Hypervalent Iodine Hot Paper

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Amphiphilic Iodine(III) Reagents for the Lipophilization of Peptides in Water

*Abhaya Kumar Mishra[+], Romain Tessier[+], Durga Prasad Hari, and Jerome Waser**

Abstract: *We report the functionalization of cysteine residues with lipophilic alkynes bearing a silyl group or an alkyl chain using amphiphilic ethynylbenziodoxolone reagents (EBXs). The reactions were carried out in buffer (pH 6 to 9), without organic co-solvent or removal of oxygen, either at 37°C or room temperature. The transformation led to a significant increase of peptide lipophilicity and worked for aromatic thiols, homocysteine, cysteine, and peptides containing 4 to 18 amino acids. His_6-Cys-Ubiquitin was also alkynylated under physiological conditions. Under acidic conditions, the thioalkynes were converted into thioesters, which could be cleaved in the presence of hydroxylamine.*

Since the use of insulin in the treatment of diabetes,[1] the importance of peptide-based drugs has constantly increased.[2] However, the high polarity and low stability of natural peptides result in unfavorable pharmacological properties, requiring chemical modifications.[3] An adequate lipophilicity is essential to control the ADMET properties (absorption, distribution, metabolism, elimination and toxicology),[4] and the lipidation of peptides has proved to be effective in this regard.[5] The lipidation of proteins, through post-translational modifications (PTMs), is an essential process to control the properties and localization of biomolecules in the cell.[6] Lipopeptides have also found numerous applications in material sciences.[7]

Peptides and proteins containing nucleophilic residues are often functionalized with electrophilic reagents.[8] Considering their low abundance and high nucleophilicity, cysteines are targets of choice.[9] To achieve lipidation, naturally occurring palmitoylation and prenylation have been the focus of most research.[10] Due to the low stability of thioesters and the importance of permanent lipidation in multiple applications, chemists have recently focused on more stable natural lipids (Figure 1a).[4,5] Brimble and co-workers reported the photoinitiated coupling of cysteines with vinyl palmitate **A** via a thiol–ene process.[11] Breinbauer and co-workers developed a palladium-catalyzed geranylation of cysteine residues using carbonate reagent **B**.[12] Reports on stable non-natural modifications of cysteine under mild physiological conditions remain scarce (Figure 1b).[9d]

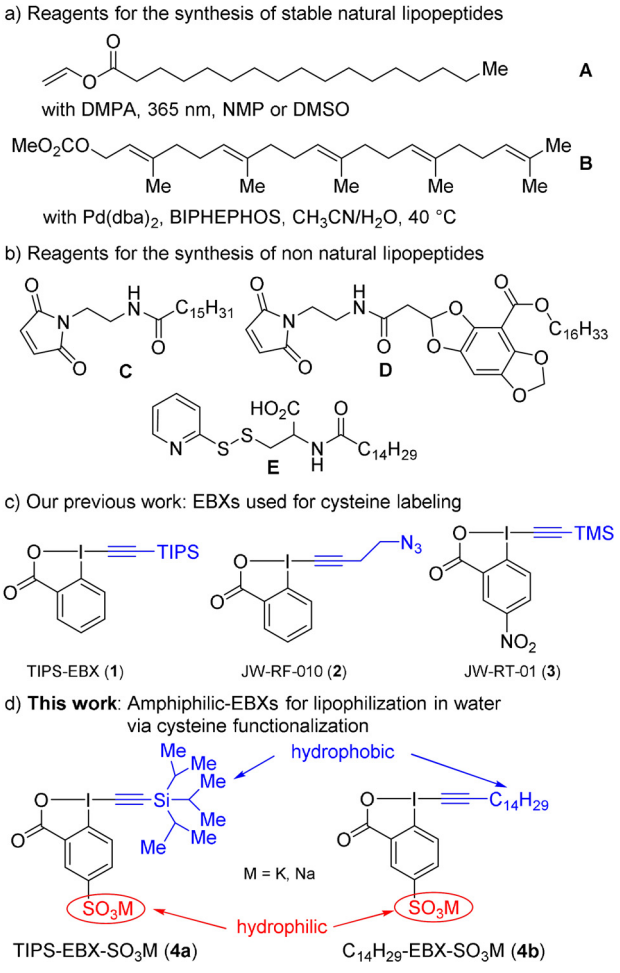


Figure 1. a) Chemical methods for stable natural lipidation. b) Reported reagents for non-natural lipidation. c) EBX reagents for cysteine labeling. d) This work: amphiphilic reagents for non-natural lipidation under physiological conditions. DMPA = 2,2-dimethoxy-2-phenylacetophenone, NMP = N-methyl-2-pyrrolidone, BIPHEPHOS = 6,6′-[(3,3′-di-tert-butyl-5,5′-dimethoxy-1,1′-biphenyl-2,2′-diyl)bis(oxy)]bis(di-benzo[d,f][1,3,2]dioxaphosphepin), TIPS = tri*iso*propylsilyl, TMS = trimethylsilyl.

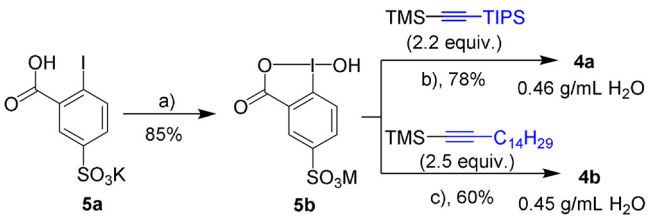
[*] Dr. A. K. Mishra,[+] Dr. R. Tessier,[+] Dr. D. P. Hari, Prof. Dr. J. Waser
Laboratory of Catalysis and Organic Synthesis, Ecole Polytechnique Fédérale de Lausanne, EPFL SB ISIC LCSO, BCH 4306
1015 Lausanne (Switzerland)
E-mail: jerome.waser@epfl.ch
Dr. R. Tessier[+]
Present address: Department of Chemical Biology, Max Planck Institute of Molecular Physiology
Otto-Hahn-Strasse 11, 44227 Dortmund (Germany)
[+] These authors contributed equally to this work.
Supporting information and the ORCID identification number(s) for the author(s) of this article can be found under: https://doi.org/10.1002/anie.202106458.

Müller, Wessig, and co-workers used maleimide derivatives **C** and **D** for the recruitment of thiol-containing peptides into the cell membrane.[13] Many reported lipidation processes use highly reactive reagents together with organic co-solvents, due to the low solubility of the lipophilic reagents, which can be an issue in presence of sensitive biomolecules. There are only few reagents for cysteine functionalization that are fully water-soluble,[14] and only reagent **E** was reported for the specific case of lipidation, based on the formation of labile disulfide bridges.[14a]

Our group previously investigated ethynylbenziodoxolone (EBX) reagents for the alkynylation of thiols (Figure 1c).[15] The non-water-soluble reagent tri*iso*propylsilylethynyl-benziodoxolone (TIPS-EBX, **1**) was used in organic solvents.[15a,16] Less lipophilic alkyl reagents, such as JW-RF-010 (**2**) gave alkynylated thiols, hypervalent iodine vinyl-benziodoxolone (VBX) addition products or a mixture of both depending on the cysteine reactivity and the reaction conditions.[15b–d] Finally, trimethysilylated reagents such as JW-RT-01 (**3**) were deprotected under physiological conditions.[15e]

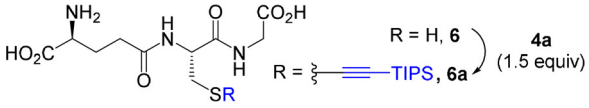
The importance of lipo-peptides and -proteins, combined with the lack of lipidation methods in absence of organic solvents and the reactivity of EBXs towards thiols, motivated us to design water-soluble amphiphilic-EBXs. Herein, we report the synthesis of sulfonylated EBXs **4a** and **4b** and their application for the lipophilization of unprotected peptides and proteins under physiological conditions (Figure 1d). Under acidic conditions, the obtained thiolakynes could be converted to cleavable thioesters.

Among the approaches to access water-soluble hypervalent iodine reagents,[17] we focused on sulfonylated derivatives[18] because the iodine precursor 2-iodo-5-potassium sulfonate (**5a**) is easily accessible (see the Supporting Information)[19] and the sulfonate group ensures good solubility in water at a broad range of pHs. **5a** was oxidized to **5b** by $NaIO_4$ in 85% yield. Installation of the alkyne on **5b** was achieved by using a large excess of Lewis acid. We first prepared TIPS-EBX-SO_3M (**4a**),[20] as silylated reagents are known to exclusively give alkynylation products (Scheme 1).[15] A slightly modified procedure ($BF_3{\cdot}OEt_2$ instead of TMSOTf as Lewis acid) then allowed us to access the alkyl reagent $C_{14}H_{29}$-EBX-SO_3M (**4b**; Scheme 1). Gratifyingly, **4a** displayed a more than fifty fold increased solubility in water compared to TIPS-EBX (**1**) (0.46 for **4a** vs. $<10\,mg\,mL^{-1}$ for **1**) and **4b** was also well soluble ($0.45\,g\,mL^{-1}$).



Scheme 1. Synthesis of TIPS-EBX-SO_3M (**4a**) and $C_{14}H_{29}$-EBX-SO_3M (**4b**). a) $NaIO_4$ (1.05 equiv), 30% aq. AcOH (v/v), reflux, 4 h; b) TMSOTf (3.0 equiv), pyridine (6.0 equiv), DCE, 40 °C, 22 h; c) $BF_3{\cdot}Et_2O$ (3.0 equiv), pyridine (1.1 equiv), CH_3CN, rt, 24 h.

Table 1: Optimization of the reaction conditions with TIPS-EBX-SO_3K (**4a**) and glutathione (**6**).

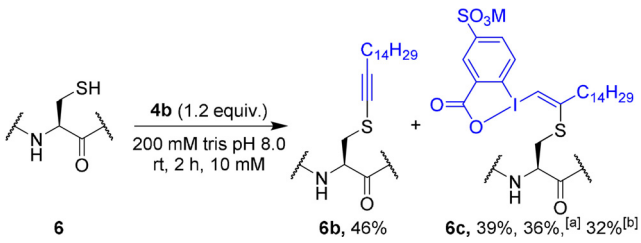


Entry	Reaction conditions[a]	**6a** Yield [%][b]
1	10 mM Tris, pH 7.4, rt, 6 h	47
2	10 mM Tris, pH 7.4, rt, 16 h	83
3	10 mM Tris, pH 7.4, 37 °C, 6 h	95
4	40 mM Tris, pH 7.4, 37 °C, 6 h	84
5	80 mM Tris, pH 7.4, 37 °C, 6 h	48
6	200 mM Tris, pH 7.4, 37 °C, 6 h	24
7	10 mM Tris, pH 7.0, 37 °C, 6	93
8	10 mM Tris, pH 8.2, 37 °C, 6 h	94
9	10 mM Tris, pH 9.0, 37 °C, 6 h	93
10	10 mM Tris, pH 6.0, 37 °C, 6 h	54
11	Water, 37 °C, 6 h	90

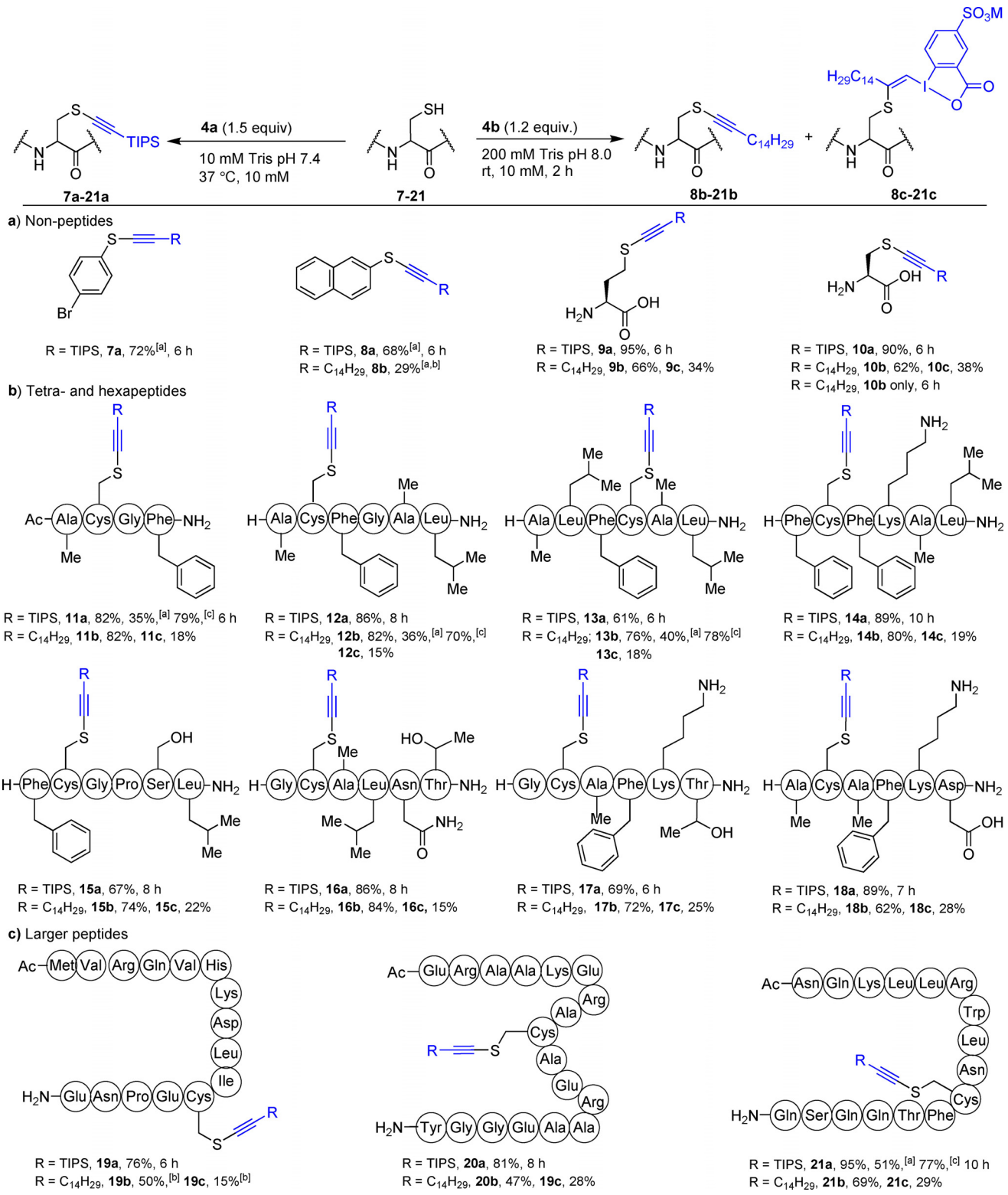
[a] Labeling condition: 16.0 µmol scale in 1.6 mL of non-degassed buffer. [b] Relative ratio of **6a** and disulfide based on HPLC-UV at 214 nm.

We then examined the alkynylation of glutathione (**6**, GSH) in buffer (Table 1). In 10 mM Tris buffer at pH 7.4 at rt,[15] S-alkynylated product **6a** was obtained in 47% yield in 6 h (entry 1).[21] After 16 h, the yield was improved to 83% (entry 2). At 37 °C, 95% yield could be obtained in 6 h (entry 3). With gradual increase of the buffer concentration, the yield decreased from 83% to 24% (entries 4 to 6). Surprisingly, the yield was almost unchanged from pH 7.0 to 9.0 (entries 7–9). Even at pH 6.0, 54% of the product was obtained (entry 10). This is unusual for cysteine functionalization, which proceeds normally better under basic conditions. Other buffers led to lower yields (see SI). The reaction proceeded also in pure water (entry 11). The reaction conditions had to be optimized again for reagent **4b**, due to increased formation of side products (see SI, Table S2). Best results were obtained with a 200 mM Tris buffer at pH 8.0 (Scheme 2). According to our experience with alkyl-EBXs, we expected to obtain VBX product **6c**.[15d] However, a mixture of alkynylated (**6b**), as major product, and VBX (**6c**), as minor product, was obtained.

With 4-bromothiophenol (**7**), alkynylation product **7a** was obtained in 72% isolated yield with **4a** (Scheme 3a). Naphthalene-2-thiol (**8**) gave 68% yield of **8a**. Both aromatic thiols **7** and **8** did not convert to alkynes **7b** and **8b** using **4b**. Nevertheless, **8b** could be obtained in 29% yield using



Scheme 2. Optimized conditions for the reaction of **4b** with glutathione (**6**). HPLC-MS yield is indicated. [a] Isolated yield. [b] Calibrated yield.



Scheme 3. Scope of the alkynylation reaction for a) non-peptides, b) tetra- and hexa-peptides and c) larger peptides. All the reactions were performed in 0.5 to 64.0 µmol scale at 10 mM concentration. Freshly prepared buffer was used without removing oxygen. Yields: relative ratio based on reverse phase HPLC-MS chromatogram unless stated otherwise. [a] Isolated yield. [b] Reactions were performed in 100 mM PB buffer at pH 8.0 at rt. [c] Calibrated yield.

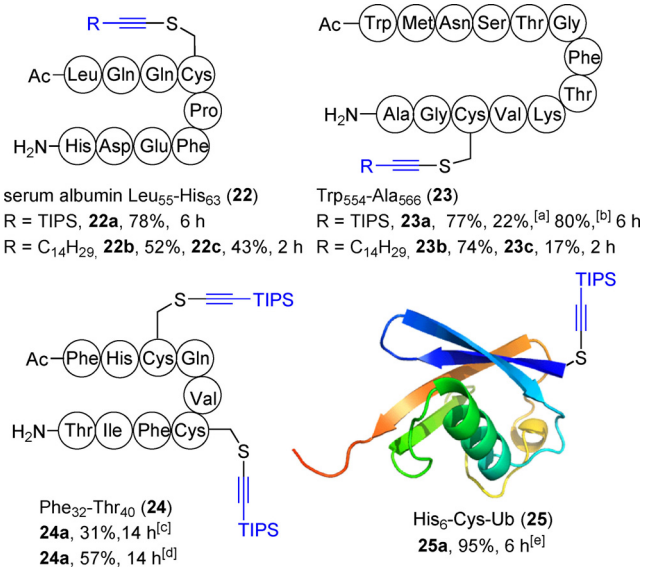
a 100 mM PB buffer. Homocysteine (**9**) gave **9a** in 95% HPLC-MS yield. Reaction with **4b** gave 66% of alkyne **9b** together with 34% of VBX **9c**. The reaction of unprotected cysteine (**10**) with **4a** and **4b** also proceeded well. After 6 h with **4b**, only **10b** was observed. With *N*-acylated peptide **11**, the alkynylation gave 82% of **11a** (79% calibrated yield and

35% isolated, Scheme 3b).[21] Using EBX **4b**, **11b** was obtained in 82% yield and 18% VBX **11c** was observed by HPLC. *N*-terminus unprotected hexapeptides **12** and **13** underwent alkynylation efficiently with both reagents. The reaction was selective for cysteine in presence of other nucleophilic amino acids such as serine, threonine, aspartic acid or lysine (peptides **14**–**18**). Larger peptides **19**–**21** (15 to 18-mer) were then investigated (Scheme 3c). Peptide **19** gave **19a** in 76% yield. Alkyne **19b** was obtained in 50% yield when using a 100 mM PB buffer. Both reagents **4a** and **4b** worked well with peptides **20** and **21** bearing nucleophilic side chains such as lysine, tryptophan, tyrosine, serine, threonine or glutamic acid.

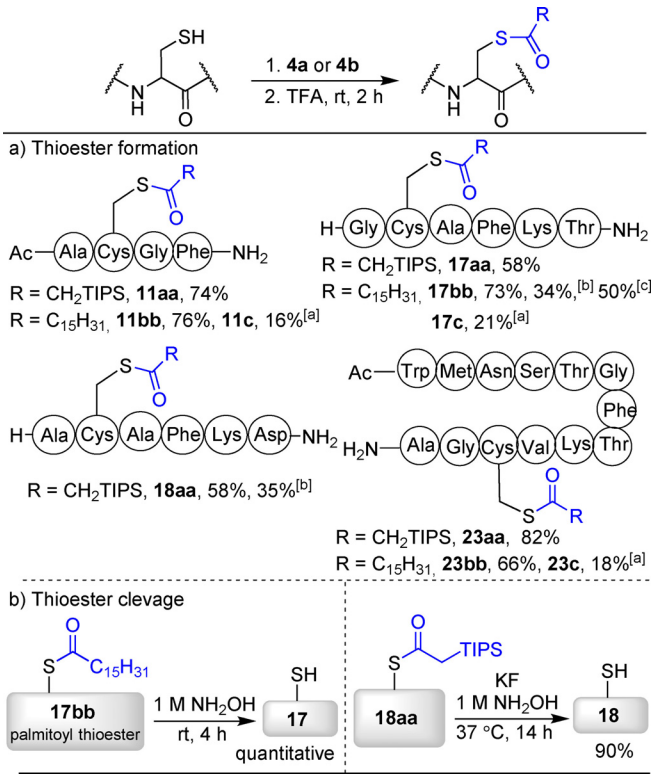
We then turned our attention to biologically relevant peptides (Scheme 4): Leu_{55}-His_{63} fragment **22** derived from human serum albumin, Trp_{554}-Ala_{566} fragment **23** derived from the hepatitis C virus (HCV) envelope glycoprotein E2[22] and Phe_{32}-Thr_{40} fragment **24** derived from the human immunodeficiency virus (HIV) tat protein.[23] Peptides **22** and **23** were successfully alkynylated with both reagents. With peptide **24** containing two cysteines, bisalkynylated product **24a** was obtained in 57% yield with 6 equivalents of **4a**. Cysteine-containing modified His_6-Cys-ubiquitin (**25**)[15d] was also alkynylated efficiently with **4a**. However, the use of reagent **4b** led to a complex mixture of products.

A significant increase of retention time in RP-HPLC was observed for all alkynylated peptides, indicating qualitatively higher lipophilicity. For example, the retention time of peptide **11** shifted from 5.8 to 13.6 min for **11a** and 20.2 min for **11b** (See SI). The partition coefficient (LogP) of product **11a** was determined to be 1.53 compared with −1.43 for **11**.[24]

The obtained thioalkynes constitute a new type of lipophilic compounds lacking the electrophilic carbonyl group



Scheme 4. Alkynylation of peptides **22**–**24** and His_6-Cys-ubiquitin (**25**). Reaction conditions: for **22a** and **23a**: **4a** (1.5 equiv), 10 mM Tris pH 7.4, 37 °C, for **22b** and **23b**: **4b** (1.2 equiv), 200 mM Tris buffer pH 8.0, rt. [a] Isolated yield. [b] Calibrated yield. [c] 4 equiv and [d] 6 equiv of **4a** was used in 10 mM Tris pH 7.4 at 37 °C, 5 mM. [e] Reaction was performed at 300 μM concentration.



Scheme 5. a) Access to thioesters from unprotected peptides in one pot via thioalkynes and b) Cleavage of the thioesters. See Supporting Information for detailed reaction conditions. [a] VBXs remain untouched under these reaction conditions. [b] Isolated yield. [c] Calibrated yield.

present in natural palmitoylated peptides, which is required for their hydrolysis. In presence of trifluoroacetic acid (TFA), clean hydration to give thioesters was observed (Scheme 5a). This hydration can be performed in a one pot protocol with silylated and alkylated alkynes on both small and larger peptides to give thioesters such as **11aa–bb**, **17aa–bb**, **18aa** and **23aa–bb** in 58–82% yield. **11bb**, **17bb** and **23bb** are then natural palmitoylated products. The VBX products **11c** and **23c** did not react under these acidic conditions. Numerous enzymatic and chemical methods have been reported for the cleavage of palmitoyl groups on cysteine.[25] Indeed, when peptide **17bb** was submitted to a 1 M solution of hydroxylamine, quantitative cleavage of the thioester was observed (Scheme 5b). In contrast, the silyl substituted thioester **18aa** reacts only very slowly under these conditions, probably due to the sterically hindered TIPS group. Nevertheless, treatment with a KF solution followed by hydroxylamine also allowed to cleave this thioester with 90% conversion. Taken together, our work therefore gives access to lipophilic peptide derivatives modifiable/cleavable under different conditions, which can be exploited depending on the desired application.

In summary, we have synthesized amphiphilic EBX hypervalent iodine reagents, which were employed for the selective lipophilization of cysteine under physiological conditions (pH 7.4–8.0, from room temperature to 37 °C in buffers without organic co-solvents). Aromatic thiols, homocysteine, cysteine and unprotected tetra- and hexapeptides

were successfully alkynylated. Larger peptides (15–18-mers) and one protein (His_6-Cys-Ubiquitin (**25**)) could also be selectively functionalized. Both retention time in reverse phase HPLC and LogP determination showed a significant increase of lipophilicity for the modified peptides, and the obtained thioalkynes could be converted into thioesters under acidic conditions. The thioesters could be easily cleaved using either hydroxylamine or a fluoride/hydroxylamine mixture.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: amphiphilic reagents · hypervalent iodine · lipidation · lipopeptide · ubiquitin

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[20] According to ICP-MS, potassium (K) was in major part exchanged with sodium (Na) during the synthesis; see the Supporting Information.
[21] The yield of **6a** was estimated using HPLC-UV spectra, based on the fact that only the desired product and disulfide were formed. The yield of **6b** and **6c** were estimated based on HPLC-MS spectra, as **6c** has a significantly enhanced absorbance when compared with the starting peptide. Owing to co-elution on preparative HPLC between **6a** and **6b** and the iodobenzoate formed during the reaction, pure isolated compounds could not be obtained and no calibration was performed. Nevertheless, isolation and calibration was performed on products **6c**, **11a**, **12b**, **13b**, **21a** and **23a**, showing that the estimated yields were a good approximation.
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[24] Determined using the octanol/water method according to: J. A. Arnott, S. L. Planey, *Expert Opin. Drug Discovery* **2012**, *7*, 863 – 875.
[25] Examples of cleavage using enzymes: a) Ref. [10d]; b) M. D. Resh, *Prog. Lipid Res.* **2016**, *63*, 120 – 131; c) M.-E. Zaballa, F. G. van der Goot, *Crit. Rev. Biochem. Mol. Biol.* **2018**, *53*, 420 – 451; Examples using hydroxylamine: d) P. J. O'Brien, R. S. St. Jules, T. S. Reddy, N. G. Bazan, M. Zat, *J. Biol. Chem.* **1987**, *262*, 5210 – 5215; e) C. T. M. B. Tom, B. R. Martin, *ACS Chem. Biol.* **2013**, *8*, 46 – 57.

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SUPPORTING INFORMATION

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1. General procedures

All reactions using anhydrous conditions were performed with oven-dried glassware, under an atmosphere of nitrogen, unless stated otherwise. Anhydrous acetonitrile was purchased from Sigma-Aldrich. All the Fmoc-protected amino acids, Rink Amide MBHA resin and 2-chlorotrityl chloride resin were purchased from GL Biochem. O-Benzotriazole-N,N,N',N'-etramethyluronium-hexafluoro-phosphate (HBTU, GL Biotech), N,N-di*iso*propylethylamine (DIPEA, Iris Biotech GmbH) and hydroxybenzotriazole (HOBt, GL Biotech) were used as received. All the other reagents were purchased from ABCR, Acros, Aldrich, AlfaAesar, Apollo Scientific, Fluorochem, Fluka, Roth and TCI and were used without additional purification. Melting points were measured on a Büchi B-540 melting point apparatus using open glass capillaries. The data is uncorrected. ^{1}H-NMR spectra were recorded on a Brucker DPX-400 400 MHz spectrometer in $CDCl_3$, DMSO-d_6 or D_2O. All signals are reported in ppm with the internal $CHCl_3$ signal at 7.26 ppm, the internal DMSO signal at 2.50 ppm or the internal H_2O signal at 4.79 ppm, MeOD at 4.35 as standard. The data is being reported as: s= singlet, d = doublet, t = triplet, q = quadruplet, qi = quintet, m = multiplet or unresolved, br = broad signal, app = apparent, coupling constant(s) in Hz, integration, interpretation.^{13}C-NMR spectra were recorded with ^{1}H-decoupling on a Brucker DPX-400 100 MHz spectrometer in $CDCl_3$, DMSO-d_6 or D_2O. All signals are reported in ppm with the internal $CHCl_3$ signal at 77.0 ppm or the internal DMSO signal at 39.5 ppm as standard. 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 a MICROMASS (ESI) Q-TOF Ultima API.

All reactions related to the peptide/protein alkynylation process were set up on the benchtop and carried out in 1.5 mL vial without oxygen exclusion. Buffers were not degassed and prepared with milliQ water. All the reactions were replicated three times and the reported yield is an average of these replicates.

2. Analytical HPLC and preparative HPLC information

a. Analytical

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 + 0.1% formic acid (solvent A), water:acetonitrile 5:95 + 0.1% formic acid (solvent B) were used as the mobile phase, at a flow rate of 0.6 mL/min. The column temperature was set up to 25 °C. 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. To obtain high-resolution mass spectrometric measurements, the desired fraction was recovered after separation on a Waters XBridge C18 column (250 x 4.6 mm, 5 µm) and submitted to the mass spectrometry service of ISIC at the EPFL that uses a MICROMASS (ESI) Q-TOF Ultima API.

Method 1: 100% A to 100% B 0-20 minutes, then 100% B 20 – 30 minutes.

Method 2: 100% A for 5 minutes isocratic, 100% A to 100% B 0-20 minutes, then 100% B 20 – 30 minutes.

Method 3: 100% A to 50% A in 5 minutes, then 50% A for 5-30 minutes.

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, 100% B to 100% A for 38-40 minutes.

b. 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 + 0.1% TFA (solvent C), water: acetonitrile 5:95 + 0.1% TFA (solvent D), water (solvent E) or water:acetonitrile 5:95 (solvent F) were used as the mobile phase at a flow rate of 20 $mL.min^{-1}$. Following methods were used.

Method 5: 100% C to 100% D in 0-20, then 100% B 20-30 minutes.

Method 6: 100% C to 50% C for 5 min, then 50% C to 50% D in 5 - 30 minutes.

Method 7: 100% E to 100% F in 30 minutes.

3. Peptide preparation

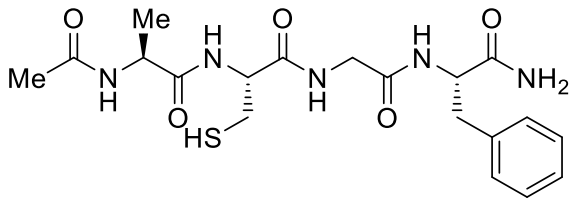
Solid-Phase Peptide Synthesis (SPPS): Peptides were synthesized on an Advanced ChemTech 348-Ω parallel peptide synthesizer (AAPPTec) using standard Fmoc SPPS-chemistry and Rink Amide MBHA resin (0.26 mmol/g resin, 0.05 mmol scale) for C-terminal amide. The coupling was carried out by shaking the resin with a Fmoc-protected monomer (4.0 equiv.), 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU, 4.0 equiv.), 4-Methylmorpholine (NMM, 6.0 equiv.), in dimethylformamide (1.3 mL), at 400 rpm, over 30 minutes. This step was accomplished twice. Capping was performed at the end of each coupling using mixture of Ac_2O:2,6-lutidine:DMF (5:6:89),

followed by dimethylformamide wash (4 x 3 mL). Fmoc groups were then removed by shaking the resin with 20% v/v piperidine in dimethylformamide at 400 rpm, over 5 minutes. This step was carried out twice. Next, washing steps were achieved with dimethylformamide (5 x 3 mL). Finally, resin was dried with dichloromethane (5 x 3 mL).

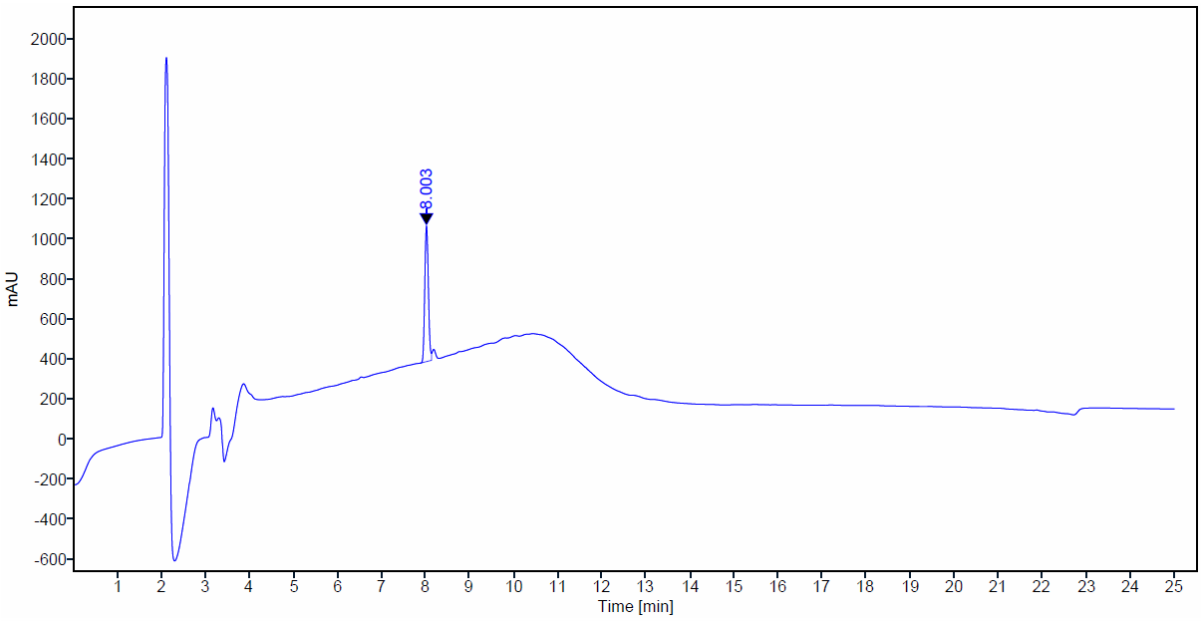
Peptide cleavage and deprotection: Peptides were deprotected and cleaved from the resin under reducing conditions, by treatment with 2.5% v/v water and 2.5% v/v triisopropylsilane (TIPS) in neat trifluoroacetic acid (5 mL). The resulting mixture was shaken for 2 hours at 400 rpm, at room temperature. The resin was removed by filtration and peptides were precipitated in cold diethyl ether (20 mL), followed by a 2 hours incubation at -20 °C. Peptides were pelleted by centrifugation at 4000 rpm, at 4 °C, for 5 minutes. Finally, the mother liquors were carefully removed and crude peptides were dried under vacuum.

Peptide purification and analyses: Peptides were dissolved in water with a minimum amount of organic co-solvent (acetonitrile, dimethylformamide or dimethyl sulfoxide). Peptides were then purified on preparative RPHPLC using method A. Fractions containing the desired peptide were lyophilized. The purity was assessed by analyzing a 10-20 mM peptide solution by RP-HPLC (HPLC gradient: 100% A to 100% B in 30 minutes. At the same time, low-resolution mass spectrometric measurements were also acquired.

Ac-Ala-Cys-Gly-Phe-NH_2 (**11**)

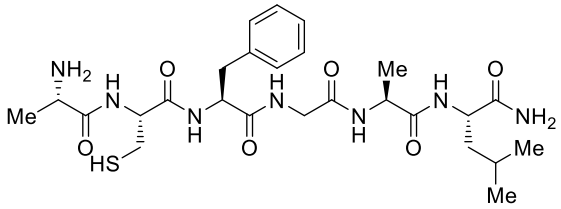


LRMS (ESI) m/z: $[M + H]^+$ Calcd for $C_{19}H_{28}N_5O_5S^+$ 438.18; Found 438.4. HPLC gradient: Method 1.

