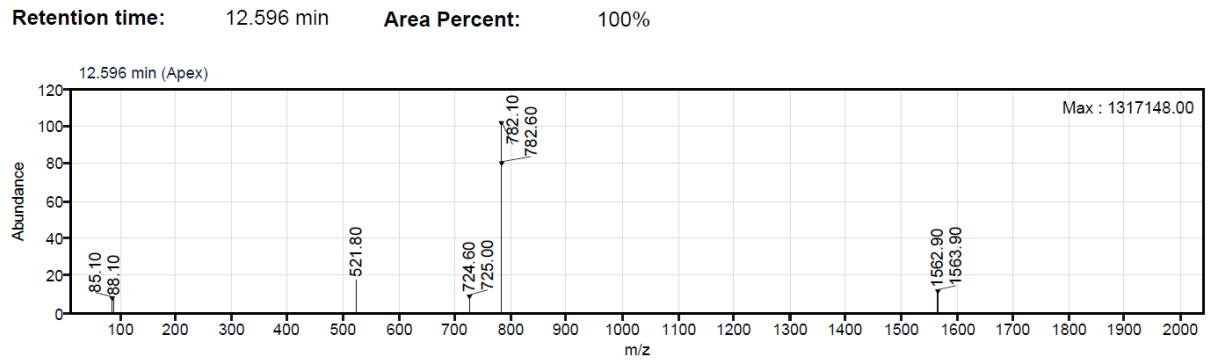
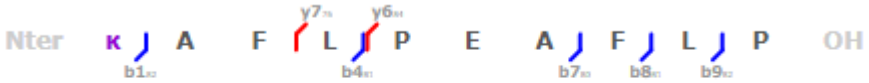


Figure S33: HPLC-UV chromatogram (210 nm) and MS(ESI) of **3r** by **Method 1.**
HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H_2]^{+2}$ Calcd for $C_{76}H_{98}IN_{11}O_{17}^{+2}$ 781.8088; Found 781.8083.

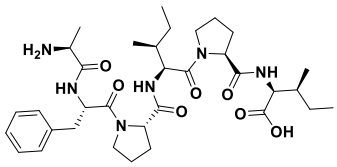
Table S16: MS/MS fragmentation of **3r**:



κ = Lys(C17H9IO3)
Nter = C2H3O

Sequence	Type	MF	MF Mass	m/z	Intensity	Similarity
PEAFLP	y6	C33H49N6O9(+1)	673.3561	673.3556	7.37	83.60%
KAFLPEAFL	b9	C71H88IN10O15(+1)	1447.548	1447.547	1.77	83.37%
K	b1	C25H24IN2O5(+1)	559.073	559.0724	0.51	81.65%
KAFL	b4	C43H49IN5O8(+1)	890.2626	890.262	6.96	81.47%
KAFLPEAF	b8	C65H77IN9O14(+1)	1334.464	1334.463	4.35	81.23%
KAFLPEAFL	b9	C71H88IN10O15(+1)	1447.548	724.2771	100.45	81.22%
KAFLPEA	b7	C56H68IN8O13(+1)	1187.395	1187.395	0.68	80.23%
LPEAFLP	y7	C39H60N7O10(+1)	786.4402	786.4396	0.67	76.07%
KAFLPEAFLP		C76H96IN11O17	1561.603	781.8088	30.41	75.26%

Peptide EBX of AFPIPI (3s)



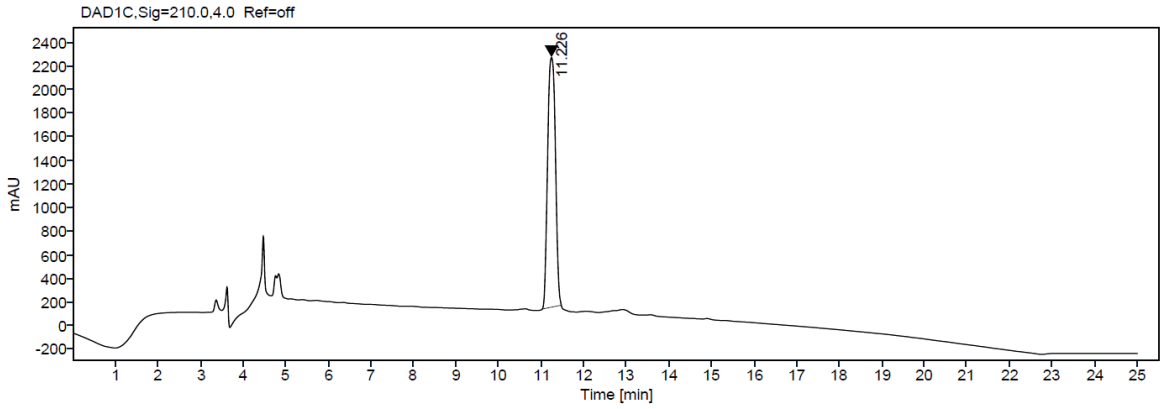
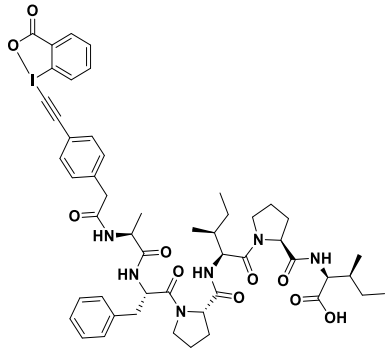
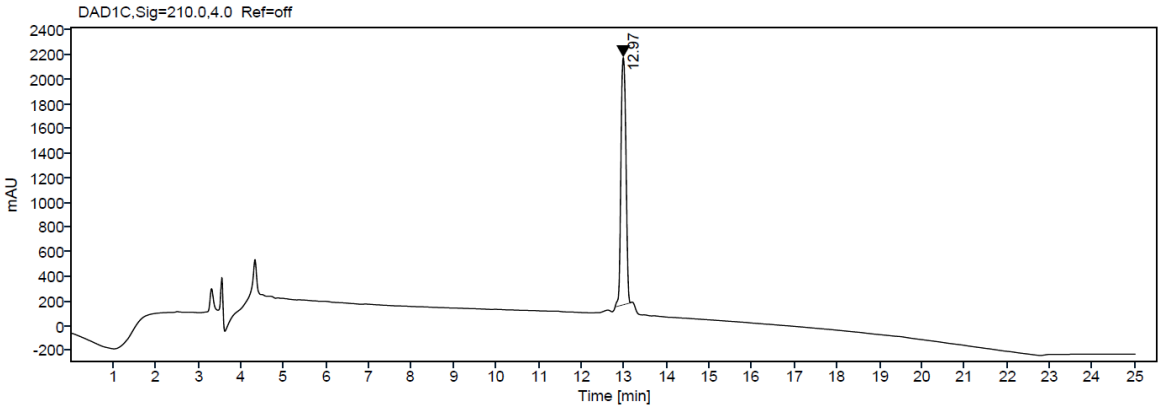


Figure S34: HPLC-UV chromatogram (210 nm) of AFPIPI by **Method 1.**

HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{34}H_{53}N_6O_7^+$ 657.3970; Found 657.3956.



Following the general procedure, the reaction was conducted on a 0.03 mmol scale. The desired product **3s** (14 mg, 0.013 mmol, 43% yield) was isolated by **Method 5**.



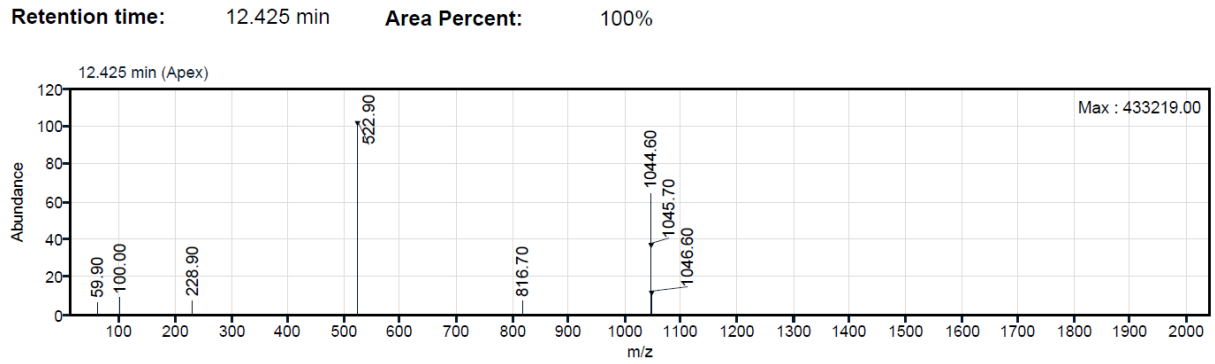
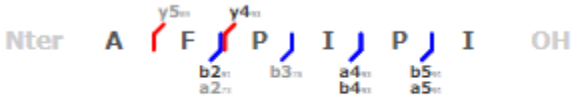


Figure S35: HPLC-UV chromatogram (210 nm) and MS(ESI) of **3s** by **Method 1.**

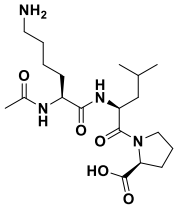
^{1}H NMR (500 MHz, CD_3OD**)** δ 8.39 (d, *J* = 8.1 Hz, 1H), 8.29 (d, *J* = 7.6 Hz, 1H), 7.90 (t, *J* = 7.6 Hz, 1H), 7.84 (t, *J* = 7.3 Hz, 1H), 7.65 (d, *J* = 7.6 Hz, 2H), 7.44 (dd, *J* = 19.4, 7.9 Hz, 2H), 7.33 – 7.18 (m, 5H), 4.58 – 4.47 (m, 3H), 4.40 – 4.29 (m, 2H), 3.93 (dt, *J* = 9.8, 6.7 Hz, 1H), 3.73 (ddt, *J* = 16.5, 9.2, 6.5 Hz, 2H), 3.62 (d, *J* = 3.8 Hz, 1H), 3.51 (dt, *J* = 12.6, 5.7 Hz, 1H), 3.16 (dd, *J* = 14.1, 5.2 Hz, 1H), 2.89 (dd, *J* = 14.0, 8.6 Hz, 1H), 2.24 – 1.86 (m, 11H), 1.77 – 1.63 (m, 2H), 1.56 (ttt, *J* = 11.3, 7.7, 4.1 Hz, 2H), 1.27 (d, *J* = 7.2 Hz, 3H), 1.04 (t, *J* = 6.6 Hz, 3H), 0.98 (d, *J* = 6.9 Hz, 3H), 0.93 (tt, *J* = 8.1, 4.3 Hz, 6H).
^{13}C NMR (201 MHz, CD_3OD**)** δ 174.8, 174.5, 174.2, 174.1, 172.8, 172.6, 172.1, 170.3, 140.16, 138.2, 136.5, 134.1, 133.0, 132.7, 130.9, 130.6, 130.5, 129.7, 129.5, 128.8, 127.8, 120.4, 117.1, 107.6, 61.5, 61.5, 58.3, 57.0, 53.8, 50.5, 48.3, 43.2, 38.5, 38.4, 38.3, 30.3, 26.3, 26.2, 26.0, 26.0, 25.7, 18.1, 17.9, 16.1, 16.1, 15.8, 15.7, 11.9, 11.4.
HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{51}H_{62}IN_6O_{10}^+$ 1045.3567; Found 1045.3564.
Table S17: MS/MS fragmentation of **3s**:



Nter = C17H10IO3

Sequence	Type	MF	MF Mass	m/z	Intensity	Similarity
AFPI	a4	C39H42IN4O6(+1)	789.2149	789.2144	6.22	92.77%
AFPI	b4	C40H42IN4O7(+1)	817.2098	817.2093	100.09	92.64%
PIPI	y4	C22H39N4O5(+1)	439.292	439.2915	1.6	92.50%
AFPIP	b5	C45H49IN5O8(+1)	914.2626	914.262	0.54	91.22%
AFPIP	a5	C44H49IN5O7(+1)	886.2677	886.2671	1.76	91.19%
AF	b2	C29H24IN2O5(+1)	607.073	607.0724	5.95	91.07%
FPIPI	y5	C31H48N5O6(+1)	586.3605	586.3599	1.03	88.65%
AFP	b3	C34H31IN3O6(+1)	704.1258	704.1252	0.67	78.16%
AF	a2	C28H24IN2O4(+1)	579.0781	579.0775	0.81	72.97%

Peptide EBX of Ac-KLP (3v)



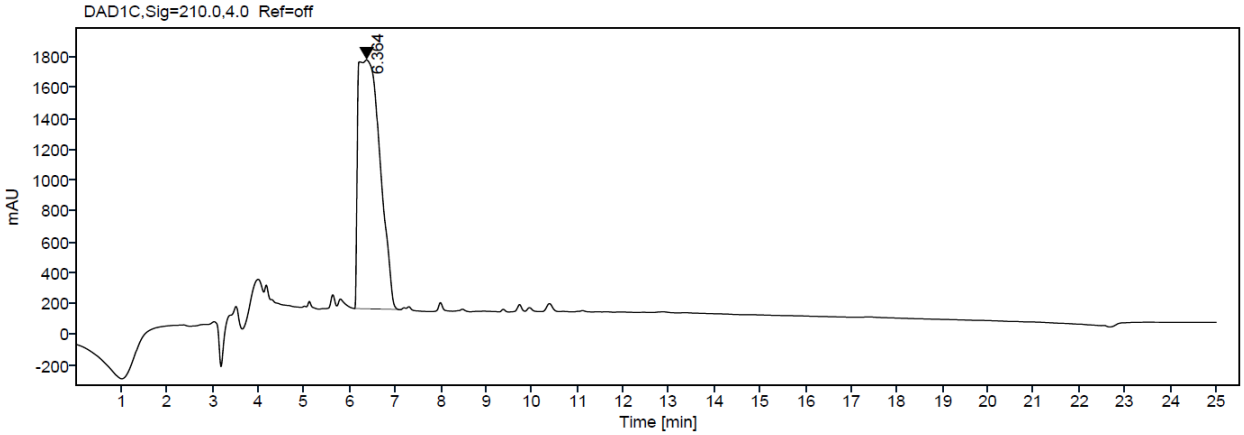
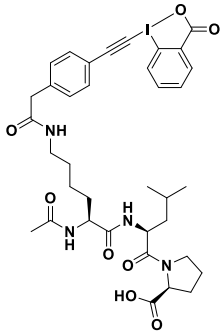


Figure S36: HPLC-UV chromatogram (210 nm) of Ac-KLP by **Method 1**.
HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{19}H_{35}N_4O_5^+$ 399.2602; Found 399.2598.



Following the general procedure, the reaction was conducted in 0.05 mmol scale. The desired product **3v** (13 mg, 0.016 mmol, 32% yield) was isolated by **Method 6** (Pure compound can be obtained by SPPS. See Section 5).

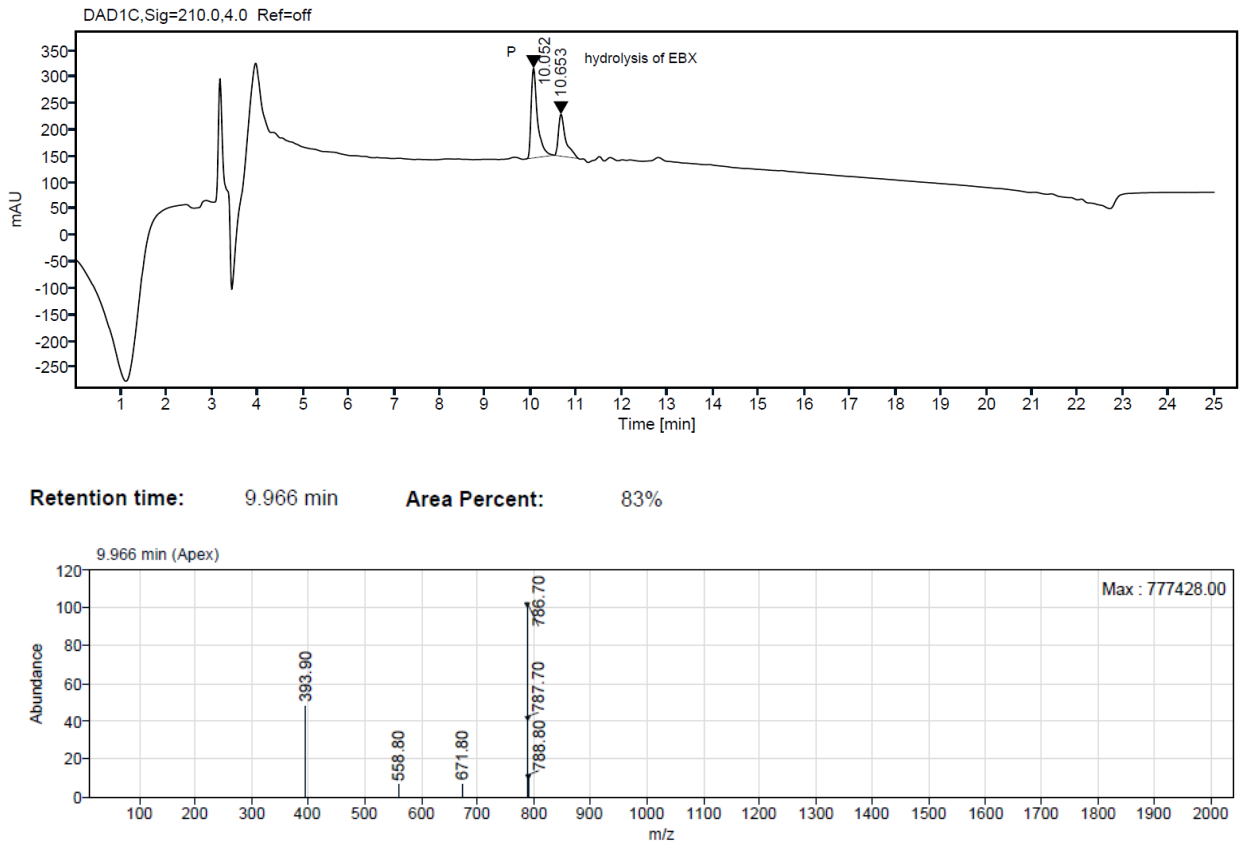


Figure S37: HPLC-UV chromatogram (210 nm) and MS(ESI) of **3v** by **Method 1**.
HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{36}H_{44}IN_4O_8^+$ 787.2198; Found 787.2169.

Table S18: MS/MS fragmentation of **3v**:

κ = Lys(C17H9IO3)
Nter = C2H3O

Sequence	Type	MF	MF Mass	m/z	Intensity	Similarity
LP	y2	C11H21N2O3(+1)	229.1552	229.1547	25.79	96.17%
K	b1	C25H24IN2O5(+1)	559.073	559.0724	28.77	95.29%
P	y1	C5H10NO2(+1)	116.0712	116.0706	5.72	93.88%
KL	b2	C31H35IN3O6(+1)	672.1571	672.1565	75.89	93.72%
KL	b2	C31H35IN3O6(+1)	672.1571	336.5819	2.92	81.05%

Peptide EBX of Ac-KLFP (3w)

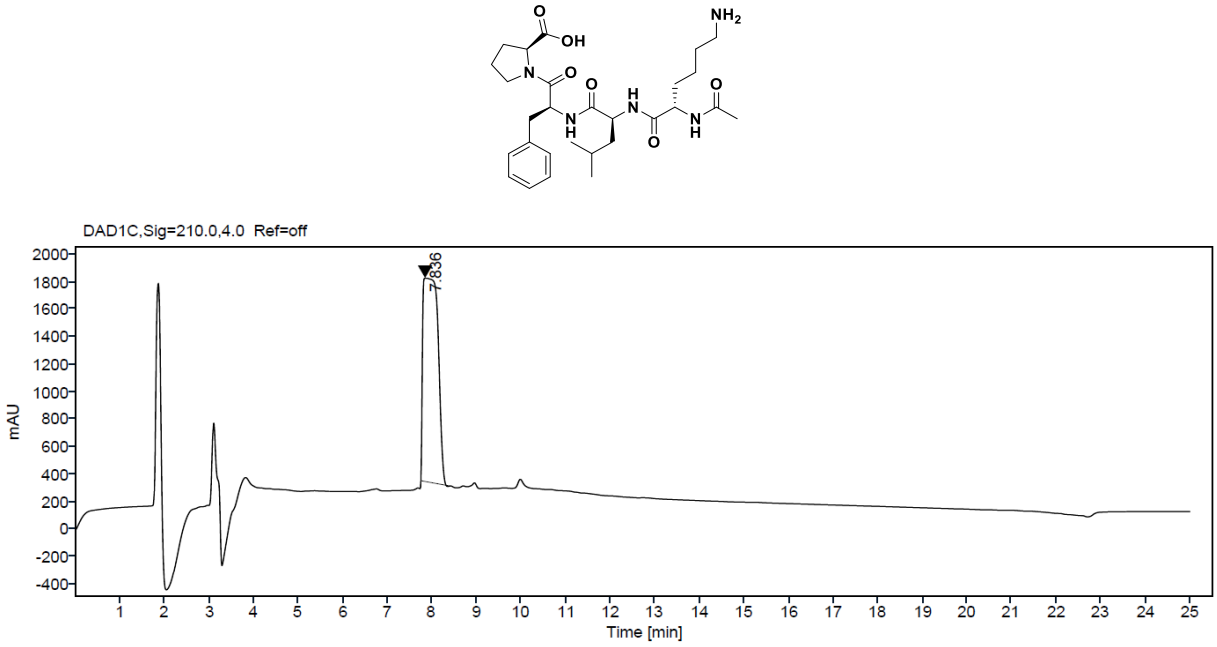
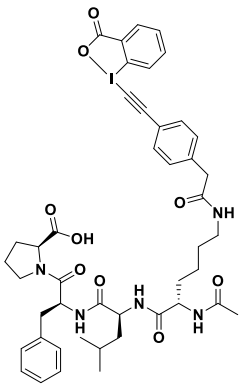
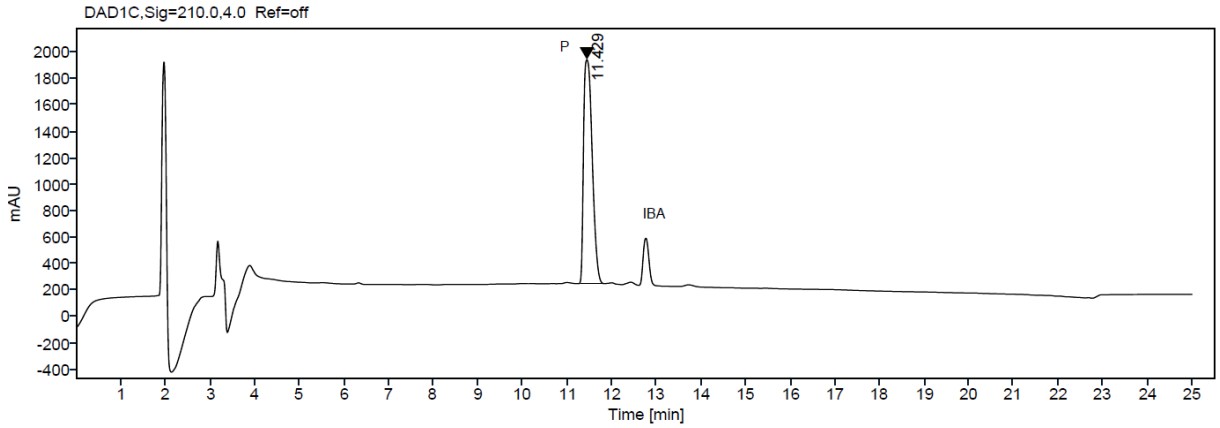


Figure S38: HPLC-UV chromatogram (210 nm) of Ac-KLFP by **Method 1.**



Following the general procedure, the reaction was conducted in 0.046 mmol scale. The desired product **3w** (17 mg, 0.018 mmol, 39% yield) was isolated by **Method 5**.



Retention time: 11.497 min **Area Percent:** 100%

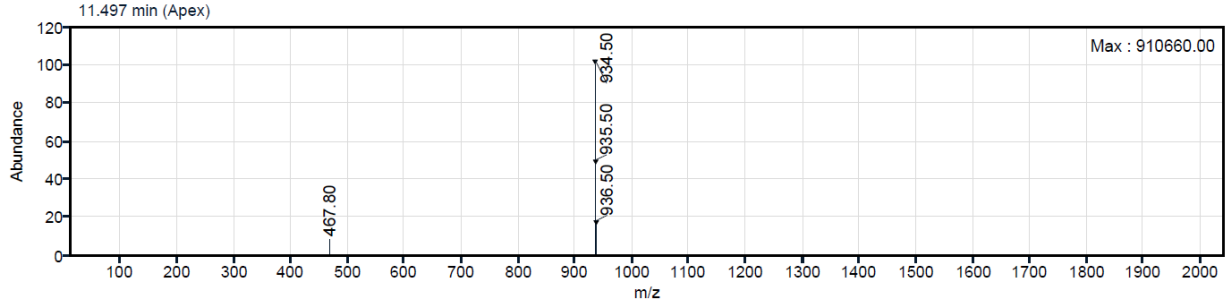
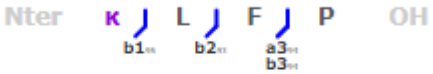


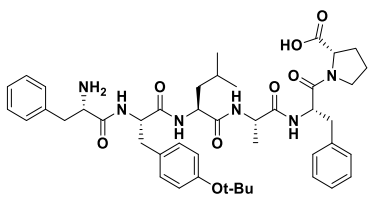
Figure S39: HPLC-UV chromatogram (210 nm) and MS(ESI) of **3w** by **Method 1.**
HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{45}H_{53}IN_5O_9^+$ 934.2883; Found 934.2880.
Table S19: MS/MS fragmentation of **3w**:



κ = Lys(C17H9IO3)
Nter = C2H3O

Sequence	Type	MF	MF Mass	m/z	Intensity	Similarity
K	b1	C25H24IN2O5(+1)	559.073	559.0724	3.08	94.59%
KLF	a3	C39H44IN4O6(+1)	791.2306	791.23	8.44	94.18%
KLF	b3	C40H44IN4O7(+1)	819.2255	819.2249	100.24	93.52%
KL	b2	C31H35IN3O6(+1)	672.1571	672.1565	4.69	93.21%

Peptide EBX of FYLAFP (3x)



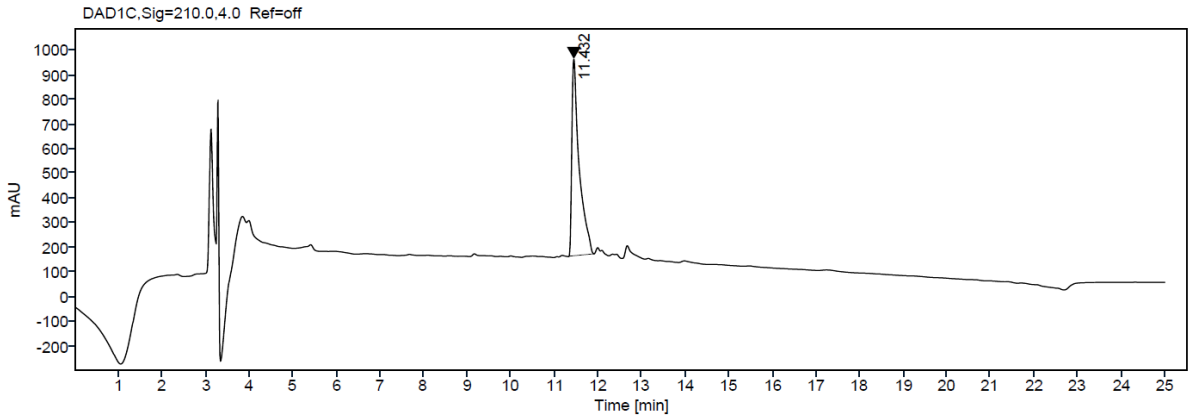
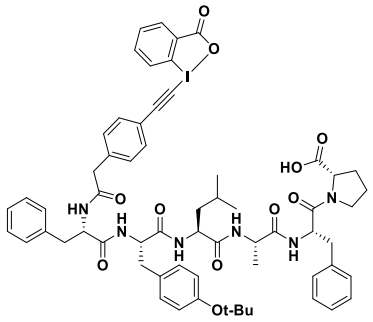
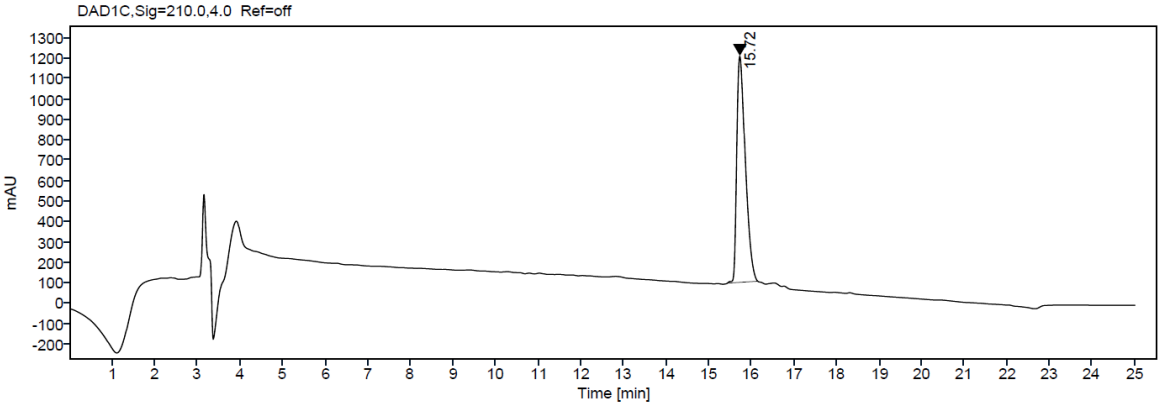


Figure S40: HPLC-UV chromatogram (210 nm) of FYLAFP by **Method 1.**
HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{45}H_{61}N_6O_8^+$ 813.4545; Found 813.4557.



Following the general procedure, the reaction was conducted in 0.037 mmol scale. The desired product **3x** (23 mg, 0.019 mmol, 53% yield) was isolated with **Method 5**.



Retention time: 15.881 min **Area Percent:** 100%

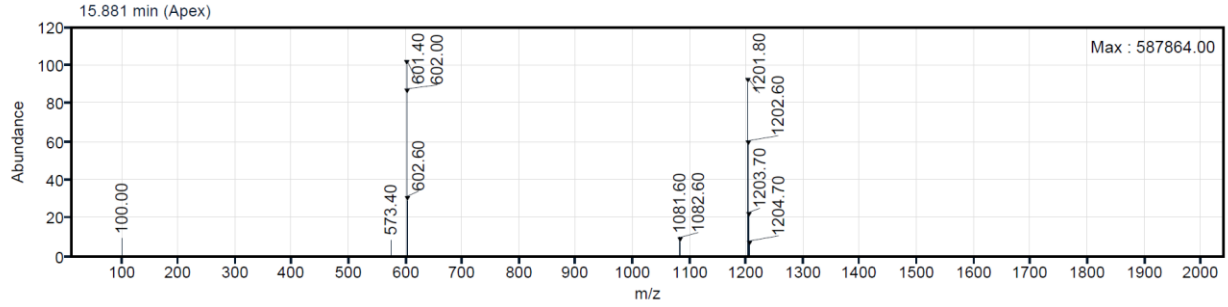
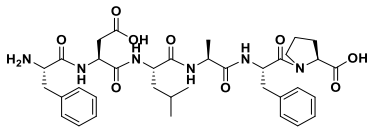


Figure S41: HPLC-UV chromatogram (210 nm) and MS(ESI) of **3x** by **Method 1.**
HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{62}H_{70}IN_6O_{11}^+$ 1201.4142; Found 1201.4123.
Table S20: MS/MS fragmentation of **3x**:

ψ = Tyr(C4H8)
Nter = C17H10IO3

Sequence	Type	MF	MF Mass	m/z	Intensity	Similarity
FYLAF	b5	C57H61IN5O9(+1)	1086.351	1086.351	102.61	88.76%
FYLA	b4	C48H52IN4O8(+1)	939.283	939.2824	7.87	87.95%
FYL	b3	C45H47IN3O7(+1)	868.2459	868.2453	1.91	87.29%
FYLAF	a5	C56H61IN5O8(+1)	1058.357	1058.356	17.63	84.14%

Peptide EBX of FDLAFP (3y)



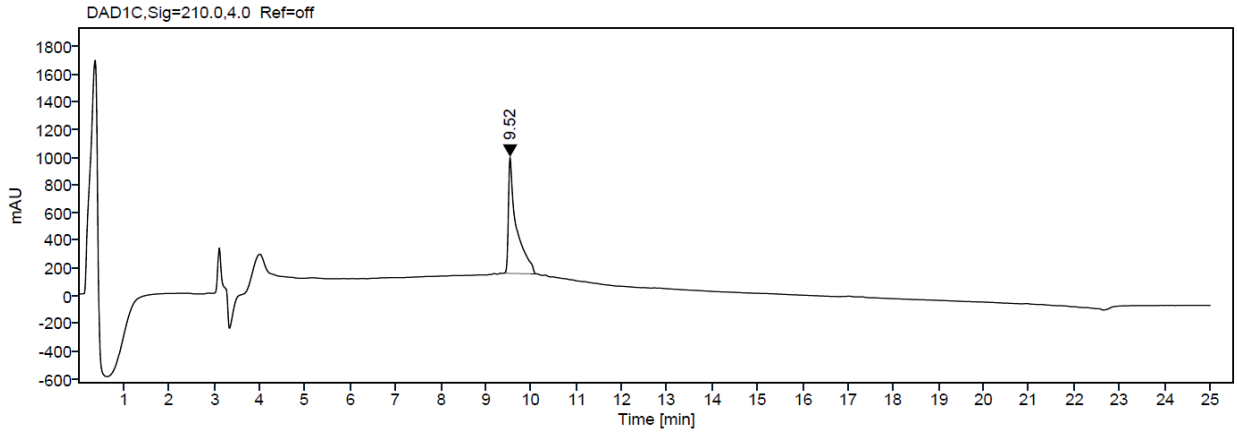
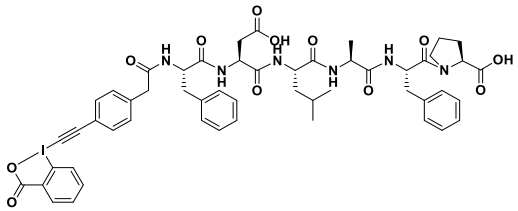
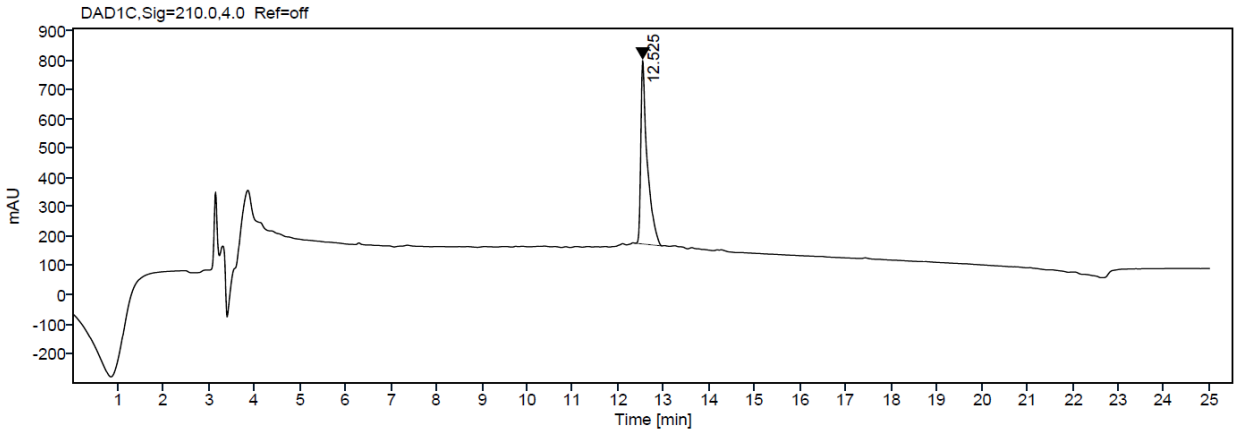


Figure S42: HPLC-UV chromatogram (210 nm) of FDLAFP by **Method 1**.
HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{36}H_{49}N_6O_9^+$ 709.3556; Found 709.3552.



Following the general procedure, the reaction was conducted on a 0.028 mmol scale. The desired product **3y** (9.6 mg, 0.0087 mmol, 31% yield) was isolated by **Method 6.**



Retention time: 12.555 min **Area Percent:** 100%

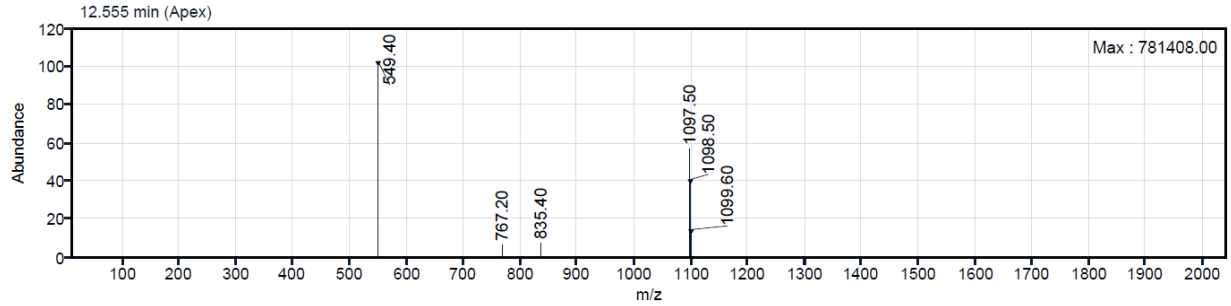
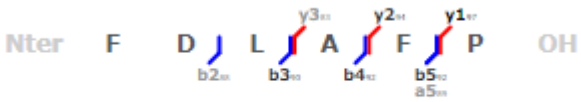


Figure S43: HPLC-UV chromatogram (210 nm) and MS(ESI) of **3y** by **Method 1**.
HRMS (Nanochip-based ESI/LTQ-Orbitrap) m/z: $[M + H_2]^{+2}$ Calcd for $C_{53}H_{59}IN_6O_{12}^{+2}$ 549.1612; Found 549.1607.

Table S21: MS/MS fragmentation of **3y**:

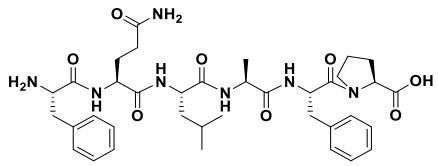


Nter = C17H10IO3

Sequence	Type	MF	MF Mass	m/z	Intensity	Similarity
P	y1	C5H10NO2(+1)	116.0712	116.0706	102.7	97.40%
FP	y2	C14H19N2O3(+1)	263.1396	263.139	28.34	94.19%
FDLAF	b5	C48H49IN5O10(+1)	982.2524	982.2519	57.2	92.60%
FDLA	b4	C39H40IN4O9(+1)	835.184	418.0954	28.86	92.21%
FDLAF	b5	C48H49IN5O10(+1)	982.2524	491.6296	34.12	92.04%
FDLAF	a5	C47H49IN5O9(+1)	954.2575	954.257	10.98	91.95%
FDLA	b4	C39H40IN4O9(+1)	835.184	835.1835	76.31	91.86%
FDLAFP		C53H57IN6O12	1096.308	549.1612	20.01	91.85%
FDL	b3	C36H35IN3O8(+1)	764.1469	764.1463	4.29	90.36%
FDL	b3	C36H35IN3O8(+1)	764.1469	382.5768	2	90.16%
FD	b2	C30H24IN2O7(+1)	651.0628	651.0623	1.02	88.38%

FDLAF	a5	C47H49IN5O9(+1)	954.2575	477.6321	0.69	86.77%

Peptide EBX of FQLAFP (3z)



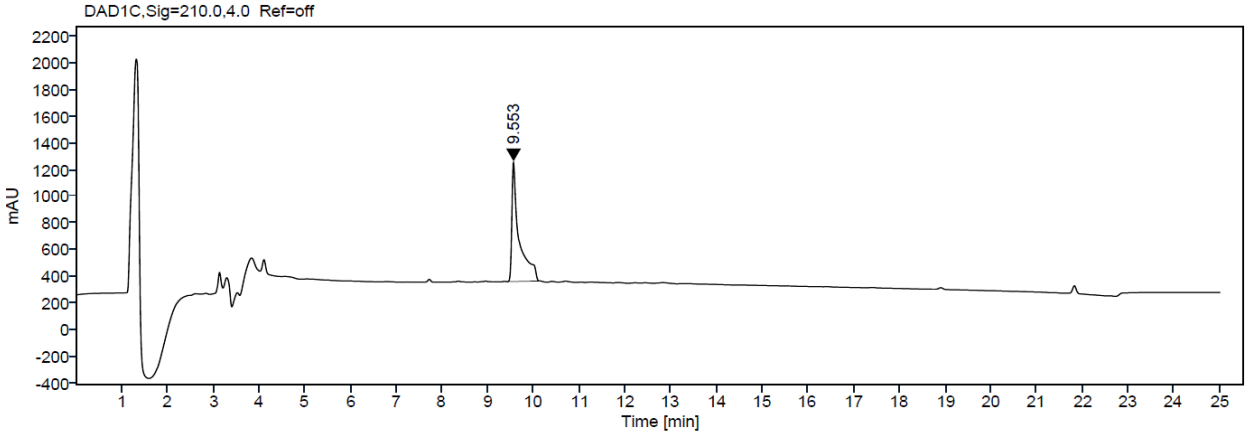
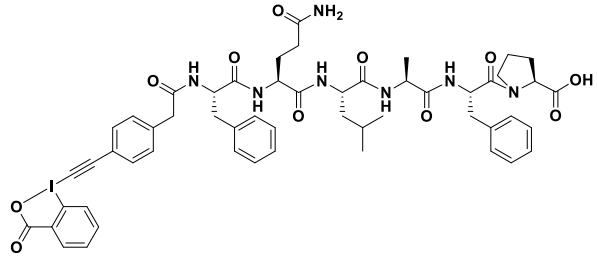
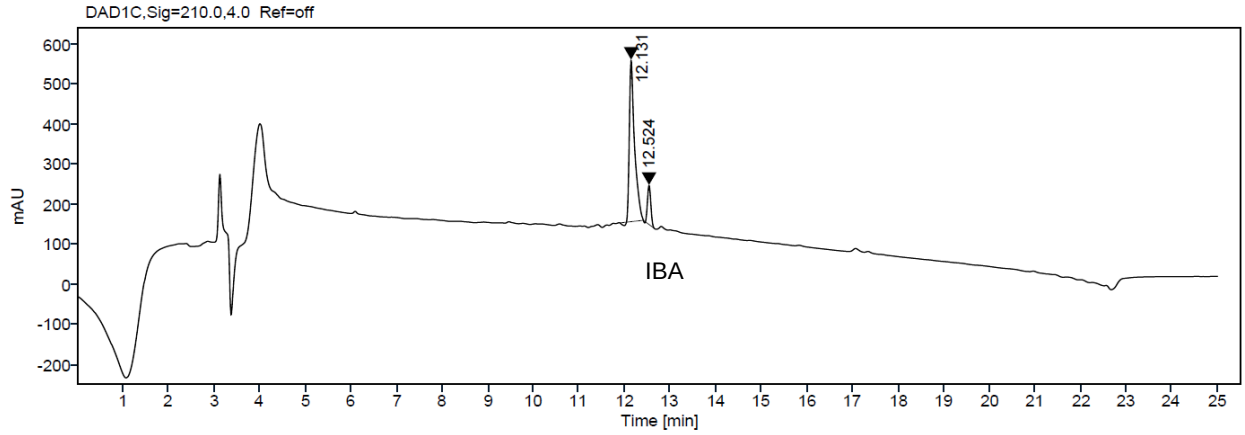


Figure S44: HPLC-UV chromatogram (210 nm) of FQLAFP by **Method 1.**
HRMS (ESI/QTOF) m/z: $[M + Na]^+$ Calcd for $C_{37}H_{51}N_7NaO_8^+$ 744.3691; Found 744.3695.



Following the general procedure, the reaction was conducted on a 0.045 mmol scale. The desired product **3z** (24 mg, 0.021 mmol, 45% yield) was isolated by **Method 6**.



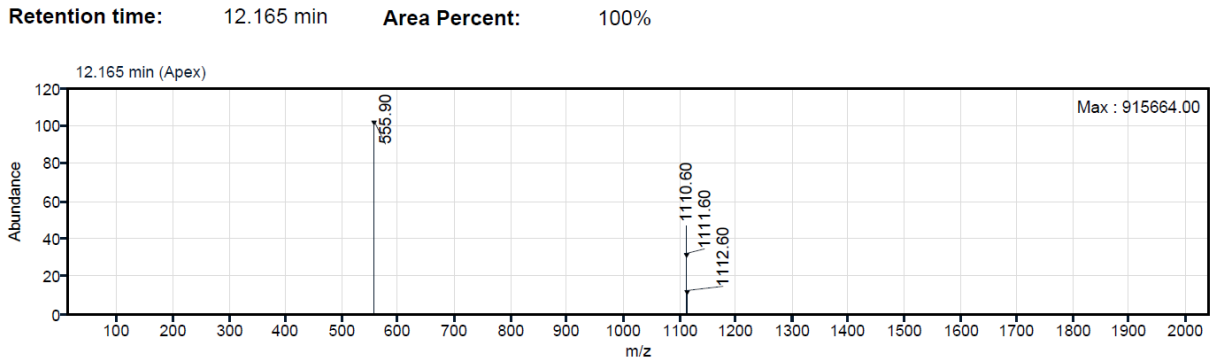
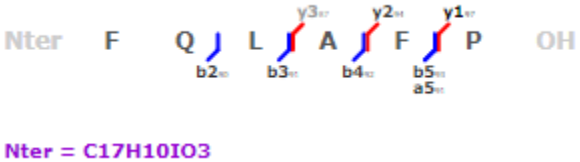


Figure S45: HPLC-UV chromatogram (210 nm) and MS(ESI) of **3z** by **Method 1**.

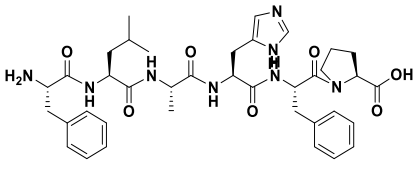
HRMS (Nanochip-based ESI/LTQ-Orbitrap) m/z: $[M + H_2]^{+2}$ Calcd for $C_{54}H_{62}IN_7O_{11}^{+2}$ 555.6771; Found 555.6761.

Table S22: MS/MS fragmentation of **3z**:



Sequence	Type	MF	MF Mass	m/z	Intensity	Similarity
P	y1	C5H10NO2(+1)	116.0712	116.0706	45.84	97.10%
FP	y2	C14H19N2O3(+1)	263.1396	263.139	27.84	94.41%
FQLAF	b5	C49H52IN6O9(+1)	995.284	498.1454	30.05	92.58%
FQLAF	b5	C49H52IN6O9(+1)	995.284	995.2835	22.27	92.52%
FQLA	b4	C40H43IN5O8(+1)	848.2156	424.6112	100.3	91.88%
FQLA	b4	C40H43IN5O8(+1)	848.2156	848.2151	48.44	91.37%
FQLAF	a5	C48H52IN6O8(+1)	967.2891	967.2886	4.47	91.35%
FQL	b3	C37H38IN4O7(+1)	777.1785	777.178	3.96	90.85%
FQL	b3	C37H38IN4O7(+1)	777.1785	389.0926	15.32	90.80%
FQLAFP		C54H60IN7O11	1109.34	555.6771	3.05	90.51%
FQ	b2	C31H27IN3O6(+1)	664.0945	664.0939	1.91	90.34%
FQLAF	a5	C48H52IN6O8(+1)	967.2891	484.1479	1.35	89.99%
AFP	y3	C17H24N3O4(+1)	334.1767	334.1761	1.08	86.73%

Peptide EBX of FLHAFP (3aa)



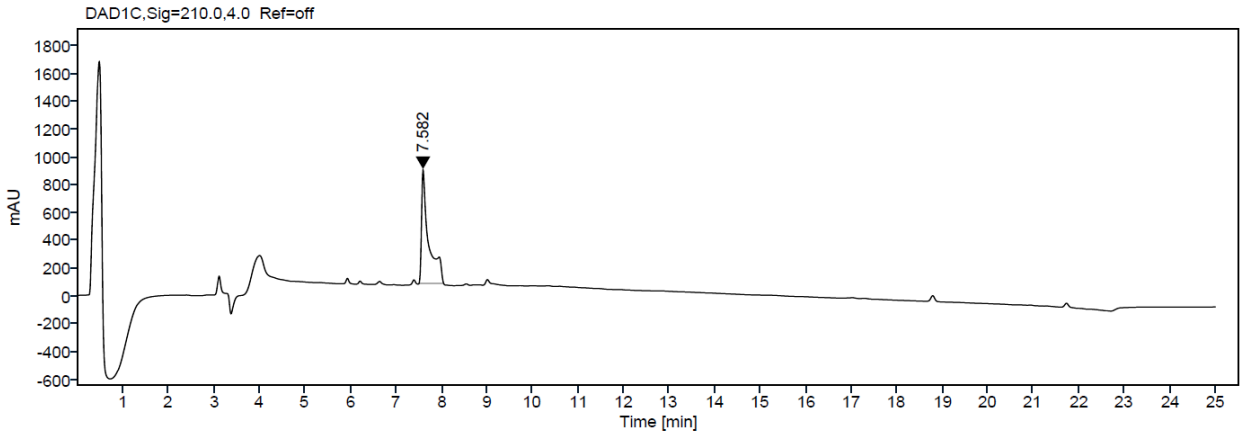
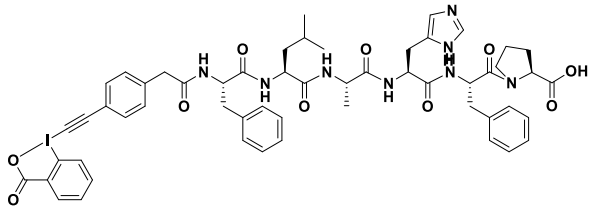
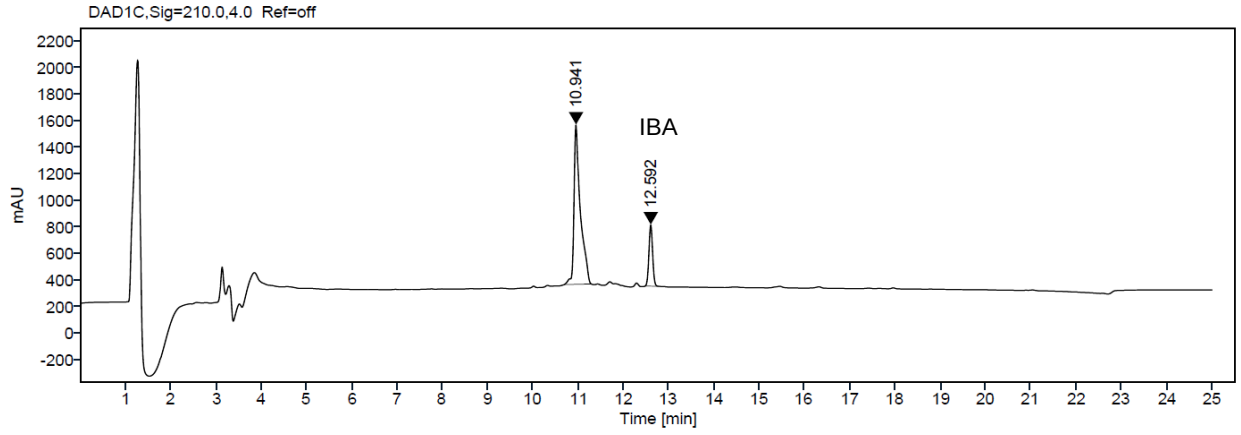


Figure S46: HPLC-UV chromatogram (210 nm) of FLHAFP by **Method 1**.
HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{38}H_{51}N_8O_7^+$ 731.3875; Found 731.3876.



Following the general procedure, the reaction was conducted on a 0.045 mmol scale. The desired product **3aa** (24 mg, 0.021 mmol, 45% yield) was isolated by **Method 6**.



Retention time: 10.983 min **Area Percent:** 100%

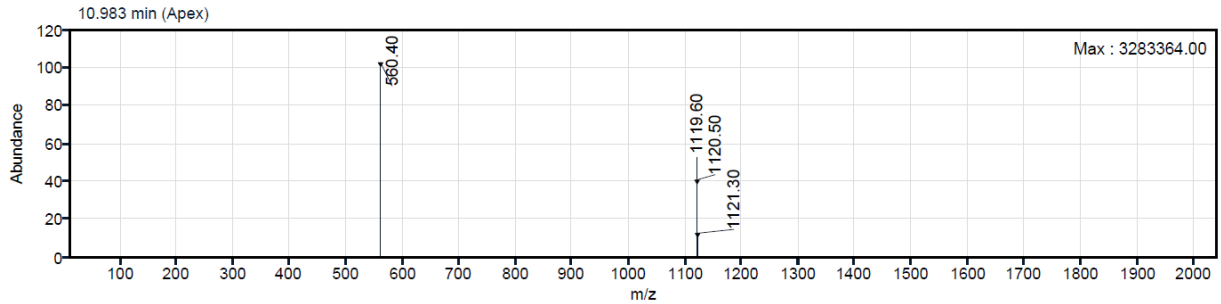
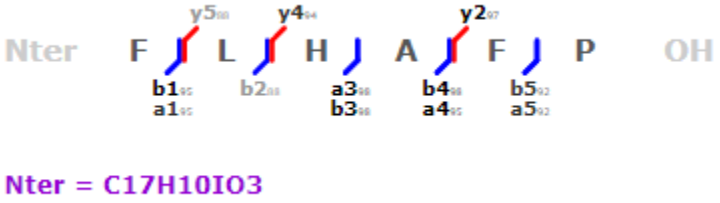


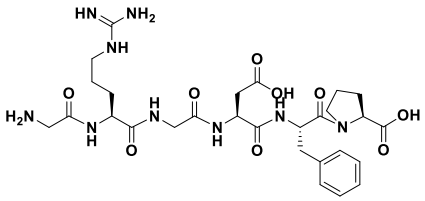
Figure S47: HPLC-UV chromatogram (210 nm) and MS(ESI) of **3aa** by **Method 1**.
HRMS (Nanochip-based ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{55}H_{60}IN_8O_{10}^+$ 1119.3472; Found 1119.3485.

Table S23: MS/MS fragmentation of **3aa**:



Sequence	Type	MF	MF Mass	m/z	Intensity	Similarity
FLH	a3	C37H37IN5O5(+1)	758.1839	758.1834	19.36	98.11%
FLHA	b4	C41H42IN6O7(+1)	857.216	857.2154	9.56	98.05%
FLH	b3	C38H37IN5O6(+1)	786.1789	786.1783	29.73	97.78%
FP	y2	C14H19N2O3(+1)	263.1396	263.139	4.55	97.13%
F	b1	C26H19INO4(+1)	536.0359	536.0353	4.91	95.15%
FLHA	a4	C40H42IN6O6(+1)	829.2211	829.2205	2.55	95.07%
F	a1	C25H19INO3(+1)	508.041	508.0404	4.38	94.95%
HAFP	y4	C23H31N6O5(+1)	471.2356	471.235	6.97	94.31%
FLHAF	b5	C50H51IN7O8(+1)	1004.284	1004.284	1.28	92.41%
FLHAF	a5	C49H51IN7O7(+1)	976.2895	976.2889	0.84	91.54%
FL	b2	C32H30IN2O5(+1)	649.1199	649.1194	0.67	88.50%
LHAFP	y5	C29H42N7O6(+1)	584.3197	584.3191	1.05	88.26%

Peptide EBX of GRGDFP (3ab)



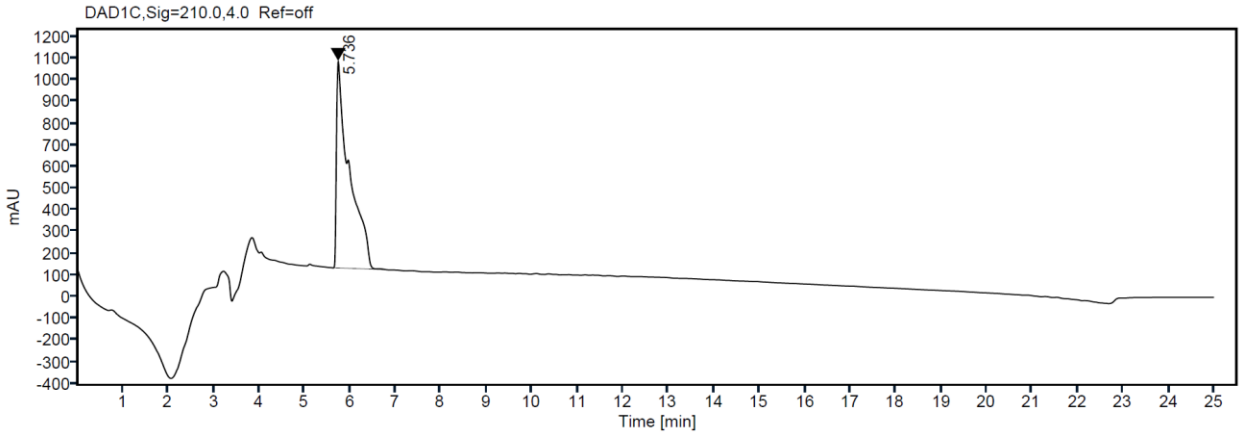
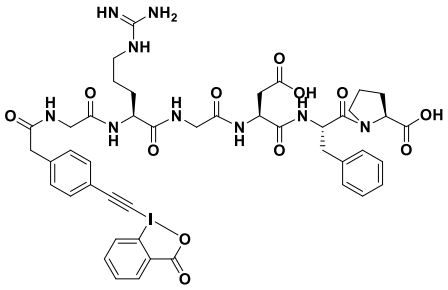


Figure S48: HPLC-UV chromatogram (210 nm) of **GRGDFP** by **Method 1**.
HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{28}H_{42}N_9O_9^+$ 648.3100; Found 648.3099.



Following the general procedure, the reaction was conducted on a 0.03 mmol scale. The desired product **3ab** (6.8 mg, 0.0065 mmol, 21% yield) was isolated by **Method 6**.

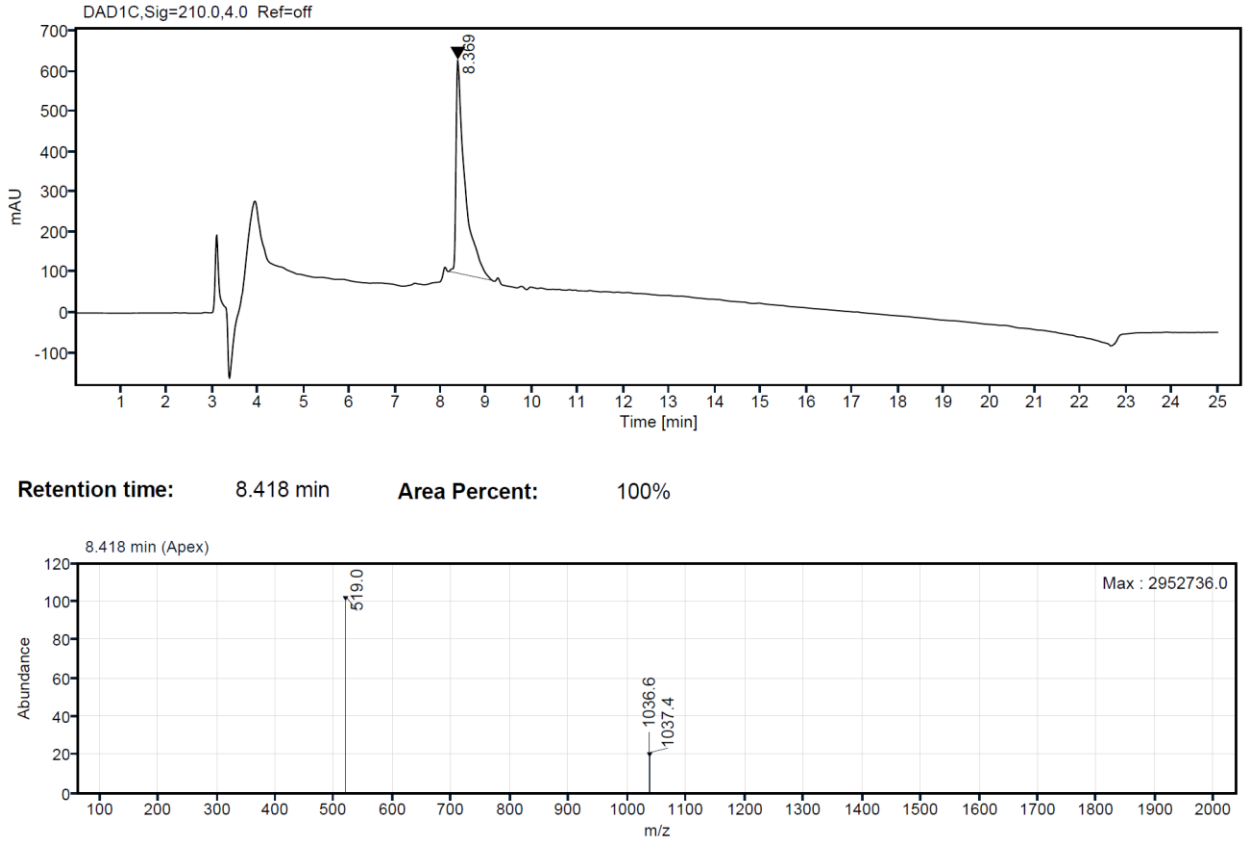
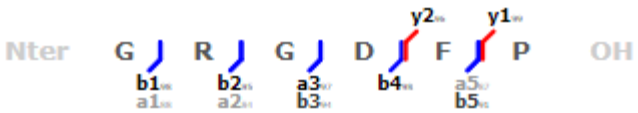


Figure S49: HPLC-UV chromatogram (210 nm) and MS(ESI) of **3ab** by **Method 1**.
HRMS (Nanochip-based ESI/LTQ-Orbitrap) m/z: $[M + H_2]^{+2}$ Calcd for $C_{45}H_{52}IN_9O_{12}^{+2}$ 518.6385; Found 518.6385.

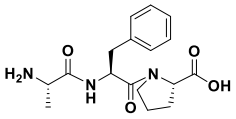
Table S24: MS/MS fragmentation of **3ab**:



Nter = C17H10IO3

Sequence	Type	MF	MF Mass	m/z	Intensity	Similarity
P	y1	C5H10NO2(+1)	116.07	116.07	74.4	98.83%
GRGD	b4	C31H33IN7O9(+1)	774.14	387.57	54.21	98.55%
GRGDFP		C45H50IN9O12	1035.3	518.64	19.4	97.94%
G	b1	C19H13INO4(+1)	445.99	445.99	11.43	97.82%
GRG	a3	C26H28IN6O5(+1)	631.12	316.06	2.13	96.82%
GRGD	b4	C31H33IN7O9(+1)	774.14	774.14	7.44	96.51%
GRG	a3	C26H28IN6O5(+1)	631.12	631.12	7.54	96.26%
FP	y2	C14H19N2O3(+1)	263.14	263.14	5.31	95.80%
GRG	b3	C27H28IN6O6(+1)	659.11	659.11	3.32	95.67%
GR	b2	C25H25IN5O5(+1)	602.09	602.09	8.61	95.31%
GR	b2	C25H25IN5O5(+1)	602.09	301.55	6.07	94.74%
GRGDF	a5	C39H42IN8O9(+1)	893.21	447.11	2.1	92.60%
GR	a2	C24H25IN5O4(+1)	574.1	574.09	5	92.28%
GRG	b3	C27H28IN6O6(+1)	659.11	330.06	1.31	92.20%
GRGDF	b5	C40H42IN8O10(+1)	921.21	461.11	1.64	91.47%
G	a1	C18H13INO3(+1)	417.99	417.99	1.72	88.04%
GRGDF	a5	C39H42IN8O9(+1)	893.21	893.21	0.62	81.21%
GR	a2	C24H25IN5O4(+1)	574.1	287.55	0.81	74.92%

Peptide EBX of AFP (3ac)



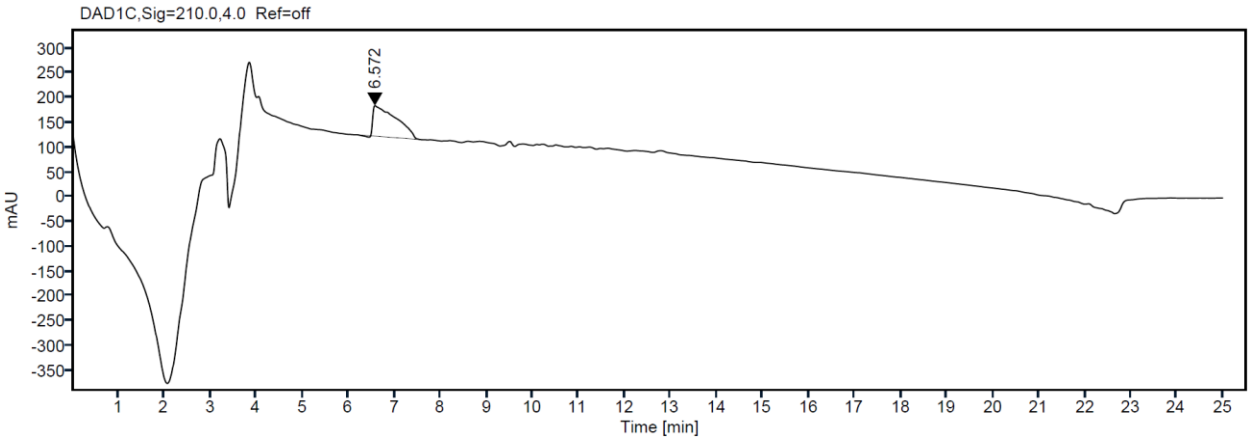
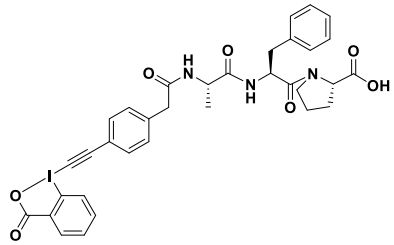
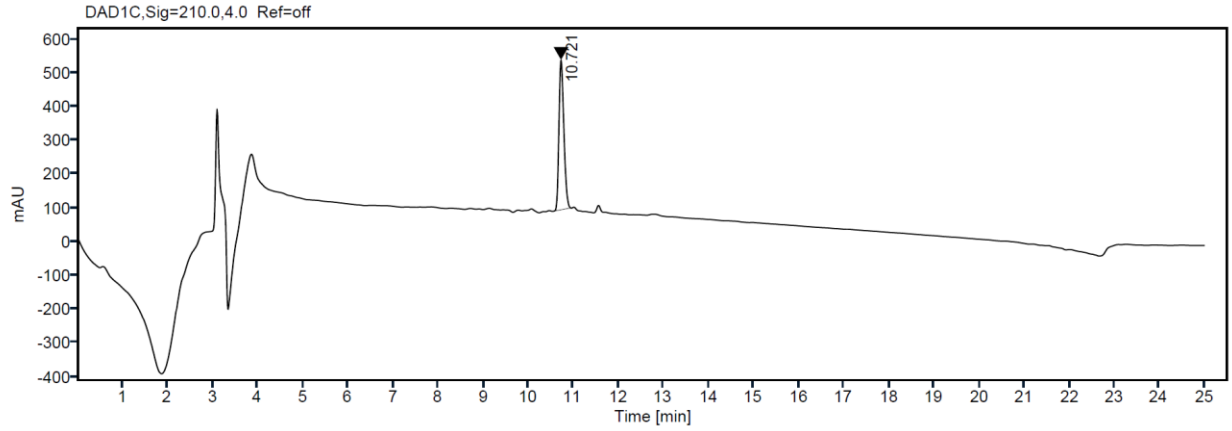


Figure S50: HPLC-UV chromatogram (210 nm) of **AFP** by **Method 1**.
HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{17}H_{24}N_3O_4^+$ 334.1761; Found 334.1760.



Following the general procedure, the reaction was conducted on 8.1 μmol scale. The desired product **3ac** (2.4 mg, 0.0033 mmol, 41% yield) was isolated by **Method 6**.



Retention time: 10.753 min **Area Percent:** 100%

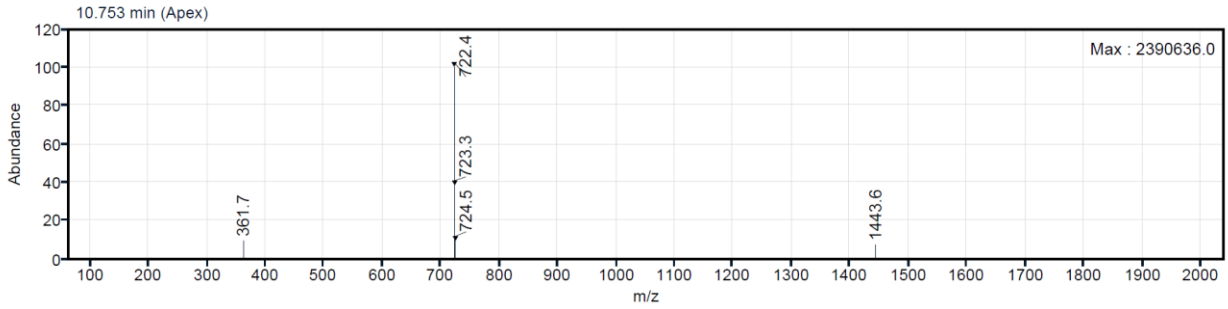


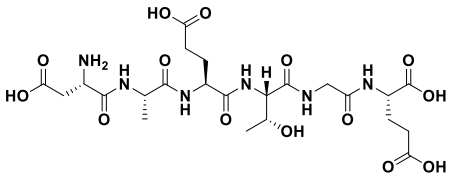
Figure S51: HPLC-UV chromatogram (210 nm) and MS(ESI) of **3ac** by **Method 1**.
HRMS (Nanochip-based ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{34}H_{33}IN_3O_7^+$ 722.1358; Found 722.1351.

Table S25: MS/MS fragmentation of **3ac**:

Nter = C17H10IO3

Sequence	Type	MF	MF Mass	m/z	Intensity	Similarity
P	y1	C5H10NO2(+1)	116.07	116.07	48.37	98.43%
AF	a2	C28H24IN2O4(+1)	579.08	579.08	7.07	98.01%
AFP		C34H32IN3O7	721.13	722.14	24.85	97.82%
AF	b2	C29H24IN2O5(+1)	607.07	607.07	12.45	97.80%
FP	y2	C14H19N2O3(+1)	263.14	263.14	15.49	97.20%
A	b1	C20H15INO4(+1)	460	460	6.49	96.85%
A	a1	C19H15INO3(+1)	432.01	432.01	3.78	96.49%

Peptide EBX of DAETGE (3ae)



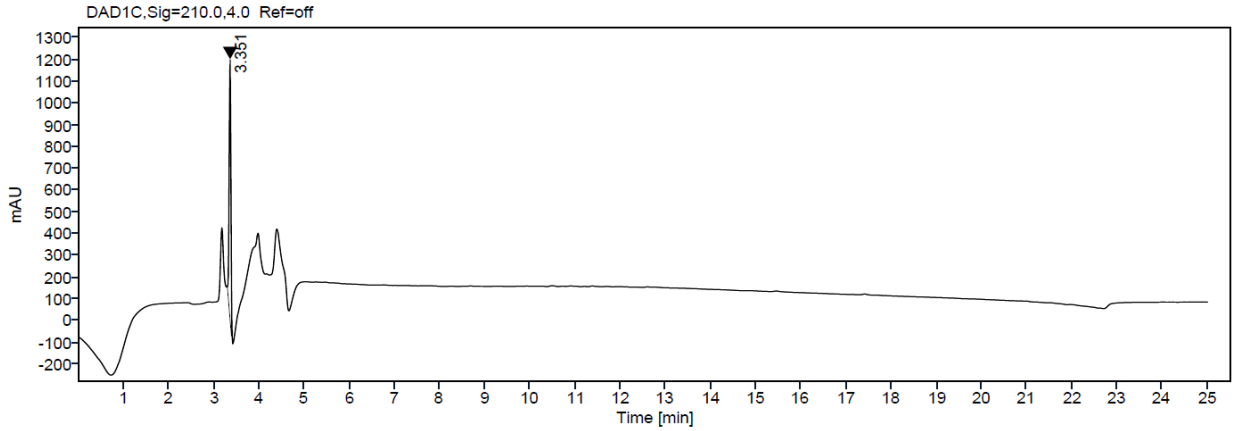
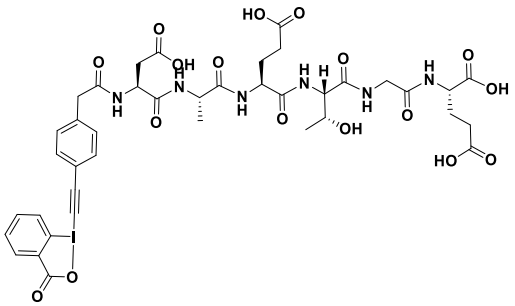


Figure S52: HPLC-UV chromatogram (210 nm) of DAETGE by **Method 1**.

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{23}H_{37}N_6O_{14}^+$ 621.2362; Found 621.2366.



Following the general procedure, the reaction was conducted on a 0.046 mmol scale. The desired product **3ae** (16 mg, 0.016 mmol, 35% yield) was isolated by **Method 6**.

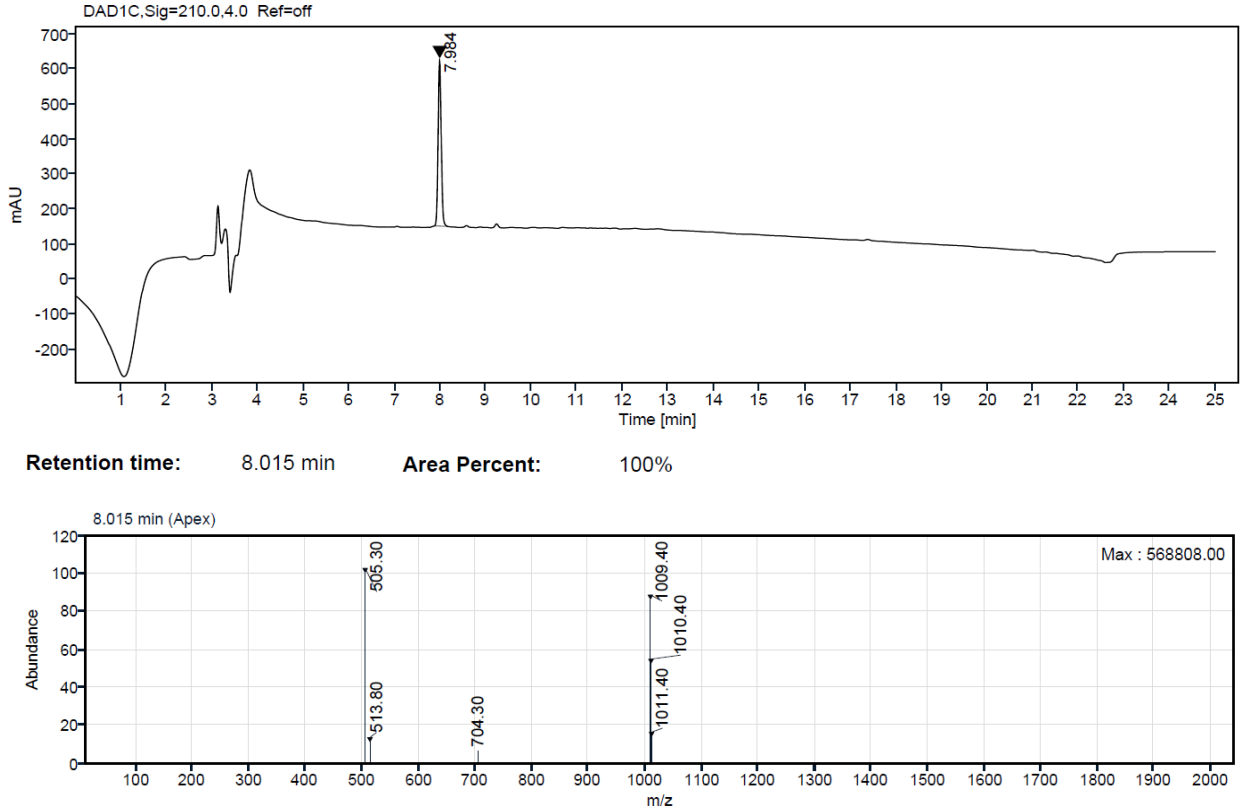


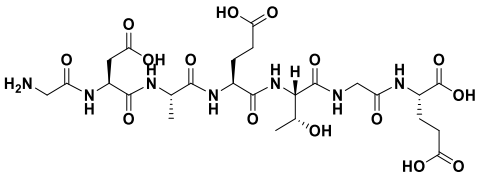
Figure S53: HPLC-UV chromatogram (210 nm) and MS(ESI) of **3ae** by **Method 1**.