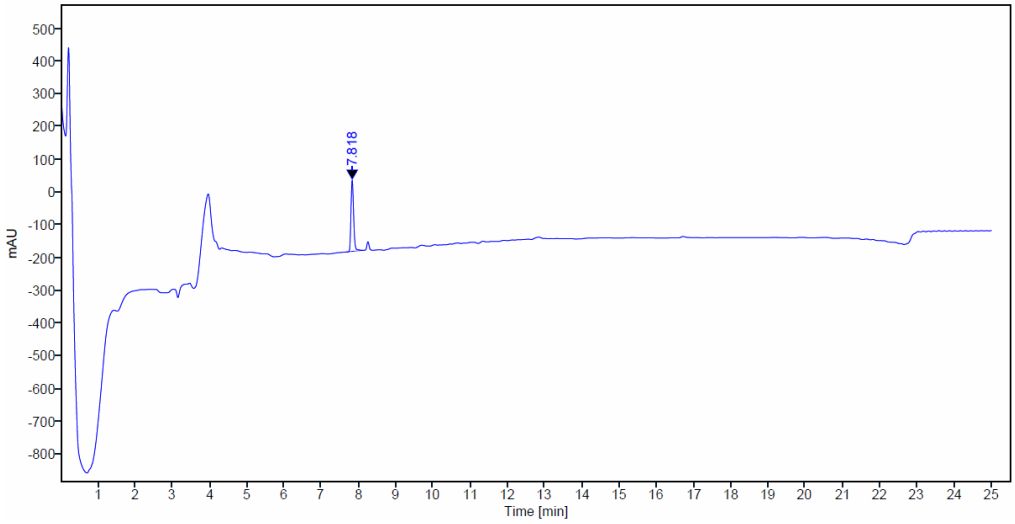


SUPPORTING INFORMATION

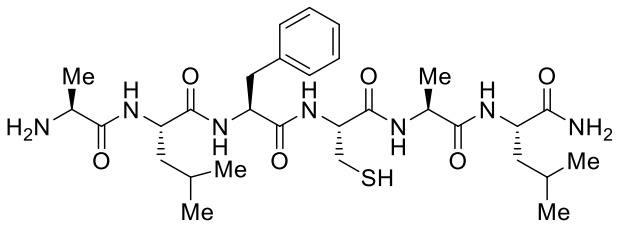
H-Ala-Cys-Phe-Gly-Ala-Leu-NH_2 (**12**)



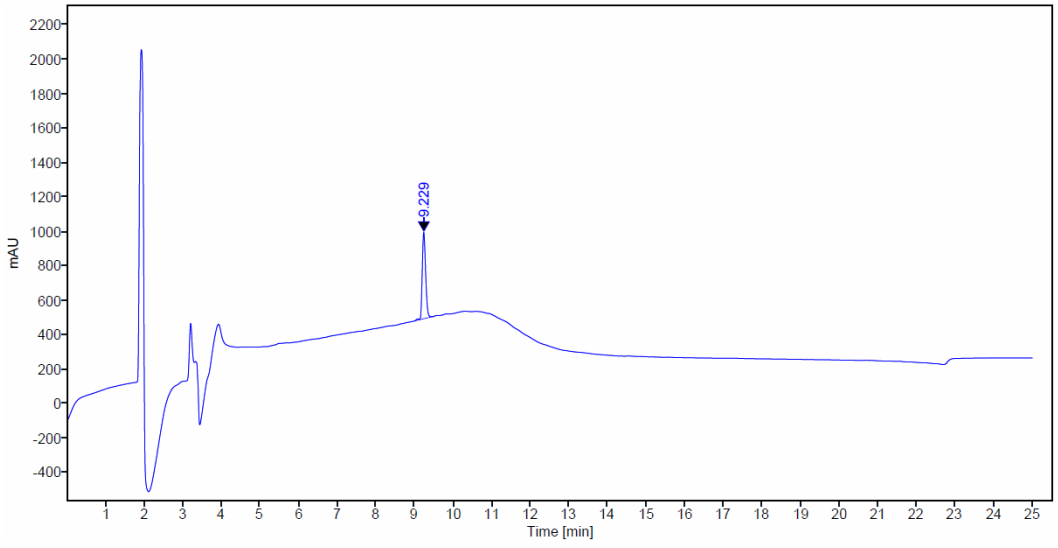
LRMS (ESI) m/z: $[M + H]^+$ Calcd for $C_{26}H_{42}N_7O_6S^+$ 580.29; Found 580.30. HPLC gradient: Method 1.



H-Ala-Leu-Phe-Cys-Ala-Leu-NH_2 (**13**)



LRMS (ESI) m/z: $[M + H]^+$ Calcd for $C_{30}H_{50}N_7O_6S^+$ 636.35; Found 636.50. HPLC gradient: Method 1.



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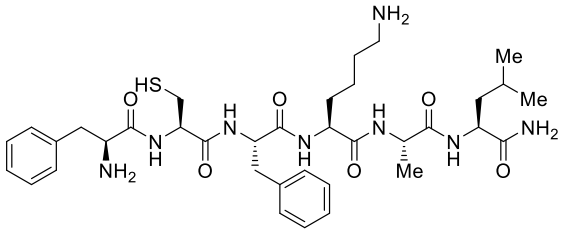
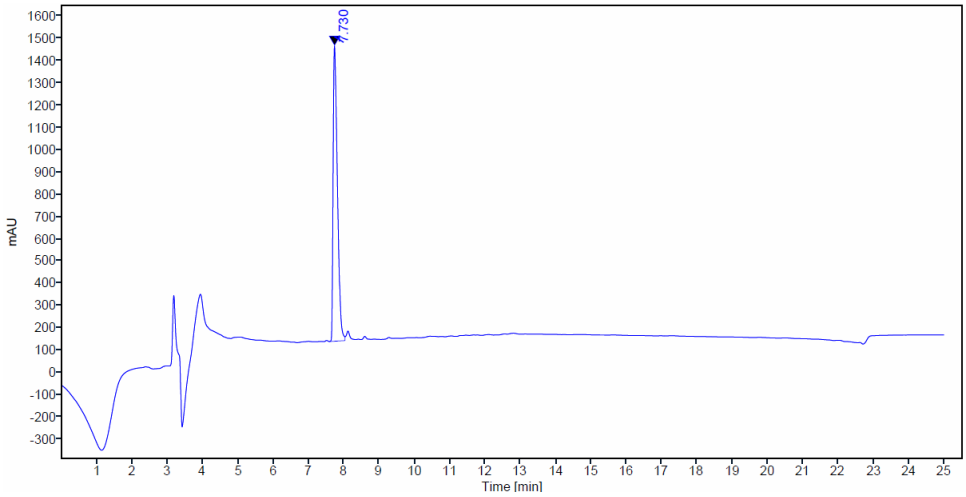
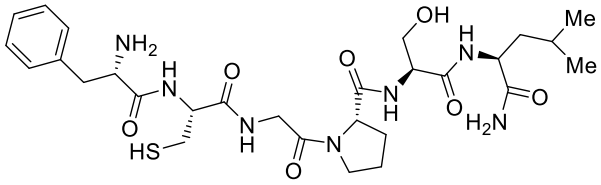
H-Phe-Cys-Phe-Lys-Ala-Leu-NH_2 (**14**)



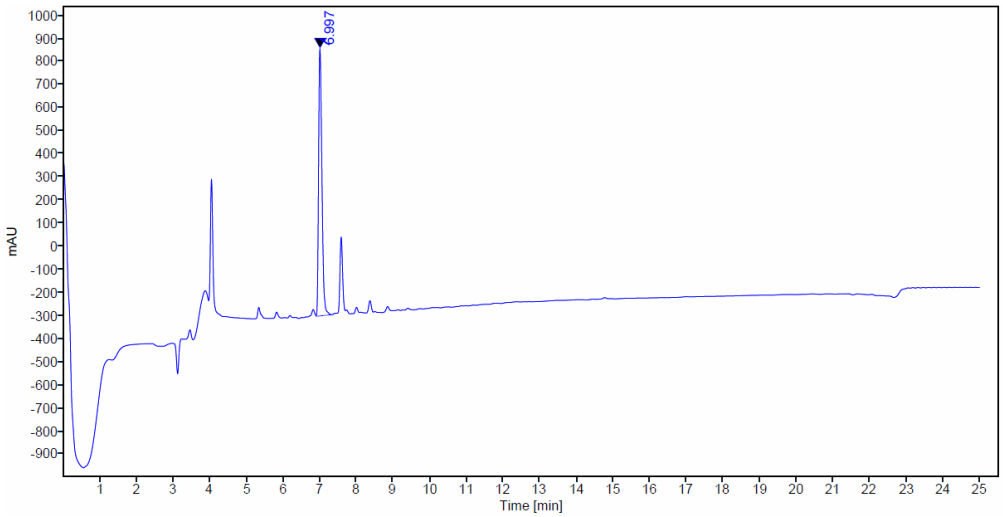
LRMS (ESI) m/z: $[M + H]^+$ Calcd for $C_{36}H_{55}N_8O_6S^+$ 726.39; Found 726.60. HPLC gradient: Method 1.



H-Phe-Cys-Gly-Pro-Ser-Leu-NH_2 (**15**)



LRMS (ESI) m/z: $[M + H]^+$ Calcd for $C_{28}H_{44}N_7O_7S^+$ 622.30; Found 622.50. HPLC gradient: Method 1.



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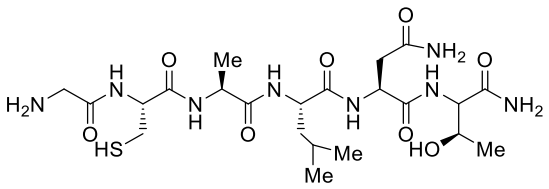
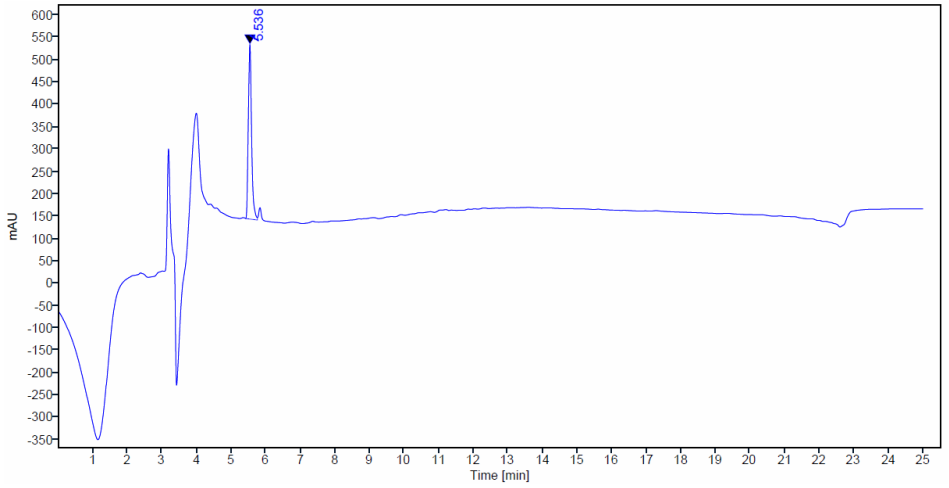
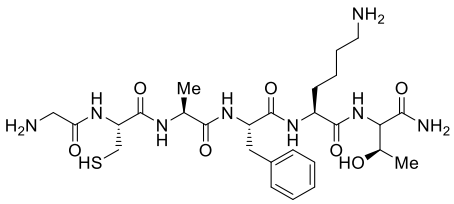
H-Gly-Cys-Ala-Leu-Asn-Thr-NH_2 (**16**)



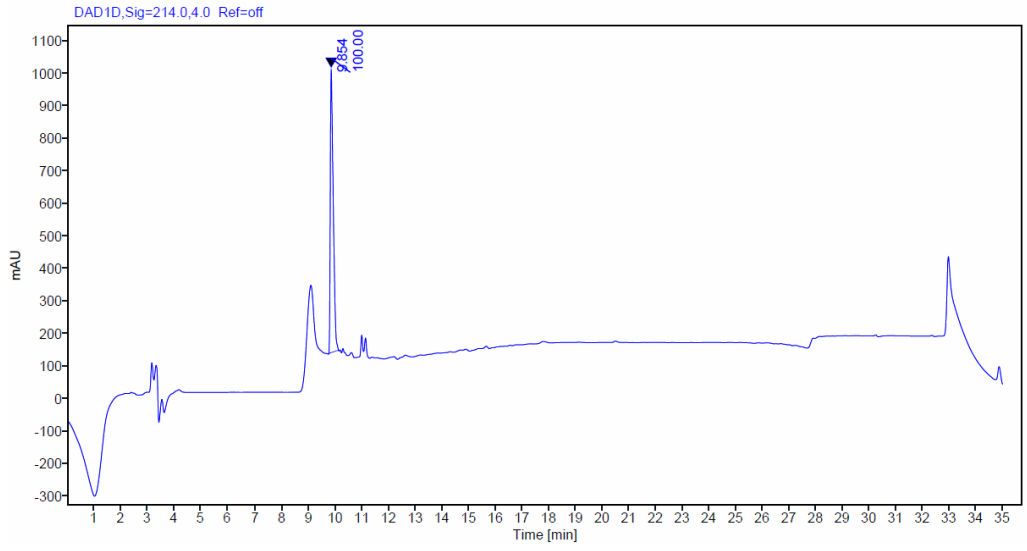
LRMS (ESI) m/z: $[M + H]^+$ Calcd for $C_{22}H_{41}N_8O_8S^+$ 577.27; Found 577.40. HPLC gradient: Method 1.



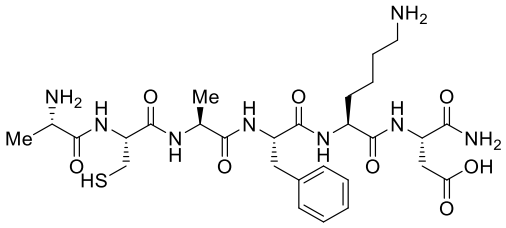
H-Gly-Cys-Ala-Phe-Lys-Thr-NH_2 (**17**)



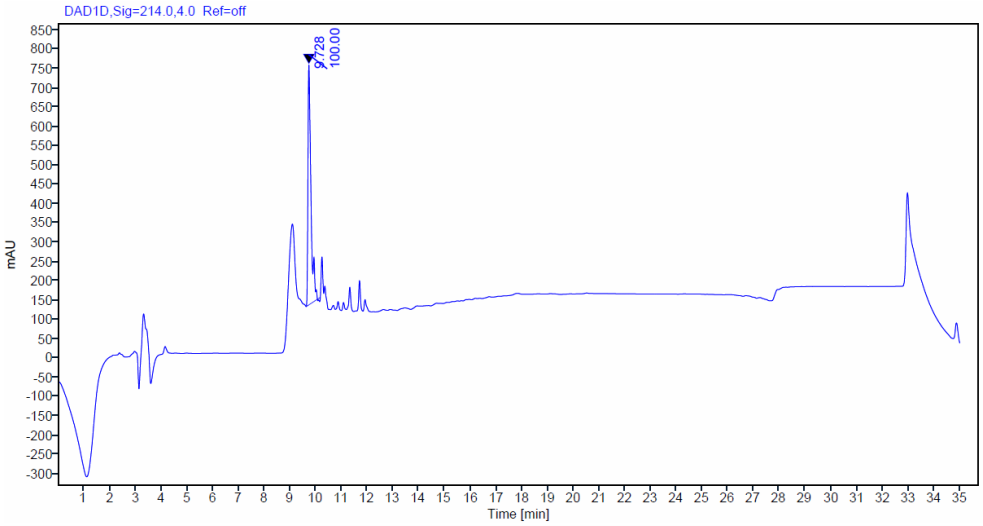
LRMS (ESI) m/z: $[M + H]^+$ Calcd for $C_{27}H_{45}N_8O_7S^+$ 625.31; Found 625.40. HPLC gradient: Method 2.



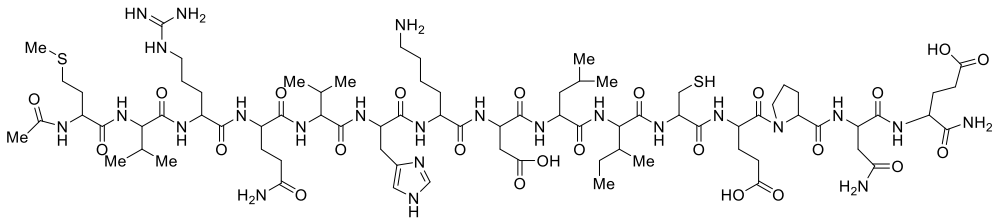
H-Ala-Cys-Ala-Phe-Lys-Asp-NH_2 (**18**)



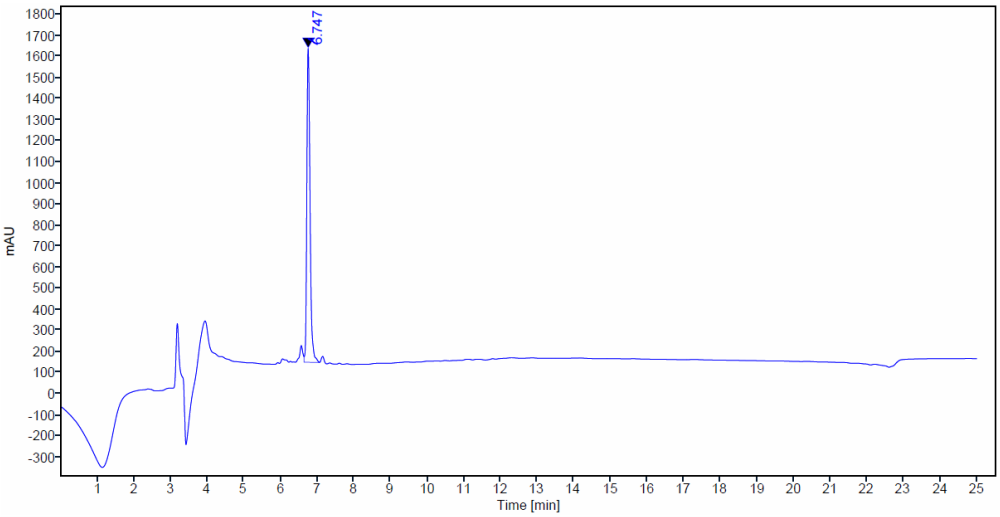
LRMS (ESI) m/z: $[M + H]^+$ Calcd for $C_{28}H_{45}N_8O_8S^+$ 653.30; Found 653.40. HPLC gradient: Method 2.



Ac-Met-Val-Arg-Gln-Val-His-Lys-Asp-Leu-Ile-Cys-Glu-Pro-Asn-Glu-NH_2 (**19**)

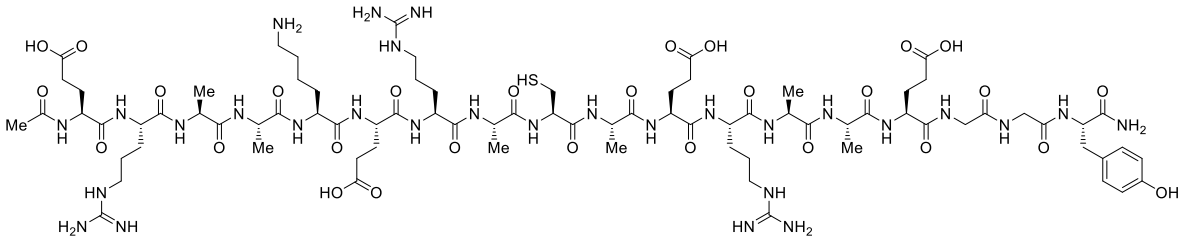


LRMS (ESI) m/z: $[M + 2H]^{2+}$ Calcd for $C_{78}H_{132}N_{24}O_{24}S_2^{2+}$ 927.46; Found 927.70. HPLC gradient: Method 1.

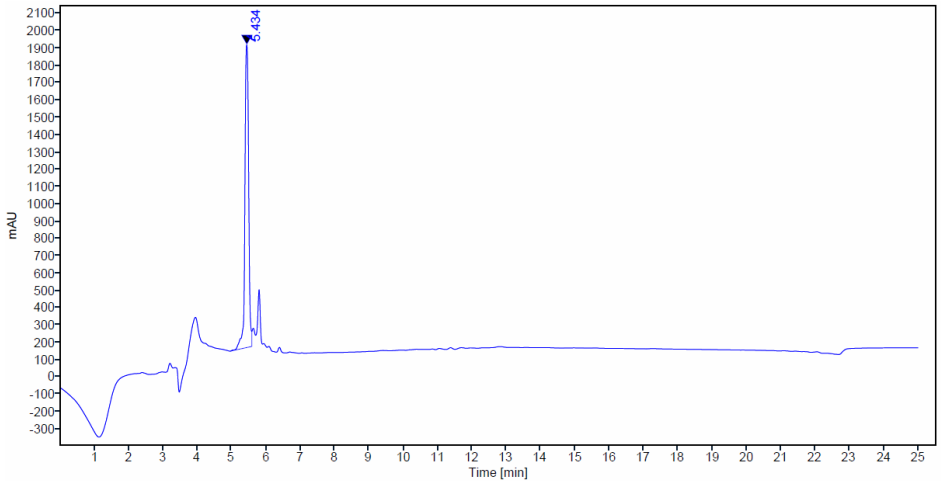


SUPPORTING INFORMATION

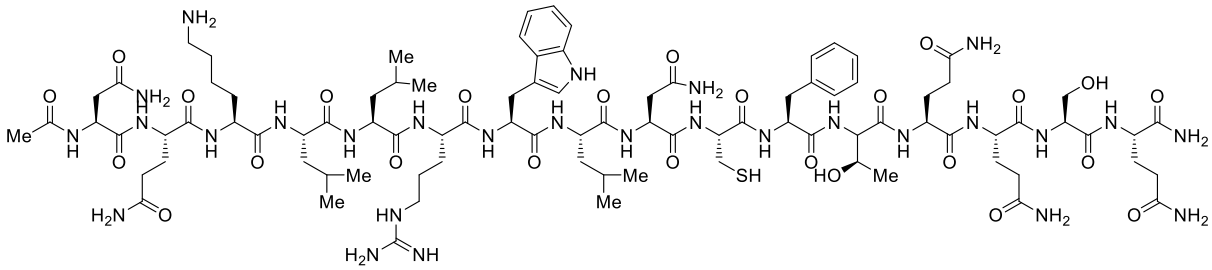
Ac-Glu-Arg-Ala-Ala-Lys-Glu-Arg-Ala-Cys-Ala-Glu-Arg-Ala-Ala-Glu-Gly-Gly-Tyr-NH_2 (**20**)



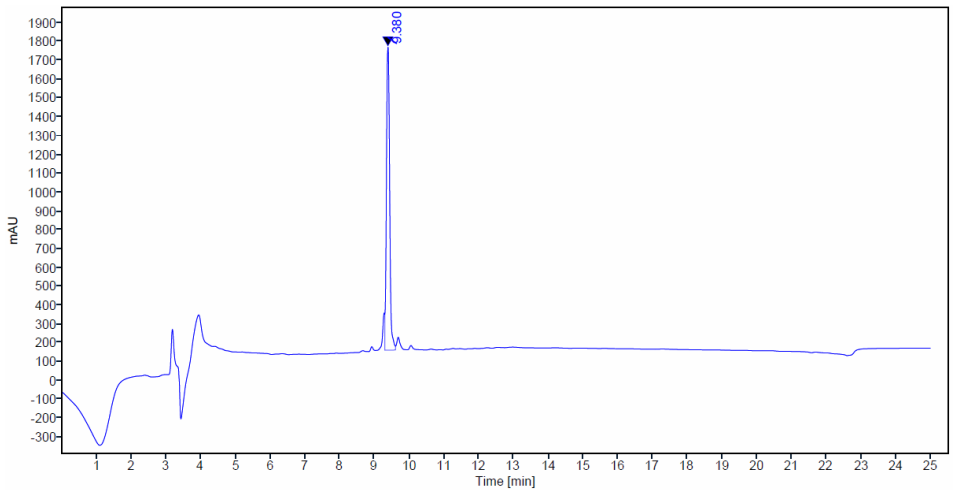
LRMS (ESI) m/z: $[M + 2H]^{2+}$ Calcd for $C_{80}H_{133}N_{29}O_{28}S^{2+}$ 990.97; Found 990.80. HPLC gradient: Method 1.



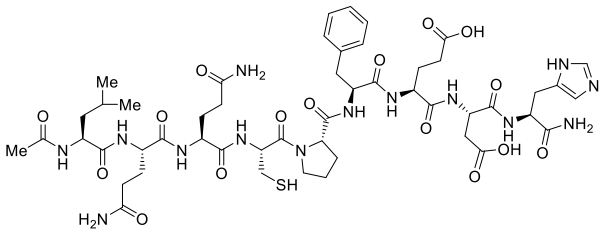
Ac-Asn-Gln-Lys-Leu-Leu-Arg-Trp-Leu-Asn-Cys-Phe-Thr-Gln-Gln-Ser-Gln-NH_2 (**21**)



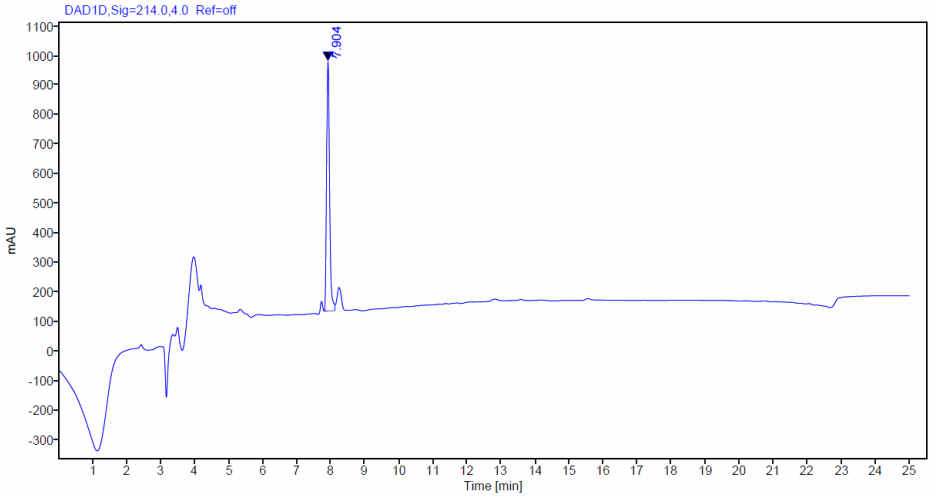
LRMS (ESI) m/z: $[M + 2H]^{2+}$ Calcd for $C_{90}H_{144}N_{28}O_{25}S^{2+}$ 1025.52; Found 1025.20. HPLC gradient: Method 1.



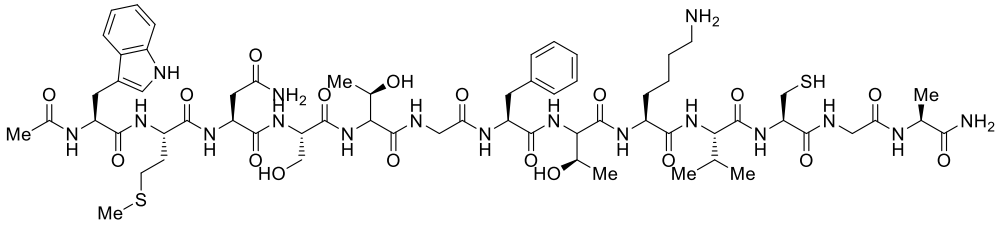
Human Serum Albumin (Leu_{55}-His_{63}): Ac-Leu-Gln-Gln-Cys-Pro-Phe-Glu-Asp-His-NH_2 (**22**)



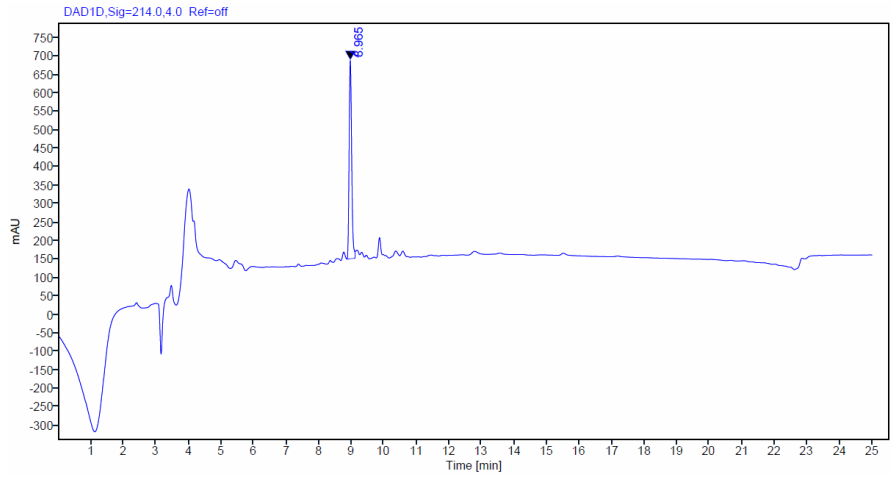
LRMS (ESI) m/z: $[M + 2H]^{2+}$ Calcd for $C_{50}H_{74}N_{14}O_{16}S^{2+}$ 580.25; Found 580.30. HPLC gradient: Method 1.



Ac-Trp-Met-Asn-Ser-Thr-Gly-Phe-Thr-Lys-Val-Cys-Gly-Ala-NH_2 (**23**)



LRMS (ESI) m/z: $[M + 2H]^{2+}$ Calcd for $C_{63}H_{97}N_{17}O_{18}S_2^{2+}$722.83; Found 722.50. HPLC gradient: Method 1.



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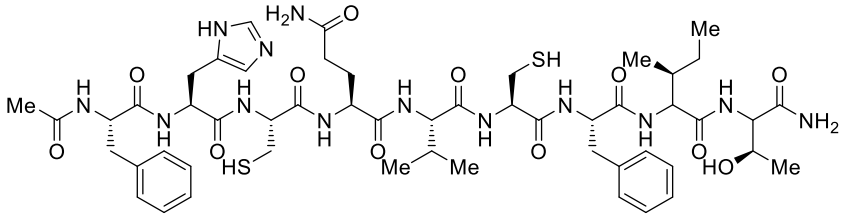
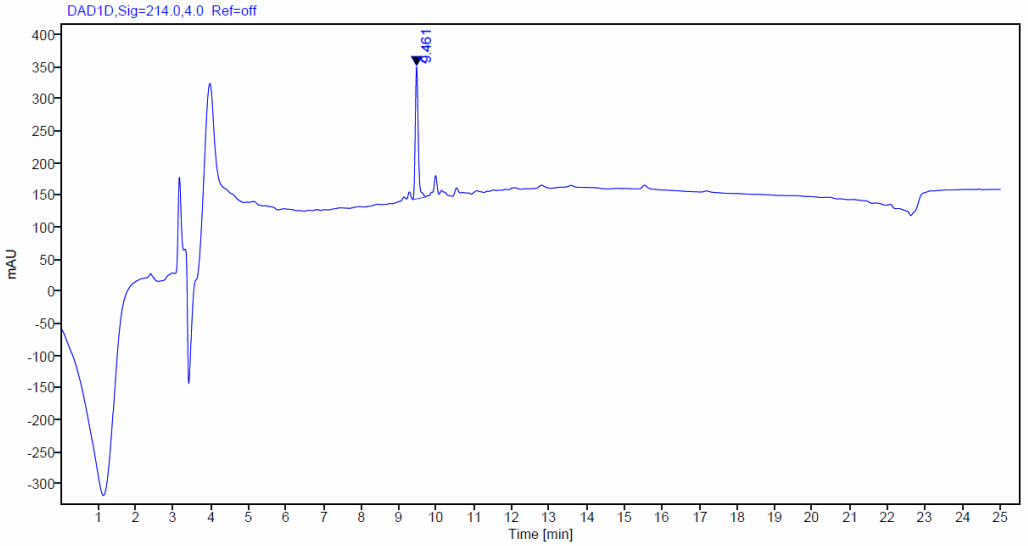
TAT-HIV (Phe$_{32}$-Thr$_{40}$) Ac-Phe-His-Cys-Gln-Val-Cys-Phe-Ile-Thr-NH$_2$ (**24**)



LRMS (ESI) m/z: $[M + H]^+$ Calcd for $C_{52}H_{76}N_{13}O_{12}S_2^+$; 1138.52, Found 1138.4. HPLC gradient: Method 1.



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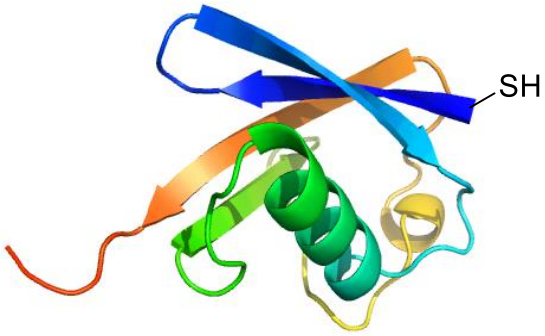
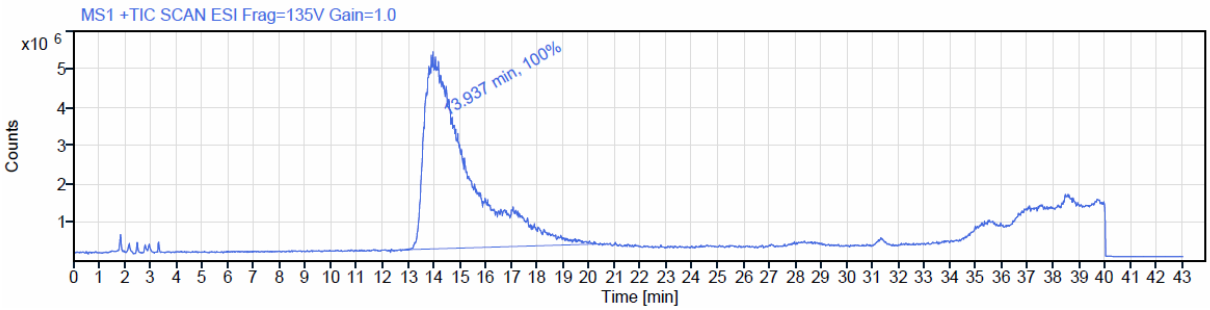
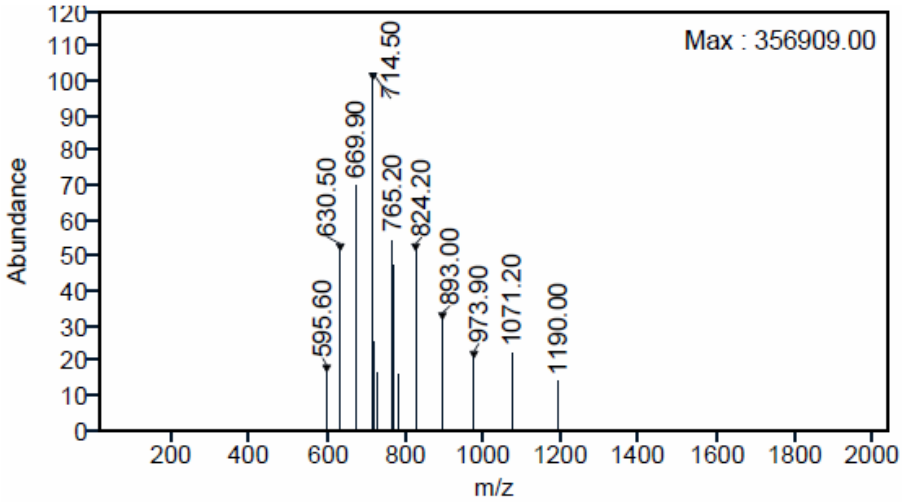
Ubiquitin (**24**)



LRMS (ESI) m/z: $[M + 15H]^{15+}$ Calcd for His_6-Cys-Ub 714.3 Found 714.5.

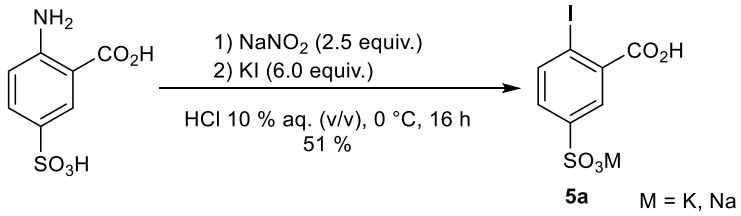
HPLC gradient: Method 4.





4. Preparation of amphiphilic reagents

Preparation of (**5a**)



Following a reported procedure,[1] 2-amino-5-sulfobenzoic acid (4.34 g, 20.0 mmol, 1.0 equiv.) was suspended in a 10% aqueous hydrochloric acid solution (100 mL) and cooled to 0 °C. A cooled solution of sodium nitrite ($NaNO_2$, 3.45 g, 50.0 mmol, 2.5 equiv.) in water (18 mL) was slowly added over a period of 45 minutes. After an additional 30 minutes stirring at this temperature, a cooled solution of potassium iodide (KI, 19.9 g, 120 mmol, 6.0 equiv.) in water (75 mL) was slowly added over a period of 1 hour at 0 °C. The resulting dark solution was allowed to warm to room temperature and stirred for 16 hours. Then, the reaction was slowly quenched by small portions of sodium bisulfite (around 14 g) until the solution persistently turned as a light-yellow[2] suspension. The resulting suspension was filtered, washed with acetone (3 x 100 mL) and dichloromethane (50 mL) to afford a yellow pale solid. The collected solid was then recrystallized from water and washed with cold water (2 x 50 mL), acetone (2 x 50 mL) and dichloromethane (2 x 50 mL) to yield pure **5a** (3.71 g, 10.1 mmol, 51% yield) as a pale-yellow solid.

^{1}H NMR (400 MHz, DMSO-d_6) δ 7.95 (d, *J* = 8.1 Hz, 1H, ArH), 7.90 (d, *J* = 2.0 Hz, 1H, ArH), 7.41 (dd, *J* = 8.1, 2.1 Hz, 1H, ArH).

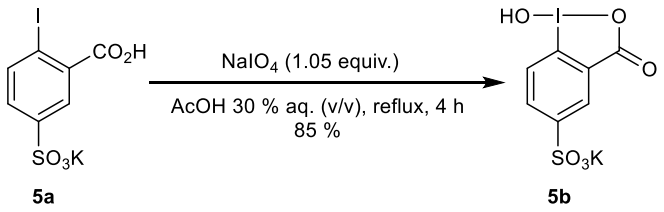
^{13}C NMR (101 MHz, DMSO-d_6) δ 167.9, 147.8, 140.5, 136.4, 129.5, 127.3, 94.8.

Spectra data was consistent with the values reported in literature.[1]

[1] A. Kommreddy, M. S. Bowsher, M. R. Gunna, K. Botha, T. K. Vinod,*Tetrahedron Lett.* **2008**, *49*, 4378.
[2] a) T. Harschneck, S. Hummel, S. Kirsch, P. Klahn, *Chem. Eur. J.* **2012**, *18*, 1187; b) A. Bredenkamp, F. Mohr, S. Kirsch, *Synthesis*, **2015**, *47*, 1937.

SUPPORTING INFORMATION

Preparation of **5b**



5a **5b**

Following a modified reported procedure, **5a** (1.75 g, 8.17 mmol, 1.00 equiv.) and sodium periodate ($NaIO_4$, 2.85 g, 7.78 mmol, 1.05 equiv.) were suspended in 30% aqueous acetic acid solution (14 mL). The vigorously stirred mixture was heated and refluxed under air for 4 h. The reaction mixture was allowed to cool to room temperature and placed under vacuum. The resulting precipitate was filtered and washed with acetone (3 x 100 mL) and dichloromethane (100 mL). The collected solid was dissolved in methanol, filtered and concentrated under pressure to afford pure potassium 2-iodosyl-5-sulfobenzoate, **5b** (2.52 g, 6.59 mmol, 85% yield) as a white solid.

m.p.: 299 – 300 °C.

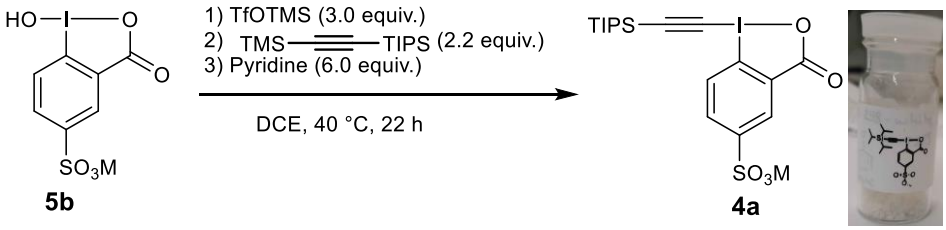
^{1}H NMR (400 MHz, DMSO-d_6) δ 8.18 (d, *J* = 1.8 Hz, 1H, ArH), 8.12 (dd, *J* = 8.3, 1.9 Hz, 1H, ArH), 7.80 (d, *J* = 8.3 Hz, 1H, ArH).

^{13}C NMR (101 MHz, DMSO-d_6) δ 167.5 (C=O), 151.1 (ArC), 132.1 (ArC), 130.7 (ArC), 128.5 (ArC), 126.3 (ArC), 119.1 (ArC).

IR ν_{max} 1648 (*m*), 1618 (*m*), 1205 (*s*), 1095 (*m*), 1041 (*m*), 1011 (*m*).

HRMS (ESI/QTOF) m/z: $[M-K]^-$ Calcd for $C_7H_4IO_6S^-$ 342.8779; Found 342.8779.

Preparation of TIPS-EBX-SO_3M (**4a**)



5b **4a**

Trimethylsilyl trifluoromethanesulfonate (TfOTMS, 2.55 mL, 14.1 mmol, 3.0 equiv.) was added dropwise to a stirred suspension of potassium 2-iodosyl-5-sulfobenzoate (1.80 g, 4.71 mmol, 1.0 equiv.) in dichloroethane (157 mL) at 40 °C. After 2 h stirring at this temperature, triisopropyl((trimethylsilyl)ethynyl)silane (2.64 g, 10.4 mmol, 2.2 equiv.) was slowly added to the solution. The reaction mixture was stirred for another 18 h and pyridine (2.29 mL, 28.3 mmol, 6.0 equiv.) was added. After 2 additional hours stirring, the mixture was diluted with dichloromethane (200 mL), washed with a 0.5 N aqueous sodium bicarbonate solution (150 mL) and a 0.5 N aqueous hydrochloric acid solution (150 mL). The organic layer was dried over magnesium sulfate, filtered and the volatiles were removed *in vacuo*. The crude orange oil was purified by column chromatography (SiO_2, Dichloromethane:Methanol gradient from 9:1 to 8:2) to yield pure K/Na-5-sulfonate TIPS-EBX-SO_3M (**4a**, 2.00 g, 3.66 mmol, 78% yield) as a white solid. Yield for **4a** calculated based on K salt.

R_f 0.50 (Dichloromethane:Methanol, 4:1).

m.p.: 325 – 326 °C,

Solubility in water (**4a)**: 0.46 g/mL

SUPPORTING INFORMATION

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.30 (d, *J* = 2.0 Hz, 1H, Ar*H*), 8.26 (d, *J* = 8.5 Hz, 1H, Ar*H*), 7.98 (dd, *J* = 8.5, 2.1 Hz, 1H, Ar*H*), 1.24 – 1.00 (m, 21H, TIPS).

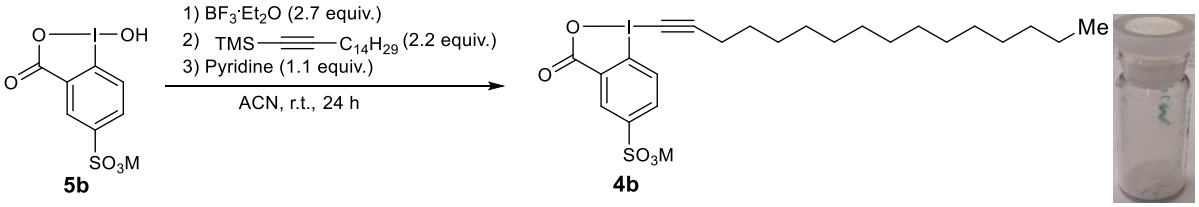
^{13}C NMR (101 MHz, DMSO-d_6) δ 165.9 (C=O), 151.7 (ArC), 132.2 (ArC), 131.2 (ArC), 128.3 (ArC), 126.6 (ArC), 115.4 (ArC), 110.7 (CC), 67.1 (CC), 18.4 (CH_3), 10.7 (CH).

IR ν_{max} 2952 (*w*), 2866 (*w*), 2372 (*w*), 2347 (*w*), 2325 (*w*), 1634 (*s*), 1238 (*s*), 1169 (*s*), 1102 (*m*), 1036 (*s*), 994 (*m*), 882 (*m*).

HRMS (ESI/QTOF) m/z: $[M\text{-}K]^-$ Calcd for $C_{18}H_{24}IO_5SSi^-$ 507.0164; Found 507.0165.

ICP-MS 53.66 µg/mg Na, 5.15 µg/mg K.

Preparation of $C_{14}H_{29}$-EBX-SO_3M (**4b**)



A flame-dried 25 mL round-bottomed flask under nitrogen was charged with potassium **5b** (0.50 g, 1.30 mmol, 1.0 equiv.) and acetonitrile (10.0 mL). A cooled solution of boron trifluoride etherate ($BF_3{\cdot}Et_2O$, 0.43 mL, 3.5 mmol, 2.7 equiv.) was added dropwise at room temperature and the reaction was stirred for 2 h. Hexadec-1-yn-1-yltrimethylsilane (0.843 g, 2.86 mmol, 2.2 equiv.) was then slowly added and the resulting mixture was stirred for an additional 18 h. Then, pyridine (115 µL, 1.43 mmol, 1.1 equiv.) was added dropwise and the reaction mixture was stirred for 2 h. The resulting precipitate was filtered and washed with acetonitrile (3 x 15 mL) and pentane (3 x 10 mL). The crude mixture was purified by RP-HPLC using C18 column (gradient: 100 H_2O to 95% ACN/H_20 for 30 mins) to obtain 60% pure **4b** (0.45 g, 0.78 mmol,) as a white solid. HPLC-Gradient: Method 7. Yield for **4b** calculated based on K salt.

R_f 0.30 (dichloromethane:methanol, 9:1).

m.p.: 184 – 185 °C.

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.29 (d, *J* = 2.1 Hz, 1H, ArH), 8.18 (d, *J* = 8.5 Hz, 1H, ArH), 8.02 (dd, *J* = 8.4, 2.1 Hz, 1H, ArH), 2.67 (t, *J* = 7.0 Hz, 2H, CH_2), 1.59 (q, *J* = 7.1 Hz, 2H, CH_2), 1.49 – 1.12 (m, 22H, 11×CH_2), 0.85 (t, *J* = 8.0 Hz , 3H, CH_3).

^{13}C NMR (101 MHz, DMSO-d_6) *δ* 166.3 (C=O), 152.0 (ArC), 132.5 (ArC), 132.0 (ArC), 128.6 (ArC), 127.3 (ArC), 115.7 (CC), 108.6 (CC), 31.7 (CH_2), 29.5 (5×CH_2), 29.4 (CH_2), 29.2 (CH_2), 28.9 (CH_2), 28.7 (CH_2), 28.1 (CH_2), 22.5 (CH_2), 20.1 (CH_2), 14.4 (CH_3).

IR ν_{max} 2918 (*s*), 2850 (*s*), 2176 (*w*), 1636 (*s*), 1469 (*m*), 1234 (*s*), 1190 (*s*), 1099 (*m*), 1039 (*s*), 998 (*s*).

HRMS (ESI/QTOF) m/z: $[M\text{-}K]^-$ Calcd for $C_{23}H_{32}IO_5S^-$ 547.1021; Found 547.1019.

ICP-MS 46.01 µg/mg Na, 2.91 µg/mg K.

Solubility in water (**4b)** 0.45 g/mL

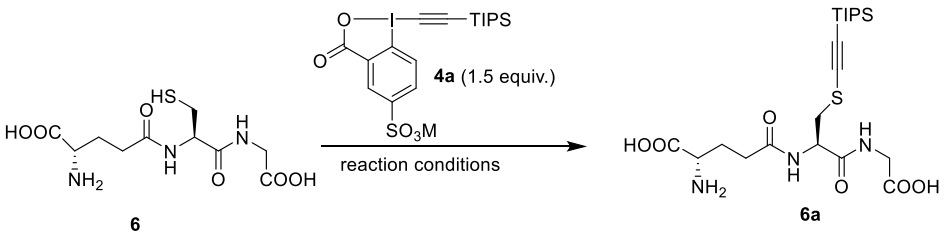
SUPPORTING INFORMATION

5. Optimization of reaction conditions

Experimental procedure for optimization of **4a**

A solution of glutathione (**6**, GSH) in non-degassed 10 mM Tris buffer pH 7.4 was prepared. Then, a solution of **4a** in non-degassed 10 mM Tris buffer pH 7.4 was added to the solution of glutathione **6** in a 1.5 mL vial. The mixture was then stirred at room temperature under "open-flask" conditions. After 6 hours, the labeling furnished a remarkable 47% yield of the alkynylated glutathione **6a** (Table S1, Entry 1). Notably, we did not observe any formation of VBX derivatives. A similar procedure adopted for the other entries of the optimization, Table S1, entry 2-20.

Table 1. Reaction optimization of glutathione with **4a**



6 **6a**

Entry	Buffer	Buffer conc. (mM)	Buffer pH	Temp (°C)	Time (h)	Yield (%)
1	Tris	10	7.4	rt	6	47
2	Tris	10	7.4	rt	16	83
3	Tris	10	7.4	37	6	83
4	Tris	20	7.4	37	6	81
5	Tris	10	7.4	37	6	95
6	Tris	40	7.4	37	6	84
7	Tris	60	7.4	37	6	78
8	Tris	80	7.4	37	6	48
9	Tris	100	7.4	37	6	33
10	Tris	200	7.4	37	6	24
11	Tris	10	7.8	37	6	94
12	Tris	10	8.2	37	6	94
13	Tris	10	8.6	37	6	95
14	Tris	10	9.0	37	6	93
15	Tris	10	7.0	37	6	93
16	PB	10	7.4	rt	6	17
17	PBS	10	7.4	rt	6	13
18	DPBS	10	7.4	rt	6	19
19	HEPES	10	7.4	rt	6	28
20	water	10	-	37	6	90

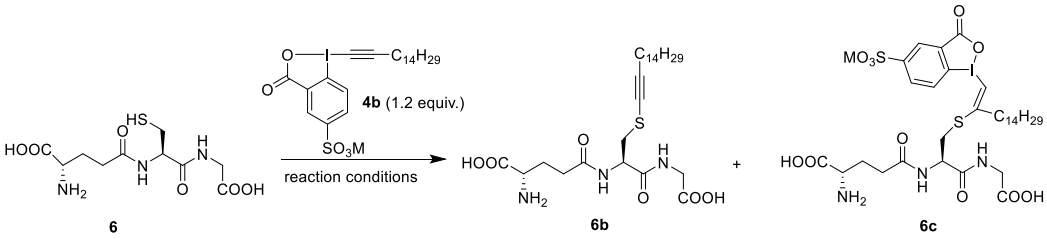
[a] Labeling condition: 16.0 µmol scale in 1.6 mL of non-degassed buffer. Equivalent of **4a** calculated based on K salt. [b] Yields were determined by relative integration based on HPLC at 214 nm.

SUPPORTING INFORMATION

Experimental procedure for optimization of **4b**

A 1.5 mL vial was charged with glutathione (**6**, 0.50 mg, 1.6 µmol) and **4b** (1.1 mg, 1.9 µmol) in 163 µL 10 mM Tris pH 7.4 with small magnetic stirring bar, and the reaction mixture was stirred at rt. After 2 h, a 25 µL aliquot of the reaction mixture was diluted with 25 µL acetonitrile to make a clear solution. Then the solution was submitted to HPLC. According to HPLC-MS chromatogram, 25% alkynylated product (**6b**), 7% VBX (**6c**) and 50% disulfide were observed (Table S2, entry 1). There was a large difference in integral ration observed in HPLC-UV and HPLC-MS chromatograms due to difference in absorption of products **6b** and **6c**, therefore the yields reported in the table S2 and manuscript are based on HPLC-MS chromatogram, which is more accurate. A similar experimental procedure was followed for the other entries of Table S2, entries 2-12.

Table S2. Reaction optimization of glutathione with **4b**



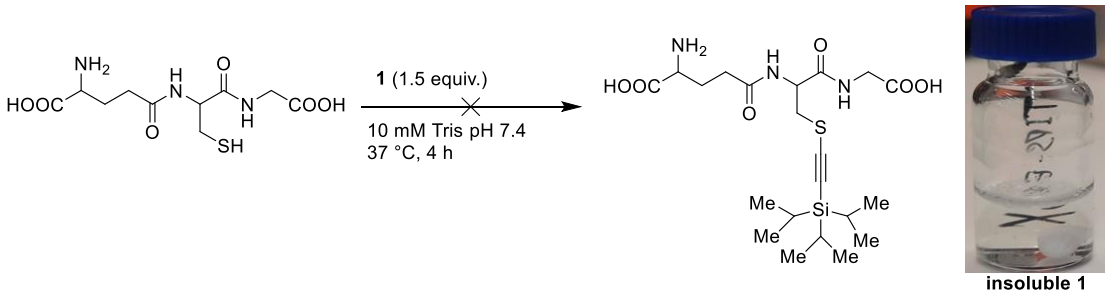
Entry	Reaction conditions	Yield %[a]		
		6b	6c	disulfide
1	10 mM Tris pH 7.4, rt, 2h	25[b]	7[b]	50
2	100 mM Tris, pH 8.0, rt, 2h	45 (8)	41 (92)	14
3	100 mM Tris, pH 8.0, 37, 2h	35 (7)	49 (93)	16
4	200 mM Tris, pH 8.0, rt, 2h	46 (9) [b]	39 (91)	4
5	200 mM Tris pH 8.0, rt, 22h	25 (6) [b]	35 (94)	5
6	200 mM Tris, pH 8.0, 37, 22h	34 (7) [b]	28 (88)	4
7	100 mM PB, pH 8.0, rt, 2h	32 (5)	60 (95)	8
8	100 mM PB, pH 8.0, 37, 2h	21 (2)	46 (98)	33
9	100 mM PB, pH 6.9, rt, 2h	22 (2)	56 (57)	22
10	100 mM PB pH 6.9, 37, 2h	14 (8)	49 (92)	37
11	100 mM HEPES, pH 8.0, rt, 2h	32 (3)	47 (97)	17
12	Water, rt, 2h	30[b]	5[b]	49

[a] Relative ratio of alkynylated (**6b**), VBX (**6c**) and disulfide based on HPLC-MS chromatogram. Equivalent of **4b** calculated based on K salt. Yields in parentheses refers to relative ratio of **6b** and **6c** based on HPLC-UV chromatogram. [b] By-product observed.

SUPPORTING INFORMATION

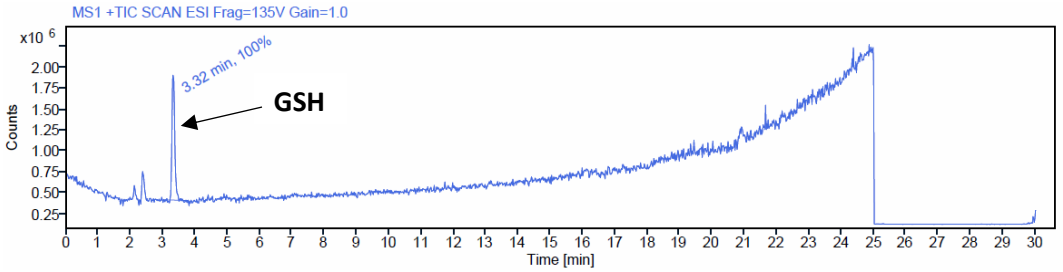
Comparison of reactivity between **1**, **4a** and **4b** with glutathione (**6**) in buffer

Reaction of TIPS-EBX (1) and glutathione (6). A 1.5 mL vial was charged with glutathione (**6**, 1.0 mg, 3.2 µmol) and **1** (2.3 mg, 5.3 µmol) in 326 µL 10 mM Tris pH 7.4 with small magnetic stirring bar, and the reaction mixture was stirred at 37 °C for 5 h. After 5 h, a 20 µL aliquot of the reaction mixture was diluted with 30 µL acetonitrile to make a clear solution. Then the solution was submitted to HPLC.

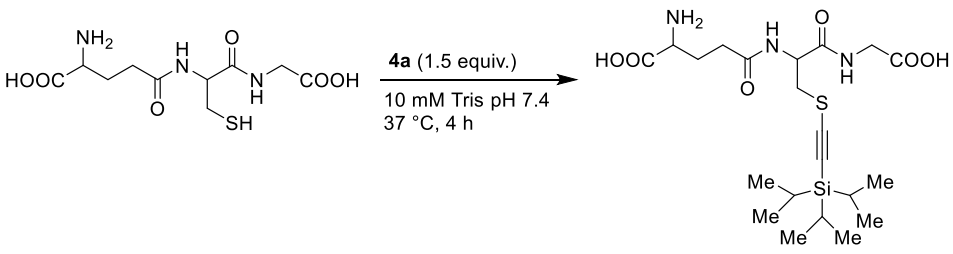


insoluble 1

HPLC-MS chromatogram of the reaction mixture

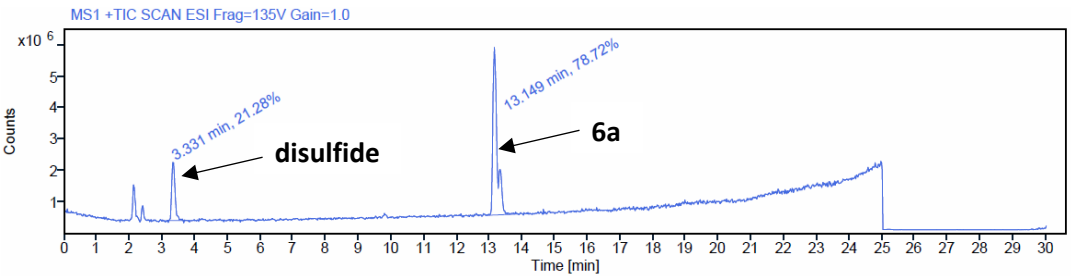


Reaction of 4a and glutathione (6). The same procedure was followed as for the reaction of TIPS-EBX (**1**) and glutathione (**6**).



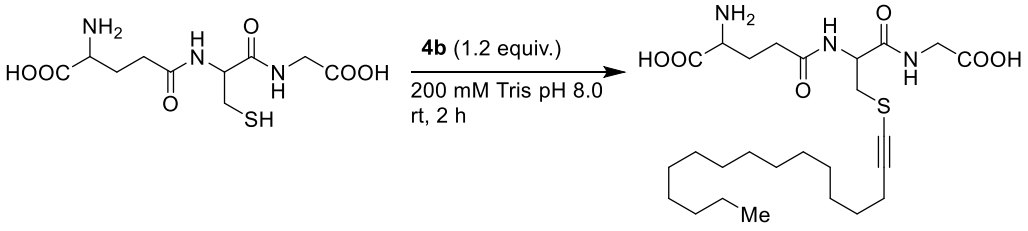
clear reaction mixture

HPLC-MS chromatogram of the reaction mixture



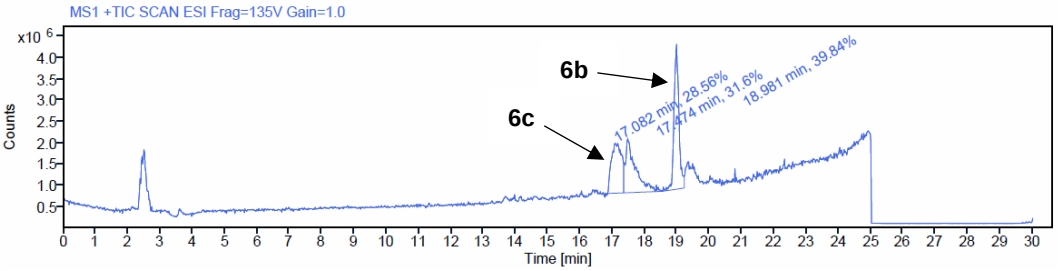
SUPPORTING INFORMATION

Reaction of 4b and glutathione (6). The same procedure was followed as for the reaction of TIPS-EBX (**1**) and glutathione (**6**).



clear reaction mixture

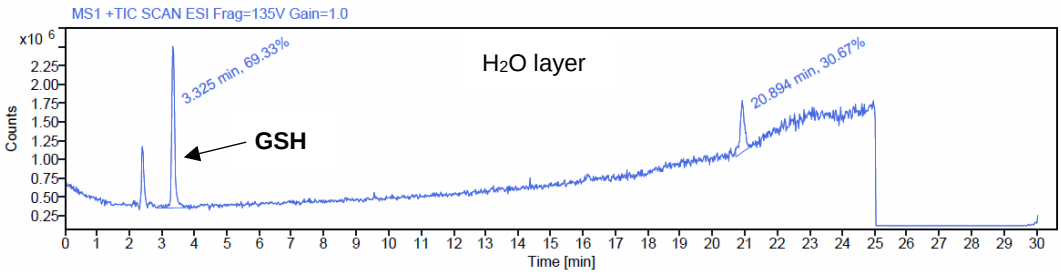
HPLC-MS chromatogram of the reaction mixture

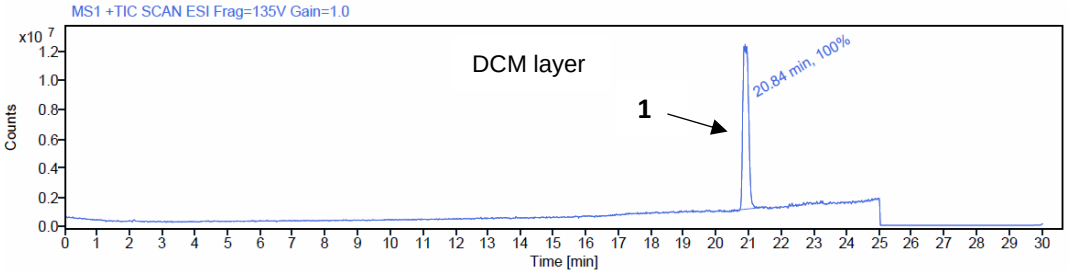


Reaction of **1** and **6** in biphasic solvents (buffer :DCM)

The same procedure was followed as for the reaction of TIPS-EBX (**1**) and glutathione (**6**), but using a 1:1 buffer/DCM mixture instead of pure buffer.

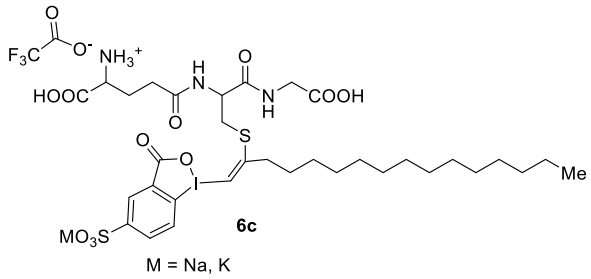
HPLC-MS chromatogram of reaction mixture of water and DCM layer





Preparation and isolation of **6c**

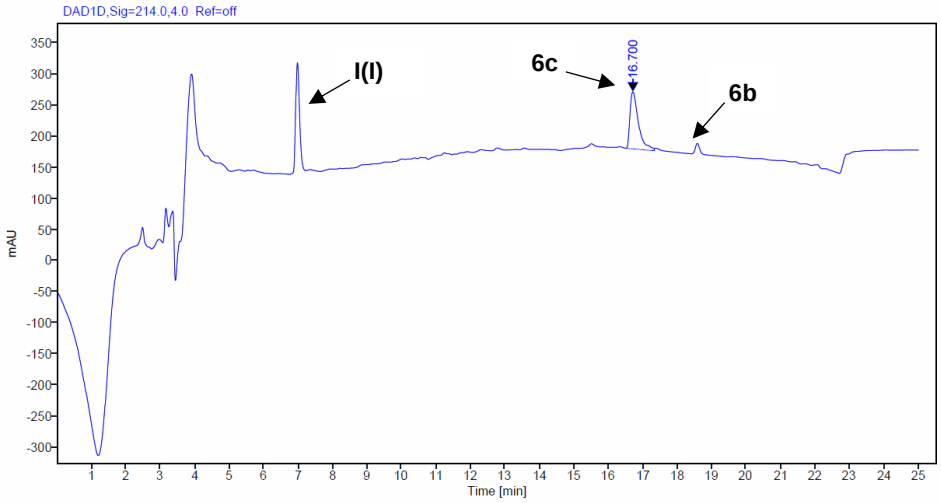
A 5 mL vial charged with glutathione (20.0 mg, 65.1 µmol) and **4b** (45.9 mg, 78.0 µmol) dissolved in 3 mL of 100 mM PB pH 8.0. The phosphate buffer was used without degassing. The reaction mixture was stirred at room temperature for two hours. After two hours a 20 µL aliquot of the reaction mixture was diluted 10 times with 50% CH_3CN: H_2O mixture to make a clear solution. The progress of the reaction was monitored by reverse phase HPLC-MS. HPLC-MS revealed a 32:60 ratio of **6b** and **6c** respectively. HPLC gradient Method 5 was used to obtained 36% (23.1 mg, 23.4 mmol) pure white solid of **6c** by preparative reverse phase HPLC. Calibrated yield 32% (retention time = 15.9).

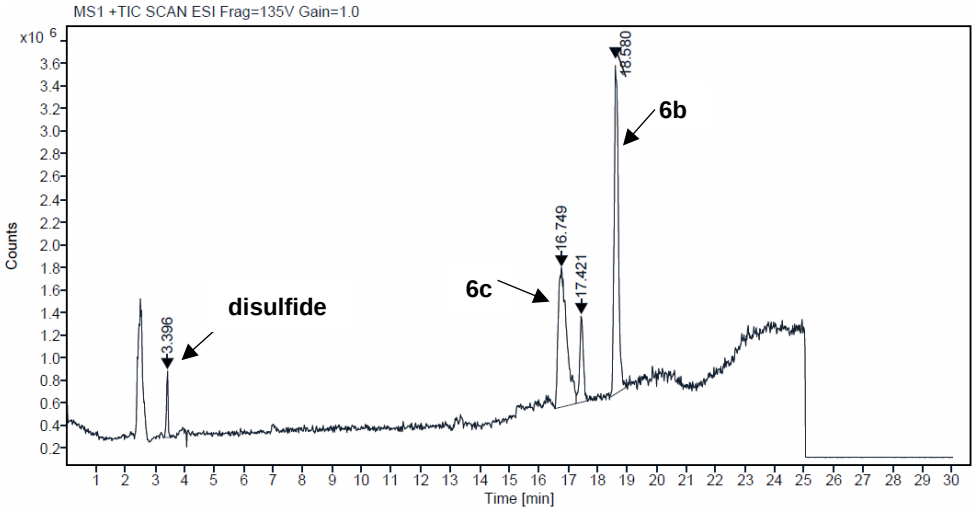


6c

M = Na, K

Analytical HPLC-UV and HPLC-MS chromatogram of reaction mixture





^{1}H NMR (400 MHz, MeOD) δ 8.74 (d, *J* = 2.1 Hz, 1H, ArH), 8.13 (dd, *J* = 8.5, 2.2 Hz, 1H, ArH), 7.71 (d, *J* = 8.5 Hz, 1H, ArH), 7.04 (s, 1H, vinylic-CH), 4.49 (dd, *J* = 10.0, 3.8 Hz, 1H, CHN), 4.03 (t, *J* = 6.4 Hz, 1H, NH), 3.93 (d, *J* = 3.4 Hz, 2H, CH_2N), 3.63 – 3.49 (m, 1H, CHN), 3.12 – 2.82 (m, 3H, CH_2 and NH), 2.51 – 2.19 (m, 2H, CH_2), 2.08 – 2.01 (m, 2H, CH_2), 1.83 – 1.72 (m, 2H, CH_2), 1.35 (d, *J* = 12.1 Hz, 24H, 12×CH_2), 0.93 (t, *J* = 6.4 Hz, 3H, CH_3).

^{19}F NMR (376 MHz, MeOD) δ -77.2.

^{13}C NMR (101 MHz, MeOD) δ 172.7 (C=O), 171.2 (C=O), 170.8 (C=O), 170.0 (C=O), 168.1 (C=O), 164.2 (ArC), 148.2 (ArC), 132.4 (ArC), 131.5 (ArC), 129.6 (ArC), 128.0 (ArC), 127.9 (ArC), 113.9 (C=C), 98.2 (vinylic-CH), 53.0 (CH), 51.9 (CH), 40.3 (CH_2), 36.3 (CH_2), 33.2 (CH_2), 31.6 (CH_2), 30.4 (CH_2), 29.4 (3×CH_2), 29.3 (3×CH_2), 29.1 (CH_2) 29.0 (CH_2), 28.7 (CH_2), 28.6 (CH_2), 25.3 (CH_2), 22.3 (CH_2), 13.0 (CH_3).

HRMS (ESI/QTOF) m/z: $[M+H]^+$ Calcd for $C_{33}H_{51}IN_3O_{11}S_2^+$ 856.2009; Found 856.2023.

Calibration **6c**

Calibration of **6a** was achieved through the preparation of several samples of different concentrations and their analysis on RP HPLC. In order to obtain average curve each analysis was performed 3 times. The following linear regression was obtained: y = 1204x + 9.8942, and R = 0.9967, where axis X is the concentration in millimolar (mM) of **6c** and Y the absorbance area of the peak at 214 nm.

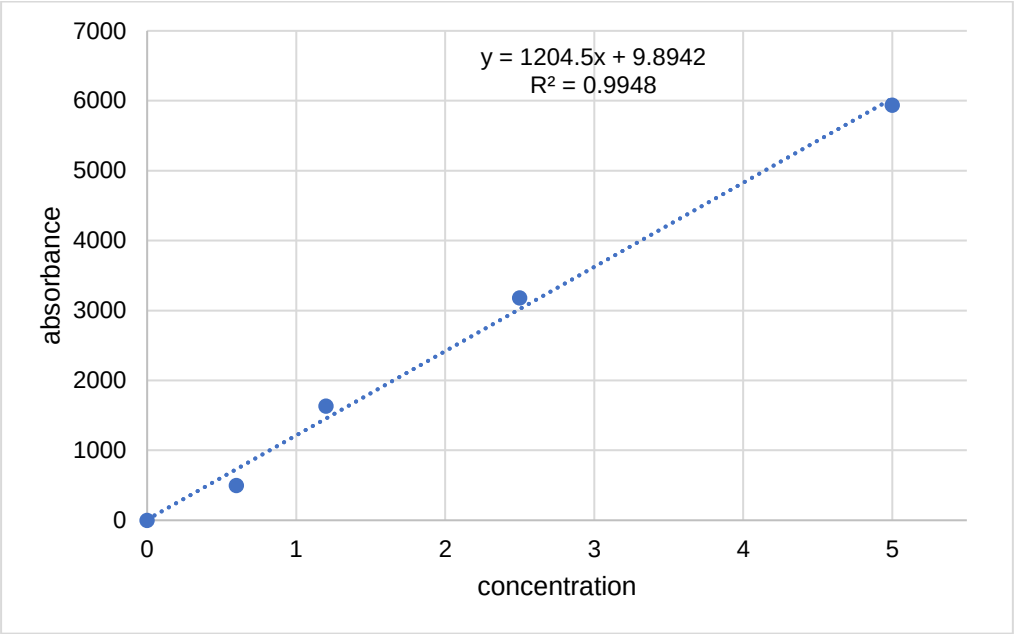
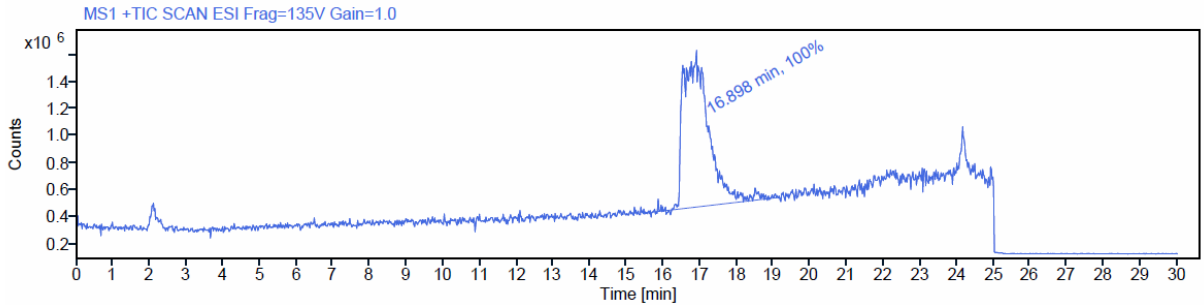


Figure S1: Calibration curve of **6c**.

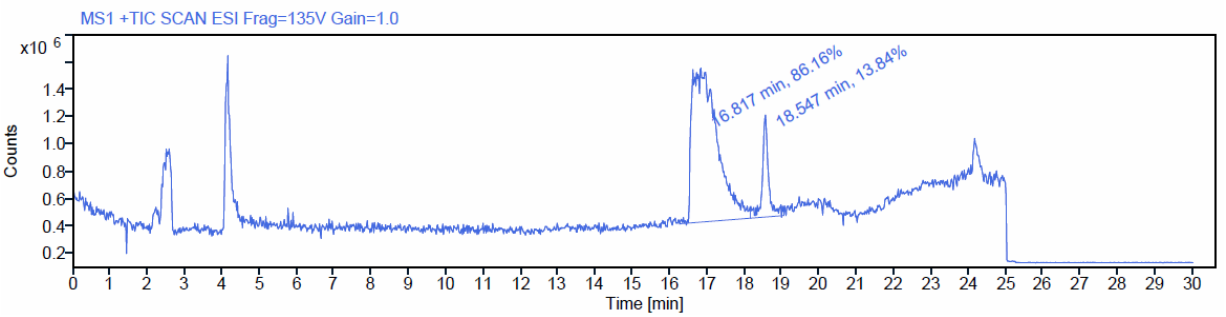
Stability of 6c at 37 °C. Isolated **6c** was dissolved in 0.1M Tris buffer pH 8.0 and stirred for 20 h at 37 °C. After 20 h HPLC-MS reveled the 14% **6b**. It means that **6c** converted in to **6b**, but rate of was very slow.

HPLC gradient: Method 1.

HPLC-MS chromatogram of isolated **6c**



HPLC-MS chromatogram of **6c** after stirring at 37 °C for 20 h



Yield calculation for final products obtained from homocysteine, cysteine, and peptides

The peak areas for all-relevant peptide-containing species on the chromatogram were integrated and the yield was determined using a slightly modified equation introduced by Li *et al.*:[3] yield % = *I* product/(*I*starting + *I*product + *I*oxidation + *I*side product), where *I*starting, *I*product, *I*oxidation and *I*side product respectively represent the average ion counts of the remaining starting material, product, oxidized starting material and side product, if any.

General reaction procedure for alkynylation with **4a**

A 1.5 mL vial was charged with thiols (1.0 equiv., concentration maintained 10 mM) and **4a** (1.5 equiv.) in 10 mM Tris pH 7.4 with small magnetic stirring bar. The reaction mixture was stirred at 37 °C for 6 h. The buffer was used directly from freshly prepared solution without degassing. After 6 h, an aliquot of 25 µL of the reaction mixture was diluted with 25 µL acetonitrile to give a clear solution. The solution was submitted to HPLC. The yields were calculated based on HPLC-MS chromatogram, unless otherwise stated.

[3] N. Li, R. Lim, S. Edwardraja, Q. Lin, *J. Am. Chem. Soc.* **2011**, *133*, 15316.