HRMS (Nanochip-based ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{40}H_{46}IN_6O_{17}^+$ 1009.1959; Found 1009.1970

Table S26: MS/MS fragmentation of **3ae**:

Sequence	Type	MF	MF Mass	m/z	Intensity	Similarity
DAETGE		C40H45IN6O17	1008.189	1009.196	46.56	98.97%
E	y1	C5H10NO4(+1)	148.061	148.0604	47.66	98.12%
GE	y2	C7H13N2O5(+1)	205.0824	205.0819	50.03	97.94%
DAE	a3	C28H27IN3O9(+1)	676.0792	676.0787	27.38	97.70%
DAE	b3	C29H27IN3O10(+1)	704.0741	704.0736	79.24	97.57%
DAET	b4	C33H34IN4O12(+1)	805.1218	805.1212	30.31	97.56%
DAETG	a5	C34H37IN5O12(+1)	834.1483	834.1478	2.4	97.54%
DAET	a4	C32H34IN4O11(+1)	777.1269	777.1263	13.42	97.48%
DAETG	b5	C35H37IN5O13(+1)	862.1433	862.1427	12.29	97.44%
D	b1	C21H15INO6(+1)	503.9944	503.9939	36.43	97.30%
DA	a2	C23H20IN2O6(+1)	547.0366	547.0361	15.27	96.29%
TGE	y3	C11H20N3O7(+1)	306.1301	306.1296	34.41	96.22%
DA	b2	C24H20IN2O7(+1)	575.0315	575.031	23.59	96.15%
D	a1	C20H15INO5(+1)	475.9995	475.9989	9.21	96.14%
AETGE	y5	C19H32N5O11(+1)	506.2098	506.2093	1.11	88.87%
ETGE	y4	C16H27N4O10(+1)	435.1727	435.1722	2	88.36%

Peptide EBX of GDAETGE (3af)



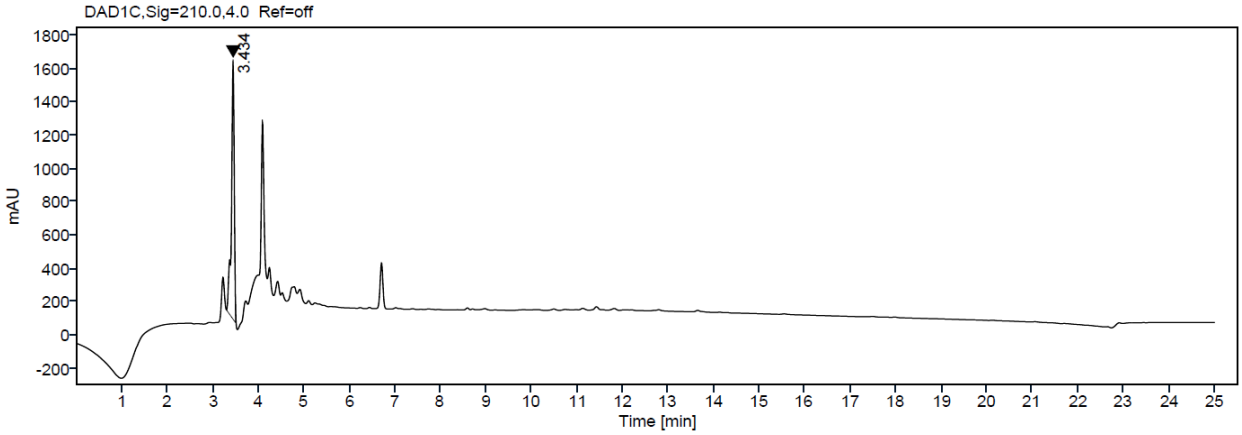
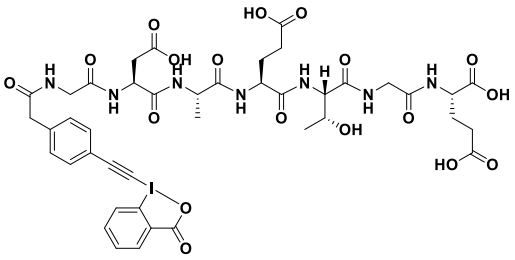


Figure S54: HPLC-UV chromatogram (210 nm) of GDAETGE by **Method 1**.
HRMS (ESI/QTOF) m/z: $[M + Na]^+$ Calcd for $C_{25}H_{39}N_7NaO_{15}^+$ 700.2396; Found 700.2392.



Following the general procedure, the reaction was conducted on a 0.032 mmol scale. The desired product **3af** (14.6 mg, 0.014 mmol, 42% yield) was isolated by **Method 6**.

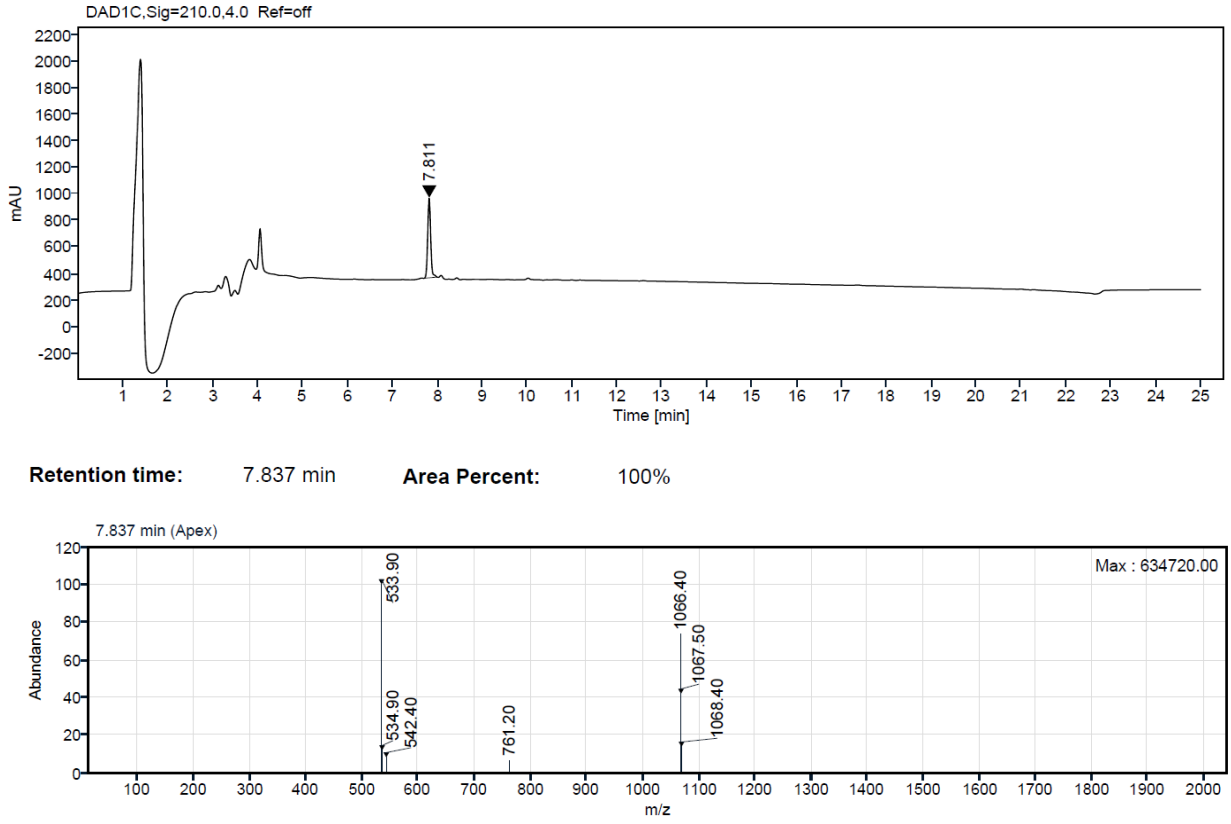
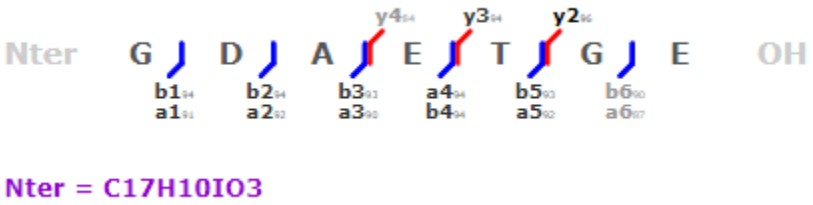


Figure S55: HPLC-UV chromatogram (210 nm) and MS(ESI) of **3af** by **Method 1**.

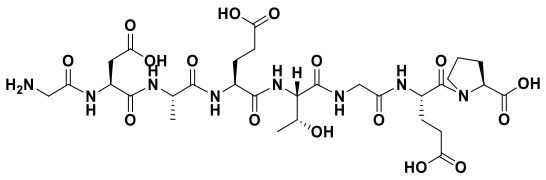
HRMS (Nanochip-based ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{42}H_{49}IN_7O_{18}^+$ 1066.2173; Found 1066.2175.

Table S27:MS/MS fragmentation of **3af**:



Sequence	Type	MF	MF Mass	m/z	Intensity	Similarity
GE	y2	C7H13N2O5(+1)	205.0824	205.0819	41.97	95.81%
G	b1	C19H13INO4(+1)	445.9889	445.9884	54.58	94.14%
TGE	y3	C11H20N3O7(+1)	306.1301	306.1296	19.57	94.04%
GDAE	a4	C30H30IN4O10(+1)	733.1007	733.1001	29.73	94.00%
GD	b2	C23H18IN2O7(+1)	561.0159	561.0153	100.57	93.74%
GDAE	b4	C31H30IN4O11(+1)	761.0956	761.095	57.51	93.67%
GDA	b3	C26H23IN3O8(+1)	632.053	632.0524	35.6	92.87%
GDAET	b5	C35H37IN5O13(+1)	862.1433	862.1427	7.78	92.72%
GDAET	a5	C34H37IN5O12(+1)	834.1483	834.1478	11.1	92.21%
GDAETGE		C42H48IN7O18	1065.21	1066.217	7.22	92.01%
GD	a2	C22H18IN2O6(+1)	533.021	533.0204	3.41	91.62%
G	a1	C18H13INO3(+1)	417.994	417.9935	9.39	90.91%
GDA	a3	C25H23IN3O7(+1)	604.0581	604.0575	5.73	90.47%
GDAETG	b6	C37H40IN6O14(+1)	919.1647	919.1642	3.69	89.90%
GDAETG	a6	C36H40IN6O13(+1)	891.1698	891.1693	1.49	87.10%
ETGE	y4	C16H27N4O10(+1)	435.1727	435.1722	1.28	83.69%

Peptide EBX of GDAETGEP (3ag)



Following the general procedure, the reaction was conducted on a 0.051 mmol scale. The desired product **3ag** (25 mg, 0.021 mmol, 41% yield) was isolated by **Method 6**.

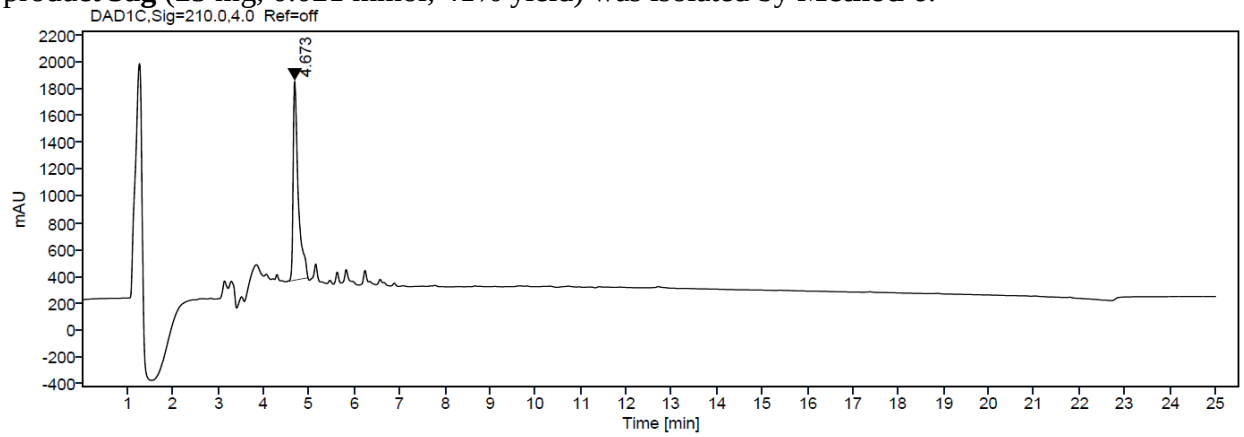


Figure S56: HPLC-UV chromatogram (210 nm) of GDAETGEP by **Method 1**.

HRMS (Nanochip-based ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{30}H_{47}N_8O_{16}^+$ 775.3105; Found 775.3095.

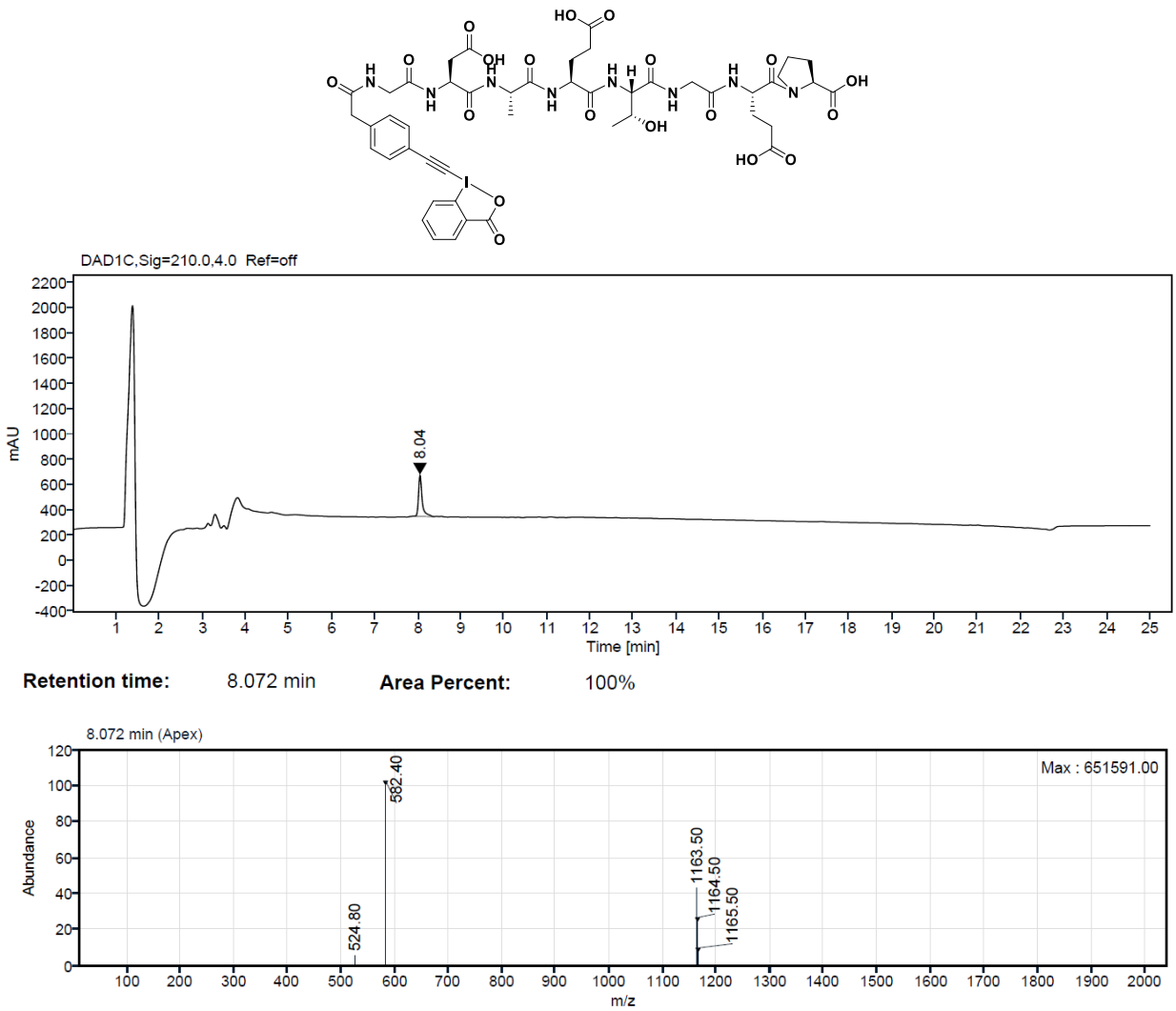
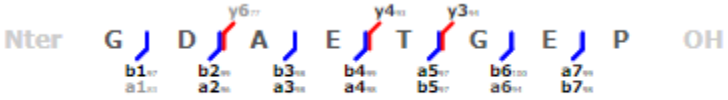


Figure S57: HPLC-UV chromatogram (210 nm) and MS(ESI) of **3ag** by **Method 1**.

HRMS (Nanochip-based ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{47}H_{56}IN_8O_{19}^+$ 1163.2701; Found 1163.2671.

Table S28: MS/MS fragmentation of **3ag**:

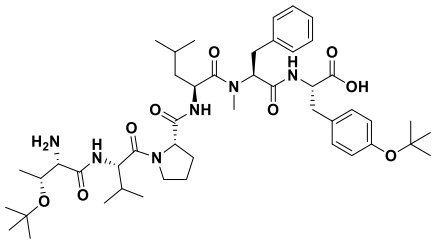


Nter = C17H10IO3

Sequence	Type	MF	MF Mass	m/z	Intensity	Similarity
GDAETG	b6	C37H40IN6O14(+1)	919.1647	919.1642	25.39	99.53%
GDAETGE	a7	C41H47IN7O16(+1)	1020.212	1020.212	49.46	99.38%
GDAE	b4	C31H30IN4O11(+1)	761.0956	761.095	67.05	99.24%
GD	b2	C23H18IN2O7(+1)	561.0159	561.0153	73.76	98.54%
GDAETGE	b7	C42H47IN7O17(+1)	1048.207	1048.207	35.28	98.34%

GDA	b3	C26H23IN3O8(+1)	632.053	632.0524	28.88	98.18%
GDAE	a4	C30H30IN4O10(+1)	733.1007	733.1001	20.64	97.80%
GDA	a3	C25H23IN3O7(+1)	604.0581	604.0575	7.5	97.74%
G	b1	C19H13INO4(+1)	445.9889	445.9884	22.97	97.37%
GDAET	a5	C34H37IN5O12(+1)	834.1483	834.1478	13.49	96.96%
GDAET	b5	C35H37IN5O13(+1)	862.1433	862.1427	23.56	96.93%
GD	a2	C22H18IN2O6(+1)	533.021	533.0204	2.87	95.65%
GDAETG	a6	C36H40IN6O13(+1)	891.1698	891.1693	7.13	94.10%
GEP	y3	C12H20N3O6(+1)	302.1352	302.1347	5.66	93.78%
TGEP	y4	C16H27N4O8(+1)	403.1829	403.1823	0.87	93.12%
G	a1	C18H13INO3(+1)	417.994	417.9935	2.17	83.27%
AETGEP	y6	C24H39N6O12(+1)	603.2626	302.1347	5.66	77.48%

Peptide EBX of TVPLFY (3ah)



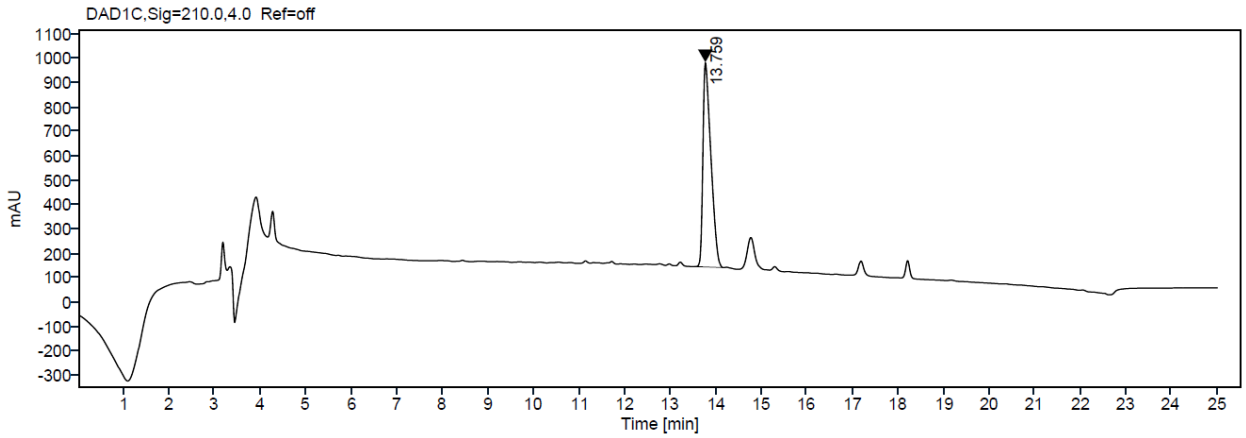
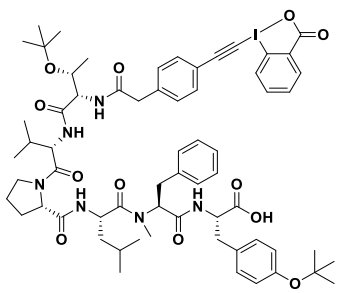


Figure S58: HPLC-UV chromatogram (210 nm) of TVPLFY by **Method 1**.

HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{47}H_{73}N_6O_9^+$ 865.5434; Found 865.5427.



Following the general procedure, the reaction was conducted in 0.025 mmol scale. The desired product **3ah** (12 mg, 0.0092 mmol, 37% yield) was isolated by **Method 6**.

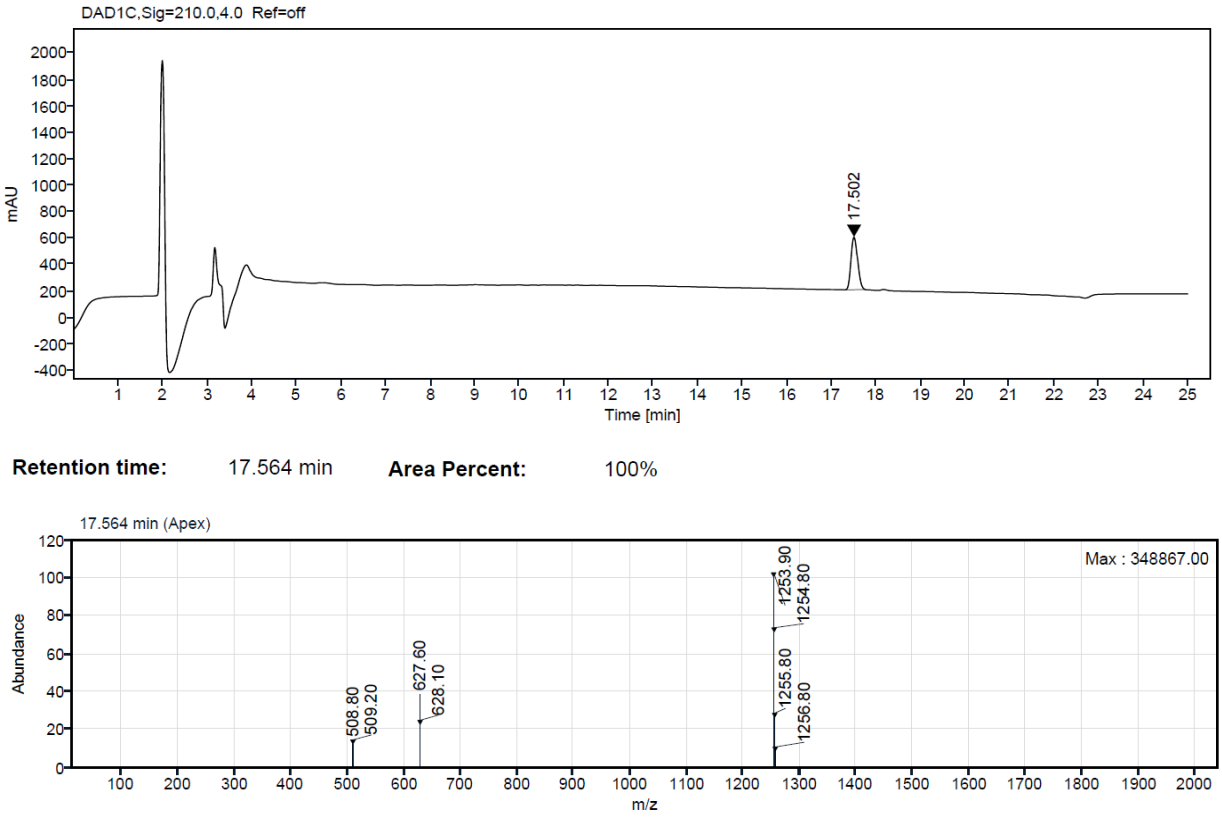
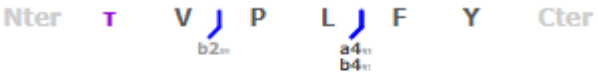


Figure S59: HPLC-UV chromatogram (210 nm) and MS(ESI) of **3ah** by **Method 1**.

^{1}H NMR (600 MHz, CD_3OD) δ 8.40 (dd, *J* = 8.3, 2.6 Hz, 1H), 8.29 (dd, *J* = 7.5, 1.7 Hz, 1H), 7.84 (t, *J* = 7.4 Hz, 2H), 7.67 (dd, *J* = 8.0, 5.5 Hz, 2H), 7.49 – 7.45 (m, 2H), 7.31 (t, *J* = 7.5 Hz, 2H), 7.25 (td, *J* = 7.2, 1.6 Hz, 1H), 7.22 – 7.08 (m, 4H), 6.92 (dd, *J* = 12.4, 8.4 Hz, 2H), 4.74 (t, *J* = 8.0 Hz, 1H), 4.61 (ddd, *J* = 11.9, 8.3, 3.2 Hz, 1H), 4.54 (ddt, *J* = 10.4, 4.7, 2.2 Hz, 1H), 4.50 – 4.41 (m, 3H), 4.08 (dtd, *J* = 9.4, 6.4, 3.3 Hz, 1H), 3.95 – 3.86 (m, 1H), 3.75 (t, *J* = 3.8 Hz, 2H), 3.65 (dt, *J* = 9.8, 6.6 Hz, 1H), 3.27 (ddd, *J* = 14.2, 9.6, 4.5 Hz, 1H), 3.23 – 3.17 (m, 1H), 3.06 – 2.98 (m, 1H), 2.94 – 2.88 (m, 1H), 2.46 (s, 3H), 2.16 (ddd, *J* = 14.8, 8.1, 4.2 Hz, 1H), 2.05 (dtt, *J* = 15.5, 9.3, 5.2 Hz, 4H), 1.92 (td, *J* = 11.6, 10.8, 5.4 Hz, 1H), 1.80 (dq, *J* = 14.0, 7.5, 7.0 Hz, 1H), 1.69 – 1.61 (m, 1H), 1.33 (d, *J* = 8.5 Hz, 9H), 1.19 (d, *J* = 10.5 Hz, 9H), 1.10 (t, *J* = 7.1 Hz, 3H), 1.03 (d, *J* = 6.4 Hz, 3H), 0.99 (d, *J* = 6.8 Hz, 3H), 0.73 (d, *J* = 6.5 Hz, 3H), 0.70 (d, *J* = 6.6 Hz, 3H).

^{13}C NMR (151 MHz, CD_3OD) δ 175.8, 175.0, 174.5, 173.2, 172.0, 171.7, 170.3, 155.4, 140.1, 139.3, 136.5, 134.5, 134.1, 134.1, 133.0, 132.7, 132.6, 131.0, 130.9, 130.8, 130.7, 130.1, 129.5, 128.8, 128.1, 125.4, 120.5, 117.1, 107.5, 79.5, 75.8, 68.8, 63.9, 61.1, 60.6, 59.3, 57.7, 56.2, 43.4, 39.1, 36.8, 34.6, 32.2, 30.2, 29.2, 29.2, 28.7, 28.7, 26.0, 25.2, 23.6, 20.6, 20.1, 19.8, 18.9.
HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{64}H_{82}IN_6O_{12}^+$ 1253.5030; Found 1253.5004.

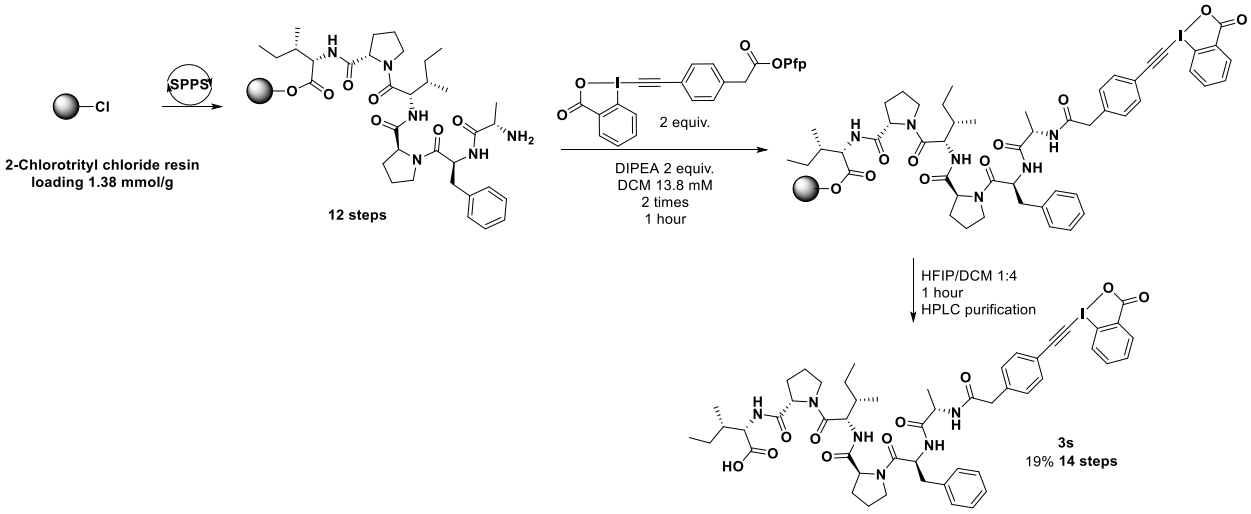
Table S29: MS/MS fragmentation of **3ah**:



T = Thr(C4H8)
Nter = C17H10IO3
Cter = C4H8

Sequence	Type	MF	MF Mass	m/z	Intensity	Similarity
TVPL	a4	C40H52IN4O7(+1)	827.2881	827.2875	0.95	92.62%
TVPL	b4	C41H52IN4O8(+1)	855.283	855.2824	10.75	90.94%
TV	b2	C30H34IN2O6(+1)	645.1462	645.1456	1.43	89.38%

5. Synthesis of peptide-EBXs on solid phase



Loading efficiency evaluation:[5]

The loading efficiency was evaluated through treatment of the resin with 20% piperidine/DMF (3 mL, 2 × 3 min) to deprotect the Fmoc group. The combined deprotection solutions were diluted to 10 mL with 20% piperidine/DMF. An aliquot of this mixture (50 μL) was diluted 200-fold with 20% piperidine/DMF and the UV absorbance of the piperidine-fulvene adduct was measured (λ = 301 nm, ε = 7800 M^{-1} cm^{-1}) to quantify the amount of amino acid loaded onto the resin. The theoretical maximum for the reported yields of all isolated peptides is based on the numerical value obtained from the resin loading (1.38 mmol/g for 2-chlorotrityl chloride resin, 0.34 mmol/g for rink amide resin).

Procedure for EBXs introductions:

[5] Lin, Y. & Malins, L. R. An Electrochemical Approach to Designer Peptide α-Amides Inspired by α-Amidating Monooxygenase Enzymes. *J. Am. Chem. Soc.* **2021**, *143*, 11811-11819,.

Peptide-EBX **3s** was prepared on a 28 μmol scale from resin-bound substrate. Bifunctional EBX (32 mg, 55 μmol, 2.0 equiv.) was weighed into the syringe reactor and dissolved in 2 mL DCM. Then DIPEA (19.0 μL, 110 μmol, 4.0 equiv.) was added into the reactor and the mixture was agitated at room temperature for 1 hour. The resin was then filtered and washed with DCM (3 × 3 mL). The procedure was repeated one more time to ensure the *N*-terminus was fully reacted. Following HFIP/DCM cleavage from resin and removal of volatiles, the crude peptide was purified by reverse-phase HPLC with **Method 5** and lyophilized to afford peptide **3s** as a fluffy white solid (5.5 mg, 19% yield based on the original resin loading).

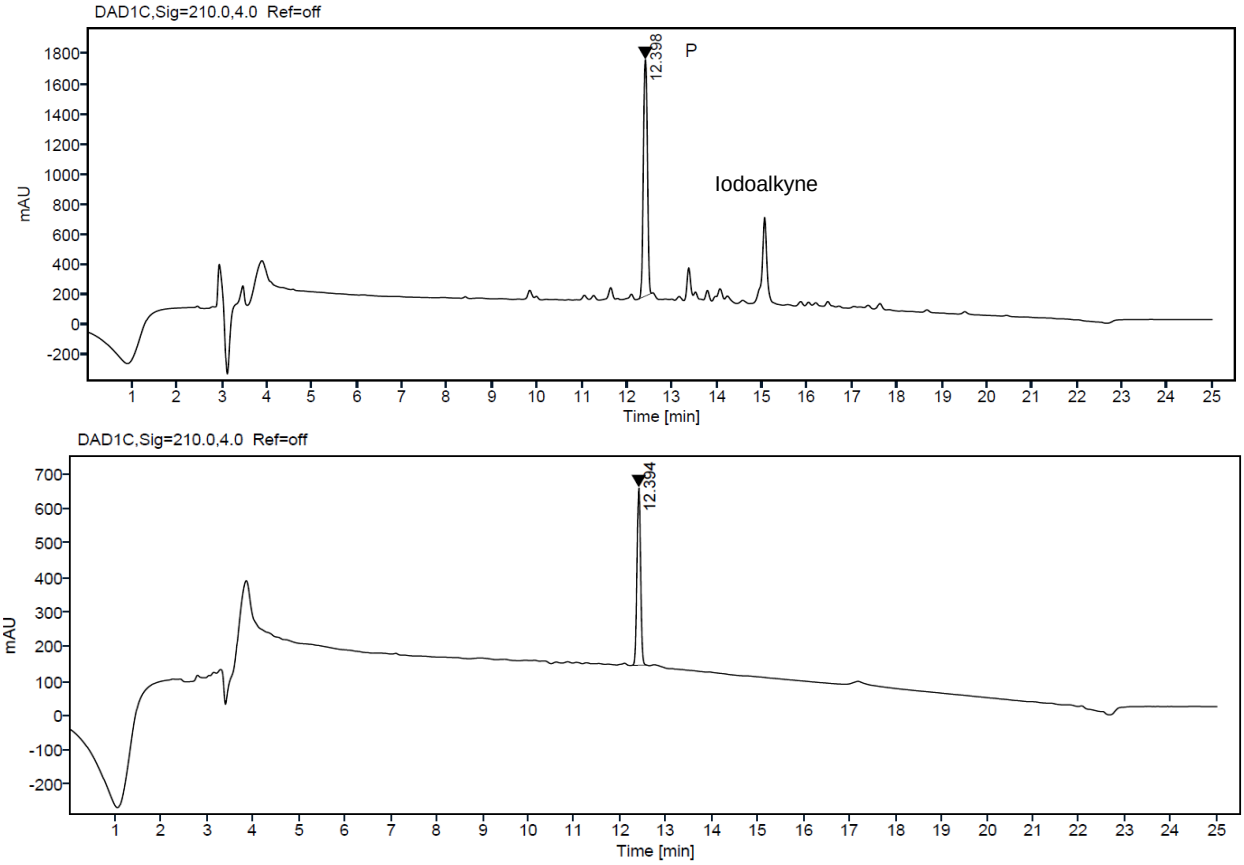
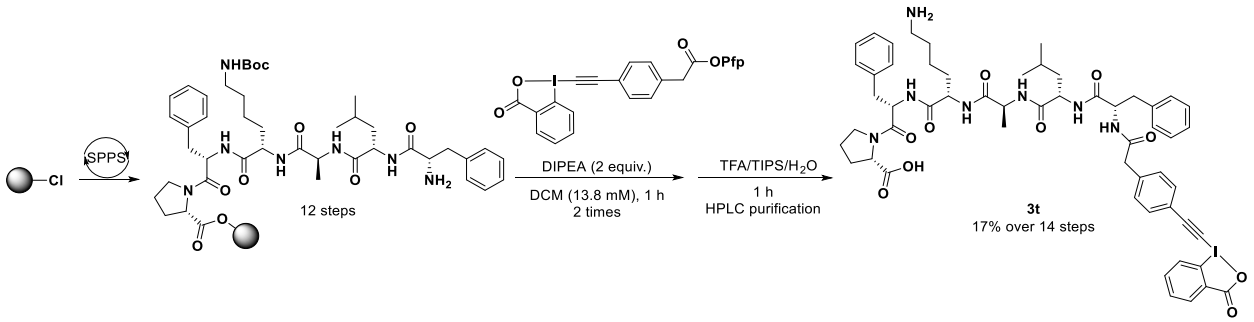


Figure S60: HPLC-UV chromatogram (210 nm) of the crude and purified product **3s** by **Method 1**.



Peptide-EBX **3t** was prepared on a 42 μmol scale from resin-bound substrate. Bifunctional EBX (49 mg, 84 μmol, 2.0 equiv.) was weighed into the syringe reactor and dissolved in 2 mL DCM. Then DIPEA (30.0 μL, 168 μmol, 4.0 equiv.) was added into the reactor and the mixture was agitated at room temperature for 1 hour. The resin was then filtered and washed with DCM (3 × 3

mL). The procedure was repeated one more time to ensure the *N*-terminus was fully reacted. Following HFIP/DCM cleavage from resin and removal of volatiles, the crude peptide was purified by reverse-phase HPLC with **Method 5** and lyophilized to afford peptide **3t** as a fluffy white solid (8.1 mg, 17% yield based on the original resin loading).

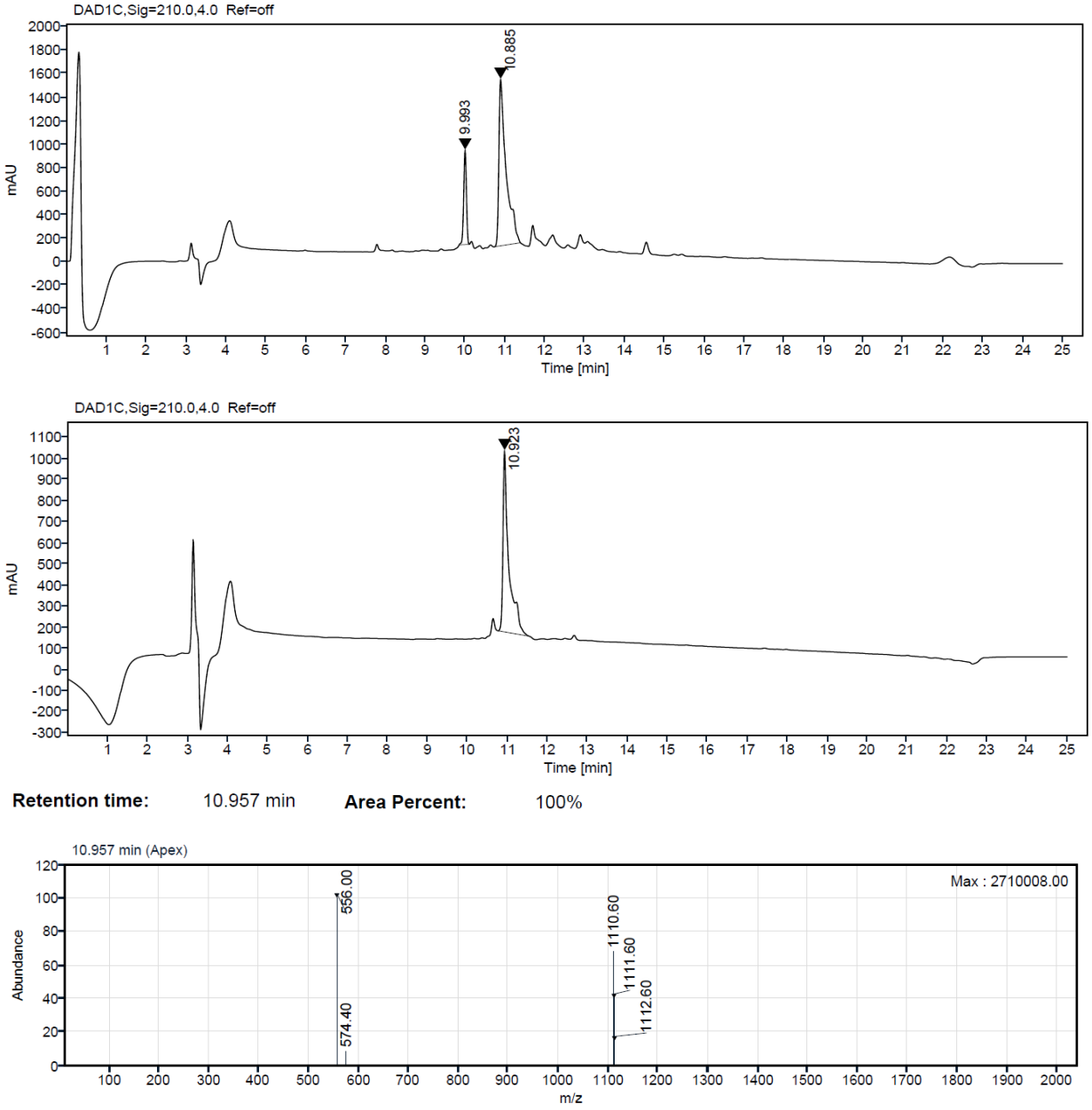


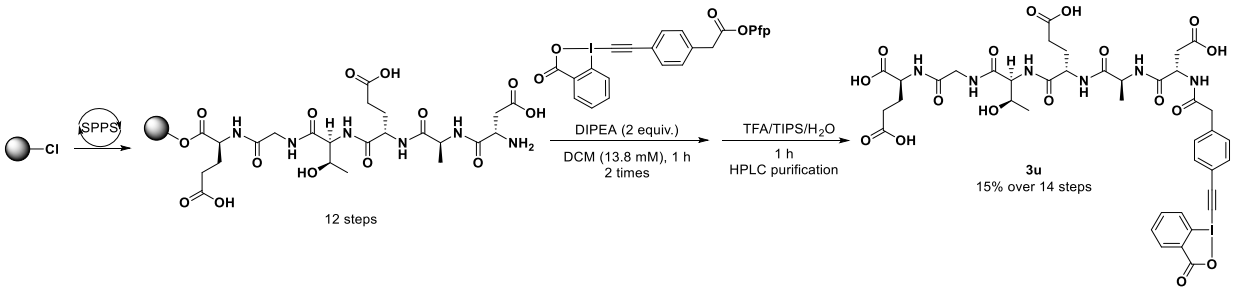
Figure S61: HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude and purified product **3t** by **Method 1**.

HRMS (Nanochip-based ESI/LTQ-Orbitrap) m/z: $[M + H_2]^{+2}$ Calcd for $C_{55}H_{66}IN_7O_{10}^{+2}$ 555.6952; Found 555.6949.

Table S30: MS/MS fragment of **3t:**

Nter = C17H10IO3

Sequence	Type	MF	MF Mass	m/z	Intensity	Similarity
P	y1	C5H10NO2(+1)	116.0712	116.0706	46.94	98.61%
FLA	b3	C35H35IN3O6(+1)	720.1571	720.1565	72.56	98.40%
FLAKF	a5	C49H56IN6O7(+1)	967.3255	484.1661	21.69	98.36%
FLAKF	b5	C50H56IN6O8(+1)	995.3204	498.1636	59.86	98.36%
FL	b2	C32H30IN2O5(+1)	649.1199	649.1194	27.51	97.73%
FP	y2	C14H19N2O3(+1)	263.1396	263.139	37.79	97.70%
FLAK	b4	C41H47IN5O7(+1)	848.252	848.2515	7.95	97.42%
FLAKFP		C55H64IN7O10	1109.3759	555.6952	2.06	97.17%
F	b1	C26H19INO4(+1)	536.0359	536.0353	8.85	96.16%
FLAK	b4	C41H47IN5O7(+1)	848.252	424.6294	2.12	95.56%
FLA	a3	C34H35IN3O5(+1)	692.1621	692.1616	4.03	94.84%
F	a1	C25H19INO3(+1)	508.041	508.0404	4.16	94.54%
FL	a2	C31H30IN2O4(+1)	621.125	621.1245	3.75	93.88%
KFP	y3	C20H31N4O4(+1)	391.2345	391.234	0.87	89.50%



Peptide-EBX **3u** was prepared on a 28 μmol scale from resin-bound substrate. Bifunctional EBX (32 mg, 56 μmol, 2.0 equiv.) was weighed into the syringe reactor and dissolved in 2 mL DCM. Then DIPEA (19.0 μL, 112 μmol, 4.0 equiv.) was added into the reactor and the mixture was agitated at room temperature for 1 hour. The resin was then filtered and washed with DCM (3 × 3 mL). The procedure was repeated one more time to ensure the *N*-terminus was fully reacted. Following HFIP/DCM cleavage from resin and removal of volatiles, the crude peptide was purified by reverse-phase HPLC with **Method 5** and lyophilized to afford peptide **3u** as a fluffy white solid (4.5 mg, 15% yield based on the original resin loading).

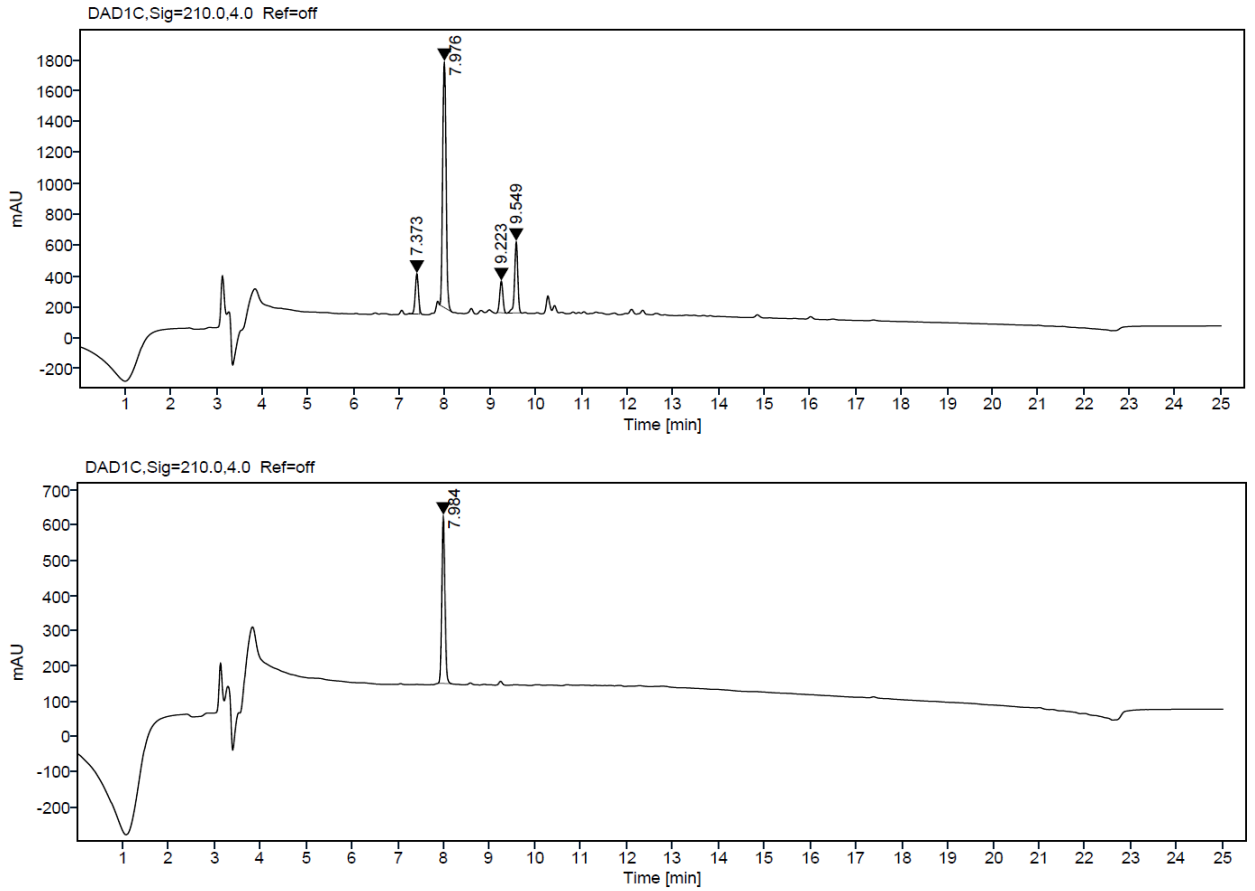
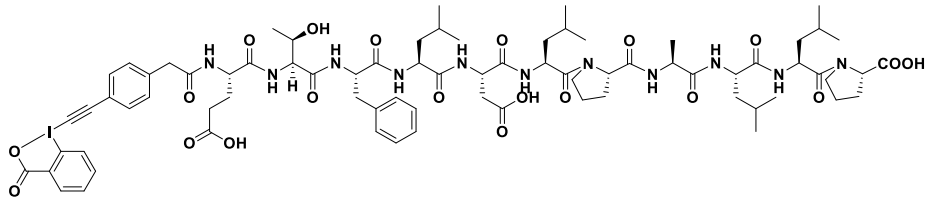


Figure S63: HPLC-UV chromatogram (210 nm) of the crude and purified product **3u** by **Method 1**.

Peptide EBX of ETFLDLPALLP (3ad)



Peptide-EBX **3ad** was prepared on a 50 μmol scale from resin-bound substrate. Bifunctional EBX (57.3 mg, 100 μmol, 2.0 equiv.) was weighed into the syringe reactor and dissolved in 2 mL DCM. Then DIPEA (35.7 μL, 200 μmol, 4.0 equiv.) was added into the reactor and the mixture was agitated at room temperature for 1 hour. The resin was then filtered and washed with DCM (3 × 3 mL). The procedure was repeated one more time to ensure the *N*-terminus was fully reacted. Following TFA cleavage from resin and removal of volatiles, the crude peptide was purified by reverse-phase HPLC with **Method 5** and lyophilized to afford peptide **3ad** as a fluffy white solid (14.5 mg, 18% yield based on the original resin loading).

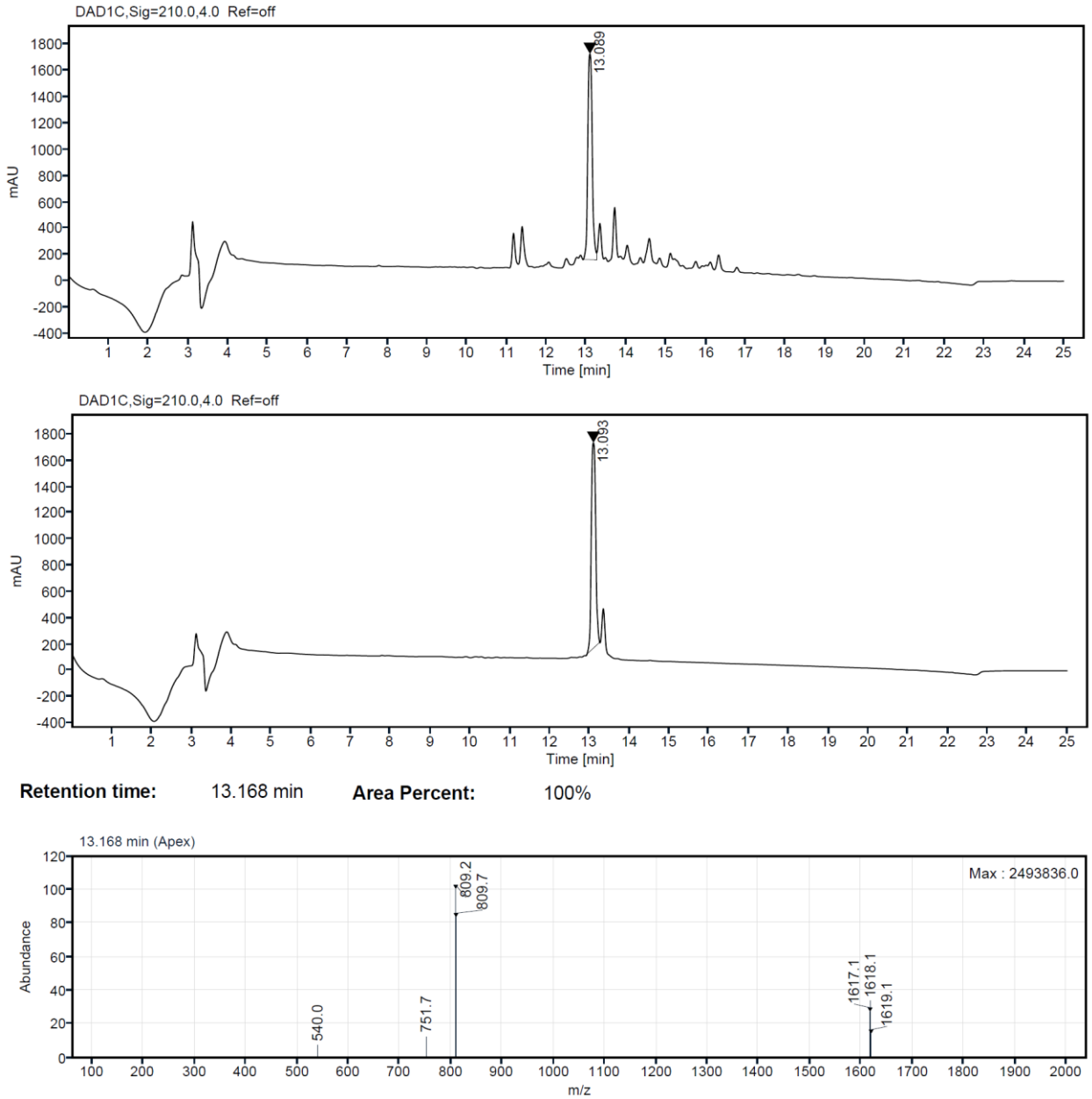
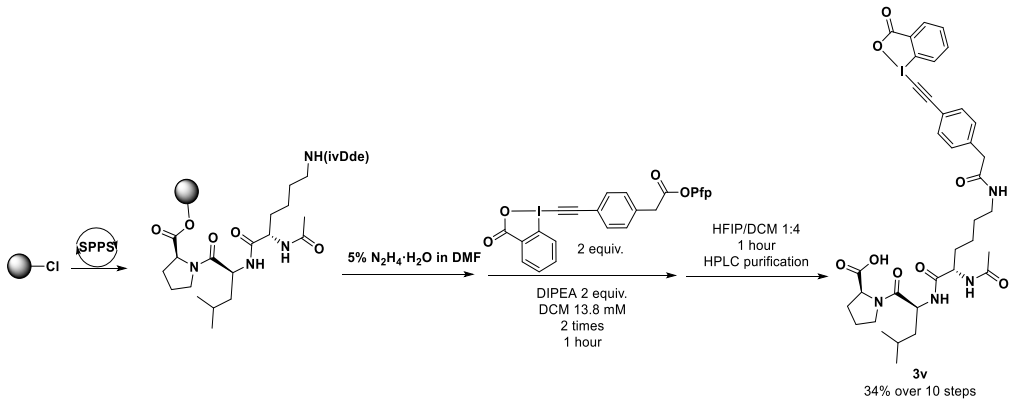


Figure S64: HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude and purified product **3ad** by **Method 1**.



Peptide-EBX **3v** was prepared on a 14 μmol scale from resin-bound substrate. Then the resin was treated with 1 mL 5% N_2H_4 H_2O in DMF and agitated for 10 min (2 times) to deprotect the Lys. Bifunctional EBX (16 mg, 28 μmol, 2.0 equiv.) was then weighed into the syringe reactor and dissolved in 2 mL DCM. Then DIPEA (9.5 μL, 56 μmol, 4 equiv.) was added into the reactor and agitated at room temperature for 1 hour. The resin was then filtered and washed with DCM (3 × 3 mL). The procedure was repeated one more time to ensure the *N*-terminus was fully reacted. Following HFIP/DCM cleavage from resin and removal of volatiles, the crude peptide was purified by reverse-phase HPLC with **Method 5** and lyophilized to afford peptide **3v** as a fluffy white solid (3.6 mg, 4.6 μmol, 34% yield based on the original resin loading).

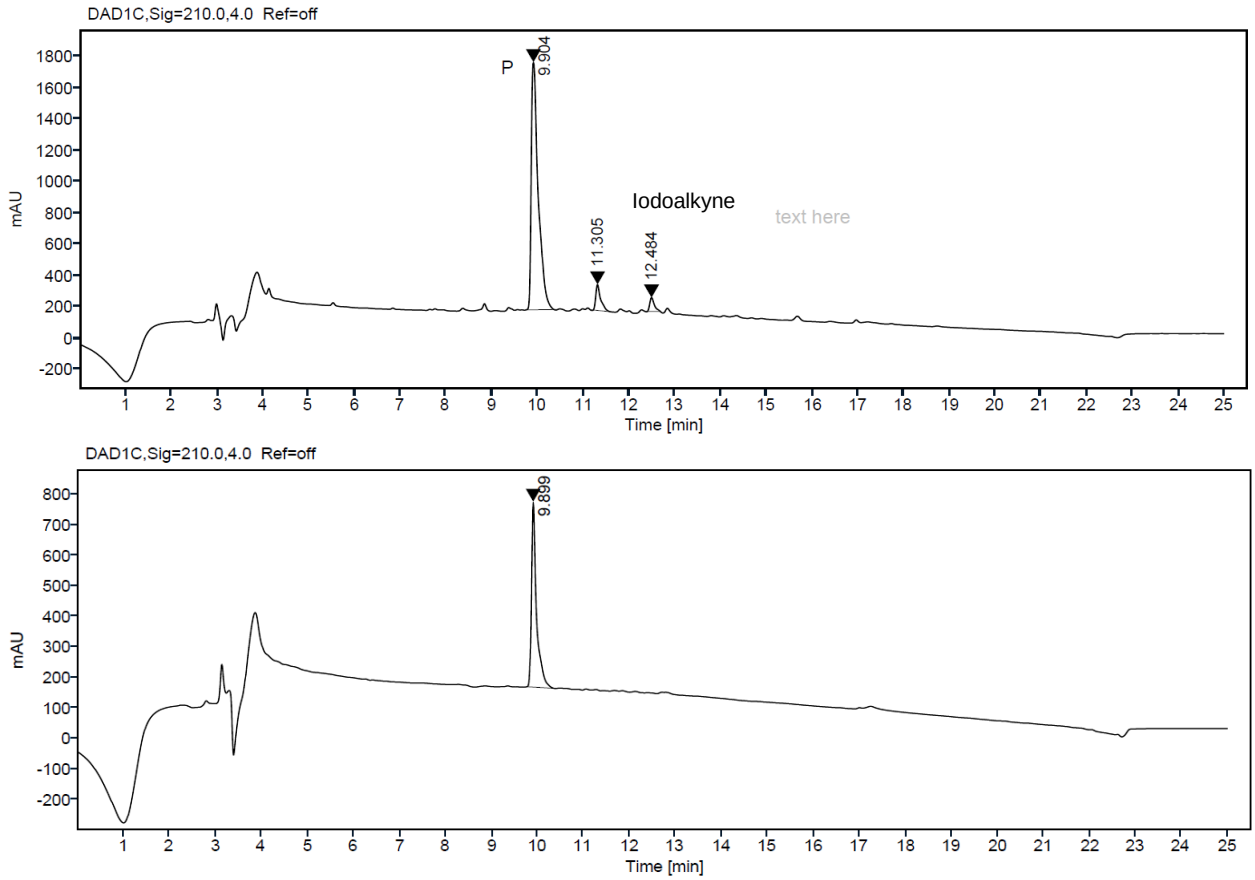
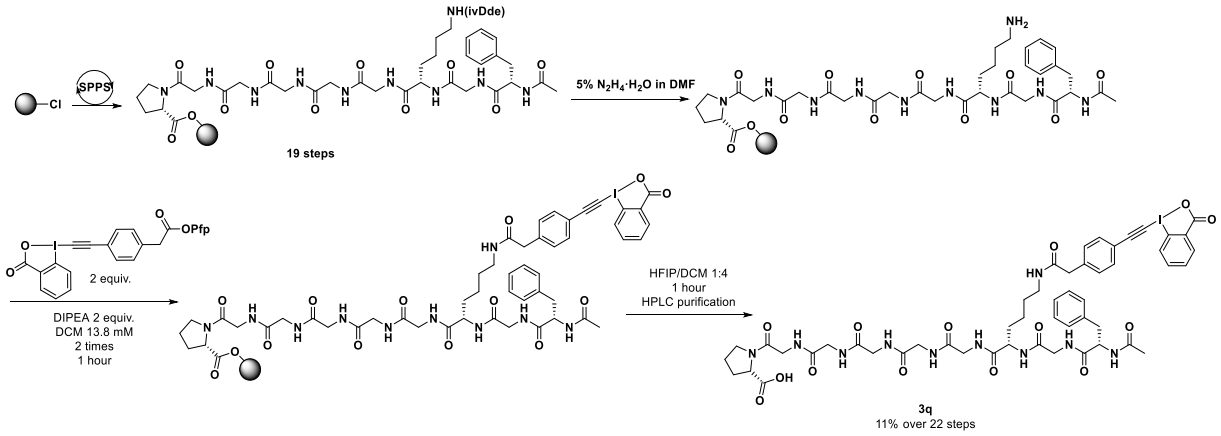
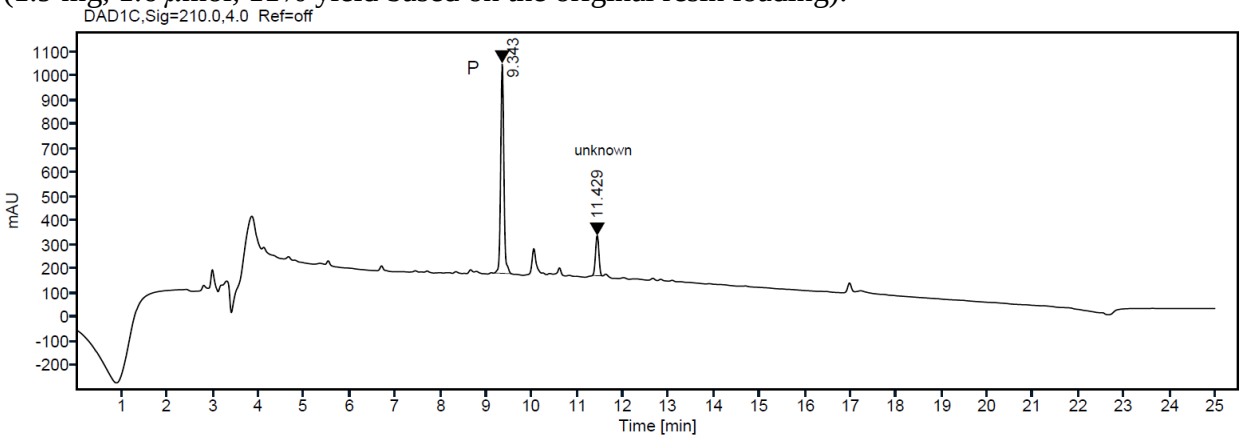


Figure S65: HPLC-UV chromatogram (210 nm) of the crude and purified product **3v** by **Method 1**.



Procedure:

Peptide-EBX **3q** was prepared on a 14 μmol scale from resin-bound substrate. Bifunctional EBX (156 mg, 28 μmol, 2.0 equiv.) was weighed into the syringe reactor and dissolved in 2 mL DCM. Then DIPEA (9.5 μL, 56 μmol, 4.0 equiv.) was added into the reactor and the mixture was agitated at room temperature for 1 hour. Then the resin was treated with 1 mL 5% $N_2H_4\,H_2O$ in DMF and agitated for 10 min (2 times) to deprotect the Lys. The resin was then filtered and washed with DCM (3 × 3 mL). The procedure was repeated one more time to ensure the Lys was fully reacted. Following HFIP/DCM cleavage from resin and removal of volatiles, the crude peptide was purified by reverse-phase HPLC with **Method 5** and lyophilized to afford peptide **3q** as a fluffy white solid (1.9 mg, 1.6 μmol, 11% yield based on the original resin loading).



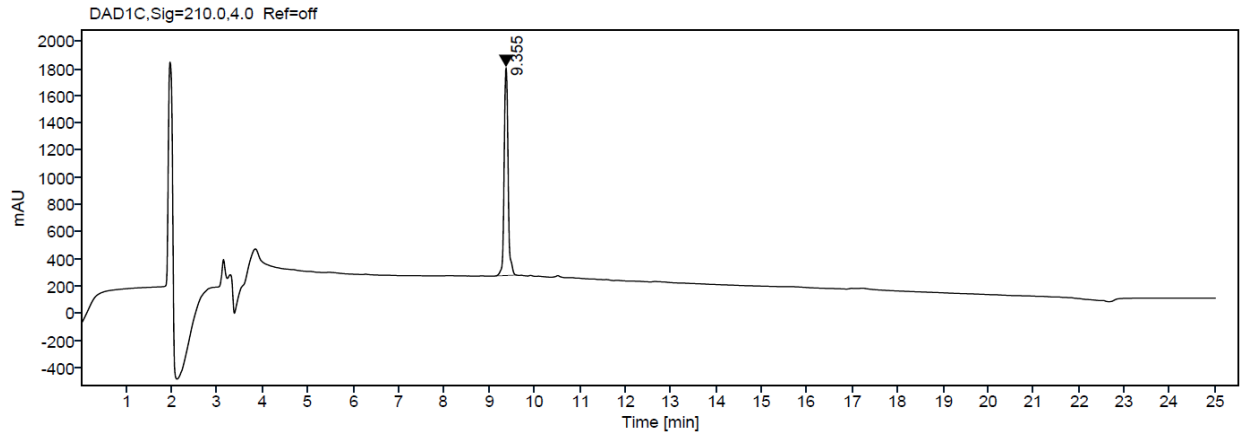


Figure S66: HPLC-UV chromatogram (210 nm) of the crude and purified product **3q** by **Method 1**.

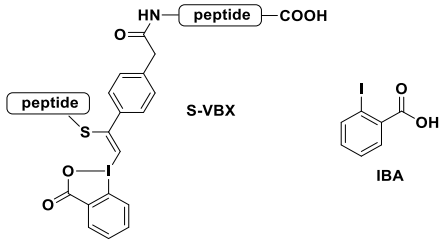
6. General procedure for S-alkynylation of peptide-EBXs:

Peptide-EBX (5 μmol, 1 equiv.) was weighed on the analytical balance and dissolved in 200 µL non-degassed DMA. Afterwards, 55 μL of a solution of Cys containing peptides (100 mM in DMF, for Glutathione 100 mM in DMF/H_2O 1:1) (5.5 μmol, 1.1 equiv.), 10 μL DIPEA (100 mM in DMF, 10 μmol, 2 equiv.) were added. The reaction was stirred for 1 h at RT without protecting from atmosphere and light.

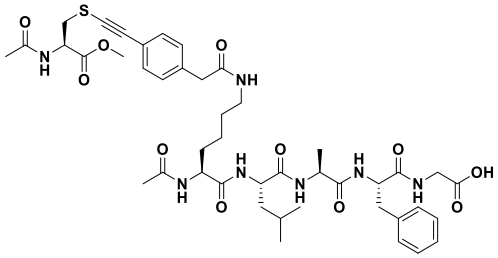
At the end of the reaction, 10 μL of the crude was diluted with 90 μL of MeCN/water 1:1 and injected in RP-HPLC. The desired products were isolated by Prep-RP-HPLC.

HPLC-UV ratio was determined by taking the ration of A_{prod}/A_{total} where A_{prod} = area in mAU of the product peak and A_{total} = area in mAU of the combined peptide containing species

Common side-products that can be observed in HPLC:



S-alkynylation product of AcKLAFG (5a)



Following the general procedure, the reaction was conducted in 5 μmol scale. HPLC ratio (210 nm) of the product: 84%. The desired product **5a** (3.3 mg, 3.7 μmol, 75% yield) was isolated by **Method 6**.

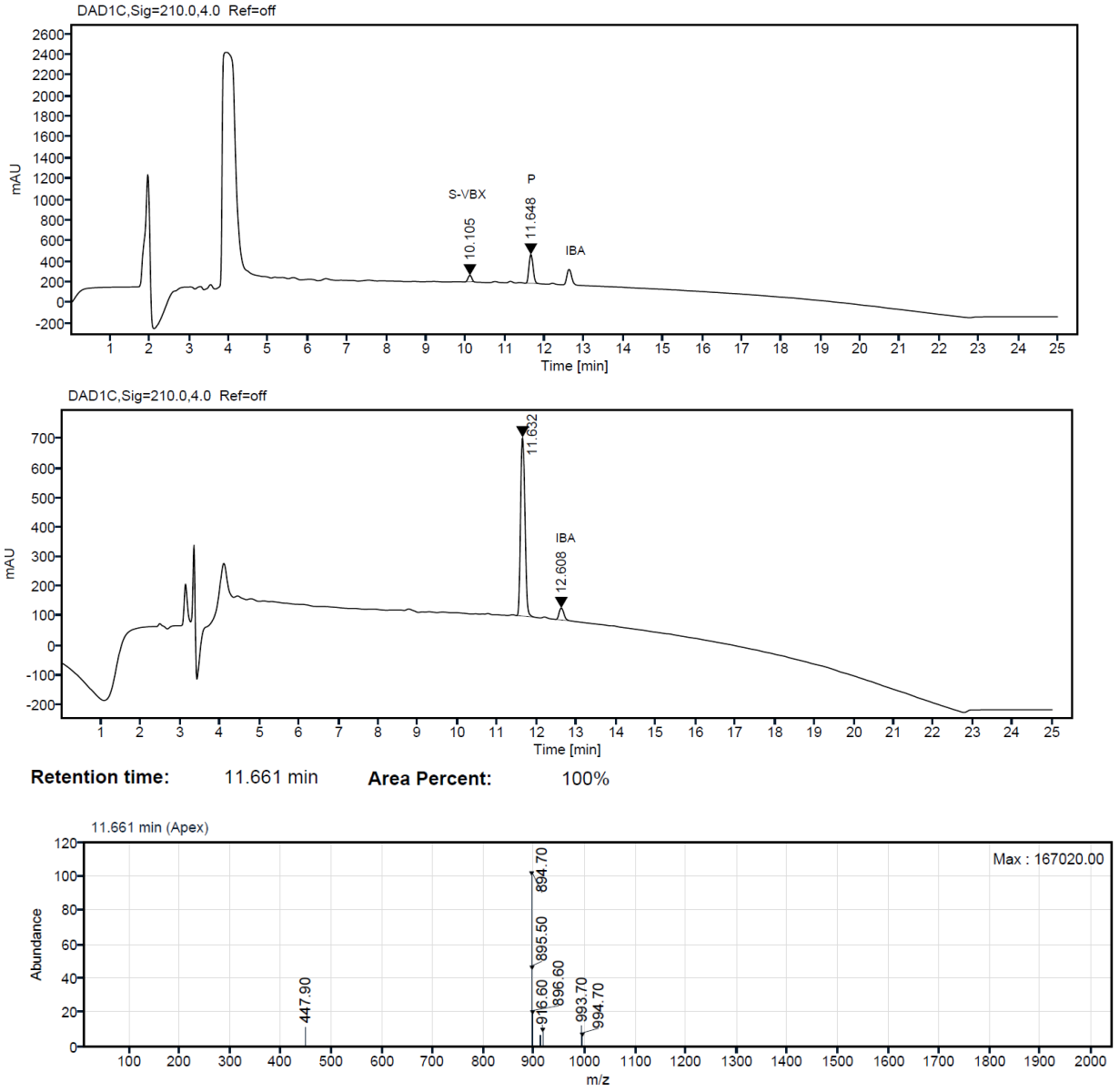


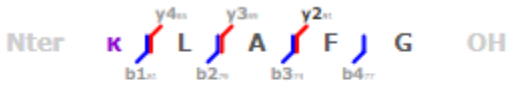
Figure S67: HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude and purified product **5a** by **Method 1**.

^{1}H NMR (800 MHz, CD_3OD) δ 7.41 – 7.37 (m, 2H), 7.29 – 7.26 (m, 6H), 7.20 (ddd, *J* = 8.5, 5.6, 2.0 Hz, 1H), 4.83 (d, *J* = 5.0 Hz, 1H), 4.66 – 4.61 (m, 1H), 4.38 (dt, *J* = 10.6, 5.6 Hz, 1H), 4.27 – 4.19 (m, 2H), 3.94 – 3.87 (m, 2H), 3.75 (d, *J* = 1.4 Hz, 3H), 3.51 (d, *J* = 6.0 Hz, 3H), 3.23 (d, *J* = 9.2 Hz, 1H), 3.20 (d, *J* = 5.9 Hz, 1H), 3.16 – 3.12 (m, 1H), 2.97 (dd, *J* = 14.0, 9.1 Hz, 1H), 2.02 (d, *J* = 1.4 Hz, 3H), 2.00 (d, *J* = 1.4 Hz, 3H), 1.79 (ddt, *J* = 15.7, 11.4, 5.9 Hz, 1H), 1.71 (m, 1H), 1.69 – 1.64 (m, 1H), 1.61 (dd, *J* = 10.4, 4.8 Hz, 1H), 1.58 (dd, *J* = 9.4, 4.7 Hz, 1H), 1.54 (m, 2H), 1.45 – 1.36 (m, 2H), 1.26 (d, *J* = 7.2 Hz, 3H), 1.21 (dd, *J* = 6.9, 1.4 Hz, 1H), 0.98 (dd, *J* = 6.6, 1.3 Hz, 3H), 0.94 – 0.92 (m, 3H).

^{13}C NMR (201 MHz, CD_3OD) δ 174.9, 174.8, 174.7, 173.7, 173.6, 173.5, 173.4, 172.8, 172.0, 138.5, 137.8, 132.7, 130.4, 130.3, 129.4, 127.7, 122.9, 94.2, 78.9, 66.9, 55.8, 55.3, 53.6, 53.3, 53.1, 50.9, 43.7, 42.0, 41.3, 40.3, 38.6, 37.7, 32.4, 30.0, 25.9, 24.2, 23.6, 22.4, 22.4, 21.8, 17.9, 15.4.

HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H_2]^{+2}$ Calcd for $C_{44}H_{61}N_7O_{11}S^{+2}$ 447.7069; Found 447.7063.

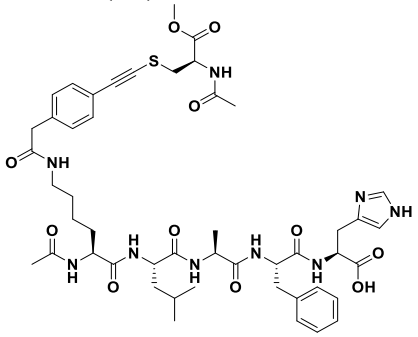
Table S31: MS/MS fragmentation of **5a**:



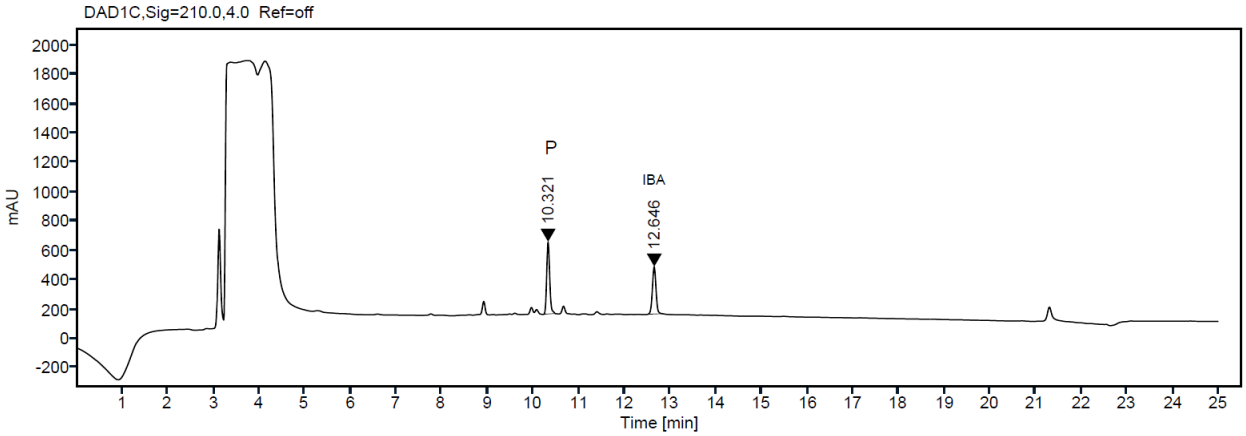
κ = Lys(C16H15NO4S)
Nter = C2H3O

Sequence	Type	MF	MF Mass	m/z	Intensity	Similarity
FG	y2	C11H15N2O3(+1)	223.1083	223.1077	21.66	91.28%
AFG	y3	C14H20N3O4(+1)	294.1454	294.1448	3.3	88.51%
LAFG	y4	C20H31N4O5(+1)	407.2294	407.2289	5.93	85.20%
K	b1	C24H30N3O6S(+1)	488.1855	488.185	3.84	81.22%
KL	b2	C30H41N4O7S(+1)	601.2696	601.269	2.59	79.08%
KLA	b3	C33H46N5O8S(+1)	672.3067	672.3062	23.13	77.19%
KLAF	b4	C42H55N6O9S(+1)	819.3751	410.1909	101.81	76.68%
KLA	b3	C33H46N5O8S(+1)	672.3067	336.6567	1.42	71.05%

S-alkynylation product of AcKLAFH (5b)



Following the general procedure, the reaction was conducted in 2 μmol scale. HPLC ratio (210 nm) of the product: > 95%. The desired product **5b** (1.2 mg, 1.3 μmol, 64% yield) was isolated by **Method 5**.



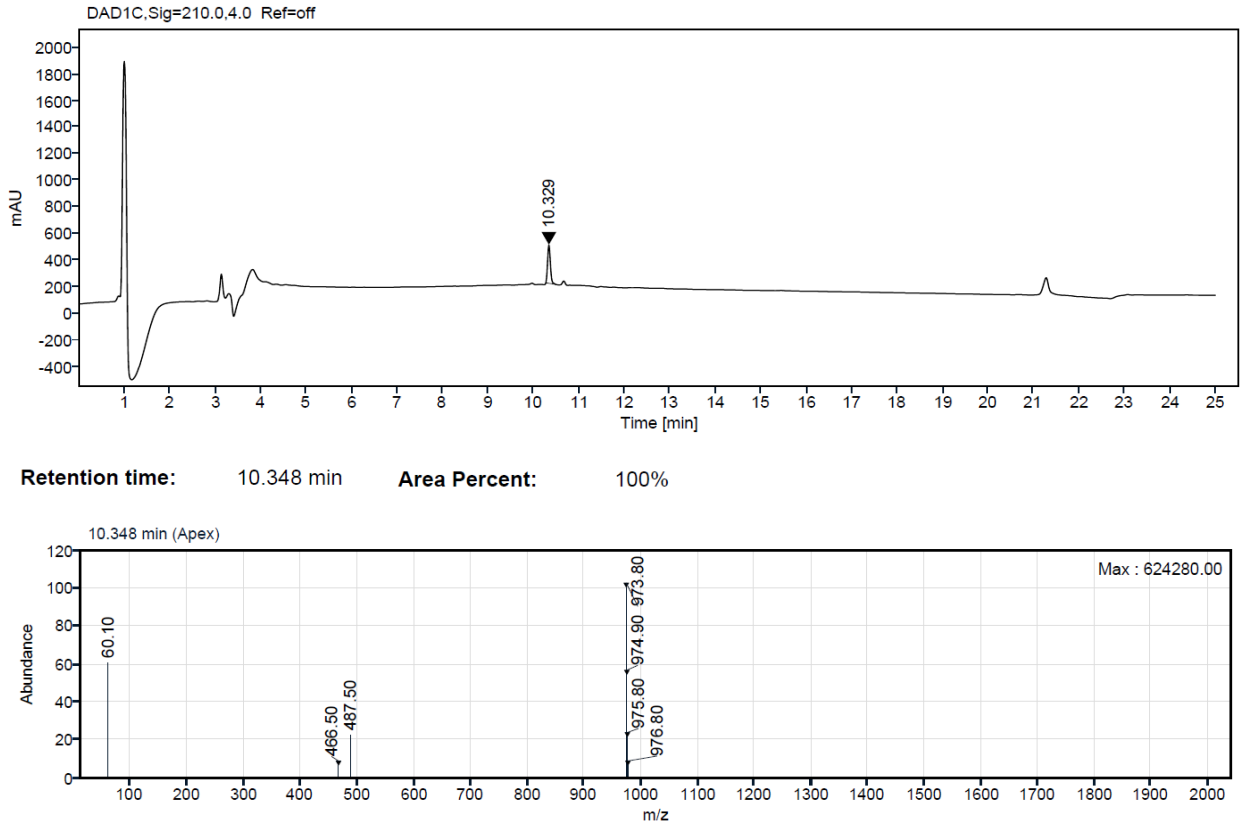
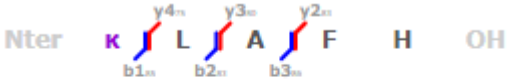


Figure S68: HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude and purified product **5b** by **Method 1**.

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{48}H_{64}N_9O_{11}S^+$ 974.4441; Found 974.4452.

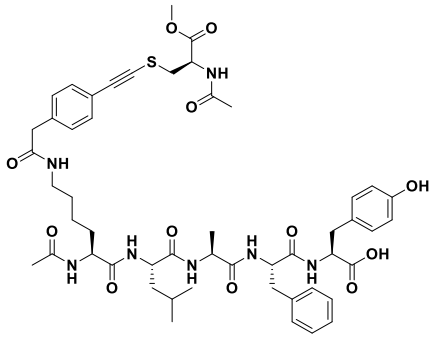
Table S32: MS/MS fragmentation of **5b**:



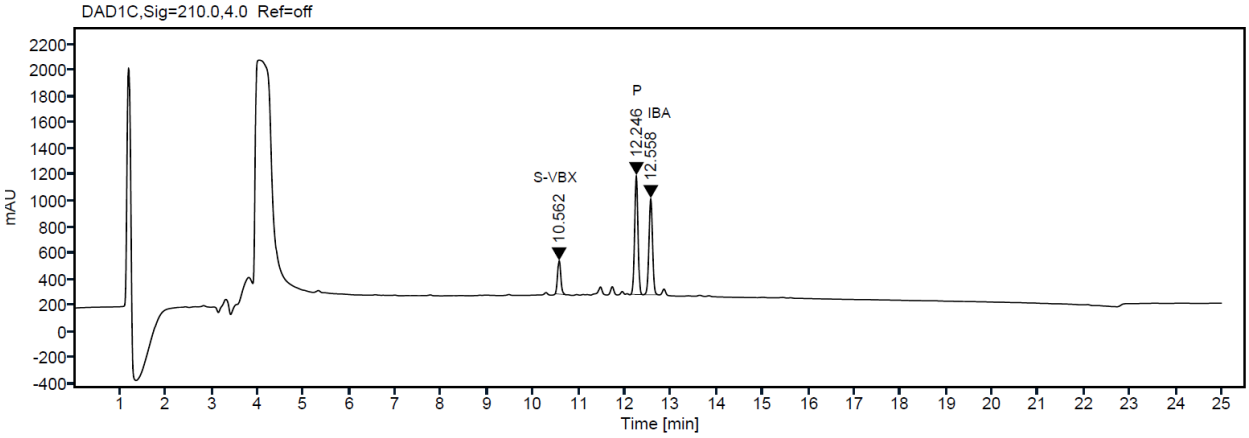
κ = Lys(C16H15NO4S)
Nter = C2H3O

Sequence	Type	MF	MF Mass	m/z	Intensity	Similarity
KLA	b3	C33H46N5O8S(+1)	672.3067	672.3062	39.68	86.38%
K	b1	C24H30N3O6S(+1)	488.1855	488.185	59.7	85.37%
FH	y2	C15H19N4O3(+1)	303.1457	303.1452	3.56	83.22%
KL	b2	C30H41N4O7S(+1)	601.2696	601.269	64.5	83.02%
AFH	y3	C18H24N5O4(+1)	374.1828	374.1823	2.76	80.13%
LAFH	y4	C24H35N6O5(+1)	487.2669	487.2663	14.27	74.76%

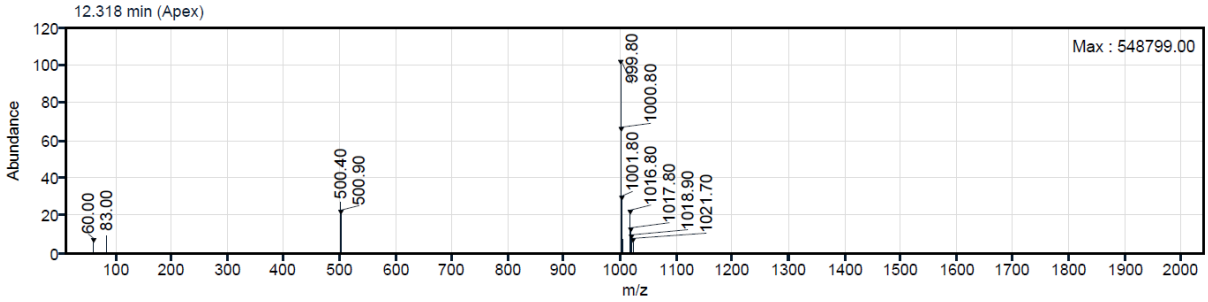
S-alkynylation product of AcKLAFY (5c)



Following the general procedure, the reaction was conducted in 3 μmol scale. HPLC ratio (210 nm) of the product: 67%. The desired product **5c** (1.8 mg, 1.8 μmol, 60% yield) was isolated by **Method 6**.



Retention time: 12.318 min **Area Percent:** 100%



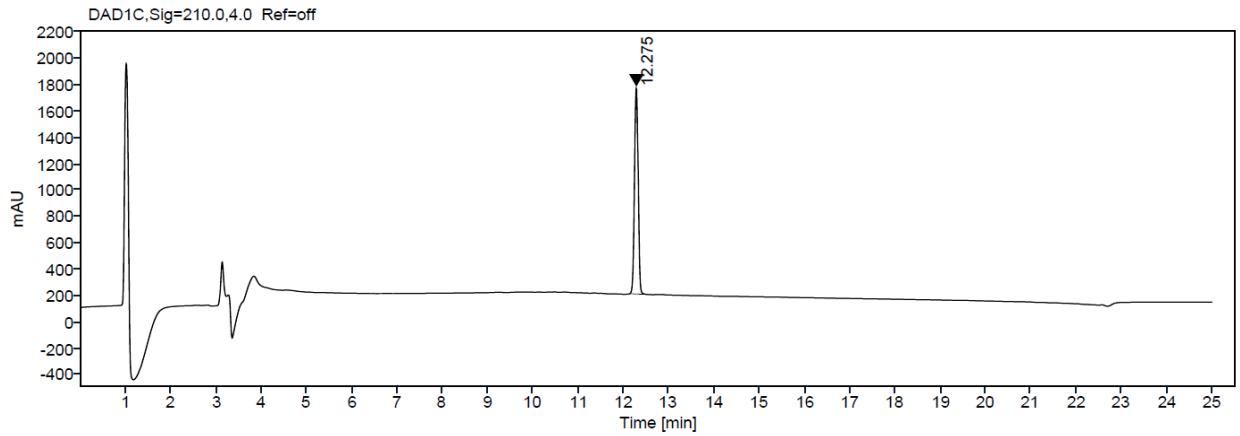
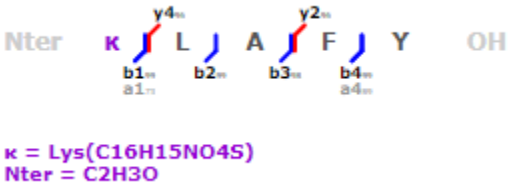


Figure S69: HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude and purified product **5c** by **Method 1**.

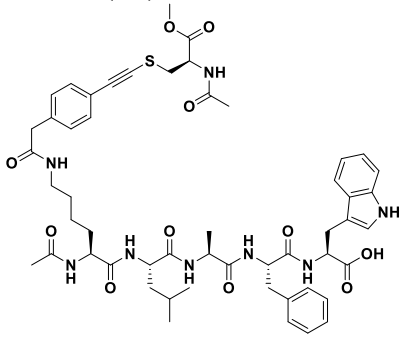
HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{51}H_{66}N_7O_{12}S^+$ 1000.4485; Found 1000.4494.

Table S33: MS/MS fragmentation of **5c**:



Sequence	Type	MF	MF Mass	m/z	Intensity	Similarity
KLAFY		C51H65N7O12S	999.4412	1000.449	12.39	99.45%
KL	b2	C30H41N4O7S(+1)	601.2696	601.269	57.45	99.26%
K	b1	C24H30N3O6S(+1)	488.1855	488.185	73.38	99.04%
KLAF	b4	C42H55N6O9S(+1)	819.3751	819.3746	34.45	98.87%
KLA	b3	C33H46N5O8S(+1)	672.3067	672.3062	58.37	98.35%
FY	y2	C18H21N2O4(+1)	329.1501	329.1496	12.63	96.29%
LAFY	y4	C27H37N4O6(+1)	513.2713	513.2708	13.63	95.61%
KLAF	a4	C41H55N6O8S(+1)	791.3802	791.3797	4.23	89.43%
K	a1	C23H30N3O5S(+1)	460.1906	460.1901	2.41	73.32%

S-alkynylation product of AcKLAFW (5d)



Following the general procedure, the reaction was conducted in 3 μmol scale. HPLC ratio (210 nm) of the product: 67%. The desired product **5d** (1.7 mg, 1.6 μmol, 54% yield) was isolated by **Method 6**.

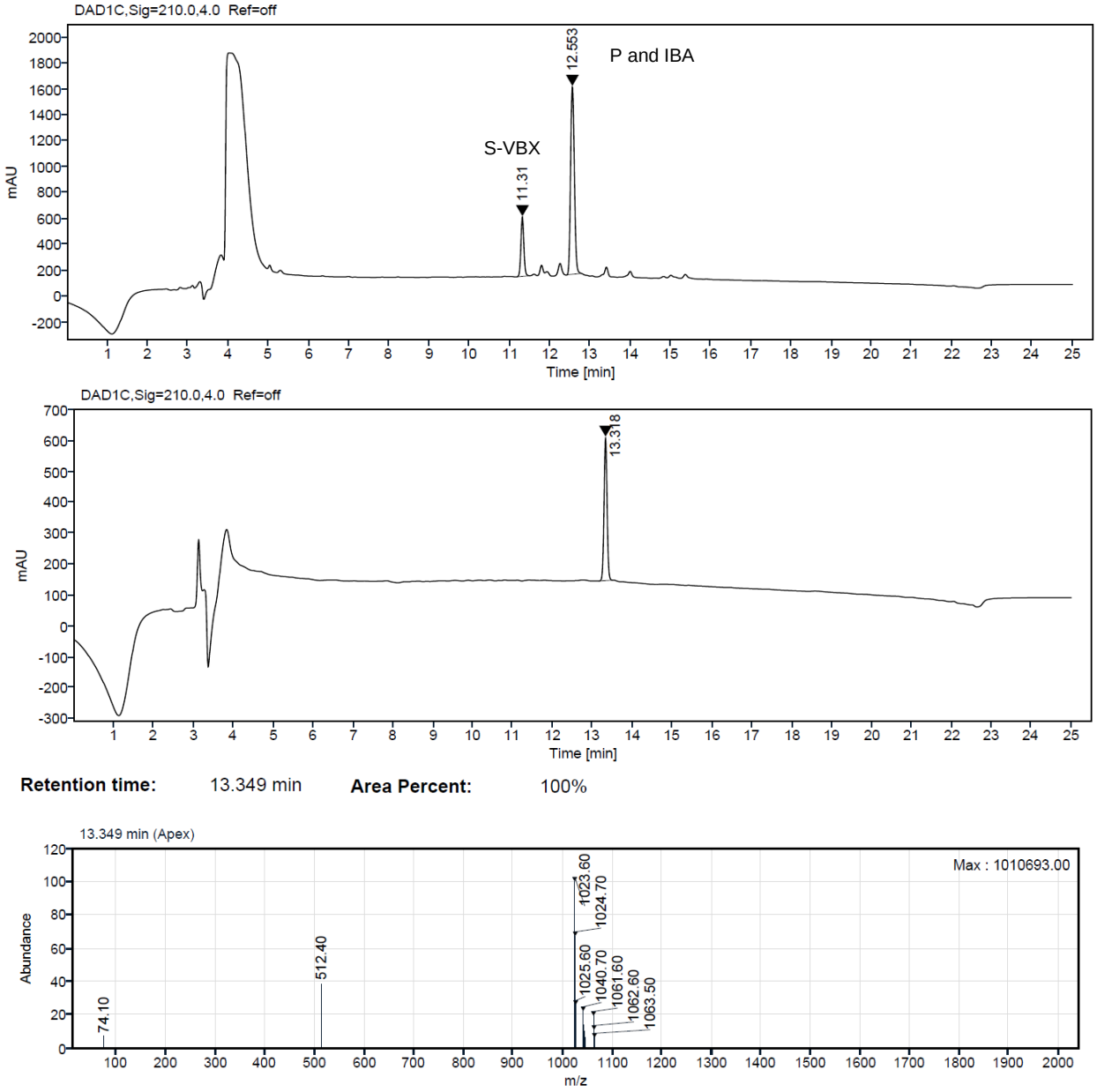
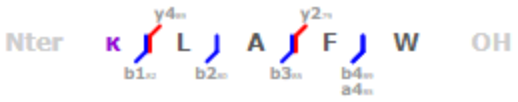


Figure S70: HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude and purified product **5d** by **Method 1**.

HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{53}H_{67}N_8O_{11}S^+$ 1023.4645; Found 1023.4676.

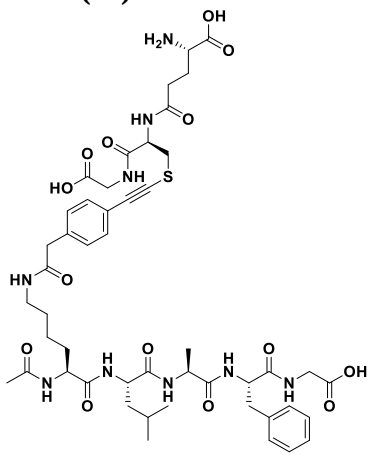
Table S34: MS/MS fragmentation of **5d**:



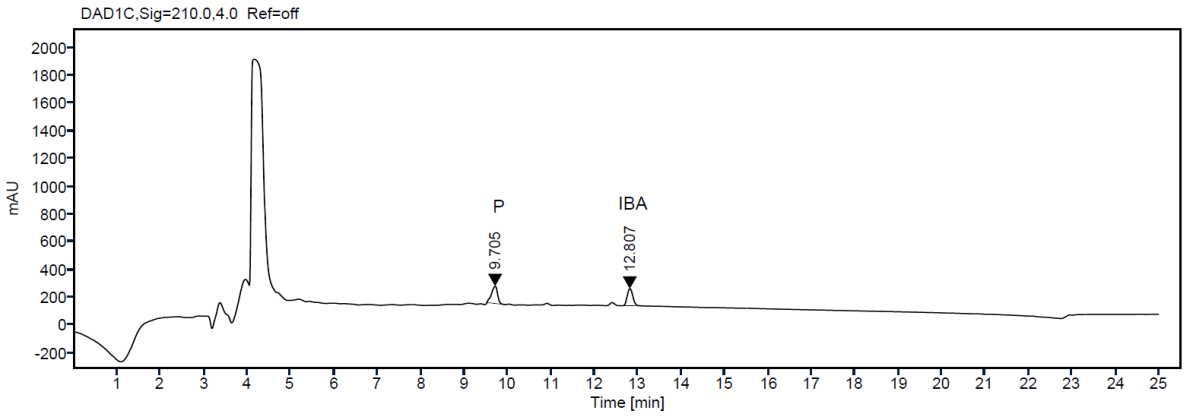
κ = Lys(C16H15NO4S)
Nter = C2H3O

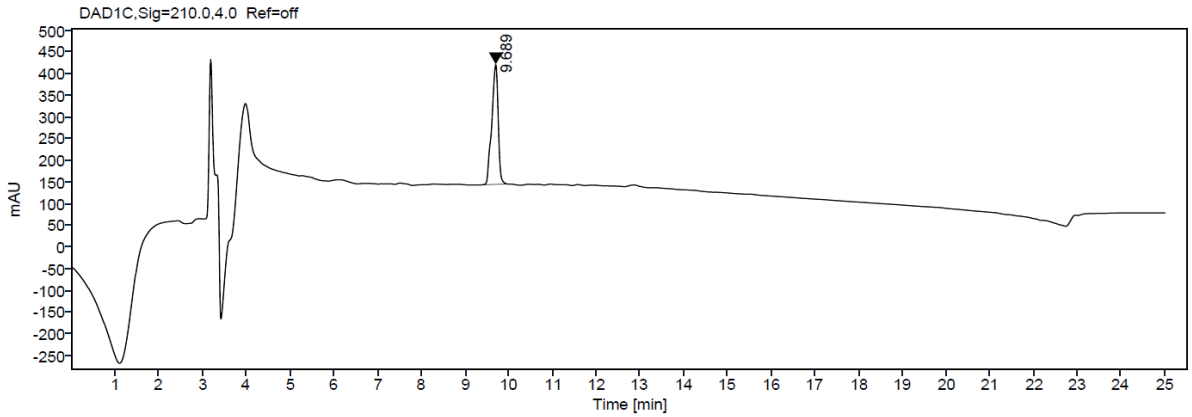
Sequence	Type	MF	MF Mass	m/z	Intensity	Similarity
LAFW	y4	C29H38N5O5(+1)	536.2873	536.2867	11.62	89.11%
KLAF	b4	C42H55N6O9S(+1)	819.3751	819.3746	100.2	89.11%
KLA	b3	C33H46N5O8S(+1)	672.3067	672.3062	48.12	85.20%
KLAF	a4	C41H55N6O8S(+1)	791.3802	791.3797	6.65	84.98%
K	b1	C24H30N3O6S(+1)	488.1855	488.185	14.76	81.52%
KL	b2	C30H41N4O7S(+1)	601.2696	601.269	20.07	79.94%
FW	y2	C20H22N3O3(+1)	352.1661	352.1656	0.86	79.20%

S-alkynylation product of AcKLAFG (5e)



Following the general procedure, the reaction was conducted in 3 μmol scale. HPLC ratio (210 nm) of the product: > 95%. The desired product **5e** (2.4 mg, 2.3 μmol, 78% yield) was isolated by **Method 5**.





Retention time: 9.714 min **Area Percent:** 100%

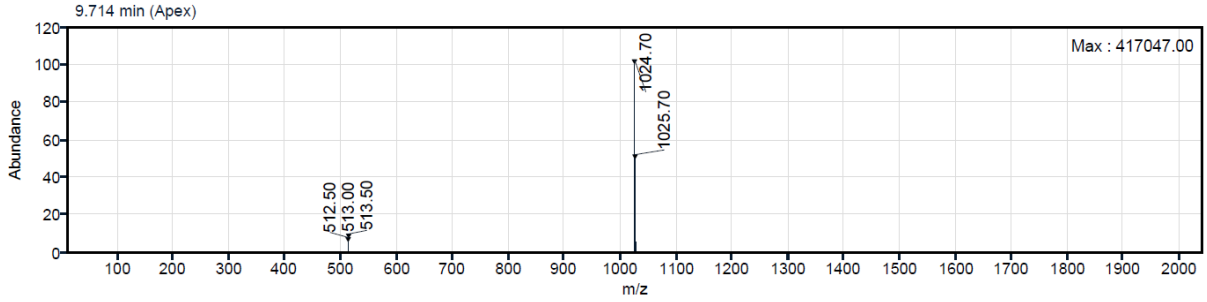
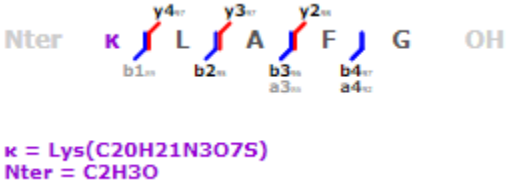


Figure S71: HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude and purified product **5e** by **Method 1**.

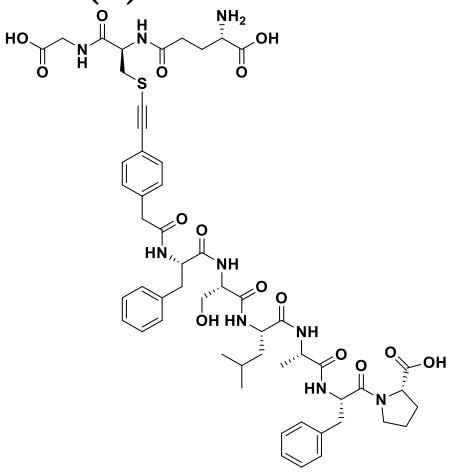
HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H_2]^{+2}$ Calcd for $C_{48}H_{67}N_9O_{14}S^{+2}$ 512.7259; Found 512.7253.

Table S35: MS/MS fragmentation of **5e**:

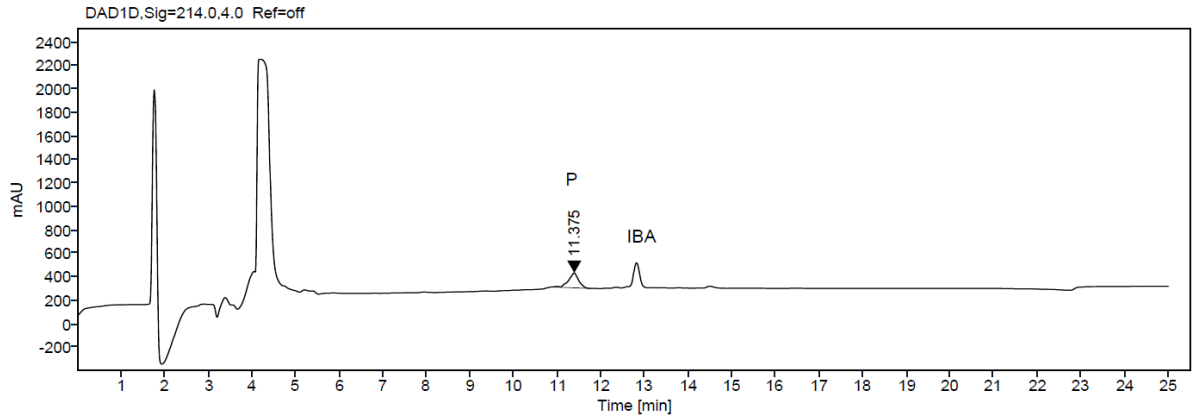


Sequence	Type	MF	MF Mass	m/z	Intensity	Similarity
FG	y2	C11H15N2O3(+1)	223.1083	223.1077	100.35	98.31%
KLAFG		C48H65N9O14S	1023.437	512.7259	9.26	96.93%
LAFG	y4	C20H31N4O5(+1)	407.2294	407.2289	14.04	96.83%
AFG	y3	C14H20N3O4(+1)	294.1454	294.1448	14.45	96.75%
KLA	b3	C37H52N7O11S(+1)	802.3446	802.344	45.29	96.71%
KLAF	b4	C46H61N8O12S(+1)	949.413	475.2098	34.61	96.50%
KL	b2	C34H47N6O10S(+1)	731.3074	366.1571	7.4	95.60%
KLA	b3	C37H52N7O11S(+1)	802.3446	401.6756	37.01	95.42%
KL	b2	C34H47N6O10S(+1)	731.3074	731.3069	9.86	94.92%
K	b1	C28H36N5O9S(+1)	618.2234	618.2228	9.12	94.68%
KLAF	a4	C45H61N8O11S(+1)	921.4181	461.2124	1.45	91.69%
KLA	a3	C36H52N7O10S(+1)	774.3496	774.3491	1.03	86.37%
K	b1	C28H36N5O9S(+1)	618.2234	309.6151	0.75	82.67%

S-alkynylation product of FSLAFP (5f)



Following the general procedure, the reaction was conducted in 2 μmol scale. HPLC ratio (210 nm) of the product: > 95%. The desired product **5f** (1.5 mg, 1.3 μmol, 65% yield) was isolated by **Method 5**.



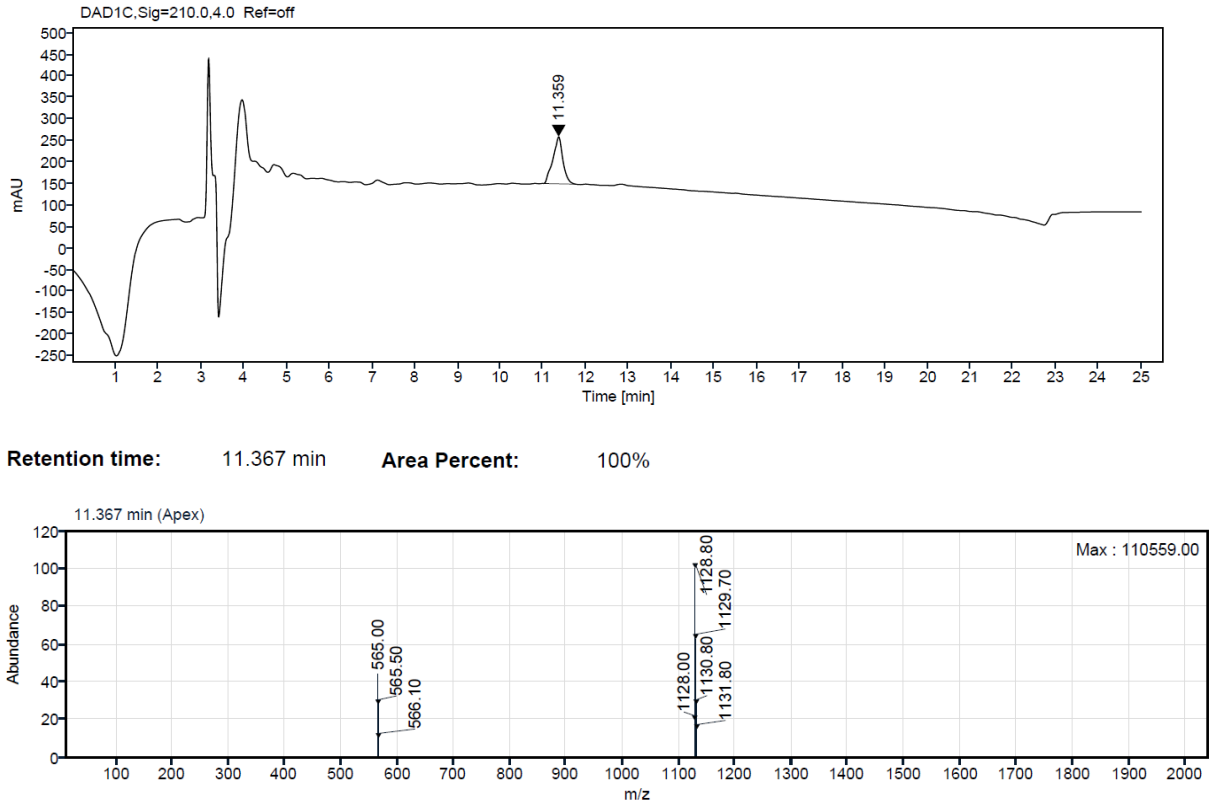
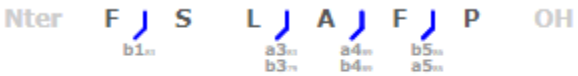


Figure S72: HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude and purified product **5f** by **Method 1**.

HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{55}H_{70}N_9O_{15}S^+$ 1128.4707; Found 1128.4689.

Table S36: MS/MS fragmentation of **5f**:

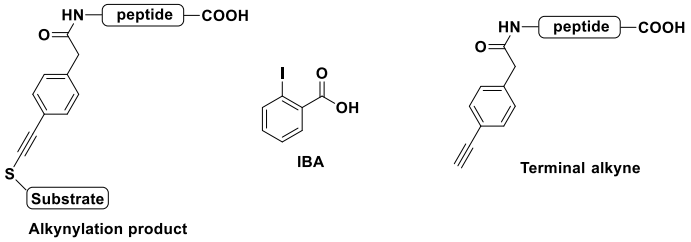


Nter = C20H22N3O7S

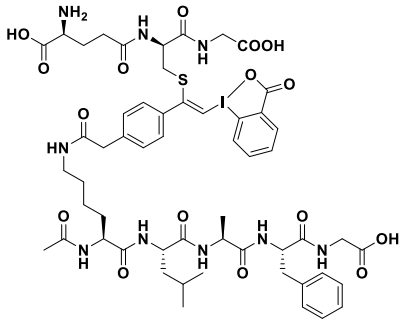
Sequence	Type	MF	MF Mass	m/z	Intensity	Similarity
FSLA	a4	C40H52N7O11S(+1)	838.3446	838.344	3.01	89.22%
FSLA	b4	C41H52N7O12S(+1)	866.3395	866.3389	8.78	88.90%
FSLAF	b5	C50H61N8O13S(+1)	1013.408	1013.407	21	86.11%
FSLAF	a5	C49H61N8O12S(+1)	985.413	985.4124	13.61	85.33%
F	b1	C29H31N4O8S(+1)	595.1863	595.1857	2.9	82.87%
FSL	a3	C37H47N6O10S(+1)	767.3074	767.3069	1.37	82.86%
FSL	b3	C38H47N6O11S(+1)	795.3024	795.3018	2.49	79.06%

7. Procedures for S-VBX formation of peptide-EBXs:

HPLC-UV ratio was determined by taking the ration of A_{prod}/A_{total} where A_{prod} = area in mAU of the product peak and A_{total} = area in mAU of the combined peptide containing species
Common side-products can be observed in HPLC:

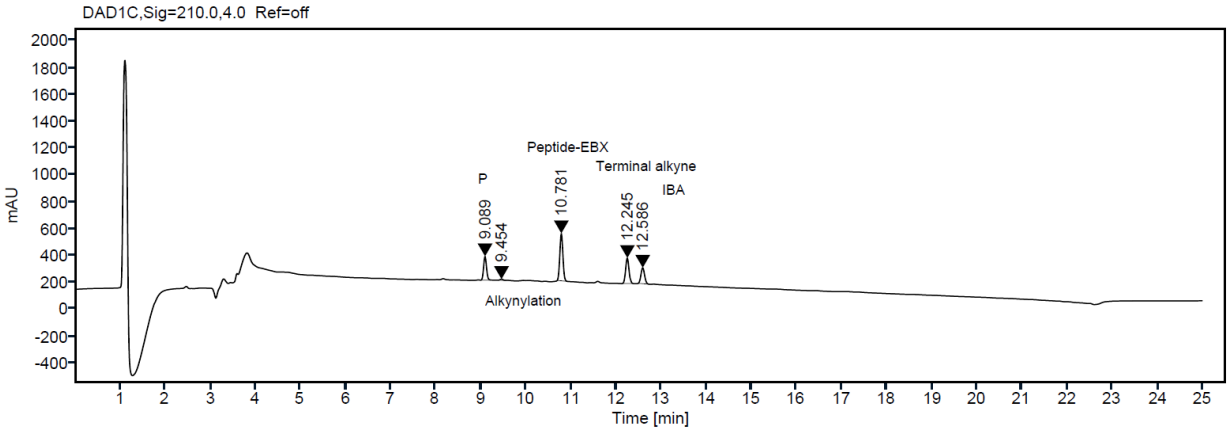


S-VBX of Ac-KLAFG (6a)



Procedure:

Peptide-EBX (Ac-KLAFG) (2.6 mg, 2.7 μmol, 1.5 equiv.) was weighed on the analytical balance in an Eppendorf tube. Afterwards, 18 μL of a solution of *L*-Glutathione (100 mM in water) (1.8 μmol, 1.0 equiv.) The reaction mixture was then diluted with 347 μL Tris buffer (pH 9.0, 100 mM), overall concentration: 5 mM, and placed on a shaker and shaken for 3 h at RT without protecting from atmosphere and light.
At the end of the reaction, 10 μL of the crude was diluted with 60 μL MeCN/water 1:1 and injected in RP-HPLC. HPLC ratio (210 nm) of the product: > 95%. The desired products were isolated by Prep-RP-HPLC. The desired product **6a** (1.6 mg, 1.2 μmol, 71% yield) was isolated by **Method 5**.



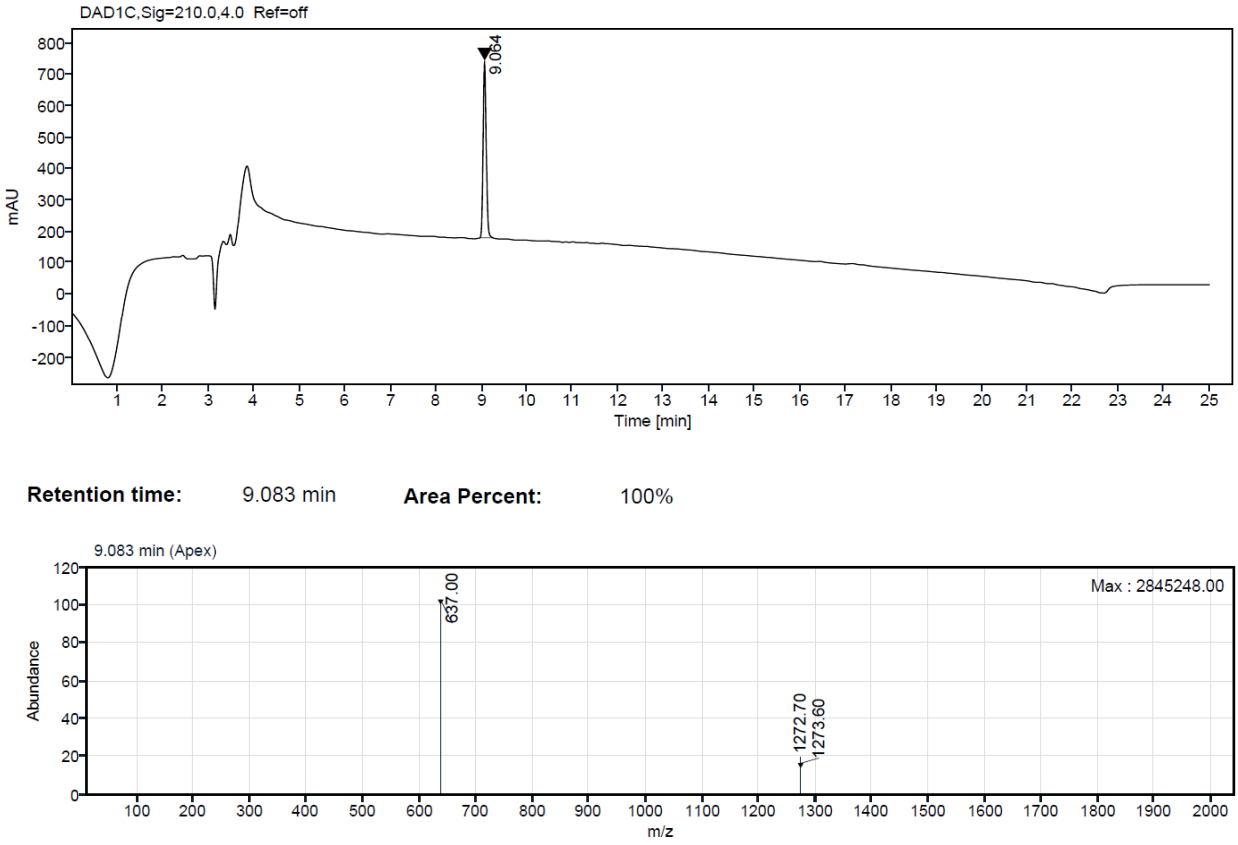
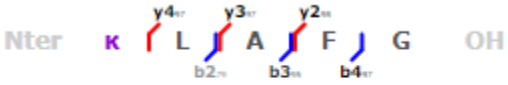


Figure S73: HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude and purified product **6a** by **Method 1**.
HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + HNa]^{+2}$ Calcd for $C_{55}H_{71}IN_9NaO_{16}S^{+2}$ 647.6835; Found 647.6875.

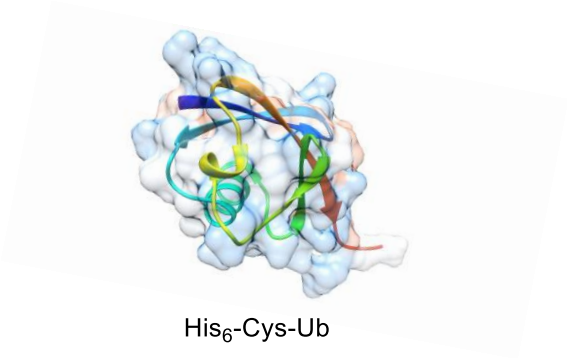
Table S37: MS/MS fragmentation of **6a**:



κ = Lys(C27H26IN3O9S)
Nter = C2H3O

Sequence	Type	MF	MF Mass	m/z	Intensity	Similarity
KLAFG		C55H70IN9O16S	1271.371	636.6926	25	98.38%
FG	y2	C11H15N2O3(+1)	223.1083	223.1077	100.35	98.31%
KLAF	b4	C53H66IN8O14S(+1)	1197.346	599.1766	5.19	97.25%
LAFG	y4	C20H31N4O5(+1)	407.2294	407.2289	14.04	96.83%
AFG	y3	C14H20N3O4(+1)	294.1454	294.1448	14.45	96.75%
KLA	b3	C44H57IN7O13S(+1)	1050.278	525.6424	5.79	96.21%
KLA	b3	C44H57IN7O13S(+1)	1050.278	1050.277	1.62	93.06%
KL	b2	C41H52IN6O12S(+1)	979.2409	490.1238	0.56	79.19%

His_6-Cys-Ub



Sequence:
GSSHHHHHHSSGLVPRGSHCMQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQ
QRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRGG

LCMS - ESI ionization, C4 column, LC 10-90% ACN+0.1% FA, flowrate 5ml/min, over 6min, TOF-MS 200-2000m/z ES+, MS continuum scan rate 200ms/scan. Deconvolution of spectrum with MaxEnt1 (5-30,000 Da, 0.5Da)

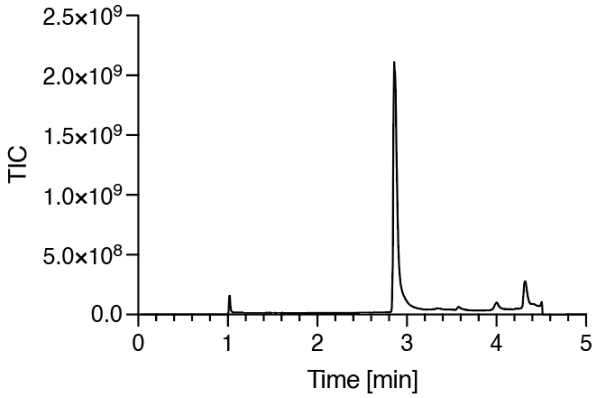


Figure S74: MS analysis on XEVO G2-XS Q-TOF.

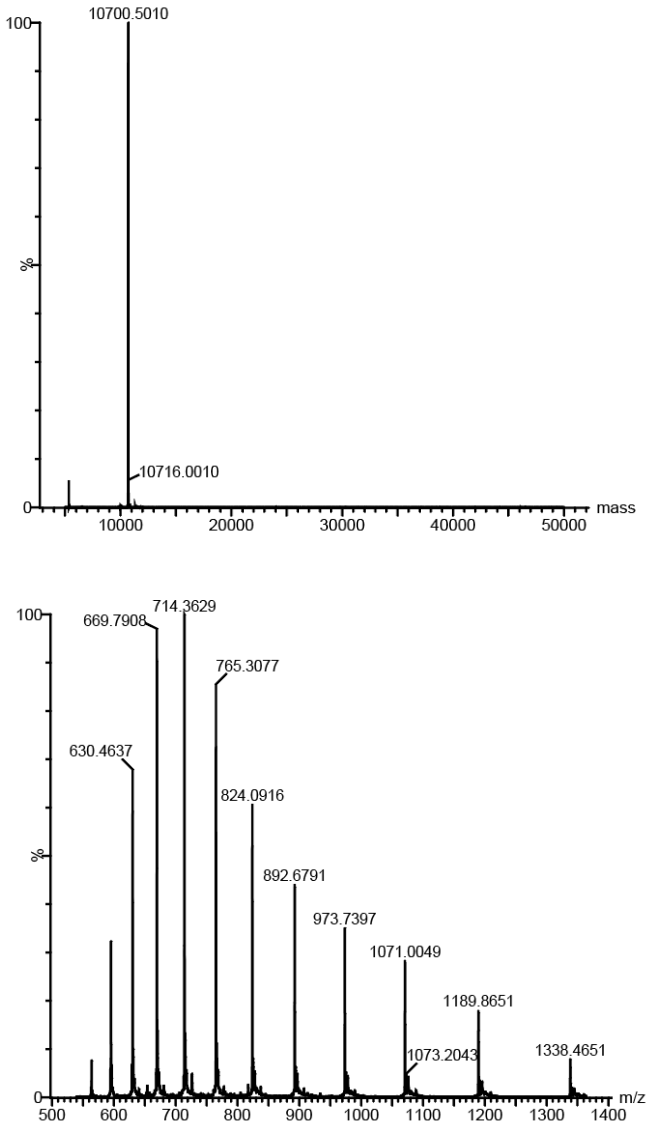
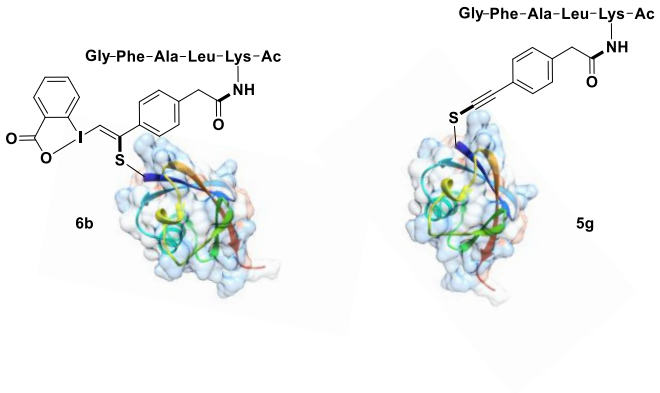


Figure S75: Mass spectrum of His_6-Cys-Ub.

HRMS (HESI/LTQ-Orbitrap) m/z: $[M + H_{15}]^{+15}$ Calcd for $C_{466}H_{771}N_{142}O_{143}S_2^{+15}$ 714.3455. Found 714.3629.

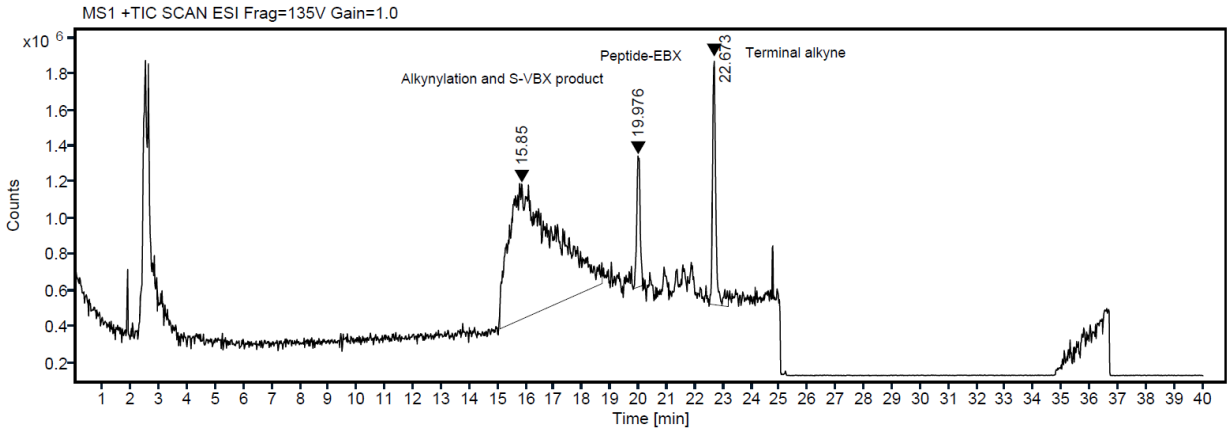
His_6-Cys-Ubiquitin S-VBX modification product (6b, 5g)



Procedure:

Protein His_6-Tag-Ub (0.44 mg, 0.004 μmol) was weighed in an Eppendorf tube. Afterwards, 10 μL of Peptide-EBX (Ac-KLAFG) (2 equiv., 7.8 mM in Tris buffer pH 9.0, 100 mM) and 260 μL Tris buffer (pH 9.0, 100 mM), overall concentration 150 μM, were added in the tube. The reaction mixture was then placed on a shaker and shaken for 3 h at RT without protecting from atmosphere and light.

At the end of the reaction, 10 μL of the crude was diluted with 40 μL water and injected in RP-HPLC using **Method 4**. The yield was determined based on the LC/MS ration as 100% for (mixture of **6b** and **5g, 6b**:**5g** = 1.4:1). The ratio of **6b** and **5g** was determined by the relative peak intensity of deconvoluted mass spectrum.



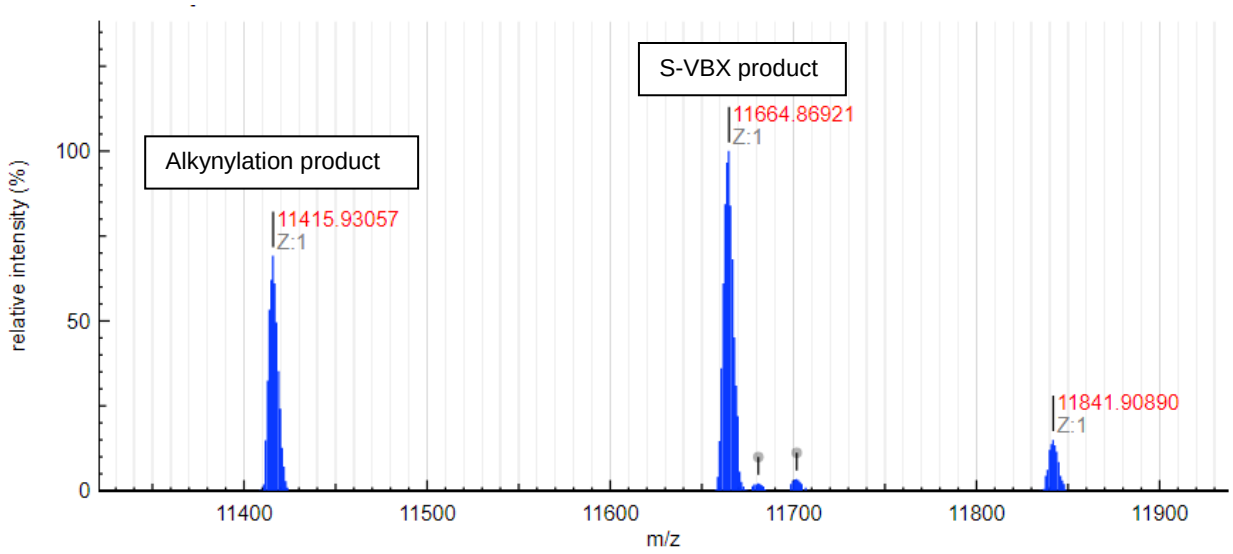


Figure S76: LC/MS of reaction crude by Method 4 and deconvoluted mass spectrum.

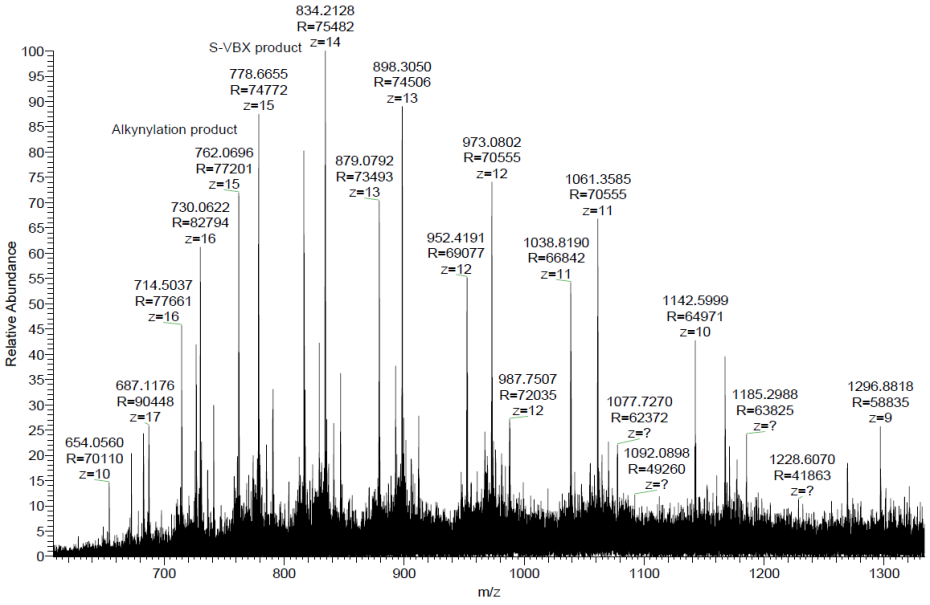


Figure S77: LC/MS chromatogram (210 nm) of the crude by **Method 1.**
For alkynylation product **5g**: HRMS (HESI/LTQ-Orbitrap) m/z: $[M + H_{15}]^{+15}$ Calcd for $C_{504}H_{819}N_{148}O_{151}S_2^{+15}$ 762.1338. Found 762.0696.

For S-VBX product **6b**: HRMS (HESI/LTQ-Orbitrap) m/z: $[M + H_{15}]^{+15}$ Calcd for $C_{511}H_{824}N_{148}O_{153}S_2^{+15}$ 778.6643. Found 778.6655.

Table S38: MS/MS fragmentation of alkynylation product (5g):

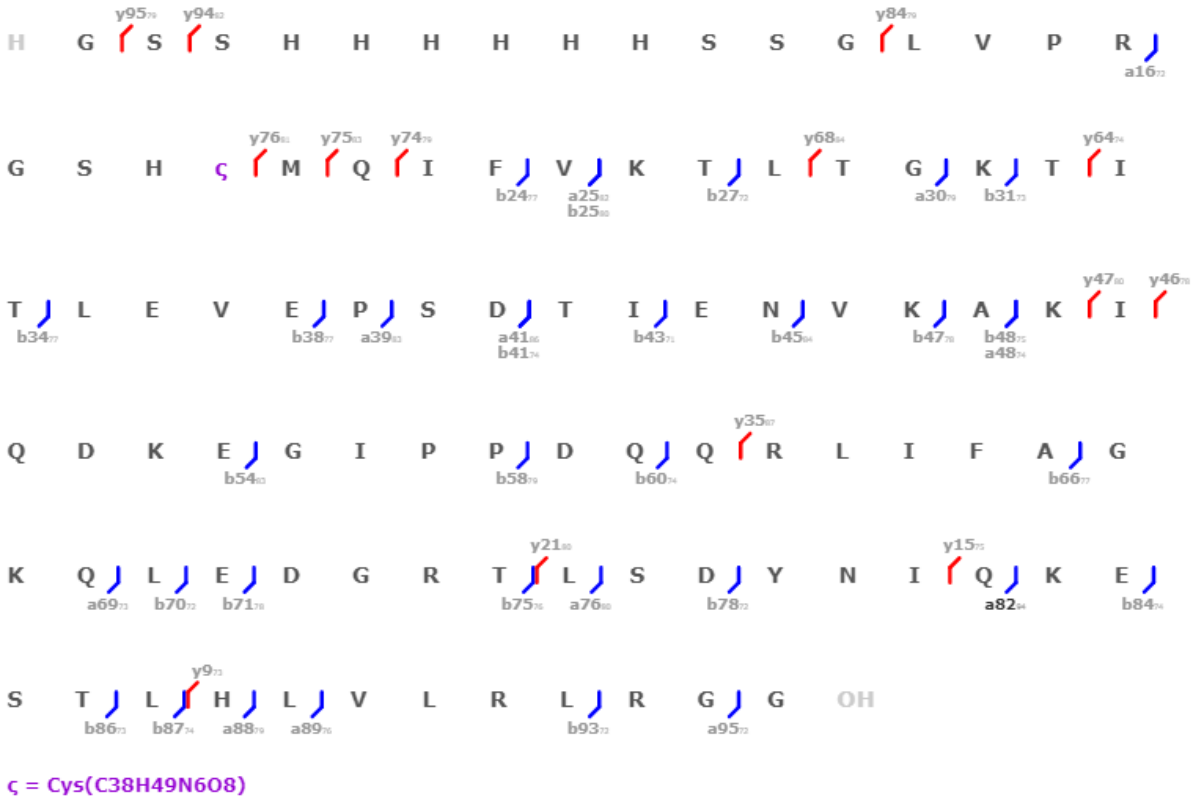
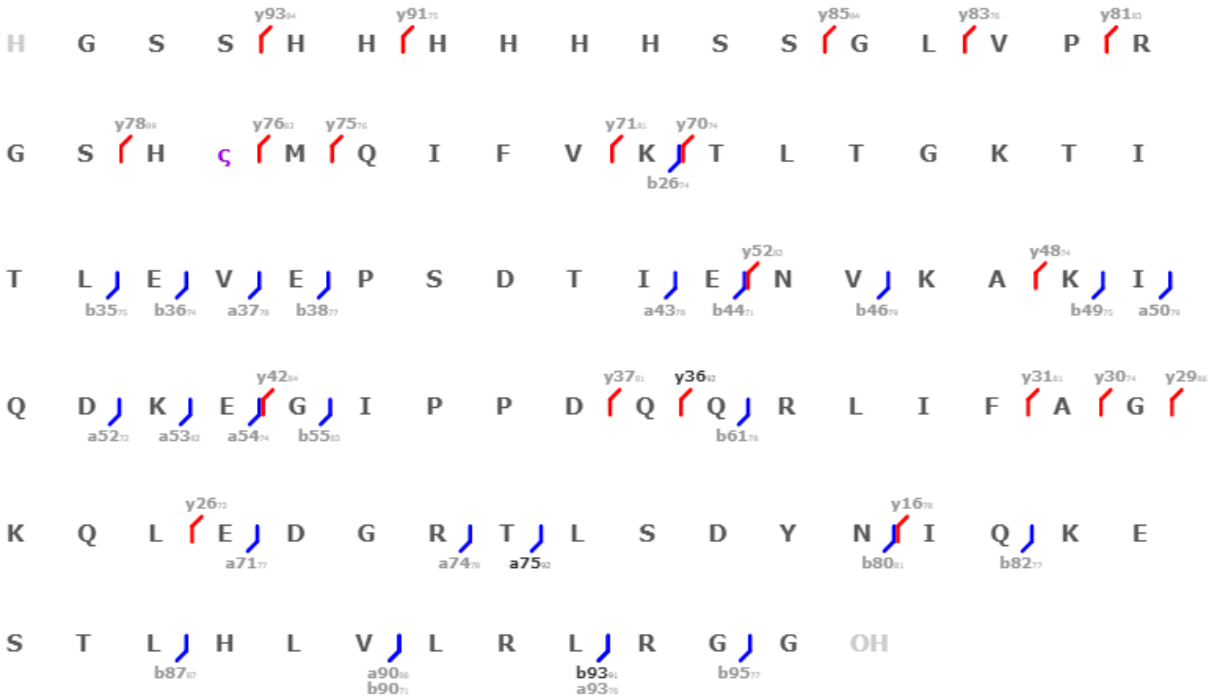


Table S39: MS/MS fragmentation of S-VBX product (**6b**):



8. Decarboxylative cross-coupling:

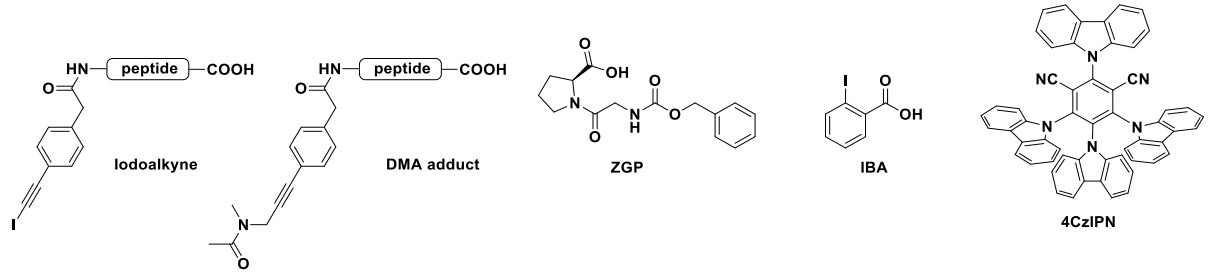
General Procedure

Peptide-EBX (5 μmol, 1 equiv.) were weighed on the analytical balance and dissolved in 200 μL non-degassed DMA in a 2 mL vial. Afterwards, 100 μL of a solution of dipeptide ZGP (10 μmol, 2 equiv.) (100 mM in DMA), the peptide solution, 60 μL of a solution of CzIPN (30 mol%, 25 mM in DMA), 25 μL of 2 M K_2HPO_4 (10 equiv.) in milli-Q purified water and 115 μL of DMA were placed into the vial, overall concentration: 10 Mm. The vial was then capped and degassed by bubbling with N_2 for 20 min. The reaction was stirring under Blue LEDs irradiation for 30 min at RT.

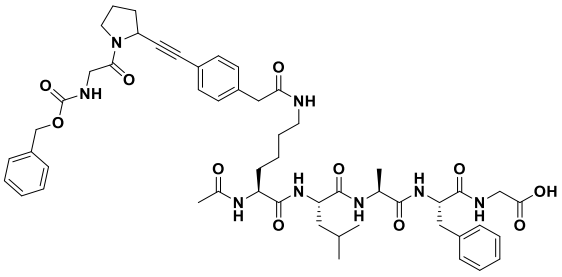
At the end of the reaction, 10 μL of the crude was diluted with 90 μL of MeCN/water 1:1 and injected in RP-HPLC. The desired products were isolated by Prep-RP-HPLC.

HPLC UV ratio was determined by taking the ration of A_{prod}/A_{total} where A_{prod} = area in mAU of the product peak and A_{total} = area in mAU of the combined peptide containing species (product, DMA adduct, iodoalkyne).

Common side-products that can be observed in HPLC.



Decarboxylative alkynylation of AcKLAFG (8a)



Following the general procedure, the reaction was conducted in 5 μmol scale. HPLC ratio (210 nm) of the product: > 95%, dr 1.2: 1 (determined by ^{13}C NMR). The desired product **8a** (2.6 mg, 2.6 μmol, 52% yield) was isolated by **Method 5**.

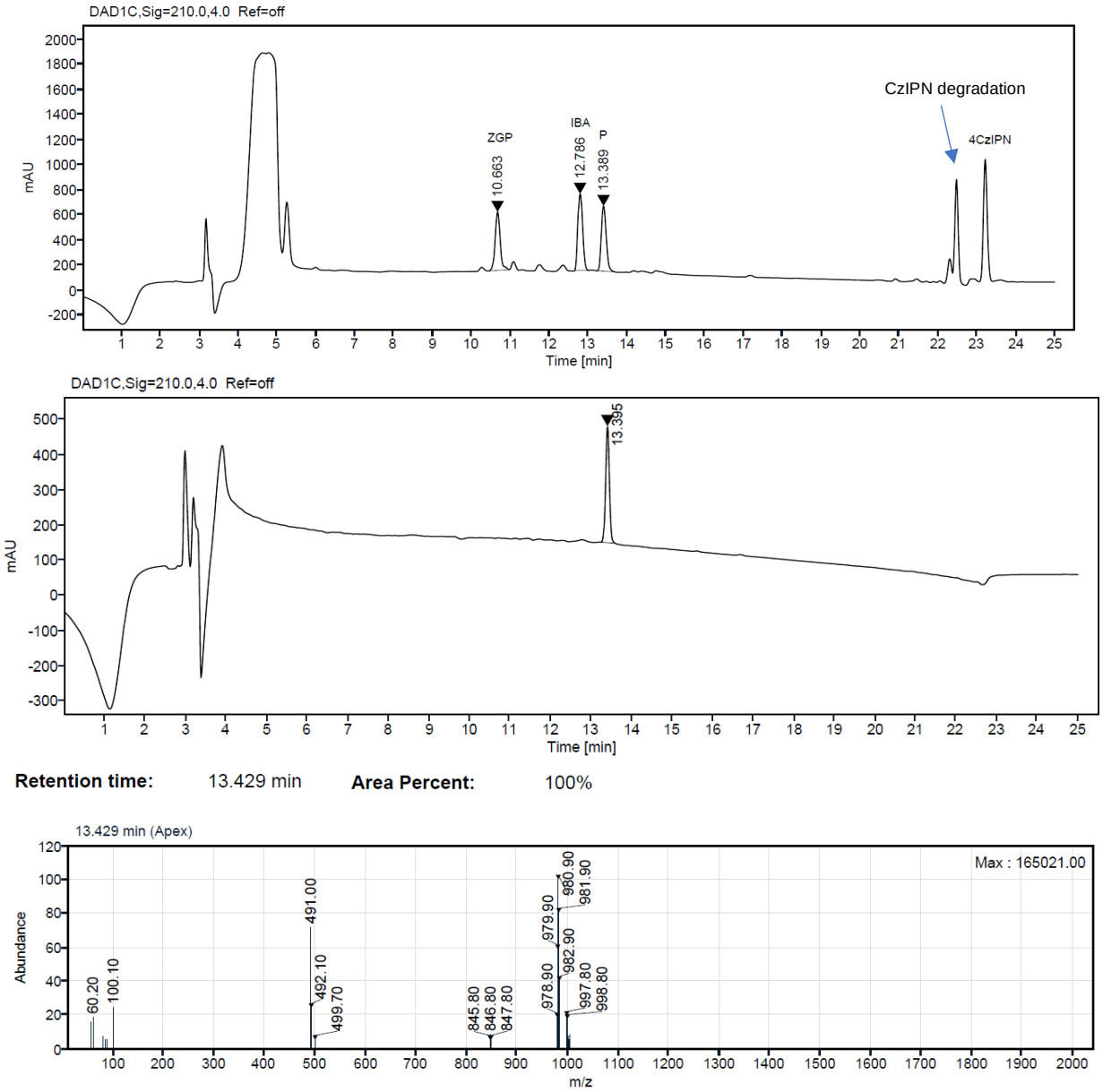
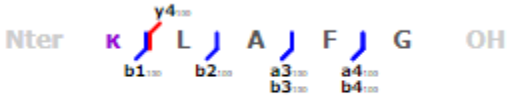


Figure S78: HPLC-UV chromatogram (210 nm) and MS(ESI) and MS(ESI) of the crude and purified product **8a** by **Method 1.**

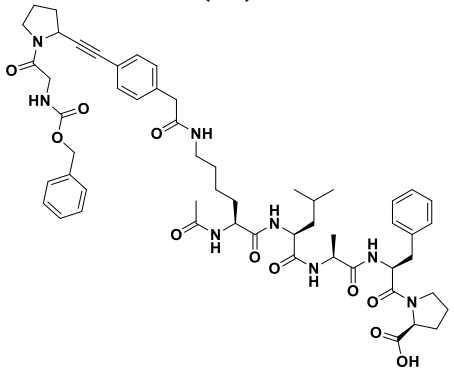
^{1}H NMR (600 MHz, CD_3OD) δ 7.44 – 7.15 (m, 14H), 5.12 (s, 2H), 4.63 (dd, *J* = 9.1, 5.2 Hz, 1H), 4.37 (dd, *J* = 10.4, 4.8 Hz, 1H), 4.31 – 4.13 (m, 3H), 4.03 – 3.93 (m, 1H), 3.89 (dd, *J* = 3.5, 1.8 Hz, 2H), 3.72 – 3.61 (m, 1H), 3.55 – 3.47 (m, 3H), 3.25 – 3.16 (m, 3H), 2.97 (ddd, *J* = 14.0, 9.1, 1.7 Hz, 1H), 2.35 – 2.28 (m, 1H), 2.27 – 2.22 (m, 1H), 2.14 (td, *J* = 12.5, 9.6, 4.7 Hz, 2H), 2.03 (dt, *J* = 6.4, 3.4 Hz, 1H), 1.99 (d, *J* = 1.5 Hz, 3H), 1.78 (ddt, *J* = 12.4, 9.4, 6.1 Hz, 1H), 1.70 (td, *J* = 13.5, 12.6, 6.3 Hz, 1H), 1.67 – 1.49 (m, 5H), 1.40 (dt, *J* = 15.7, 8.2 Hz, 2H), 1.25 (d, *J* = 7.1 Hz, 3H), 1.00 – 0.95 (m, 3H), 0.94 – 0.90 (m, 3H).
^{13}C NMR (151 MHz, CD_3OD mixture of two diastereomers) δ 174.9, 174.8, 174.7, 173.7, 173.6, 173.5, 172.9, 170.1, 169.5, 138.5, 138.2, 138.0, 137.5, 132.9, 132.8, 130.4, 130.3, 130.1, 129.5, 129.4, 129.0, 128.9, 127.7, 122.8, 122.1, 89.5, 88.4, 85.1, 82.9, 67.8, 67.8, 55.8, 55.2, 53.3, 50.9, 47.3, 46.6, 44.2, 44.1, 43.7, 42.1, 41.3, 40.3, 38.6, 35.4, 33.4, 32.4, 30.0, 25.9, 25.9, 24.2, 23.9, 23.6, 22.4, 21.8, 17.9.
HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{52}H_{67}N_8O_{11}^+$ 979.4924; Found 979.4906.
Table S40: MS/MS fragmentation of **8a**:



κ = Lys(C24H22N2O4)
Nter = C2H3O

Sequence	Type	MF	MF Mass	m/z	Intensity	Similarity
LAFG	y4	C20H31N4O5(+1)	407.2294	407.2289	0.91	99.97%
KLA	a3	C40H53N6O7(+1)	729.3976	729.397	0.55	99.97%
KLAF	a4	C49H62N7O8(+1)	876.466	876.4654	4.42	99.94%
K	b1	C32H37N4O6(+1)	573.2713	573.2708	11.34	99.93%
KL	b2	C38H48N5O7(+1)	686.3554	686.3548	15.57	99.91%
KLAF	b4	C50H62N7O9(+1)	904.4609	904.4604	100.01	99.90%
KLAFG		C52H66N8O11	978.4851	979.4924	37.38	99.88%
KLA	b3	C41H53N6O8(+1)	757.3925	757.3919	46.15	99.79%

Decarboxylative alkynylation of AcKLAFP (8b)



Following the general procedure, the reaction was conducted in 4 μmol scale. HPLC ratio (210 nm) of the product: 94%. The dr was not determined. The desired product **8b** (0.65 mg, 0.65 μmol, 16% yield) was isolated by **Method 5**.

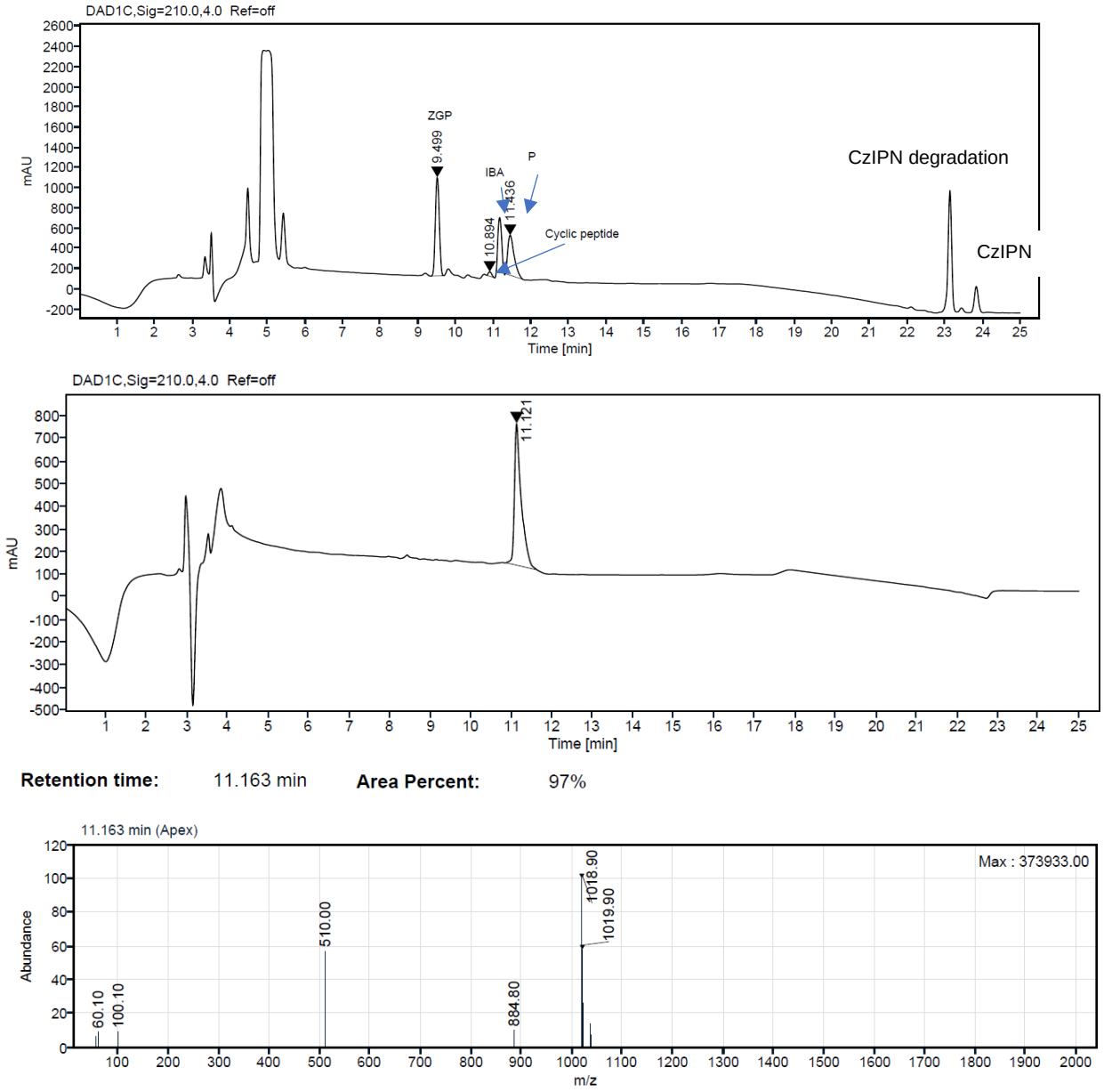
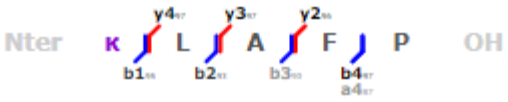


Figure S79: **HPLC-UV** chromatogram (210 nm) and MS(ESI) and MS(ESI) of the crude and purified product **8b** by **Method 1.**

HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H_2]^{+2}$ Calcd for $C_{55}H_{72}N_8O_{11}{}^{+2}$ 510.2655; Found 510.2638.

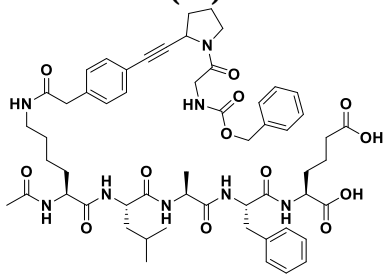
Table S41: MS/MS fragmentation of **8b**:



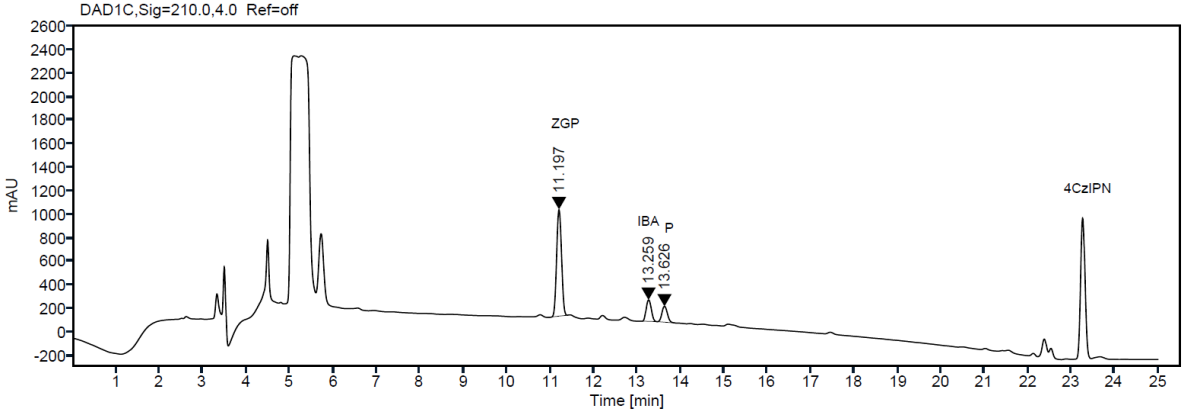
κ = Lys(C24H22N2O4)
Nter = C2H3O

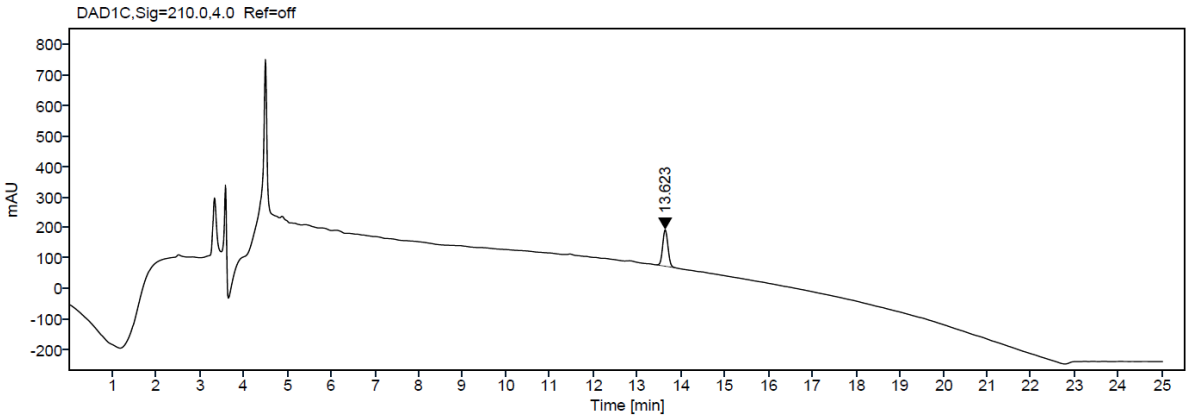
Sequence	Type	MF	MF Mass	m/z	Intensity	Similarity
KLAF	b4	C50H62N7O9(+1)	904.4609	904.4604	101.39	98.15%
AFP	y3	C17H24N3O4(+1)	334.1767	334.1761	3.23	96.75%
LAFP	y4	C23H35N4O5(+1)	447.2607	447.2602	4.04	96.74%
FP	y2	C14H19N2O3(+1)	263.1396	263.139	15.31	96.50%
KLAF	b4	C50H62N7O9(+1)	904.4609	452.7338	78.32	95.71%
K	b1	C32H37N4O6(+1)	573.2713	573.2708	4.8	94.80%
KL	b2	C38H48N5O7(+1)	686.3554	686.3548	5.3	92.98%
KLA	b3	C41H53N6O8(+1)	757.3925	757.3919	21.29	92.43%
KLAF	a4	C49H62N7O8(+1)	876.466	876.4654	2	90.19%
KLA	b3	C41H53N6O8(+1)	757.3925	379.1996	4.34	87.31%
KLAF	a4	C49H62N7O8(+1)	876.466	438.7364	0.7	84.65%

Decarboxylative alkynylation of AcKLAFE (8c)



Following the general procedure, the reaction was conducted in 2 μmol scale. HPLC ratio (210 nm) of the product: > 95%. dr was not determined. The desired product **8c** (1.1 mg, 1.1 μmol, 54% yield) was isolated by **Method 5**.