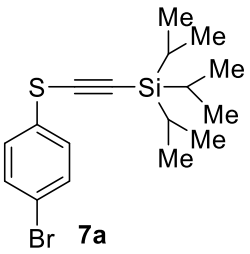
General reaction procedure for alkynylation with **4b**

A 1.5 mL vial was charged with thiols (1.0 equiv., concentration maintained 10 mM) and **4b** (1.2 equiv.) in 200 mM Tris pH 8.0 (unless otherwise stated) with small magnetic stirring bar. The reaction mixture was stirred at rt for 2 h. The buffer was used directly from freshly prepared solution without degassing. After 2 h, an aliquot of 25 µL of the reaction mixture was diluted with 25 µL acetonitrile to give a clear solution. The solution was submitted to HPLC. The reported yields were calculated based on HPLC-MS chromatogram.

6. Substrate scope for small molecules

Preparation of triisopropyl((4-bromophenyl)ethynyl)silane 7a. A 5 mL vial was charged with 4-bromobenzene thiol (10.0 mg, 52.8 µmol, 1.0 equiv.), **4a** (43.5 mg, 79.3 µmol, 1.5 equiv.) and a stirring bar. Tris buffer (10 mM, pH 7.4, 3 mL) was then added and the resulting mixture stirred vigorously over 6 h at 37 °C. After 6 h, the reaction mixture was then diluted with 10 mL DCM and the organic layer was collected using a separating funnel. The solvent was evaporated and the crude residue was purified by column chromatography on Biotage (Büchi flashpure cartridge 12 g, Pentane) to afford pure **7a**. A low melting pure **7a** was obtained in 72% (14.0 mg, 38.0 µmol) yield.

Triisopropyl((4-bromophenyl)ethynyl)silane, **7a**



7a

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.46 (d, *J* = 6.9 Hz, 2H, ArH), 7.31 (d, *J* = 8.5 Hz, 2H, ArH), 1.20 – 1.00 (m, 21H, TIPS).

^{13}C NMR (101 MHz, $CDCl_3$) δ 132.1 (ArC), 127.5 (ArC), 120.0 (ArC), 104.1 (CC), 90.2 (CC), 18.6 (CH_3), 11.3 (CH).

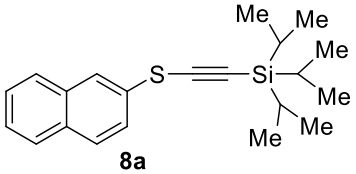
IR (ν_{max}, cm^{-1}) 2942 (s), 2926 (s), 2865 (s), 2094 (s), 1472 (s), 1388 (m), 1083 (s), 1070 (s), 1008 (s), 997 (m), 882 (s), 859 (s), 809 (s), 743 (s), 679 (s), 660 (s), 605 (s).

HRMS (APCI/QTOF) m/z: $[M]^+$ Calcd for $C_{17}H_{25}BrSSi^+$ 368.0624; Found 368.0499, 370.0487 $[M + 2]^+$.

Preparation of triisopropyl((naphthalen-2-ylthio)ethynyl)silane, 8a. A 5 mL vial was charged with naphthalene-2-thiol (10.0 mg, 62.4 µmol, 1.0 equiv.), **4a** (51.3 mg, 93.6 µmol, 1.5 equiv.) and as stirring bar. Tris buffer (10 mM, pH 7.4, 3 mL) was then added and the resulting mixture was stirred vigorously at 37 °C for 6 h. The buffer was used directly from freshly prepared bottle without degassing. Although the reaction solution was not clear due to the hydrophobic nature of naphthalene-2-thiol, no effect was observed on the rate of the reaction. After 6 h, the reaction mixture was diluted with 10 mL DCM and the organic layer was extracted using separating funnel. The organic layer dried over Na_2SO_4.The solvent was evaporated and the crude residue was purified by column chromatography on Biotage (Büchi flashpure cartridge 12 g, Pentane) to afford pure **8a** as low melting solid, yield 68% (14.5 mg, 42.5 µmol).

SUPPORTING INFORMATION

Triisopropyl((naphthalen-2-ylthio)ethynyl)silane, **8a**



8a

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^{1}H NMR (400 MHz, $CDCl_3$) δ 7.96 (d, *J* = 2.3 Hz, 1H, ArH), 7.81 (d, *J* = 8.8 Hz, 2H, ArH), 7.73 (d, *J* = 8.0 Hz, 1H, ArH), 7.52 – 7.42 (m, 3H, ArH), 1.21 – 1.10 (m, 21H, TIPS).

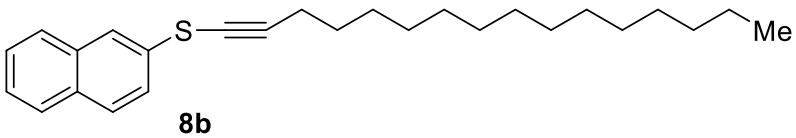
^{13}C NMR (101 MHz, $CDCl_3$) δ 133.8 (ArC), 131.9 (ArC), 130.1 (ArC), 128.8 (ArC), 127.8 (ArC), 127.0 (ArC), 126.8 (ArC), 125.8 (ArC), 124.3 (ArC), 123.9 (ArC), 103.6 (CC), 91.1 (CC), 18.7 (CH_3), 11.4 (CH).

IR (ν_{max}, cm^{-1}) 3056 (s), 2943 (s), 2865 (s), 2092 (s), 1626 (s), 1591 (s), 1503 (s), 1462 (s), 1071 (s), 996 (s), 882 (s), 859 (s), 851 (s), 742 (s), 680 (s), 661 (s), 604 (s).

HRMS $(APPI)^+$ m/z: $[M]^+$ Calcd for $C_{21}H_{28}SSi^+$ 340.1681; Found 340.1673.

Preparation of hexadec-1-yn-1-yl(naphthalen-2-yl)sulfane, 8b. A 5 mL vial was charged with naphthalene-2-thiol, **8** (10.0 mg, 62.4 µmol, 1.0 equiv.), **4b** (44.1 mg, 74.8 µmol, 1.2 equiv.) and magnetic bar. Then 3 mL 100 mM PB buffer pH 8.0 was then added and reaction mixture was stirred vigorously at rt for 2 h. The buffer was used directly from freshly prepared bottle without degassing. Although the reaction solution was not clear due to the hydrophobic nature of naphthalene-2-thiol, no effect was observed on the rate of the reaction. After 6 h, the reaction mixture was diluted with 20 mL diethyl ether and the organic layer was extracted using separating funnel. Then organic layer dried over Na_2SO_4, and diethyl ether evaporated in rotary evaporator under reduced pressure. The crude residue was purified by column chromatography on Biotage (Büchi flashpure cartridge 12 g, Pentane) to afford pure **8b** as colorless liquid, yield 29% (7.0 mg, 18 µmol).

Hexadec-1-yn-1-yl(naphthalen-2-yl)sulfane, **8b**



8b

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.87 (brs, 1H, ArH), 7.83 – 7.71 (m, 3H, ArH), 7.53 – 7.37 (m, 3H, ArH), 2.50 (t, *J* = 6.3 Hz, 2H, CH_2), 1.70 – 1.60 (m, 2H, CH_2), 1.54 – 1.20 (m, 22H, 11×CH_2), 0.88 (t, *J* = 7.4, 3H, CH_3).

^{13}C NMR (101 MHz, $CDCl_3$) δ 133.7 (ArC), 131.8 (ArC), 131.20 (ArC), 128.6 (ArC), 127.7 (ArC), 127.0 (ArC), 126.7 (ArC), 125.6 (ArC), 123.9 (ArC), 123.8 (ArC), 100.3 (CC), 64.5 (CC), 31.9 (CH_2), 29.6 (5×CH_2), 29.5 (CH_2), 29.3 (CH_2), 29.1 (CH_2), 28.9 (CH_2), 28.6 (CH_2), 22.6 (CH_2), 20.3 (CH_2), 14.1 (CH_3).

HRMS (APCI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{26}H_{37}S^+$ 381.2610; Found 381.2613.

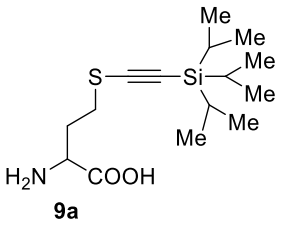
IR (ν_{max}, cm^{-1}) 3154 (m), 3058 (m), 2927 (m), 2859 (m), 2253 (m), 1793 (m), 1458 (m), 1380 (m), 903 (s), 722 (s).

Reaction procedure for alkynylation of homocysteine **9** using **4a**

SUPPORTING INFORMATION

A 1.5 mL vial was charged with homocysteine (**9**, 0.50 mg, 3.9 µmol, 1.0 equiv.) and **4a** (3.0 mg, 5.5 µmol, 1.5 equiv.) in 726 µL 10 mM Tris pH 7.4 with small magnetic stirring bar. The reaction mixture was stirred at 37 °C for 6 h. After 2 h, an aliquot of 25 µL of the reaction mixture was diluted with 25 µL acetonitrile to give a clear solution. The solution was submitted to HPLC. According to HPLC-MS chromatogram 95% alkynylated product (**9a**) and 5% disulfide were obtained.

S-((triisopropylsilyl)ethynyl)homocysteine, **9a**

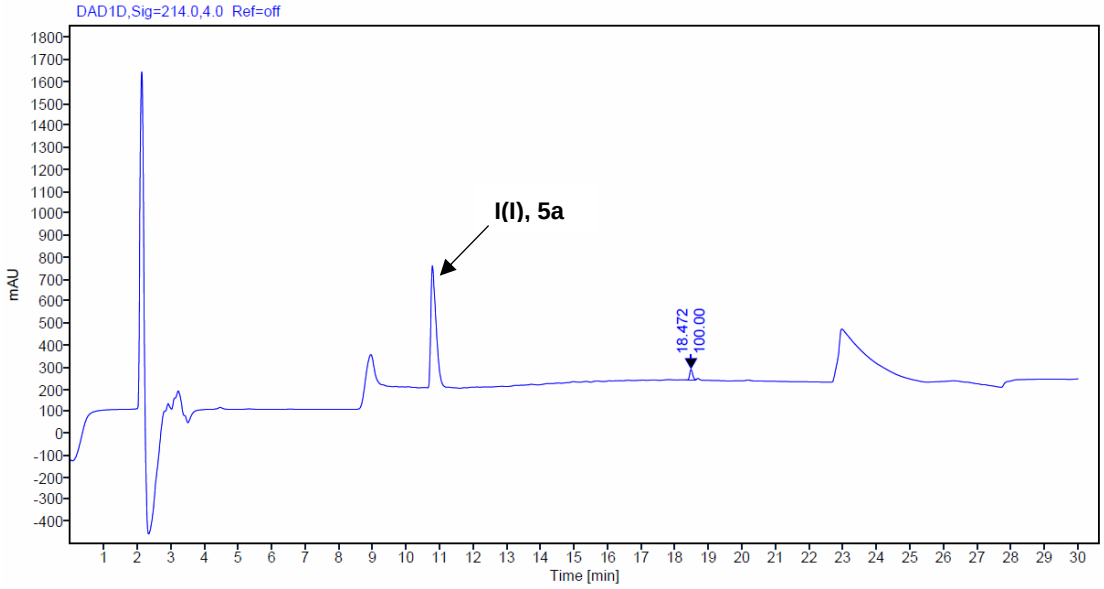


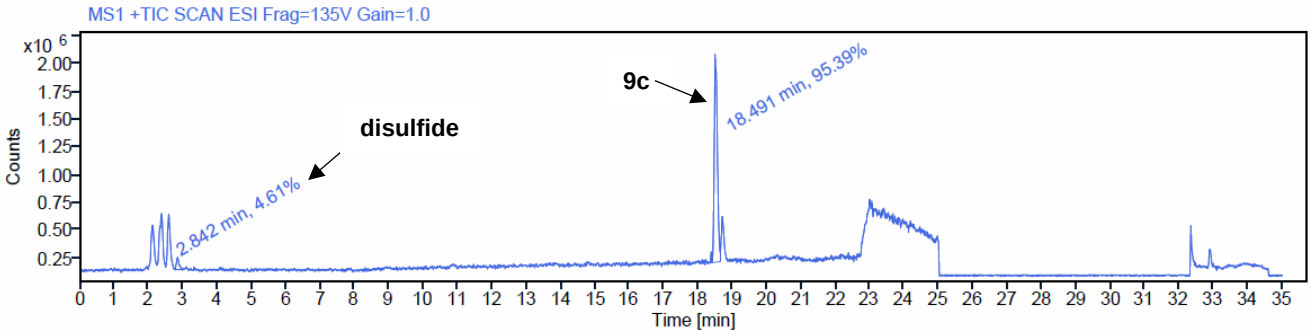
9a

HRMS (ESI/QTOF) m/z: $[M+H]^+$ Calcd for $C_{15}H_{30}NO_2SSi^+$ 316.1761; Found 316.1753.

HPLC gradient: Method 2.

HPLC-UV and HPLC-MS chromatogram



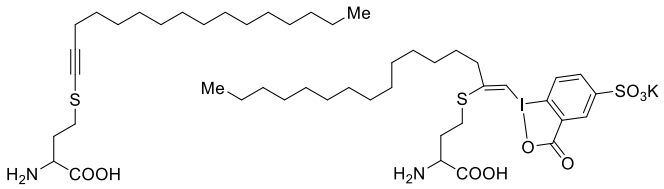


Reaction procedure for alkynylation of homocysteine **9** using **4b**

SUPPORTING INFORMATION

A 1.5 mL vial was charged with homocysteine (**9**, 0.50 mg, 3.9 µmol, 1.0 equiv.) and **4b** (2.6 mg, 4.4 µmol, 1.2 equiv.) in 726 µL 200 mM Tris pH 8.0 with small magnetic stirring bar. The reaction mixture was stirred at rt for 2 h. After 2 h, an aliquot of 25 µL of the reaction mixture was diluted with 25 µL acetonitrile to give a clear solution. The solution was submitted to HPLC. According to HPLC-MS chromatogram 66% alkynylated product **9b** (retention time = 19.0) and 34% VBX **9c** (retention time = 16.1 were obtained.

S-(hexadec-1-yn-1-yl)homocysteine, **9b** and VBX of homocysteine, **9c**



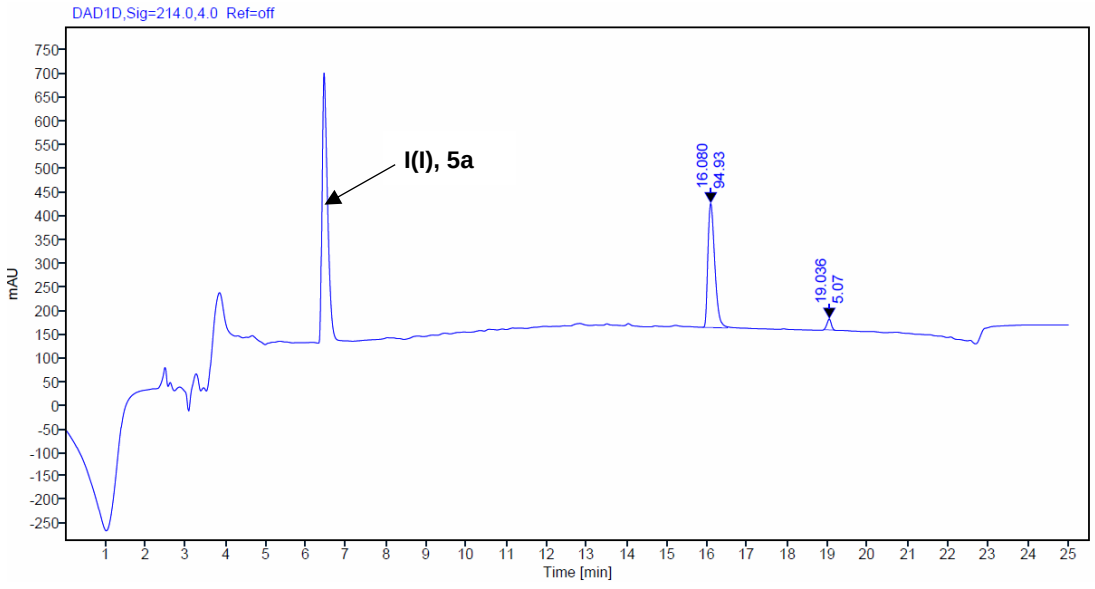
9b **9c**

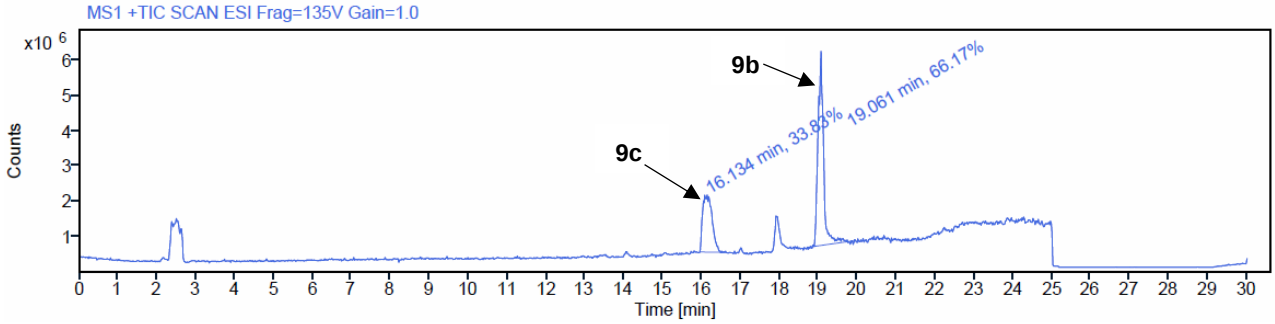
HPLC gradient: Method 1.

HRMS (ESI/QTOF) m/z for **9b**: $[M + H]^+$ Calcd for **9b** $C_{20}H_{38}NO_2S^+$ 356.2618; Found 356.2622.

HRMS (ESI/QTOF) m/z: $[M]^-$ Calcd for **9c** $C_{27}H_{41}INO_7S_2^-$ 682.1375; Found 682.1370.

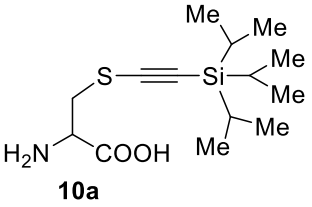
HPLC-UV and HPLC-MS chromatogram





SUPPORTING INFORMATION

S-((triisopropylsilyl)ethynyl)cysteine, **10a**



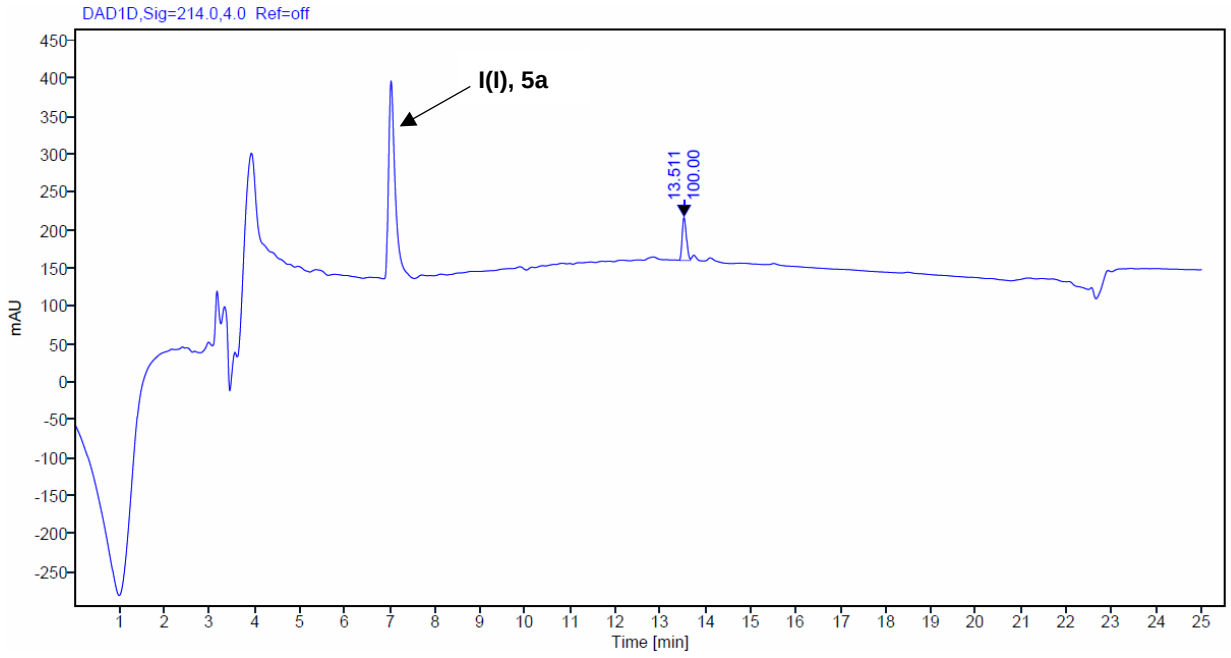
10a

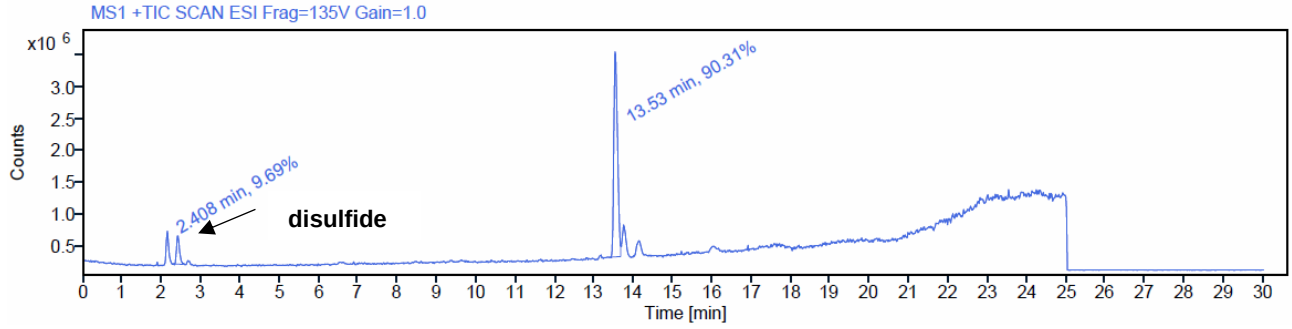
cysteine **10** (0.20 mg, 1.6 µmol) and **4a** (1.3 mg, 2.6 µmol) in 165 µL 10 mM Tris pH 7.4. Adopted General reaction procedure for **4a**. Yield 90% (retention time = 13.53)

HRMS (ESI/QTOF) m/z: $[M+H]^+$ Calcd for $C_{14}H_{28}NO_2SSi^+$ 302.1605; Found 302.1597.

HPLC gradient: Method 1.

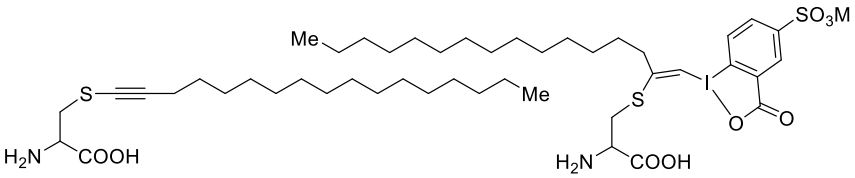
HPLC-UV and HPLC-MS chromatogram





SUPPORTING INFORMATION

S-(heptadec-1-yn-1-yl)cysteine, **10b**, VBX of cysteine, **10c**



10b **10c**

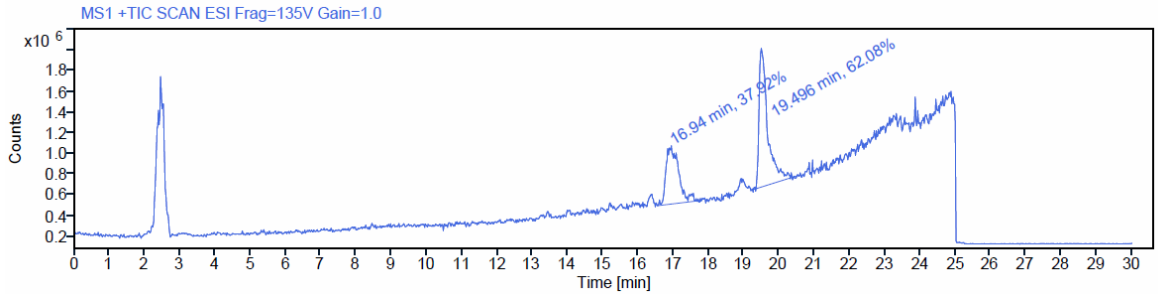
Cysteine **10** (0.20 mg, 1.6 µmol) and **4b** (1.2 mg, 1.9 µmol) in 165 µL 10 mM Tris pH 7.4. Adapted General reaction procedure. After 2 h; Yield for **10b** 62% (retention time = 19.4) and **10c,** 38% (retention time = 16.9).

HRMS (ESI/QTOF) m/: $[M + H]^+$ Calcd for **10b**, $C_{19}H_{36}NO_2S^+$ 342.2461; Found 342.2478.

HRMS (ESI/QTOF) m/z: $[M]^-$ Calcd for **10c** $C_{26}H_{39}INO_7S_2^-$ 668.1218; Found 668.1210.

HPLC-MS chromatogram 2 h

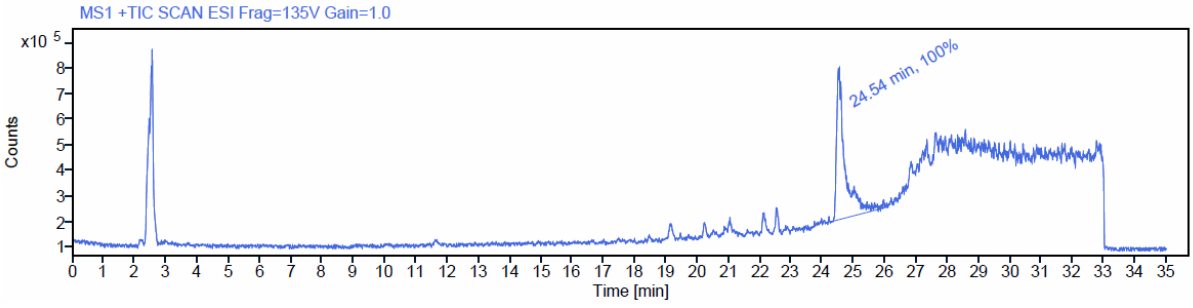
HPLC gradient: Method 1



HPLC-MS chromatogram 6 h

HPLC gradient: Method 2.

After 6 h; quantitative yield observed for **10b** (retention time = 24.5).

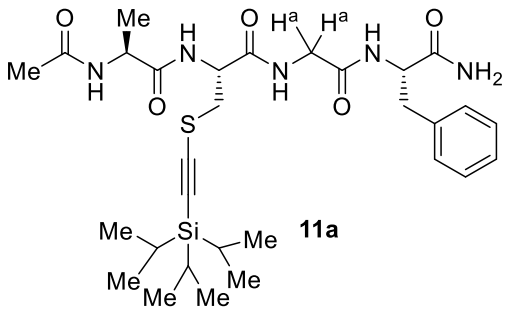


7. Substrate scope for tetra and hexapeptides

Procedure for preparation of **11a**

A 5 mL vial was charged with **11** (15.0 mg, 34.2 µmol, 1.0 equiv.) and **4a** (28.2 mg, 51.4 µmol, 1.5 equiv.) and magnetic bar. Then in 3.3 mL of Tris buffer (10 mM, pH 7.4) added, followed by reaction mixture was stirred at 37 °C for 10 h. No effort was made to exclude oxygen. The reaction mixture was diluted was directly submitted to HPLC-MS as it was clear solution. On the basis of HPLC-MS 82% (retention time = 13.7) yield was observed for **11a**. Pure **11a** was isolated by prep RP-HPLC using Method 6. The pure fraction was collected and lyophilized for two days. A white solid obtained with 36% yield (7.5 mg, 12 µmol, retention time = 14.5 min), 79% calibrated yield.

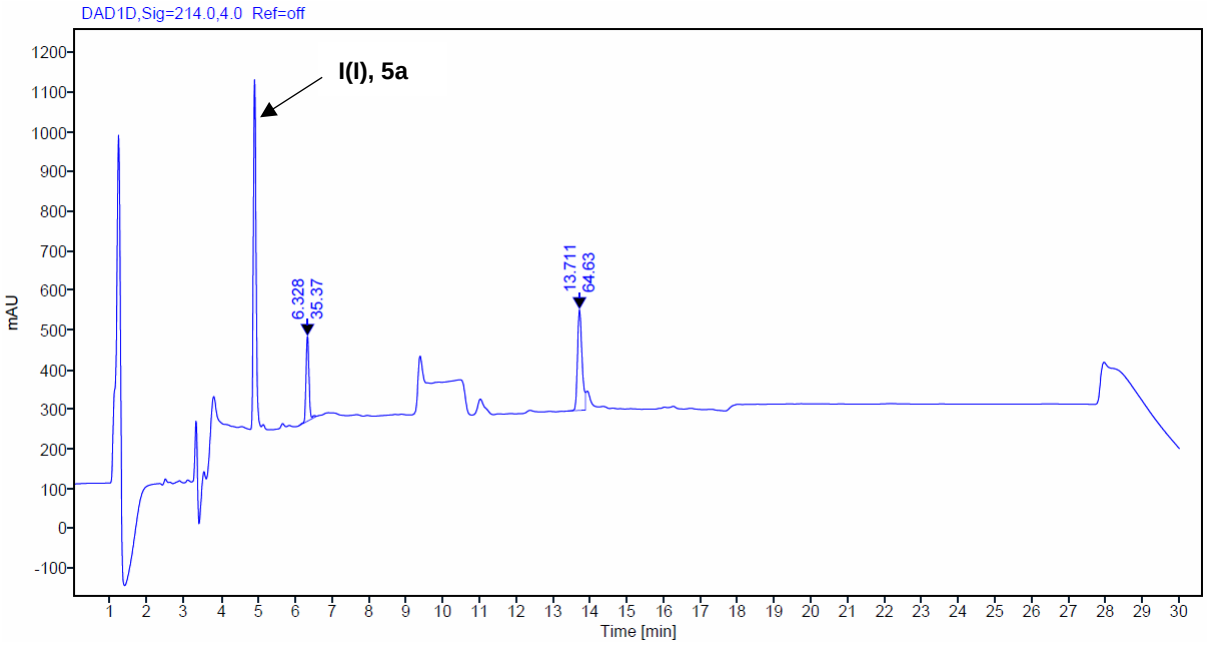
Ac-Cys-Gly-Phe-NH_2 (**11a**)

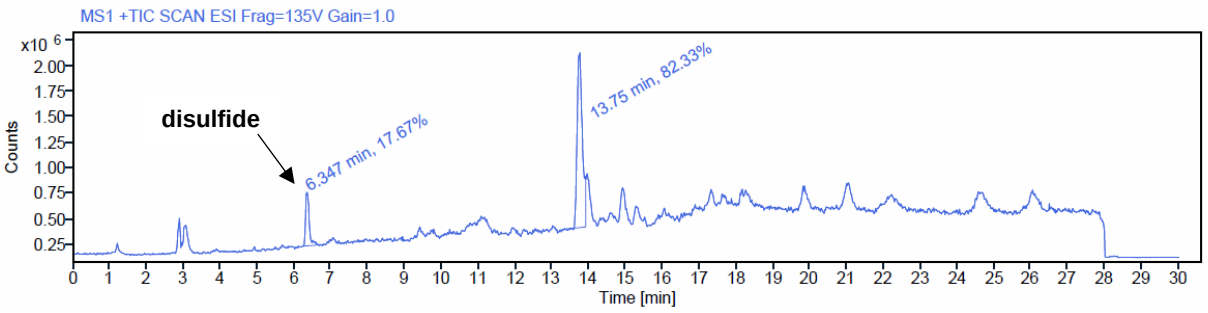


11a

HPLC Gradient: Method 3 (analytical HPLC), Method 6 (preparative HPLC).

HPLC-UV and HPLC-MS chromatogram of **11a**





^{1}H NMR (400 MHz, MeOD) δ 7.26 – 6.98 (m, 5H, ArH), 4.61 – 4.50 (m, 2H, CH_2), 4.30 – 4.15 (m, 1H, CH), 3.96 (d, *J* = 16.7 Hz, 1H, CH^a), 3.65 (d, *J* = 16.7 Hz, 1H, CH^a), 3.45 (dd, *J* = 13.3, 4.6 Hz, 1H, CH), 3.20 (dd, *J* = 14.0, 5.0 Hz, 1H, CH), 3.06 – 2.90 (m, 2H, CH_2), 1.89 (s, 3H, CH_3), 1.29 (d, *J* = 7.2 Hz, 3H, CH_3), 0.99 (d, *J* = 3.0 Hz, 21H, TIPS).

^{19}F NMR (376 MHz, MeOD) δ -77.7 (trace amount of CF_3COOH).

^{13}C NMR (101 MHz, MeOD) δ 175.4 (C=O), 175.3 (C=O), 173.7 (C=O), 171.9 (C=O), 170.7 (C=O), 137.9 (ArC), 129.6 (ArC), 128.8 (ArC), 127.0 (ArC), 98.2 (CC), 95.4 (CC), 55.4 (CH), 53.5 (CH), 50.8 (CH), 43.0 (CH_2), 38.0 (CH_2), 37.2 (CH_2), 21.9 (CH_3), 18.4 (CH_3), 16.9 (CH), 11.8 (CH_3).

HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{30}H_{48}N_5O_5SSi^+$ 618.3140; Found 618.3115.

HRMS (ESI/QTOF) m/z: $[M + Na]^+$ Calcd for $C_{30}H_{47}N_5NaO_5SSi^+$ 640.2959; Found 640.2961.

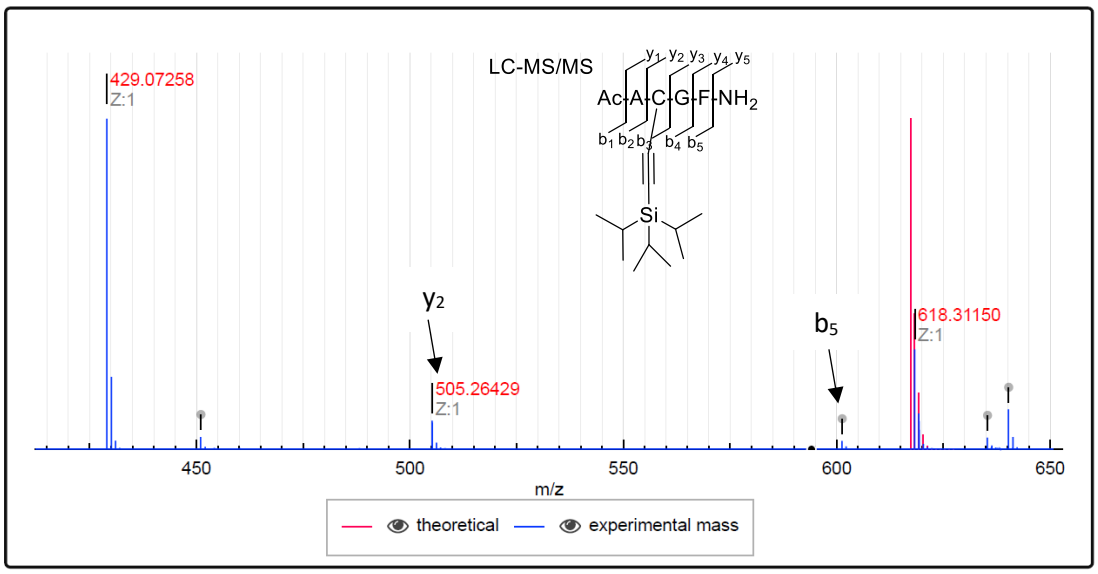


Figure S2. LC-MS/MS of **11a.**

SUPPORTING INFORMATION

Calibration curve of **11a**

Calibration with **11a** was achieved through the preparation of several samples of different concentrations and their analysis on RP HPLC. These analyses were repeated three times in order to obtain an average curve of calibration. The following linear regression was obtained: Y = 470.12 X + 284.02 and R = 0.9887, where Y is the absorption in $mAUs^{-1}$ at 214 nm and X the concentration of **11a** in mM.