Retention time: 13.637 min **Area Percent:** 100%

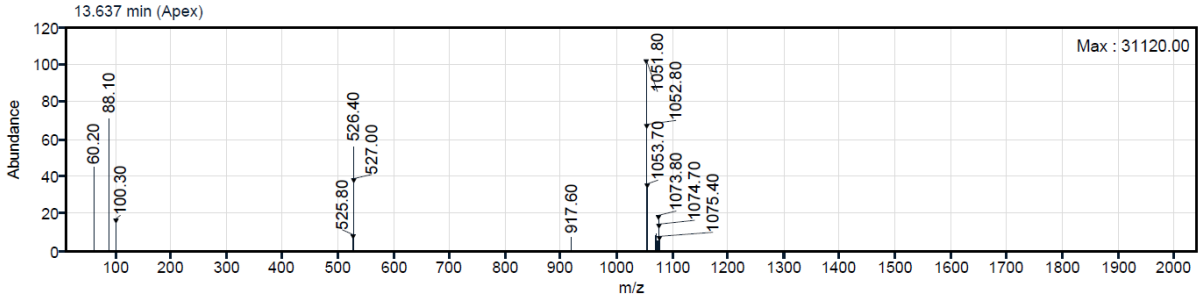
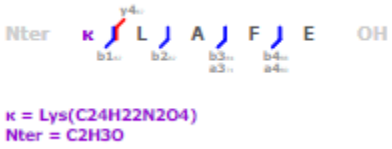


Figure S80: HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude and purified product **8c** by **Method 1.**

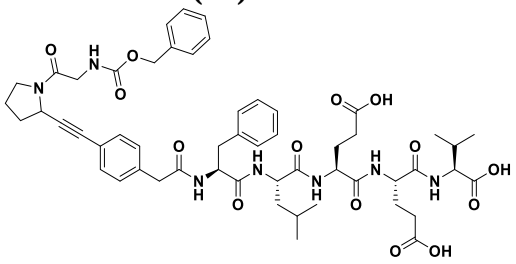
HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{55}H_{71}N_8O_{13}^+$ 1051.5135; Found 1051.5150.

Table S42: MS/MS fragmentation of **8c**:

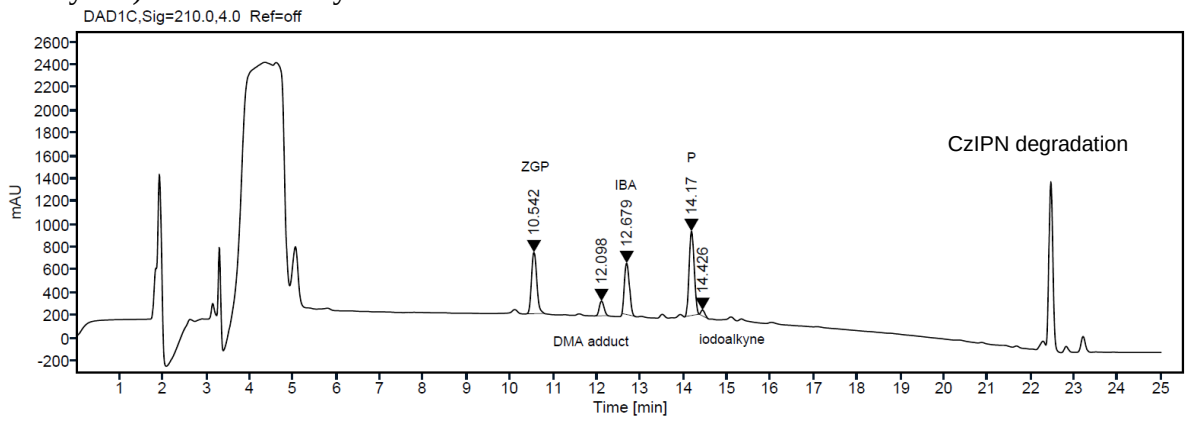


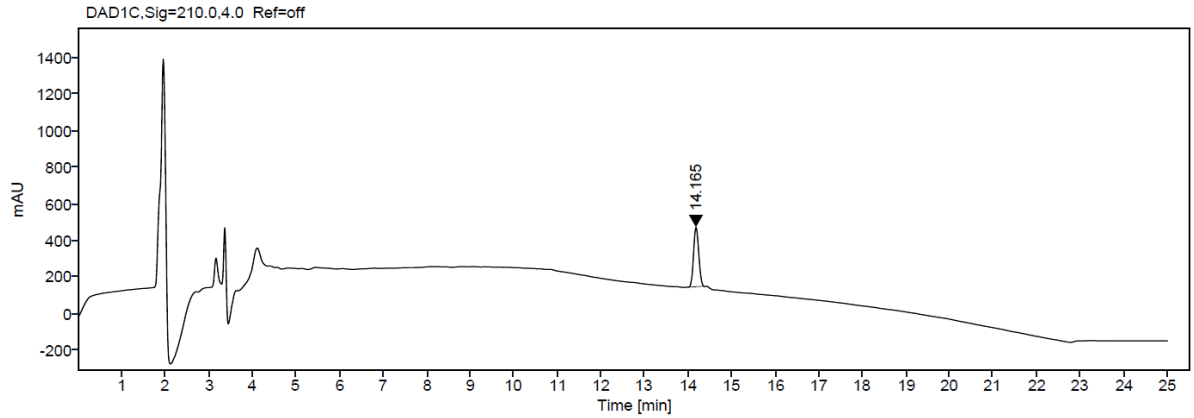
Sequence	Type	MF	MF Mass	m/z	Intensity	Similarity
KLAF	b4	C50H62N7O9(+1)	904.4609	904.4604	101.72	87.61%
LAFE	y4	C23H35N4O7(+1)	479.2506	479.25	8.81	87.29%
KLA	b3	C41H53N6O8(+1)	757.3925	757.3919	78.69	84.23%
KL	b2	C38H48N5O7(+1)	686.3554	686.3548	41.83	81.83%
K	b1	C32H37N4O6(+1)	573.2713	573.2708	33.07	81.55%
KLAF	a4	C49H62N7O8(+1)	876.466	876.4654	8.82	79.70%
KLA	a3	C40H53N6O7(+1)	729.3976	729.397	1.05	70.52%

Decarboxylative alkynylation of FLEEV (8d)



Following the general procedure, the reaction was conducted in 2 μmol scale. HPLC ratio (210 nm) of the product: 82%. The dr was not determined. The desired product **8d** (1.5 mg, 1.5 μmol, 49% yield) was isolated by **Method 5**.





Retention time: 14.21 min **Area Percent:** 100%

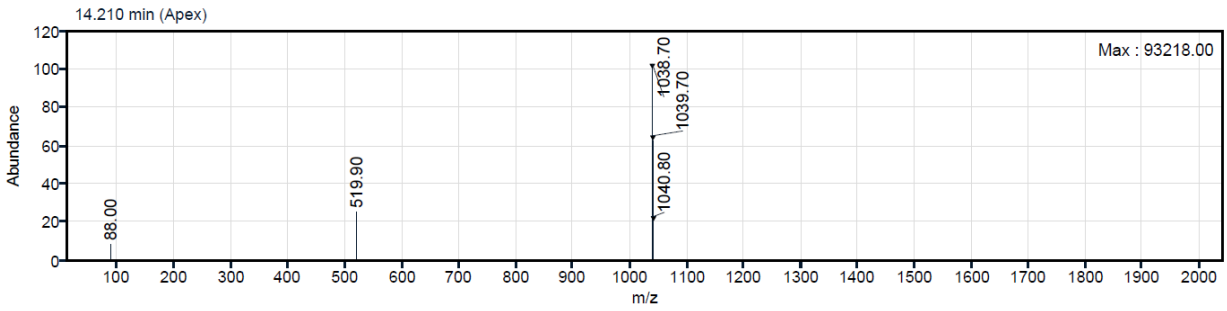
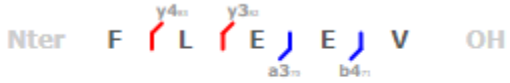


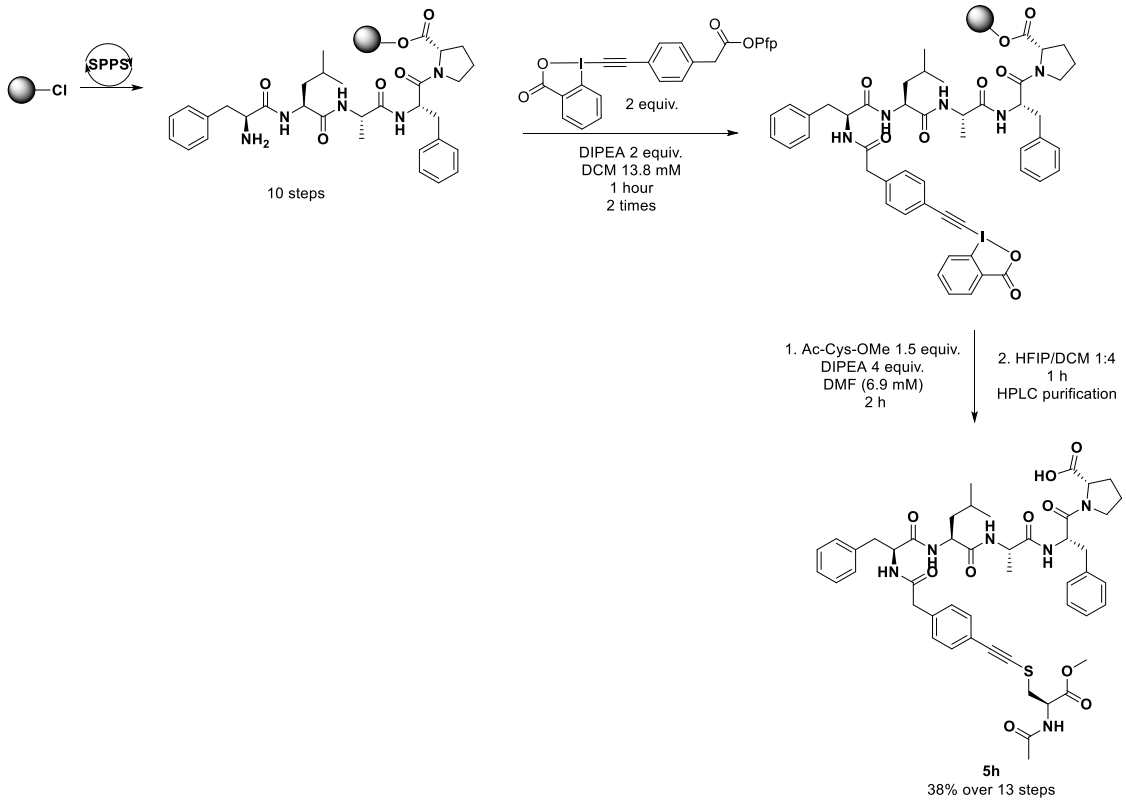
Figure S81: HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude and purified product **8d** by **Method 1.**
HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{54}H_{68}N_7O_{14}^+$ 1038.4819; Found 1038.4844.
Table S43: MS/MS fragmentation of **8d**:



Nter = C24H23N2O4

Sequence	Type	MF	MF Mass	m/z	Intensity	Similarity
LEEV	y4	C21H37N4O9(+1)	489.2561	489.2555	2.5	83.27%
EEV	y3	C15H26N3O8(+1)	376.172	376.1714	0.62	82.33%
FLEE	b4	C49H57N6O12(+1)	921.4034	921.4029	67.4	70.56%
FLE	a3	C43H50N5O8(+1)	764.3659	764.3654	1.56	70.05%

9. S-alkynylation and S-esterification on solid phase



Procedure:
Peptide EBX was prepared on a 13.8 μmol scale from resin-bound substrate. Bifunctional EBX (15.8 mg, 27.6 μmol, 2 equiv.) was weighed into the syringe reactor and dissolved in 1 mL DCM. Then DIPEA (9.6 μL, 55 μmol, 4 equiv.) was added into the reactor and agitated at room temperature for 1 hour. The resin was then filtered and washed with DCM (3 × 3 mL). The

procedure was repeated one more time to ensure the *N*-terminus was fully reacted. 91 μL of Ac-Cys-OMe solution (226 mM) in DMF (20.7 μmol, 1.5 equiv.), DIPEA (9.6 μL, 55 μmol, 4 equiv.) and DMF (1.9 mL) were added into the reactor and the mixture was agitated at room temperature without protecting from light and atmosphere for 2 hours. Following HFIP/DCM cleavage from resin and removal of volatiles, the crude peptide was purified by reverse-phase HPLC with **Method 5** and lyophilized to afford peptide **5h** as a fluffy white solid (4.8 mg, 5.2 μmol, 38% yield based on the original resin loading).

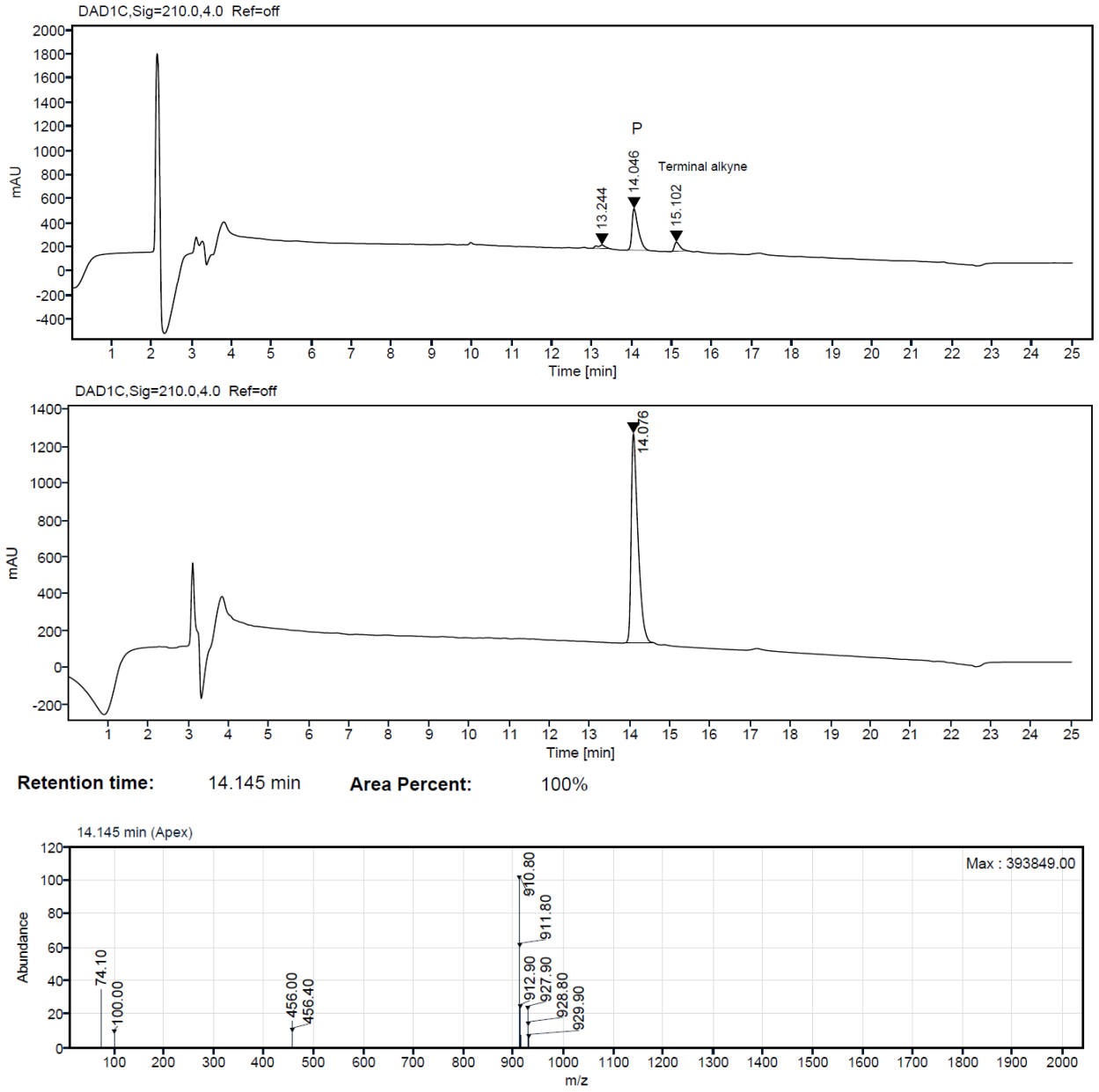
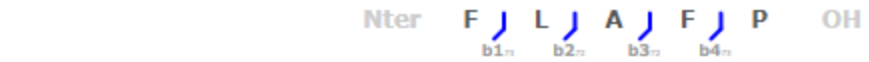


Figure S82: HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude and purified product **5h** by **Method 1.**

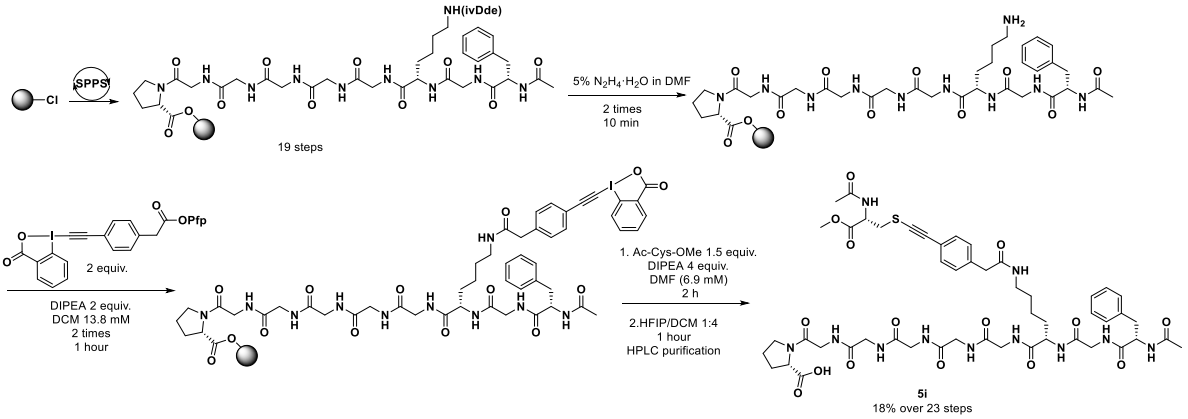
HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + Na]^+$ Calcd for $C_{48}H_{58}N_6NaO_{10}S^+$ 933.3827; Found 933.3805.

Table S44: MS/MS fragmentation of **5h**:

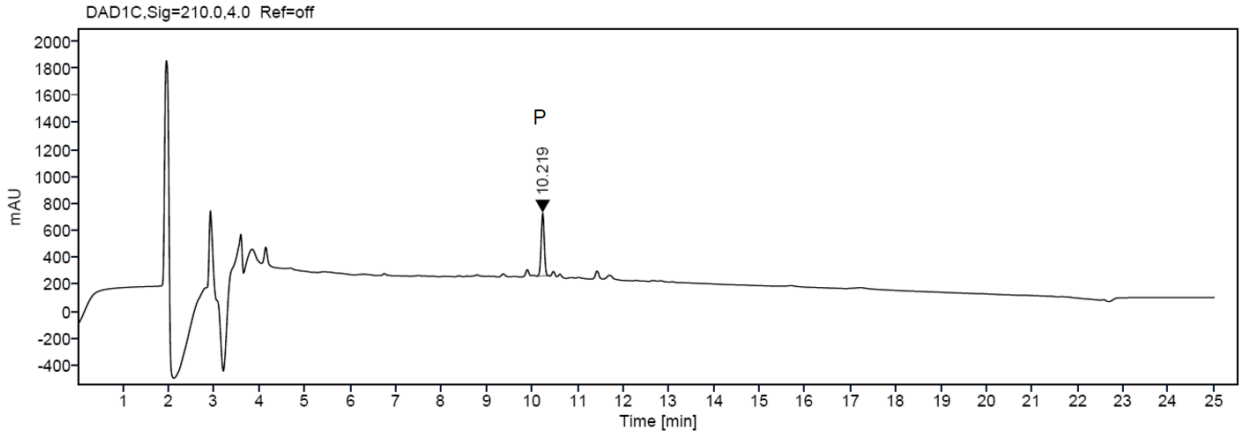


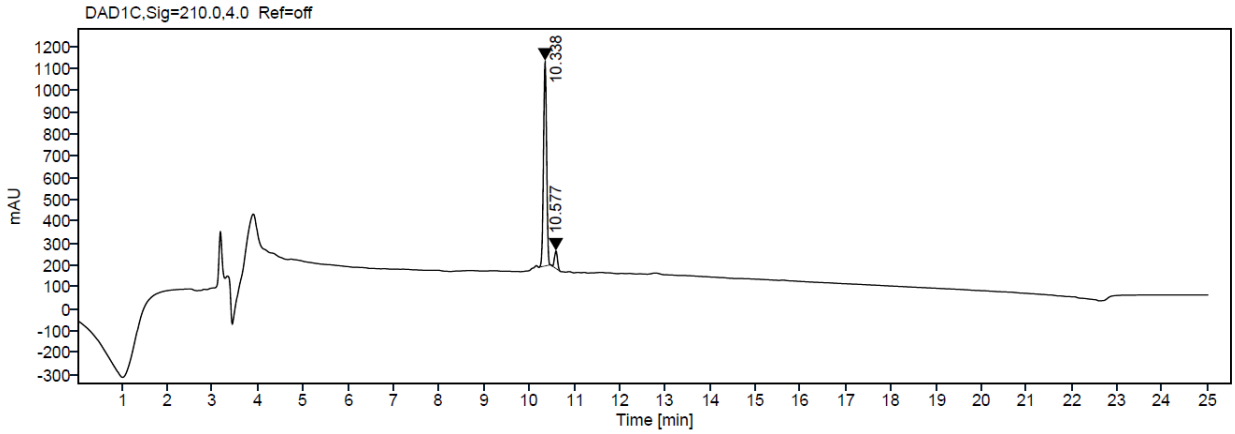
Nter = C16H16NO4S

Sequence	Type	MF	MF Mass	m/z	Intensity	Similarity
F	b1	C25H25N2O5S(+1)	465.1484	465.1479	1.01	73.27%
FLAF	b4	C43H50N5O8S(+1)	796.338	796.3375	102.18	73.13%
FLA	b3	C34H41N4O7S(+1)	649.2696	649.269	33.13	71.75%
FL	b2	C31H36N3O6S(+1)	578.2325	578.2319	5.3	71.58%



Peptide EBX was prepared on a 13.8 μmol scale from resin-bound substrate. The resin was treated with 1 mL 5% N_2H_4 H_2O in DMF and agitated for 10 min (2 times) to deprotect the Lys. At this moment, bifunctional EBX **1** (16 mg, 28 μmol, 2 equiv.) and DIPEA (9.5 μL, 55 μmol, 4 equiv.), DCM (2 mL) were then added to the resin and the mixture was shaken at room temperature for 1 hour. The resin was then filtered and washed with DCM (3 × 3 mL). The procedure was repeated one more time to ensure the *N*-terminus was fully reacted. 91 μL of Ac-Cys-OMe solution (226 mM) in DMF (20.7 μmol, 1.5 equiv.), DIPEA (9.6 μL, 55 μmol, 4 equiv.) and DMF (1.9 mL) were added into the reactor and the mixture was agitated at room temperature without protecting from light and atmosphere for 2 hours. Following HFIP/DCM cleavage from resin and removal of volatiles, the crude peptide was purified by reverse-phase HPLC with **Method 5** and lyophilized to afford **5i** as a fluffy white solid (2.7 mg, 2.4 μmol, 18% yield based on the original resin loading).





Retention time: 10.388 min **Area Percent:** 92%

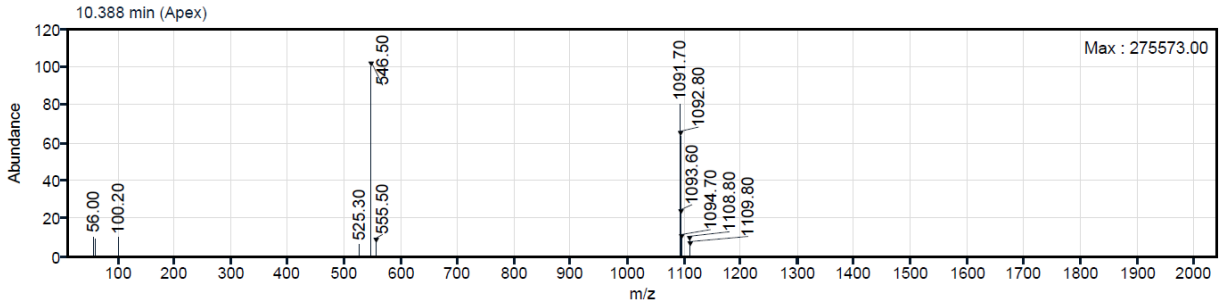


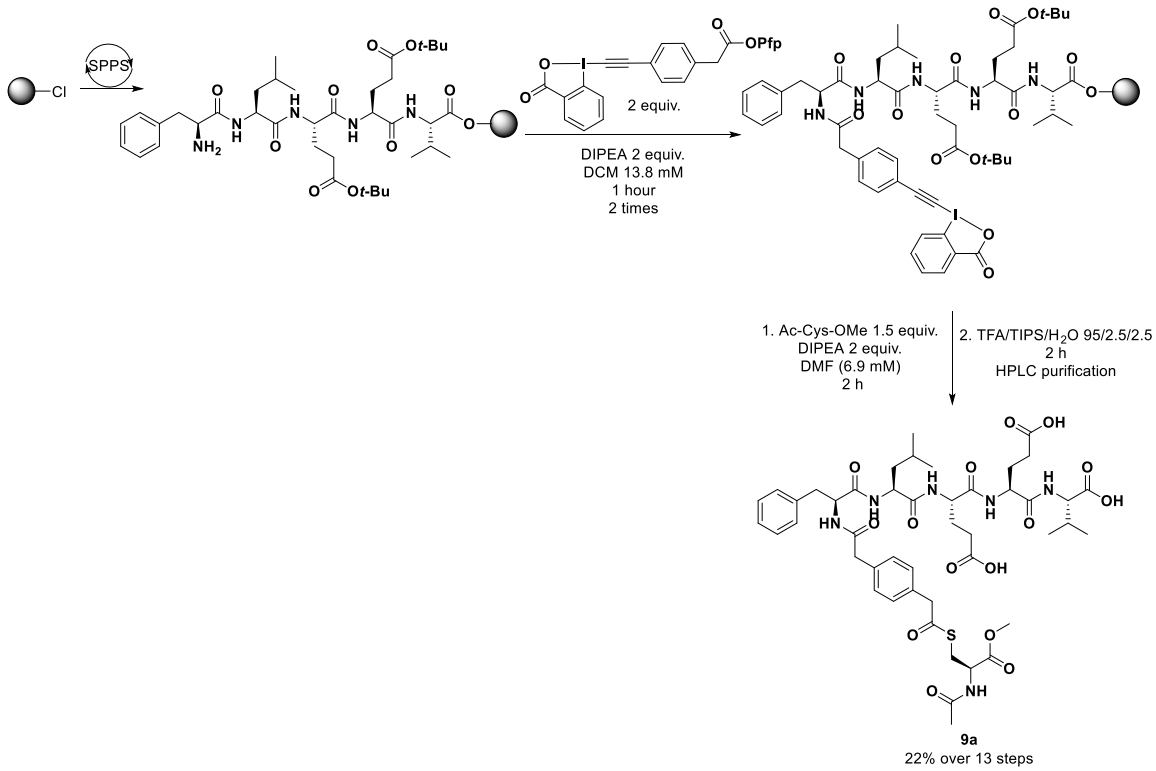
Figure S83: HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude and purified product **5i** by **Method 1.**

HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H_2]^{+2}$ Calcd for $C_{50}H_{67}N_{11}O_{15}S^{+2}$ 546.7264; Found 546.7256.

Table S45: MS/MS fragmentation of **5i**:

Sequence	Type	MF	MF Mass	m/z	Intensity	Similarity
GGGGGP	y6	C15H25N6O7(+1)	401.1785	401.1779	13.33	93.65%
GGP	y3	C9H16N3O4(+1)	230.1141	230.1135	1.64	93.04%
GP	y2	C7H13N2O3(+1)	173.0926	173.0921	2.19	91.27%
FGK	b3	C35H42N5O8S(+1)	692.2754	692.2749	3.57	90.25%
GGGP	y4	C11H19N4O5(+1)	287.1355	287.135	1.71	90.05%
F	a1	C10H12NO(+1)	162.0919	162.0913	0.55	89.13%
F	b1	C11H12NO2(+1)	190.0868	190.0863	0.57	87.97%
GKGGGGGP	y8	C39H55N10O13S(+1)	903.3671	903.3665	3.97	86.99%
GGGGP	y5	C13H22N5O6(+1)	344.157	344.1565	3.15	86.34%
FGKGGGGG	b8	C45H57N10O13S(+1)	977.3827	489.1947	5.43	85.87%
FGKGGGGG	b8	C45H57N10O13S(+1)	977.3827	977.3822	7.02	85.65%

GKGGGGGP	y8	C39H55N10O13S(+1)	903.3671	452.1869	15.64	83.12%
FGKGGGG	b7	C43H54N9O12S(+1)	920.3613	920.3607	3.1	81.84%
FGKGGG	b6	C41H51N8O11S(+1)	863.3398	863.3393	1.1	81.53%



Procedure:

Peptide EBX was prepared on a 13.8 μmol scale from resin-bound substrate. Bifunctional EBX (15.8 mg, 27.6 μmol, 2 equiv.) was added into the syringe reactor and dissolved in 1 mL DCM. Then DIPEA (9.6 μL, 55 μmol, 4 equiv.) was added into the reactor and the mixture was agitated at room temperature for 1 hour. The resin was then filtered and washed with DCM (3 × 3 mL). The procedure was repeated one more time to ensure the *N*-terminus was fully reacted. 91 μL of Ac-Cys-OMe solution (226 mM) in DMF (20.7 μmol, 1.5 equiv.), DIPEA (9.6 μL, 55 μmol, 4 equiv.) and DMF (1.9 mL) were added into the reactor and the mixture was agitated at room temperature without protecting from light and atmosphere for 2 hours. Resin was cleaved by using TFA/TIPS/H_2O (95/2.5/2.5) and the remaining solution was diluted with water and removed by lyophilization. The crude peptide was purified by reverse-phase HPLC with **Method 5** and lyophilized to afford **9a** as a fluffy white solid (2.9 mg, 3.0 μmol, 22% yield based on the original resin loading).

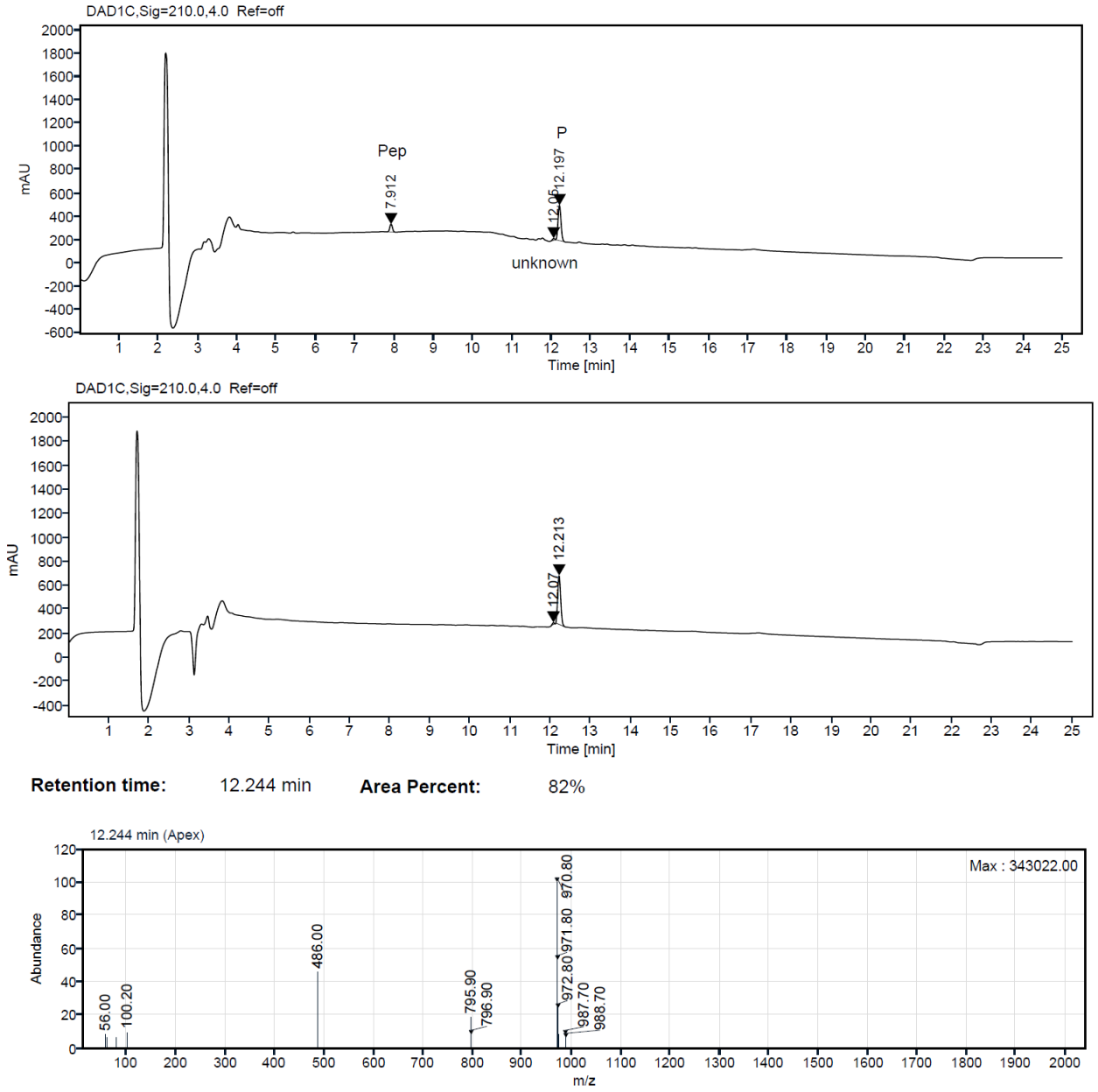
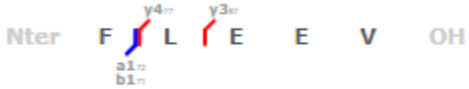


Figure S84: HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude and purified product **9a** by **Method 1.**

^{1}H NMR (500 MHz, CD_3OD) δ 7.18 (dddd, *J* = 22.0, 18.8, 9.7, 5.6 Hz, 7H), 7.07 (d, *J* = 6.7 Hz, 2H), 4.65 (dt, *J* = 11.2, 5.4 Hz, 1H), 4.56 (td, *J* = 7.5, 4.4 Hz, 1H), 4.45 (d, *J* = 7.1 Hz, 1H), 4.41 – 4.27 (m, 3H), 3.84 – 3.79 (m, 1H), 3.70 – 3.63 (m, 2H), 3.51 – 3.37 (m, 3H), 3.15 (pd, *J* = 10.0, 7.7, 4.1 Hz, 2H), 2.88 (ddd, *J* = 16.4, 11.0, 5.1 Hz, 1H), 2.59 – 2.54 (m, 1H), 2.49 – 2.29 (m, 4H), 2.14 (ddq, *J* = 36.5, 13.8, 6.3 Hz, 3H), 2.05 – 1.97 (m, 1H), 1.90 (q, *J* = 5.1, 3.6 Hz, 3H), 1.65 – 1.51 (m, 3H), 0.95 (h, *J* = 2.7 Hz, 7H), 0.94 – 0.84 (m, 7H).
^{13}C NMR (201 MHz, CD_3OD) δ 200.2, 197.9, 176.6, 174.7, 173.9, 173.9, 173.6, 173.5, 173.1, 173.3, 172.0, 138.3, 135.8, 133.6, 130.9, 130.4, 130.4, 129.5, 127.8, 59.2, 56.0, 56.0, 54.1, 53.9, 53.5, 53.4, 53.0, 50.6, 43.1, 41.6, 38.6, 31.7, 31.2, 28.5, 28.1, 26.7, 25.8, 23.5, 22.3, 22.0, 19.7, 18.4.

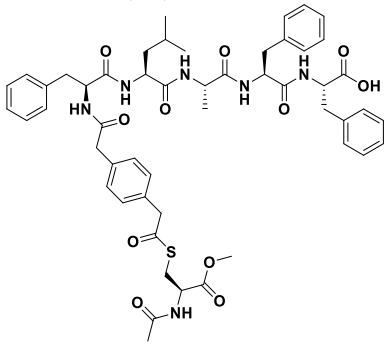
HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{46}H_{63}N_6O_{15}S^+$ 971.4067; Found 971.4075.

Table S46: MS/MS fragmentation of **9a**:



Nter = C16H18NO5S

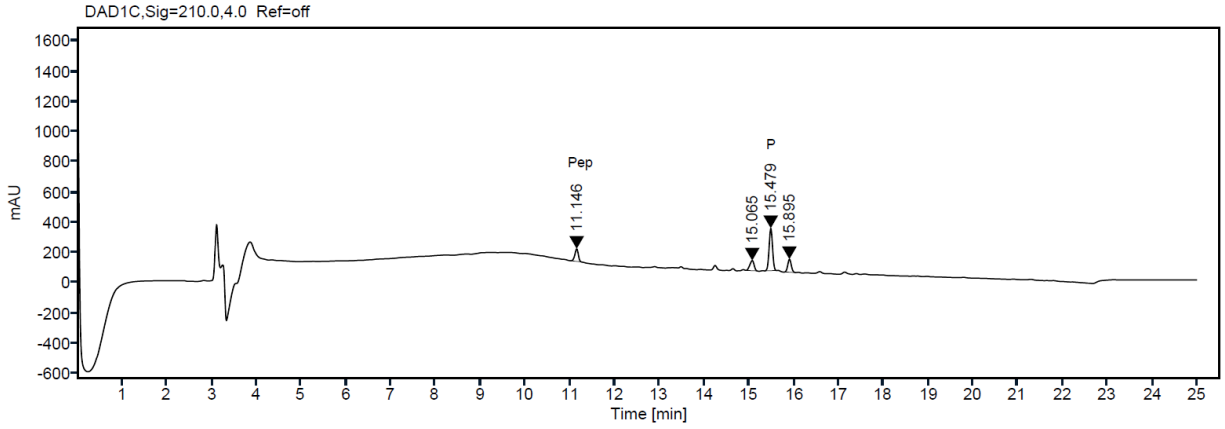
Sequence	Type	MF	MF Mass	m/z	Intensity	Similarity
EEV	y3	C15H26N3O8(+1)	376.172	376.1714	0.55	87.22%
LEEV	y4	C21H37N4O9(+1)	489.2561	489.2555	5.19	77.26%
F	a1	C24H27N2O5S(+1)	455.1641	455.1635	9.79	71.90%
F	b1	C25H27N2O6S(+1)	483.159	483.1584	31.02	71.10%



9b
19% over 13 steps

Procedure:

Following the same procedure as product **9b**: The crude peptide was purified by reverse-phase HPLC with **Method 5** and lyophilized to afford **9b** as a fluffy white solid (3.8 mg, 3.9 μmol, 19% yield based on the original resin loading).



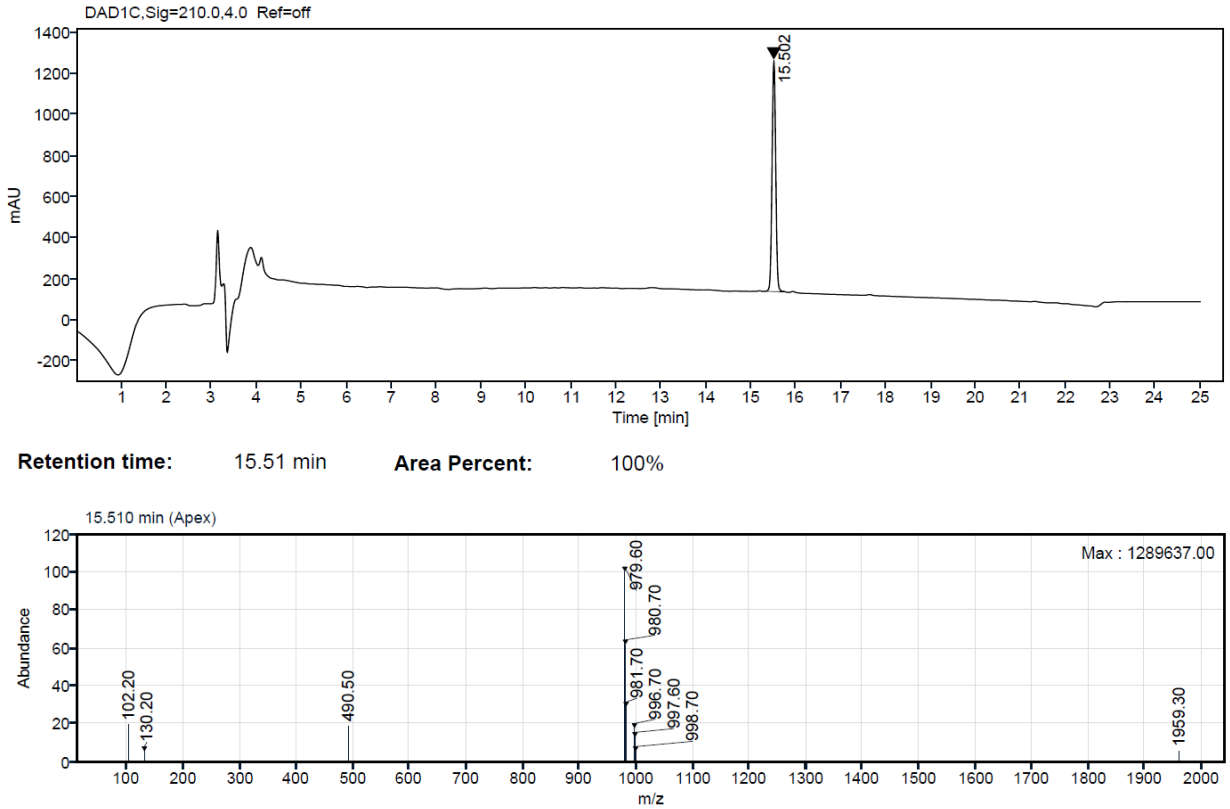


Figure S85: HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude and purified product **9b** by **Method 1.**

HRMS (Nanochip-based ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{52}H_{63}N_6O_{11}S^+$ 979.4270; Found 979.4273.

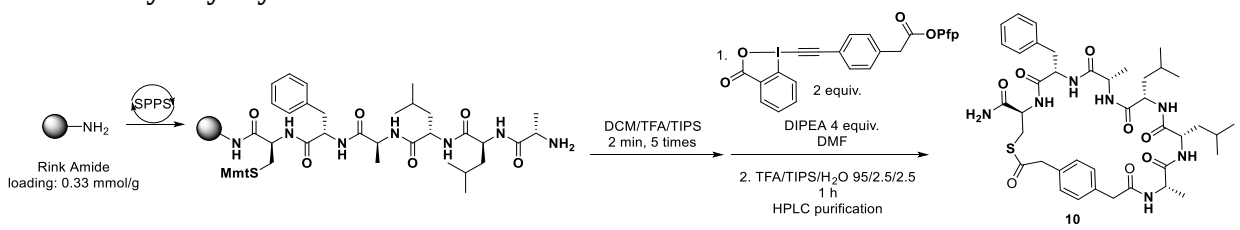
Table S47: MS/MS fragmentation of **9b**:

Nter = C16H18NO5S

Sequence	Type	MF	MF Mass	m/z	Intensity	Similarity
FF	y2	C18H21N2O3(+1)	313.1552	313.1547	0.88	92.59%
LAFF	y4	C27H37N4O5(+1)	497.2764	497.2758	9.52	90.54%
AFF	y3	C21H26N3O4(+1)	384.1923	384.1918	1.77	90.07%
FLAF	b4	C43H52N5O9S(+1)	814.3486	814.348	95.19	89.18%
FLAF	a4	C42H52N5O8S(+1)	786.3537	786.3531	8.44	88.59%
FLA	b3	C34H43N4O8S(+1)	667.2802	667.2796	101.38	88.39%
FLAFF		C52H62N6O11S	978.4197	979.427	10.26	88.24%
FL	b2	C31H38N3O7S(+1)	596.243	596.2425	44.2	88.17%
FLA	a3	C33H43N4O7S(+1)	639.2852	639.2847	1.28	88.01%

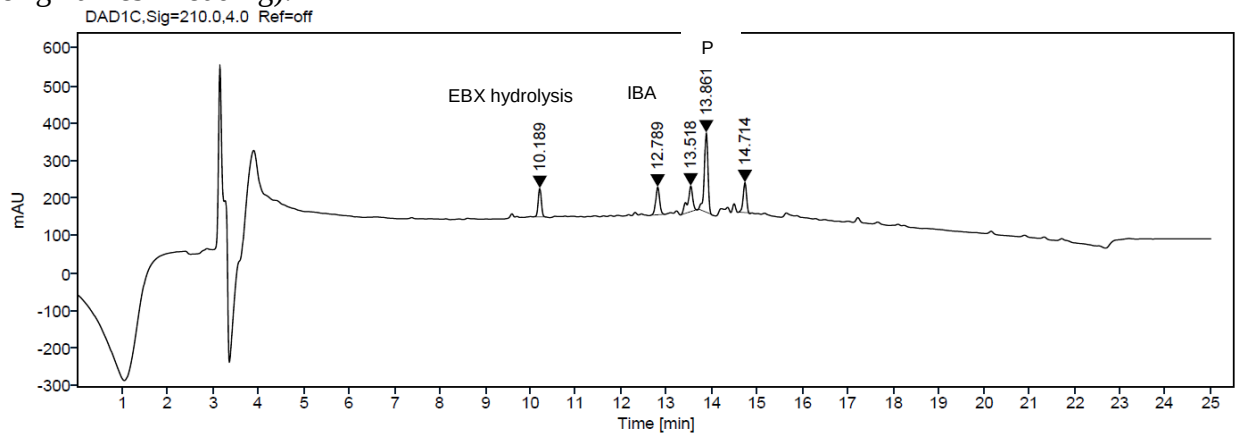
F	b1	C25H27N2O6S(+1)	483.159	483.1584	9.25	87.55%
F	a1	C24H27N2O5S(+1)	455.1641	455.1635	0.58	86.40%
FL	a2	C30H38N3O6S(+1)	568.2481	568.2476	1.33	85.85%

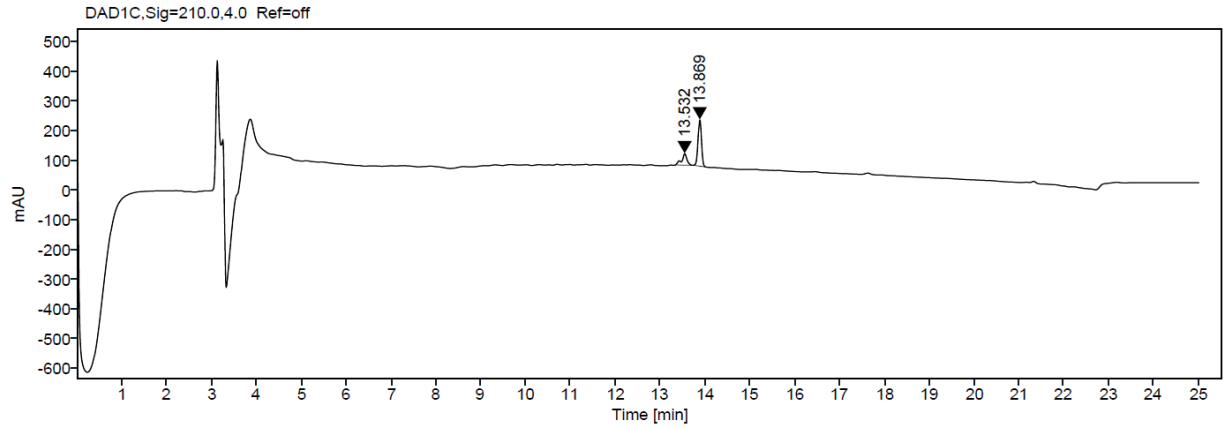
On resin Cys-Lys cyclization:



Procedure:

Peptide EBX was prepared on a 20 μmol scale from resin-bound substrate. After SPPS synthesis, the Mmt protecting group was removed by treating with 1 mL of DCM/TFA/TIPS (94:1:5) for 5 times, each time for 2 min. The resin was then washed with DCM (3 × 3 mL). Bifunctional EBX (22 mg, 40 μmol, 2 equiv.) was added into the syringe reactor and dissolved in 2 mL DMF. Then DIPEA (14 μL, 80 μmol, 4 equiv.) was added into the reactor and the mixture was agitated at room temperature for 2 hours. The resin was then filtered and washed with DCM (3 × 3 mL). Resin was cleaved by using TFA/TIPS/H_2O (95/2.5/2.5) for 1 hour and the remaining solution was diluted with water and removed by lyophilization. The crude peptide was purified by reverse-phase HPLC with **Method 6** and lyophilized to afford **10** (2.5 mg, 3.1 μmol, 16% yield) (yield based on the original resin loading).





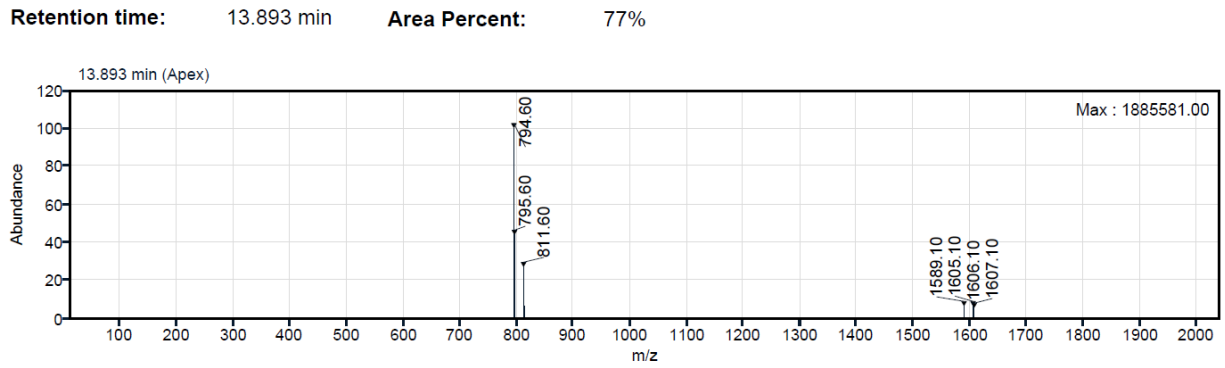
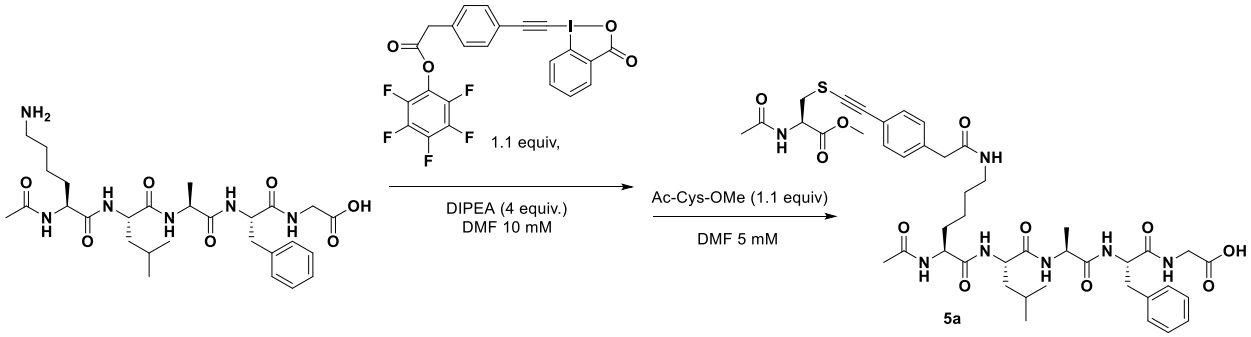


Figure S86: HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude and purified product **10** by **Method 1.**

HRMS (Nanochip-based ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{40}H_{56}N_7O_8S^+$ 794.3906; Found 794.3898.

10. Procedure for one-pot S-alkynylation of peptide:



Peptide AcKLAFG (8.0 μmol, 4.6 mg, 1 equiv.) was weighed on the analytical balance and dissolved in 200 μL non-degassed DMF. Afterwards, the bifunctional EBX reagent (5.0 mg, 8.8 μmol, 1.1 equiv.), DIPEA (5.7 μL, 32 μmol, 4 equiv.) and 600 μL DMF were added into the reaction solution (concentration: 10 mM) and it was stirred for 20 min without protecting from the atmosphere. 166 μL of a solution of Ac-Cys-OMe (1.1 equiv., 50 mM in DMF) and 634 μL DMF (concentration: 5 mM) were placed into a 2 mL vial and the reaction mixture was stirred for 1 hour without protection of light or atmosphere. The desired product **5a** was isolated by Prep-RP-HPLC as a white fluffy solid (34% yield, 2.4 mg, 2.7 μmol).

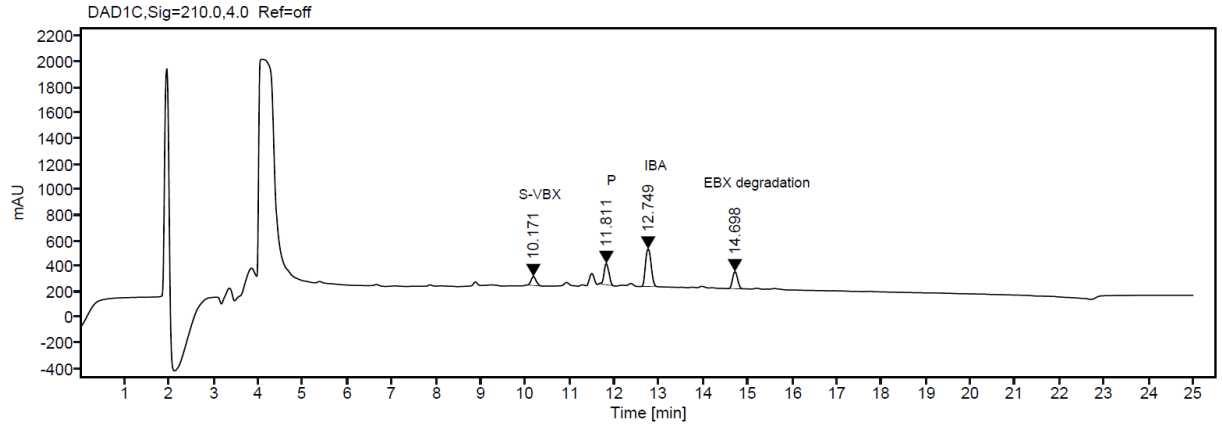
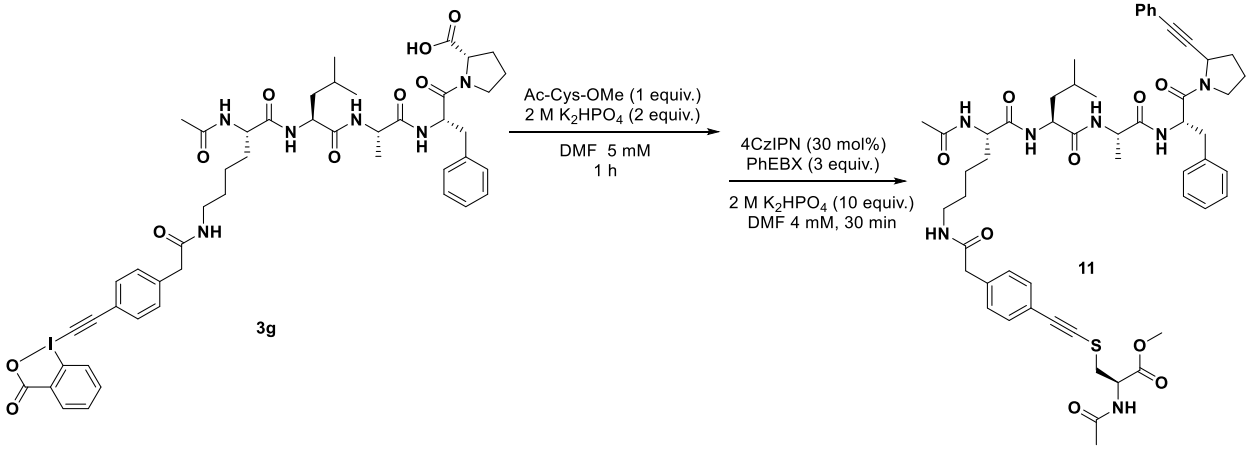


Figure S87: HPLC-UV chromatogram (210 nm) of the crude by **Method 1.**

11. Procedure for one-pot dual functionalization of peptide-EBX:

EBX of AcKLAFP **3g** (5 mg, 5 μmol, 1 equiv.) was weighed on the analytical balance and dissolved in 200 μL non-degassed DMF. Afterwards, 100 μL of a solution of Ac-Cys-OMe (1 equiv., 50 mM in DMF), 5 μL 2M K_2HPO_4 (2 equiv.) in milli-Q purified water and 685 μL DMF were placed into a 2 mL vial and the reaction mixture was stirred for 1 hour without protection of light or atmosphere. Then 214 μL of PhEBX (70 mM in DMF), 60 μL of as solution of CzIPN (30 mol%, 25 mM in DMF) and 20 μL 2 M K_2HPO_4 (8 equiv.) in milli-Q purified water were added into the reaction mixture. The vial was then capped and degassed by bubbling with N_2 for 20 min. The reaction was stirred under Blue LEDs irradiation for 30 min at RT.

At the end of the reaction, the crude was injected in RP-HPLC. The desired product **11** (2.1 mg, 2.1 μmol, 42% yield) was isolated by Prep-RP-HPLC with **Method 5**.



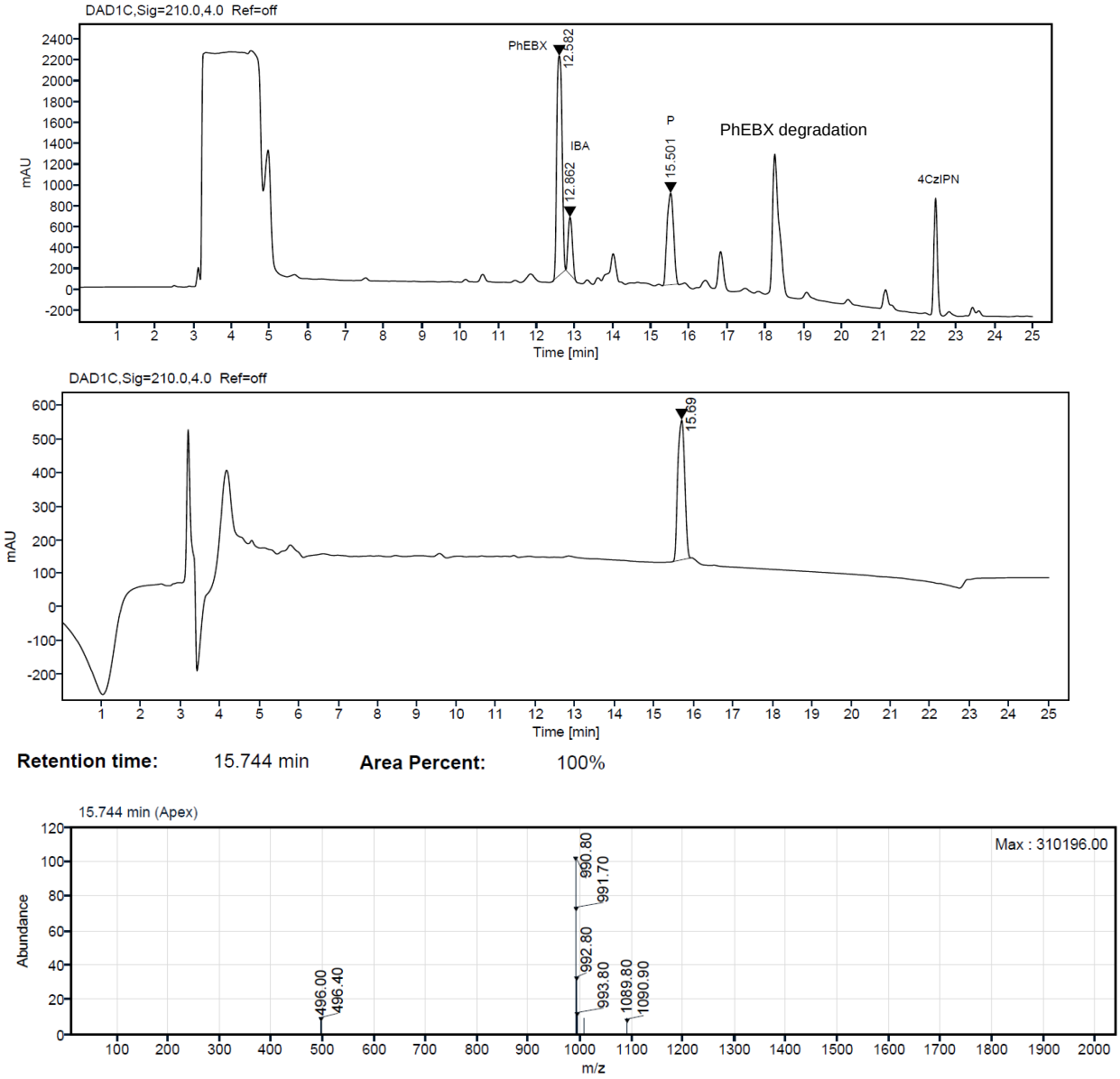
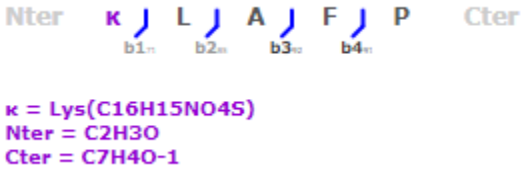


Figure S88: HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude and purified product **11** by **Method 1.**

HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H_2]^{+2}$ Calcd for $C_{54}H_{69}N_7O_9S^{+2}$ 495.7433; Found 495.7426.

Table S48: MS/MS fragmentation of **11**:

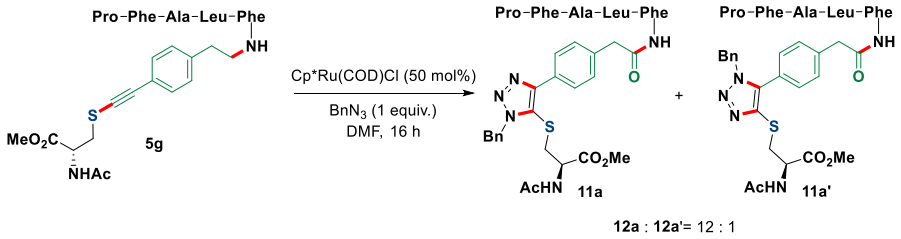


Sequence	Type	MF	MF Mass	m/z	Intensity	Similarity

KLAF	b4	C42H55N6O9S(+1)	819.3751	819.3746	17.48	92.61%
KLA	b3	C33H46N5O8S(+1)	672.3067	672.3062	2.42	92.12%
KLAF	b4	C42H55N6O9S(+1)	819.3751	410.1909	3.45	88.90%
KL	b2	C30H41N4O7S(+1)	601.2696	601.269	0.6	85.49%
K	b1	C24H30N3O6S(+1)	488.1855	488.185	0.68	71.46%

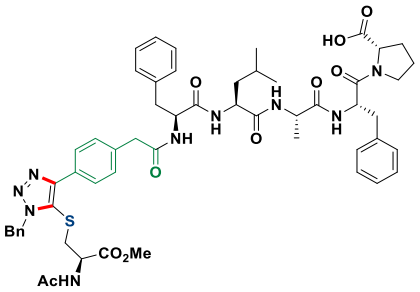
12. RuAtAC of S-alkynylation products

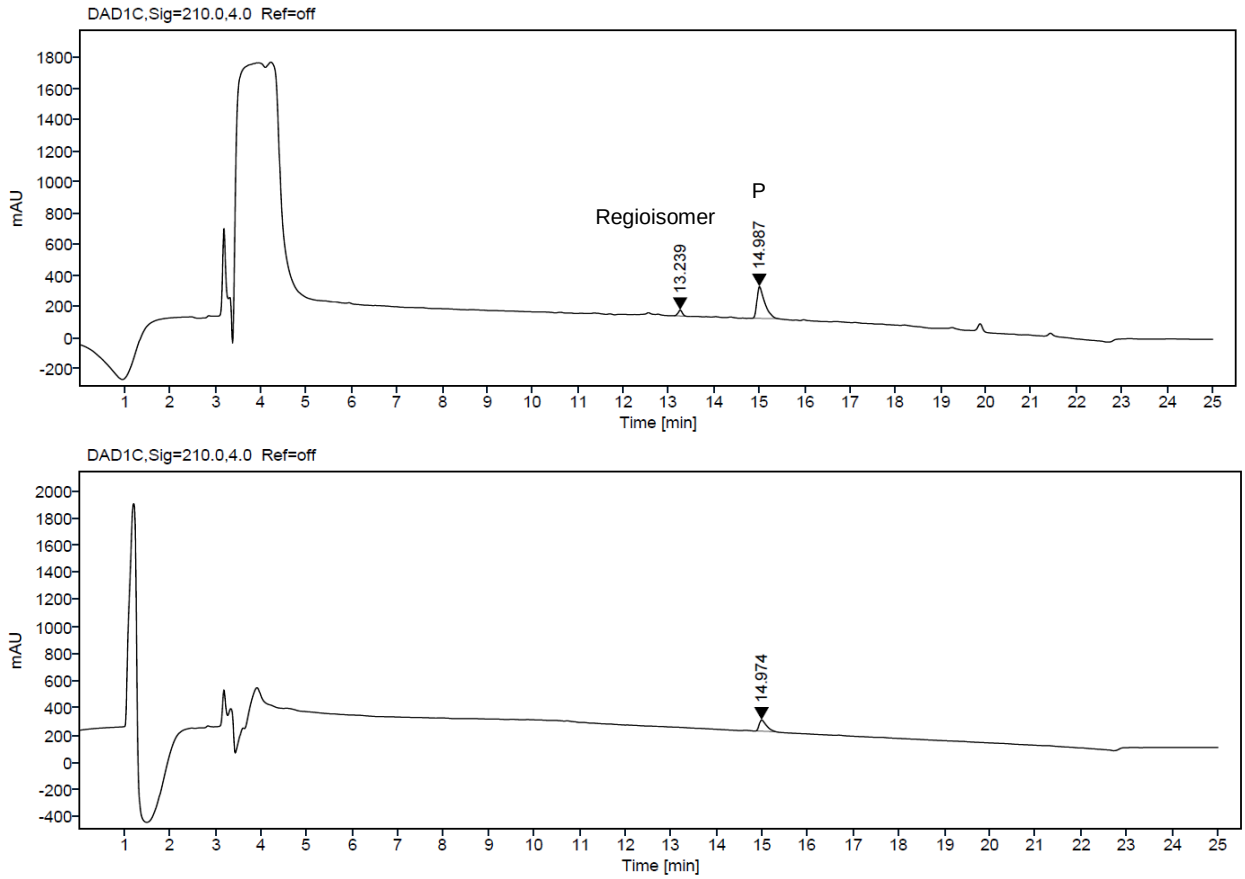
RuAtAC product of FLAFP S-alkynylation product (12a)



Procedure:

S-alkynylation product **5g** (3.0 mg, 3.2 μmol, 1.0 equiv.) was weighed on the analytical balance in an eppendorf. 50 μL of 32 mM solution of Cp*Ru(cod)Cl (1.6 μmol, 50 mol%) in DMF and 32 μL of 10 mM solution of (azidomethyl)benzene (3.2 μmol, 1.0 equiv.) in DMF were added. Both solutions were prepared by using degassed DMF. Then the reaction mixture was further diluted with 118 μL DMF (concentration: 16.5 mM). The reaction vessel was sealed with parafilm and shaken for 16 h under air. At the end of the reaction, 5 μL of the crude was diluted with 45 μL of MeCN and injected in RP-HPLC. HPLC ratio (210 nm) of the product: > 95% **12a**: **12a'**= 12: 1. The desired product **12a** (major regioisomer) was isolated by RP-HPLC by **Method 5** (2.1 mg, 2.0 μmol, 61%) as purple fluffy solid.





Retention time: 15.016 min **Area Percent:** 100%

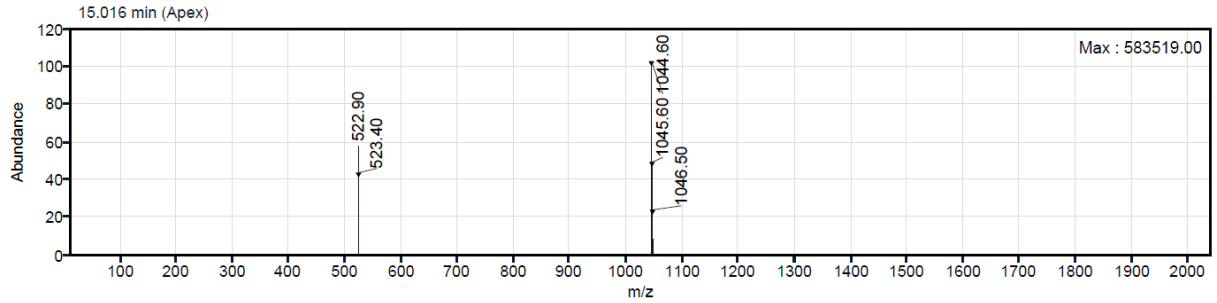
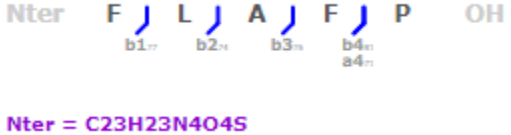


Figure S89: HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude and purified product **12a** by **Method 1.**
HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{55}H_{66}N_9O_{10}S^+$ 1044.4648; Found 1044.4627.

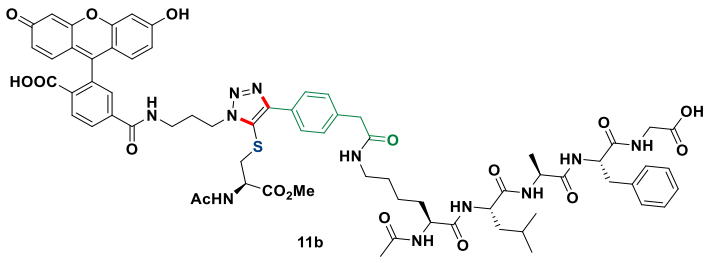
Table S49: MS/MS fragmentation of **12a**:



Nter = C23H23N4O4S

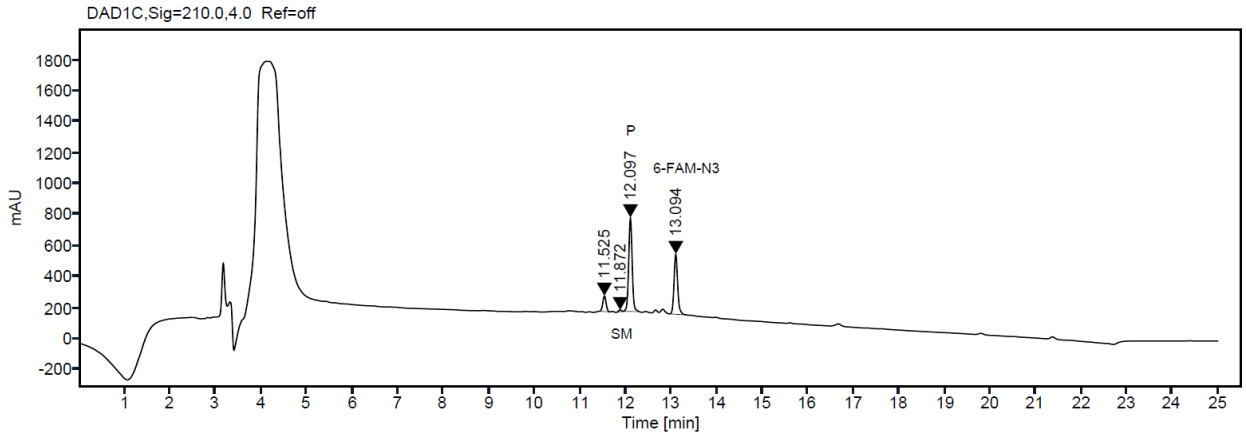
Sequence	Type	MF	MF Mass	m/z	Intensity	Similarity
FLAF	b4	C50H57N8O8S(+1)	929.402	929.4015	100.23	81.29%
F	b1	C32H32N5O5S(+1)	598.2124	598.2119	1.36	77.04%
FLA	b3	C41H48N7O7S(+1)	782.3336	782.333	16.87	76.01%
FL	b2	C38H43N6O6S(+1)	711.2965	711.2959	4.1	74.02%
FLAF	a4	C49H57N8O7S(+1)	901.4071	901.4065	11.71	70.81%

RuAtAC product of KLAFG S-alkynylation product (12b)



Procedure:

S-alkynylation product **5a** (0.2 mg, 0.2 μmol, 1.0 equiv.) was weighed on the analytical balance in an eppendorf. 10 μL of 10 mM solution of Cp*Ru(cod)Cl (0.1 μmol, 50 mol%) in DMF and 12 μL of 10 mM solution of 6-FAM-N_3 (0.12 μmol, 1.2 equiv.) in DMF were added. Both solutions were prepared by using degassed DMF. Then the reaction mixture was further diluted with 28 μL DMF (concentration: 4 mM). The reaction vessel was sealed with parafilm and shaken for 16 h under air. At the end of the reaction, 5 μL of the crude was diluted with 45 μL of MeCN and injected in RP-HPLC. HPLC ratio (210 nm) of the product **12b**: 98%.



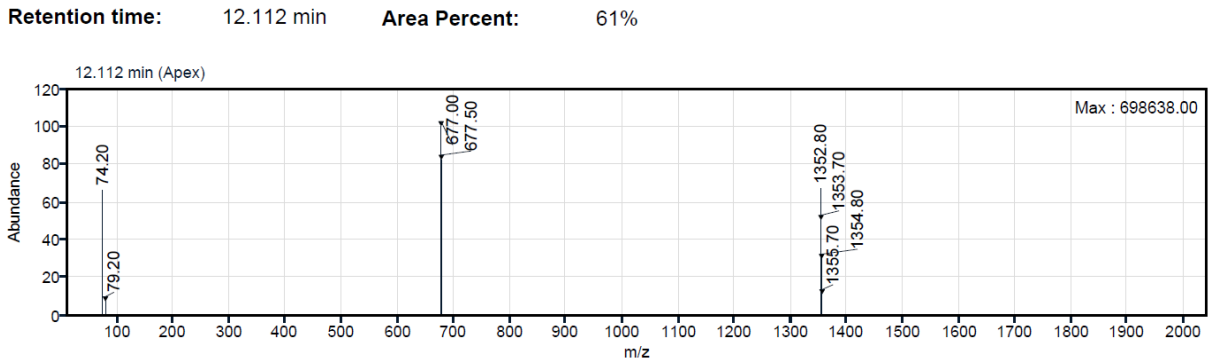
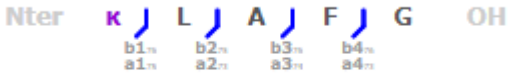


Figure S90: HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude by **Method 1.** HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{68}H_{78}N_{11}O_{17}S^+$ 1352.5292; Found 1352.5292.

Table S50: MS/MS fragmentation of **12b**:



κ = Lys(C40H33N5O10S)
Nter = C2H3O

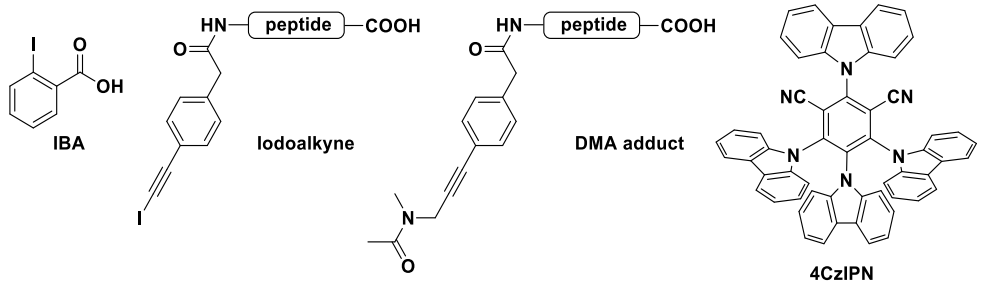
Sequence	Type	MF	MF Mass	m/z	Intensity	Similarity
K	b1	C48H48N7O12S(+1)	946.3082	946.3076	76.48	76.46%
KLAF	b4	C66H73N10O15S(+1)	1277.498	1277.497	36.23	76.17%
KLA	b3	C57H64N9O14S(+1)	1130.429	1130.429	58.35	75.57%
K	a1	C47H48N7O11S(+1)	918.3133	918.3127	5.28	75.20%
KL	b2	C54H59N8O13S(+1)	1059.392	1059.392	44.67	74.98%
KLA	a3	C56H64N9O13S(+1)	1102.434	1102.434	2.94	73.71%
KL	a2	C53H59N8O12S(+1)	1031.397	1031.397	4.64	72.88%
KLAF	a4	C65H73N10O14S(+1)	1249.503	1249.502	13.48	72.59%

13. Peptide macrocyclization:

General Procedure:

Peptide-EBX (0.01 mmol) were weighed on the analytical balance and dissolved in 830 μL non-degassed DMA in a 2 mL vial. Afterwards, 120 μL of CzIPN solution (3 μmol, 0.3 equiv.) (25 mM in DMA), 50 μL 2 M K_2HPO_4 (0.10 mmol, 10 equiv.) in milli-Q purified water were added (concentration: 10 mM). The vial was then capped and degassed by bubbling with N_2 for 20 min. The reaction was stirring under Blue LEDs irradiation for 30 min at RT. The cyclic peptides were isolated by Prep-RP-HPLC, followed by lyophilization.

Common side-products that can be observed in HPLC:



13.1 Calibration of the cyclization reaction

Absorbance (mAU) versus concentration (mM) of the cyclic peptide Ac-KLAFP (**13a**)

Conc. (mM)	Absorbance (mAU)
0.5	1261.93
1	2071.25
1.5	3535.31
2	6247.10

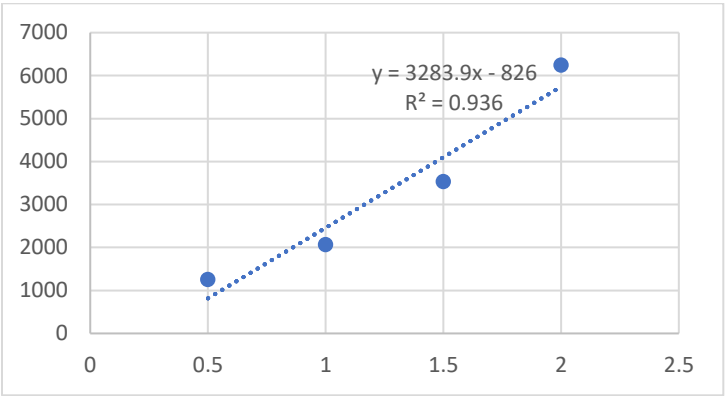
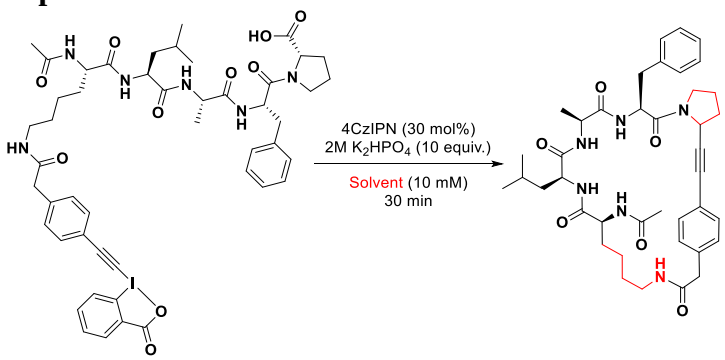


Figure S91: Linear equation of the absorbance (mAU) versus concentration (mM) of the cyclic peptide (Ac-KLAFP)

Table S51: Reaction optimization:



a. Solvent optimization

Using DMF as the reaction solvent resulted in more by-product for other substrates. Therefore, DMA was chosen as the optimal solvent.

Table S51: Solvent optimization.

Solvent	Absorbance (mAU)	Yield (%)

DMSO	876.98	52
DMF	1192.41	61
DMA	1111.11	59
MeCN	369.33	36

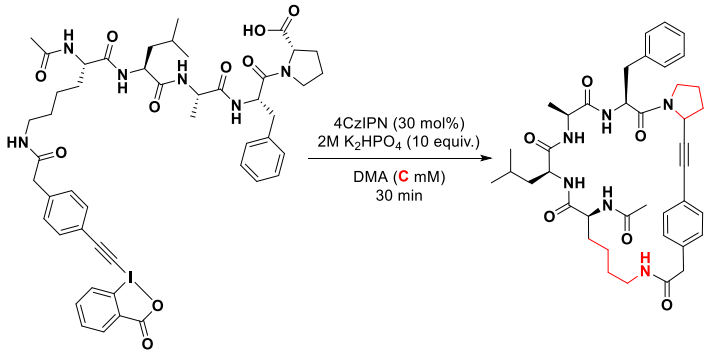


Table S52: Concentration optimization

Conc. (mM)	Absorbance (mAU)	Yield (%)
10	1111.11	59
2.5	361.30	36
5	745.87	48
20	819.92	50

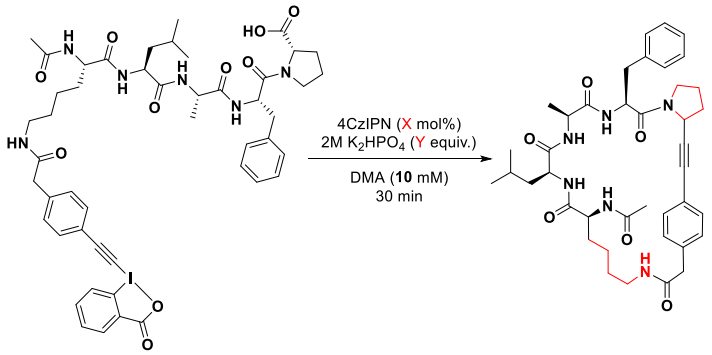


Table S53: Catalyst loading and base amount optimization

Catalyst loading (mol%)	Base (equiv.)	Absorbance (mAU)	Yield (%)
30	5	910.53	53
15	10	863.71	51

13.2 Scope of the peptide macrocyclization

HPLC UV ratio was determined by taking the ration of A_{prod}/A_{total} where A_{prod} = area in mAU of the product peak and A_{total} = area in mAU of the combined peptide containing species (product, DMA adduct, iodoalkyne). The dr of the product was determined by the HPLC UV ratio of the reaction crude.

Cyclic peptide of Ac-KLAFP (13a)

Following the general procedure, the reaction was conducted in 0.01 mmol scale. HPLC ratio (210 nm) of the product: > 95%. The dr was not determined. The desired product **13a** (3.2 mg, 4.5 μmol, 45% yield) was isolated as diastereomers (dr 1.7:1 by ^{1}H NMR of isolated product) by **Method 5**.

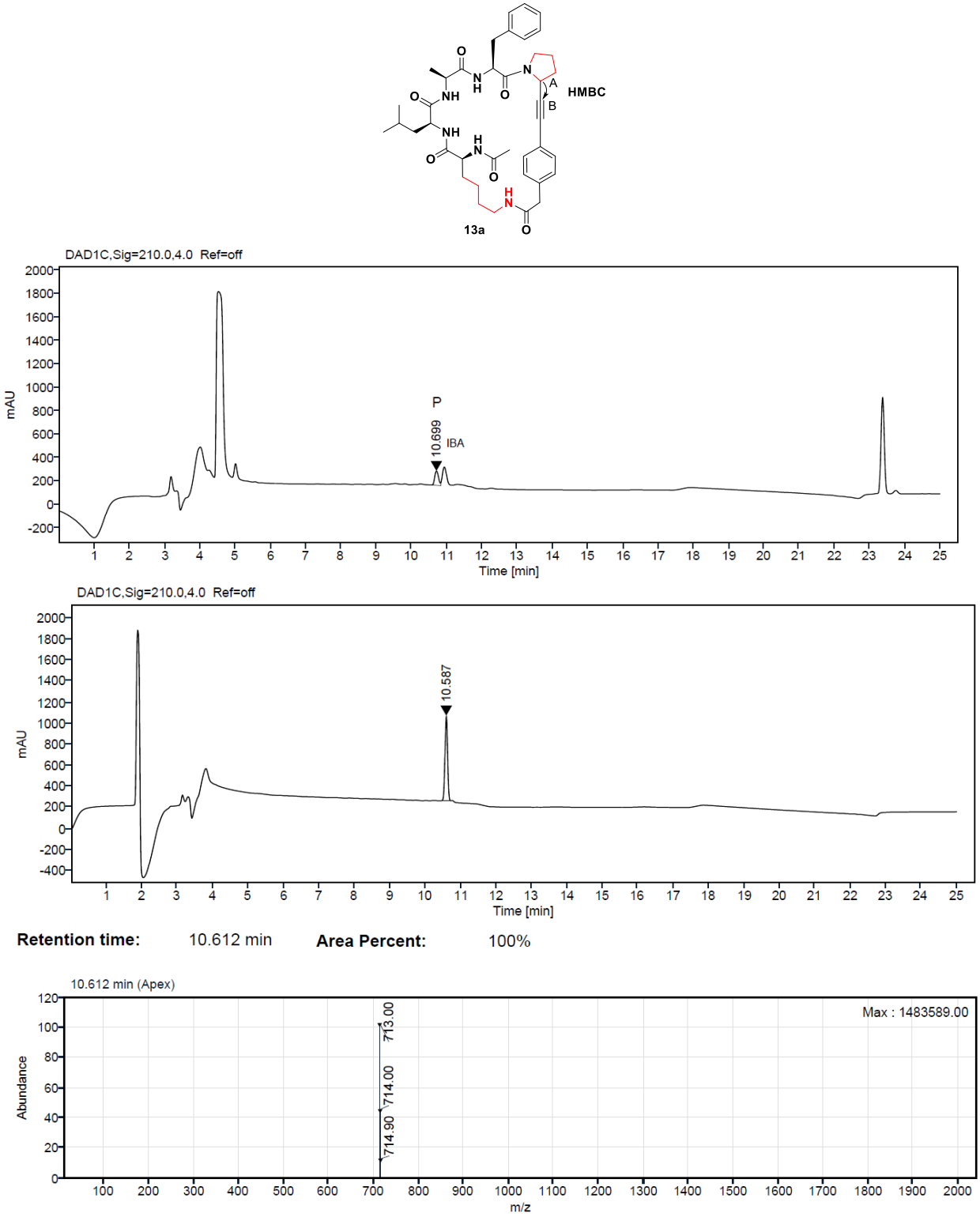
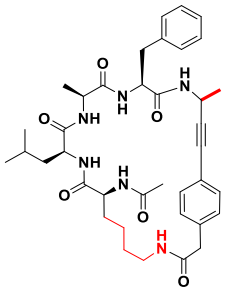


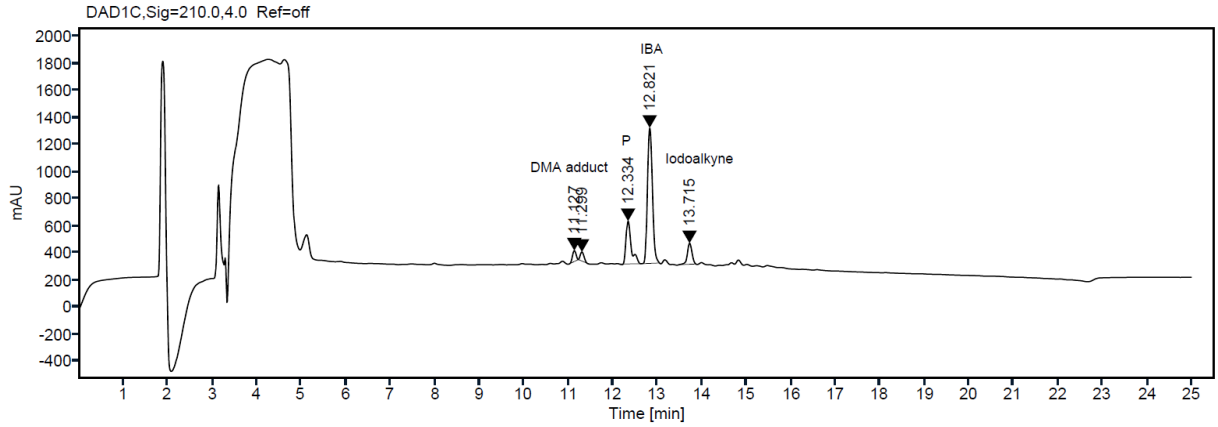
Figure S92: HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude and purified product **13a** by **Method 1.**

^{1}H NMR (600 MHz, DMSO-d_6 (dr 1.7:1, only one was resolved)) δ 7.45 (dd, *J* = 26.9, 7.4 Hz, 1H), 7.34 – 7.26 (m, 3H), 7.25 – 7.19 (m, 5H), 5.04 (q, *J* = 7.5 Hz, 1H), 4.33 (p, *J* = 6.9 Hz, 1H), 4.25 – 4.18 (m, 2H), 4.19 – 4.12 (m, 1H), 4.06 (dd, *J* = 7.3, 2.6 Hz, 1H), 3.44 (ddt, *J* = 12.6, 7.5, 3.6 Hz, 1H), 3.22 – 3.15 (m, 1H), 3.12 – 3.02 (m, 1H), 3.00 – 2.91 (m, 2H), 2.87 (dd, *J* = 13.0, 8.0 Hz, 1H), 2.00 (dd, *J* = 18.4, 9.1 Hz, 1H), 1.90 (ddt, *J* = 9.4, 6.2, 3.4 Hz, 1H), 1.82 (d, *J* = 2.7 Hz, 3H), 1.75 – 1.68 (m, 1H), 1.60 – 1.53 (m, 1H), 1.49 (dq, *J* = 9.8, 4.7 Hz, 2H), 1.45 – 1.37 (m, 2H), 1.35 – 1.29 (m, 1H), 1.22 (q, *J* = 7.5 Hz, 2H), 1.12 (d, *J* = 7.0 Hz, 2H), 1.01 (d, *J* = 6.9 Hz, 1H), 0.85 (dd, *J* = 17.5, 6.3 Hz, 3H), 0.79 (dd, *J* = 17.2, 6.1 Hz, 3H).
^{13}C NMR (201 MHz, DMSO-d_6 (mixture of two diastereomers)) δ 172.3, 172.1, 171.4, 171.2, 170.9, 169.9, 169.8, 169.3, 169.2, 168.8, 137.7, 137.3, 137.3, 136.9, 131.6, 131.3, 129.2, 128.6, 128.6, 128.4, 128.2, 126.8, 126.6, 120.5, 119.7, 89.6, 87.9, 83.4, 81.5, 52.4, 51.9, 51.9, 51.8, 50.8, 50.1, 48.2, 47.9, 47.8, 47.7, 45.5, 45.4, 43.0, 42.7, 39.0, 38.1, 37.8, 36.9, 33.5, 31.8, 31.6, 28.8, 28.3, 24.3, 24.1, 23.1, 23.0, 22.5, 22.5, 22.4, 22.1, 21.9, 21.6, 21.3, 18.9, 18.6.
HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{40}H_{53}N_6O_6^+$ 713.4021; Found 713.4024.

Cyclic peptide of Ac-KLAFA (13b)

Following the general procedure, the reaction was conducted in 0.01 mmol scale. HPLC ratio (210 nm) of the product: 56%. The dr was not determined. The desired product **13b** (2.1 mg, 3.1 μmol, 31% yield) was isolated by **Method 5**.





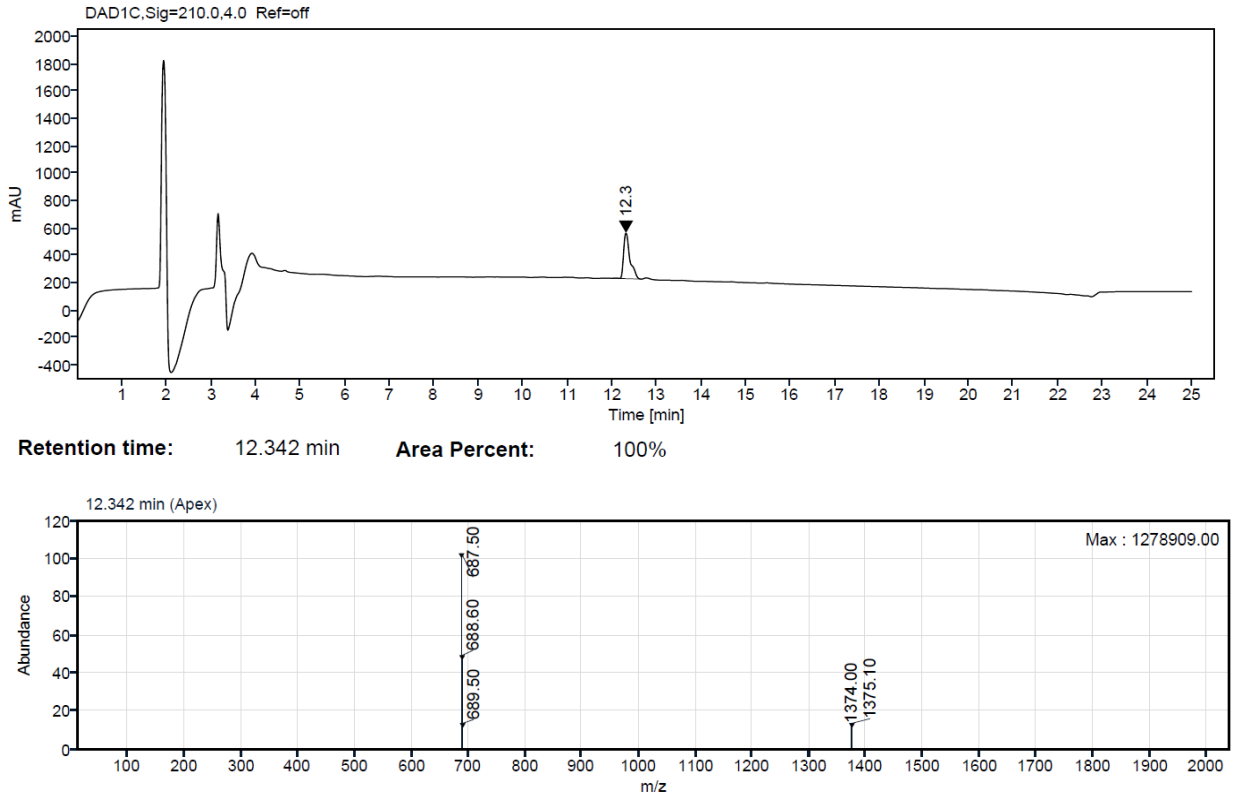
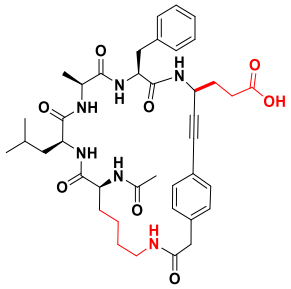


Figure S93: HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude and purified product **13b** by **Method 1.**

HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{38}H_{51}N_6O_6^+$ 687.3865; Found 687.3866.

Cyclic peptide of Ac-KLAFE (13c)

Following the general procedure, the reaction was conducted in 0.01 mmol scale. HPLC ratio (210 nm) of the product: > 95% (dr 2.3:1). The desired product **13c** (2.2 mg, 2.8 μmol, 28% yield) (dr 1:1.8) was isolated by **Method 5**.



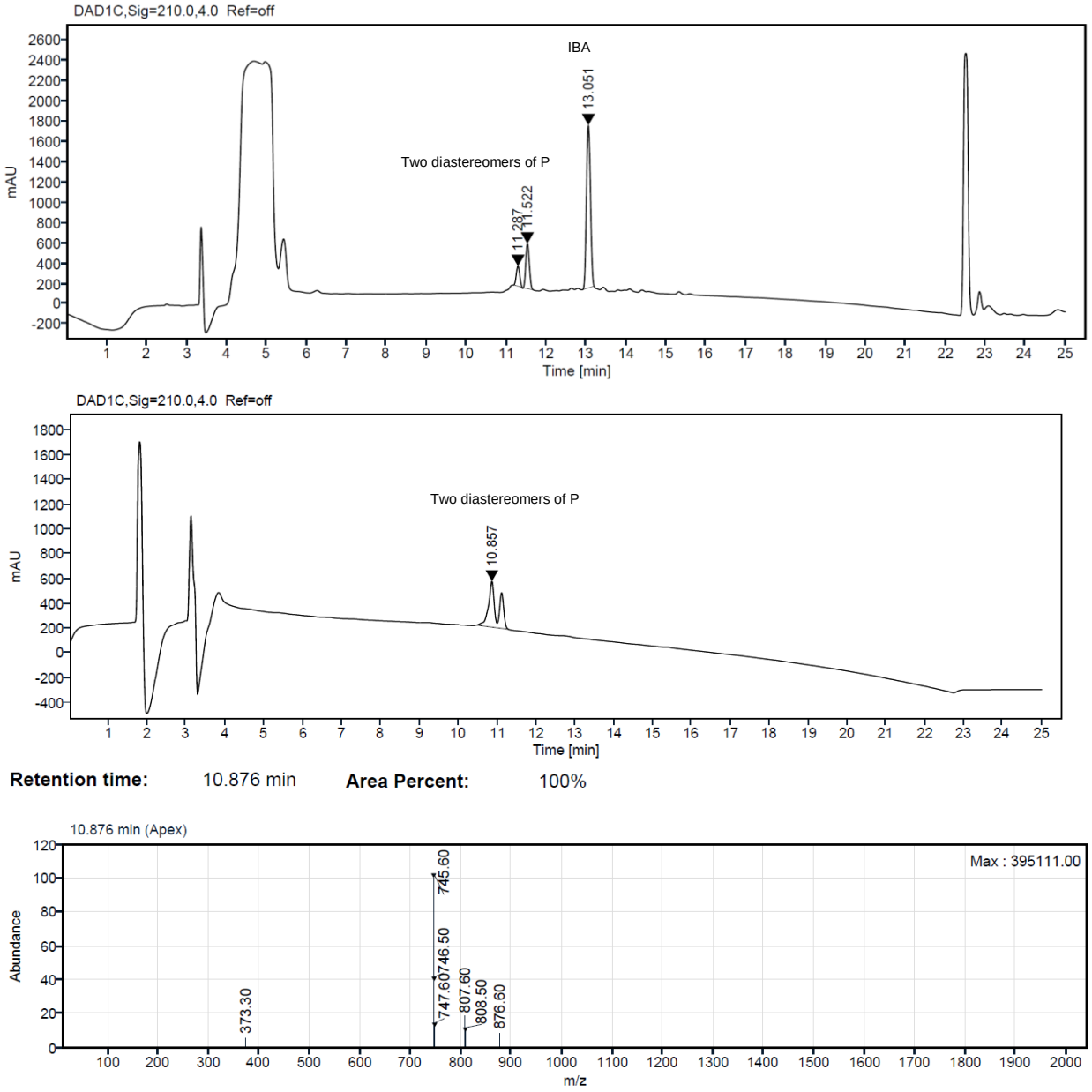


Figure S94: HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude and purified product **13c** by **Method 1.**

HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{40}H_{53}N_6O_8^+$ 745.3919; Found 745.3919.

Cyclic peptide of Ac-KLAFF (13d)

Following the general procedure, the reaction was conducted in 0.01 mmol scale. HPLC ratio (210 nm) of the product: 62%. dr was not determined. The desired product **13d** (2.1 mg, 2.7 μmol, 27% yield) was isolated by **Method 5**.

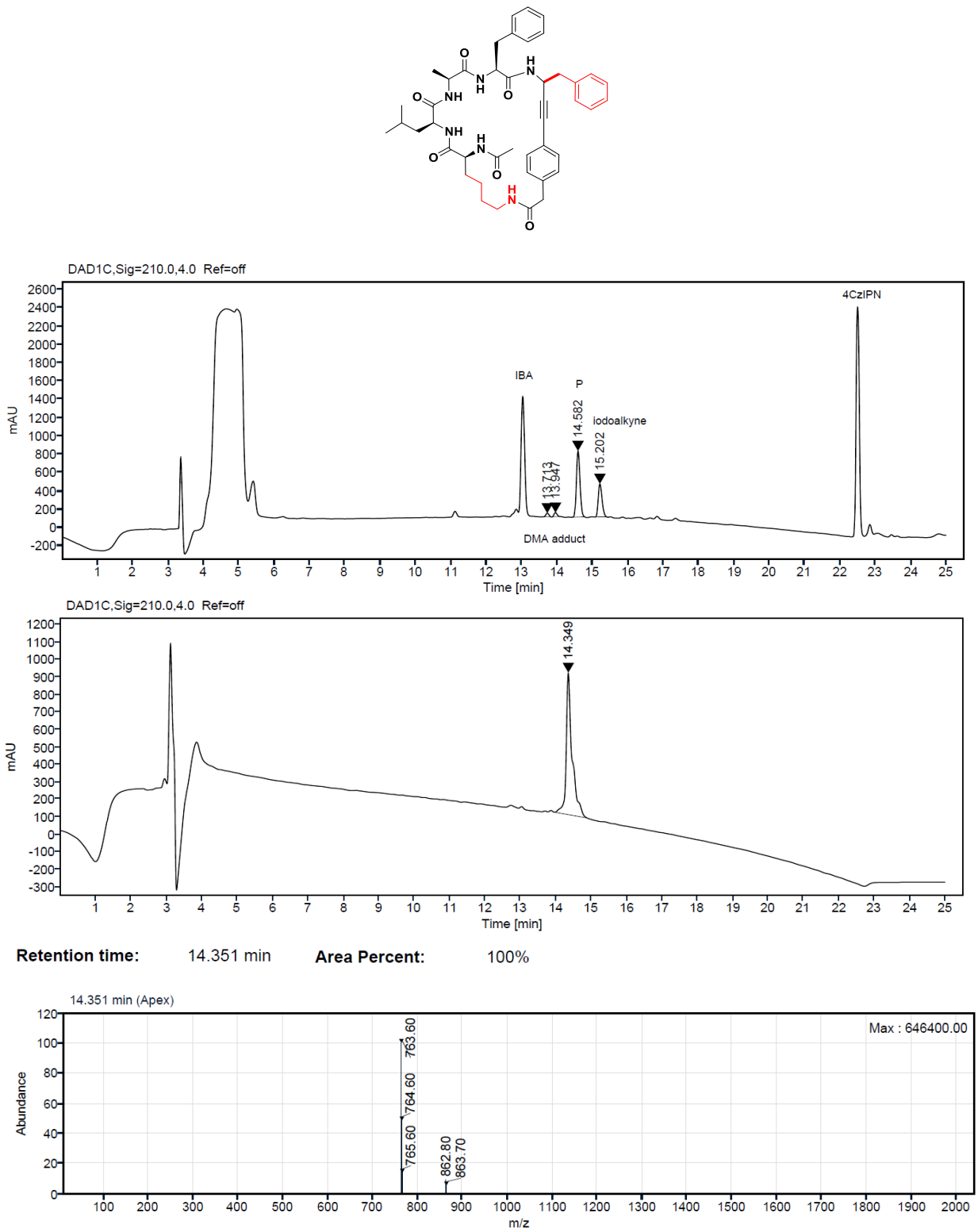
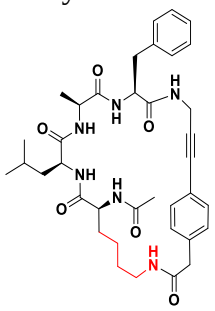


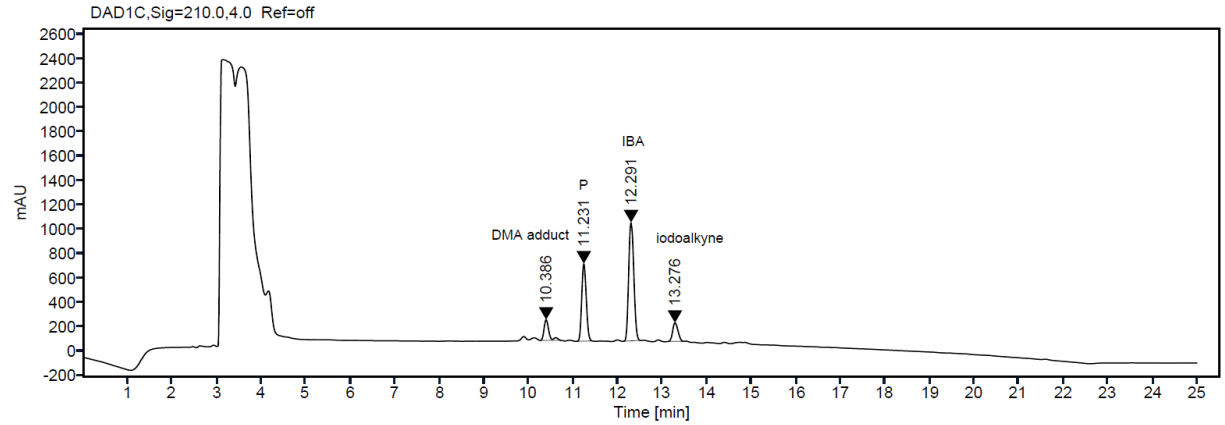
Figure S95: HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude and purified product **13d** by **Method 1.**

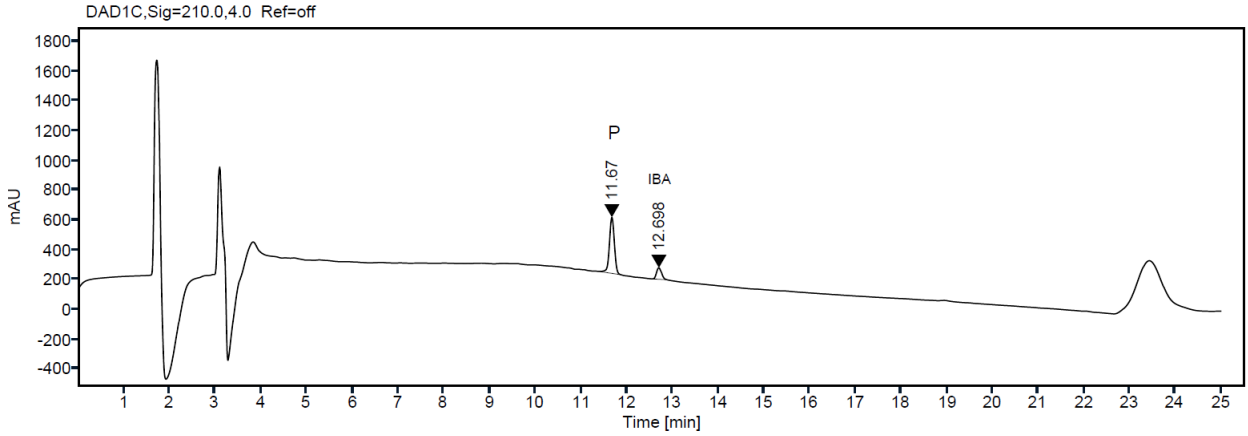
HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{44}H_{55}N_6O_6^+$ 763.4178; Found 763.4184.

Cyclic peptide of Ac-KLAFG (13e)

Following the general procedure (4-Cl-CzIPN was used instead of 4CzIPN), the reaction was conducted in 0.01 mmol scale. HPLC ratio (210 nm) of the product: 66%. The desired product **13e** (1.2 mg, 1.7 μmol, 17% yield) was isolated by **Method 5**.







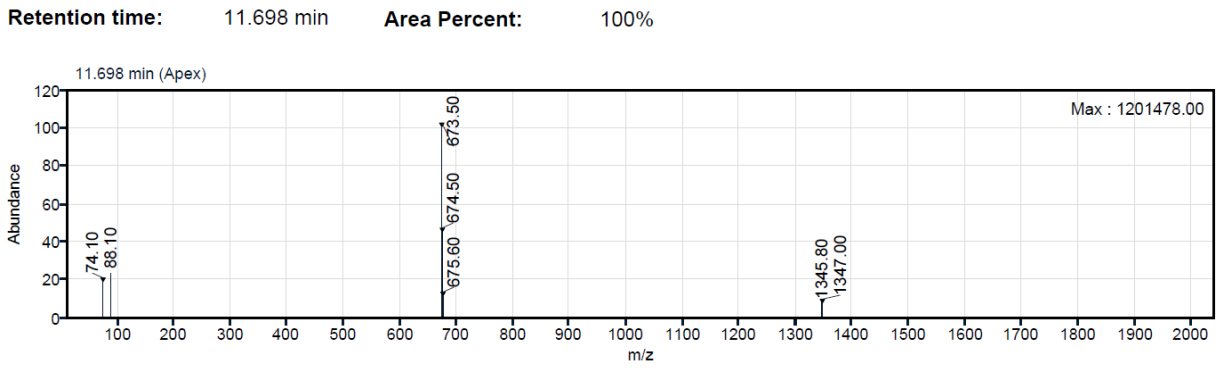
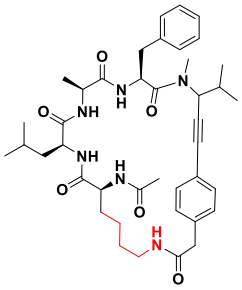
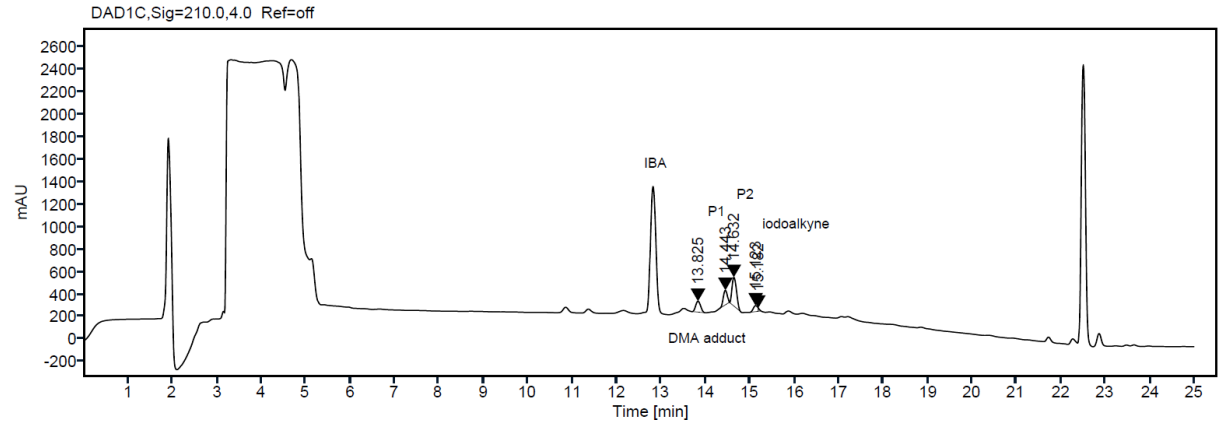


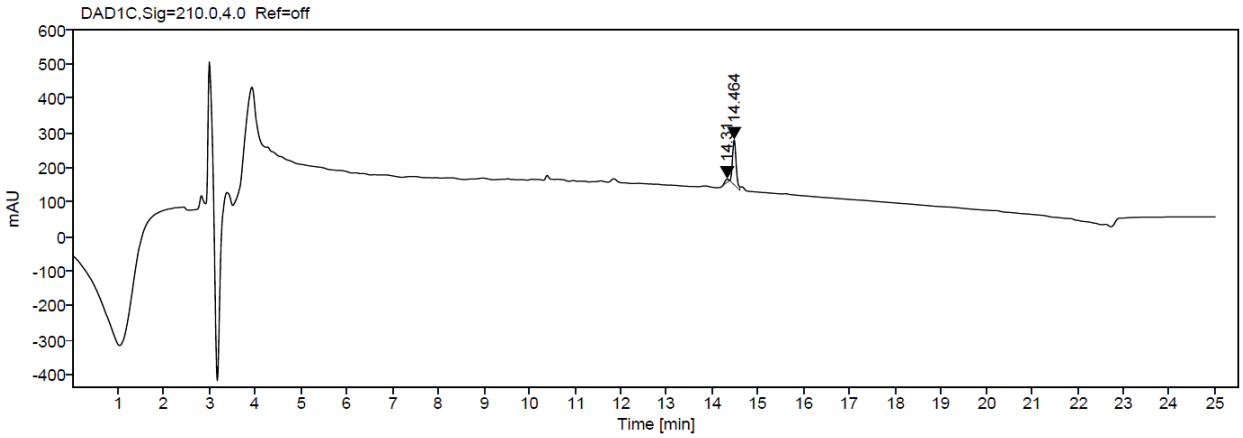
Figure S96: HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude and purified product **13e** by **Method 1.**
HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{37}H_{49}N_6O_6^+$ 673.3708; Found 673.3710.

Cyclic peptide of Ac-KLAFV (13f)
Following the general procedure, the reaction was conducted in 7 μmol scale. HPLC ratio (210 nm) of the product: 66% (dr 2.3:1). The desired products **13f** were isolated as two separable diostereomers (dr 1.8:1) (P1 0.68 mg, 0.93 μmol, 13% yield, P2 1.2 mg, 1.7 μmol, 24% yield, 37% yield in total) by **Method 5**.

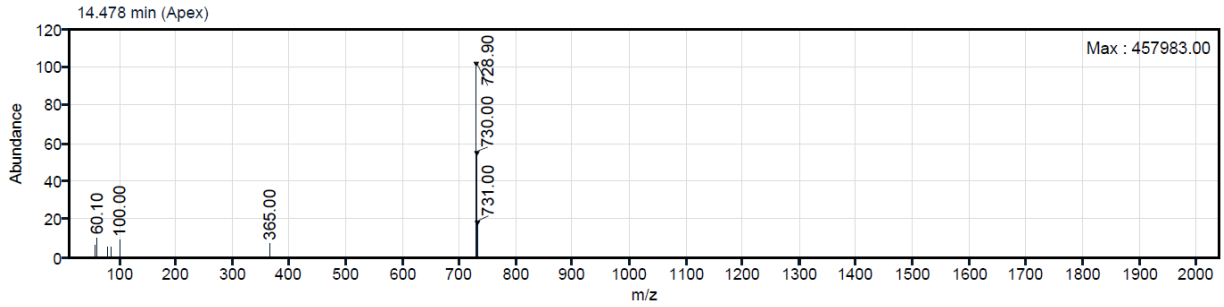




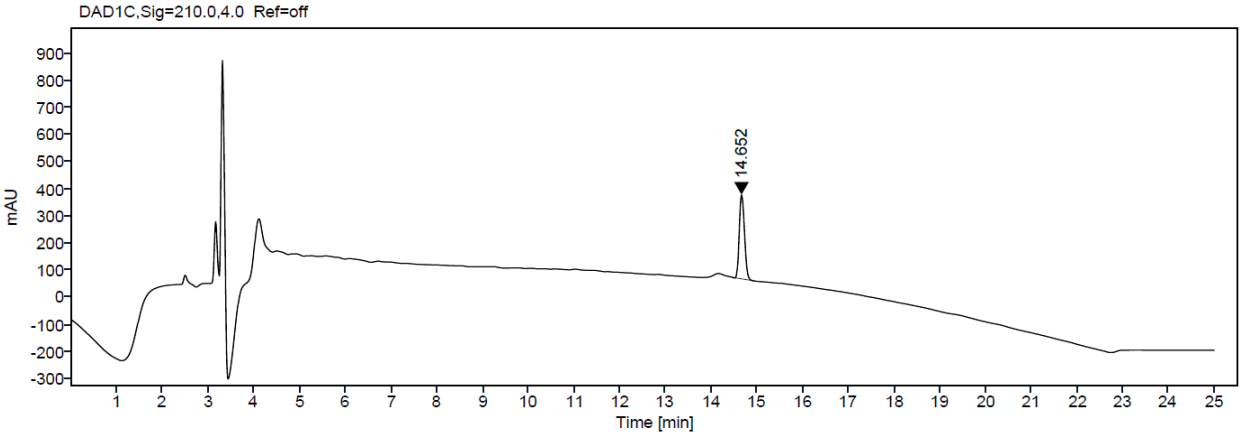
HPLC-UV chromatogram (210 nm) and MS(ESI) of **13f** P1 by **Method 1.**



Retention time: 14.478 min **Area Percent:** 100%



HPLC-UV chromatogram (210 nm) and MS(ESI) of **13f** P2 by **Method 1.**



Retention time: 14.452 min **Area Percent:** 100%

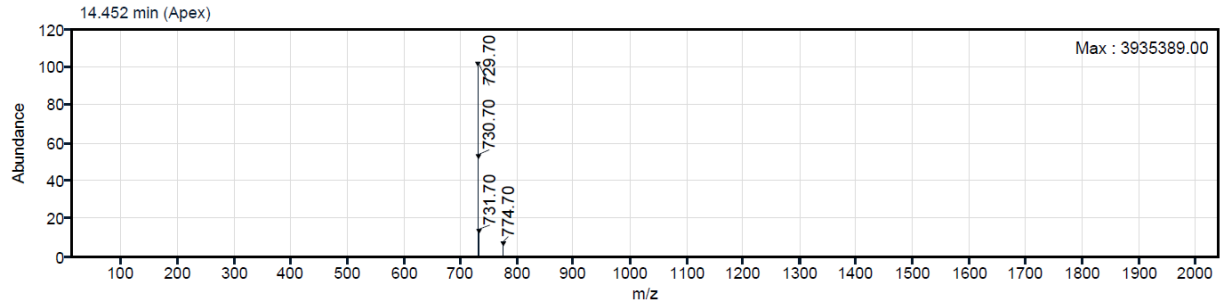
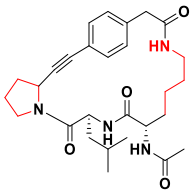


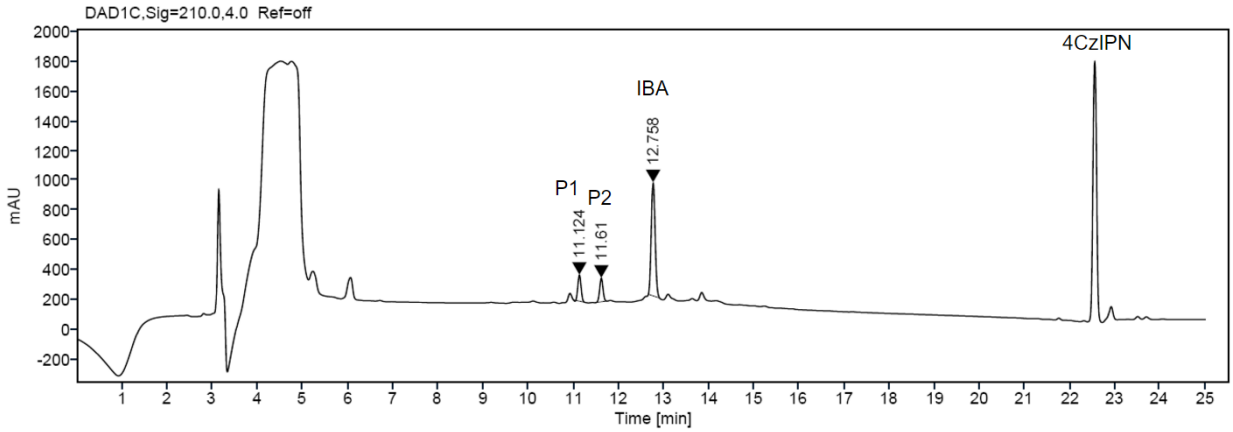
Figure S97: HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude and purified product **13f** P1 and P2 by **Method 1.**

HRMS (ESI/QTOF) m/z: $[M + Na]^+$ Calcd for $C_{41}H_{56}N_6NaO_6^+$ 751.4154; Found 751.4170.

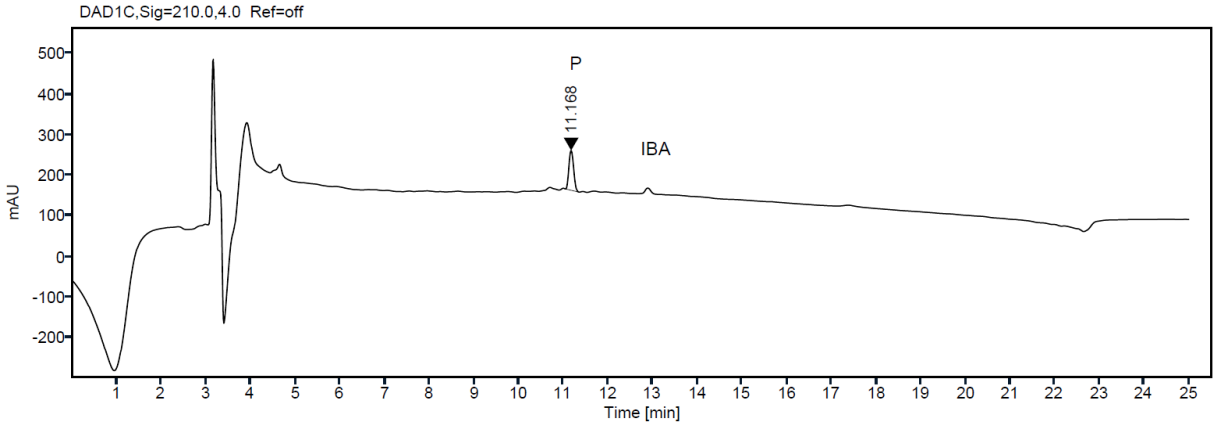
Cyclic peptide of Ac-KLP (13g)

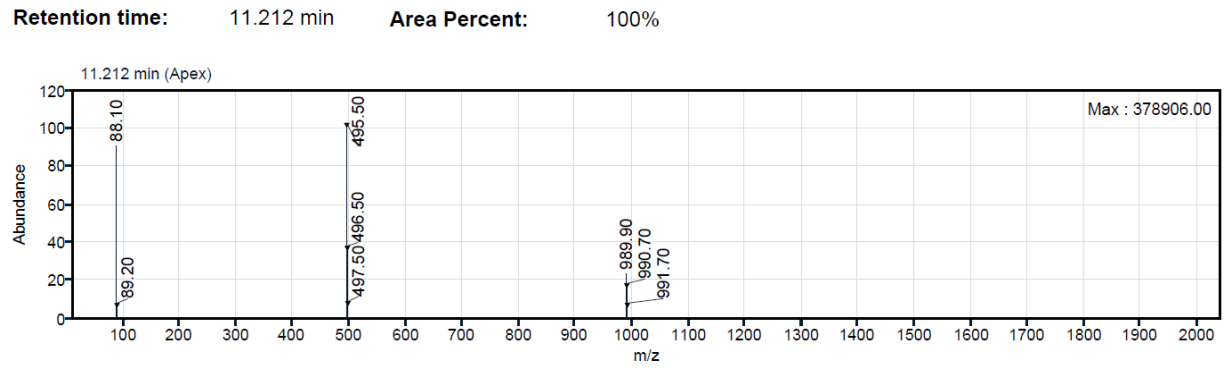
Following the general procedure, the reaction was conducted in 0.01 mmol scale. HPLC ratio (210 nm) of the product: >95% (dr 1.4:1). The desired products **13g** were isolated by **Method 5** as two separable diastereomers (P1 0.43 mg, 0.87 μmol, 9% yield, P2 1.1 mg, 2.1 μmol, 21% yield, 30% yield in total). (mixed with 2-iodobenzoic acid)





HPLC-UV chromatogram (210 nm) and MS(ESI) of cyclic **13g** P1 by **Method 1.**





HPLC-UV chromatogram (210 nm) and MS(ESI) of cyclic **13g** P2 by **Method 1.**

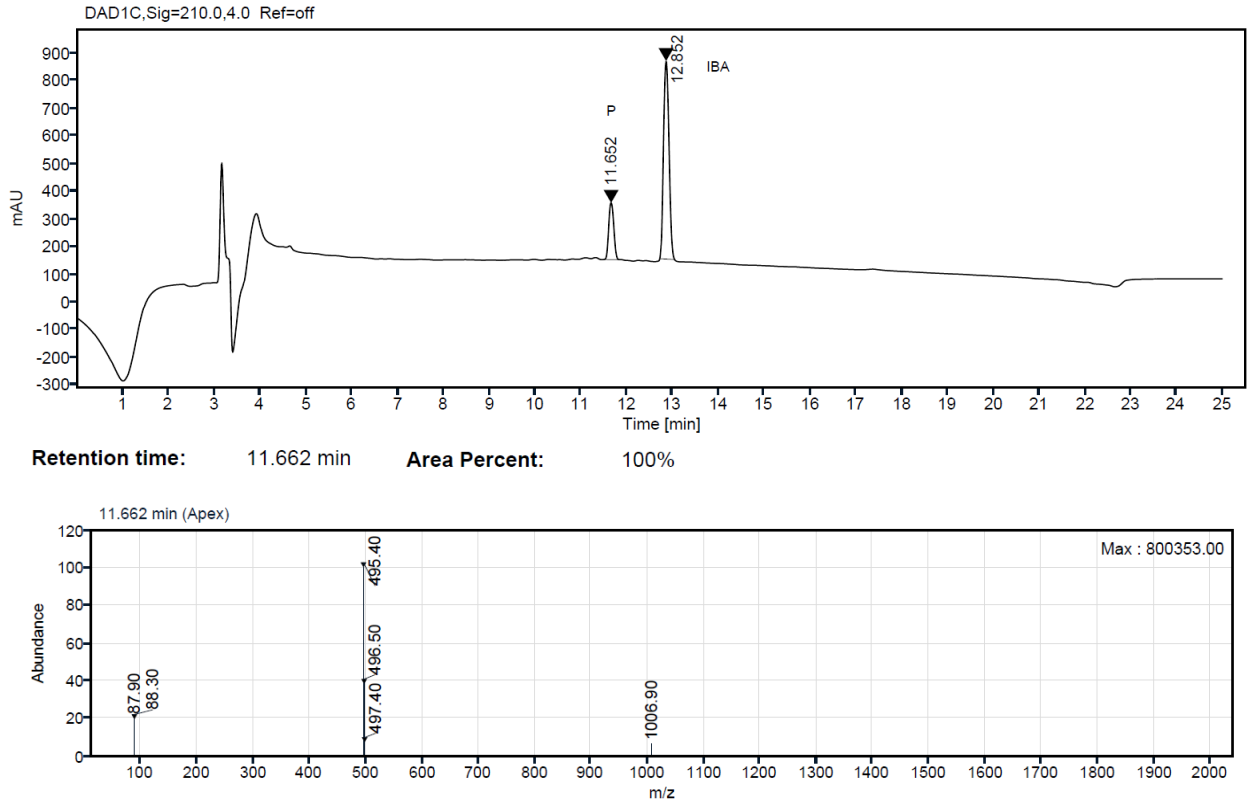
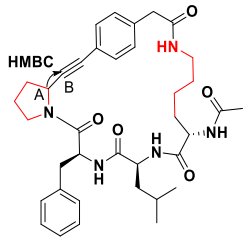


Figure S98: HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude and purified product **13g** P1 and P2 by **Method 1.**

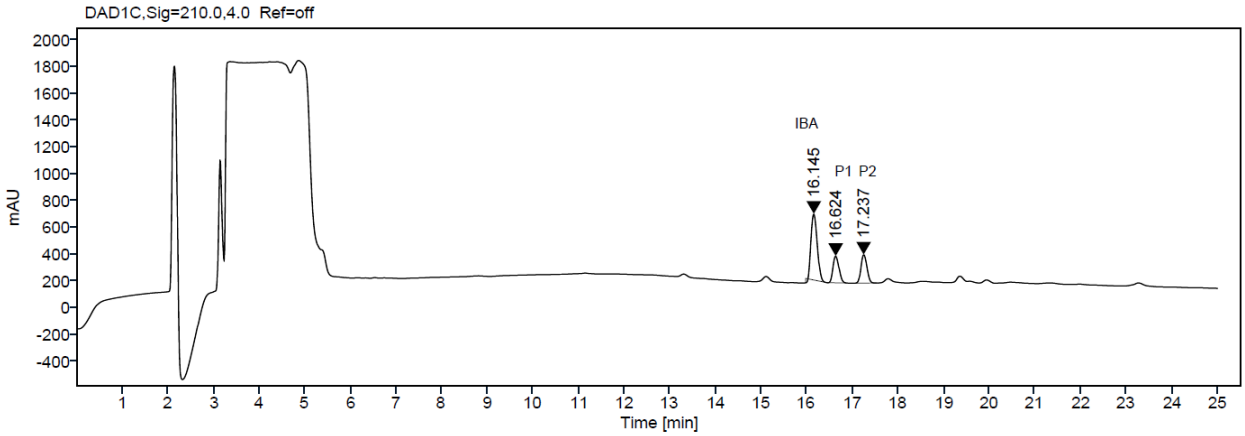
HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{28}H_{39}N_4O_4^+$ 495.2966; Found 495.2951.

Cyclic peptide of Ac-KLFP (13h)

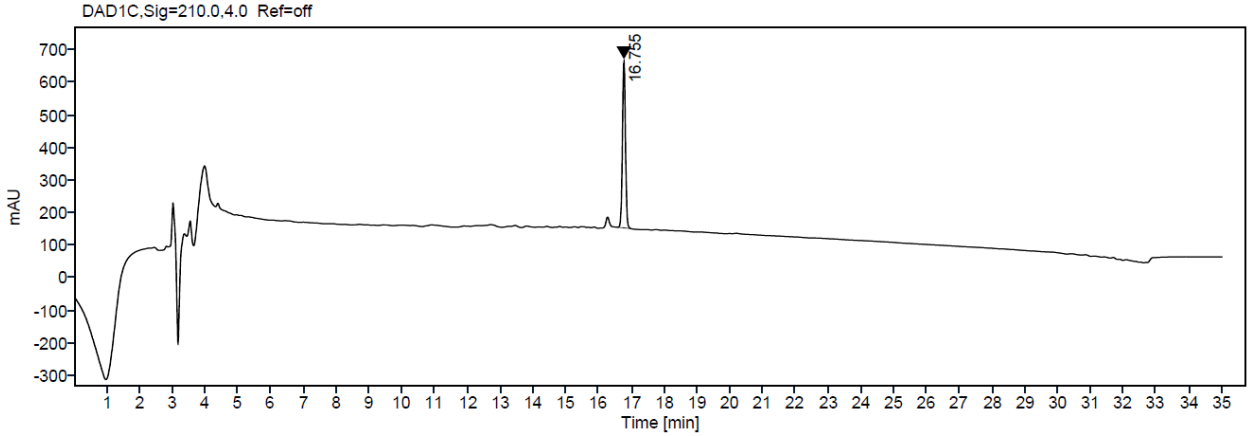
Following the general procedure, the reaction was conducted in 0.01 mmol scale. HPLC ratio (210 nm) of the product: >95% (dr 1:1). The desired products **13h** were isolated as two separable diastereomers (P1 1.8 mg, 2.9 μmol, 29% yield. P2 1.9 mg, 3.0 μmol, 30% yield, 59% yield in total) by **Method 5**.



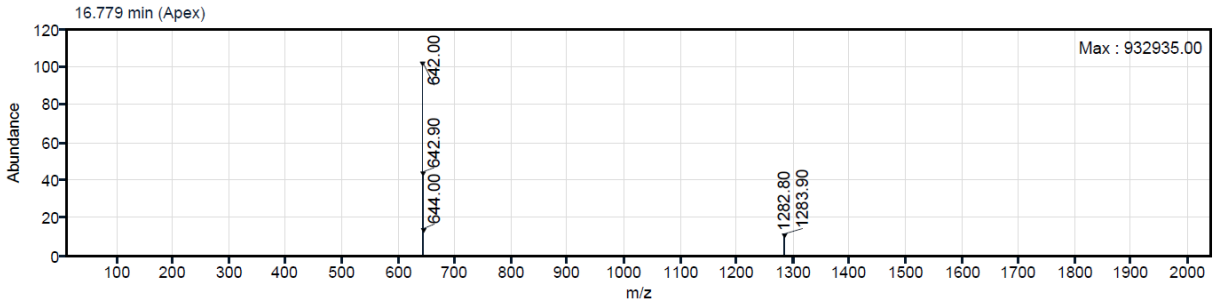
HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude reaction mixture after 30 min by **Method 2**



HPLC-UV chromatogram (210 nm) and MS(ESI) of **13h** P1 by **Method 2**:



Retention time: 16.779 min **Area Percent:** 100%



HPLC-UV chromatogram (210 nm) and MS(ESI) of **13h** P2 by **Method 2**:

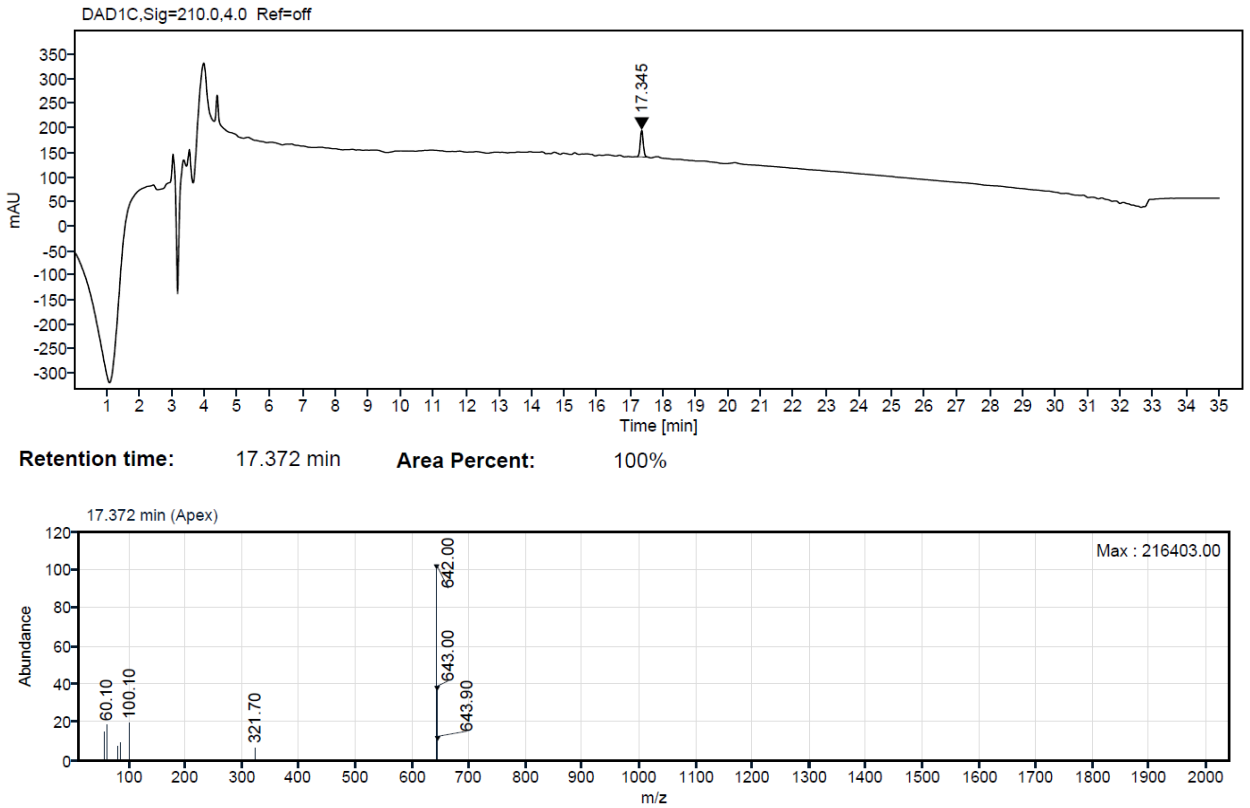


Figure S99: HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude and purified product **13h** P1 and P2 by **Method 1.**

NMR of **13h** P1

^{1}H NMR (500 MHz, CD_3OD) δ 7.45 (d, *J* = 7.8 Hz, 2H), 7.35 – 7.29 (m, 2H), 7.26 (dd, *J* = 13.7, 7.4 Hz, 5H), 5.05 (dt, *J* = 10.8, 5.7 Hz, 1H), 4.51 (dq, *J* = 9.7, 4.3 Hz, 1H), 4.22 (q, *J* = 6.4 Hz, 1H), 3.75 (dd, *J* = 7.7, 2.6 Hz, 1H), 3.54 (ddt, *J* = 11.7, 8.0, 4.8 Hz, 1H), 3.47 (s, 1H), 3.39 (d, *J* = 13.4 Hz, 2H), 3.29 – 3.23 (m, 2H), 3.06 (tt, *J* = 12.9, 6.2 Hz, 2H), 3.01 – 2.94 (m, 1H), 1.98 (s, 3H), 1.95 – 1.83 (m, 2H), 1.74 – 1.65 (m, 2H), 1.61 (ddd, *J* = 13.4, 8.9, 4.3 Hz, 2H), 1.54 (td, *J* = 10.0, 8.8, 5.2 Hz, 4H), 1.45 – 1.38 (m, 1H), 1.30 (q, *J* = 7.6, 6.3 Hz, 3H), 0.94 (d, *J* = 6.5 Hz, 6H).
^{13}C NMR (201 MHz, CD_3OD) δ 174.1, 174.0, 173.5, 173.5, 172.4, 137.9, 137.8, 133.3, 130.5, 129.8, 129.6, 128.4, 122.8, 88.0, 85.5, 54.9, 54.8, 52.2, 49.9, 49.5, 47.3, 44.3, 42.8, 39.4, 35.1, 32.7, 29.7, 25.7, 23.8, 23.7, 23.6, 22.4, 22.1.

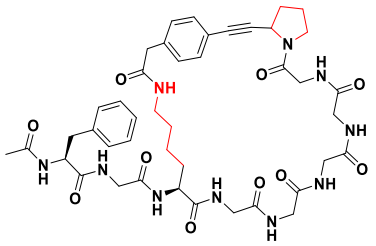
NMR of **13h** P2

^{1}H NMR (500 MHz, CD_3OD) δ 7.36 (d, *J* = 8.1 Hz, 2H), 7.28 (d, *J* = 8.3 Hz, 2H), 7.28 – 7.18 (m, 5H), 4.84 – 4.77 (m, 2H), 4.25 (dd, *J* = 10.3, 4.8 Hz, 1H), 3.82 (dd, *J* = 10.7, 3.4 Hz, 1H), 3.65 – 3.59 (m, 2H), 3.49 (q, *J* = 7.1 Hz, 2H), 3.17 (q, *J* = 4.4, 3.5 Hz, 2H), 3.11 – 3.05 (m, 1H), 3.01 – 2.94 (m, 2H), 2.19 – 2.10 (m, 1H), 2.01 (s, 3H), 2.00 – 1.93 (m, 2H), 1.83 (tq, *J* = 7.2, 3.5 Hz, 1H), 1.53 (dt, *J* = 21.3, 7.1 Hz, 1H), 1.43 (ddt, *J* = 13.8, 7.5, 4.3 Hz, 2H), 1.37 – 1.28 (m, 2H), 1.18 (t, *J* = 7.0 Hz, 1H), 1.10 (td, *J* = 12.9, 7.3 Hz, 1H), 0.90 (d, *J* = 6.6 Hz, 3H), 0.83 (d, *J* = 6.5 Hz, 3H).
^{13}C NMR (151 MHz, CD_3OD) δ 174.4, 174.2, 174.1, 174.1, 170.5, 138.4, 137.9, 133.1, 130.7, 129.7, 129.4, 127.8, 123.2, 89.5, 83.1, 66.9, 55.7, 54.0, 53.8, 47.1, 44.6, 41.6, 39.5, 38.8, 32.9, 31.6, 30.4, 25.9, 25.6, 24.4, 23.5, 22.6, 21.5.

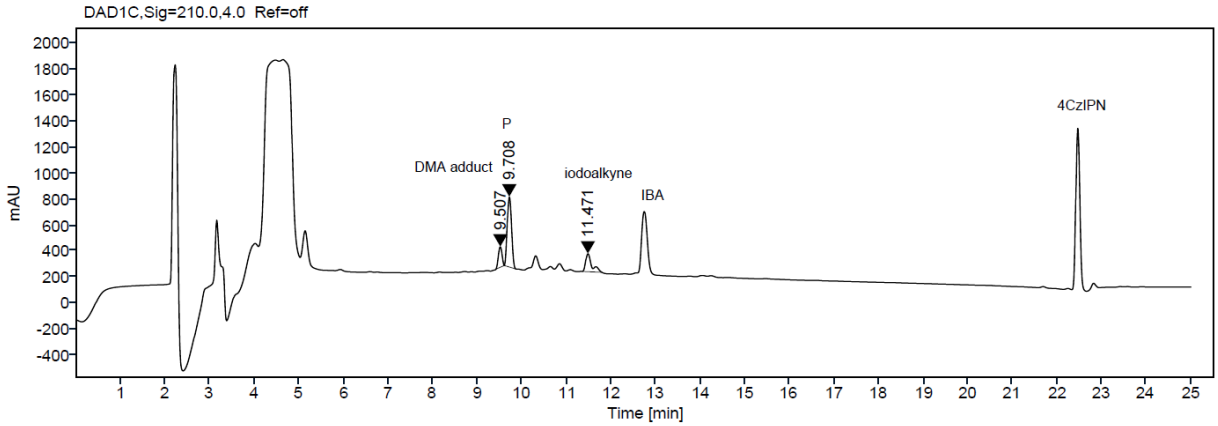
HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{37}H_{48}N_5O_5^+$ 642.3650; Found 642.3660.

Cyclic peptide of Ac-FGKGGGGGP (13i)

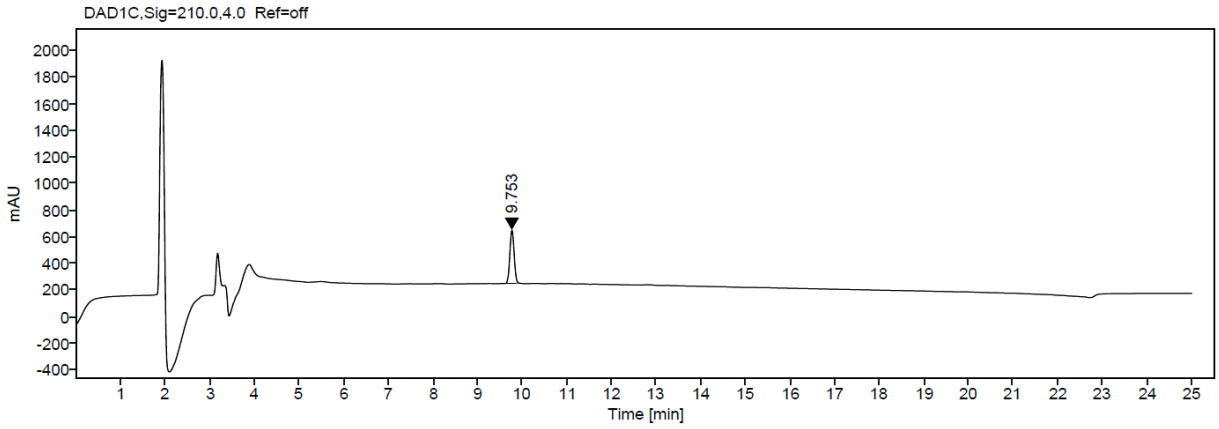
Following the general procedure, the reaction was conducted on a 6.4 μmol scale. HPLC ratio (210 nm) of the product: 68%. dr was not determined. The desired product **13i** (2.5 mg, 2.9 μmol, 45% yield) was isolated by **Method 7**.



HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude reaction mixture after 30 min by **Method 1**



HPLC-UV chromatogram (210 nm) and MS(ESI) of **13i** by **Method 1.**



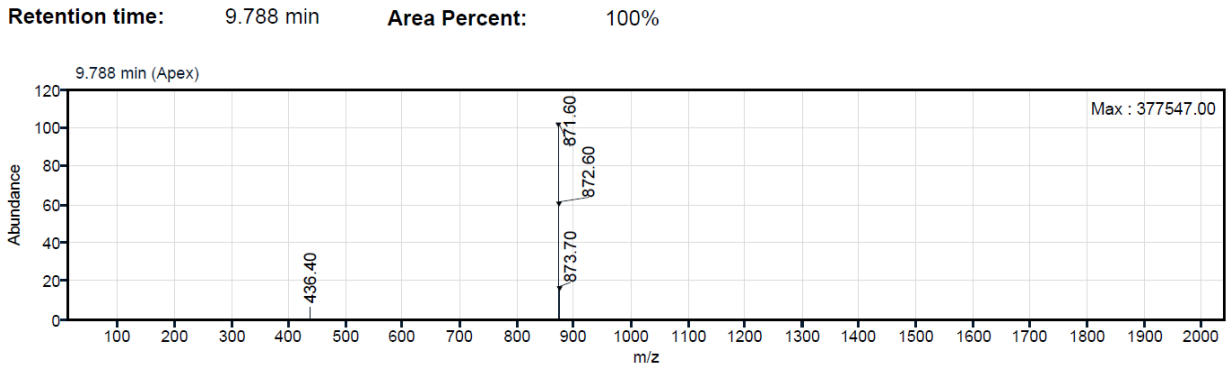
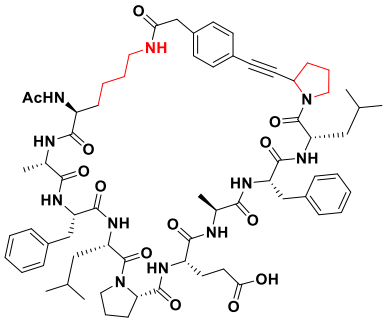


Figure S100: HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude and purified product **13i** P1 and P2 by **Method 1.**

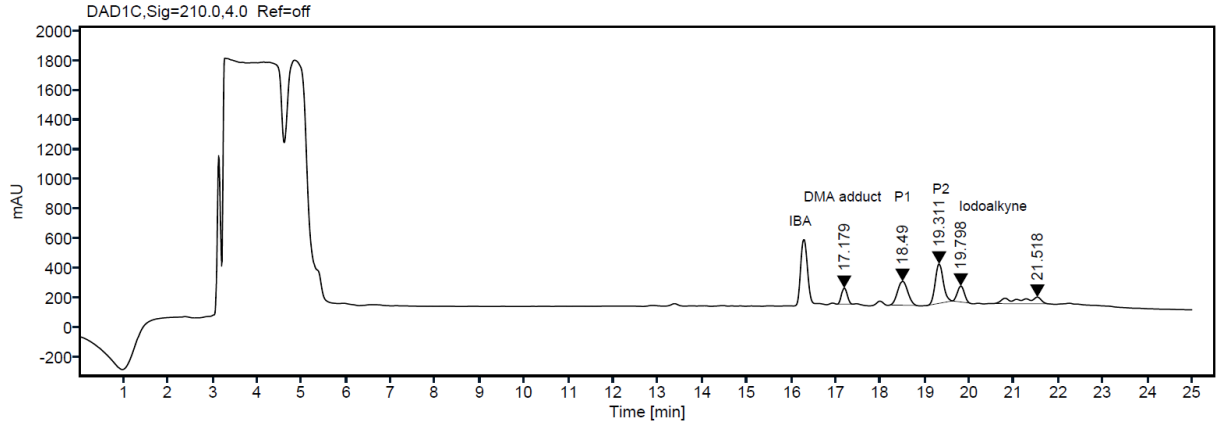
HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{43}H_{55}N_{10}O_{10}^+$ 871.4097; Found 871.4079.

Cyclic peptide of Ac-KAFLPEAFLP (13j)

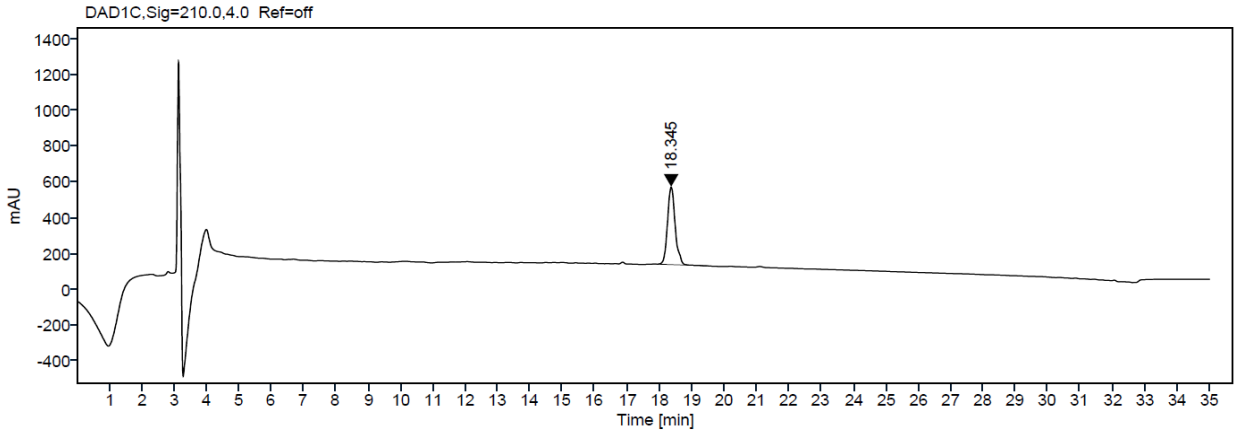
Following the general procedure, the reaction was conducted in 7.7 μmol scale. HPLC ratio (210 nm) of the product: 68% (dr 1.4:1). The desired products **13j** were isolated as two separable diastereomers (P1 0.66 mg, 0.52 μmol, 7% yield. P2 0.95 mg, 0.75 μmol, 10% yield, 17% yield in total) by **Method 6**.



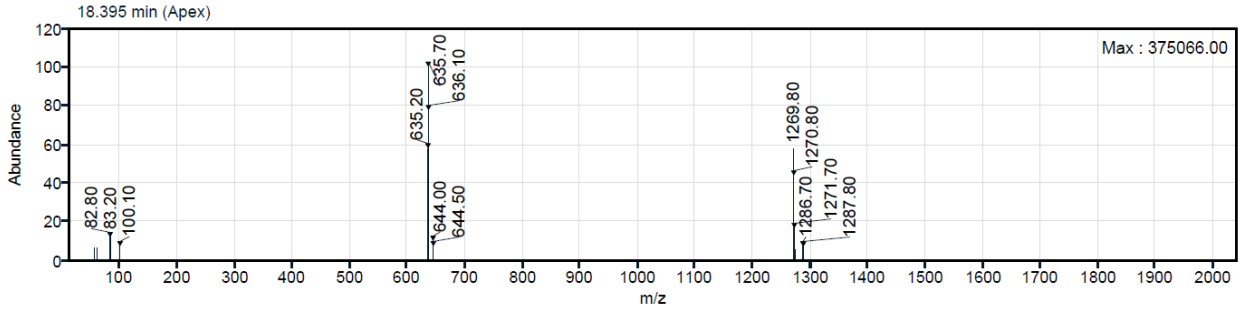
HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude reaction mixture after 30 min with **Method 2**



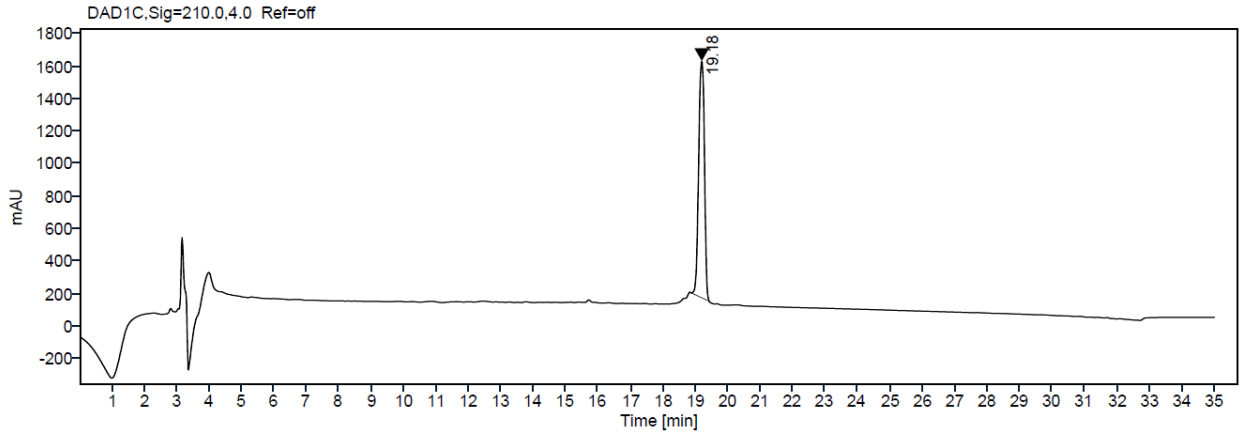
HPLC-UV chromatogram (210 nm) and MS(ESI) of **13j** P1with **Method 2**:



Retention time: 18.395 min **Area Percent:** 100%



HPLC-UV chromatogram (210 nm) and MS(ESI) of **13j** P2 with **Method 2**:



Retention time: 19.259 min **Area Percent:** 100%

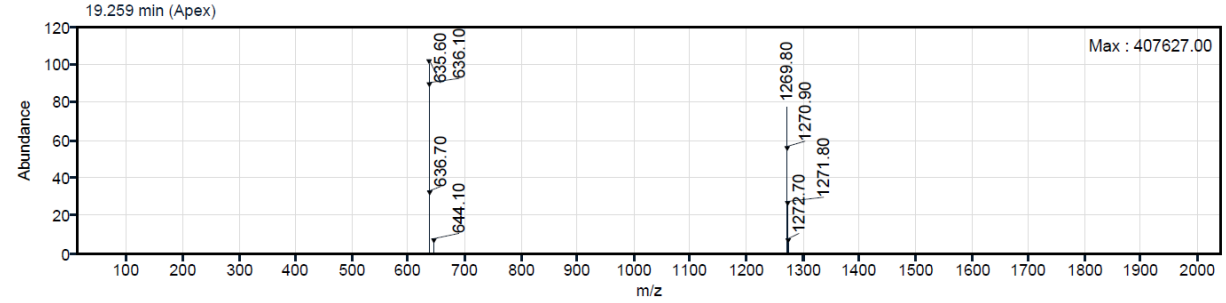
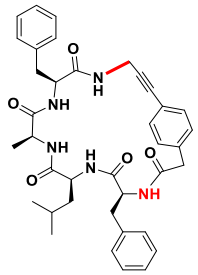


Figure S101: HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude and purified product **13j** P1 and P2 by **Method 1.**

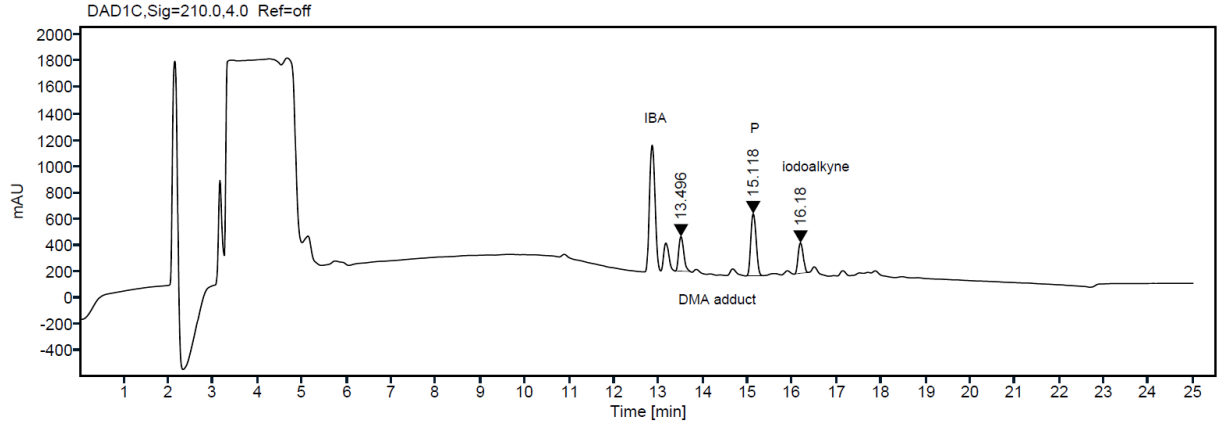
HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H_2]^{+2}$ Calcd for $C_{68}H_{93}N_{11}O_{13}^{+2}$ 635.8472; Found 635.8460.