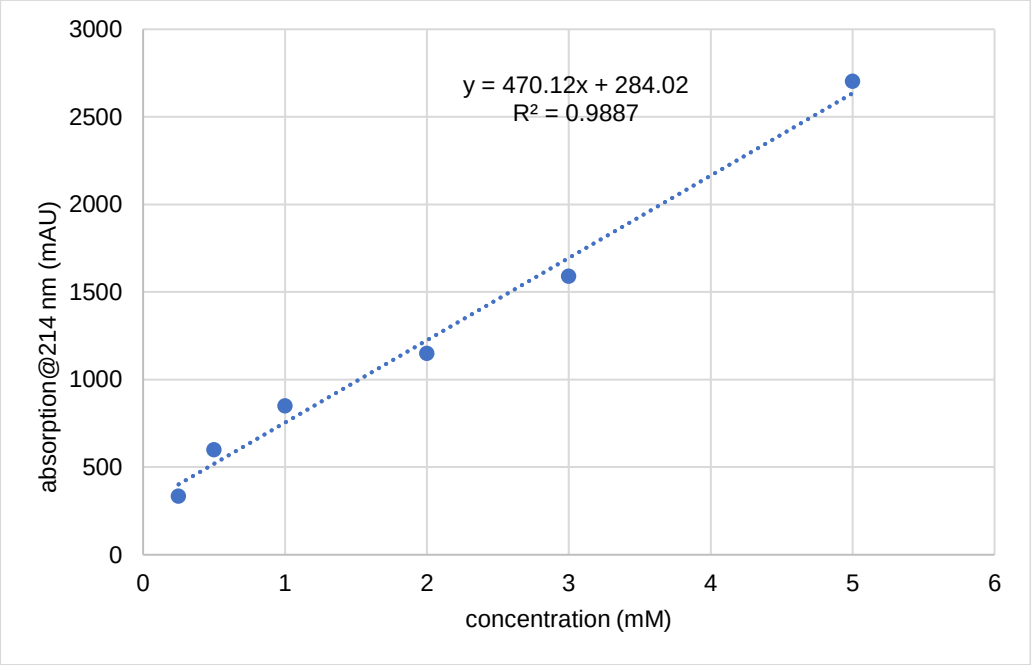


Figure S3. calibration curve of **11a.**

Calculation of Partition coefficient (LogP)[4]

2.5 mg (4.0 µmol) **11a** was dissolved in 1.7 mL octanol-H_2O mixture (0.85 mL 1-Octanol + 0.85 mL milli-Q H_2O) in a 5 mL vial. In order to mix the layers, the vial was shaken on vertex for 1 min. Then the mixture was transferred to a separating funnel. The separating funnel was closed and left standing for 3 h to separate the organic and the H_2O layer. Both layers were taken separately and submitted tonreverse phase HPLC. The concentration of both layers was determined by HPLC-UV (absorption 214 nm).

Amount of **11a** in 1-octanol = 2.41 mg/mL

Amount of **11a** in H_2O = 0.09 mg/mL

Partition coefficient (P) = amount of **11a** in 1-octanol/ amount of **11a** in H_2O

P = (2.41mg/mL)/(0.09mg/mL)

P = 26.78 or LogP = Log(26.78)

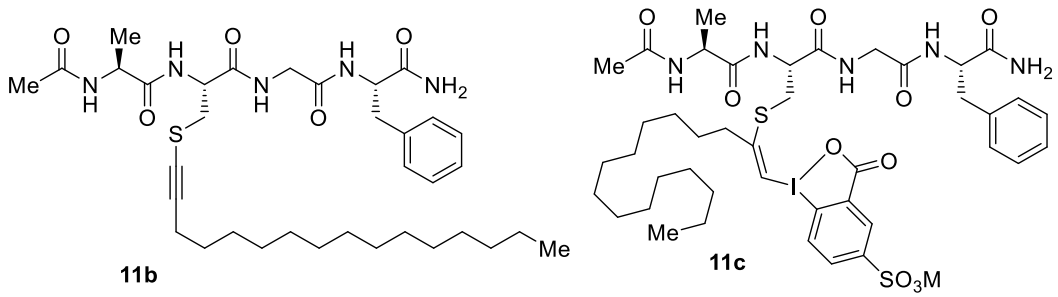
LogP = **1.43**

Following the same procedure for starting material **11**: LogP = -1.53

[4] J. R. Espinosa, C. R. Wand, C. Vega, E. Sanz, D. Frenkel, *J. Chem. Phys*. **2018**, *149*, 224501.

SUPPORTING INFORMATION

Alkynylated Ac-Ala-Cys-Gly-Phe-NH_2 (**11b**)



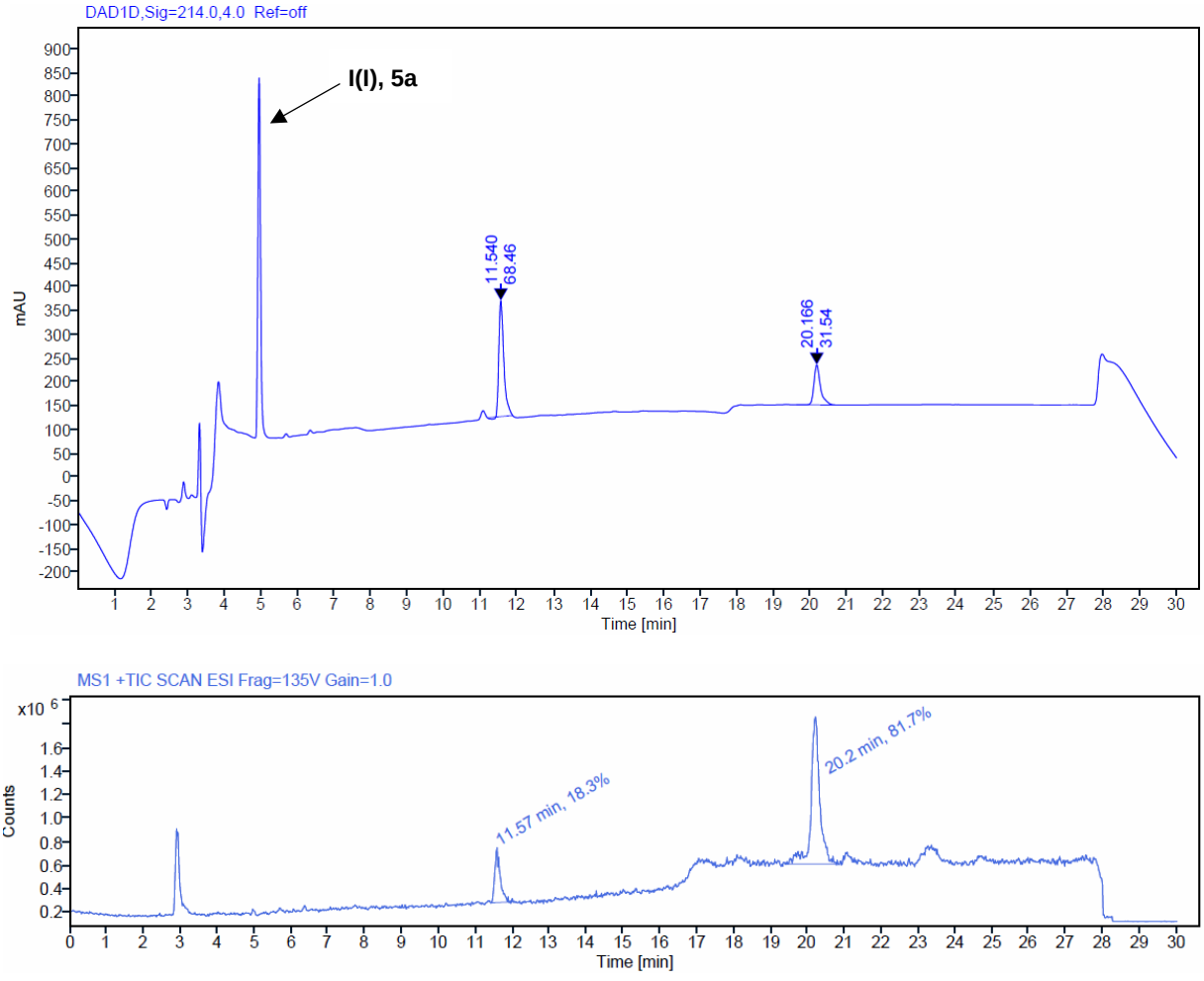
11 (1.0 mg, 2.3 µmol) and **4b** (1.6 mg, 2.7 µmol) in 115 µL 200 mM Tris pH 7.4. Adapted General reaction procedure for **4b**. Yield for **11b** 82% (retention time = 20.2), **11c** 18% (retention time = 11.54)

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for **11b** $C_{14}H_{28}NO_2SSi^+$ 302.1605; Found 302.1597.

HRMS (ESI/QTOF) m/z: $[M]^-$ Calcd for **11c** $C_{42}H_{59}IN_5O_{10}S_2^-$ 984.2754; Found 984.2731.

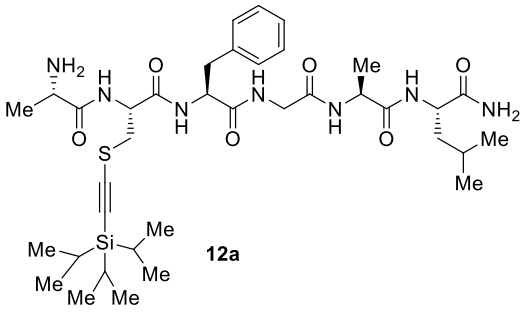
HPLC gradient: Method 3.

HPLC-UV and HPLC-MS chromatogram



SUPPORTING INFORMATION

Alkynylated H-Ala-Cys-Phe-Gly-Ala-Leu-NH_2 with (**12a**)



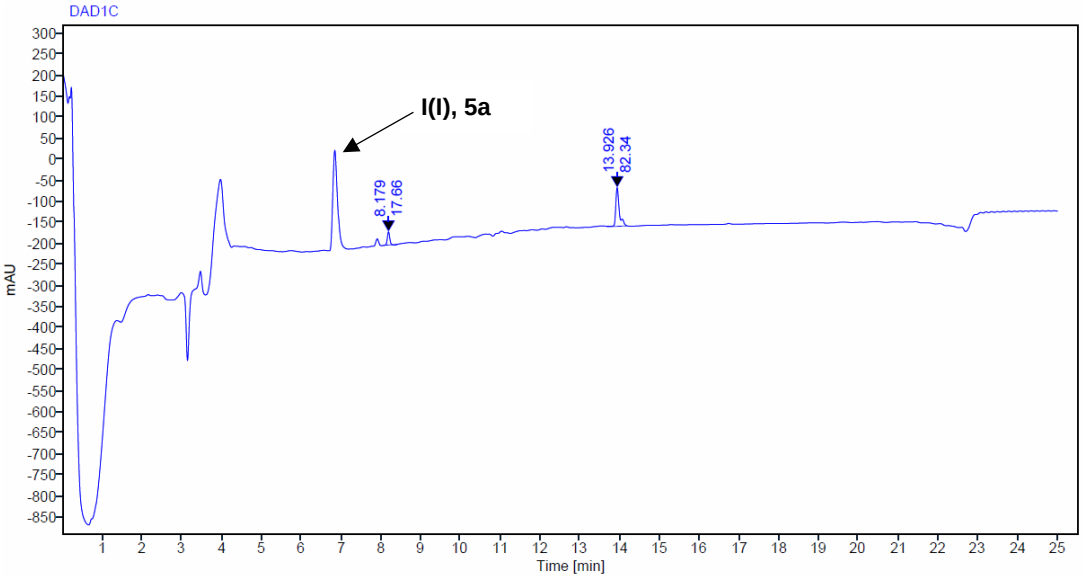
12a

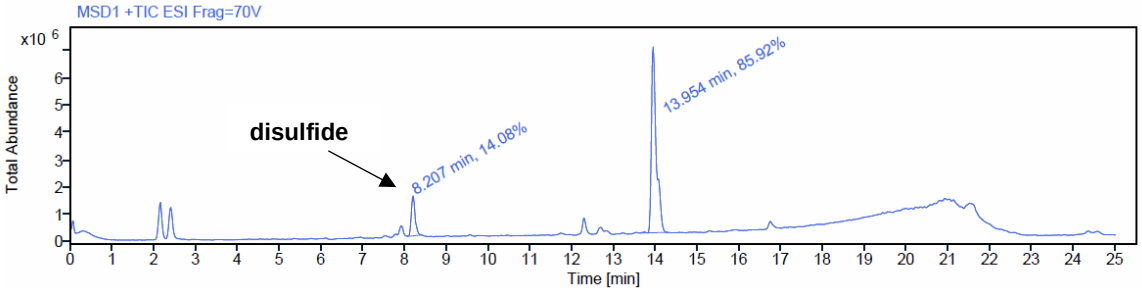
12 (0.50 mg, 0.86 µmol) and **4a** (0.70 mg, 1.3 µmol) in 86 µL 10 mM Tris pH 7.4. Adapted General reaction procedure for **4a**. Yield for **12a** 86% (retention time = 13.9)

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for **12a** $C_{37}H_{62}N_7O_6SSi^+$ 760.4246; Found 760.4253.

HPLC gradient: Method 1.

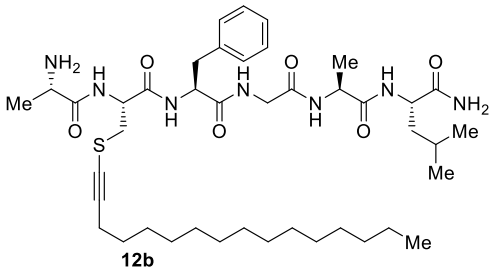
HPLC-UV and HPLC-MS chromatogram

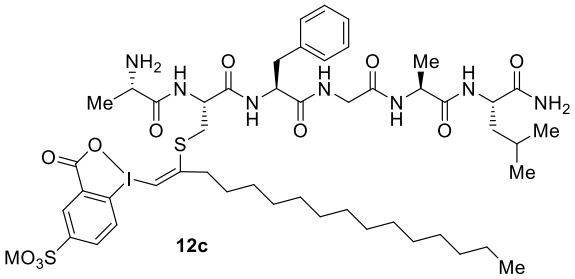




SUPPORTING INFORMATION

Alkynylated H-Ala-Cys-Phe-Gly-Ala-Leu-NH_2 with (**12b)** and corresponding VBX (**12c**)





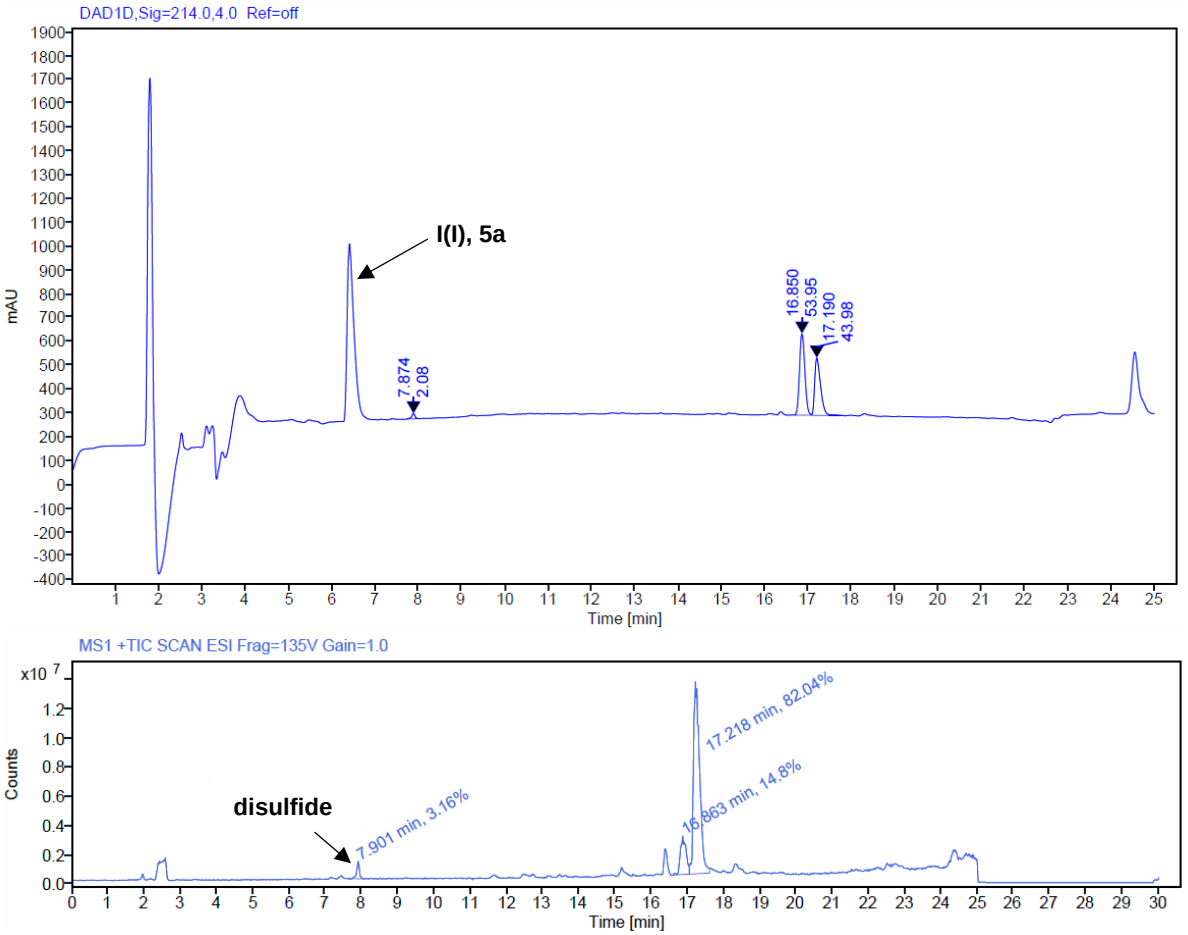
12 (5.0 mg, 8.0 µmol) and **4b** (6.1 mg, 10.0 µmol) in 0.85 mL 200 mM Tris pH 8.0. Adapted general reaction procedure for **4b**. Yield for **12b** 82% (retention time = 17.2), **12c** 15 % (retention time = 16.8). Isolated yield of **12b**: 36% (2.5 mg, 2.8 µmol, retention time 17-19 min).

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for **12b** $C_{42}H_{70}N_7O_6S^+$ 800.5103; Found 800.5098.

HRMS (ESI/QTOF) m/z: $[M]^-$ Calcd for **12c** $C_{49}H_{73}IN_7O_{11}S_2^-$ 1126.3860; Found 1126.3880.

HPLC gradient: Method 1. **Prep HPLC gradient**: Method 7.

HPLC-UV and HPLC-MS chromatogram



SUPPORTING INFORMATION

Calibration curve of **12b**

Preparation of stock solution. Pure **12b** (0.5 mg) dissolved in 183 uL of CH_3CN: H_2O (1:1). Stock solution was diluted separately and submitted for RP-HPLC. Calibrated yield 70% (based on HPLC-UV), 90% (based on HPLC-MS).

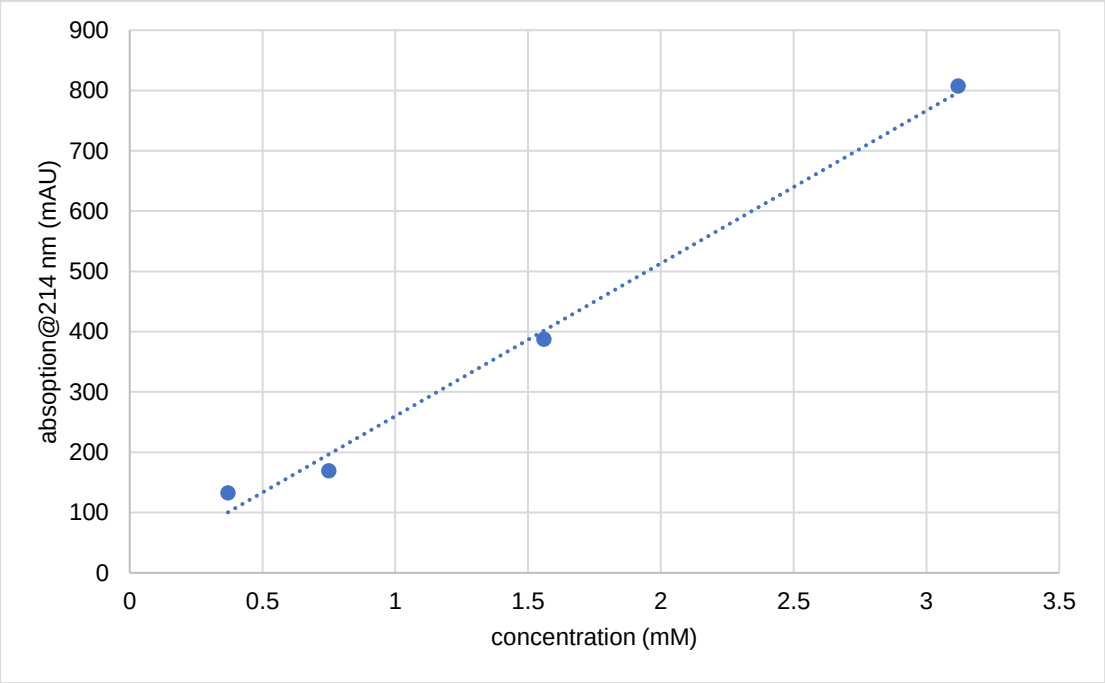


Figure S4. calibration curve of **12b** based on absorption at 214 nm.

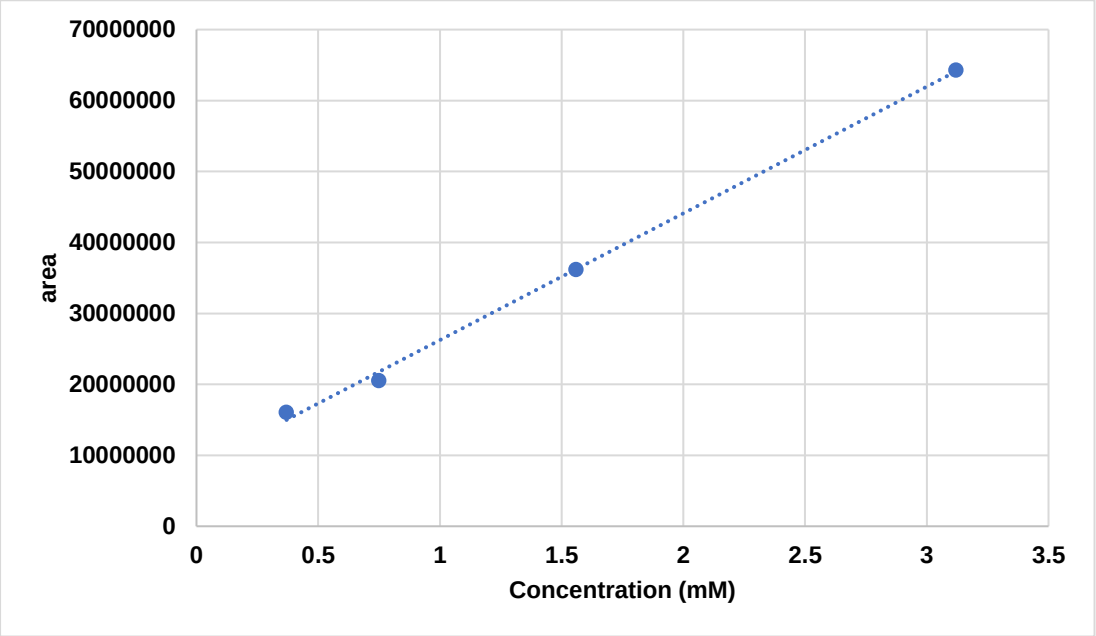
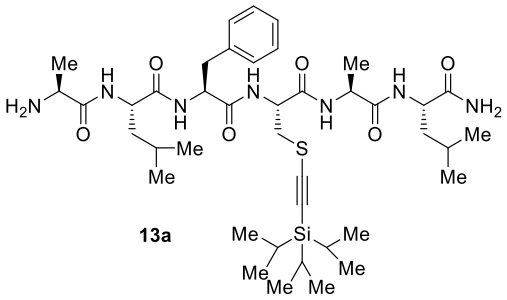


Figure S5. calibration curve of **12b** based on HPLC-MS.

SUPPORTING INFORMATION

Alkynylated H-Ala-Leu-Phe-Cys-Ala-Leu-NH_2 (**13a**)



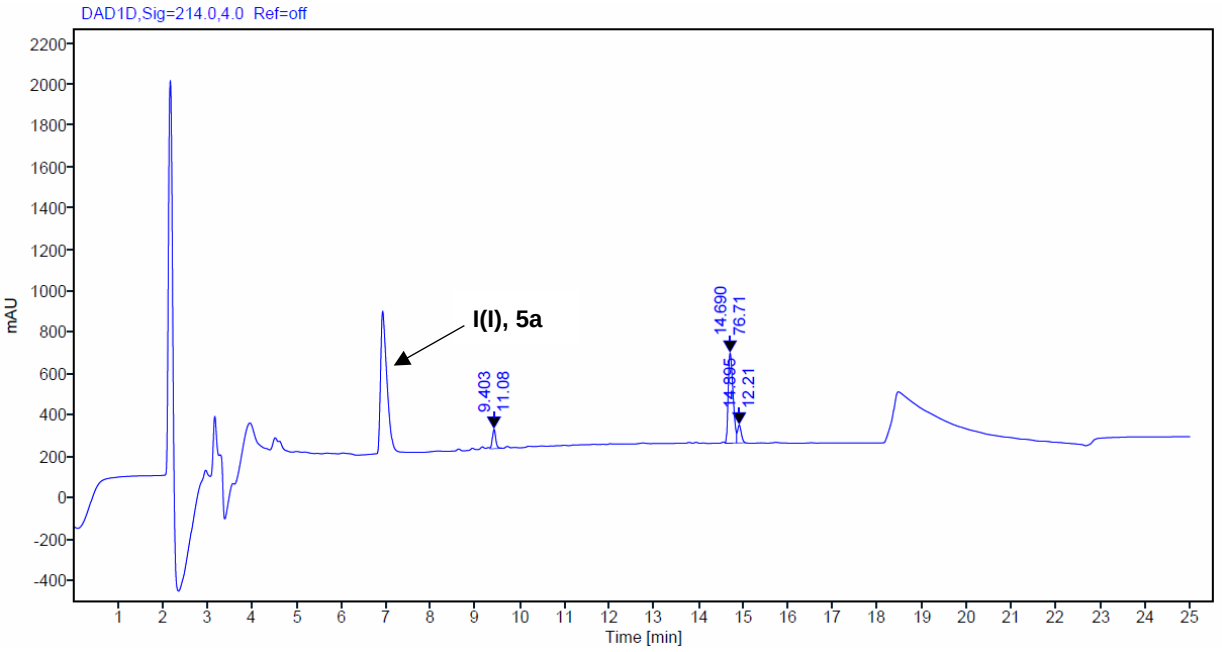
13a

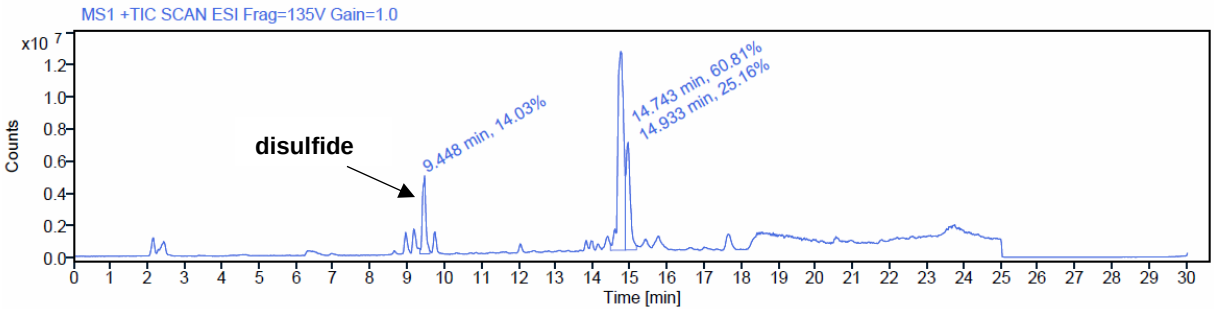
13 (0.50 mg, 0.78 µmol) and **4a** (0.64 mg, 1.2 µmol) in 79 µL 10 mM Tris pH 7.4. Adapted general reaction procedure for **4a**. Yield for **13a** 61% (retention time = 14.7)

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for **13a** $C_{41}H_{70}N_7O_6SSi^+$ 816.4872; Found 816.4877.

HPLC gradient: Method 1.

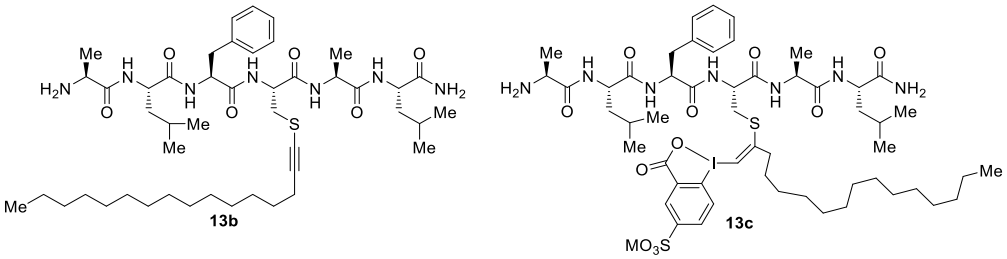
HPLC-UV and HPLC-MS chromatogram





SUPPORTING INFORMATION

Alkynylated H-Ala-Leu-Phe-Cys-Ala-Leu-NH_2 (**13b**) and corresponding VBX (**13c**)



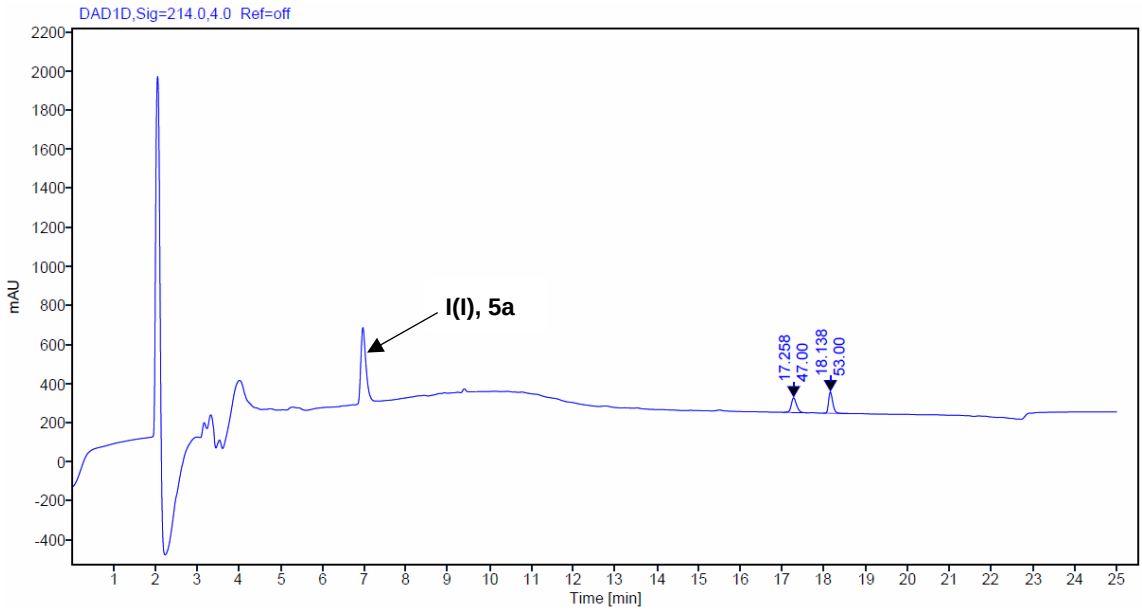
13 (15.0 mg, 23.5 µmol) and **4b** (11.1 mg, 18.8 µmol) in 1.5 mL 200 mM Tris pH 8.0. Adapted general reaction procedure for **4b**. Yield for **13b** 76% (retention time = 18.1), **13c** 18% (retention time = 17.2). Isolated yield of **13b**: 40% (7.8 mg, 9.4 µmol retention time = 16-18 min.)

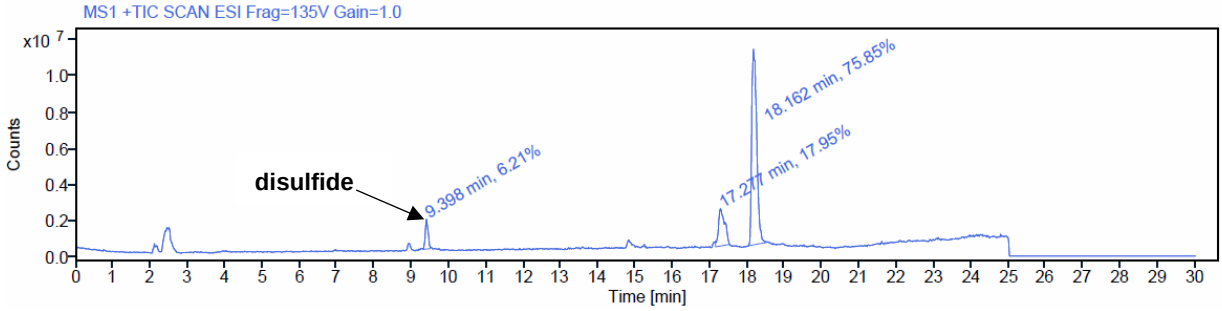
HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for **13b** $C_{46}H_{78}N_7O_6S^+$ 856.5729; Found 856.5723.

HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M]^-$ for **13c** Calcd for $C_{53}H_{81}IN_7O_{11}S_2^-$ 1182.4486; Found 1182.4523.

HPLC gradient: Method 1. **Prep HPLC gradient**: Method 7.

HPLC-UV and HPLC-MS chromatogram





SUPPORTING INFORMATION

Calibration curve of **13b**

Preparation of stock solution. Pure **13b** (0.5 mg) dissolved in 73 uL of CH_3CN: H_2O (1:1). Stock solution was diluted separately and submitted for RP-HPLC. Calibrated yield 78% (based on HPLC-UV), 69% (based on HPLC-MS).

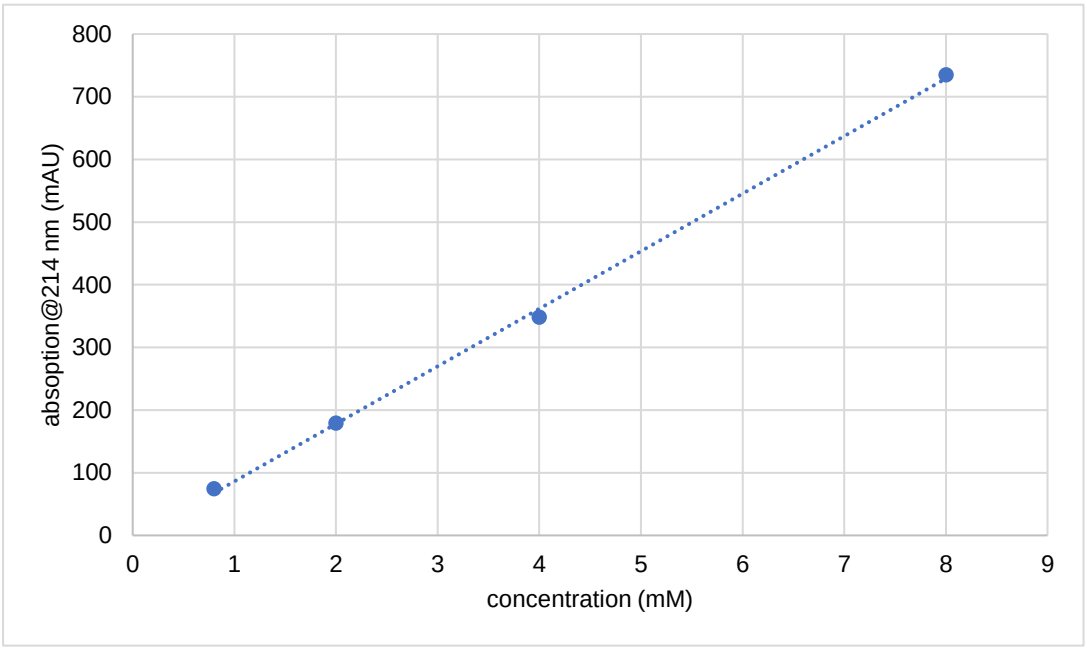


Figure S6. calibration curve of **13b** based HPLC-UV absorption at 214 nm.

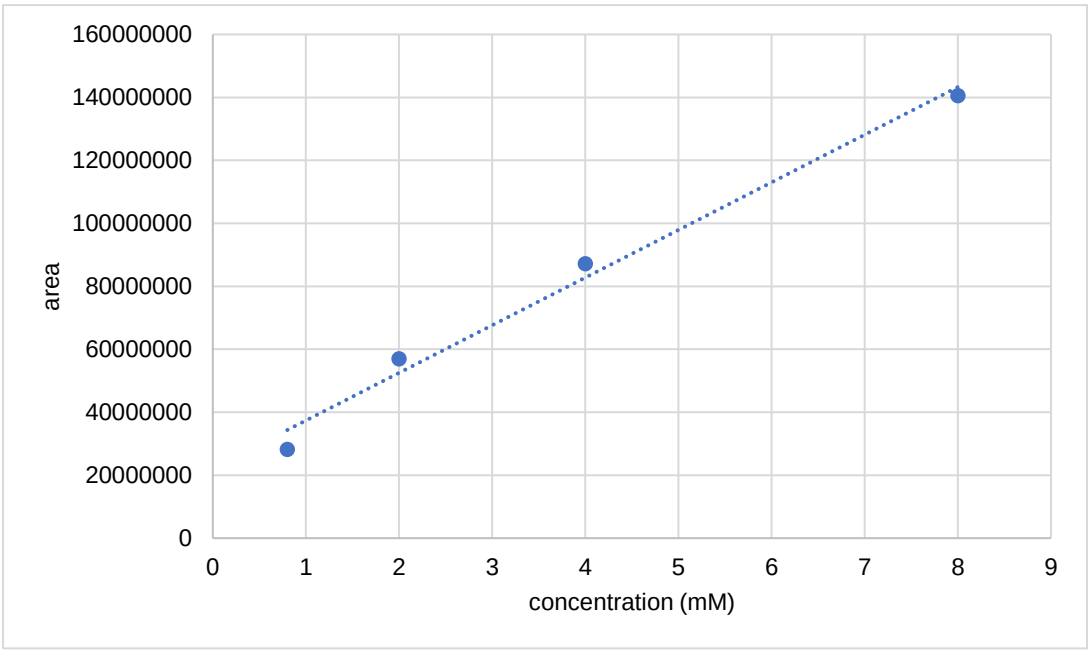
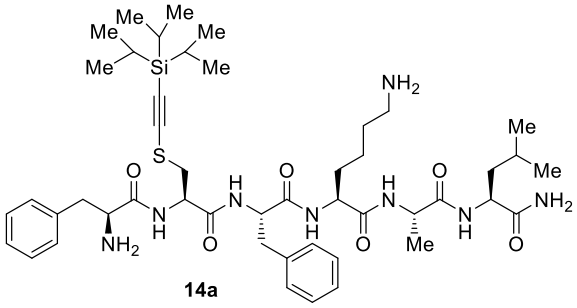


Figure S7. calibration curve of **13b** based HPLC-MS.