Cyclic peptide of FLAFG (13k)

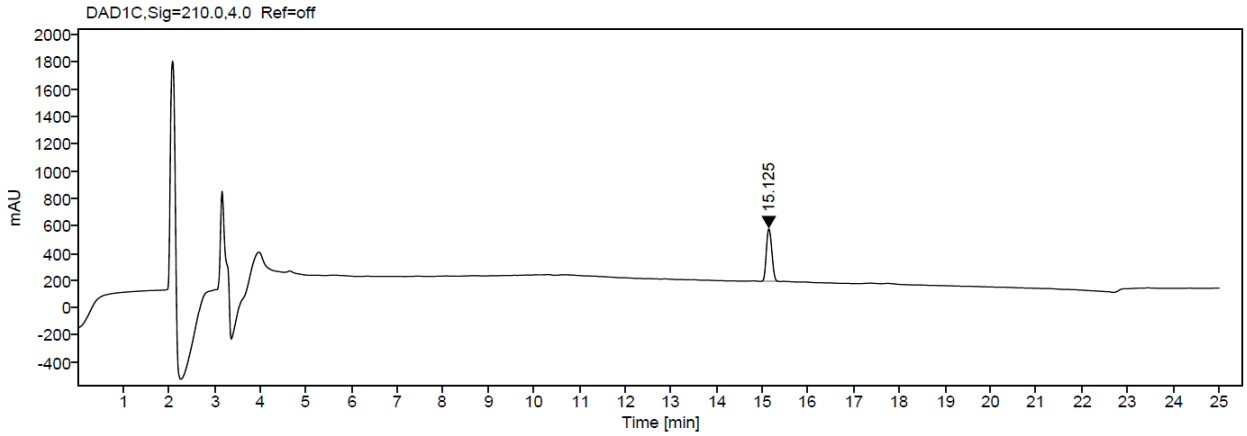
Following the general procedure, the reaction was conducted on a 5 μmol scale. HPLC ratio (210 nm) of the product: 51%. The desired product **13k** (0.59 mg, 0.91 μmol, 18% yield) was isolated by **Method 5**.



HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude reaction mixture after 30 min by **Method 1**



HPLC-UV chromatogram (210 nm) and MS(ESI) of **13k** by **Method 1.**



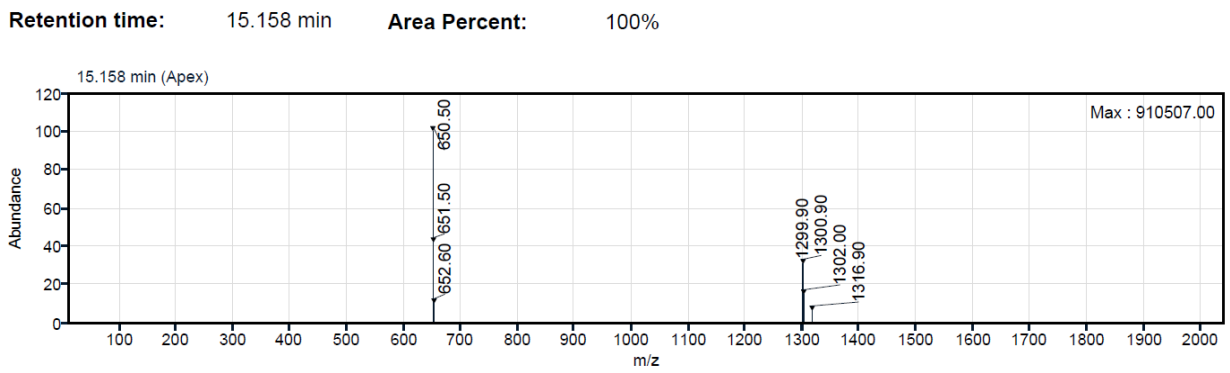
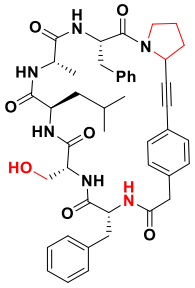


Figure S102: HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude and purified product **13k** by **Method 1.**

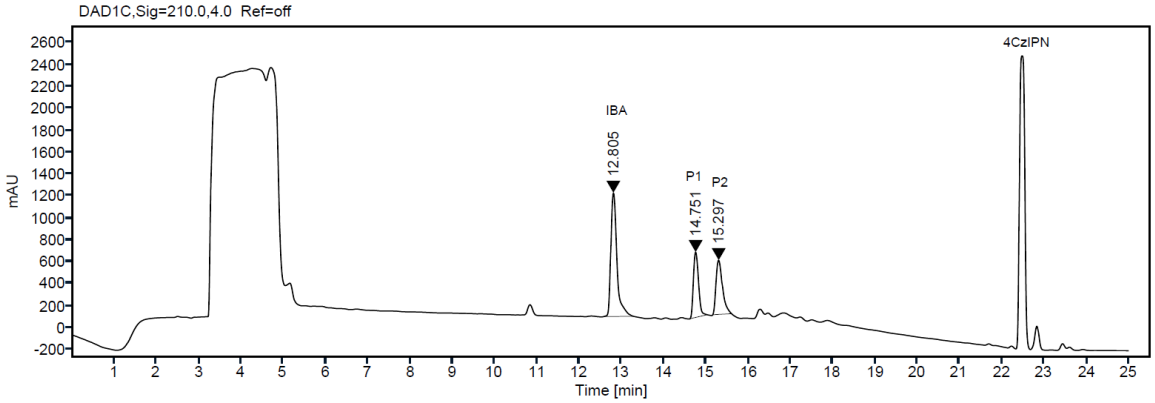
HRMS (ESI/QTOF) m/z: $[M + Na]^+$ Calcd for $C_{38}H_{43}N_5NaO_5^+$ 672.3156; Found 672.3161.

Cyclic peptide of FSLAFP (13l)

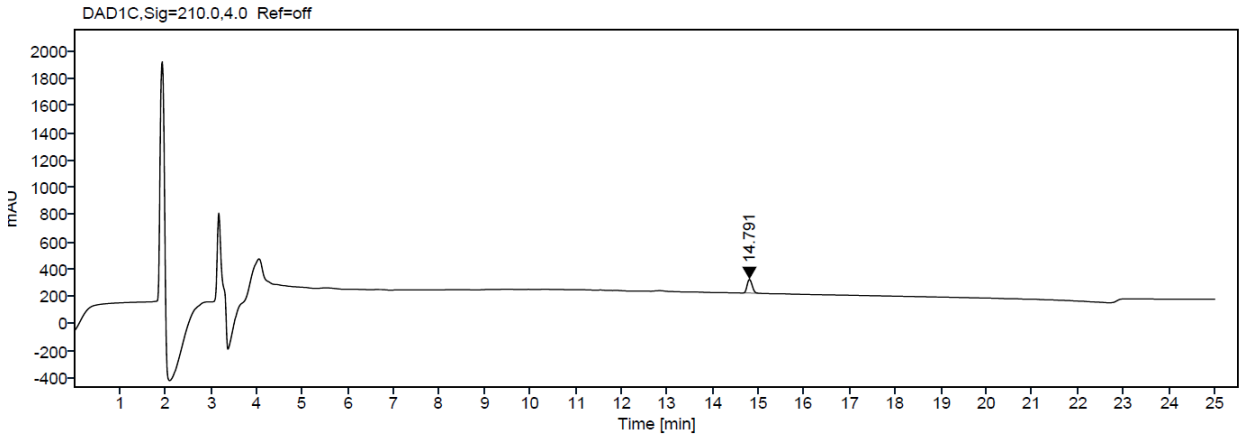
Following the general procedure, the reaction was conducted on a 0.01 mmol scale. HPLC ratio (210 nm) of the product: >95% (dr 1.2:1). The desired products **13l** were isolated as two separable diastereomers (P1 1.1 mg, 1.5 μmol, 15% yield, P2, 1.3 mg, 1.7 μmol, 17% yield, 32% yield in total) by **Method 5**.



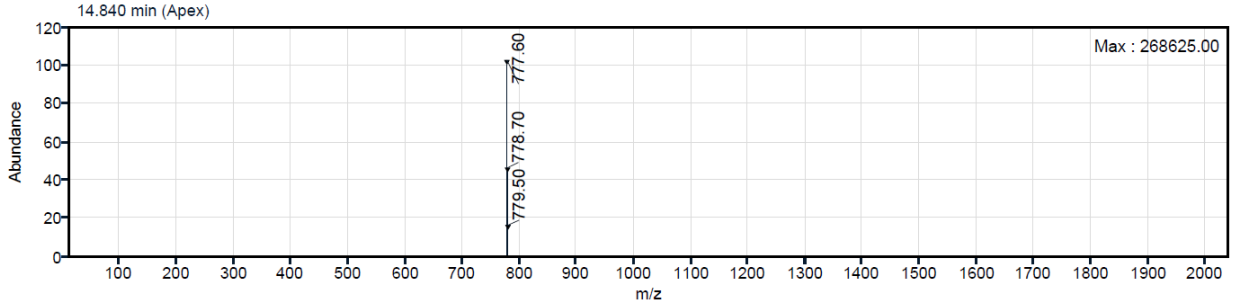
HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude reaction mixture after 30 min by **Method 1**



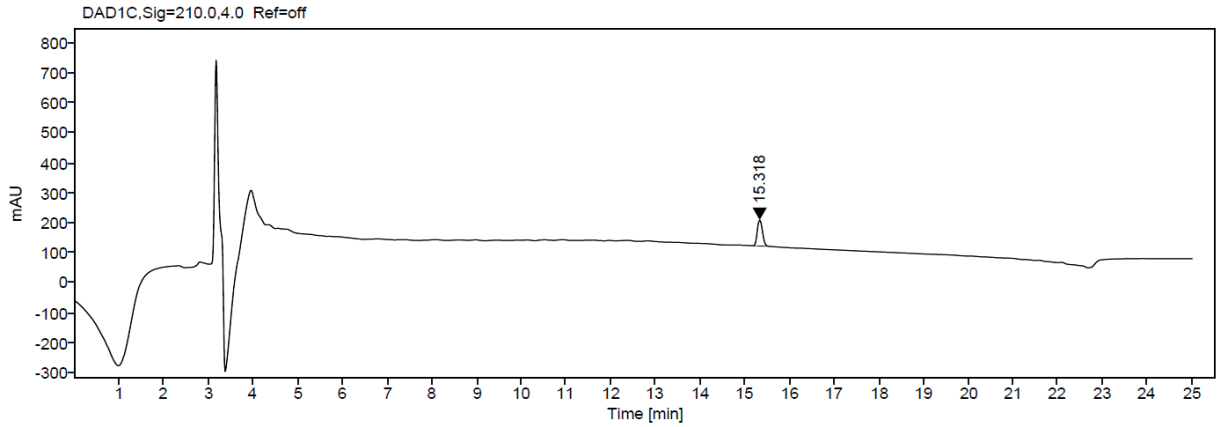
HPLC-UV chromatogram (210 nm) and MS(ESI) of **13l** P1 by **Method 1.**



Retention time: 14.84 min **Area Percent:** 100%



HPLC-UV chromatogram (210 nm) and MS(ESI) of **13l** P2 by **Method 1.**



Retention time: 15.349 min **Area Percent:** 100%

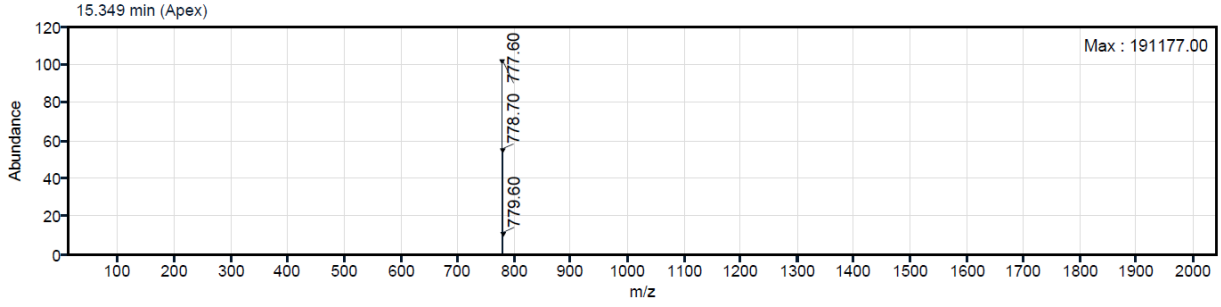
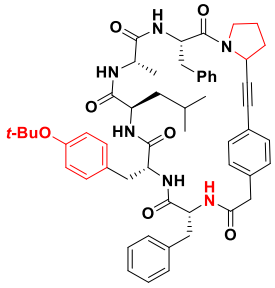


Figure S103: HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude and purified product **13l** P1 and P2 by **Method 1.**

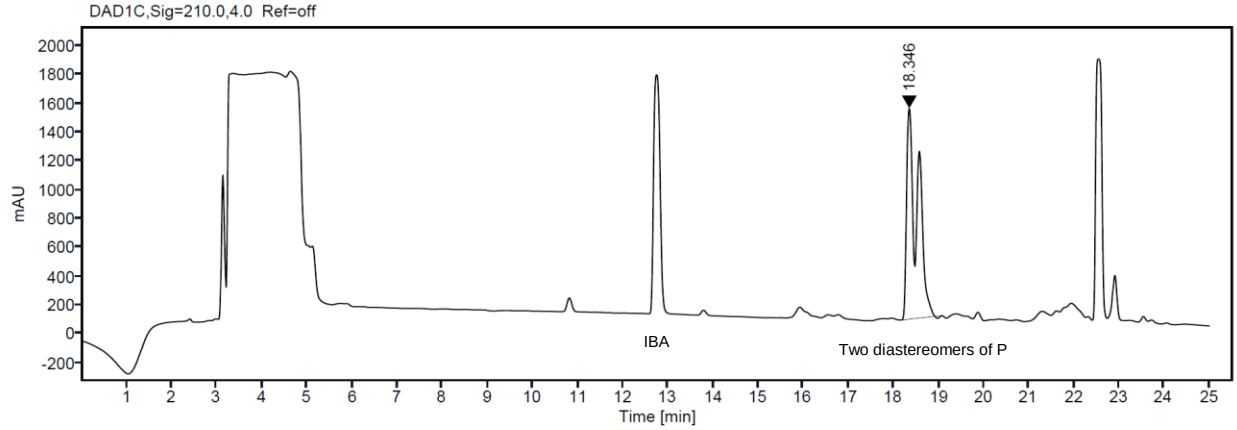
HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + Na]^+$ Calcd for $C_{44}H_{52}N_6NaO_7^+$ 799.3790; Found 799.3754.

Cyclic peptide of FYLAFP (13m)

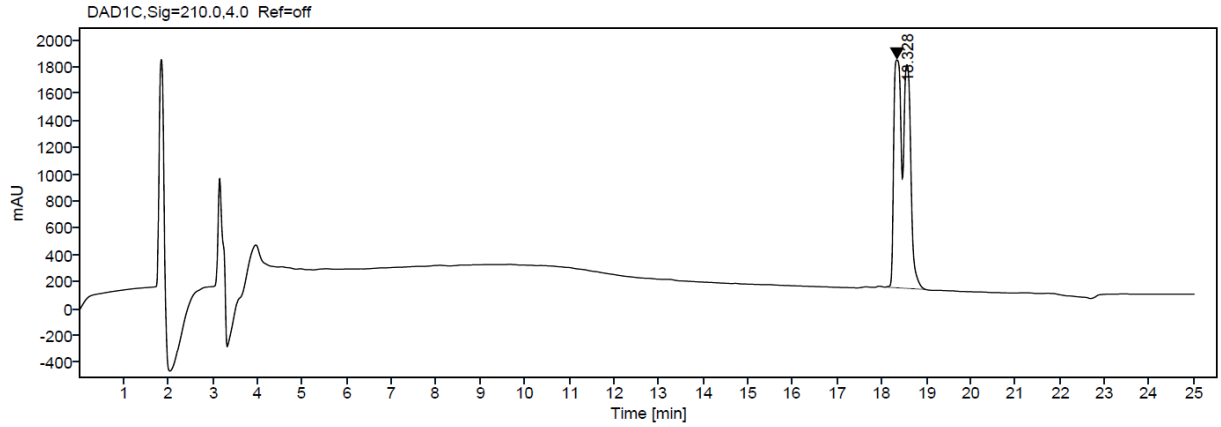
Following the general procedure, the reaction was conducted in 0.01 mmol scale. HPLC ratio (210 nm) of the product: >95% (dr 1.5:1). The desired product **13m** (3.6 mg, 4.0 μmol, 40% yield) was isolated by **Method 5**.



HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude reaction mixture after 30 min by **Method 1**



HPLC-UV chromatogram (210 nm) and MS(ESI) of **13m** by **Method 1.**



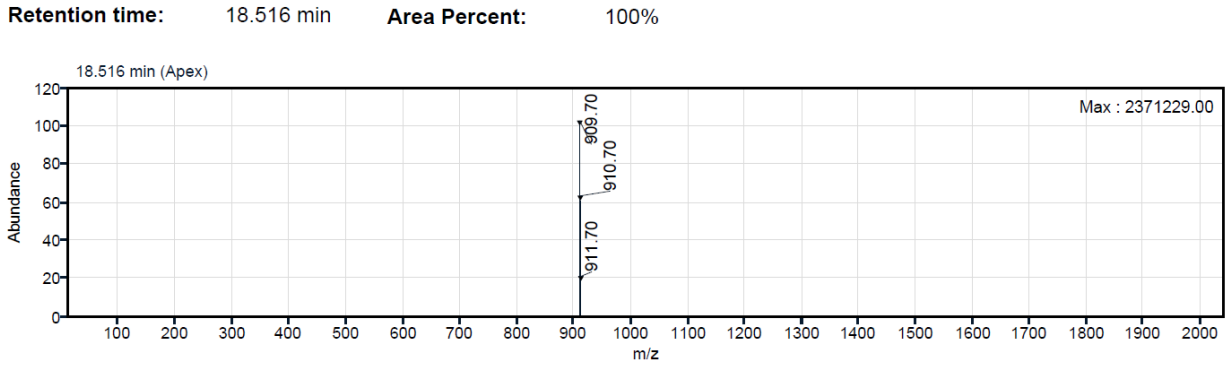
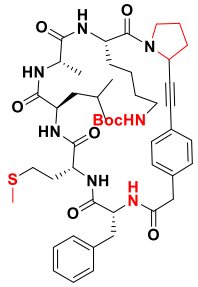


Figure S104: HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude and purified product **13m** by **Method 1.**

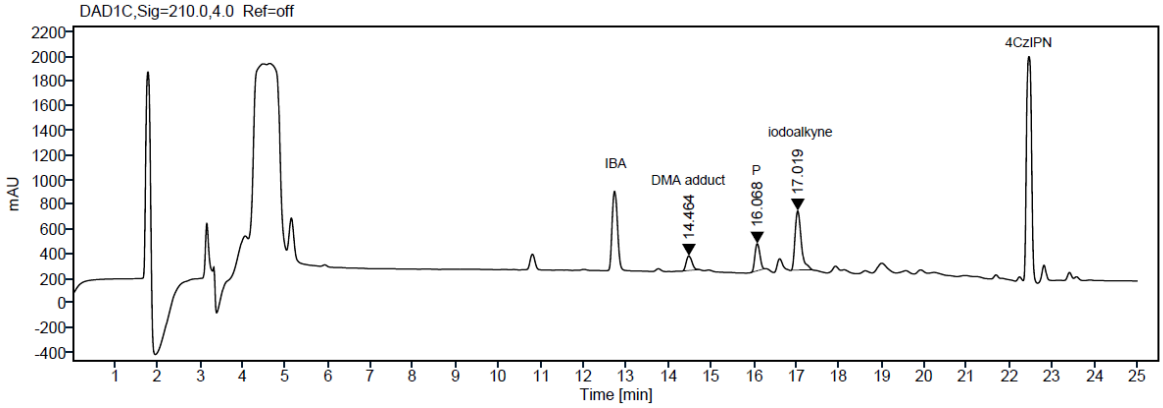
HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{54}H_{65}N_6O_7^+$ 909.4909; Found 909.4883.

Cyclic peptide of FMLAKP (13n)

Following the general procedure, the reaction was conducted on a 0.01 mmol scale. HPLC ratio (210 nm) of the product: 28%. The dr was not determined. The desired product **13n** (0.74 mg, 0.82 μmol, 8% yield) was isolated by **Method 5**.



HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude reaction mixture after 30 min by **Method 1**



HPLC-UV chromatogram (210 nm) and MS(ESI) of **13n** by **Method 1.**

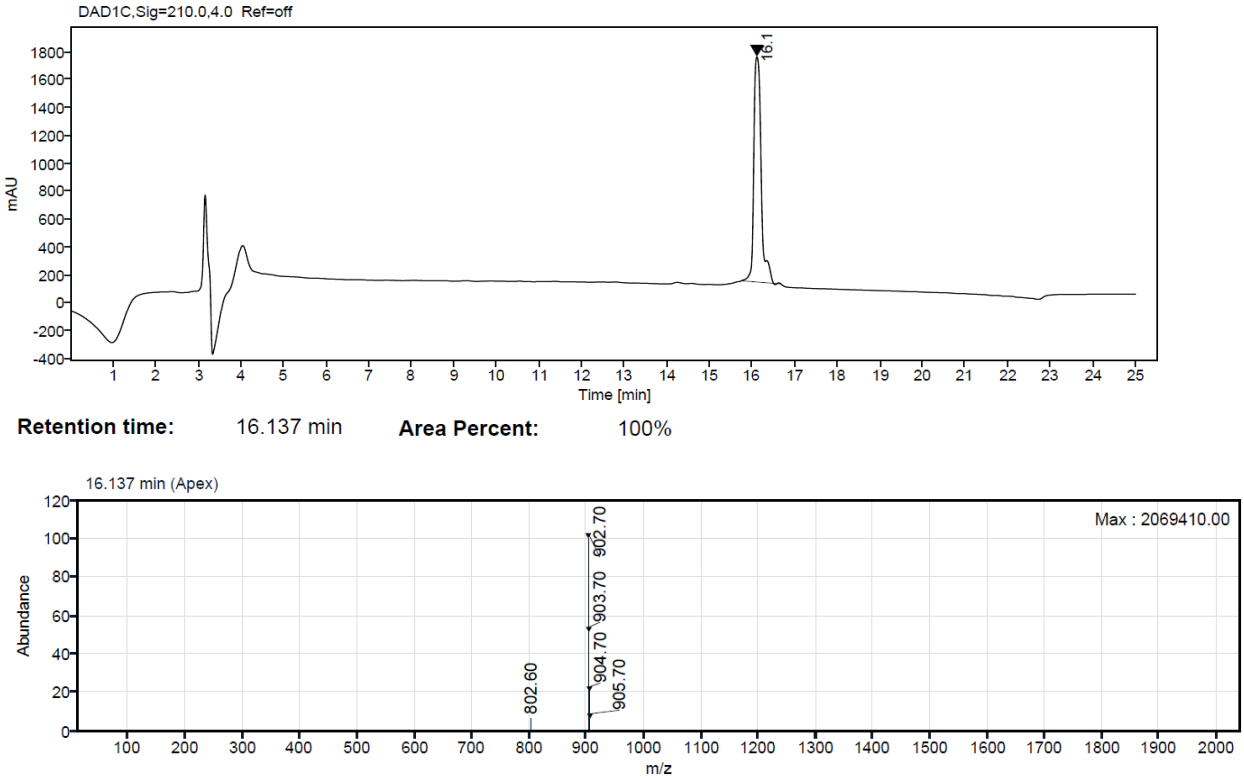
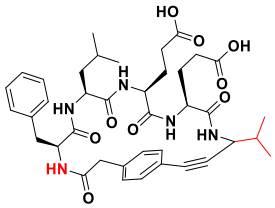


Figure S105: HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude and purified product **13n** by **Method 1.**

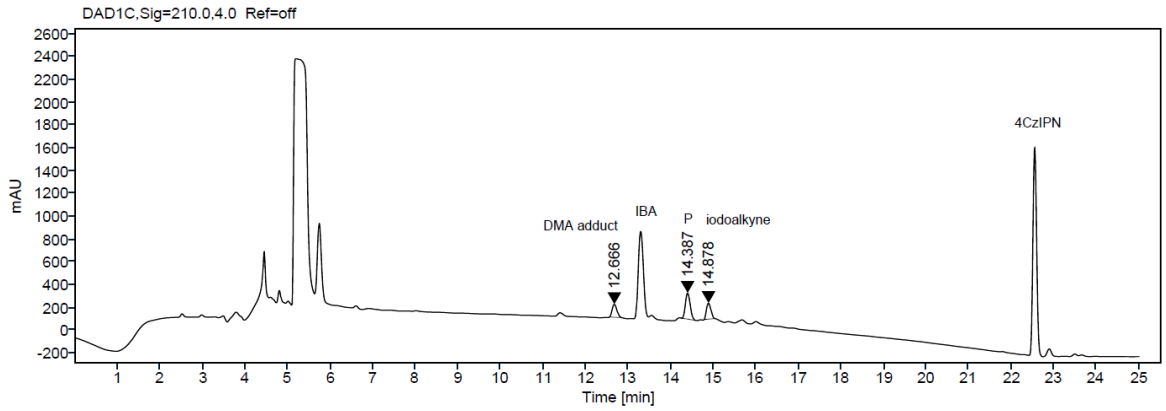
HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{39}H_{64}N_7O_9S^+$ 902.4845; Found 902.4845.

Cyclic peptide of FLEEV (13o)

Following the general procedure, the reaction was conducted in 6.0 μmol scale. HPLC ratio (210 nm) of the product: 49%. The dr was not determined. The desired product **13o** (1.4 mg, 1.9 μmol, 33% yield) was isolated by **Method 6**.



HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude reaction mixture after 30 min by **Method 1**



HPLC-UV chromatogram (210 nm) and MS(ESI) of **13o** by **Method 1.**

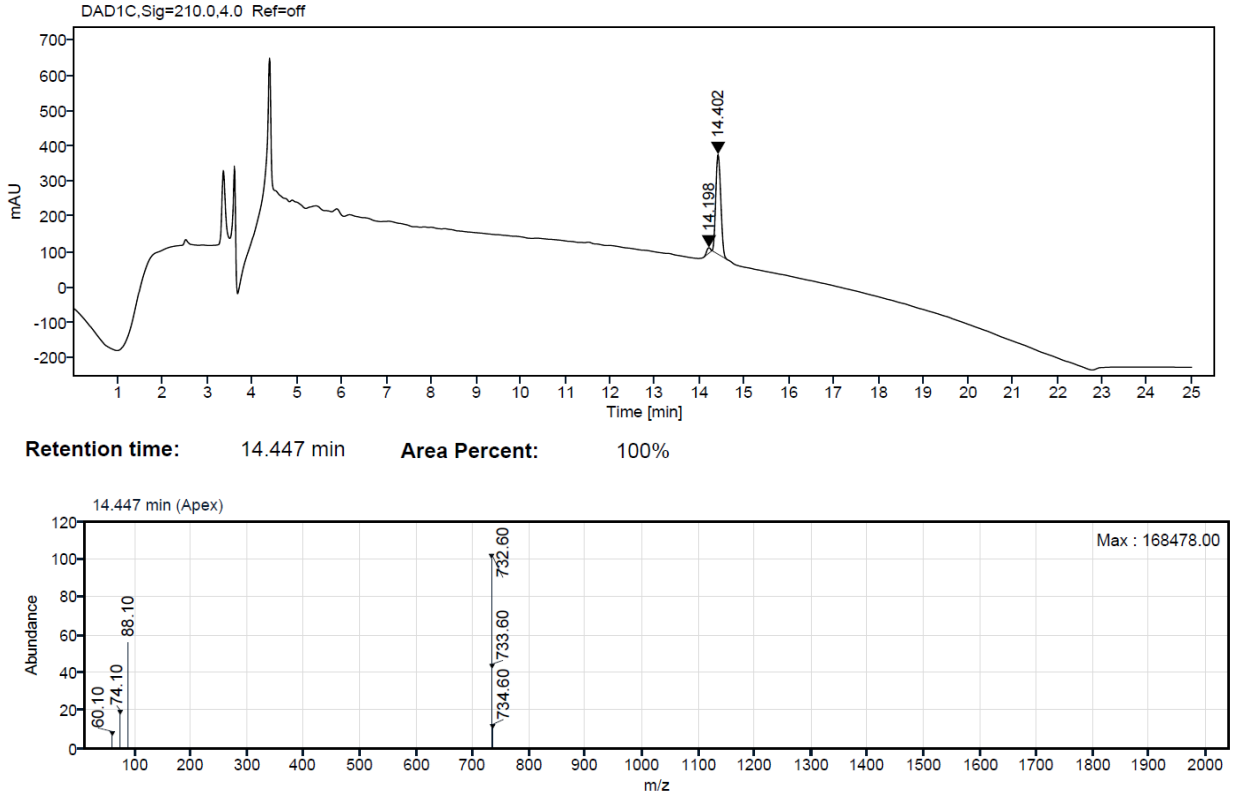
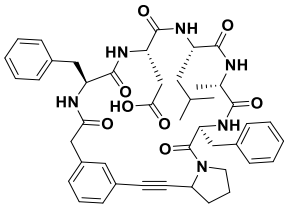


Figure S106: HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude and purified product **13o** by **Method 1.**

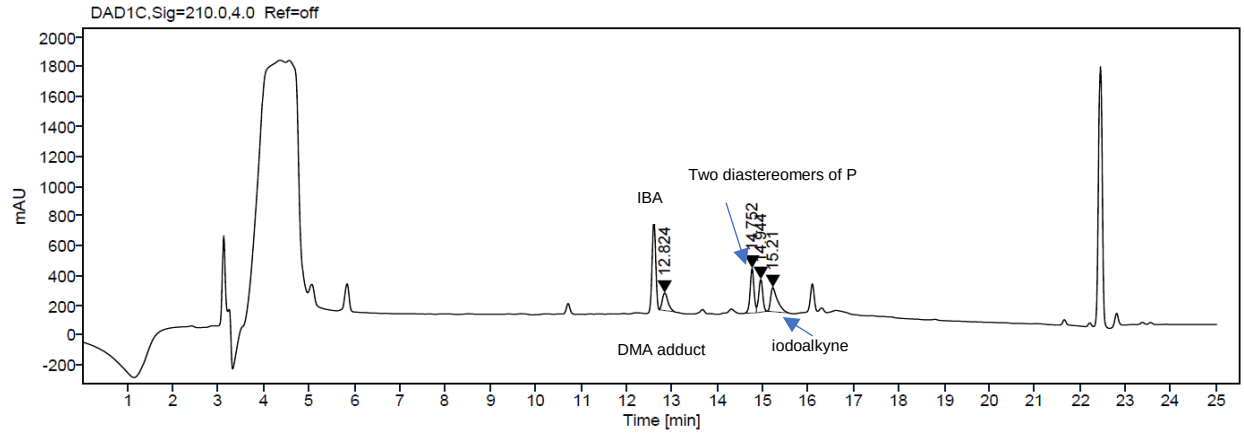
HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{39}H_{50}N_5O_9^+$ 732.3603; Found 732.3591.

Cyclic peptide of FDLAFP (13p)

Following the general procedure, the reaction was conducted on a 0.008 mmol scale. HPLC ratio (210 nm) of the product: 53% (dr 1.4:1). The desired product **13p** (1.7 mg, 2.1 μmol, 26% yield) was isolated by **Method 6**.



HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude reaction mixture after 30 min by **Method 1**



HPLC-UV chromatogram (210 nm) and MS(ESI) of **13p** by **Method 1.**

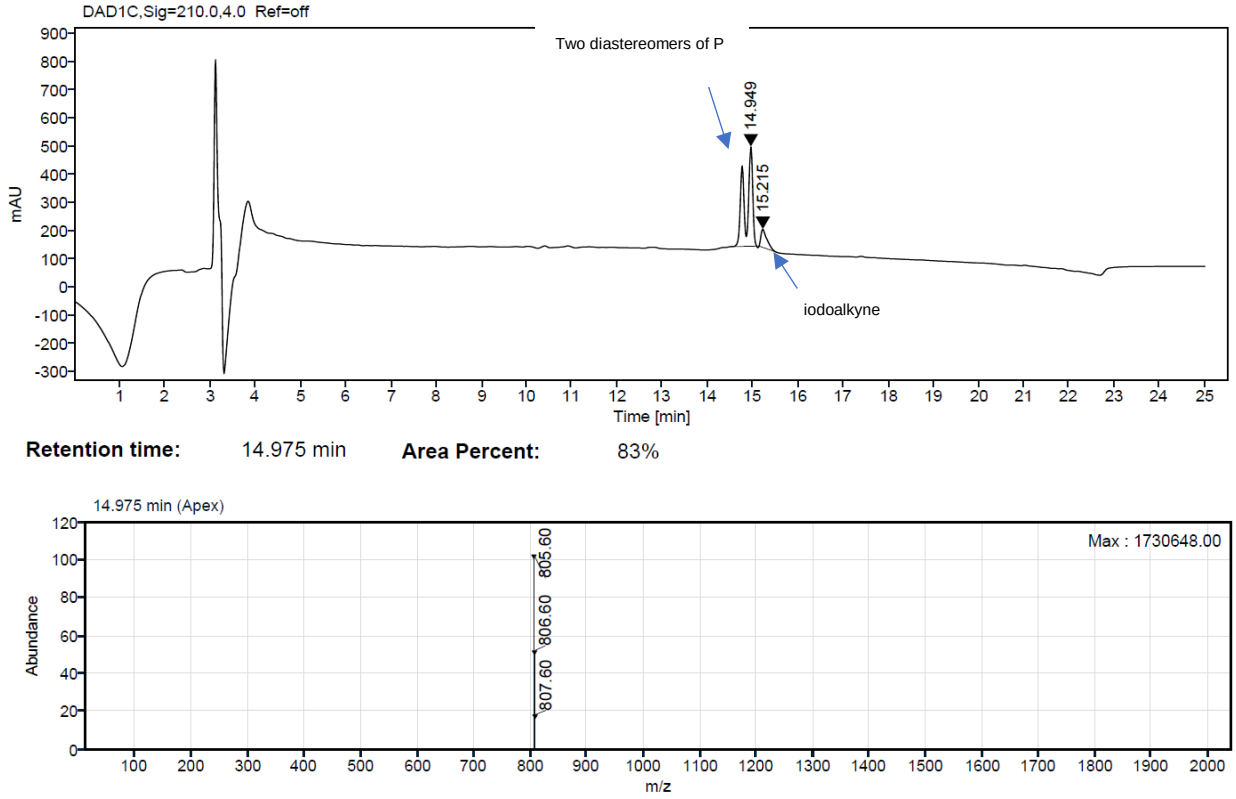
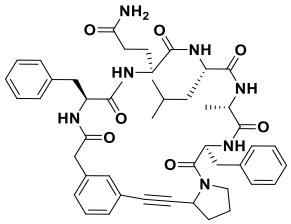


Figure S107: HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude and purified product **13p** by **Method 1.**

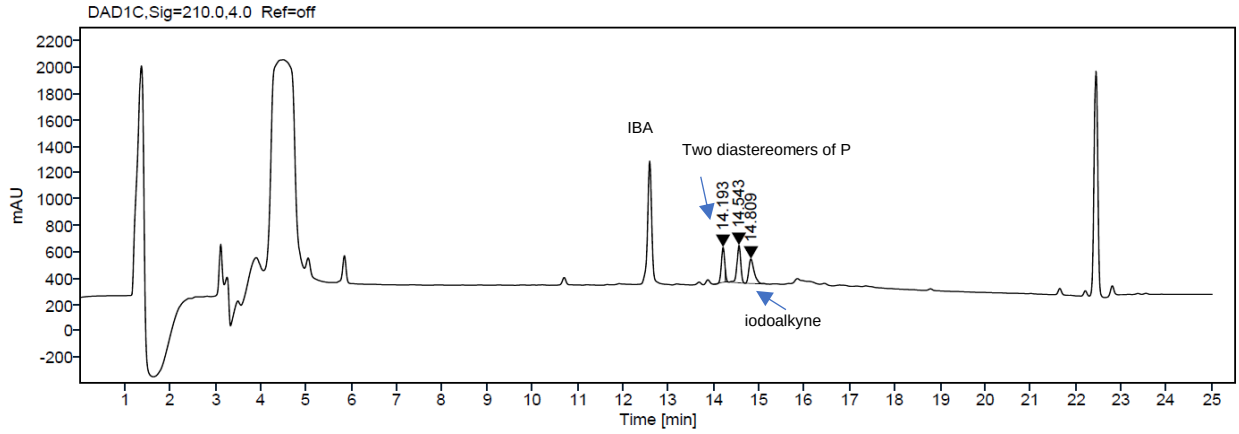
HRMS (Nanochip-based ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{45}H_{53}N_6O_8^+$ 805.3919; Found 805.3915.

Cyclic peptide of FQLAFP (13q)

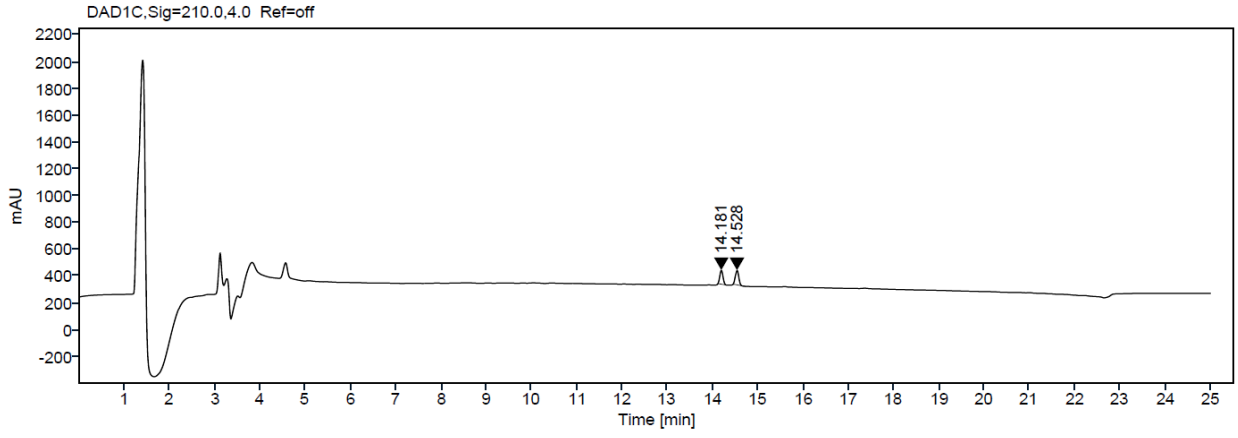
Following the general procedure, the reaction was conducted on a 0.007 mmol scale. HPLC ratio (210 nm) of the product: 66% (dr 1.4:1). The desired product **13q** (1.4 mg, 1.7 μmol, 25% yield) was isolated by **Method 5**.



HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude reaction mixture after 30 min by **Method 1**



HPLC-UV chromatogram (210 nm) and MS(ESI) of **13q** by **Method 1.**



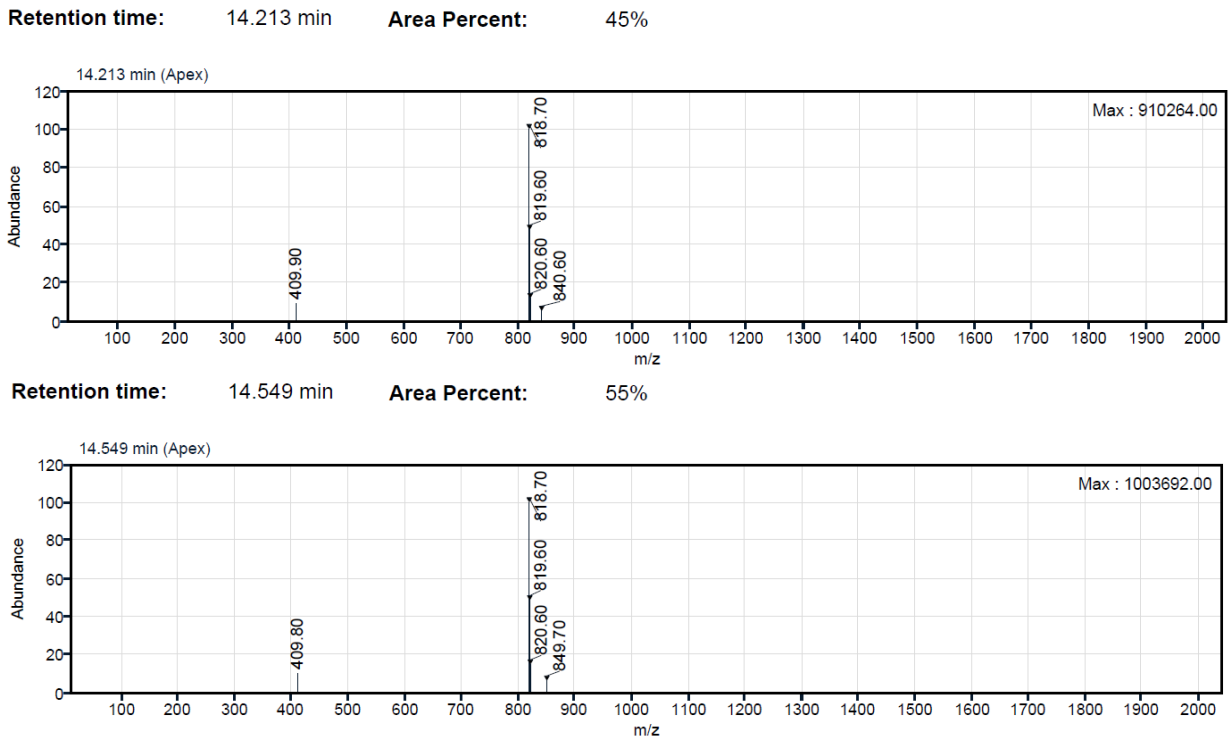
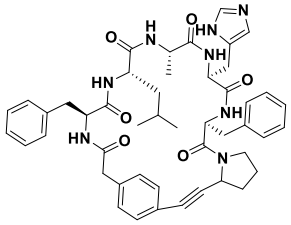
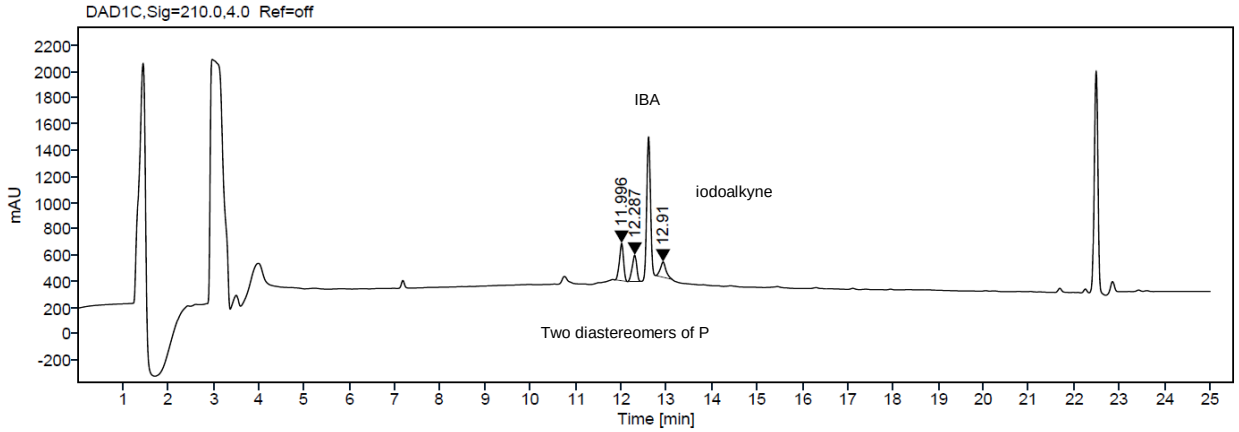


Figure S108: HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude and purified product **13q** by **Method 1.**
HRMS (ESI/QTOF) m/z: $[M + Na]^+$ Calcd for $C_{46}H_{55}N_7NaO_7^+$ 840.4055; Found 840.4066

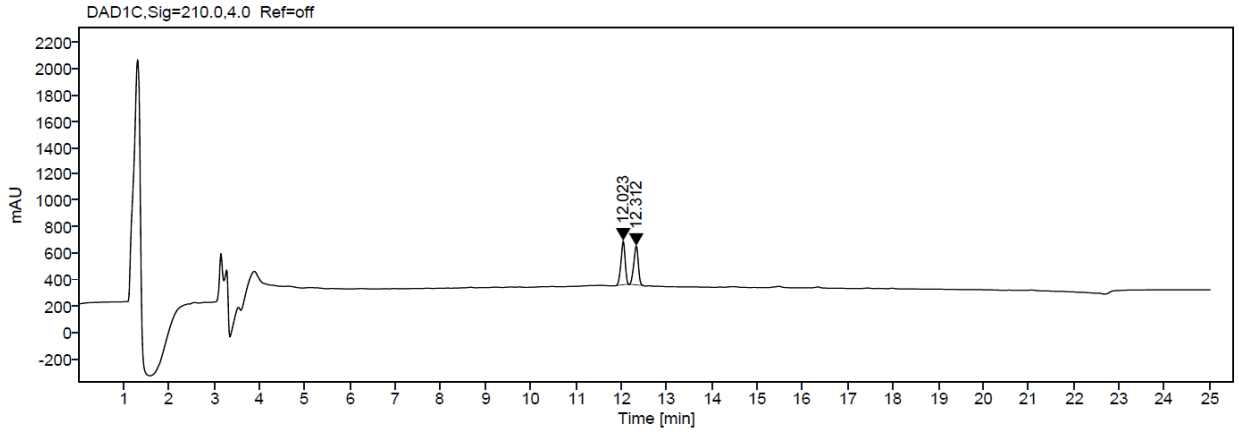
Cyclic peptide of FLHAFP (13r)
Following the general procedure, the reaction was conducted on a 0.01 mmol scale. HPLC ratio (210 nm) of the product: 75% (dr 1.4:1). The desired product **13r** (2.4 mg, 2.9 μmol, 32% yield) was isolated by **Method 5**.



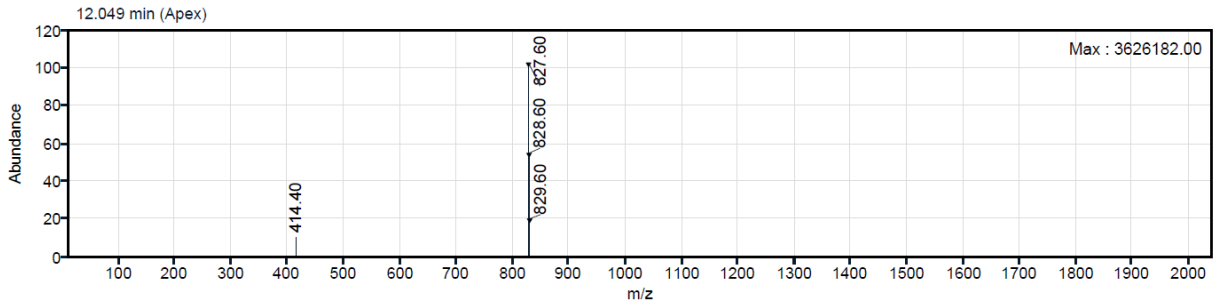
HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude reaction mixture after 30 min by **Method 1**



HPLC-UV chromatogram (210 nm) and MS(ESI) of **13r** by **Method 1.**



Retention time: 12.049 min **Area Percent:** 53%



Retention time: 12.326 min **Area Percent:** 47%

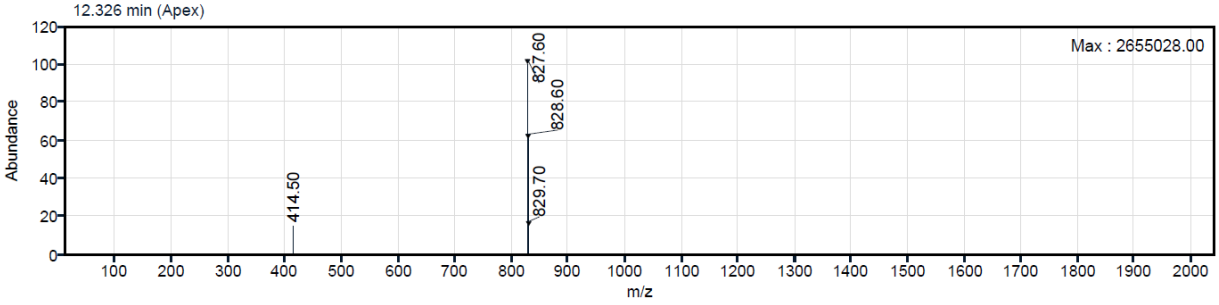
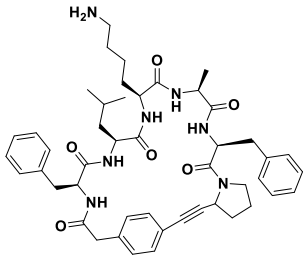


Figure S109: HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude and purified product **13r** by **Method 1.**

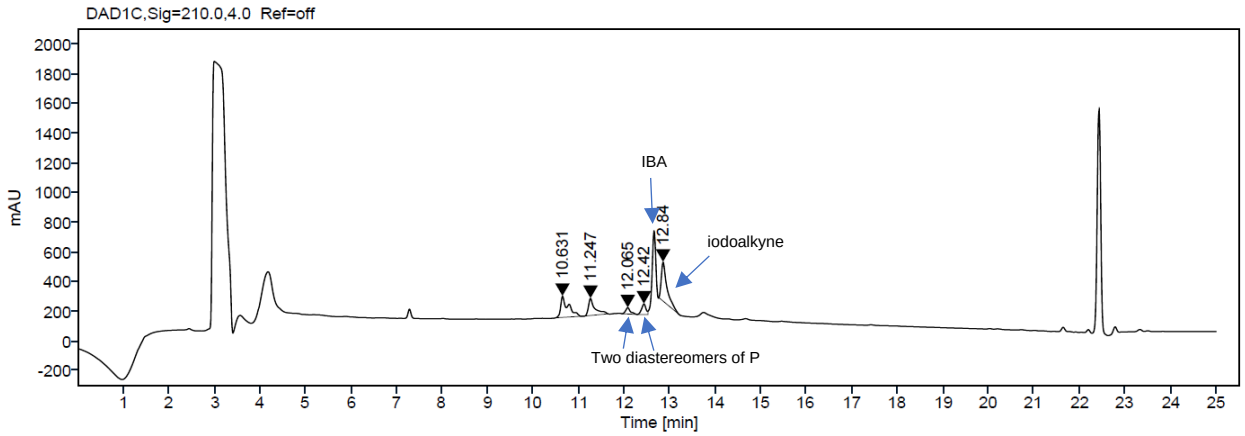
HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{47}H_{55}N_8O_6^+$ 827.4239; Found 827.4236.

Cyclic peptide of FLKAFP (13s)

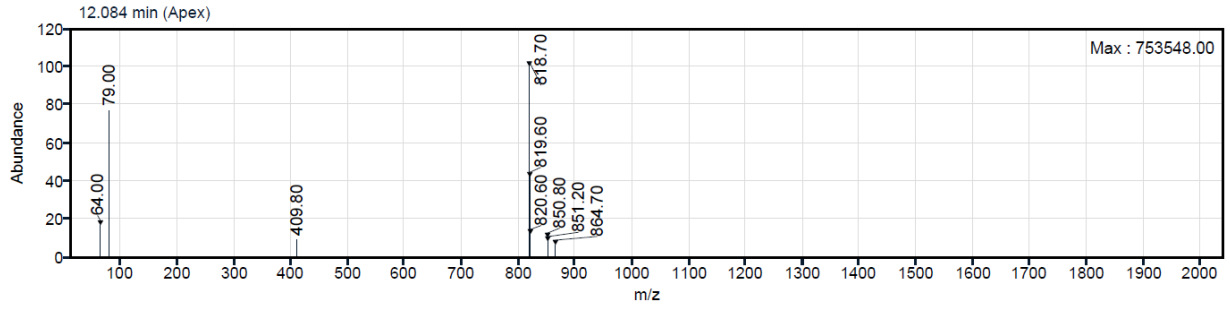
Following the general procedure, the reaction was conducted on a 4 μmol scale. HPLC ratio (210 nm) of the product 17%. Isolation was not conducted due to the low yield.



HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude reaction mixture after 30 min by **Method 1**



Retention time: 12.084 min **Area Percent:** 7%



Retention time: 12.454 min **Area Percent:** 10%

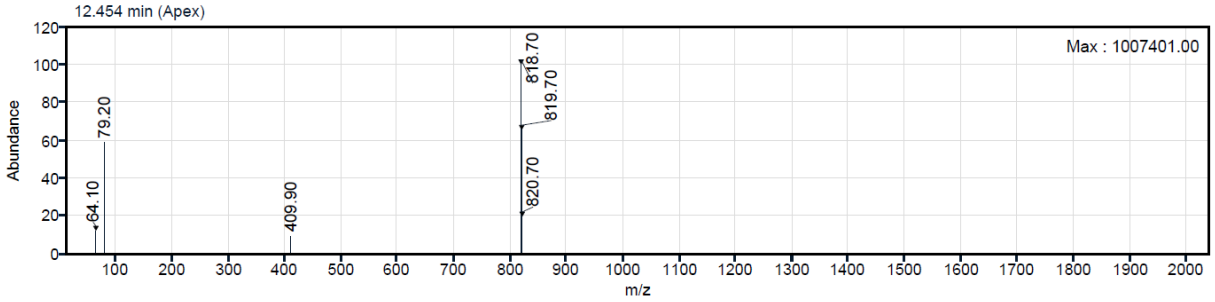
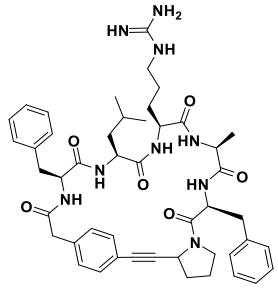
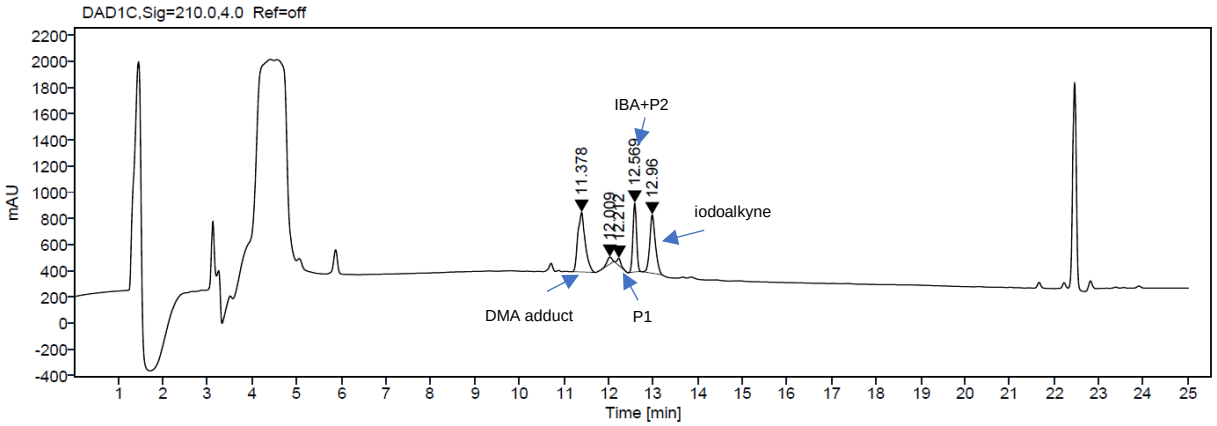


Figure S110: HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude by **Method 1.**

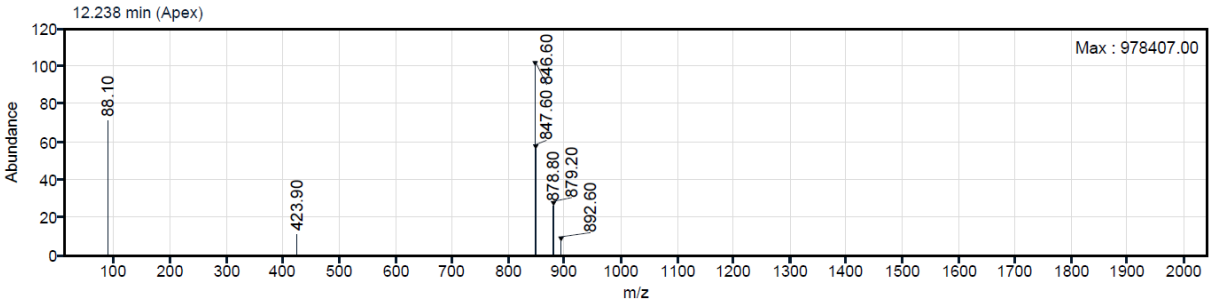
Cyclic peptide of FLRAFP (13t)



Following the general procedure, the reaction was conducted on a 4 μmol scale. HPLC ratio (210 nm) of the product 14%. Isolation was not conducted due to the low yield.



Retention time: 12.238 min **Area Percent:** 7%



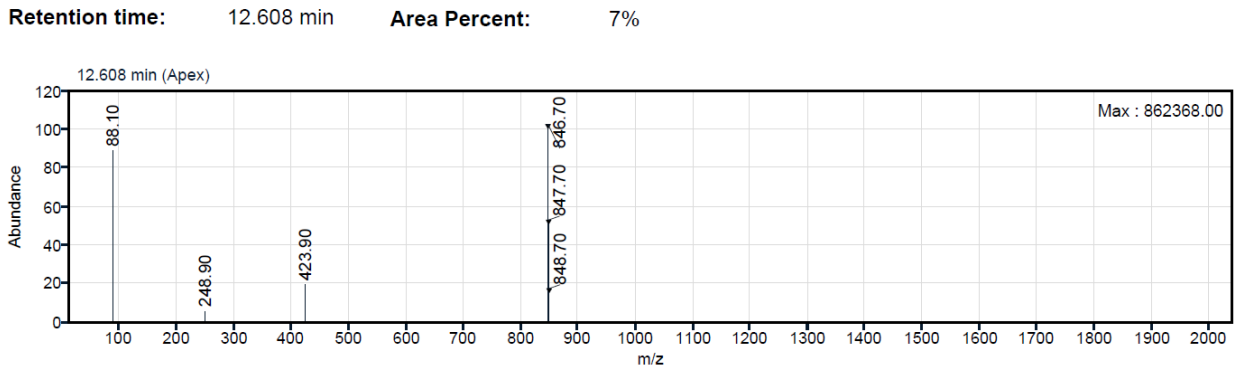
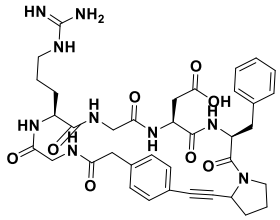


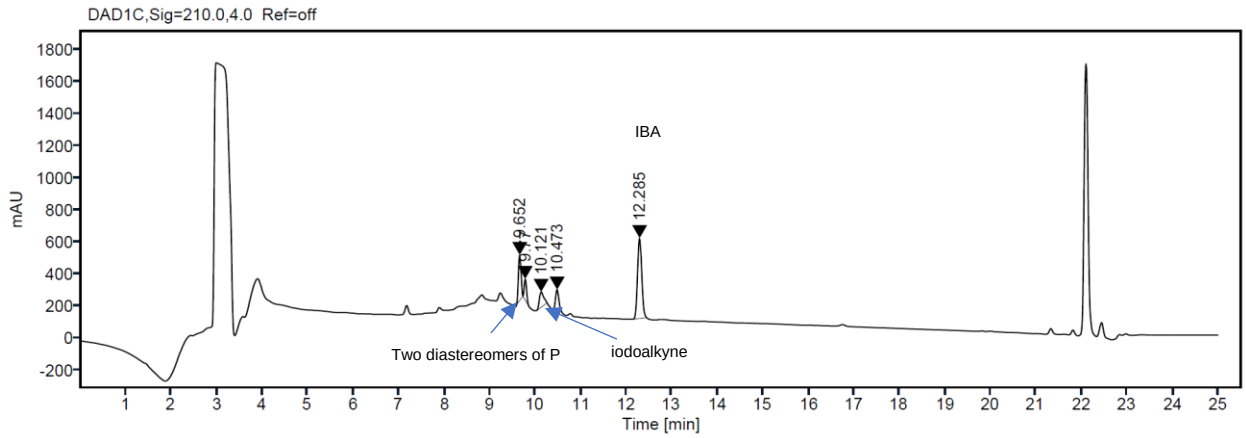
Figure S111: HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude by **Method 1.**

Cyclic peptide of GRGDFP (13u)

Following the general procedure (DMSO as the solvent), the reaction was conducted on a 0.004 mmol scale. HPLC ratio (210 nm) of the product: 65% (dr 2.1:1). The desired product **13u** (1.1 mg, 1.5 µmol, 37% yield) was isolated by **Method 6**.



HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude reaction mixture after 30 min by **Method 2.**



HPLC-UV chromatogram (210 nm) and MS(ESI) of **13u** by **Method 2**:

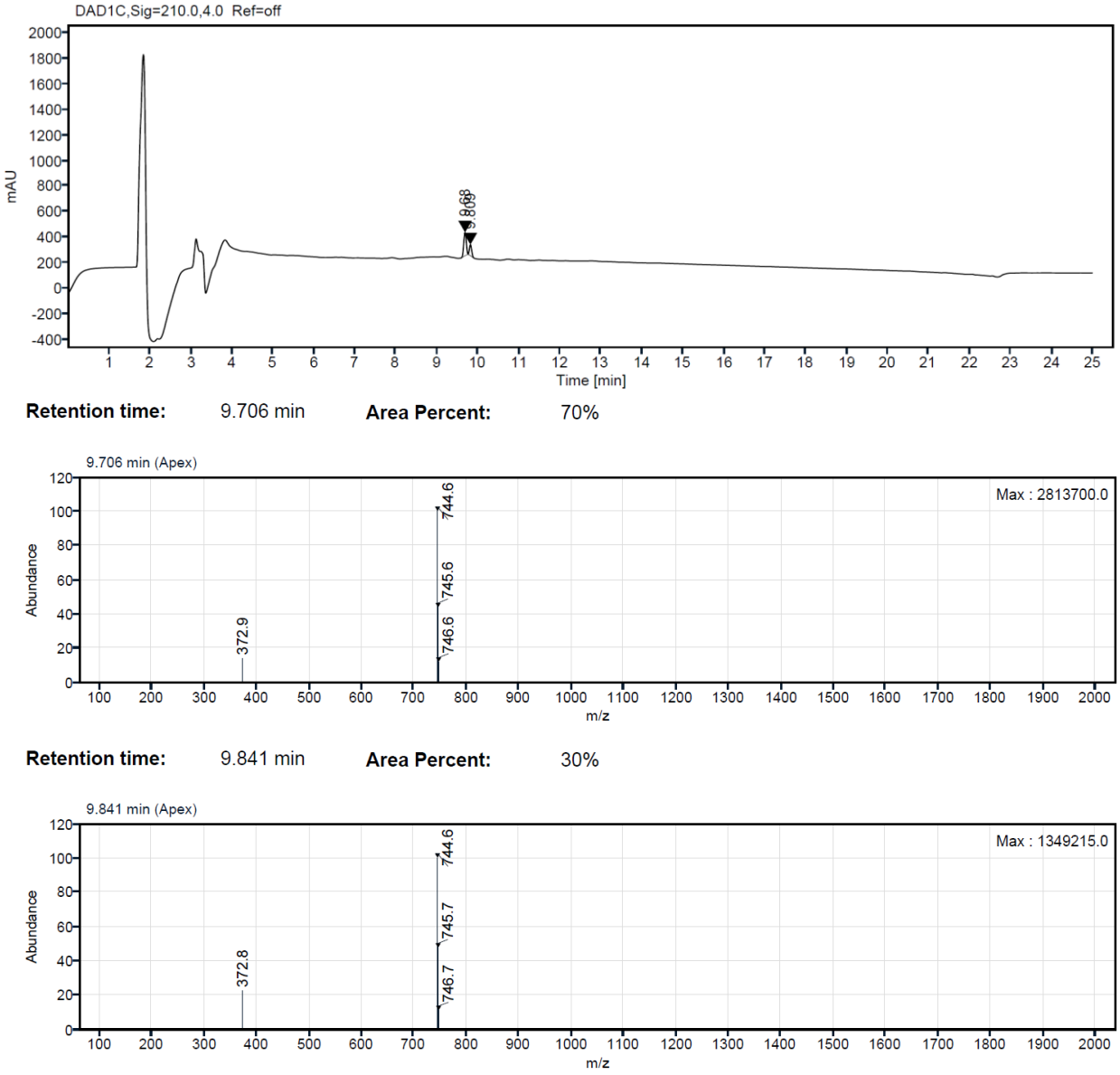


Figure S112: HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude and purified product **13u** by **Method 1.**

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{37}H_{46}N_9O_8^+$ 744.3464; Found 744.3464.

Cyclic peptide of ETFLDLPALLP(13w)

Following the general procedure (DMSO as the solvent), the reaction was conducted on a 0.005 mmol scale. HPLC ratio (210 nm) of the product: 85% (dr n.d.). The desired product **13w** (2.3 mg, 1.7 µmol, 39% yield) was isolated by **Method 6**.

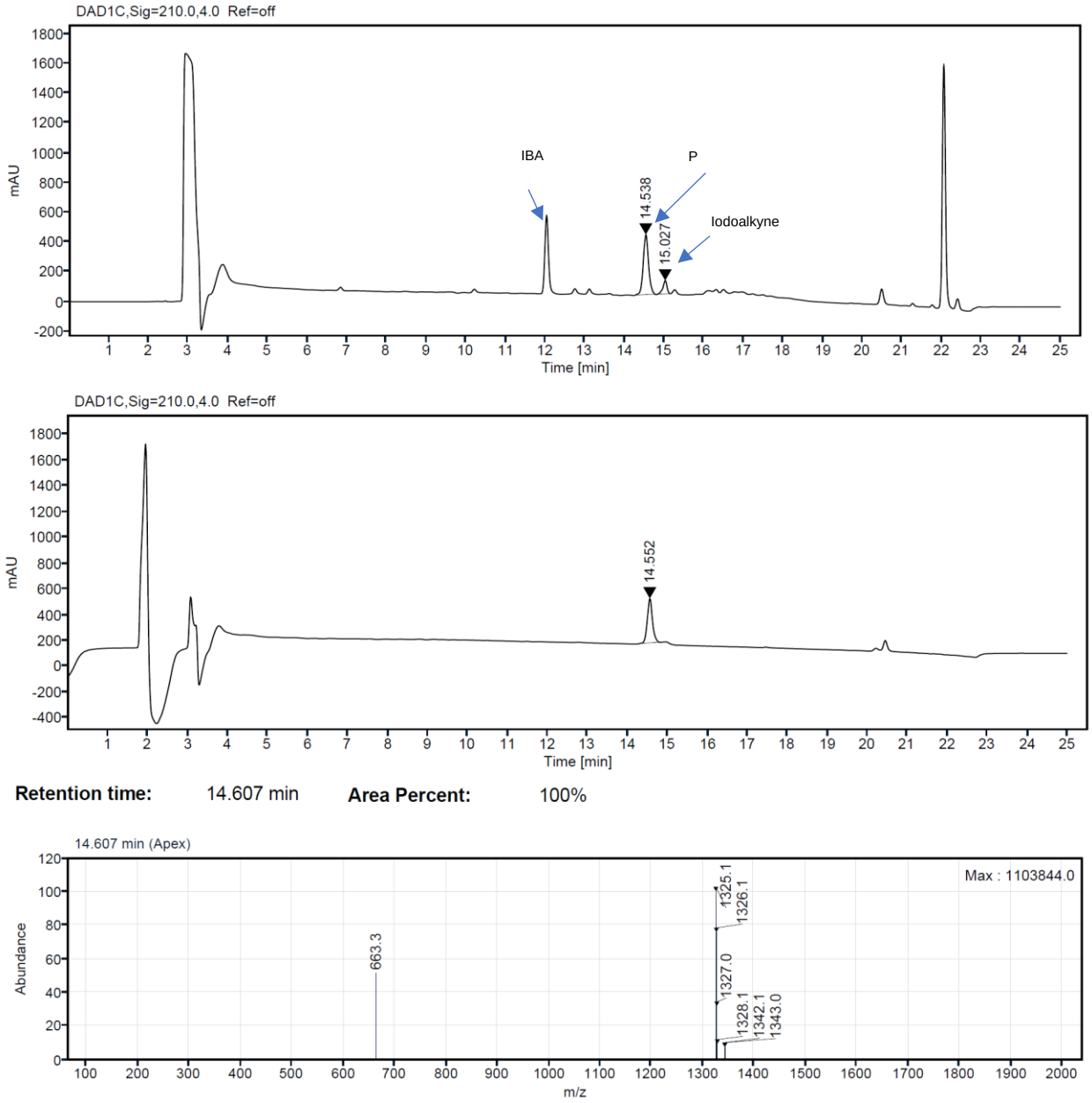
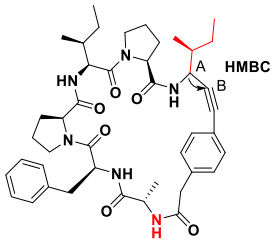


Figure S113: HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude and purified product **13w** by **Method 1.**

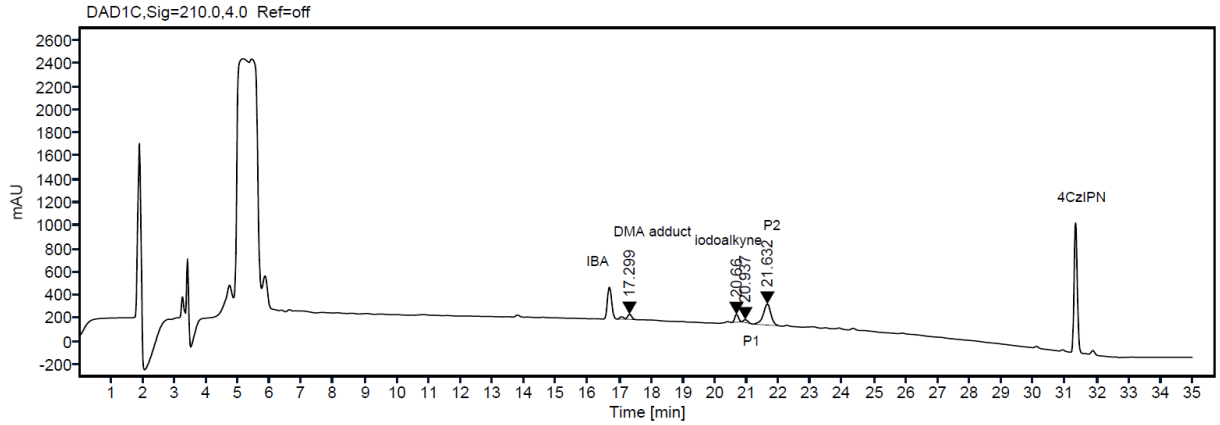
HRMS (ESI/QTOF) m/z: $[M + H_2]^{+2}$ Calcd for $C_{68}H_{99}N_{11}O_{16}^{+2}$ 662.8630; Found 662.8631.

Cyclic peptide of AFPIPI (13x)

Following the general procedure, the reaction was conducted on a 0.014 mmol scale. HPLC ratio (210 nm) of the product: 81% (dr 13:1). The desired product **13x** P2 (4.3 mg, 5.6 μmol, 41% yield) was isolated by **Method 6 (pure P1 could not be isolated)**.



HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude reaction mixture after 30 min by **Method 2.**



HPLC-UV chromatogram (210 nm) and MS(ESI) of **13x** P2 by **Method 2**:

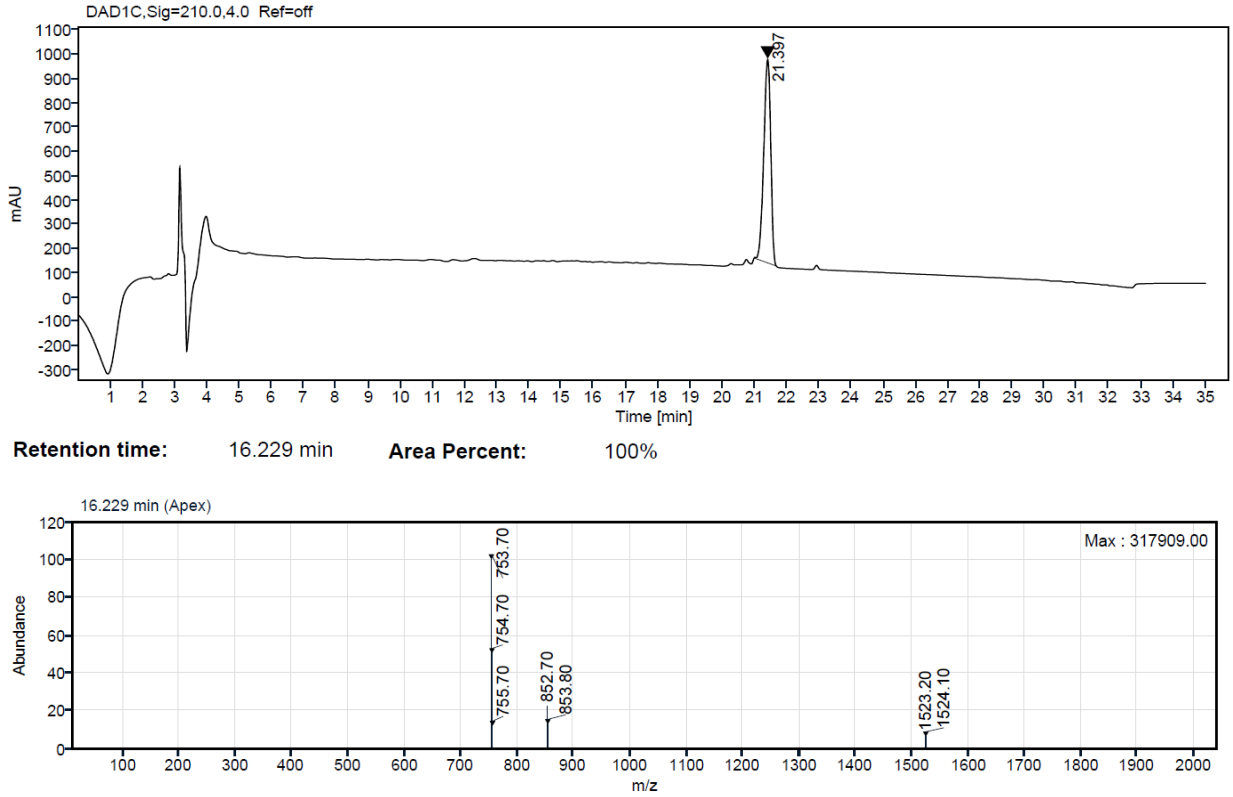
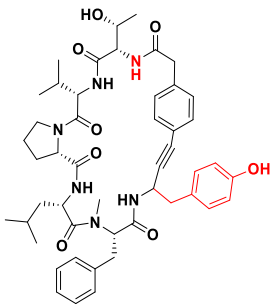


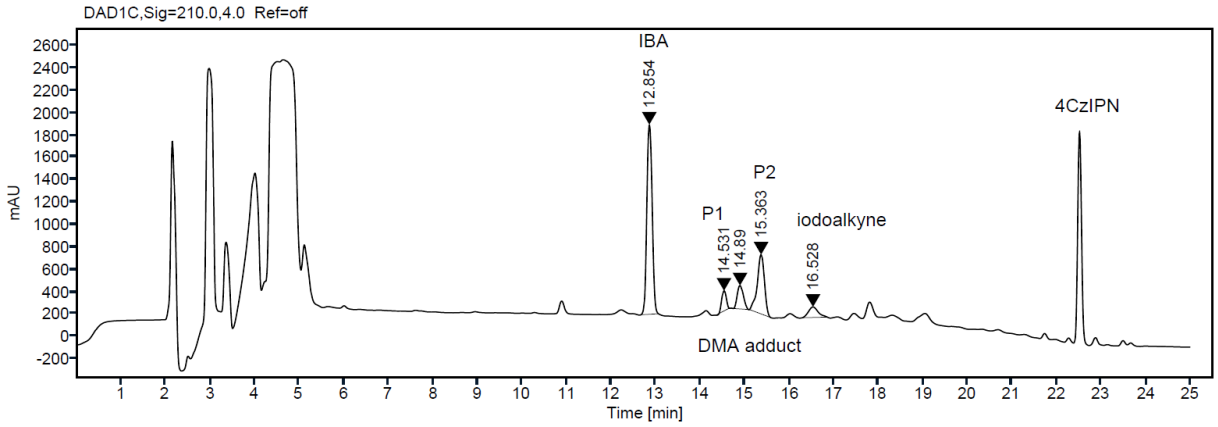
Figure S114: HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude and purified product **13x** by **Method 1.**

^{1}H NMR (500 MHz, CD_3OD) δ 7.59 (d, *J* = 7.9 Hz, 2H), 7.40 (d, *J* = 8.0 Hz, 2H), 7.26 (p, *J* = 8.8, 8.0 Hz, 5H), 5.09 – 5.04 (m, 1H), 4.77 – 4.66 (m, 2H), 4.65 – 4.60 (m, 1H), 4.45 (t, *J* = 7.4 Hz, 1H), 4.21 (q, *J* = 7.3 Hz, 1H), 4.01 – 3.92 (m, 1H), 3.78 (d, *J* = 15.7 Hz, 2H), 3.74 – 3.63 (m, 2H), 3.58 (d, *J* = 15.8 Hz, 1H), 3.47 (td, *J* = 11.0, 6.7 Hz, 2H), 3.09 (dd, *J* = 13.8, 5.3 Hz, 1H), 2.79 (dd, *J* = 13.8, 9.4 Hz, 1H), 2.36 (s, 1H), 2.19 (ddt, *J* = 18.6, 14.5, 7.1 Hz, 2H), 2.10 – 1.91 (m, 6H), 1.76 (dq, *J* = 12.1, 7.2, 6.4 Hz, 2H), 1.64 (ddd, *J* = 13.5, 7.5, 3.2 Hz, 1H), 1.37 (dd, *J* = 8.4, 5.9 Hz, 2H), 1.17 (d, *J* = 7.4 Hz, 3H), 1.07 (d, *J* = 6.6 Hz, 4H), 1.00 (dd, *J* = 7.2, 3.0 Hz, 4H), 0.83 (t, *J* = 7.4 Hz, 2H).
^{13}C NMR (201 MHz, CD_3OD) δ 174.5, 174.4, 174.1, 173.3, 173.0, 171.9, 137.8, 136.7, 133.2, 130.5, 129.7, 129.5, 128.0, 123.1, 89.2, 84.4, 62.1, 61.5, 52.8, 51.9, 49.9, 48.3, 43.3, 40.0, 29.7, 26.9, 26.9, 26.8, 26.2, 17.7, 16.6, 15.8, 12.0, 11.6.
HRMS (ESI/QTOF) m/z: $[M + Na]^+$ Calcd for $C_{43}H_{56}N_6NaO_6^+$ 775.4154; Found 775.4176.

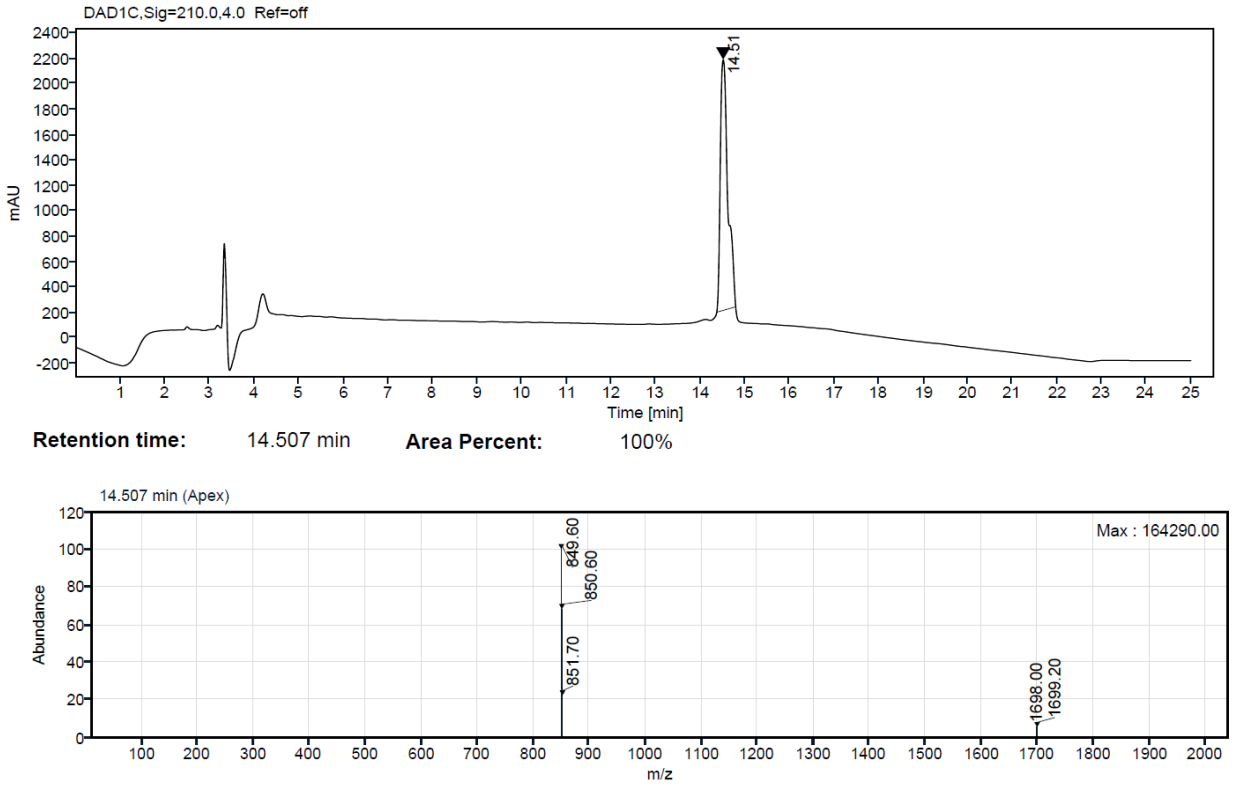
Cyclic peptide of TVPIFY (13y)
Following the general procedure, the reaction was conducted in 6.0 μmol scale. After the reaction, 1 mL TFA was added into the reaction mixture to deprotect the protecting group on the peptides. The reaction mixture was then quenched with excess amount of Na_2CO_3 until no CO_2 was formed, followed by the isolation on Prep HPLC. HPLC ratio (210 nm) of the product: 68% (dr 4.5:1). The desired products **13y** were isolated as two separable diastereomers (P1 0.29 mg, 0.34 μmol, 6% yield, P2, 1.1 mg, 1.3 μmol, 22% yield, 28% yield in total) by **Method 6**.



HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude reaction mixture after deprotection by **Method 1**



HPLC-UV chromatogram (210 nm) and MS(ESI) of **13y** P1 by **Method 1.**



HPLC-UV chromatogram (210 nm) and MS(ESI) of **13y** P2 by **Method 1.**

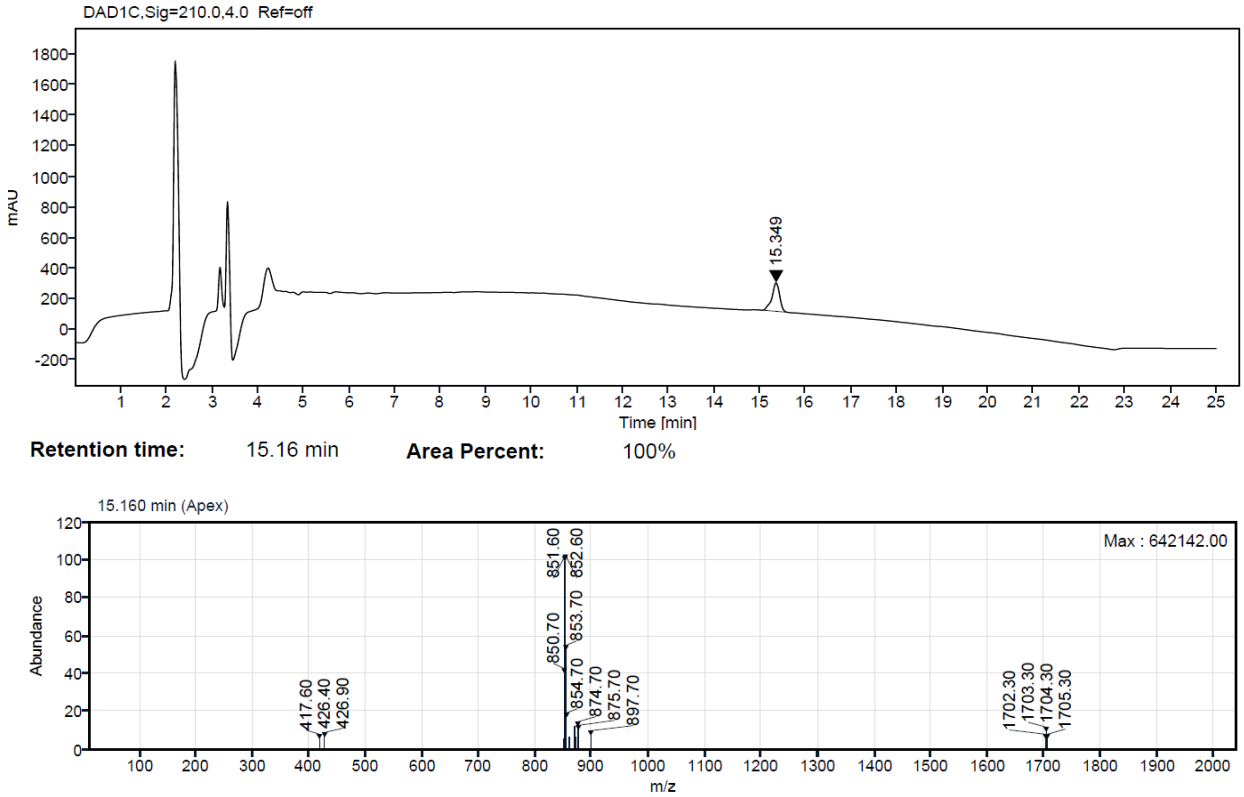
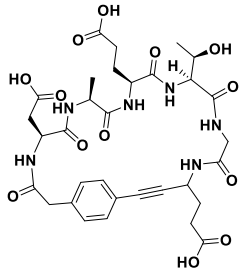


Figure S115: HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude and purified product **13y** P1 and P2 by **Method 1.**

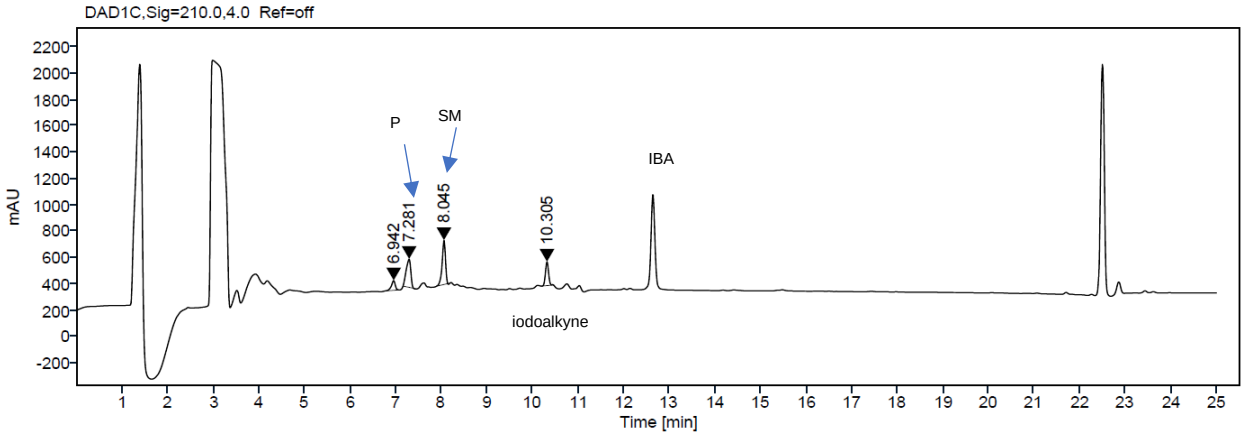
HRMS (ESI/QTOF) m/z: $[M + Na]^+$ Calcd for $C_{48}H_{60}N_6NaO_8^+$ 871.4365; Found 871.4386.

Cyclic peptide of DAETGE (13aa)

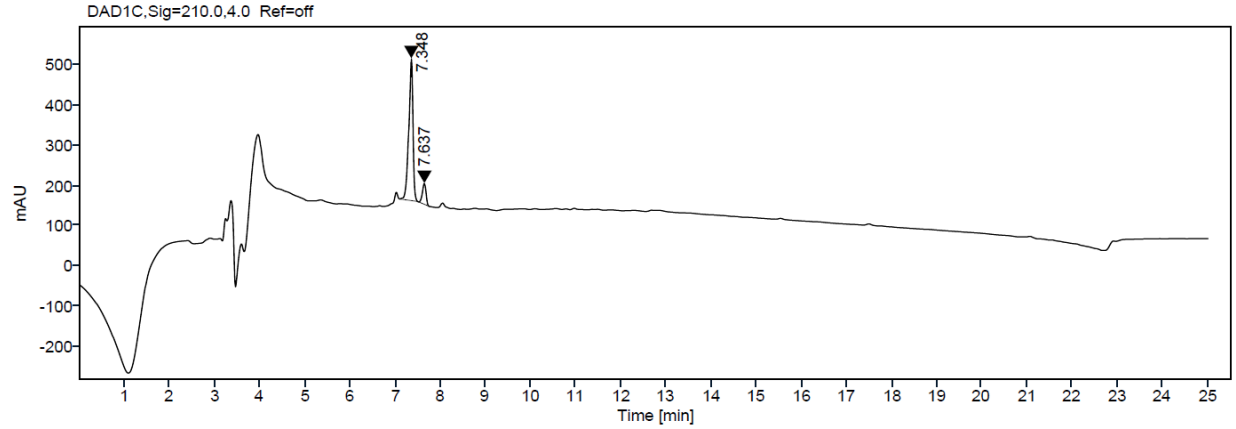
Following the general procedure (DMSO as the solvent), the reaction was conducted on a 0.01 mmol scale. HPLC ratio (210 nm) of the product: 37%. Dr was not determined. The desired product **13aa** (1.5 mg, 2.1 μmol, 21% yield) was isolated by **Method 5**.



HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude reaction mixture by **Method 1**



HPLC-UV chromatogram (210 nm) and MS(ESI) of **13aa** by **Method 1.**



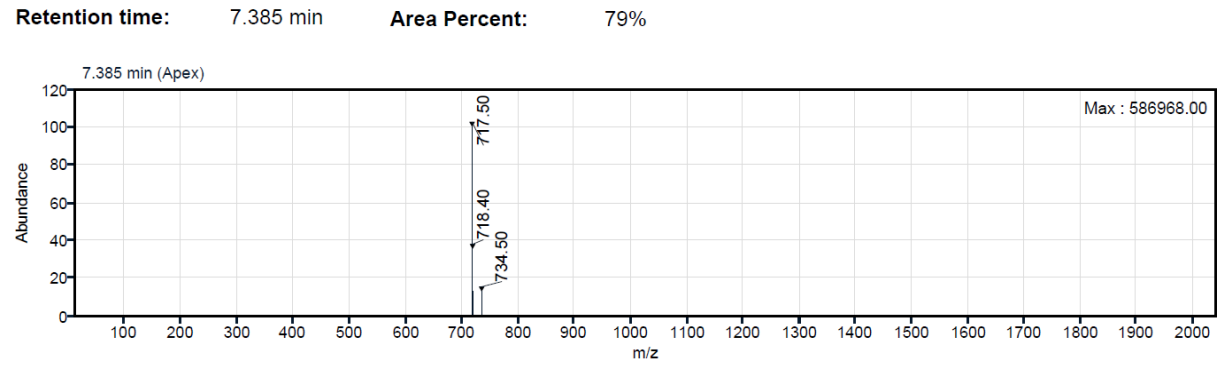
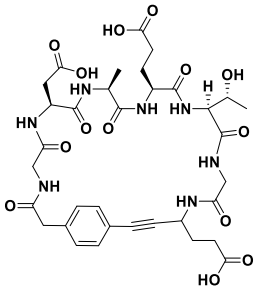


Figure S116: HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude and purified product **13aa** by **Method 1.**

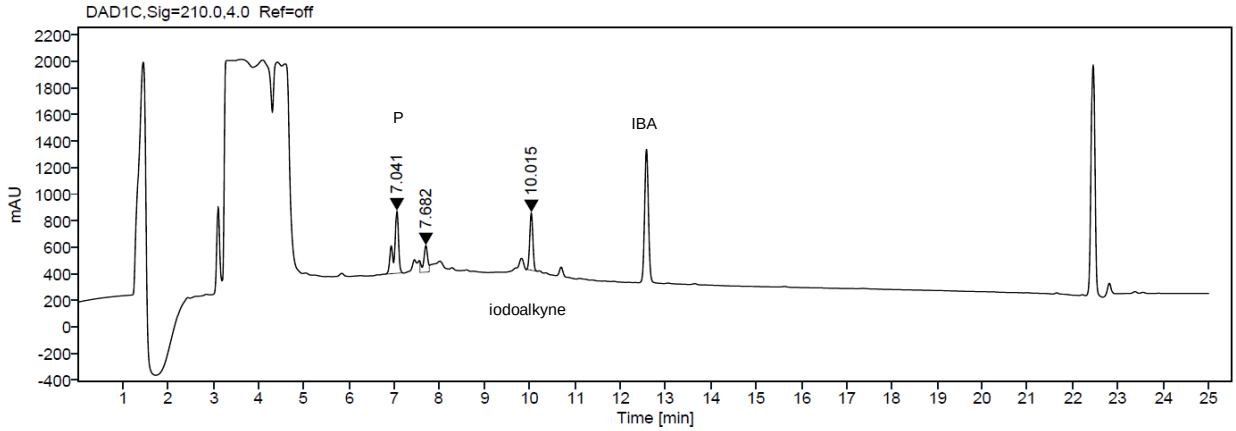
HRMS (ESI/QTOF) m/z: $[M + Na]^+$ Calcd for $C_{32}H_{40}N_6NaO_{13}^+$ 739.2546; Found 739.2552.

Cyclic peptide of GDAETGE (13ab)

Following the general procedure (DMSO as the solvent), the reaction was conducted on a 0.01 mmol scale. HPLC ratio (210 nm) of the product: 49% (dr 2.4:1). The desired product **13ab** (1.7 mg, 2.1 μmol, 27% yield) was isolated by **Method 6**.



HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude reaction mixture by **Method 1**



HPLC-UV chromatogram (210 nm) and MS(ESI) of **13ab** by **Method 1.**

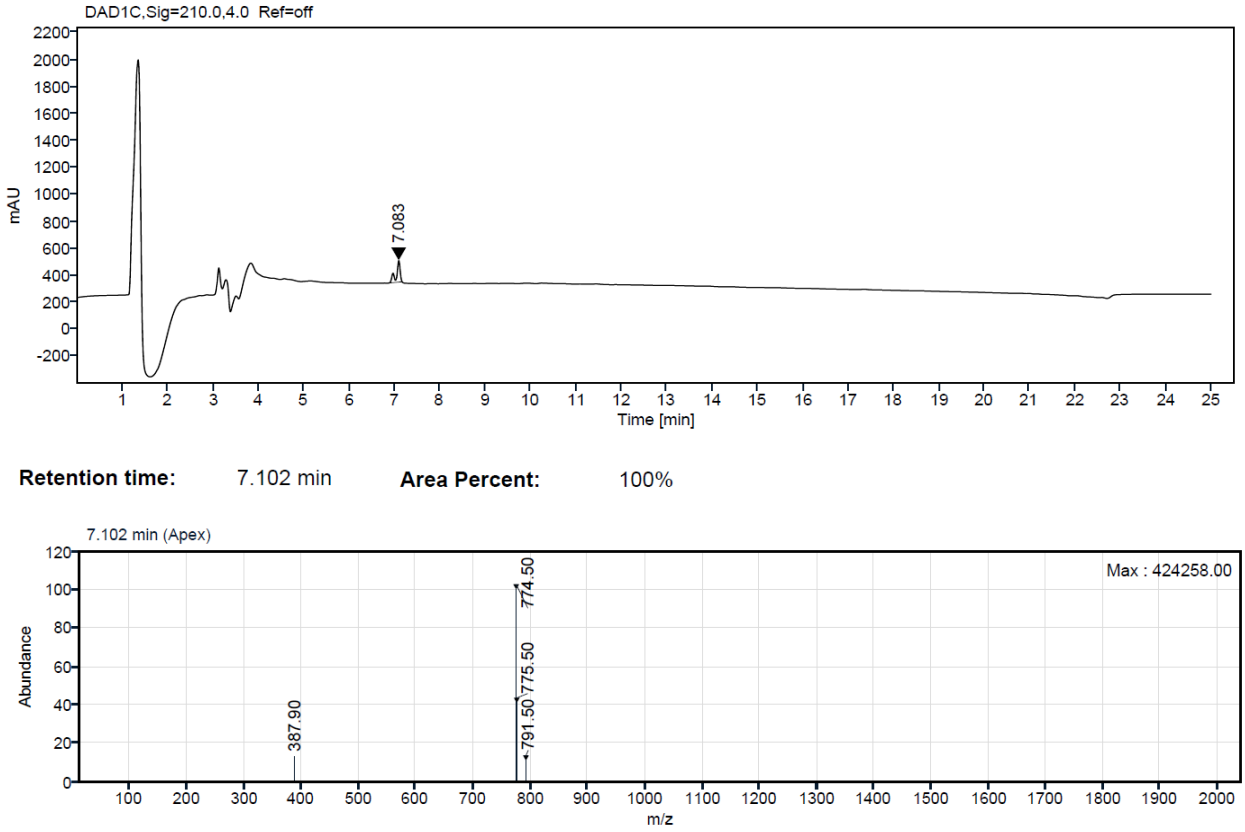
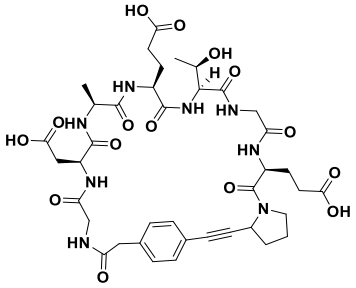
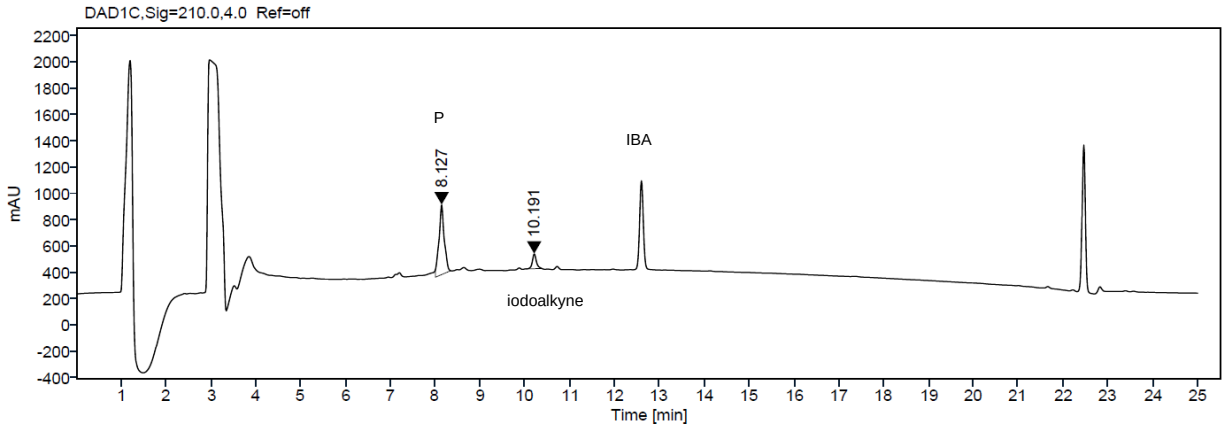


Figure S117: HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude and purified product **13ab** by **Method 1.**
HRMS (ESI/QTOF) m/z: $[M + Na]^+$ Calcd for $C_{34}H_{43}N_7NaO_{14}^+$ 796.2760; Found 796.2770.

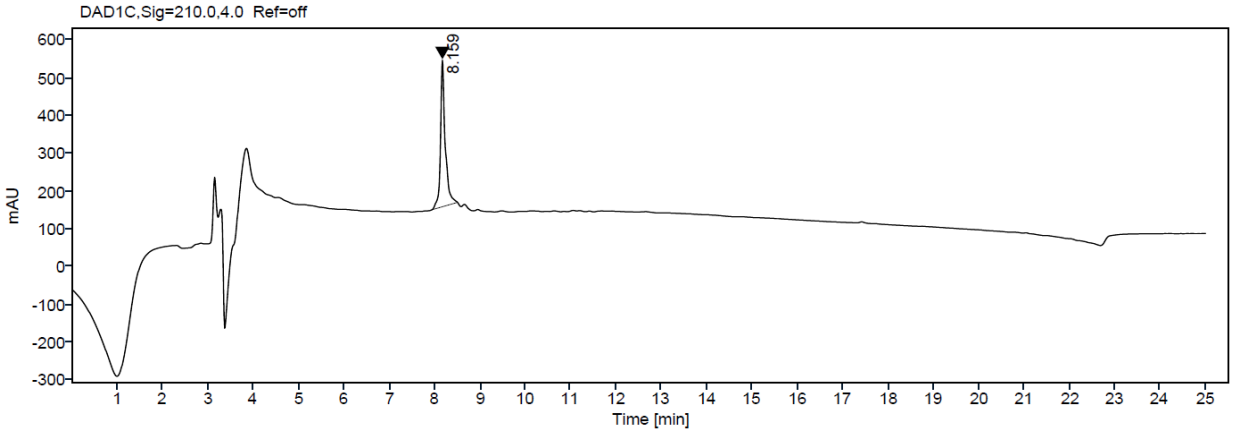
Cyclic peptide of GDAETGEP (13ac)
Following the general procedure (DMSO as the solvent), the reaction was conducted on a 0.014 mmol scale. HPLC ratio (210 nm) of the product: 79%. The desired product **13ac** (5.1 mg, 6.0 μmol, 43% yield) was isolated by **Method 6**.



HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude reaction mixture by **Method 1**



HPLC-UV chromatogram (210 nm) and MS(ESI) of **13ac** by **Method 1.**



Retention time: 8.201 min **Area Percent:** 100%

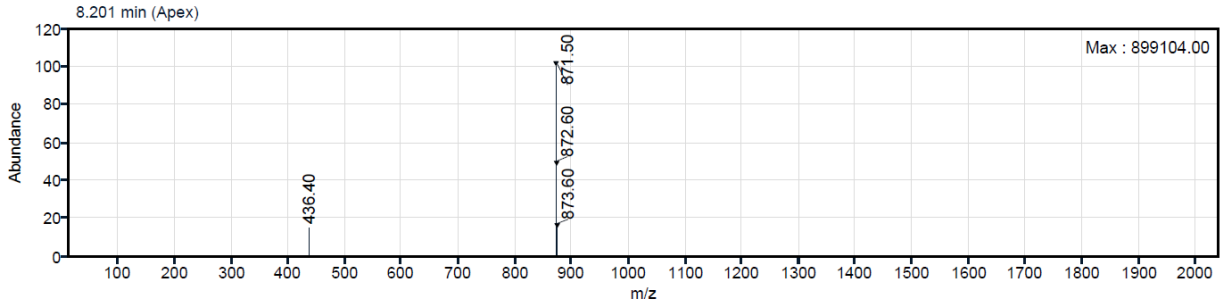
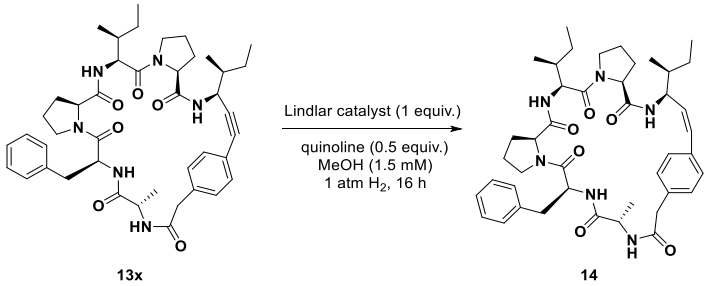


Figure S118: HPLC-UV chromatogram (210 nm) and MS(ESI) of the crude and purified product **13ac** by **Method 1.**

HRMS (Nanochip-based ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{39}H_{51}N_8O_{15}^+$ 871.3468; Found 871.3459.

14. Procedure for reduction of cyclic peptides:



Lindlar catalyst (5% Pd) (3.2 mg, 1.5 μmol, 1 equiv. based on the loading of Pd) was weighed in a 2 mL vial, the cyclic peptide AFPIPI **13x** (1.2 mg, 1.5 μmol, 1 equiv.) was dissolved in 0.5 mL dry MeOH and added into the vial. 10 μL of quinoline (0.75 μmol, 0.5 equiv., 7.5 μM in MeOH solution) was added into the vial. Then the vial was capped and degassed by bubbling with N_2 for 10 min. Then, a hydrogen balloon was connected to the flask through a needle and the mixture was vigorously stirred at room temperature for 16 h. The crude mixture was filtrated through the syringe filter and washed with MeOH. The crude material was purified by Prep-HPLC with **Method 6** to afford the desired product **14** (0.91 mg, 1.2 μmol, 80% yield).

HPLC-UV chromatogram (210 nm) and MS(ESI) of **14** by **Method 1.**

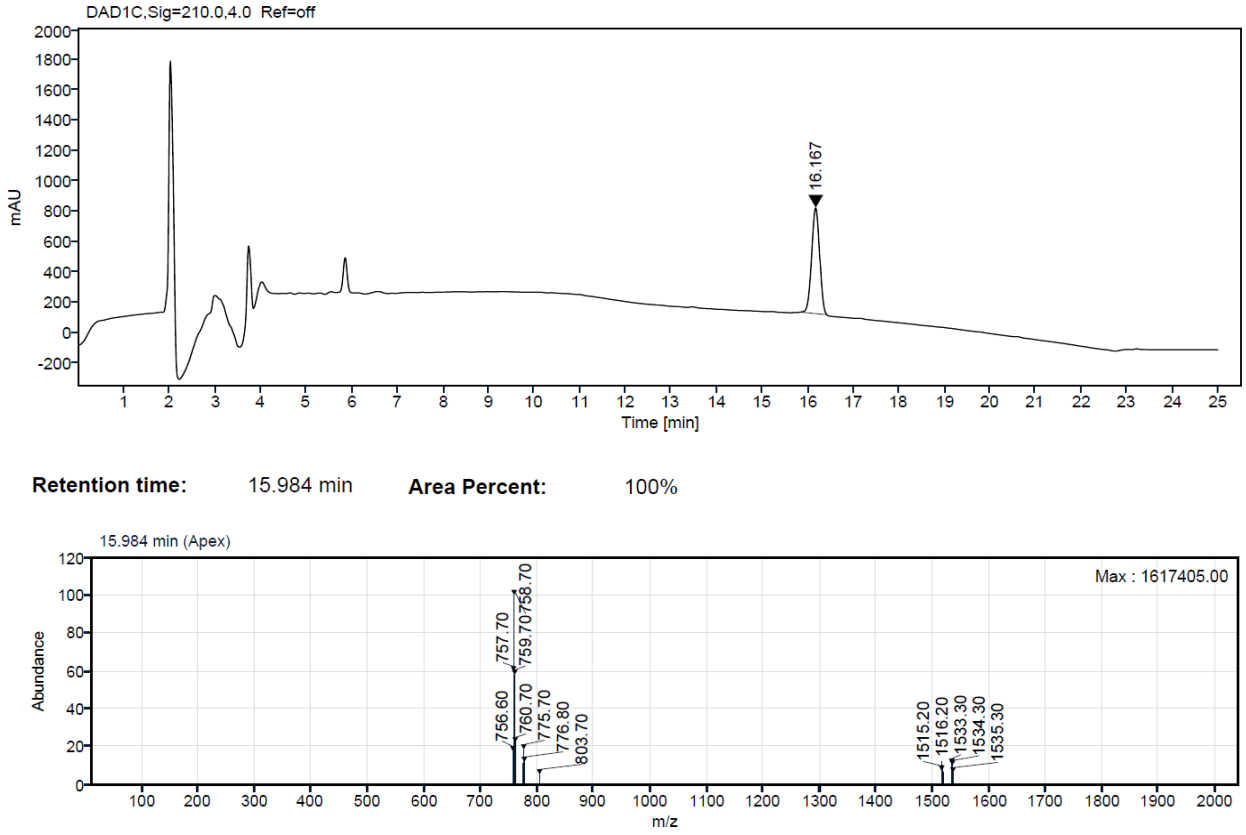
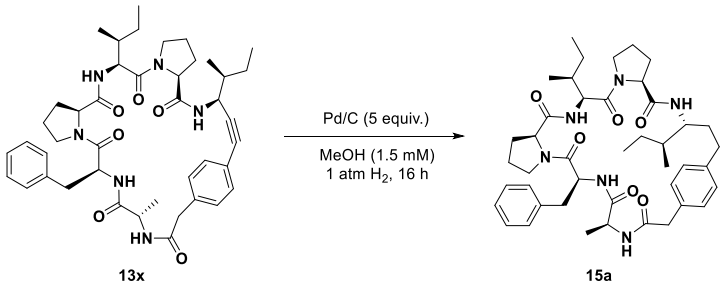


Figure S119: HPLC-UV chromatogram (210 nm) and MS(ESI) of **14** by **Method 1.**

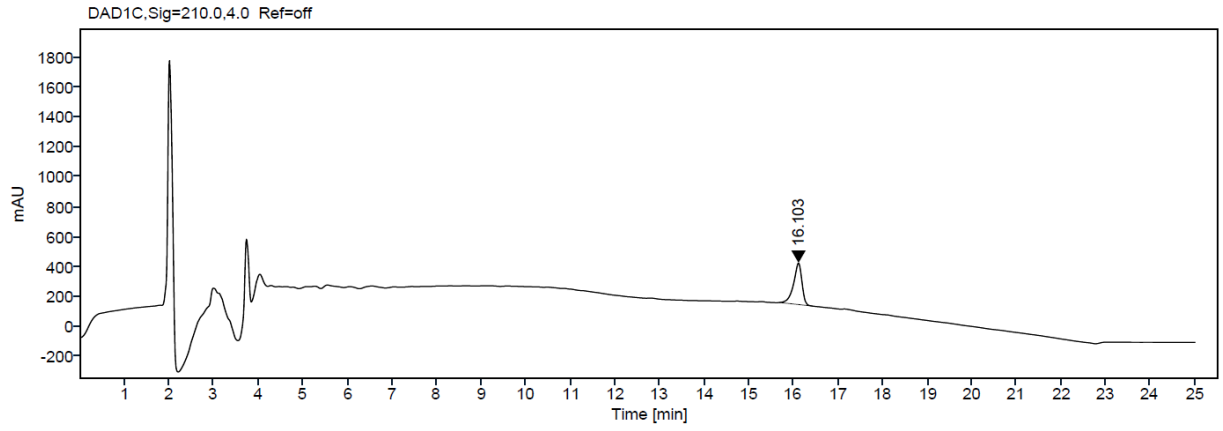
^{1}H NMR (600 MHz, CD_3OD) δ 7.38 – 7.26 (m, 5H), 7.23 – 7.18 (m, 4H), 6.67 (d, J = 11.6 Hz, 1H), 5.70 (dd, J = 11.6, 9.7 Hz, 1H), 4.83 (s, 1H), 4.53 (dd, J = 9.6, 6.0 Hz, 1H), 4.49 (q, J = 7.1

Hz, 1H), 4.40 (dd, *J* = 8.1, 6.7 Hz, 1H), 4.11 (ddd, *J* = 9.4, 7.0, 4.8 Hz, 1H), 4.07 (d, *J* = 10.9 Hz, 1H), 3.81 (d, *J* = 6.9 Hz, 1H), 3.72 – 3.65 (m, 2H), 3.52 – 3.46 (m, 1H), 3.27 (d, *J* = 13.0 Hz, 1H), 3.14 (ddd, *J* = 12.2, 9.6, 2.3 Hz, 1H), 3.07 (dd, *J* = 12.6, 5.0 Hz, 1H), 2.89 (t, *J* = 12.3 Hz, 1H), 2.53 (dtd, *J* = 10.6, 7.8, 7.2, 2.3 Hz, 1H), 2.29 (ddt, *J* = 12.2, 8.2, 6.0 Hz, 1H), 2.12 (td, *J* = 9.9, 7.7, 4.0 Hz, 1H), 2.00 (ddd, *J* = 20.3, 12.1, 6.7 Hz, 2H), 1.95 – 1.88 (m, 1H), 1.71 (dtd, *J* = 15.2, 7.6, 2.8 Hz, 1H), 1.60 (dddd, *J* = 16.6, 14.1, 8.4, 2.6 Hz, 3H), 1.44 – 1.37 (m, 5H), 1.25 (d, *J* = 7.0 Hz, 3H), 1.18 – 1.10 (m, 1H), 1.00 (t, *J* = 7.4 Hz, 4H), 0.94 – 0.83 (m, 7H), 0.36 – 0.26 (m, 1H). HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{43}H_{59}N_6O_6^+$ 755.4491; Found 755.4492.



Pd/C (10% Pd) (7.9 mg, 7.5 μmol, 5 equiv. based on the loading of Pd) was weighed in a 2 mL vial, the cyclic peptide AFPIPI **13x** (1.2 mg, 1.5 μmol, 1 equiv.) was dissolved in 0.5 mL dry MeOH and added into the vial. Then the vial was capped and degassed by bubbling with N_2 for 10 min. Then, a hydrogen balloon was connected to the flask through a needle and the mixture was vigorously stirred at room temperature for 16 h. The crude mixture was filtrated through the syringe filter and washed with MeOH and the solvent was removed under vacuum to afford the desired product **15a** (1.1 mg, 1.4 μmol, 97% yield) without further purification.

HPLC-UV chromatogram (210 nm) and MS(ESI) of **15a** by **Method 1.**



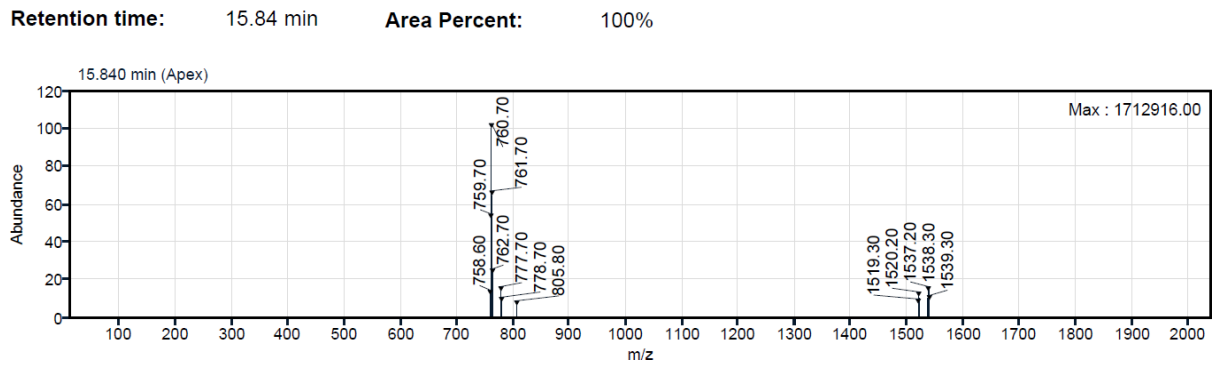
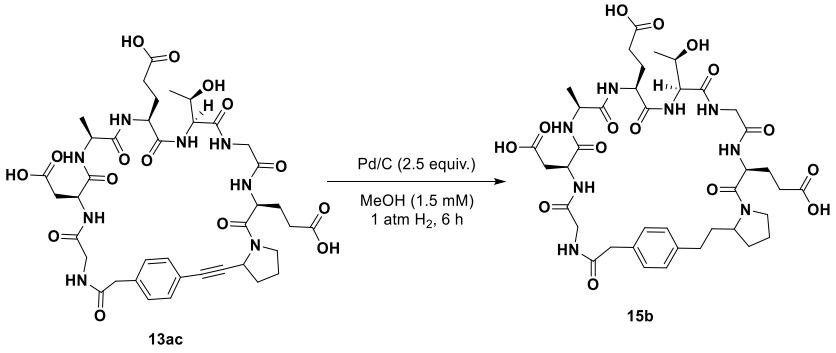
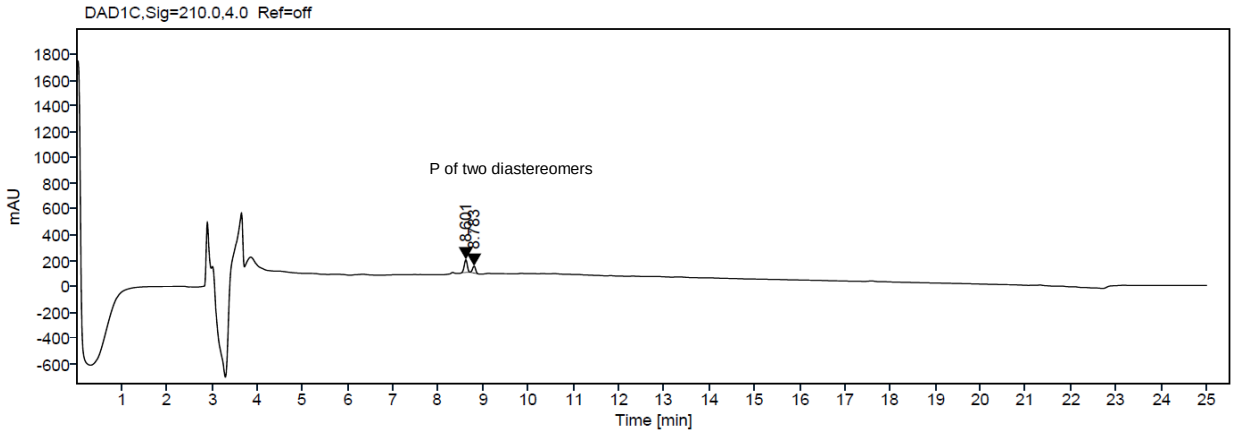


Figure S120: HPLC-UV chromatogram (210 nm) and MS(ESI) of **15a** by **Method 1.**
HRMS (ESI/QTOF) m/z: $[M + Na]^+$ Calcd for $C_{43}H_{60}N_6NaO_6^+$ 779.4467; Found 779.4476.
^{1}H NMR (600 MHz, CD_3OD) δ 7.54 (d, *J* = 7.7 Hz, 2H), 7.35 (d, *J* = 7.8 Hz, 2H), 7.28 – 7.25 (m, 2H), 7.22 (d, *J* = 6.4 Hz, 1H), 7.17 – 7.14 (m, 2H), 4.25 (d, *J* = 7.4 Hz, 1H), 3.87 – 3.78 (m, 2H), 3.72 – 3.64 (m, 3H), 3.54 (d, *J* = 14.9 Hz, 1H), 3.49 – 3.43 (m, 1H), 3.01 (dd, *J* = 13.9, 4.9 Hz, 1H), 2.94 – 2.87 (m, 1H), 2.62 (ddd, *J* = 25.1, 13.1, 8.2 Hz, 2H), 2.42 (dd, *J* = 13.1, 6.3 Hz, 1H), 2.30 – 2.23 (m, 1H), 2.18 (tq, *J* = 12.7, 7.5, 7.1 Hz, 1H), 2.01 – 1.93 (m, 5H), 1.87 – 1.80 (m, 1H), 1.63 – 1.47 (m, 5H), 1.39 (d, *J* = 6.9 Hz, 1H), 1.21 (dt, *J* = 14.0, 7.4 Hz, 1H), 1.12 (d, *J* = 7.4 Hz, 3H), 1.06 (ddd, *J* = 17.8, 10.5, 5.1 Hz, 1H), 0.99 – 0.94 (m, 7H), 0.92 (d, *J* = 6.6 Hz, 4H), 0.90 – 0.87 (m, 1H), 0.84 (t, *J* = 7.4 Hz, 3H).

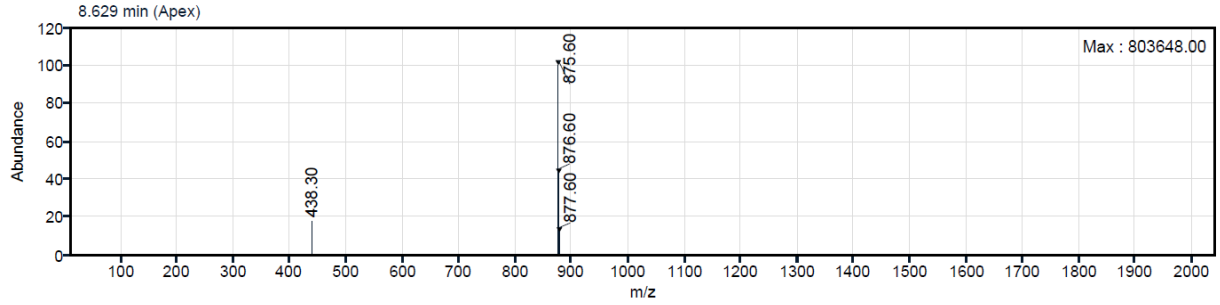


Pd/C (10% Pd) (8.5 mg, 8.0 μmol, 5 equiv. based on the loading of Pd) was weighed in a 2 mL vial, the cyclic peptide GDAETGEP **13ac** (1.4 mg, 1.6 μmol, 1 equiv.) was dissolved in 0.5 mL dry MeOH and added into the vial. Then the vial was capped and degassed by bubbling with N_2 for 10 min. Then, a hydrogen balloon was connected to the flask through a needle and the mixture was vigorously stirred at room temperature for 6 h. The crude mixture was filtrated through the syringe filter and washed with MeOH and the solvent was removed under vacuum to afford the desired product **15b** (1.2 mg, 1.4 μmol, 85% yield) without further purification.

HPLC-UV chromatogram (210 nm) and MS(ESI) of **15b** by **Method 1.**



Retention time: 8.629 min **Area Percent:** 64%



Retention time: 8.807 min **Area Percent:** 36%

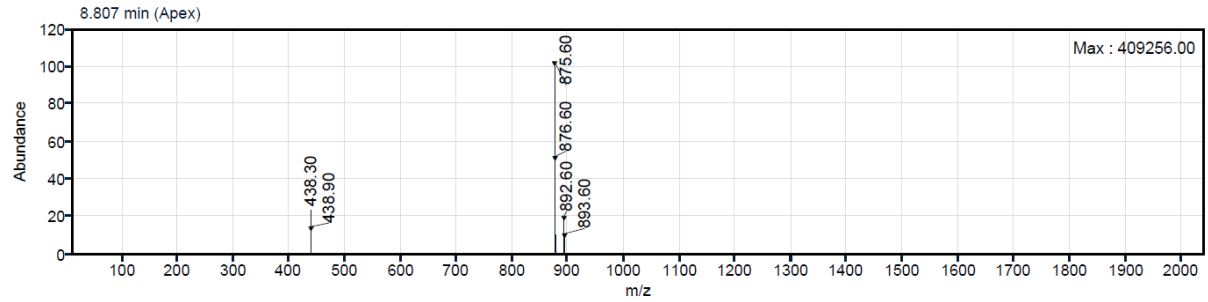


Figure S121: HPLC-UV chromatogram (210 nm) and MS(ESI) of **15b** by **Method 1.**
HRMS (ESI/QTOF) m/z: $[M + Na]^+$ Calcd for $C_{39}H_{54}N_8NaO_{15}^+$ 897.3601; Found 897.3583.

15. TR-FRET competition assay

To establish the TR-FRET assay, N-Terminal 6×His-tagged human KEAP1 Kelch repeat domain (residues 312−624) was expressed as a tagged fusion protein in E. coli and subsequently purified via Ni-NTA resin purification, and size-exclusion chromatography. For TR-FRET, N-Terminal 6×His-tagged KEAP1 was incubated with a Europium-labeled anti-6×His antibody (LANCE-Eu-W1024 Anti 6xHis, PerkinElmer) and a Sulfo-Cy5-N_3-labeled peptide probe (sequence: Ac-Propargylglycine-Peg_2-LDEETGEFL-NH_2) allowing the assembly of donor and acceptor dye pairs for use in protein-binding assay.

The assay was measured by adding 2-fold dilutions of testing peptides (final concentration from 2666.7 nM to 0.17 nM) to premixed N-Terminal 6×His-tagged KEAP1 (final concentration 20 nM), Sulfo-Cy5-N3-labeled peptide probe (final concentration 40nM) and Europium-labeled anti-6×His antibody (final concentration 0.5 nM) in HEPES buffer (10 mM HEPES, 150 mM NaCl, 0.005% (w/v) Tween-20, 0.05% (w/v) BSA, pH 7.4). The reagents were added to wells of a 384-microwell plate (ThermoFischer NUNC™ 384 shallow well std height plates non-sterile, black) to reach a total assay volume of 15 µl. The plate was sealed and centrifuged at 800 g for 1 minute to eliminate bubbles in the wells. After 30 min incubation at RT, the TR-FRET signal was measured using a plate reader (PHERAstar FSX, BMG) exciting at 337 nm and measuring with a delay of 60 μs the emission at both, 620 nm (from europium) and 665 nm (from Cy5) with a measurement window of 400 μs. Ratio of fluorescent emission intensity at 665-620 nm was calculated for each reaction (equation 1). Percent inhibition was calculated based upon Min (control compound - KI696) vs. Max (DMSO) according to equation 2 & 3. Sigmoidal curves were fitted to the data using Graphpad Prism 5 software.

To test the feasibility of this assay, we measured the binding affinity of the unlabeled linear peptide sequence (Ac-LDEETGEFL-NH_2), and a similar affinity as reported in the literature was observed (IC_{50} = 71 nM vs 74 nM[6]).

[6] Ortet, P. C.; Muellers, S. N.; Viarengo-Baker, L. A.; Streu, K.; Szymczyna, B. R.; Beeler, A. B.; Allen, K. N.; Whitty, A., Recapitulating the Binding Affinity of Nrf2 for KEAP1 in a Cyclic Heptapeptide, Guided by NMR, X-ray Crystallography, and Machine Learning. *J. Am. Chem. Soc.* **2021,** *143* (10), 3779-3793.

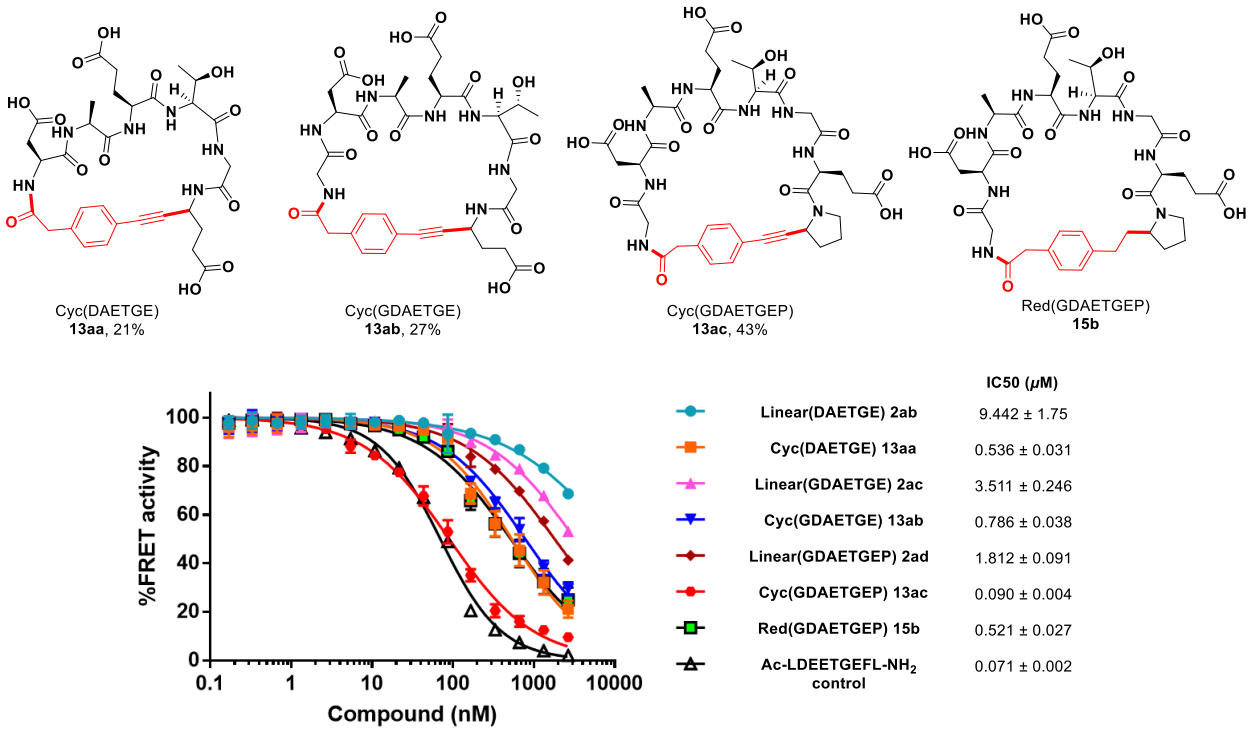


Figure S122: Binding affinity of peptidic macrocycles to the Keap1-Nrf2 PPIs site, measured in TR-FRET competition assays. Average values of three independent measurements are shown.

$$Test\ ratio\ =\ Em_{665}\ /\ Em_{620} * \ 1{,}0000 \quad (1)$$

$$\%\ FRET\ activity = \frac{Test\ ratio - Min\ (control\ compound)}{Max(DMSO)\ - Min\ (control\ compound)} * 100 \quad (2)$$

$$\%inhibition = 100 - \%\ FRET\ activity \quad (3)$$

16. NMR spectra

Figure S123: **S2** ^{1}H NMR (400 MHz, $CDCl_3$)

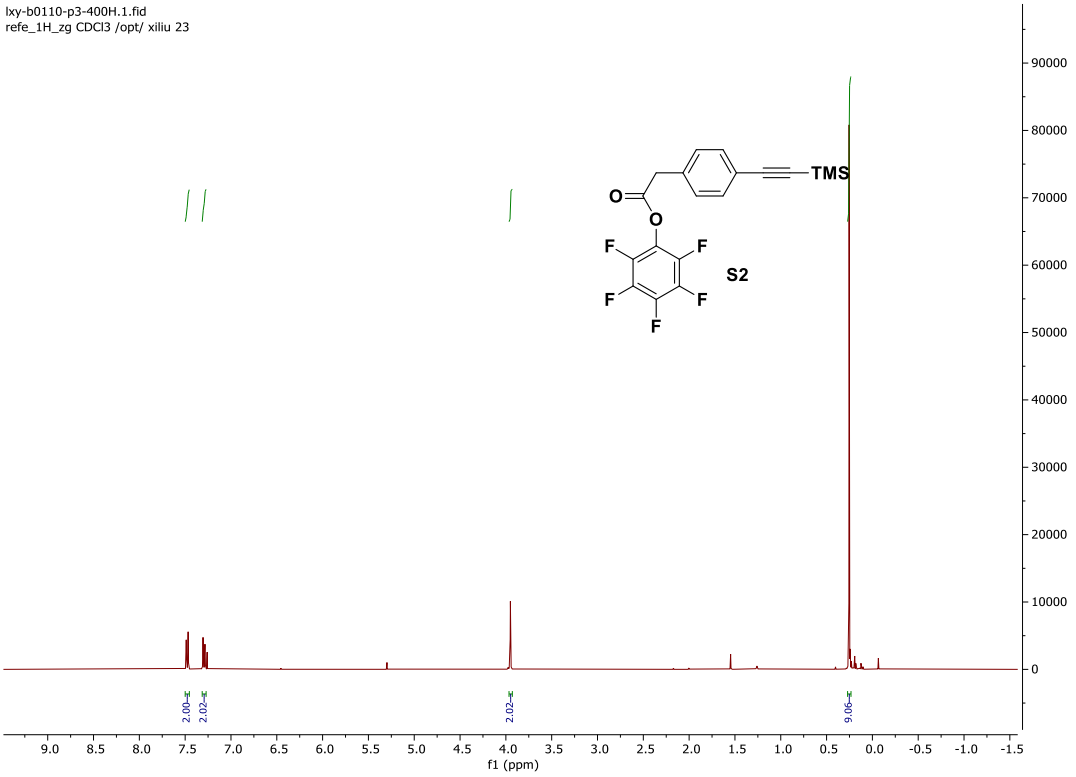


Figure S124: ^{13}C NMR (400 MHz, $CDCl_3$)

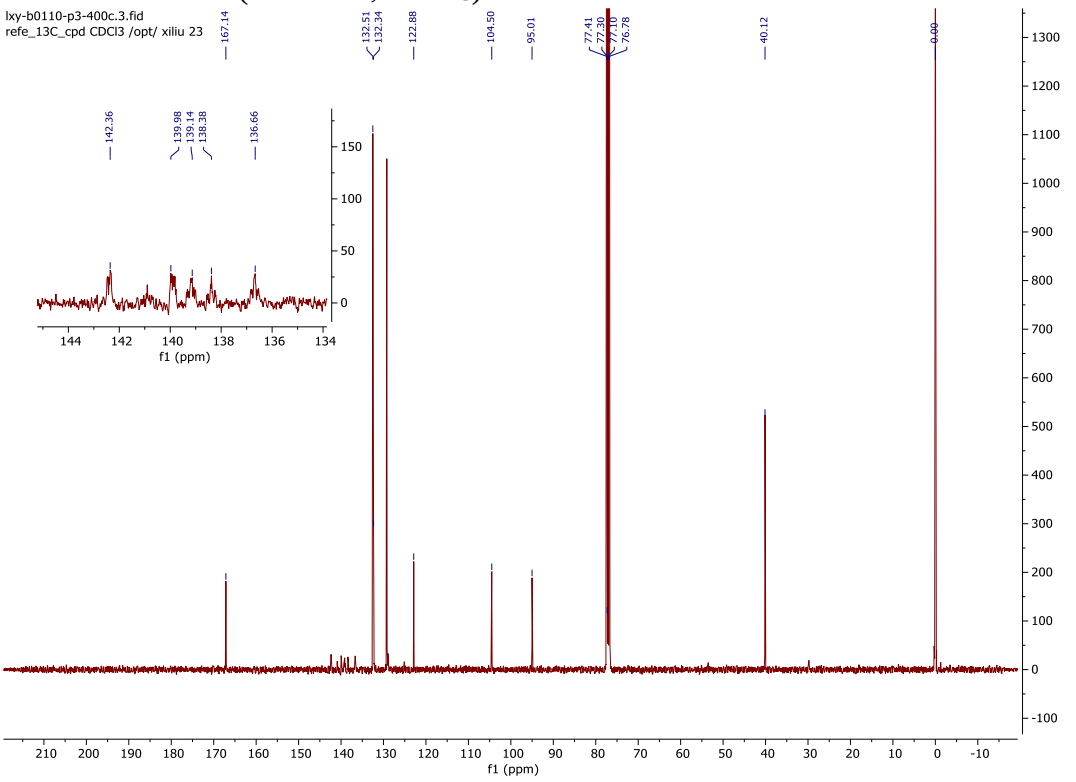


Figure S125: S5 ^{1}H NMR (400 MHz, $CDCl_3$)

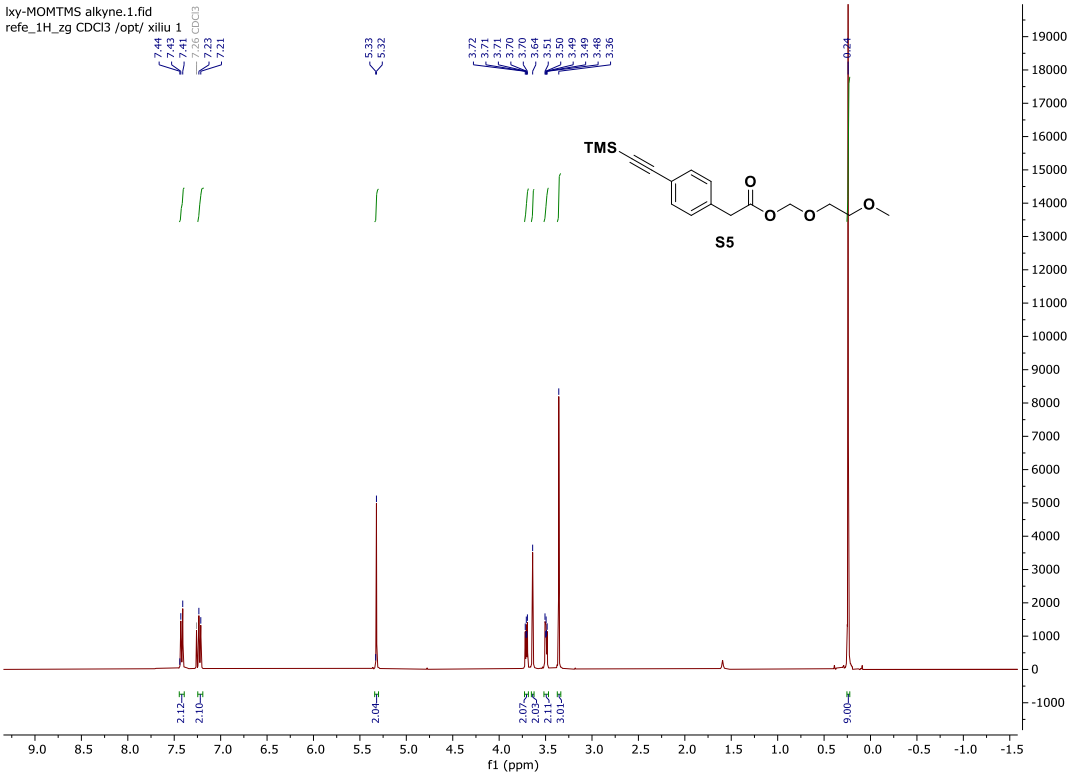


Figure S126: ^{13}C NMR (126 MHz, $CDCl_3$)

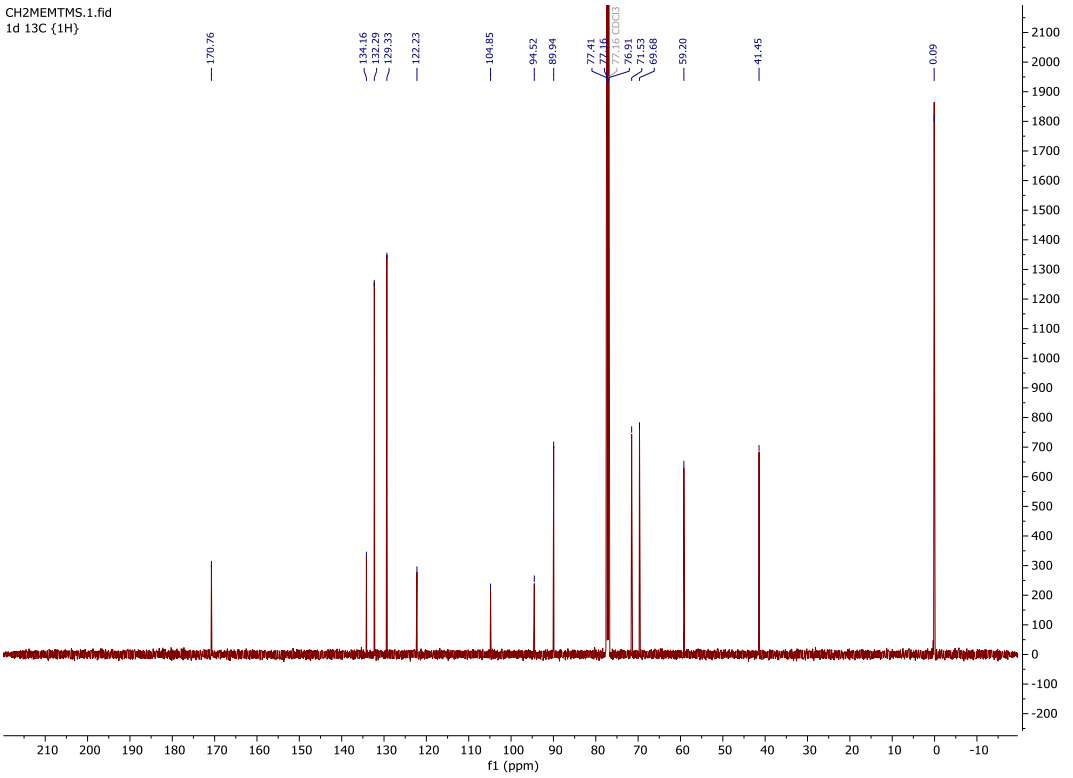


Figure S127: 1b ^{1}H NMR (800 MHz, $CDCl_3$)

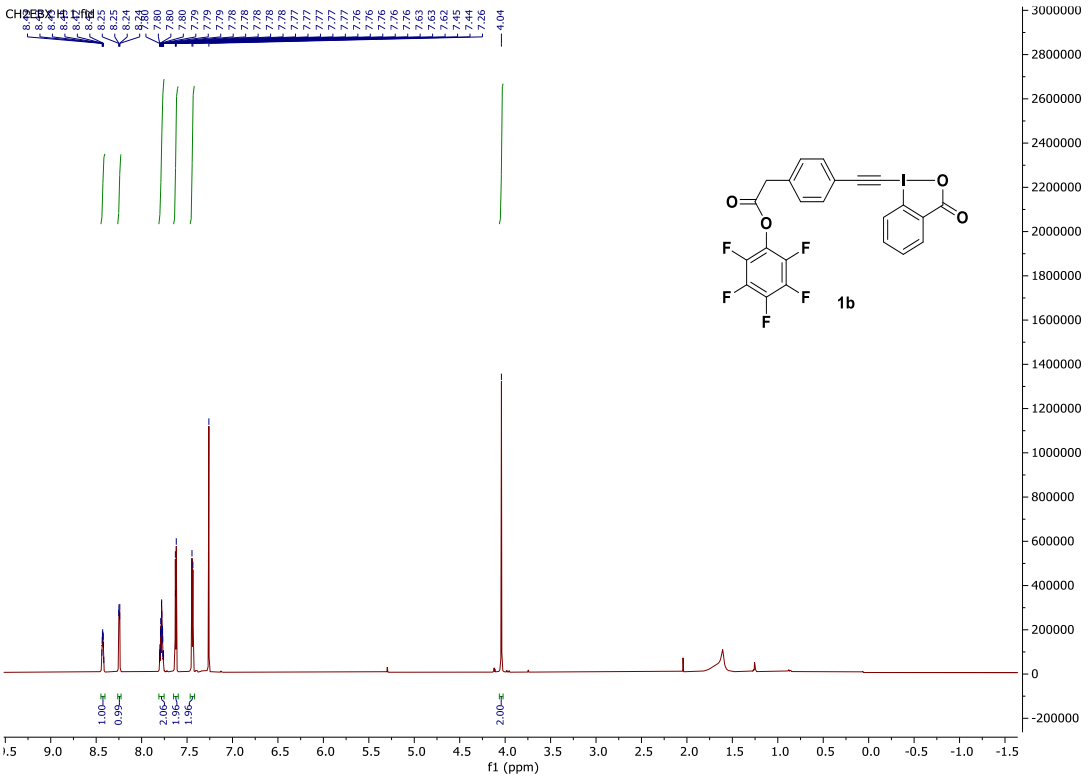


Figure S128: ^{13}C NMR (201 MHz, $CDCl_3$)

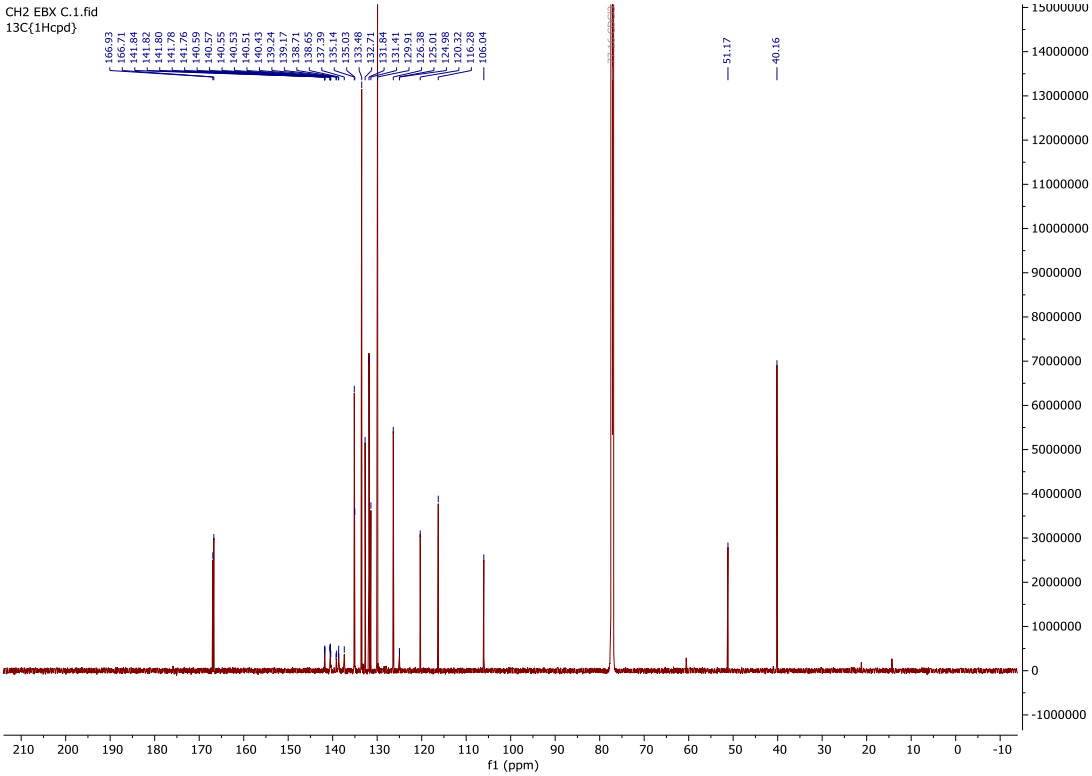


Figure S129: 3 ^{1}H NMR (400 MHz, DMSO-d_6)

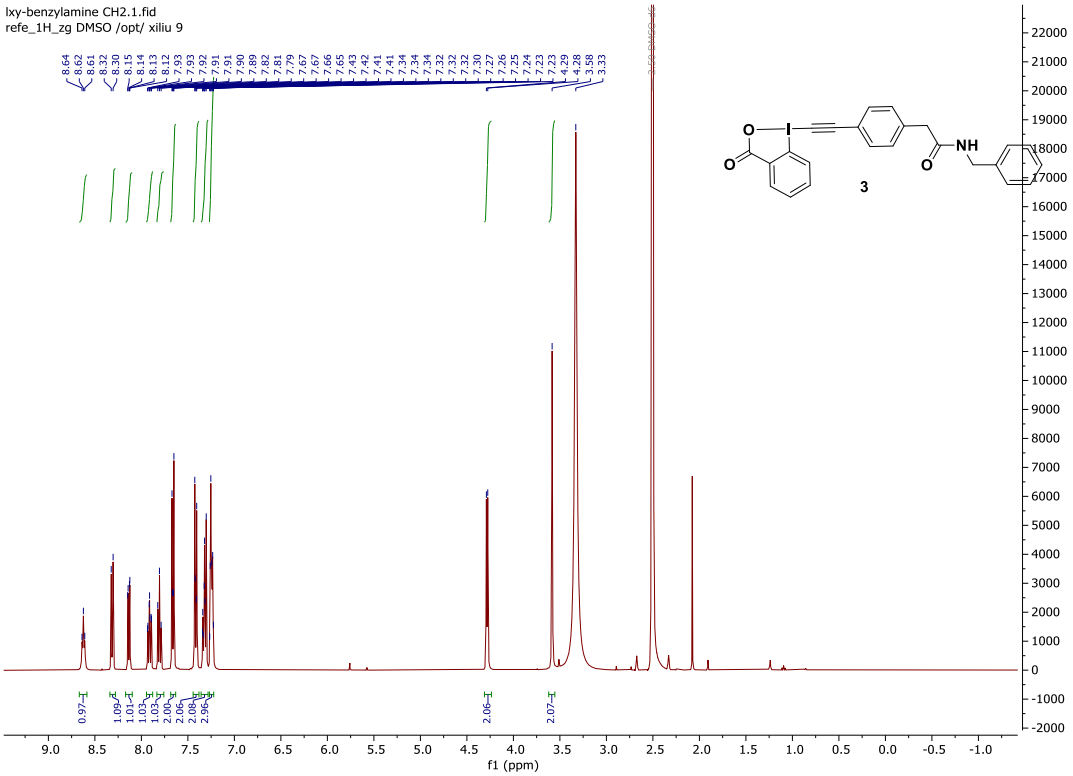


Figure S130: ^{13}C NMR (151 MHz, DMSO-d_6)

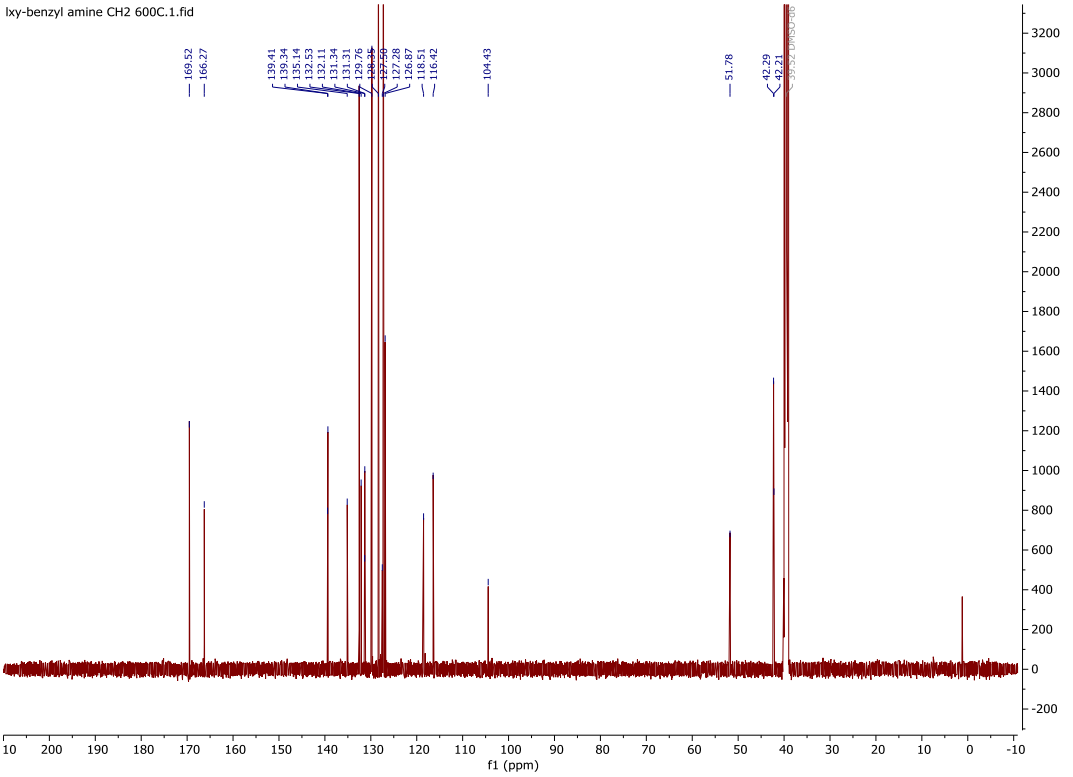


Figure S131: 3g ^{1}H NMR (800 MHz, DMSO-d_6)

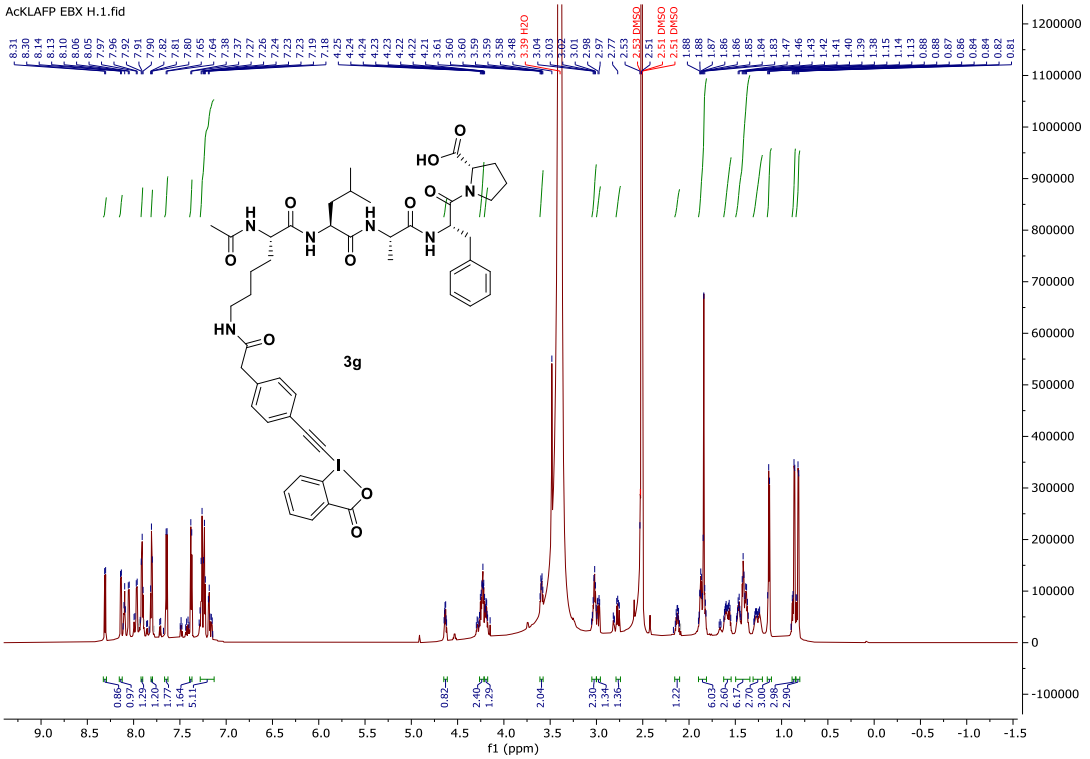


Figure S132: ^{13}C NMR (201 MHz, DMSO-d_6)