SUPPORTING INFORMATION

Alkynylated H-Phe-Cys-Phe-Lys-Ala-Leu-NH_2 (**14a**)

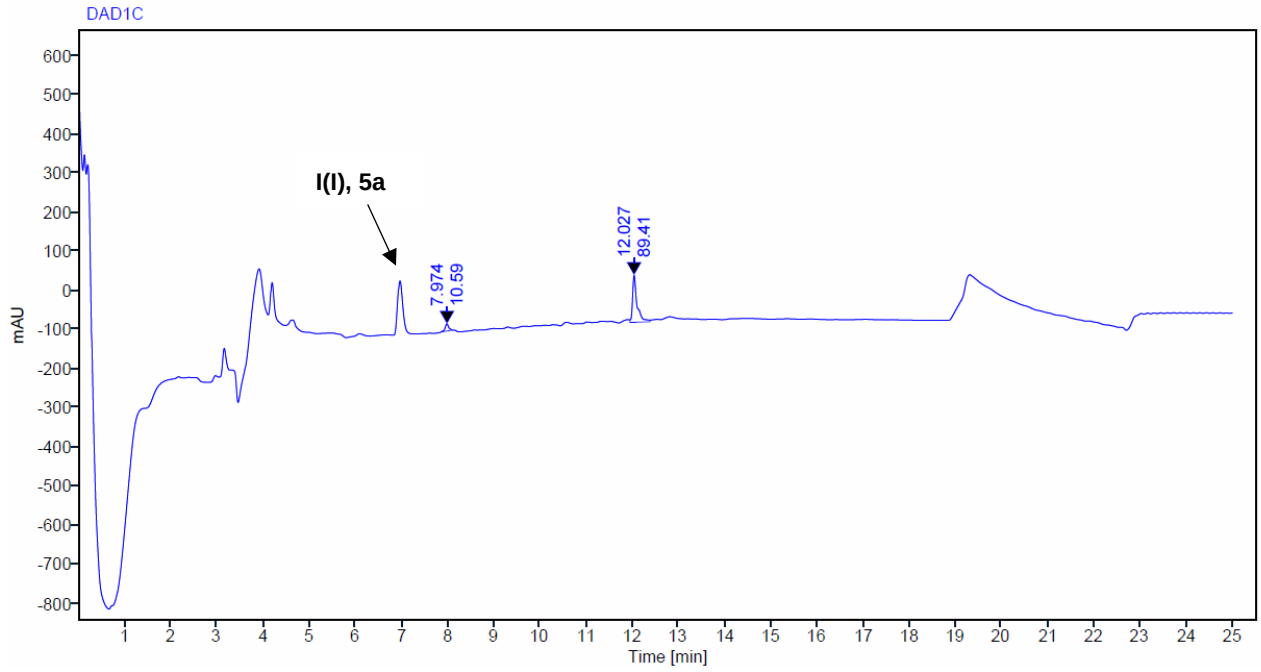


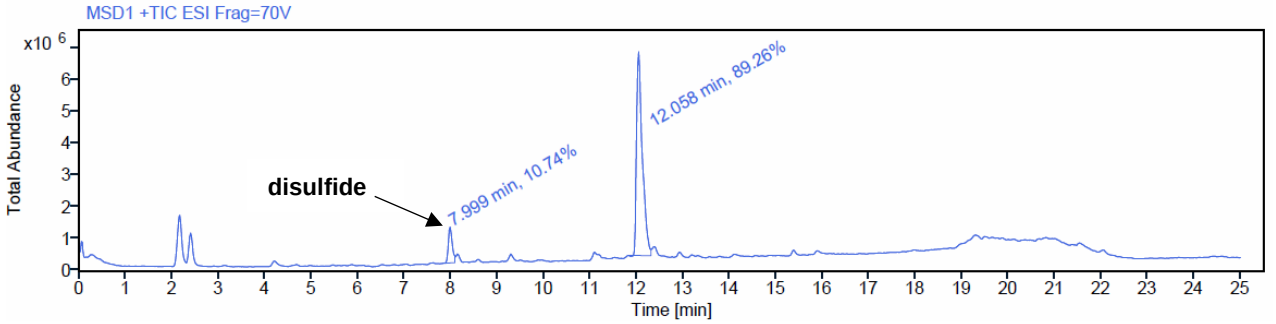
14 (1.0 mg, 1.4 µmol) and **4a** (1.1 mg, 2.0 µmol) in 137 µL 10 mM Tris pH 7.4. Adapted general reaction procedure for **4a**. Yield for **14a** 89% (retention time = 12.0)

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for **14a** $C_{47}H_{75}N_8O_6SSi^+$ 907.5294; Found 907.5328.

HPLC gradient: Method 1.

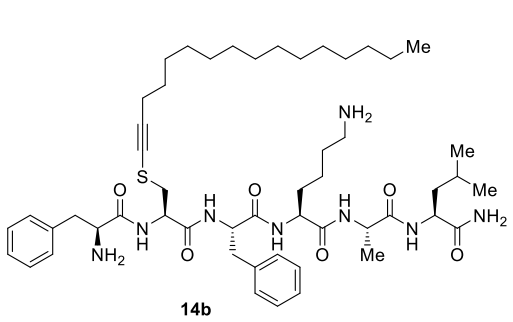
HPLC-UV and HPLC-MS chromatogram

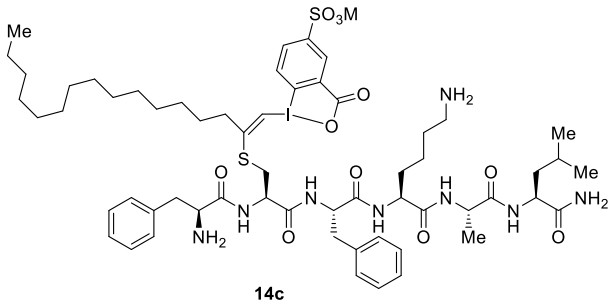




SUPPORTING INFORMATION

Alkynylated H-Phe-Cys-Phe-Lys-Ala-Leu-NH_2 (**14b**) and corresponding VBX (**14c**)





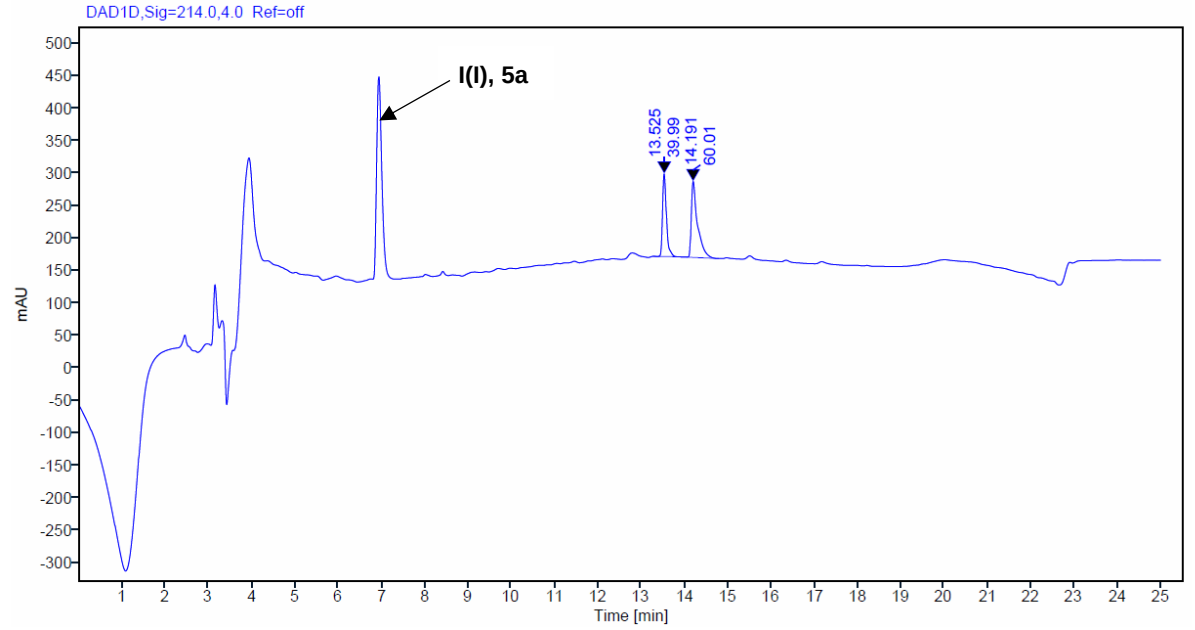
14 (0.50 mg, 0.68 µmol) and **4b** (0.48 mg, 0.82 µmol) in 68 µL 200 mM Tris pH 8.0. Adapted general reaction procedure for **4b**. Yield for **14b** 80% (retention time = 14.2), **14c** 19% (retention time = 13.5).

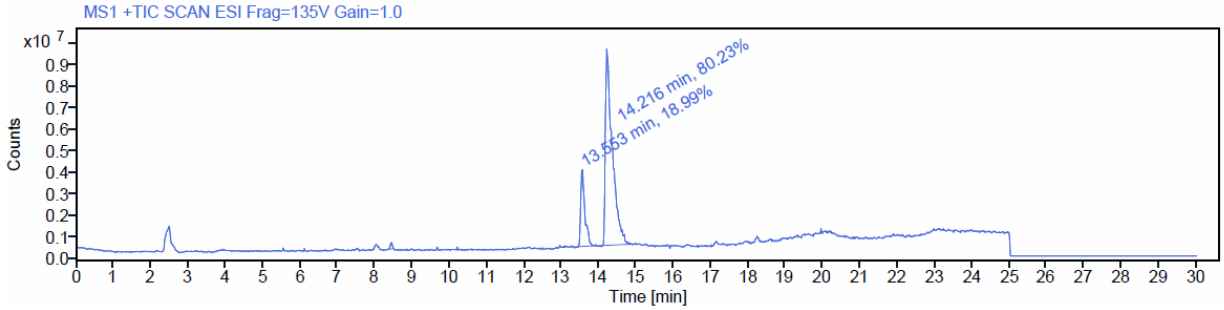
HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for **14b** $C_{52}H_{83}N_8O_6S^+$ 947.6151; Found 947.6159.

HRMS (ESI/QTOF) m/z: $[M]^-$ Calcd for **14c** $C_{59}H_{86}IN_8O_{11}S_2^-$ 1273.4908; Found 1273.4963.

HPLC gradient: Method 1.

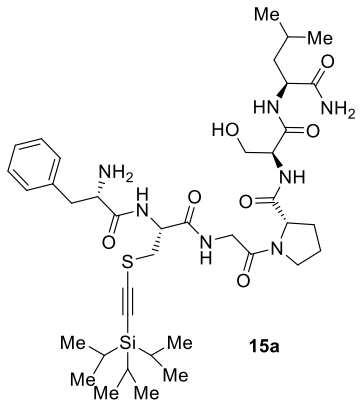
HPLC-UV and HPLC-MS chromatogram





SUPPORTING INFORMATION

Alkynylated H-Phe-Cys-Gly-Pro-Ser-Leu-NH_2 (**15a**)



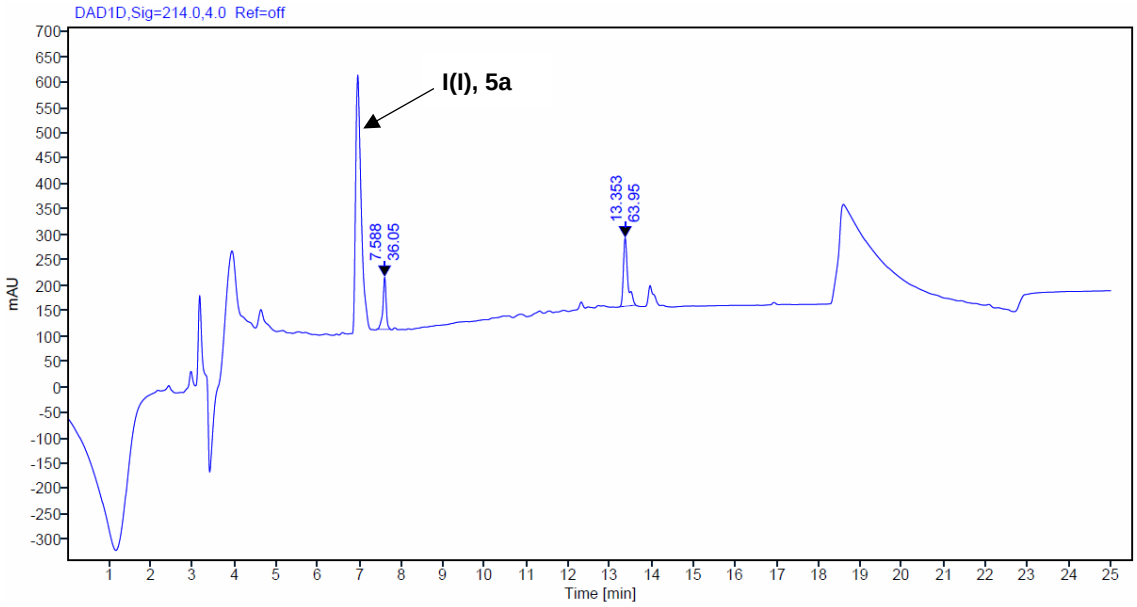
15a

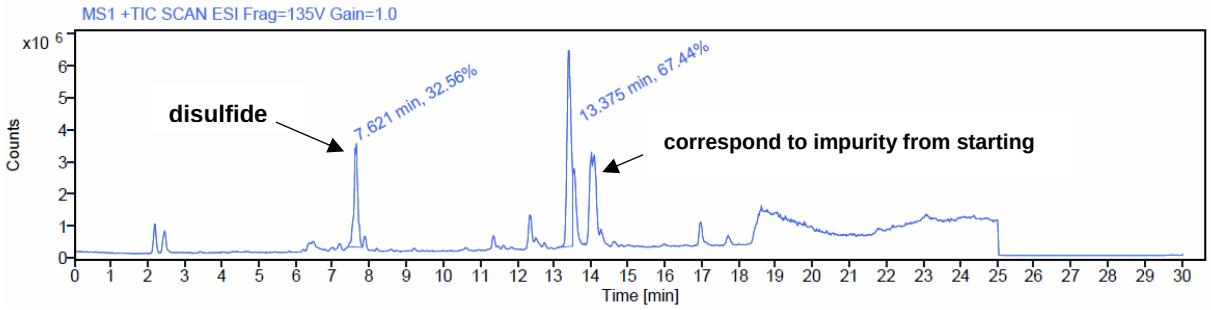
15 (0.50 mg, 0.80 µmol) and **4a** (0.60 mg, 1.2 µmol) in 80 µL 10 mM Tris pH 7.4. Adapted general reaction procedure for **4a**. Yield for **15a** 67% (retention time = 13.3).

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for **15a** $C_{39}H_{64}N_7O_7SSi^+$ 802.4352; Found 802.4318.

HPLC gradient: Method 1.

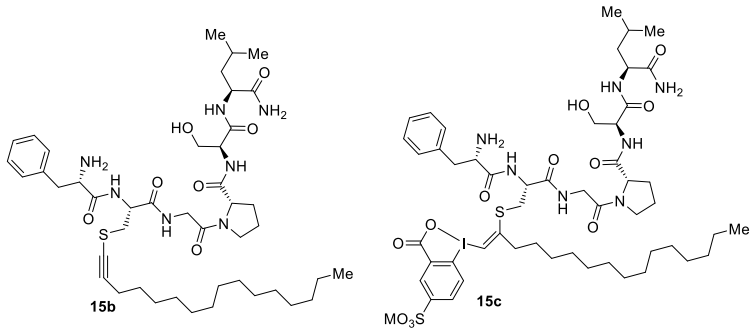
HPLC-UV and HPLC-MS chromatogram





SUPPORTING INFORMATION

Alkynylated H-Phe-Cys-Gly-Pro-Ser-Leu-NH_2 (**15b**) and corresponding VBX (**15c**)



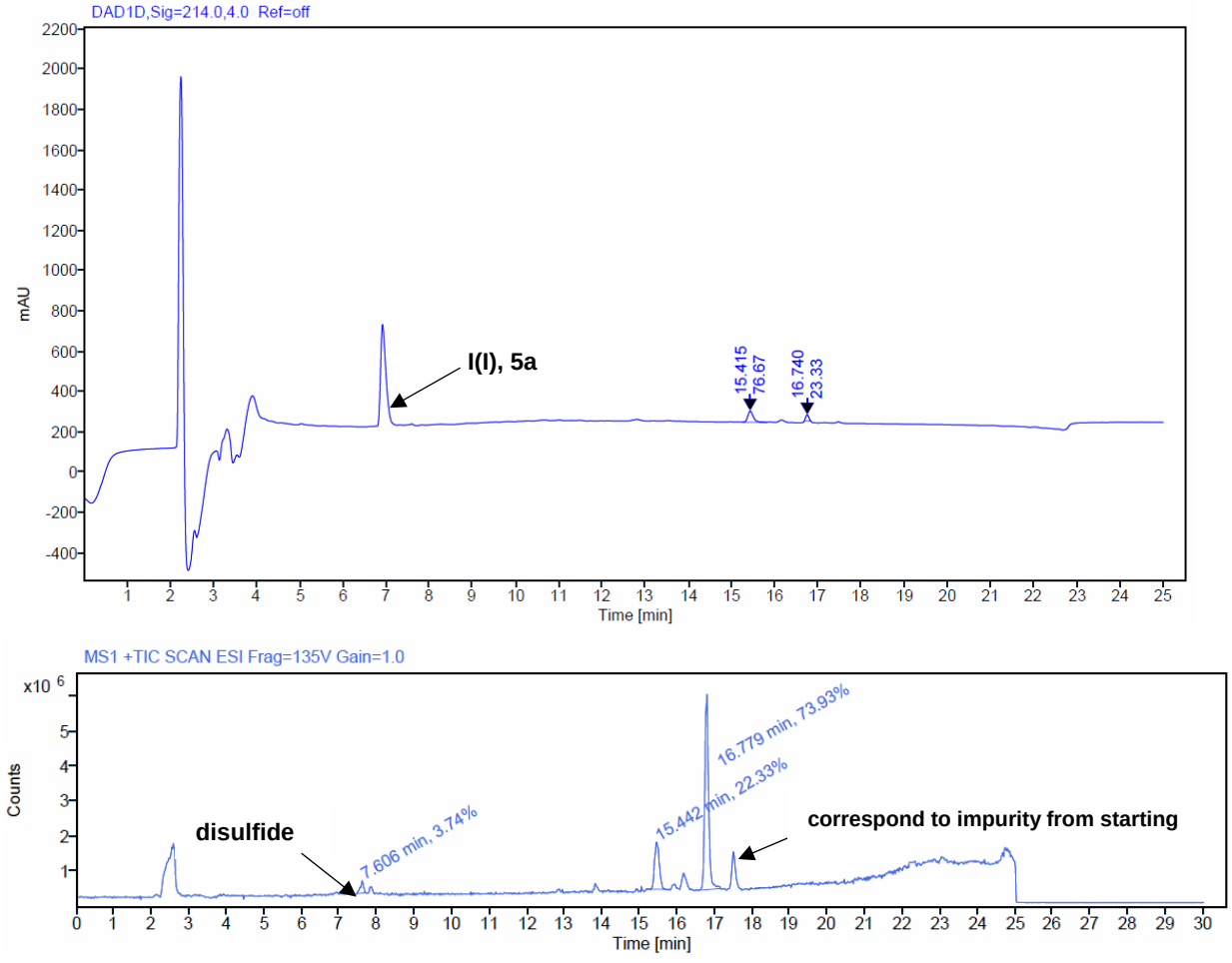
15 (0.50 mg, 0.80 µmol) and **4b** (0.56 mg, 0.82 µmol) in 80 µL 200 mM Tris pH 8.0. Adapted general reaction procedure for **4b.** Yield for **15b** 74% (retention time = 16.7), **15c** 22% (retention time = 15.4).

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for **15b** $C_{44}H_{72}N_7O_7S^+$ 842.5208; Found 842.5201.

HRMS (ESI/QTOF) m/z: $[M]^-$ Calcd for **15c** $C_{51}H_{75}IN_7O_{12}S_2^-$ 1168.3965; Found 1168.3991.

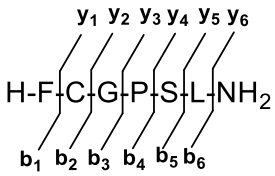
HPLC gradient: Method 1.

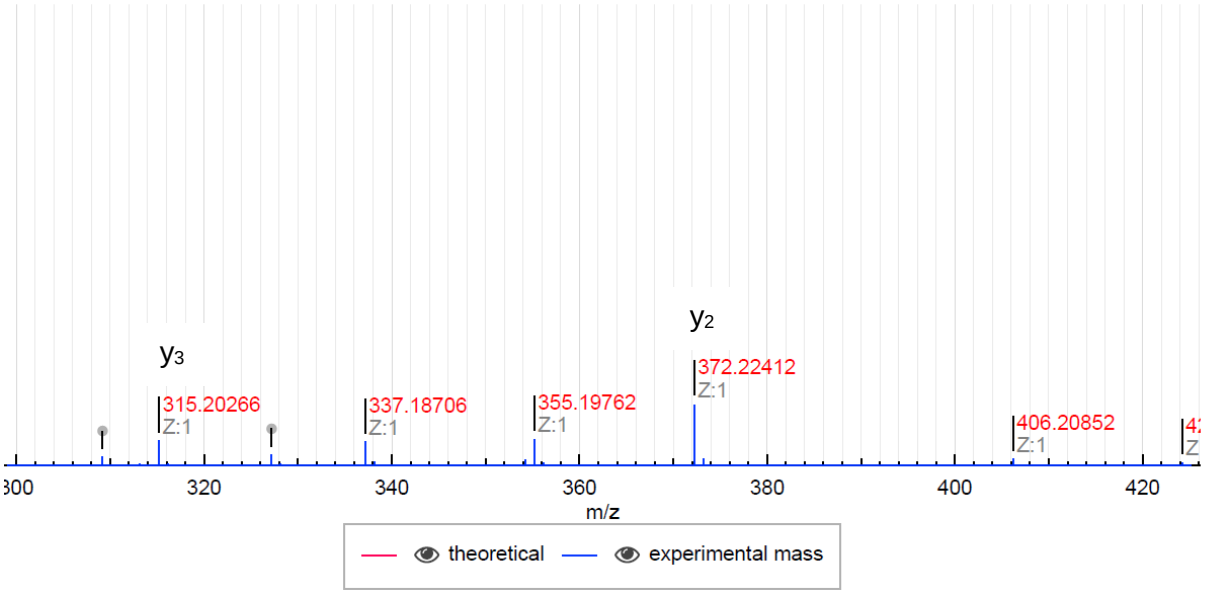
HPLC-UV and HPLC-MS chromatogram

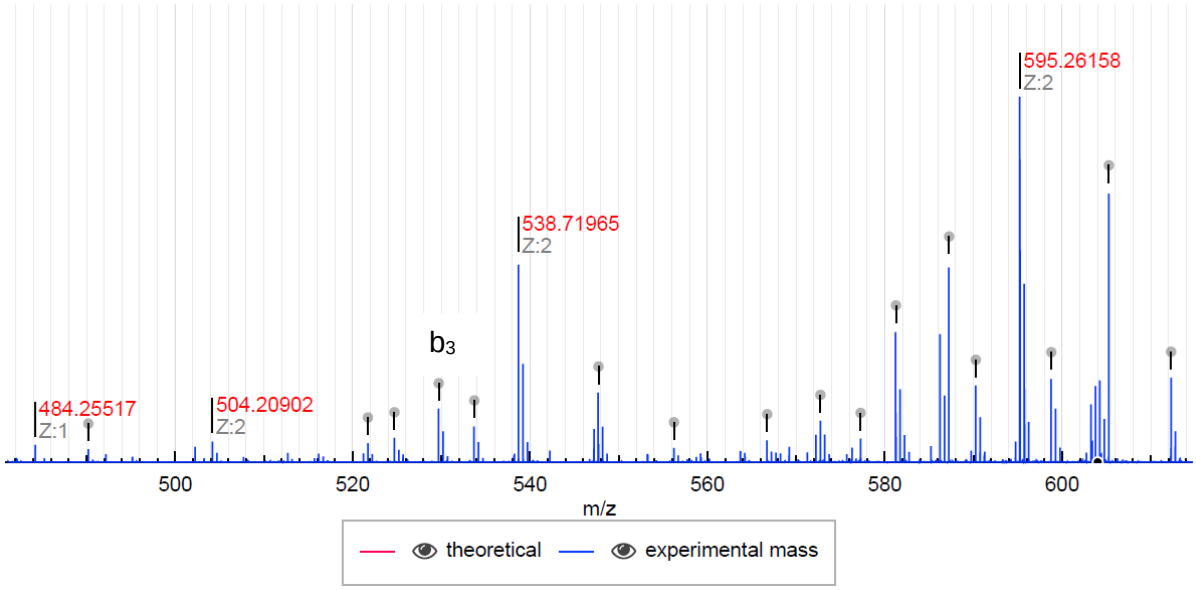


SUPPORTING INFORMATION

LC-MS/MS of **15b**

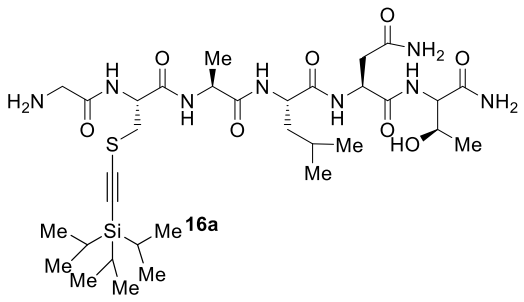






SUPPORTING INFORMATION

Alkynylated H-Gly-Cys-Ala-Leu-Asn-Thr-NH_2 (**16a**)

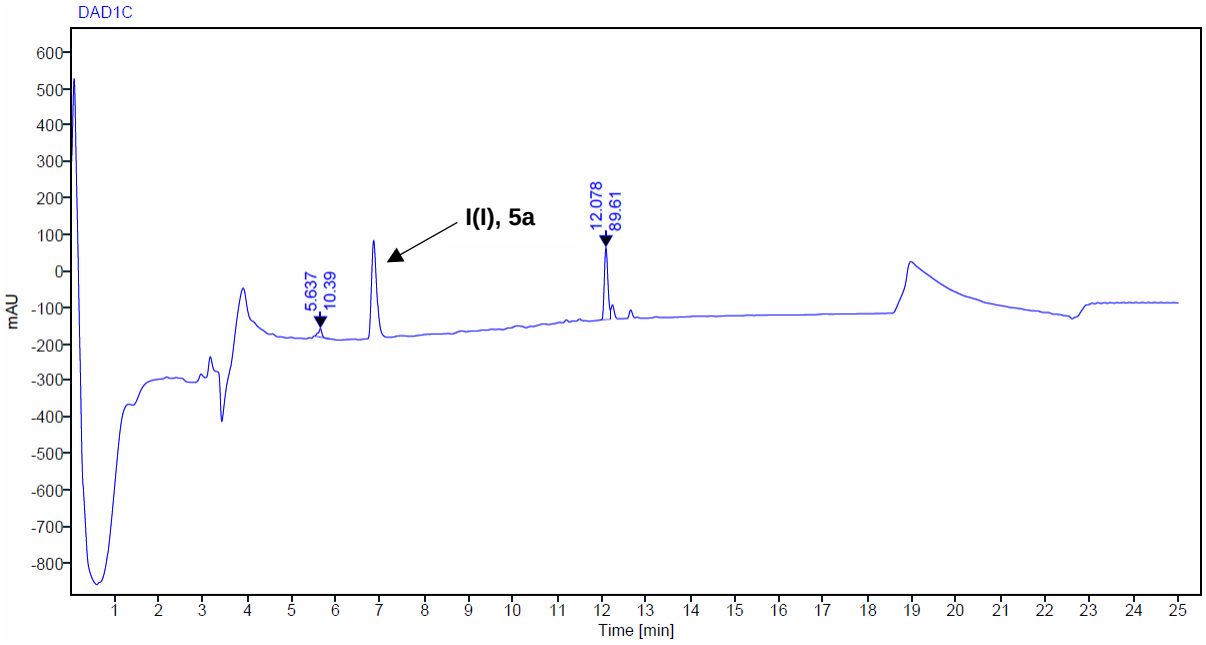


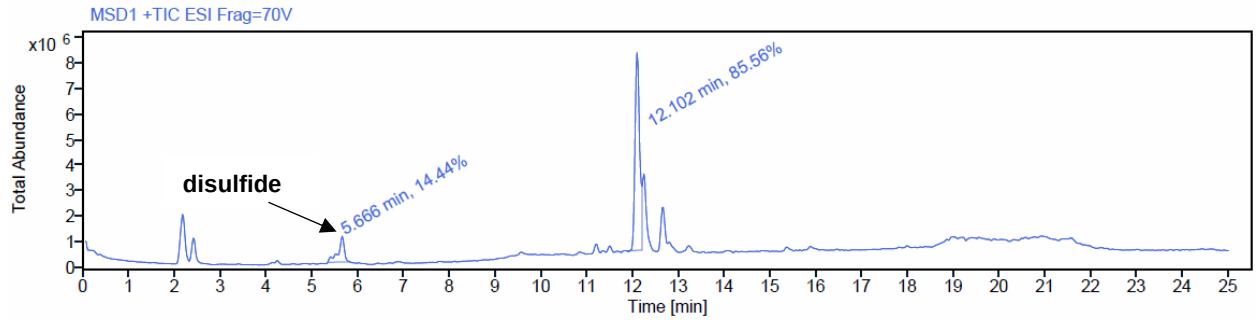
16 (1.0 mg, 1.7 µmol) and **4a** (1.4 mg, 2.6 µmol) in 173 µL 10 mM Tris pH 7.4. Adapted general reaction procedure for **4a**. Yield for **16a** 86% (retention time = 12.1)

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for **16a** $C_{33}H_{61}N_8O_8SSi^+$ 757.4097; Found 757.4130.

HPLC gradient: Method 1.

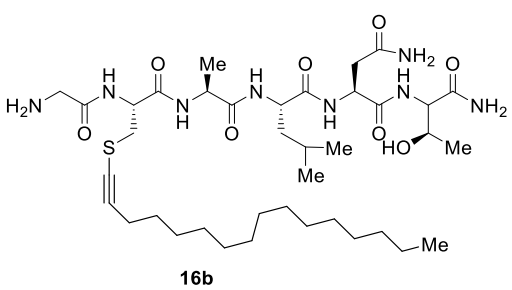
HPLC-UV and HPLC-MS chromatogram



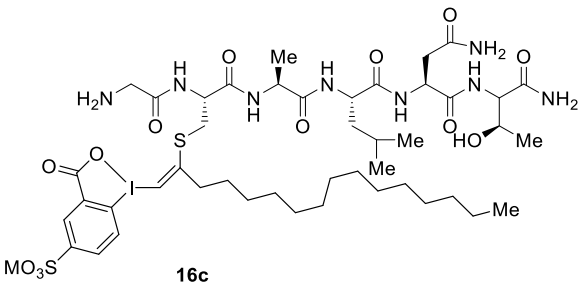


SUPPORTING INFORMATION

Alkynylated H-Gly-Cys-Ala-Leu-Asn-Thr-NH_2 (**16b**) and corresponding VBX (**16c**)



16b



16c

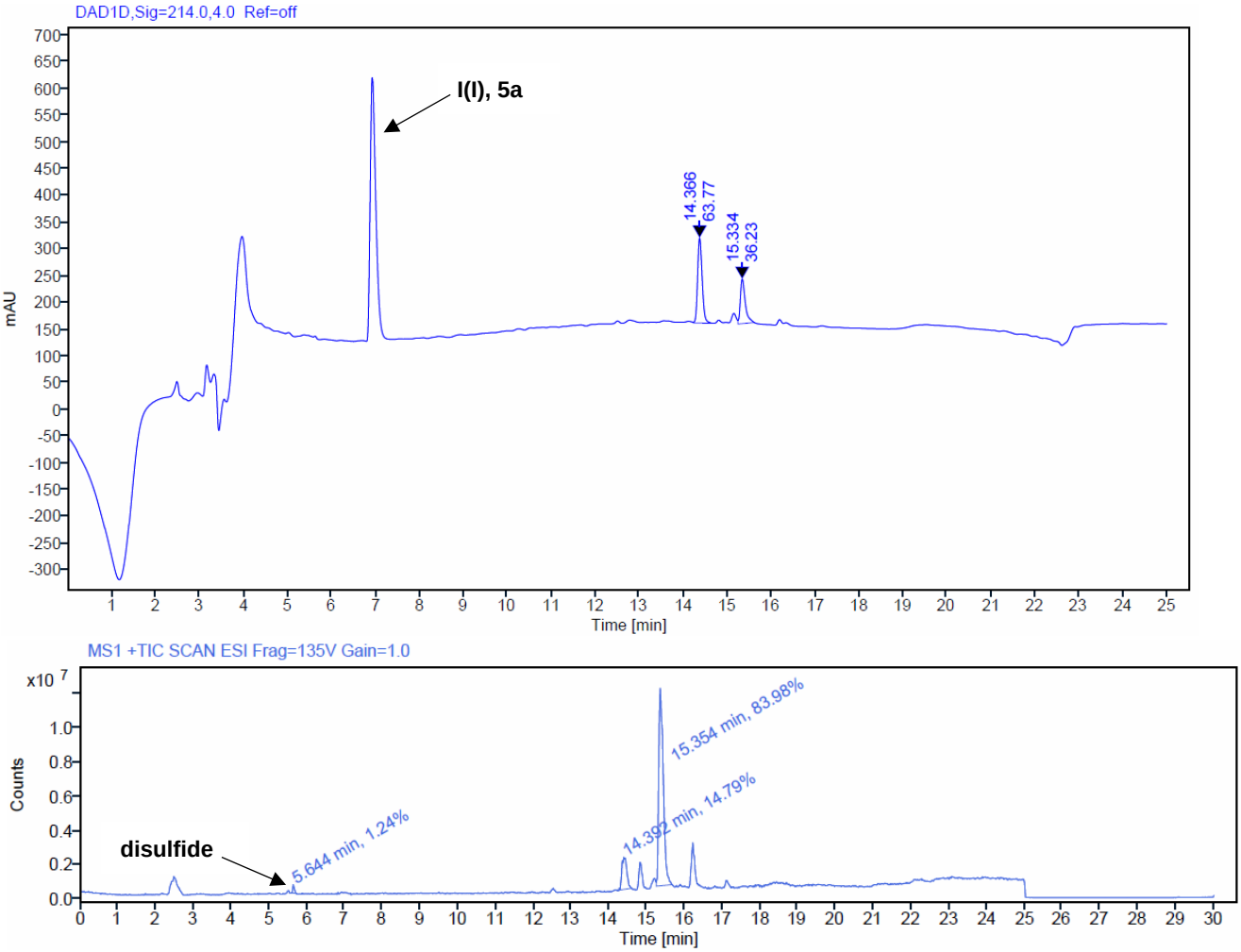
16 (0.50 mg, 0.86 µmol) and **4b** (0.61 mg, 1.0 µmol) in 86 µL 200 mM Tris pH 8.0. Adapted general reaction procedure for **4b.** Yield for **16b** 84% (retention time = 15.3), **16c** 15% (retention time = 14.4).

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for **16b** $C_{38}H_{69}N_8O_8S^+$ 797.4954; Found 797.4978.

HRMS (ESI/QTOF) m/z: $[M]^-$ Calcd for **16c** $C_{45}H_{72}IN_8O_{13}S_2^-$ 1123.3710; Found 1123.3750.