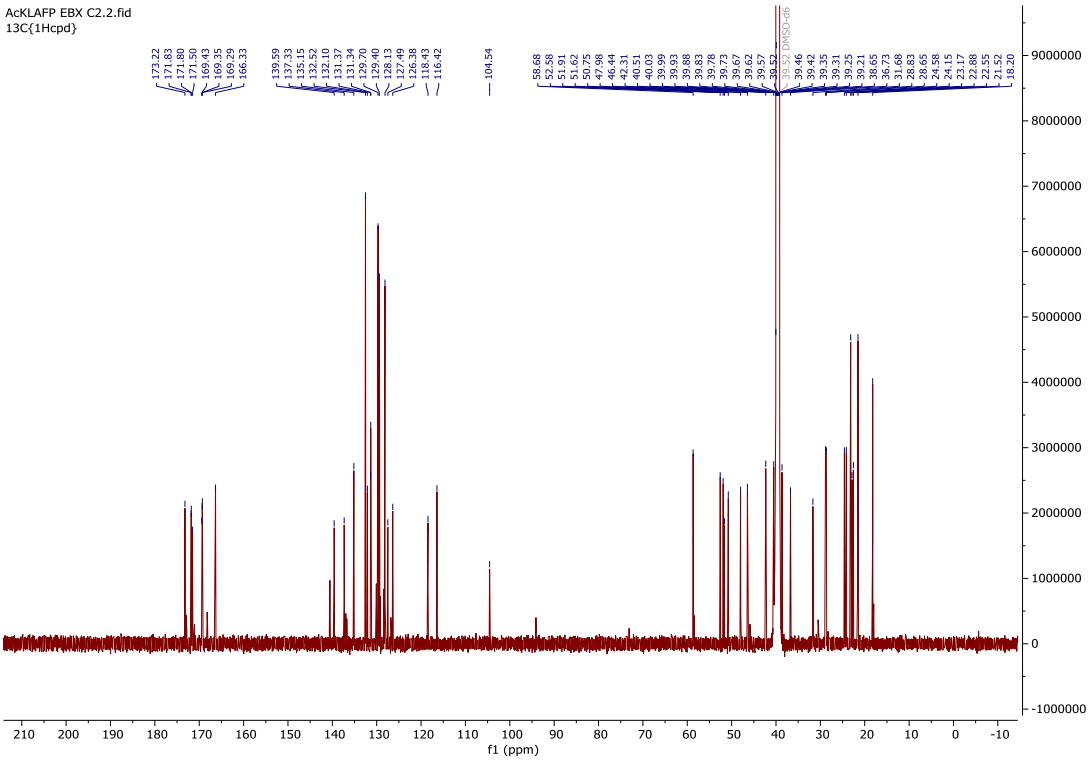


Figure S133: 3s ^{1}H NMR (500 MHz, CD_3OD)

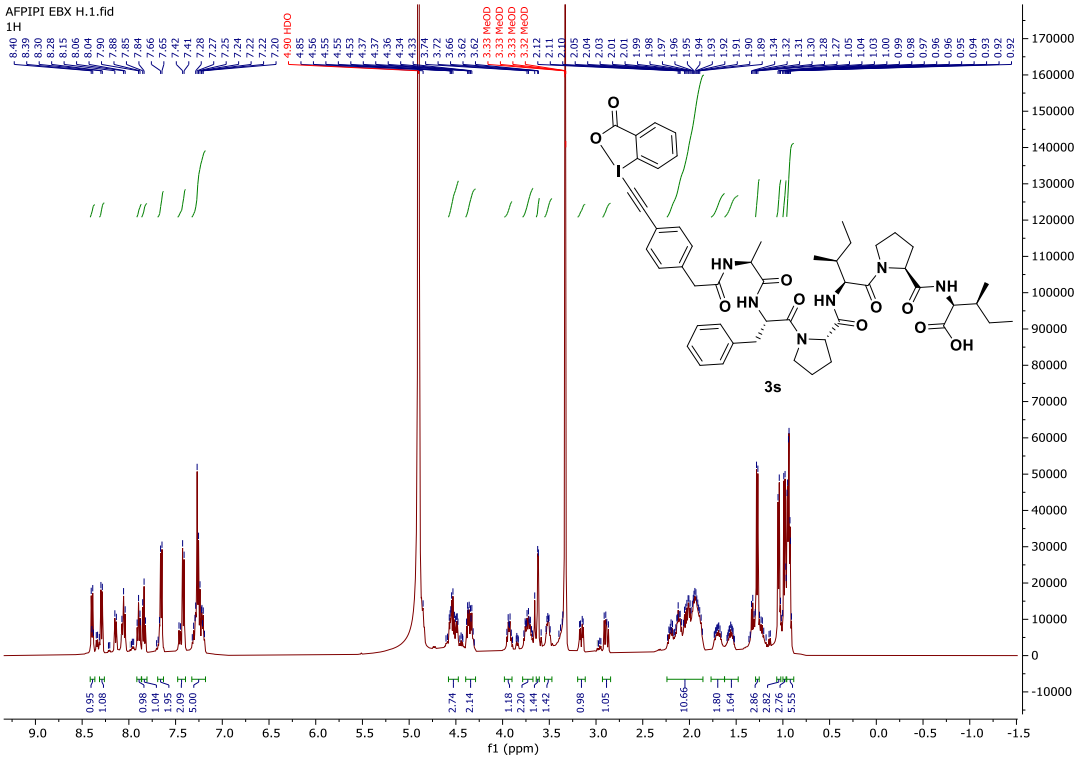


Figure S134: ^{13}C NMR (201 MHz, CD_3OD)

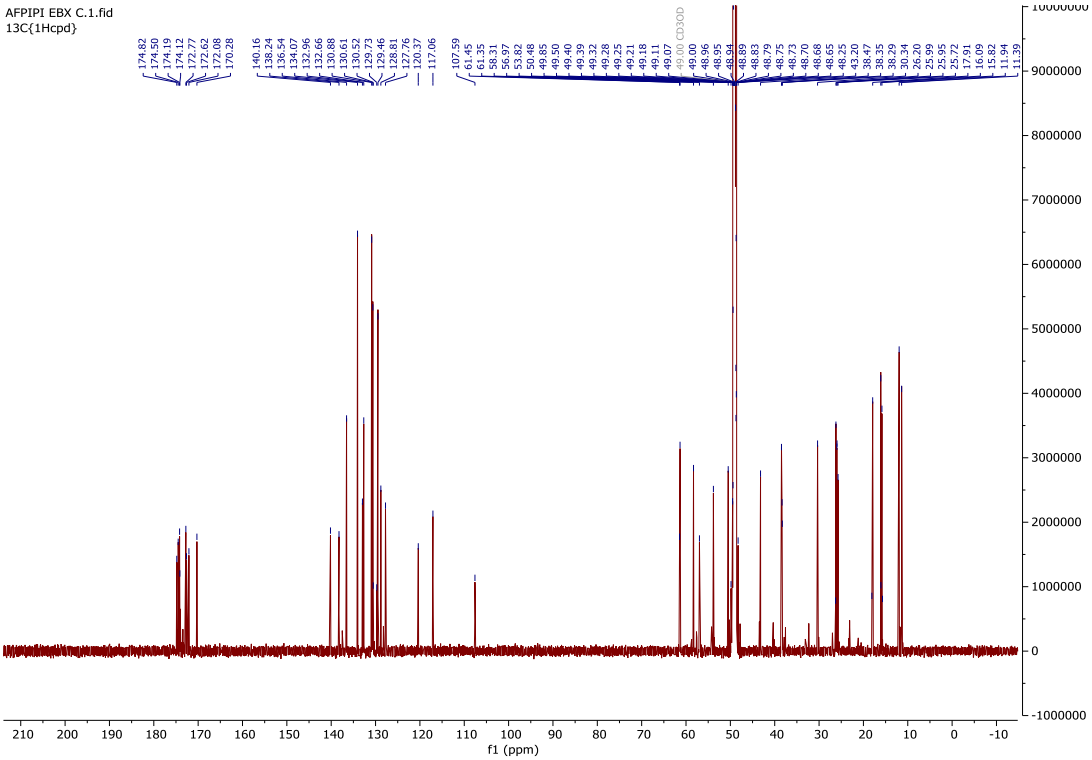


Figure S135: 3w ^{1}H NMR (600 MHz, CD_3OD)

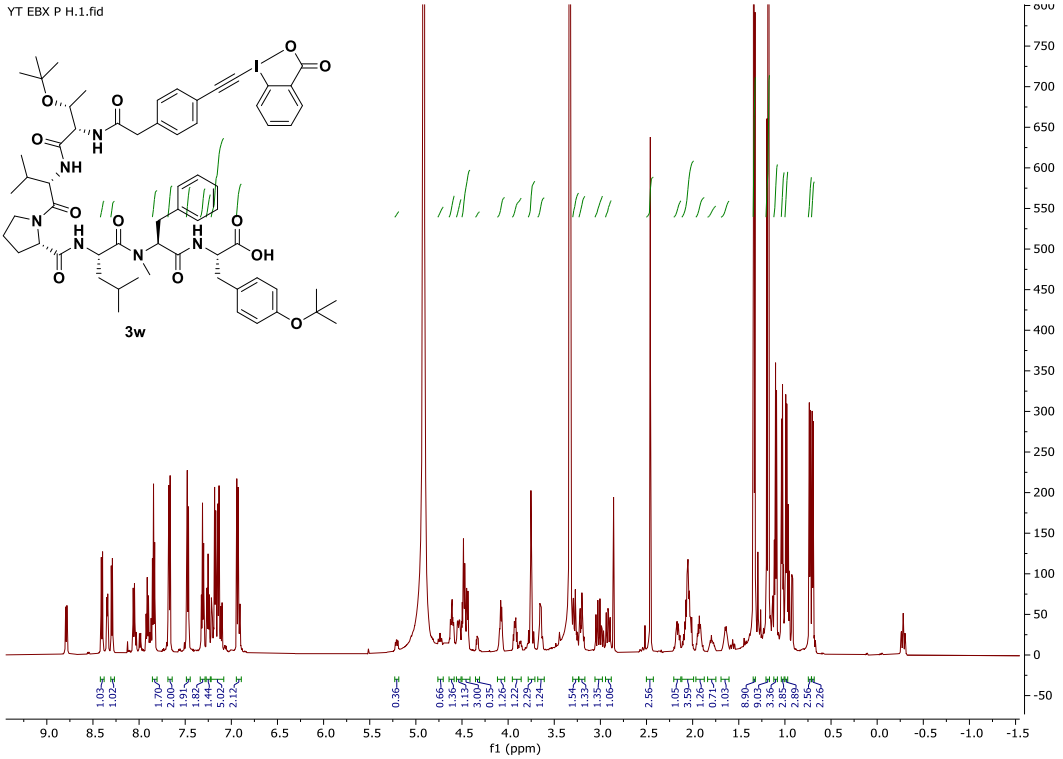


Figure S136: ^{13}C NMR (151 MHz, CD_3OD)

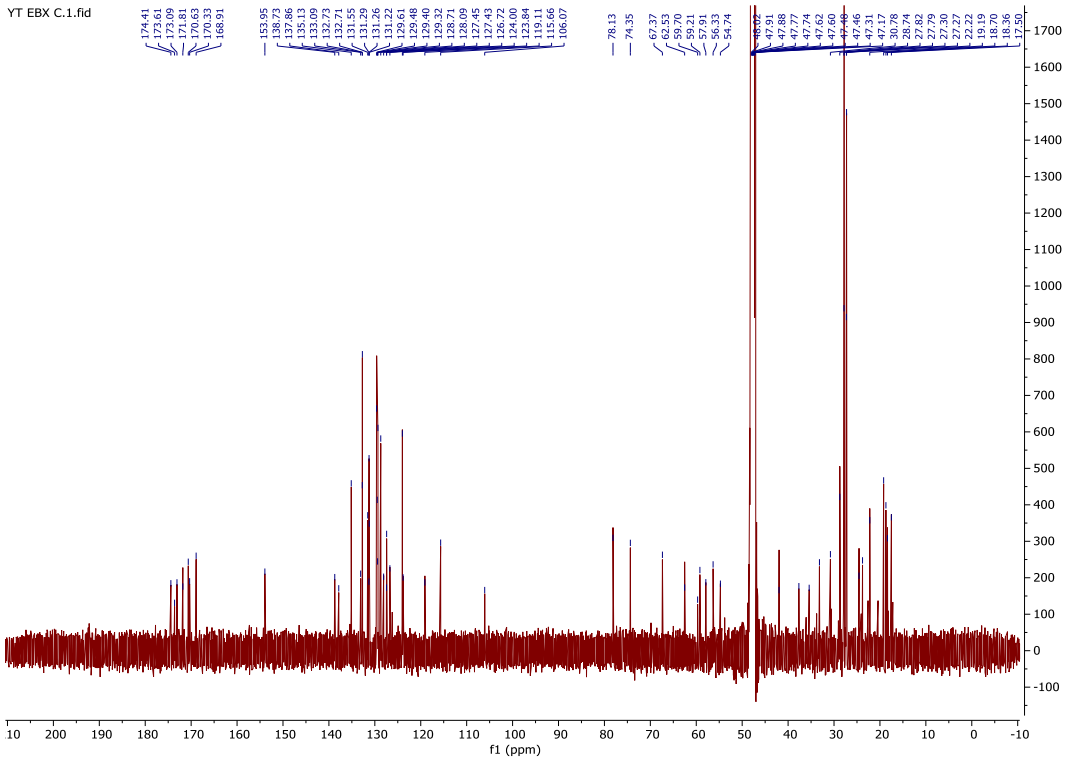


Figure S137: 5a ^{1}H NMR (800 MHz, CD_3OD)

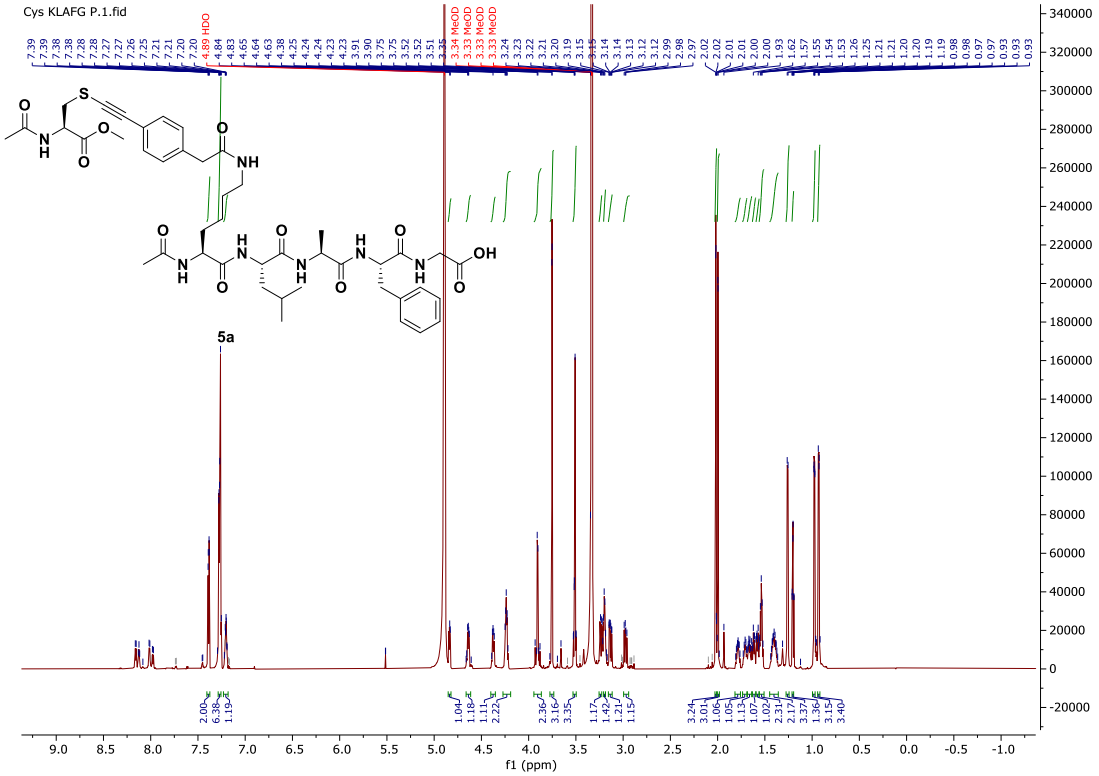


Figure S138: ^{13}C NMR (201 MHz, CD_3OD)

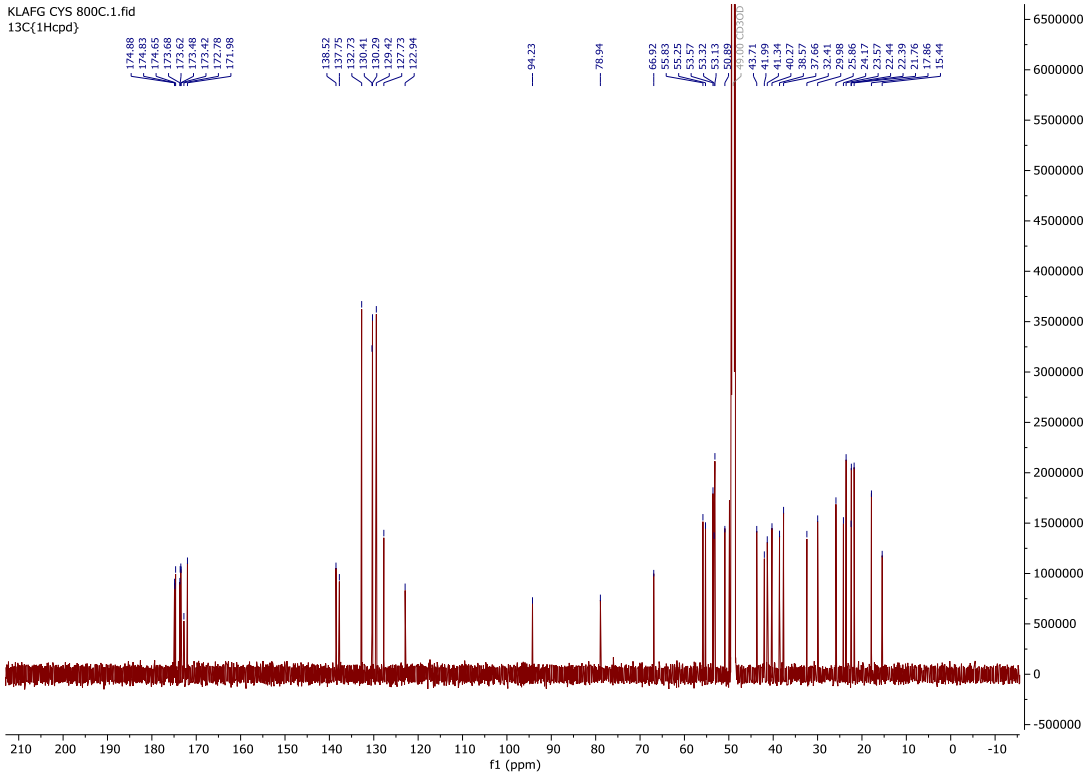


Figure S139: 8a ^{1}H NMR (600 MHz, CD_3OD)

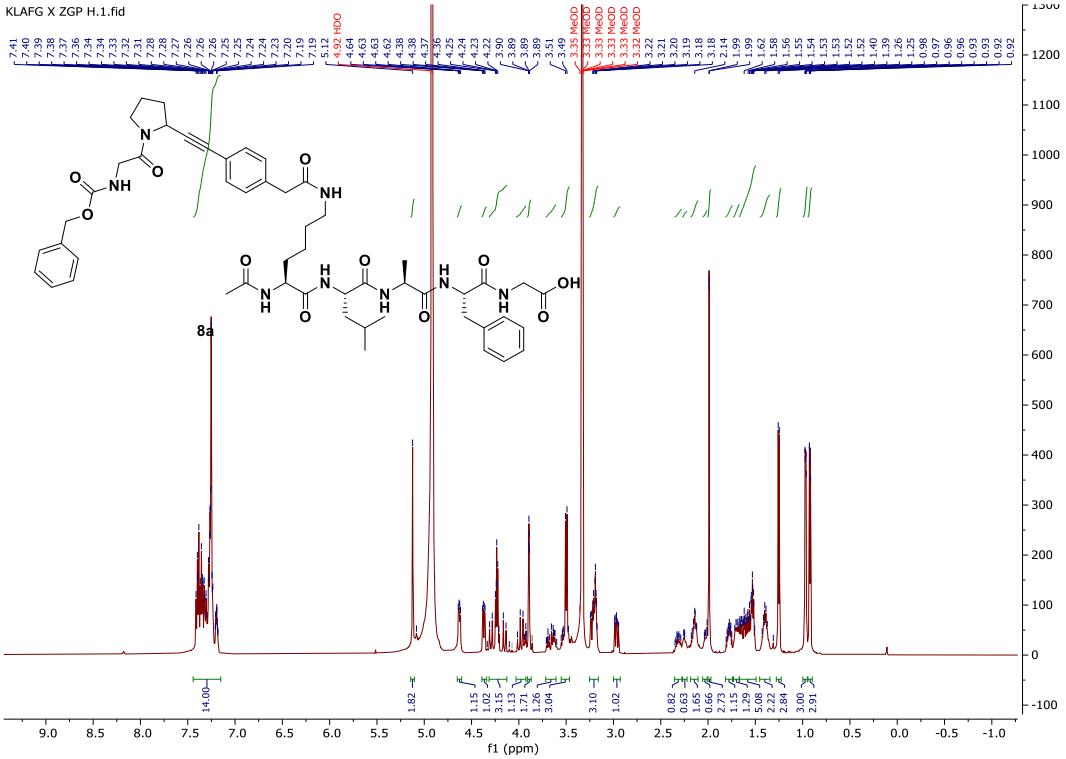


Figure S140: ^{13}C NMR (151 MHz, CD_3OD)

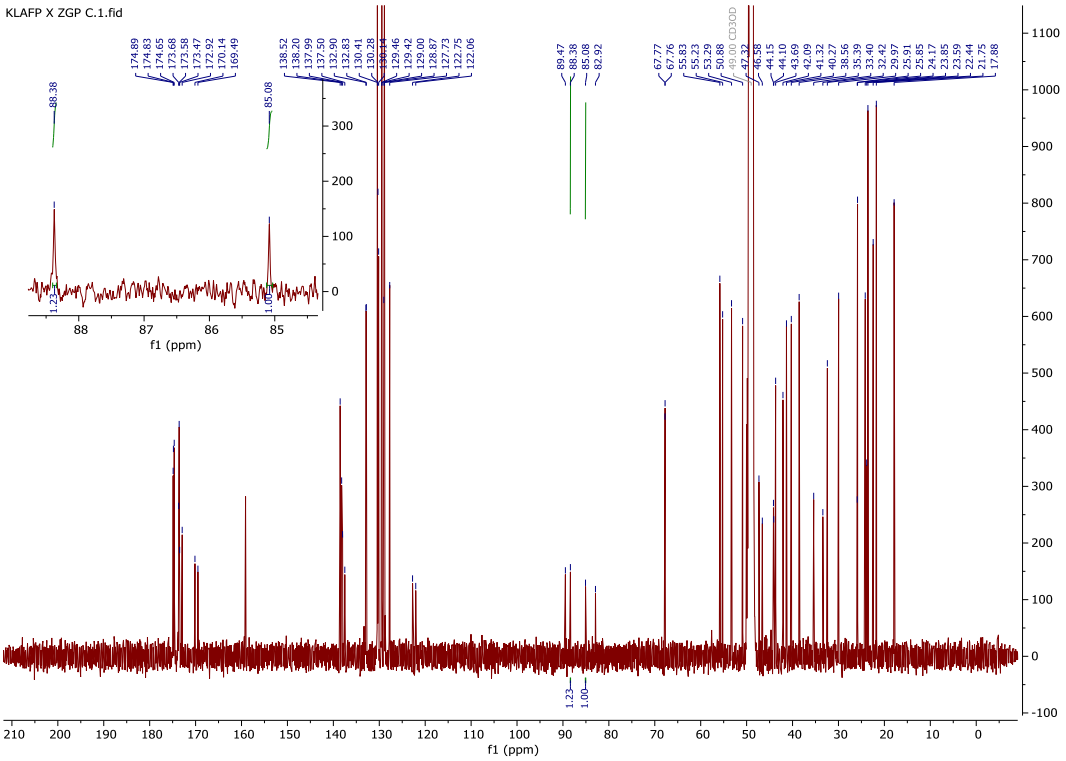


Figure S141: HMBC of **8a**

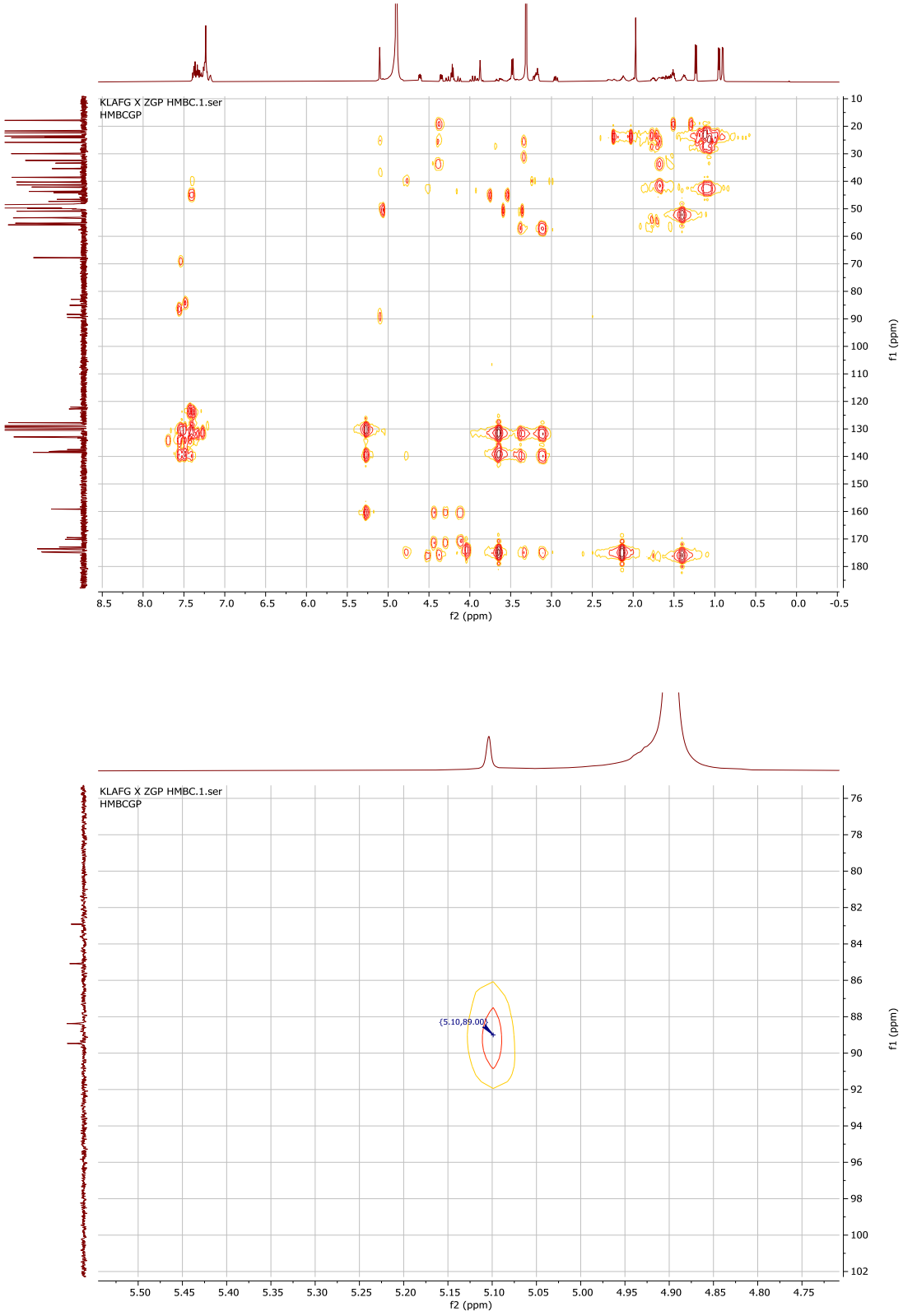


Figure S142: 9 ^{1}H NMR (500 MHz, CD_3OD)

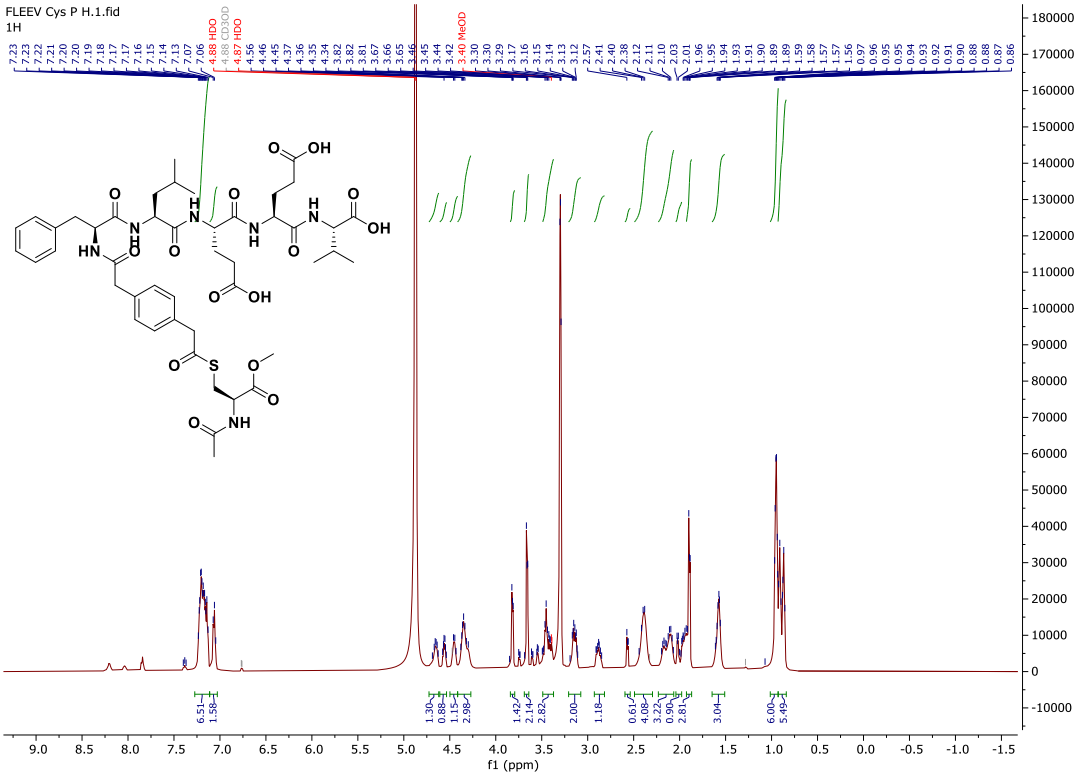


Figure S143: ^{13}C NMR (201 MHz, CD_3OD)

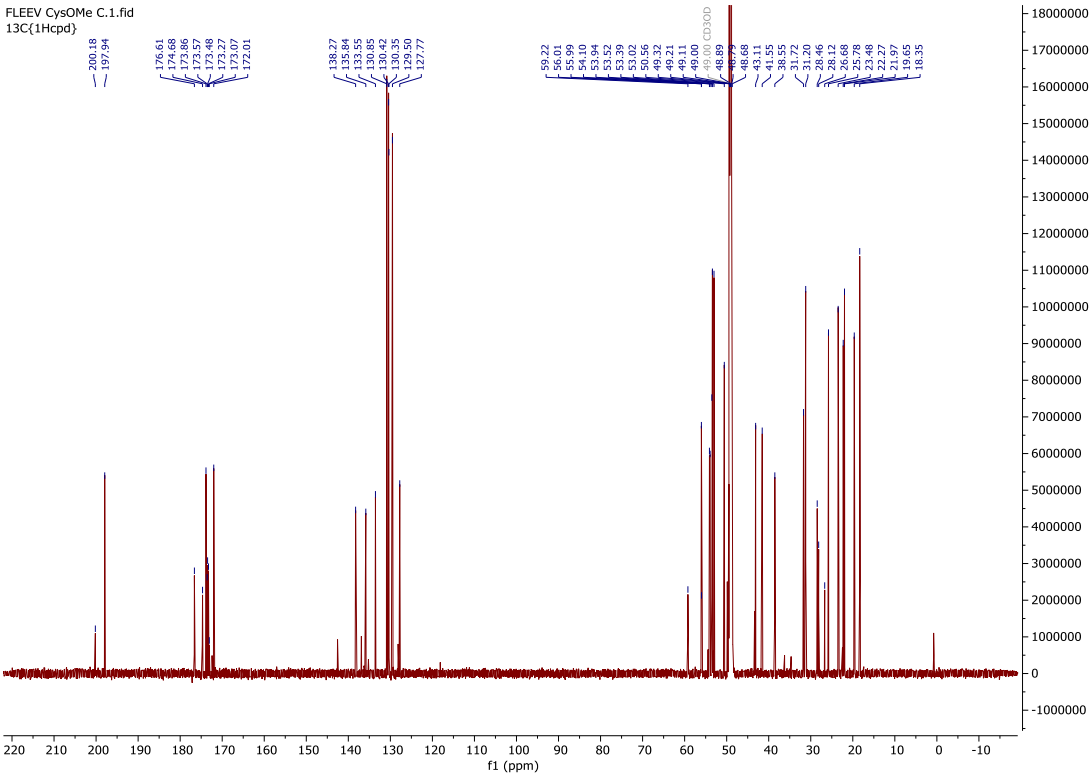


Figure S144: 13a, ^{1}H NMR (600 MHz, DMSO-d_6)

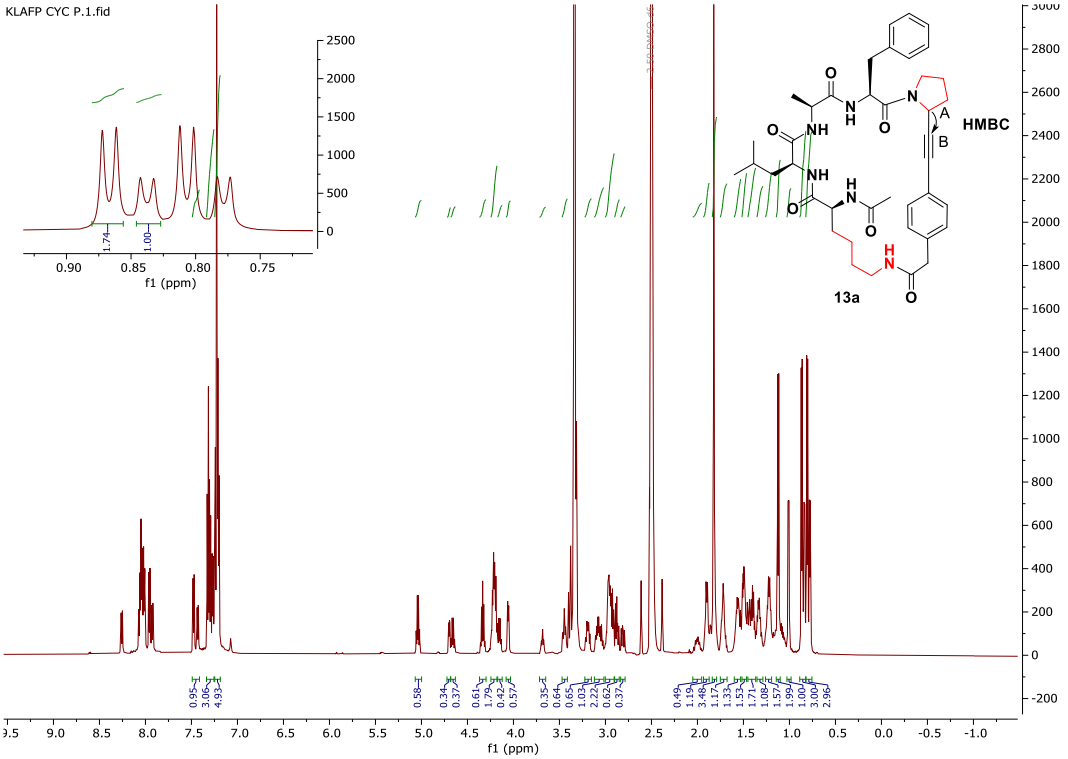


Figure S145: ^{13}C NMR (201 MHz, CD_3OD)

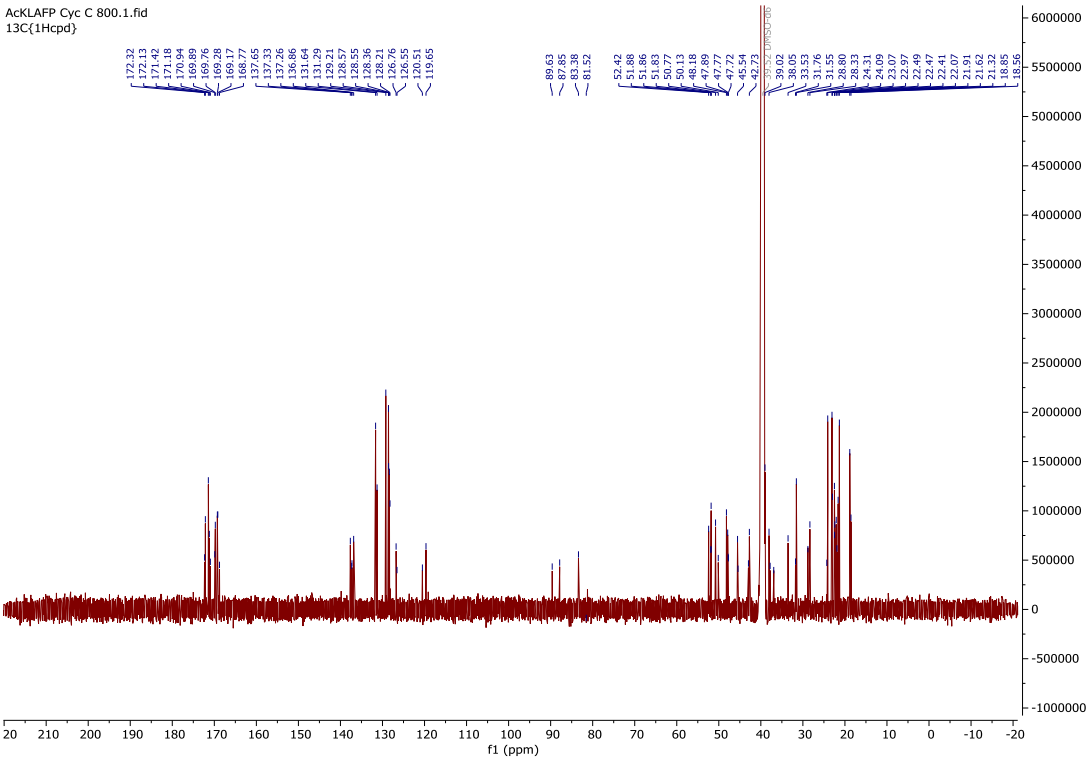
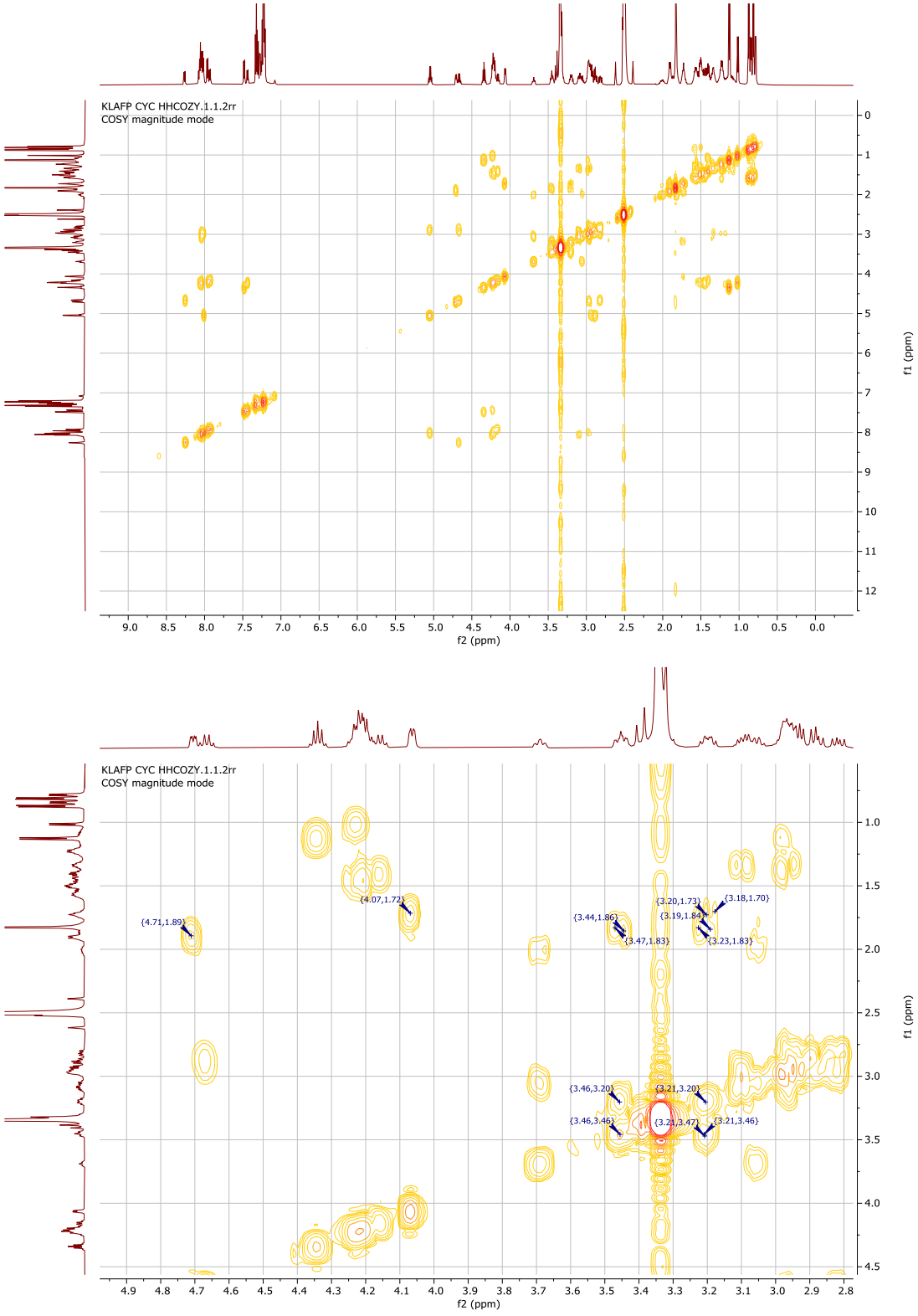


Figure S146: H-H COSY of **13a**



Based on HMBC, we were able to distinguish the proton interacting with the alkyne moiety. This allowed us to confirm that the macrocycle was formed.

Based on the H-H Cosy, we were able to assign the proton on the Pro ring.

Figure S147: HMBC of **13a**

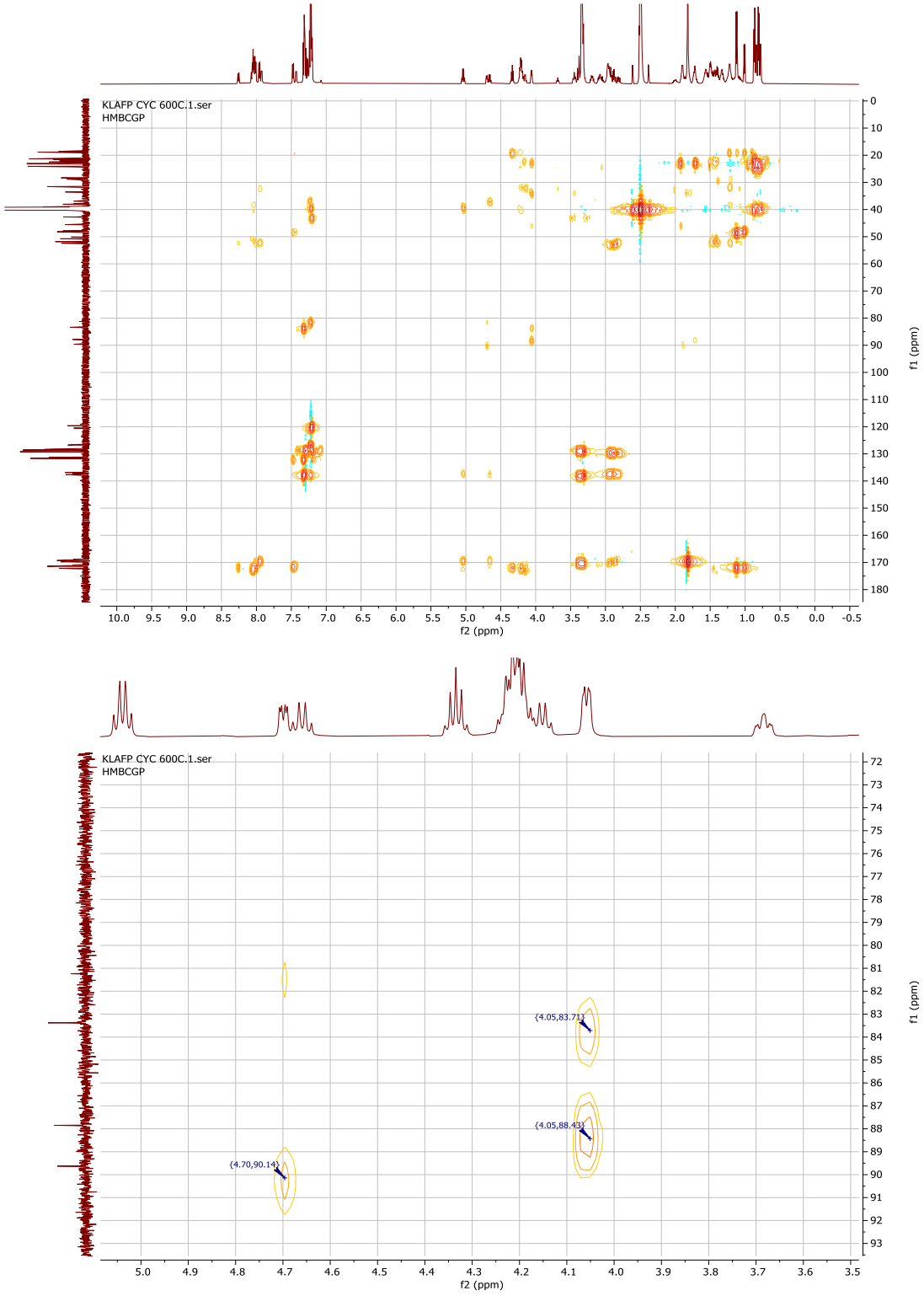


Figure S148: 13h P1 ^{1}H NMR (500 MHz, CD_3OD)

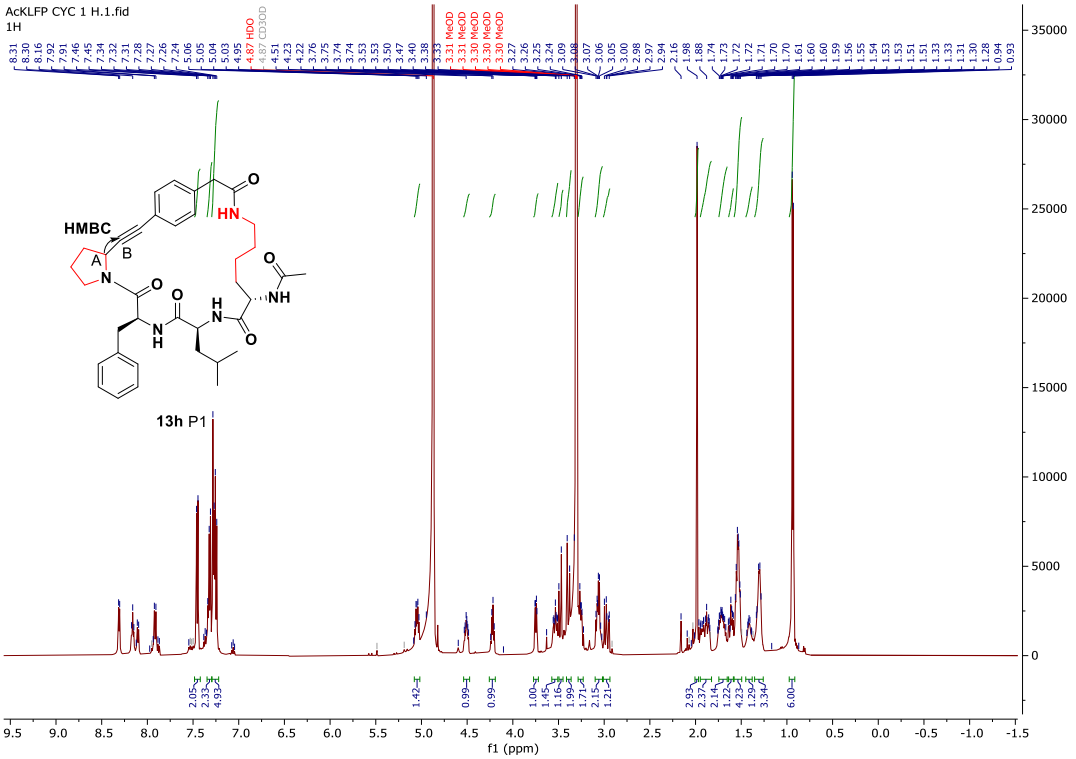


Figure S149: ^{13}C NMR (201 MHz, CD_3OD)

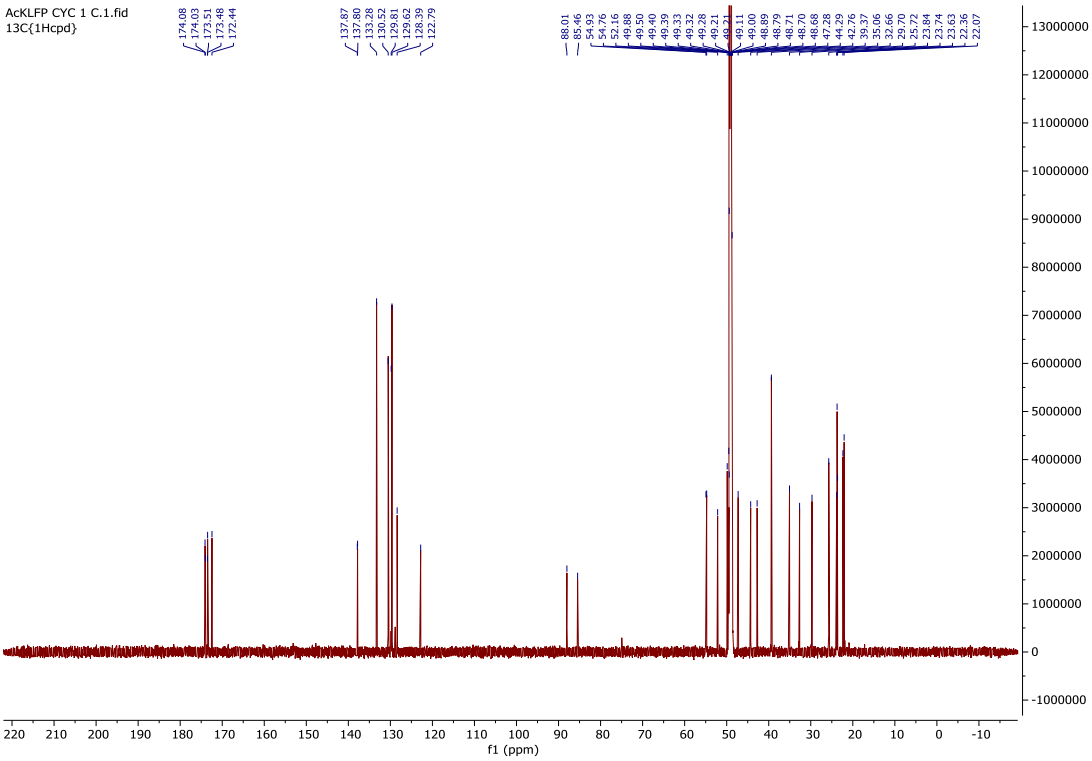


Figure S150: HMBC of **13h** P1

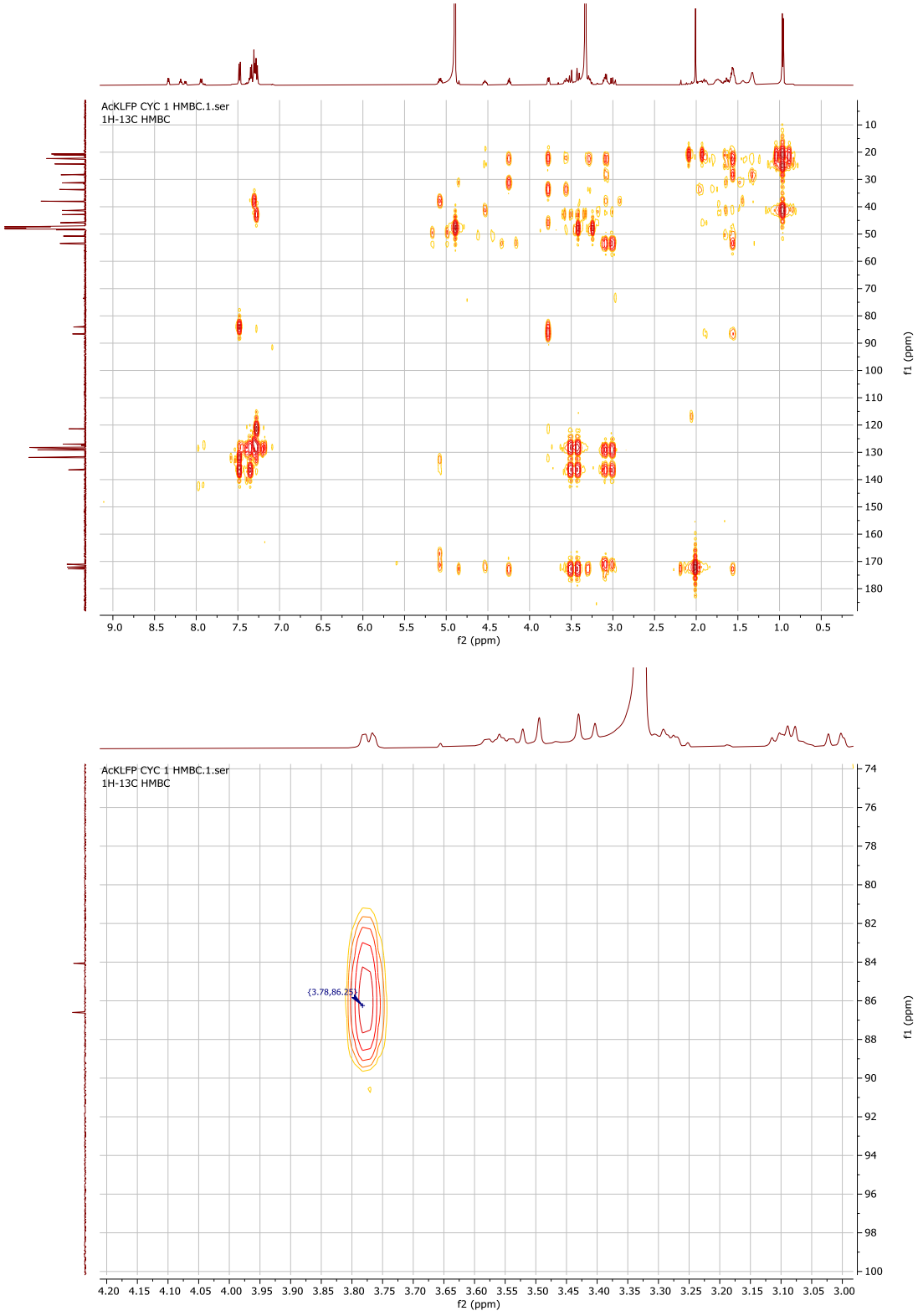


Figure S151: 13h P2 ^{1}H NMR (500 MHz, CD_3OD)

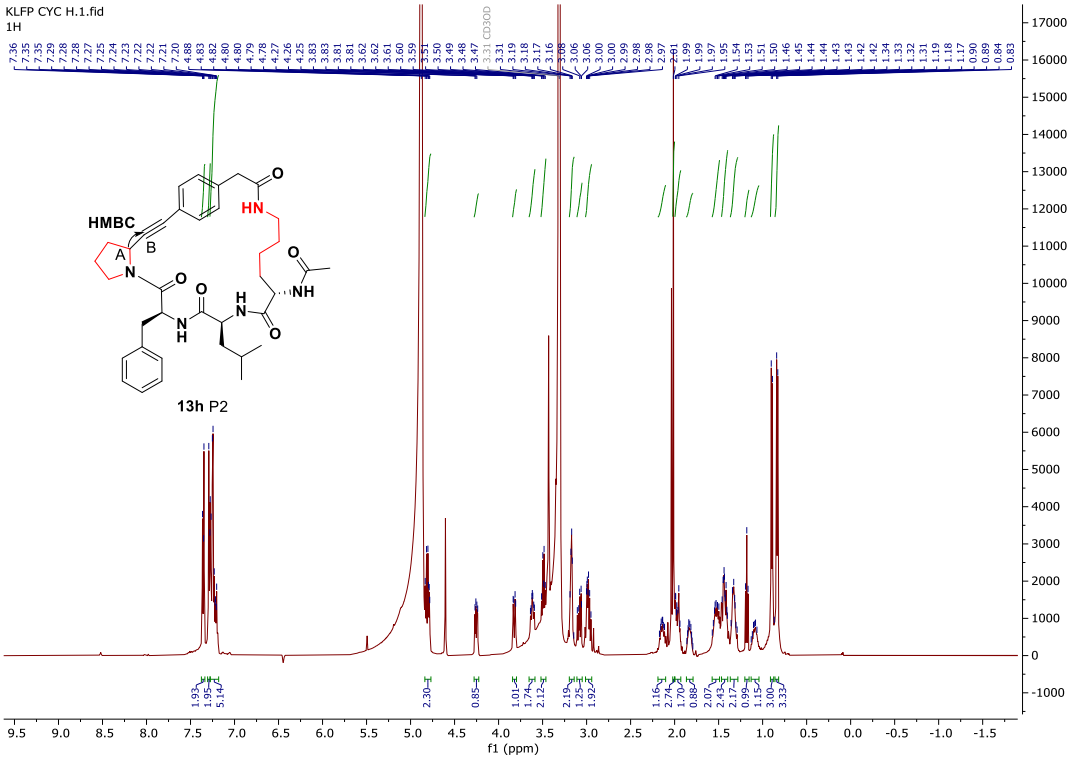


Figure S152: ^{13}C NMR (151 MHz, CD_3OD)

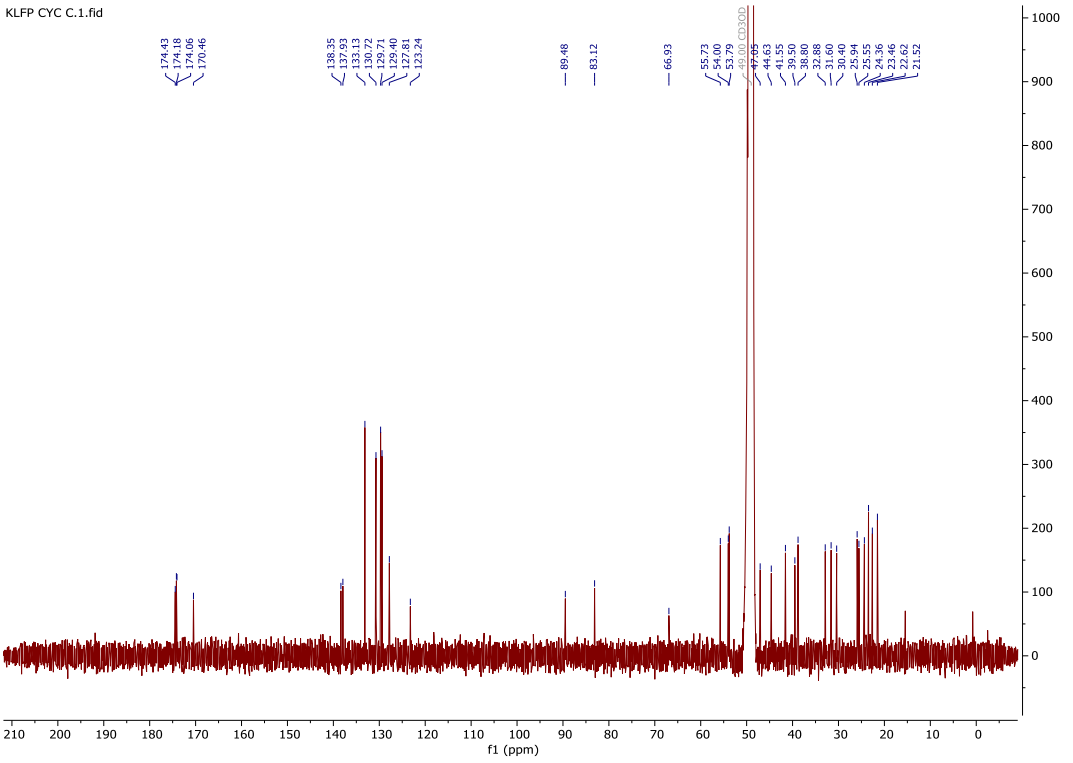


Figure S153: HMBC of **13h** P2

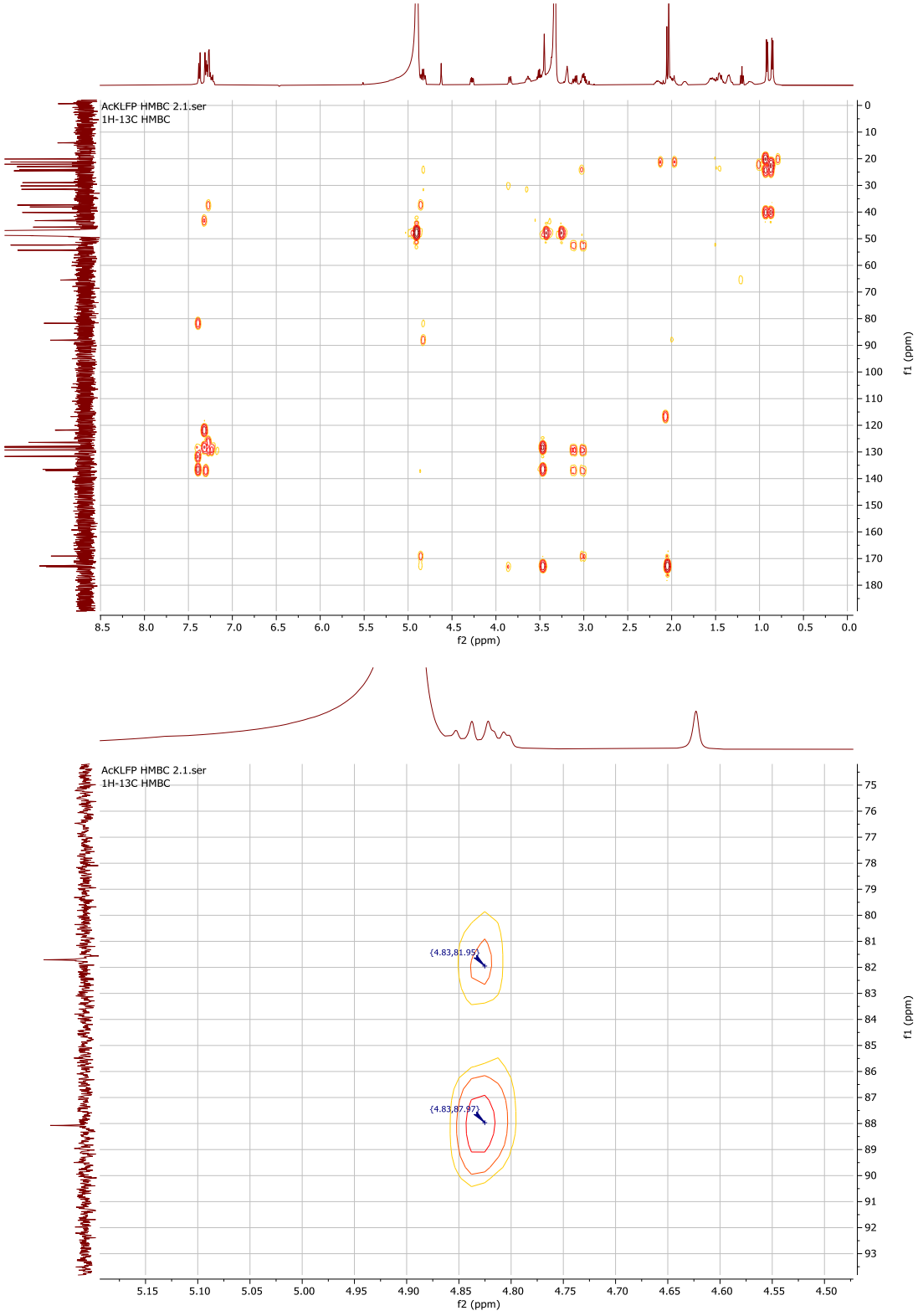


Figure S154: 13x ^{1}H NMR (500 MHz, CD_3OD)

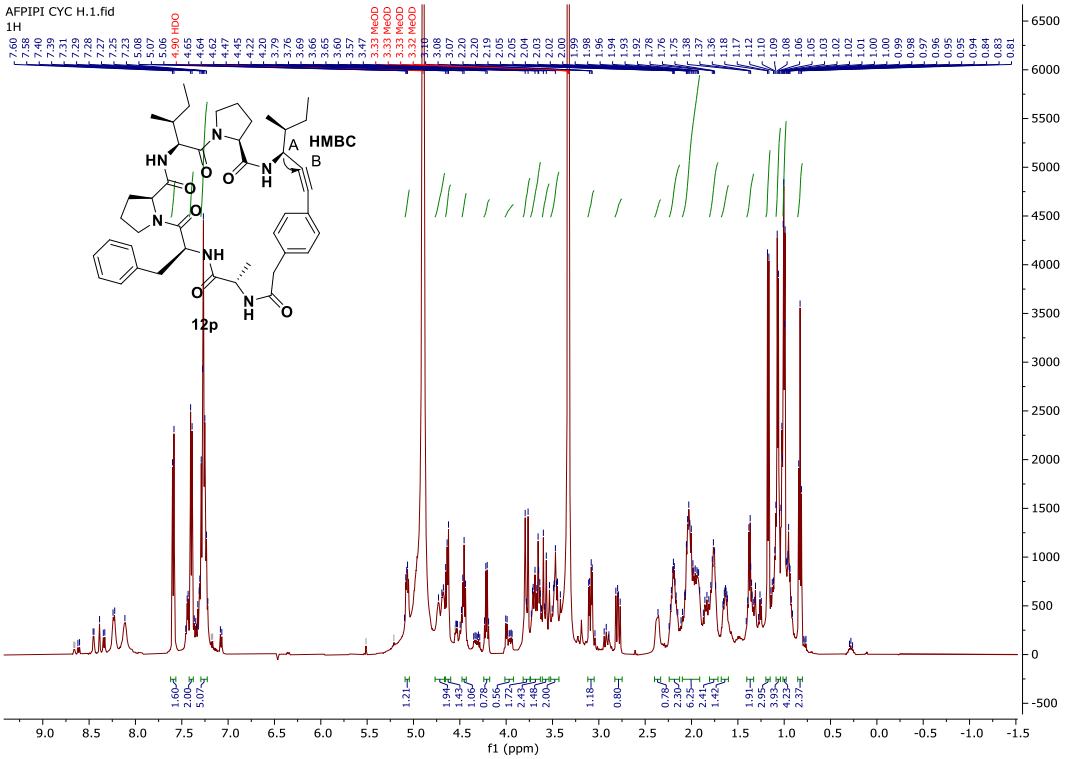


Figure S155: ^{13}C NMR (201 MHz, CD_3OD)

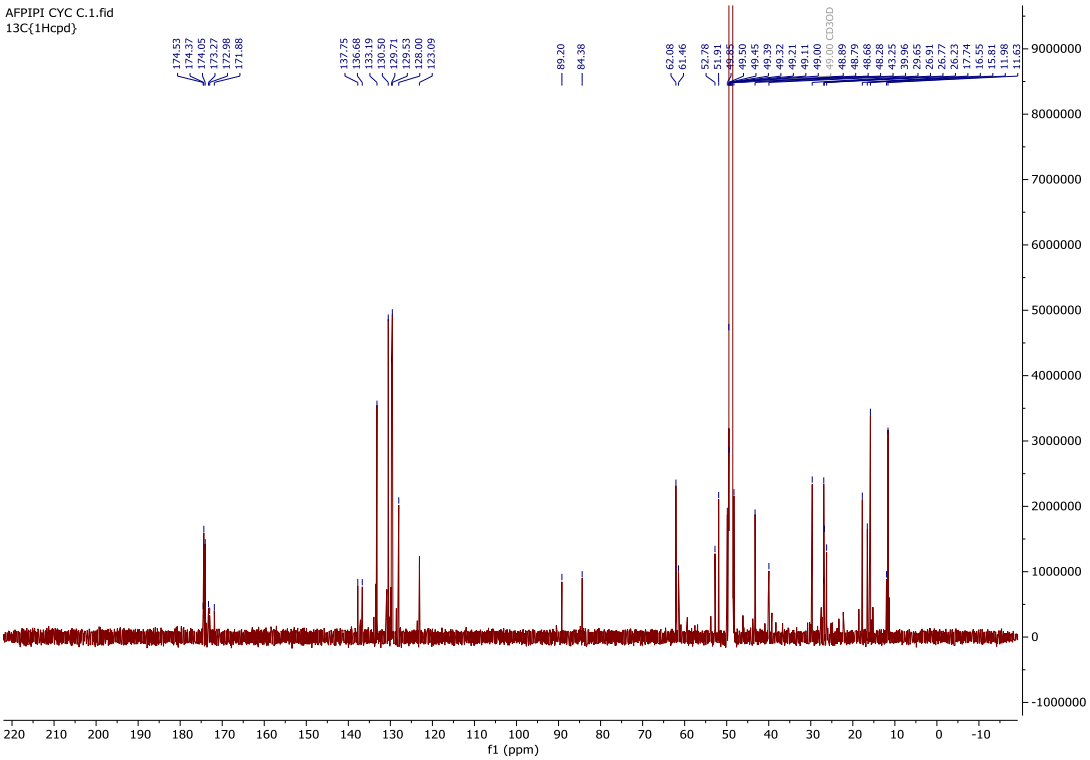


Figure S156: HMBC of **13x**

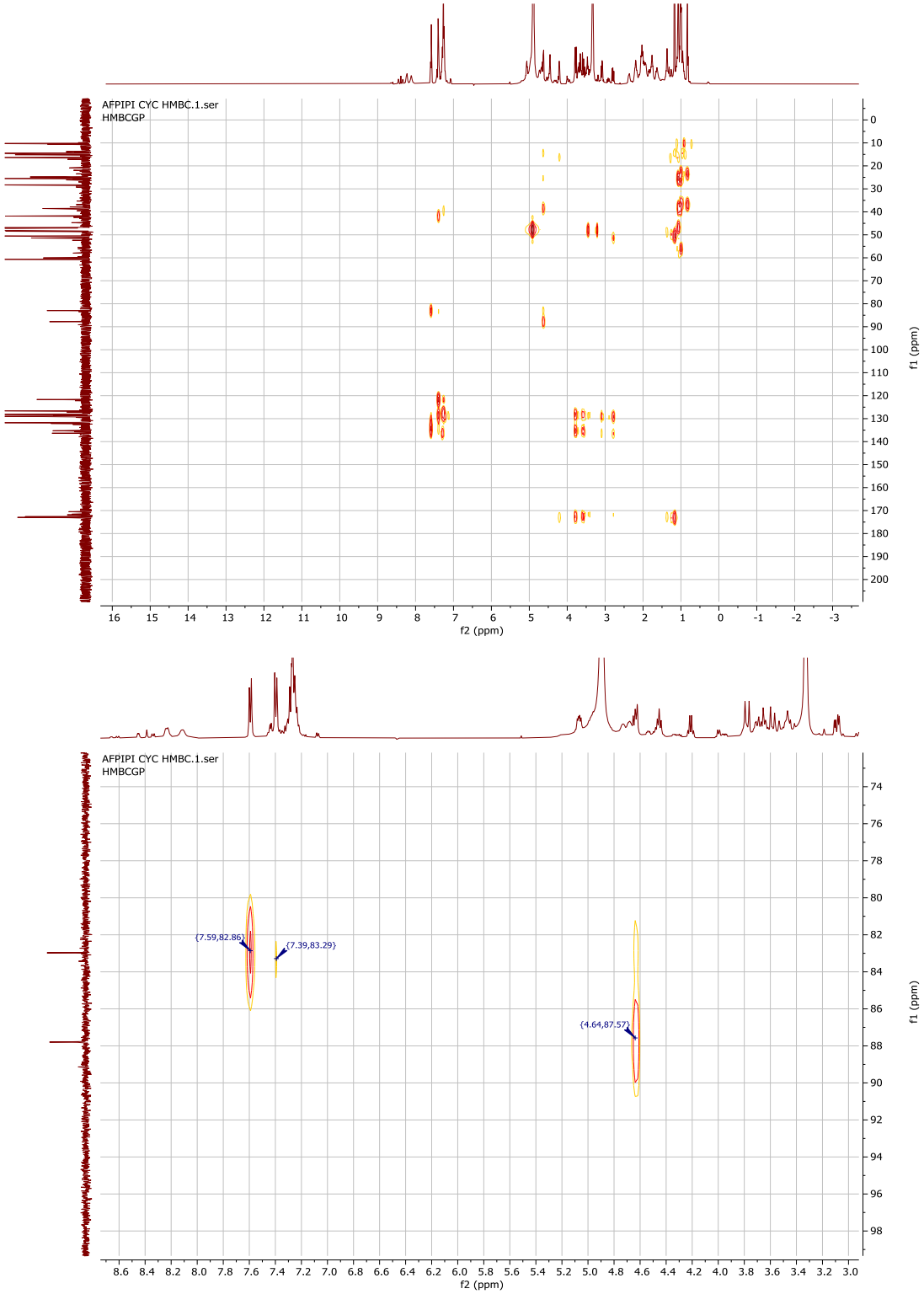


Figure S157: 14 ^{1}H NMR (600 MHz, CD_3OD)

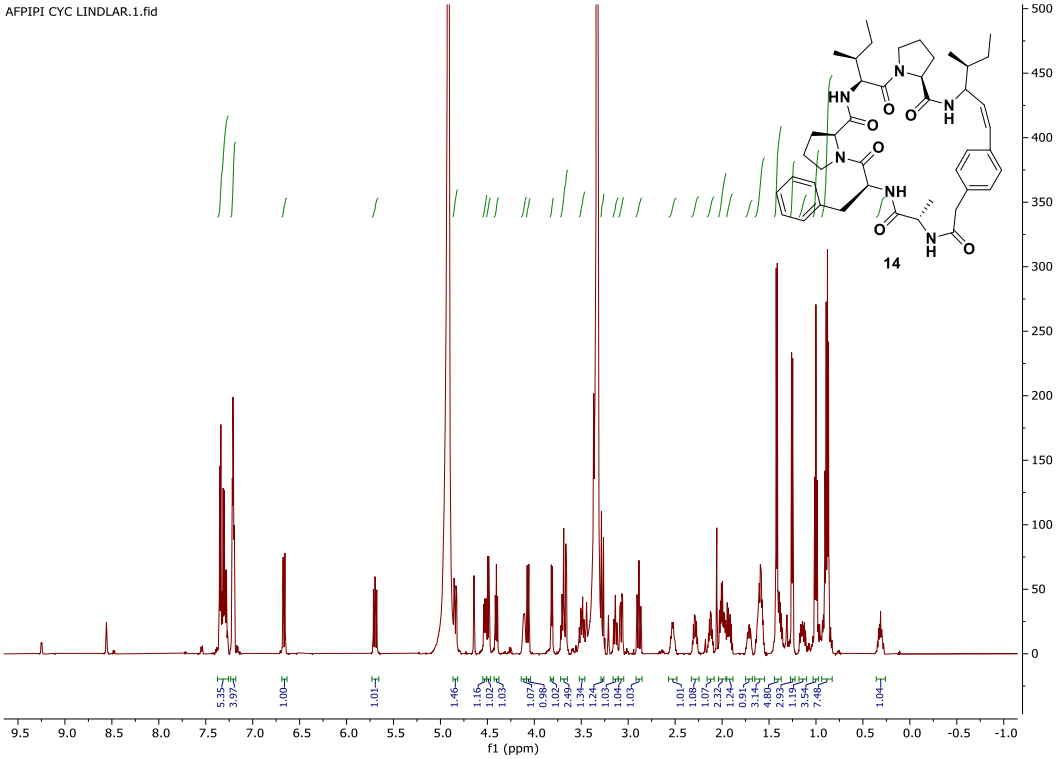


Figure S158: 15a ^{1}H NMR (600 MHz, CD_3OD)