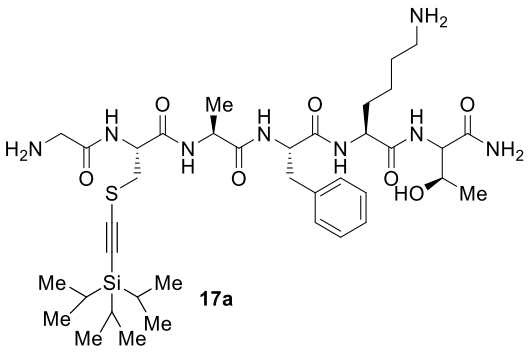
HPLC gradient: Method 1.

HPLC-UV and HPLC-MS chromatogram



SUPPORTING INFORMATION

Alkynylated H-Gly-Cys-Ala-Phe-Lys-Thr-NH_2 (**17a**)



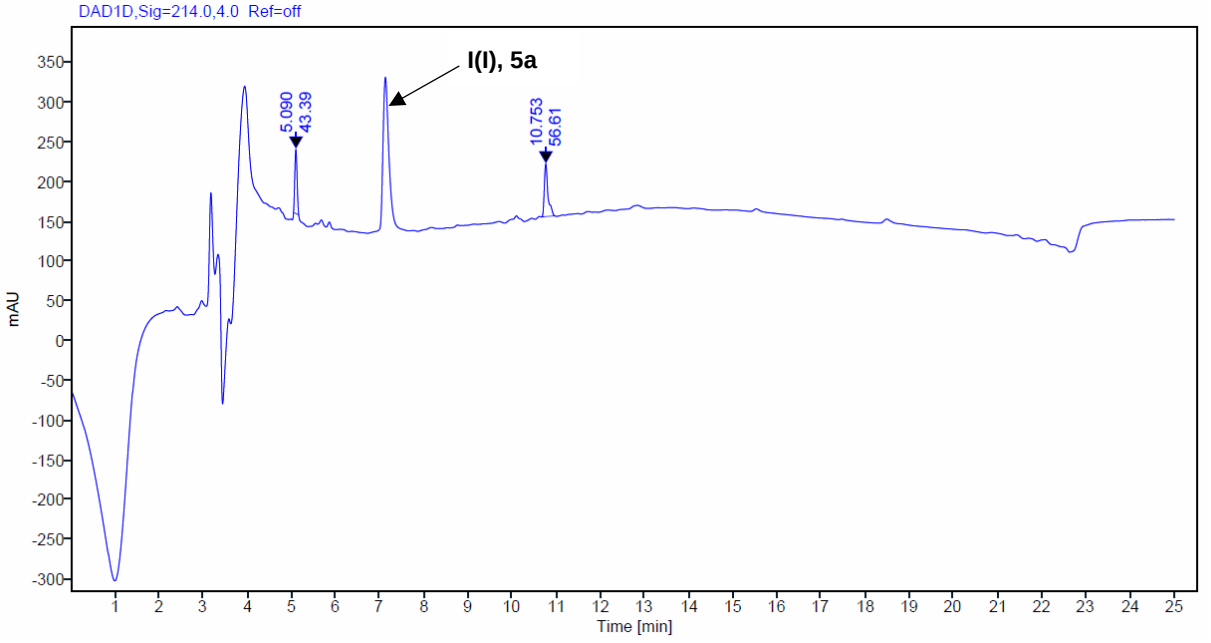
17a

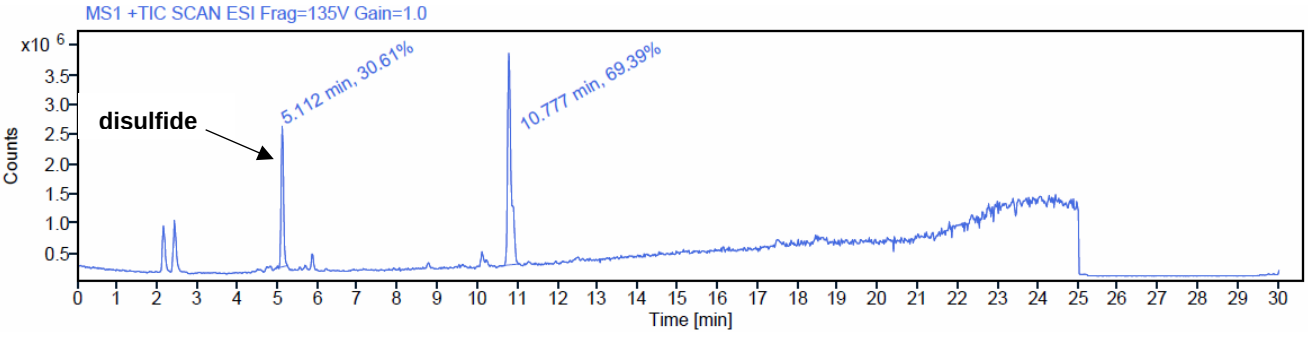
17 (1.0 mg, 1.6 µmol) and **4a** (1.3 mg, 2.4 µmol) in 160 µL 10 mM Tris pH 7.4. Adapted general reaction procedure for **4a**. Yield for **17a** 69% (retention time = 10.7)

HRMS (ESI/QTOF) m/z: $[M+H]^+$ Calcd for **17a** $C_{38}H_{65}N_8O_7SSi^+$ 805.4461; Found 805.4462.

HPLC gradient: Method 1.

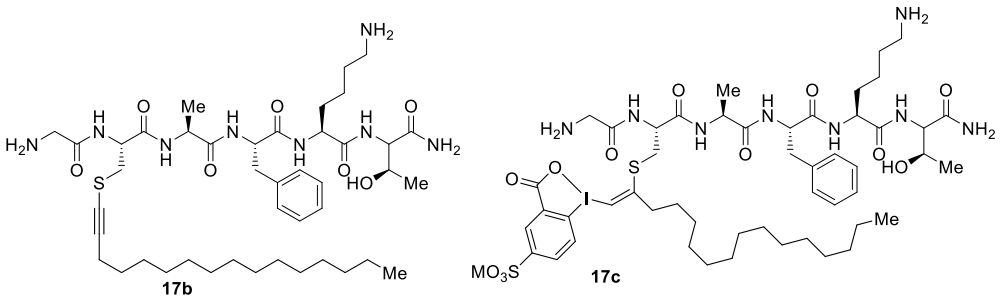
HPLC-UV and HPLC-MS chromatogram





SUPPORTING INFORMATION

Alkynylated H-Gly-Cys-Ala-Phe-Lys-Thr-NH_2 (**17b**) and corresponding VBX (**17c**)



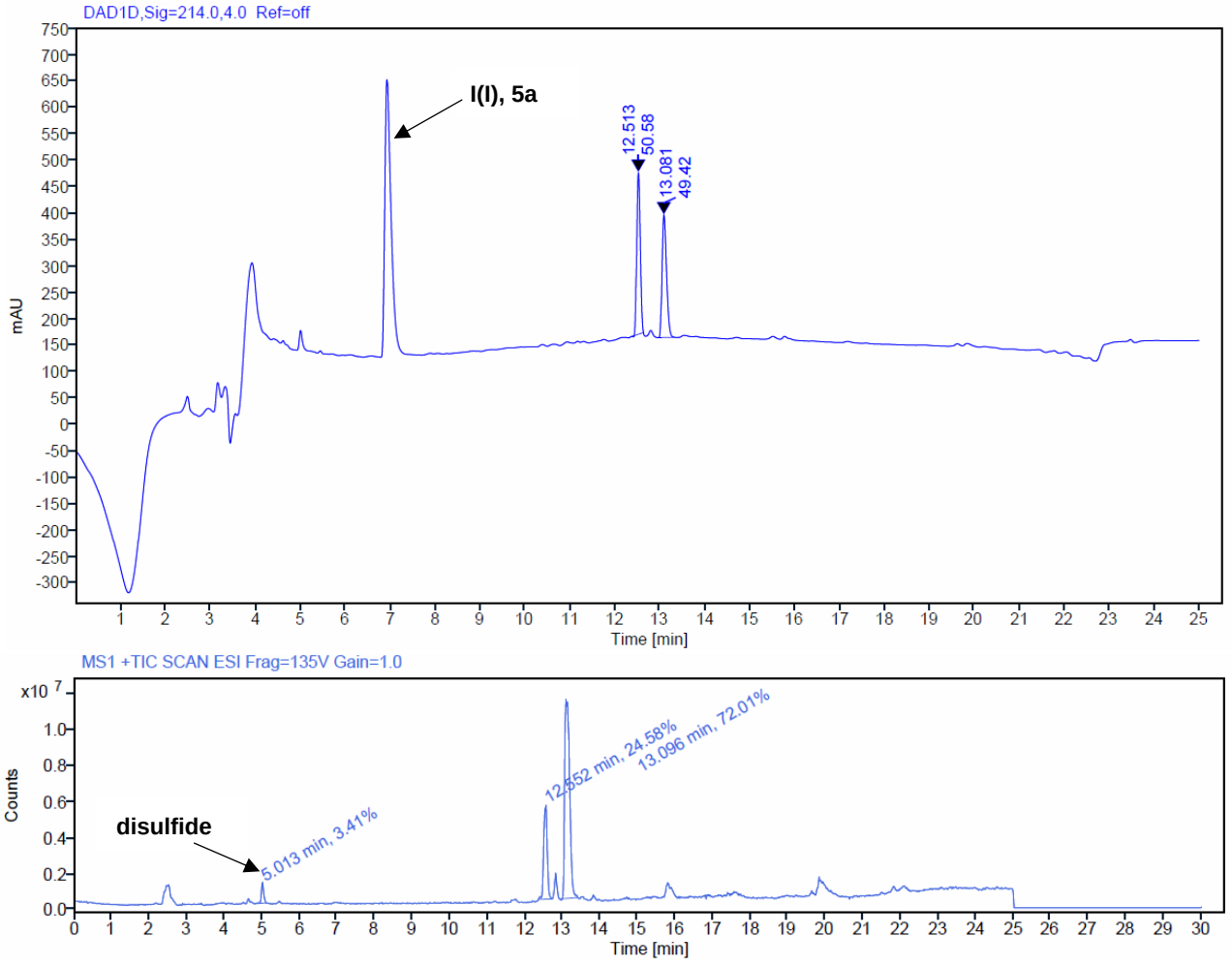
17 (0.50 mg, 0.80 µmol) and **4b** (0.56 mg, 0.96 µmol) in 80 µL 200 mM Tris pH 8.0. Adapted general reaction procedure for **4b.** Yield for **17b** 72% (retention time = 13.1), **17c** 25% (retention time = 12.5).

HRMS (QTOF) m/z: $[M + H]^+$ Calcd for **17b** $C_{43}H_{73}N_8O_7S^+$ 845.5317; Found 845.5336.

HRMS (ESI/QTOF) m/z: $[M]^-$ Calcd for **17c** $C_{50}H_{76}IN_8O_{12}S_2^-$ 1171.4074; Found 1171.4124.

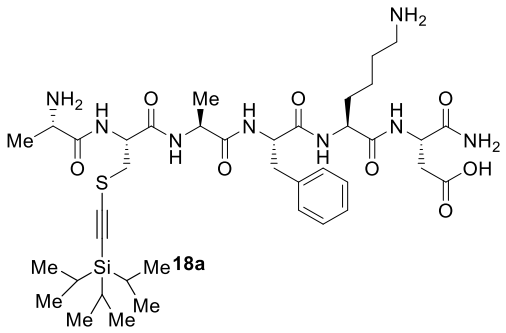
HPLC gradient: Method 1.

HPLC-UV and HPLC-MS chromatogram



SUPPORTING INFORMATION

Alkynylated H-Ala-Cys-Ala-Phe-Lys-Asp-NH_2 (**18a**)

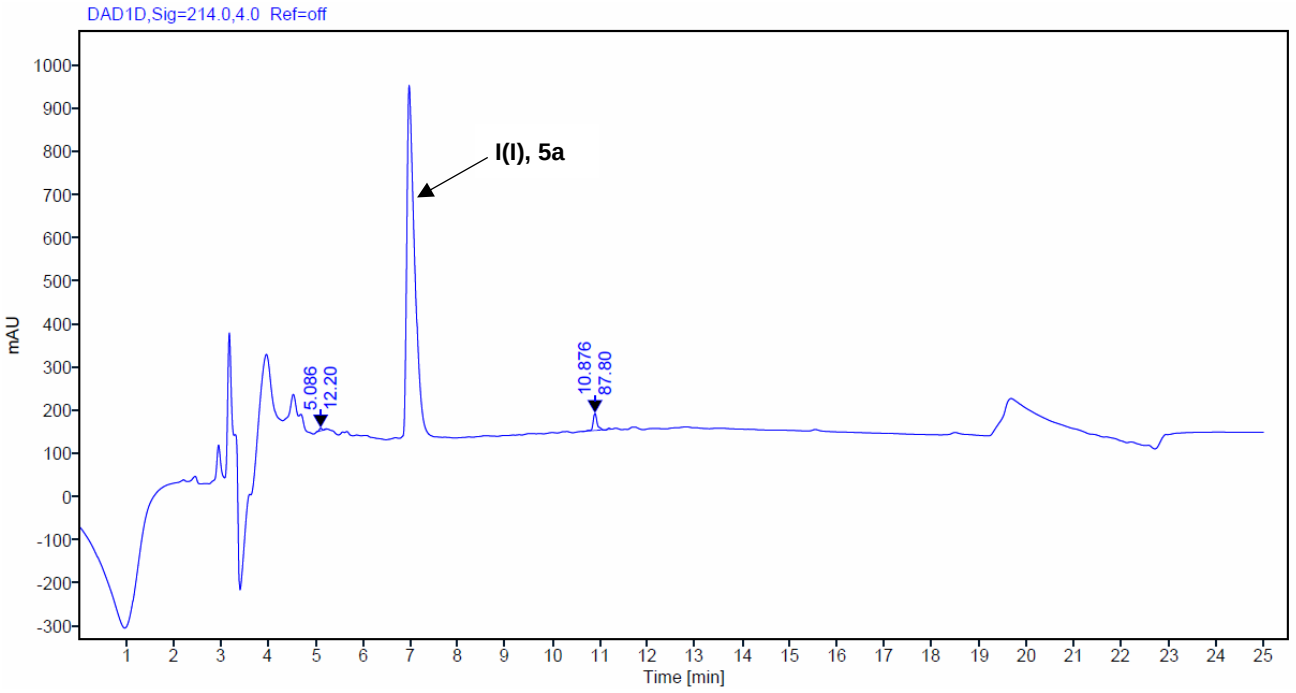


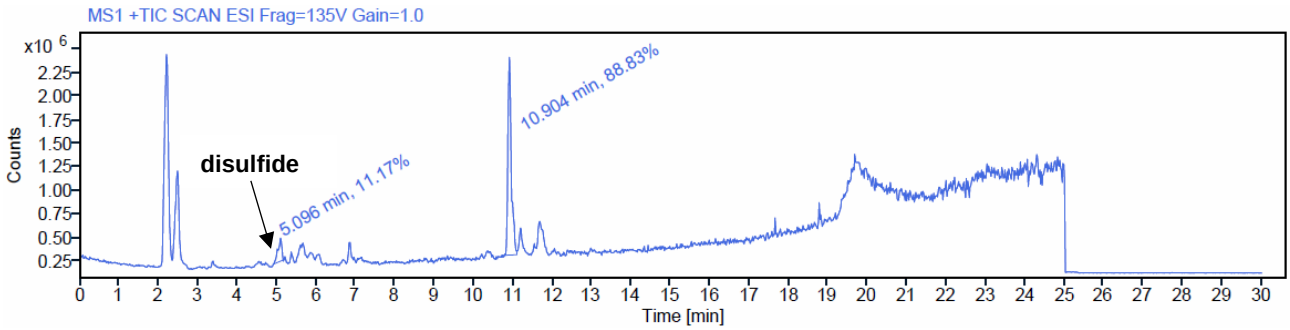
18 (1.0 mg, 1.5 µmol) and **4a** (1.3 mg, 2.3 µmol) in 153 µL 10 mM Tris pH 7.4. Adapted general reaction procedure for **4a**. Yield for **18a** 89% (retention time = 10.9).

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for **18a** $C_{39}H_{65}N_8O_8SSi^+$ 833.4410; Found 833.4400.

HPLC gradient: Method 1.

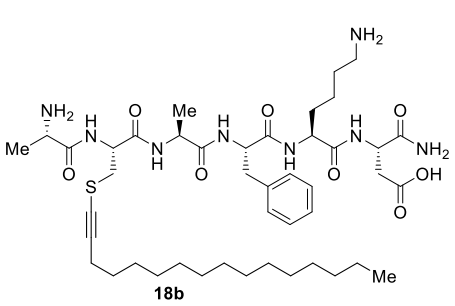
HPLC-UV and HPLC-MS chromatogram

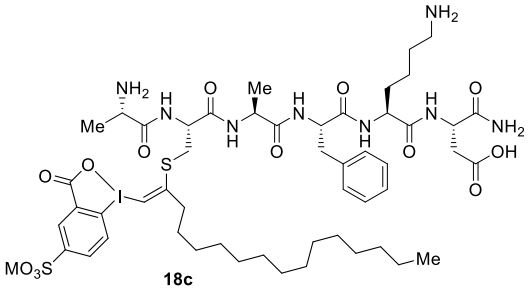




SUPPORTING INFORMATION

Alkynylated H-Ala-Cys-Ala-Phe-Lys-Asp-NH_2 (**18b**) and corresponding VBX (**18c**)





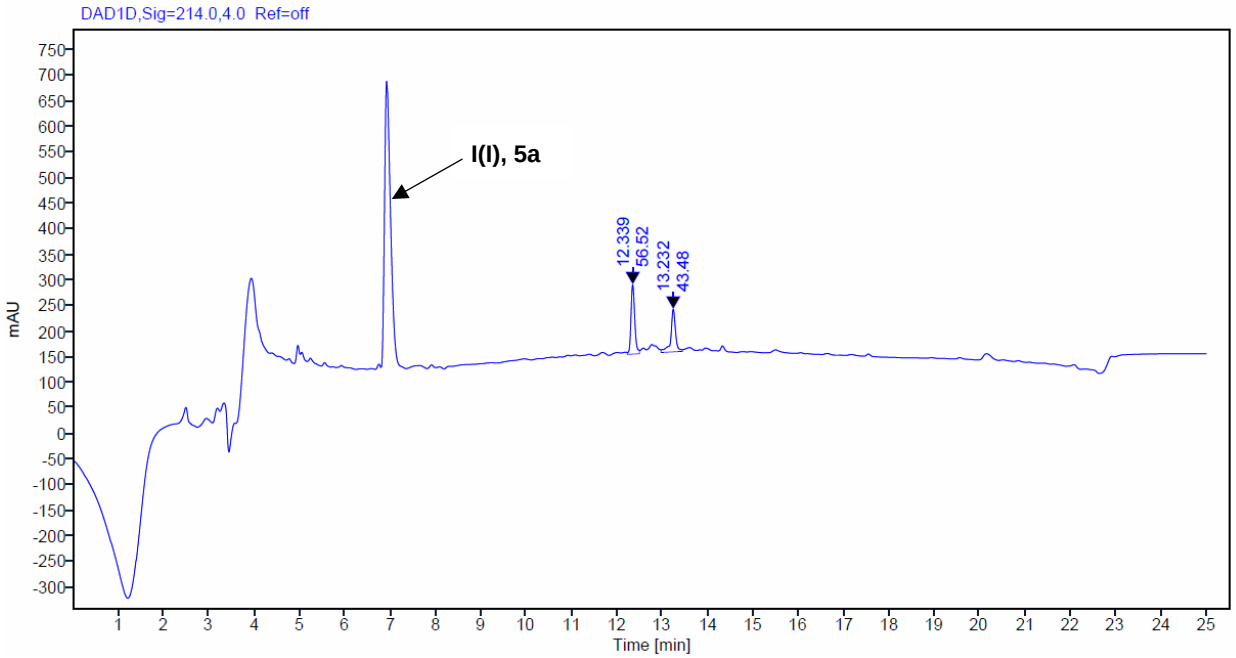
18 (0.50 mg, 0.76 µmol) and **4b** (0.54 mg, 0.91 µmol) in 77 µL 200 mM Tris pH 8.0. Adapted general reaction procedure for **4b**. Yield for **18b** 62% (retention time = 13.2), **18c** 28% (retention time = 12.3).

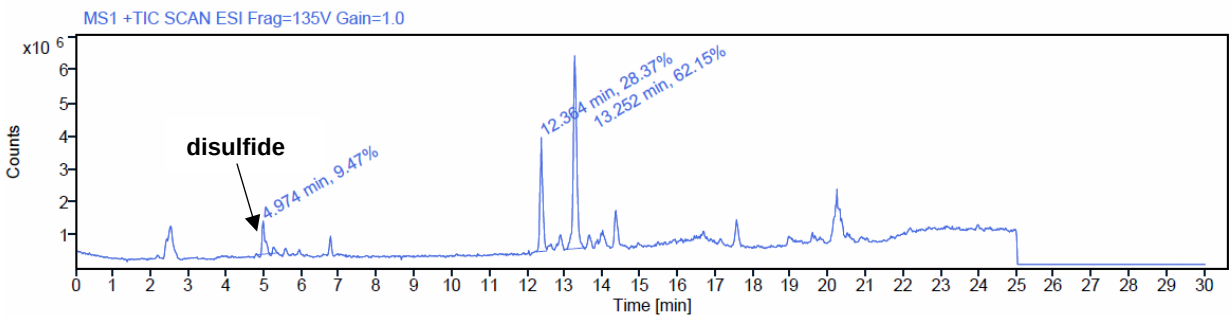
HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for **18b** $C_{44}H_{73}N_8O_8S^+$ 873.5267; Found 873.5278.

HRMS (ESI/QTOF) m/z: $[M]^-$ Calcd for **18c** $C_{51}H_{76}IN_8O_{13}S_2^-$ 1199.4023; Found 1199.4075.

HPLC gradient: Method 1.

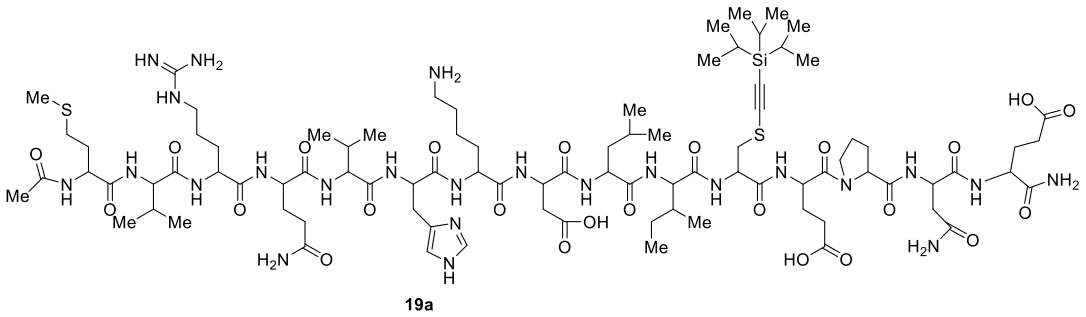
HPLC-UV and HPLC-MS chromatogram





8. Substrate scope for long peptides

Alkynylated Ac-Met-Val-Arg-Gln-Val-His-Lys-Asp-Leu-Ile-Cys-Glu-Pro-Asn-Glu-NH_2 (**19a**)



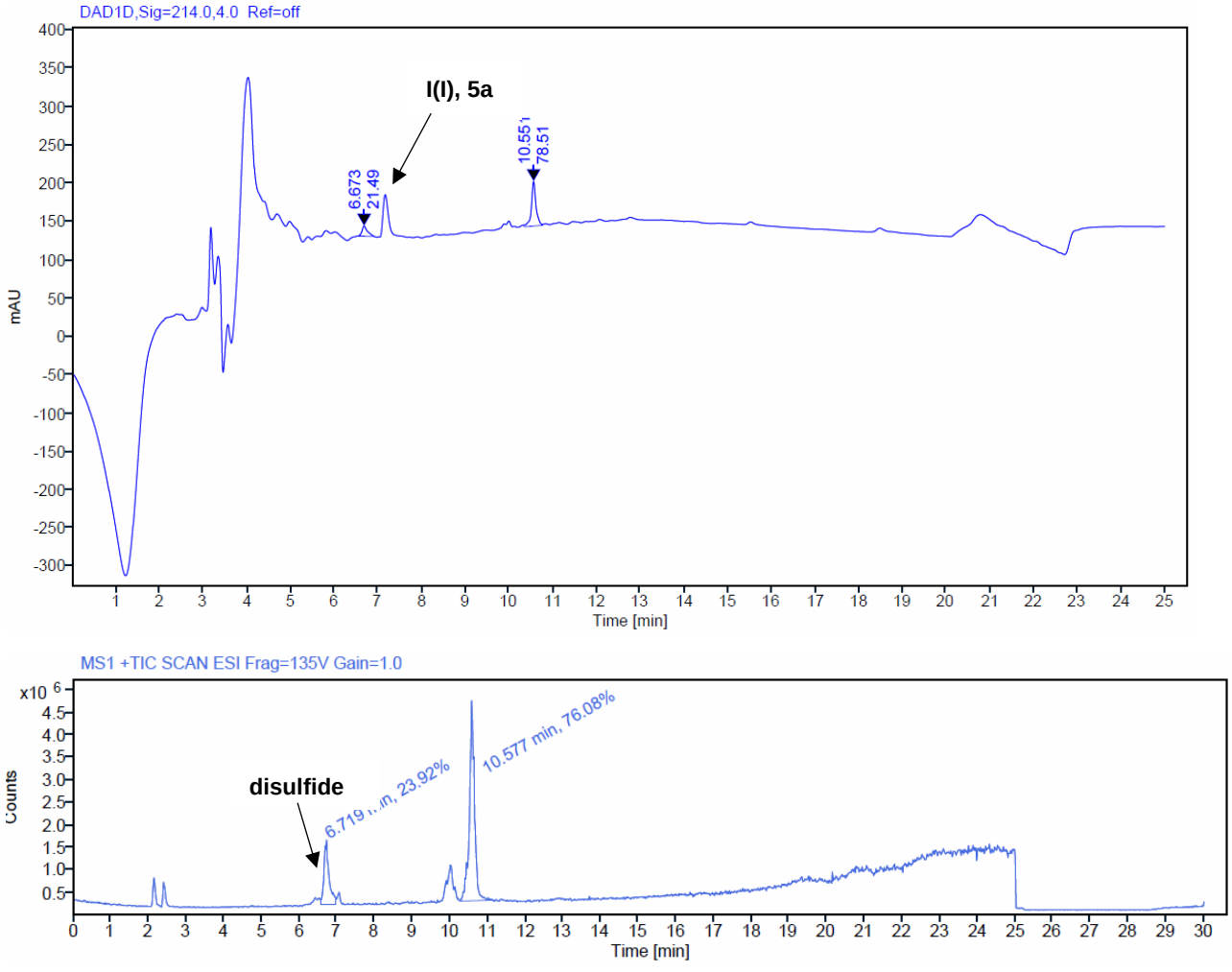
19a

19 (1.0 mg, 0.54 µmol) and **4a** (0.44 mg, 0.80 µmol) in 55 µL 10 mM Tris pH 7.4. Adapted general reaction procedure for **4a.** Yield for **19a** 76% (retention time = 10.5).

HRMS (ESI/QTOF) m/z: $[M + H_3]^{+3}$ Calcd for $C_{89}H_{153}N_{24}O_{24}S_2Si^{+3}$ 678.0228; Found 678.0254.

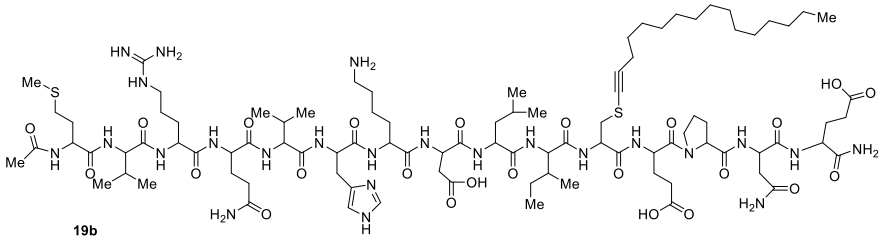
HPLC gradient: Method 1.

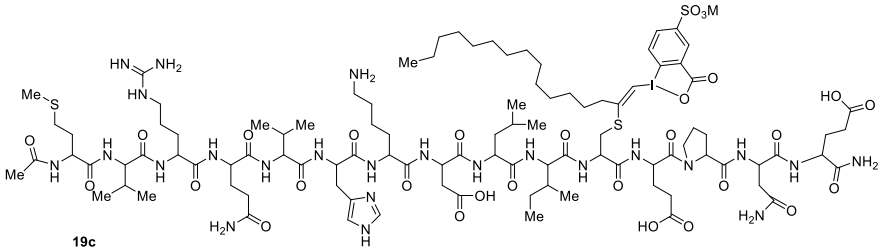
HPLC-UV and HPLC-MS chromatogram



SUPPORTING INFORMATION

Alkynylated Ac-Met-Val-Arg-Gln-Val-His-Lys-Asp-Leu-Ile-Cys-Glu-Pro-Asn-Glu-NH_2 (**19b**) and corresponding VBX (**19c**)





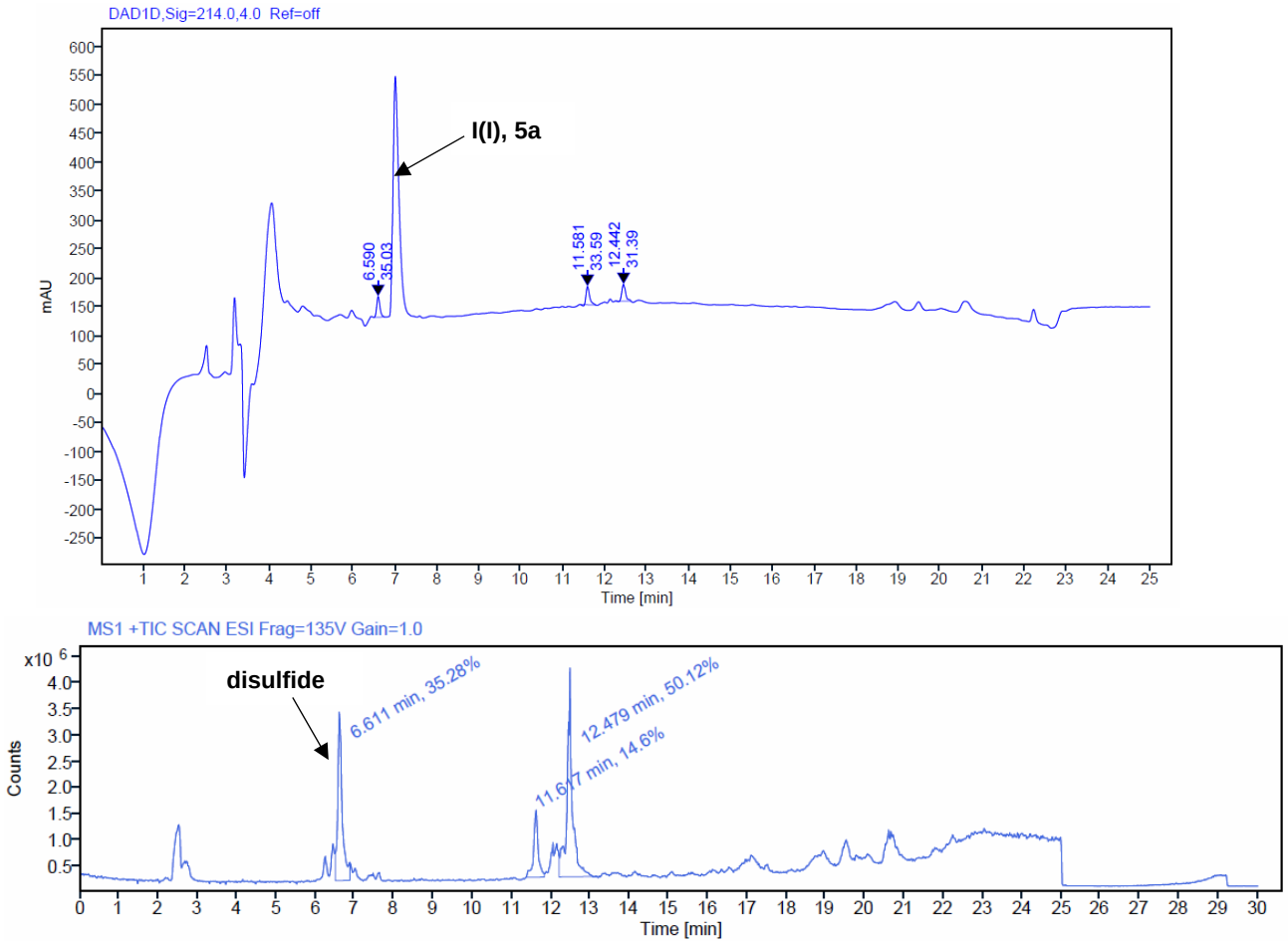
19 (1.0 mg, 0.54 µmol) and **4b** (0.38 mg, 0.64 µmol) in 77 µL 100 mM PB pH 8.0. Adapted general reaction procedure for **4b**. Yield for **19b** 50% (retention time = 12.4), **19c** 15% (retention time = 11.6).

HRMS (ESI/QTOF) m/z: $[M + 2H]^{+2}$ Calcd for **19b** $C_{94}H_{160}N_{24}O_{24}S_2^{+2}$ 1036.5734; Found 1036.5774.

HRMS (ESI/QTOF) m/z: $[M + 2H]^{+2}$ Calcd for **19c** $C_{101}H_{165}IN_{24}O_{29}S_3^{+2}$ 1200.5190; Found 1200.5153.

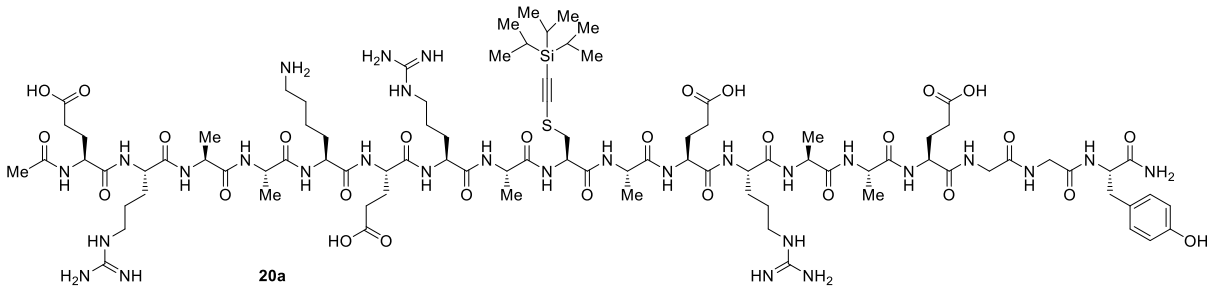
HPLC gradient: Method 1.

HPLC-UV and HPLC-MS chromatogram



SUPPORTING INFORMATION

Alkynylated Ac-Glu-Arg-Ala-Ala-Lys-Glu-Arg-Ala-Cys-Ala-Glu-Arg-Ala-Ala-Glu-Gly-Gly-Tyr-NH_2 (**20a**)

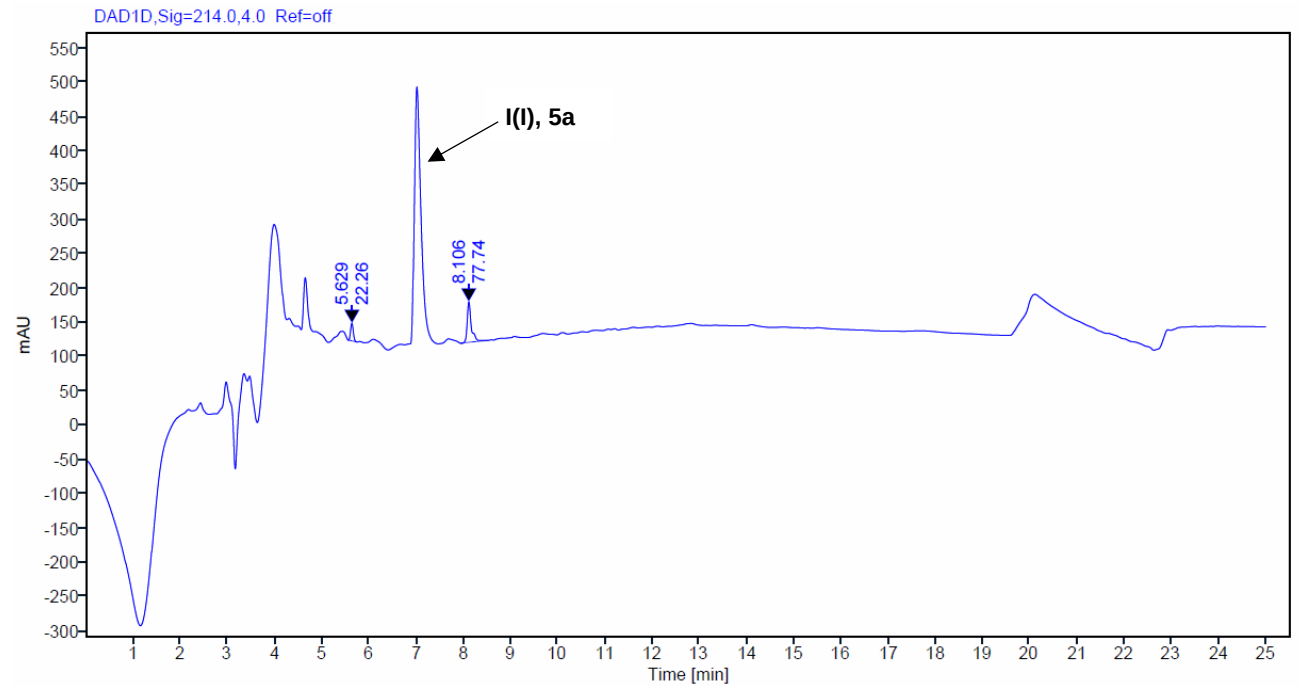


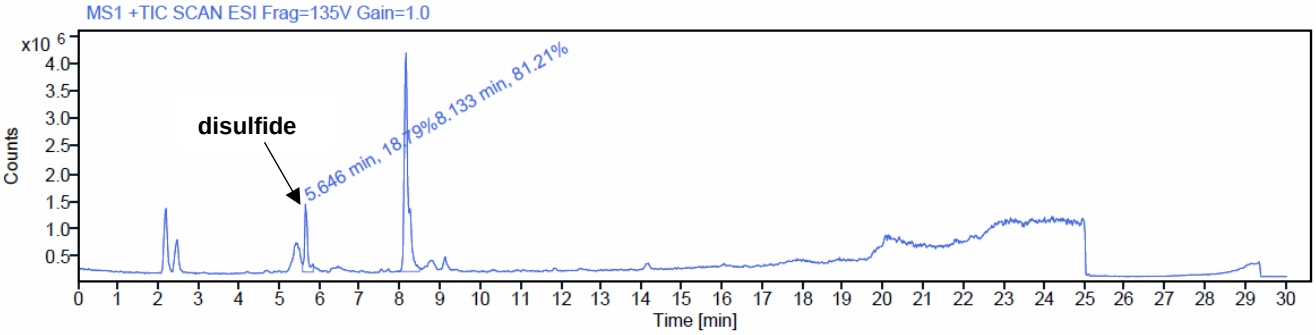
20 (1.0 mg, 0.50 µmol) and **4a** (0.41 mg, 0.75 µmol) in 50 µL 10 mM Tris pH 7.4. Adapted general reaction procedure for **4a**. Yield for **20a** 81% (retention time = 8.1).

HRMS (ESI/QTOF) m/z: $[M + 3H]^{+3}$ Calcd for $C_{91}H_{154}N_{29}O_{28}SSi^{+3}$ 720.3664; Found 720.3661.

HPLC gradient: Method 1.

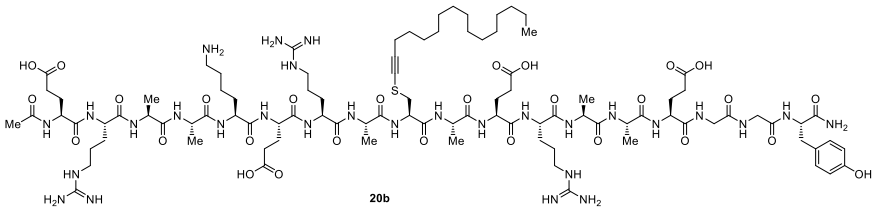
HPLC-UV and HPLC-MS chromatogram

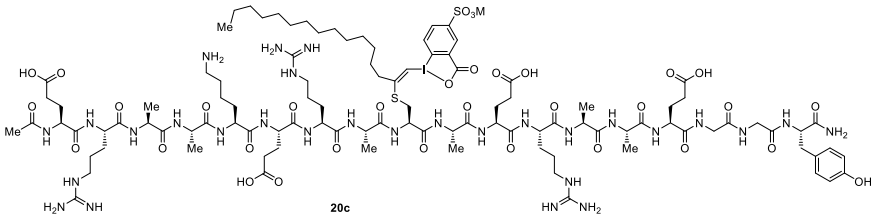




SUPPORTING INFORMATION

Alkynylated Ac-Glu-Arg-Ala-Ala-Lys-Glu-Arg-Ala-Cys-Ala-Glu-Arg-Ala-Ala-Glu-Gly-Gly-Tyr-NH_2 (**20b**) and corresponding VBX (**20c**)





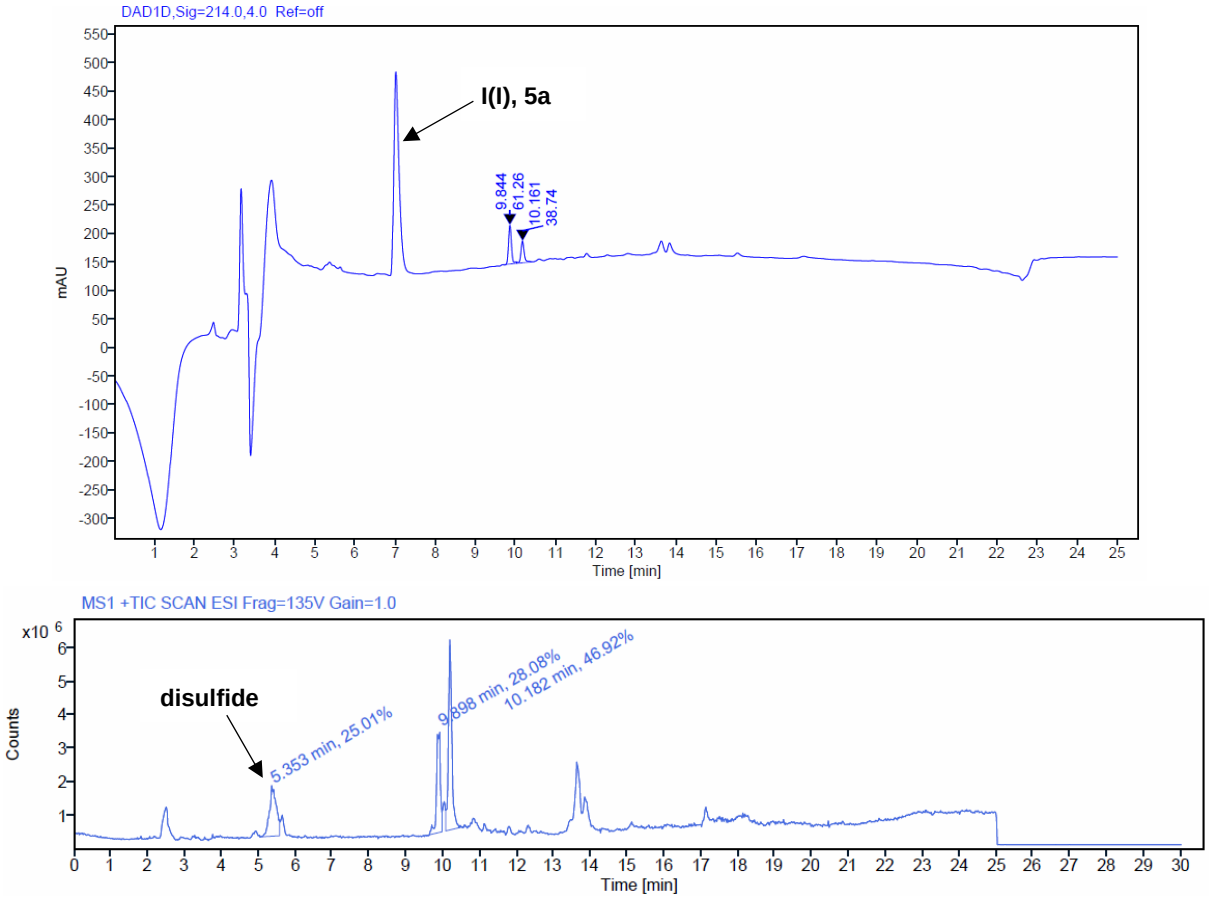
20 (1.0 mg, 0.50 µmol) and **4b** (0.60 mg, 0.35 µmol) in 51 µL 200 mM Tris pH 8.0. Adapted general reaction procedure for **4b.** Yield for **20b** 47% retention time = 10.2), **20c** 28% (retention time = 9.8).

HRMS (ESI/QTOF) m/z: $[M + 2H]^{+2}$ Calcd for **20b** $C_{96}H_{161}N_{29}O_{28}S^{+2}$ 1100.0888; Found 1100.0937.

HRMS (ESI/QTOF) m/z: $[M]^{-2}$ Calcd for **20c** $C_{103}H_{162}IN_{29}O_{33}S_2^{-2}$ 1262.0193; Found 1262.0238.

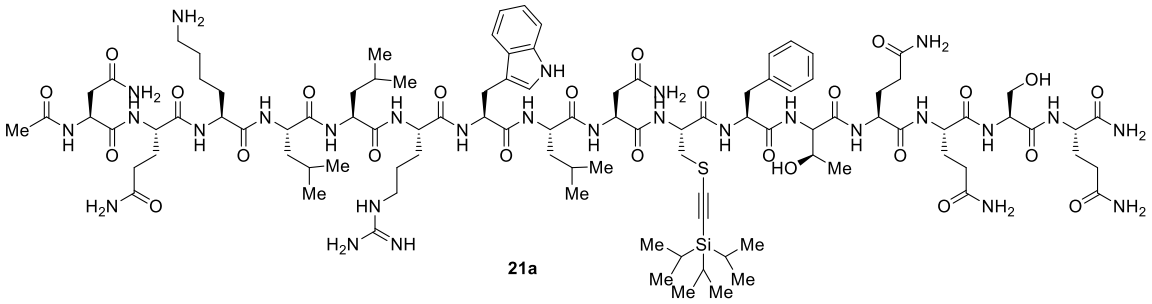
HPLC gradient: Method 1.

HPLC-UV and HPLC-MS chromatogram



SUPPORTING INFORMATION

Alkynylated Ac-Asn-Gln-Lys-Leu-Leu-Arg-Trp-Leu-Asn-Cys-Phe-Thr-Gln-Gln-Ser-Gln-NH_2 (**21a**)



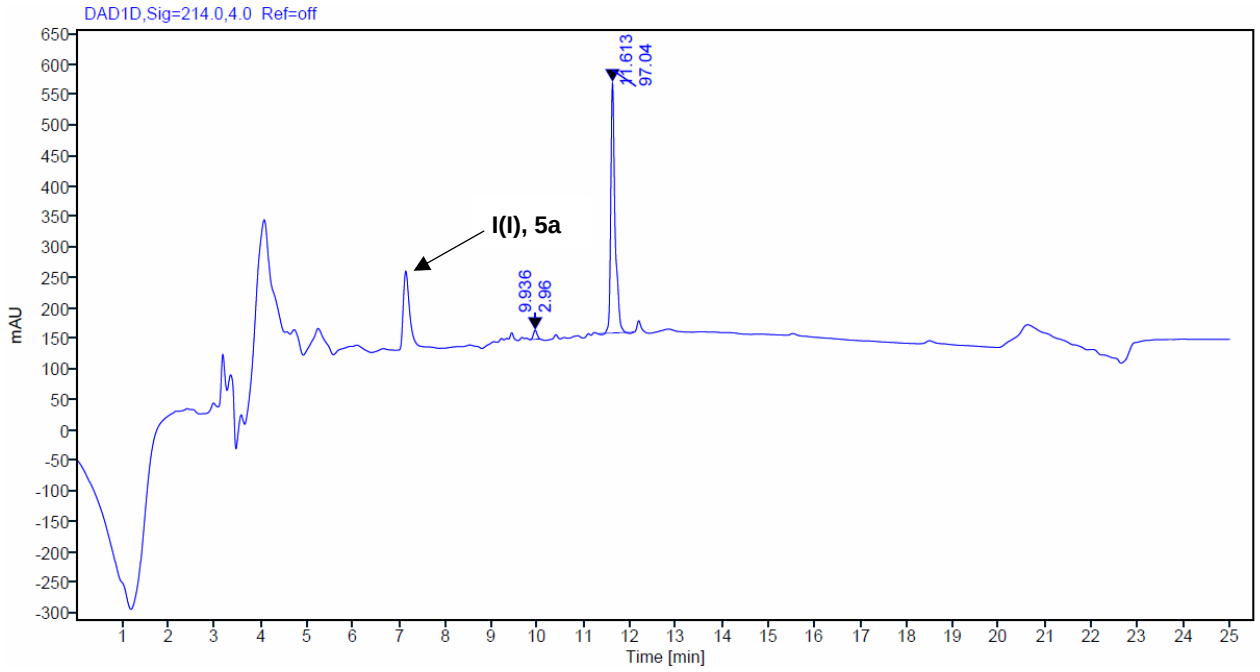
21a

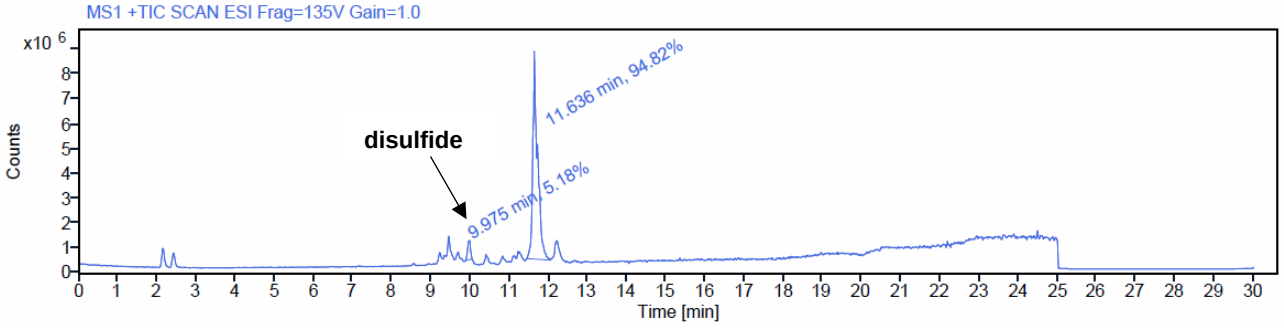
21 (10.0 mg, 4.8 µmol) and **4a** (3.4 mg, 7.3 µmol) in 500 µL 10 mM Tris pH 7.4. Adapted general reaction procedure for **4a**. Yield for **21a** 95% (retention time = 11.6). Isolated yield 51% (5.5 mg, 2.5 µmol, retention time = 14-16 min).

HRMS (ESI/QTOF) m/z: $[M + 3H]^{+3}$ Calcd for **21a** $C_{101}H_{165}N_{28}O_{25}SSi^{+3}$ 743.3991; Found 743.3979.

HPLC gradient: Method 1. **Prep HPLC gradient**: Method 7.

HPLC-UV and HPLC-MS chromatogram





SUPPORTING INFORMATION

Calibration curve of **21a**

Preparation of stock solution. Pure **21a** (0.6 mg) dissolved in 400 uL of MeOH. Stock solution was diluted separately and submitted for RP-HPLC. Calibrated yield 77% (based on HPLC-UV), 38% (based on HPLC-MS).

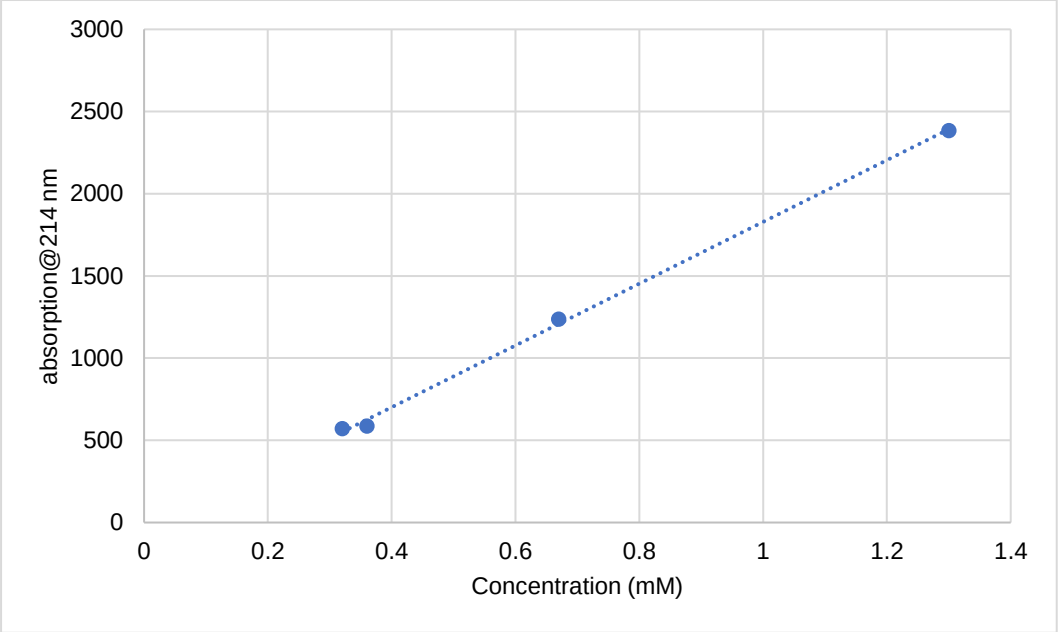


Figure S9. Calibration curve of **21a** based on HPLC-UV at 214nm.

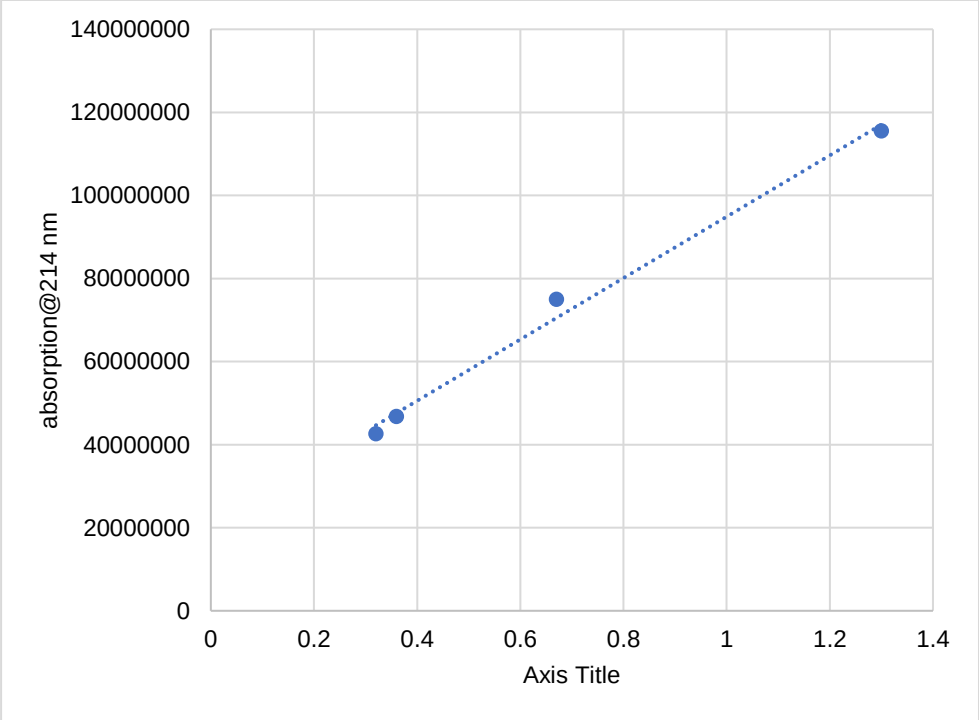
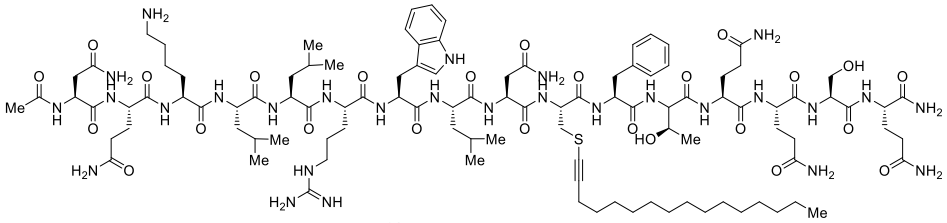


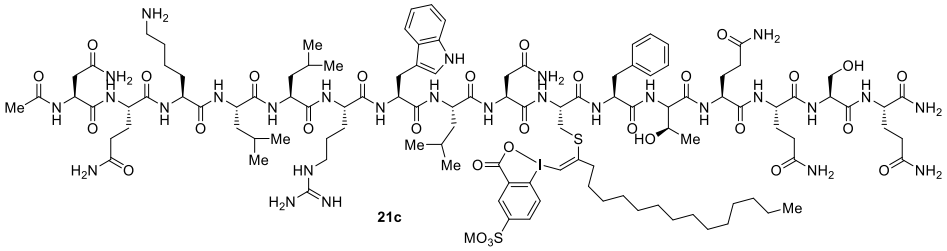
Figure S10. Calibration curve of **21a** based on HPLC-MS.

SUPPORTING INFORMATION

Alkynylated Ac-Asn-Gln-Lys-Leu-Leu-Arg-Trp-Leu-Asn-Cys-Phe-Thr-Gln-Gln-Ser-Gln-NH_2 (**21b**) and corresponding VBX (**21c**)



21b



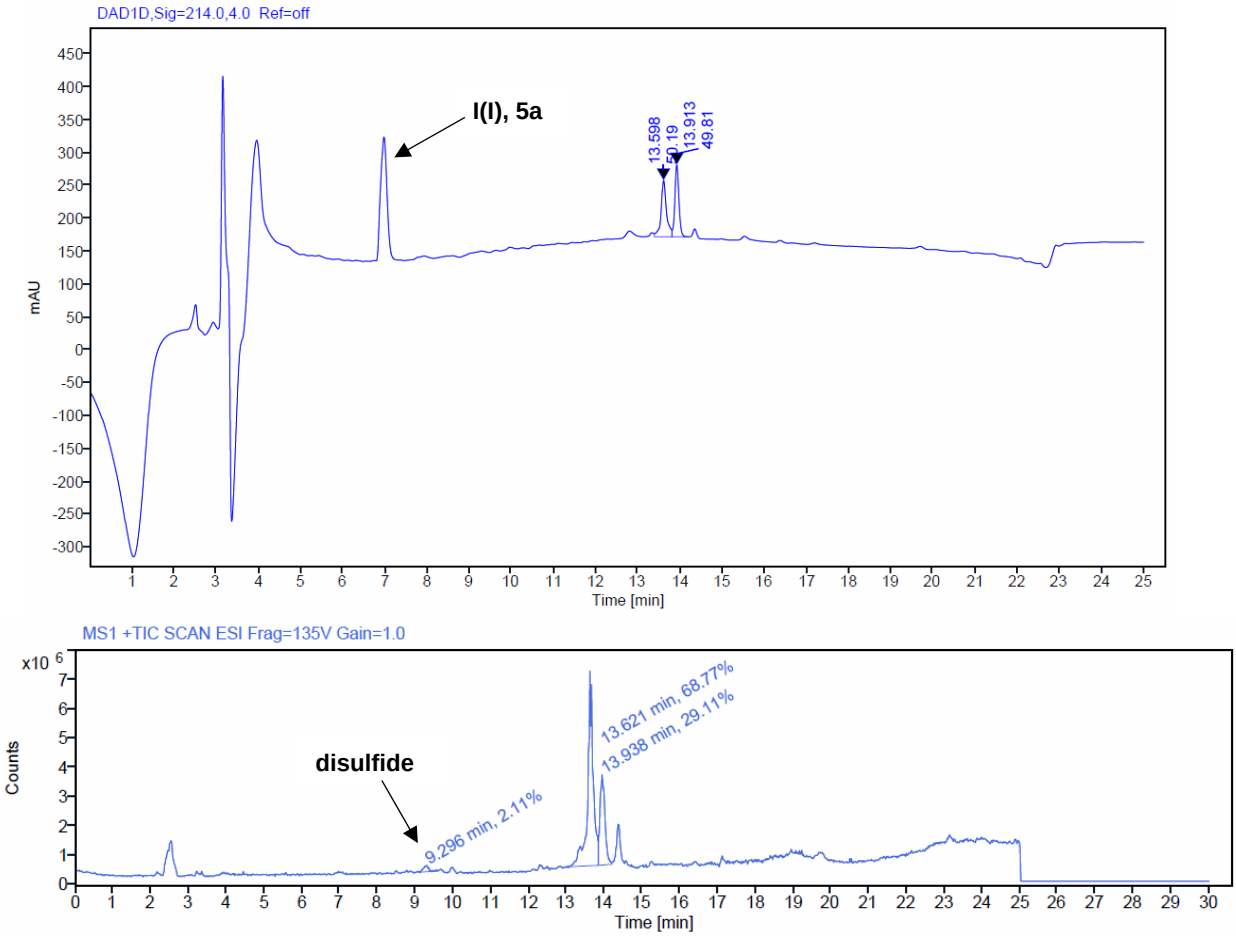
21c

21 (10.0 mg, 4.8 µmol) and **4b** (3.4 mg, 7.3 µmol) in 0.5 mL 200 mM Tris pH 8.0. Adapted general reaction procedure for **4b**. Yield for **21b** 69% (retention time = 13.6), **21c** 29% (retention time = 13.9). Isolated yield 51% (5.5 mg, retention time = 12-14 min.)

HRMS (QTOF) m/z: $[M + H_2]^{+2}$ Calcd for $C_{106}H_{172}N_{28}O_{25}S^{+2}$ 1134.6379; Found 1134.6402.

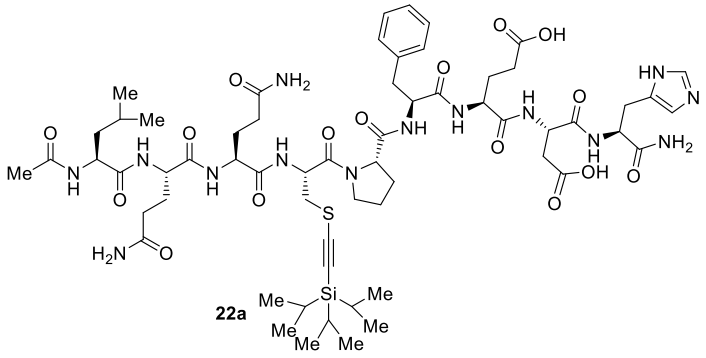
HPLC gradient: Method 1. **Pep-HPLC gradient**: Method 7.

HPLC-UV and HPLC-MS chromatogram



9. Substrate scope for bio-active fragments

Alkynylated Ac-Leu-Gln-Gln-Cys-Pro-Phe-Glu-Asp-His-NH_2 (**22a**)

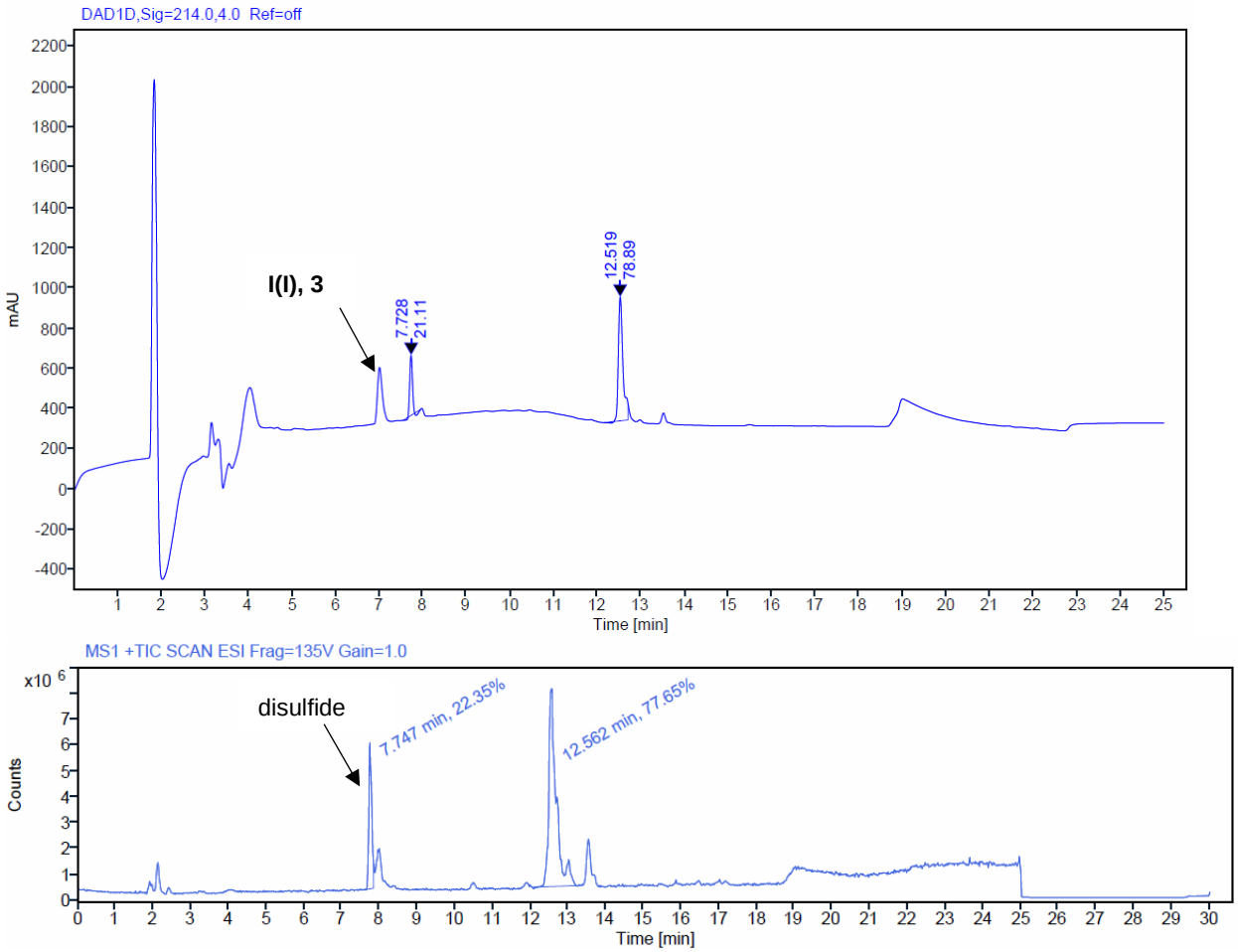


22 (1.0 mg, 0.86 µmol) and **4a** (0.71 mg, 0.73 µmol) in 86 µL 10 mM Tris pH 7.4. Adapted general reaction procedure for **4a**. Yield for **22a** 78% (retention time = 12.5).

HRMS (ESI/QTOF) m/z: $[M + 2H]^{+2}$ Calcd for $C_{61}H_{94}N_{14}O_{16}SSi^{+2}$ 669.3226; Found 669.3252.

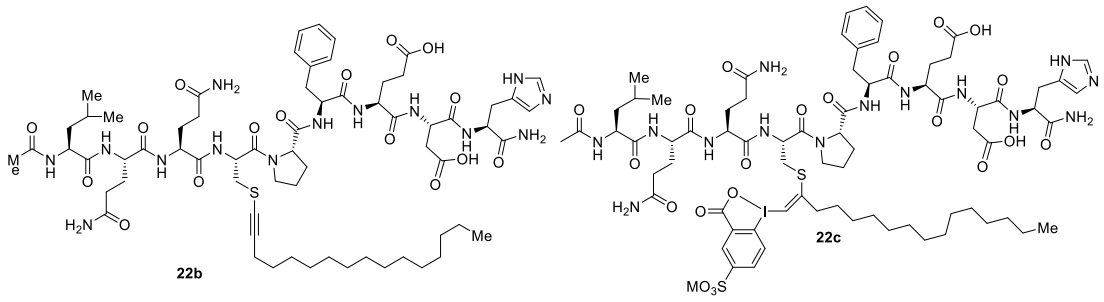
HPLC gradient: Method 1.

HPLC-UV and HPLC-MS chromatogram



SUPPORTING INFORMATION

Alkynylated Ac-Leu-Gln-Gln-Cys-Pro-Phe-Glu-Asp-His-NH_2 (**22b**) and corresponding VBX (**22c**)



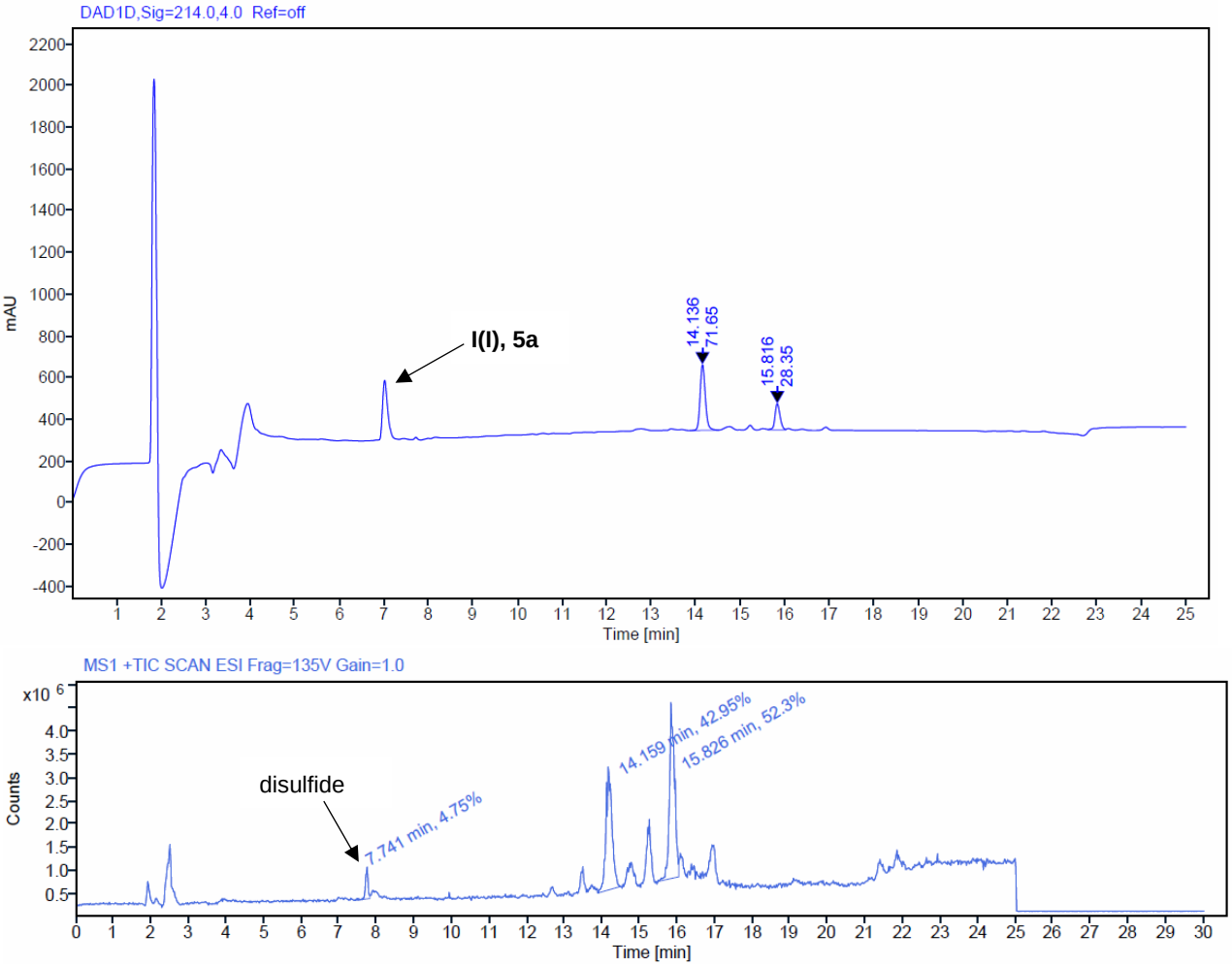
22 (1.0 mg, 0.86 µmol) and **4b** (0.61 mg, 1.0 µmol) in 86 µL 200 mM Tris pH 8.0. Adapted general reaction procedure for **4b**. Yield for **22b** 52% (retention time = 15.8), **22c** 43% (retention time = 14.1).

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for **22b** $C_{66}H_{101}N_{14}O_{16}S^+$ 1377.7235; Found 1377.7200.

HRMS (ESI/QTOF) m/z: $[M]^-$ Calcd for **22c** $C_{73}H_{104}IN_{14}O_{21}S_2^-$ 1703.5992; Found 1703.5969.

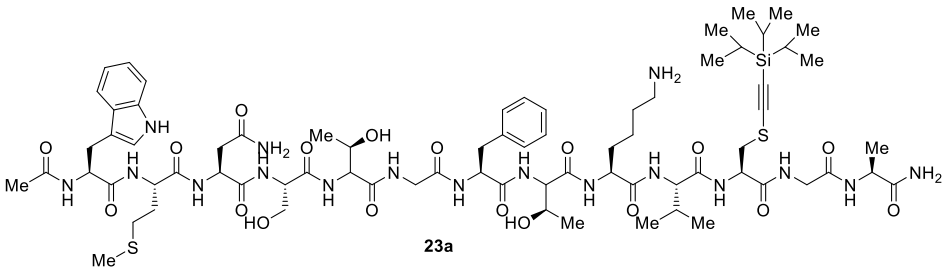
HPLC gradient: Method 1.

HPLC-UV and HPLC-MS chromatogram



SUPPORTING INFORMATION

Alkynylated Ac-Trp-Met-Asn-Ser-Thr-Gly-Phe-Thr-Lys-Val-Cys-Gly-Ala-NH_2 (**23a)**



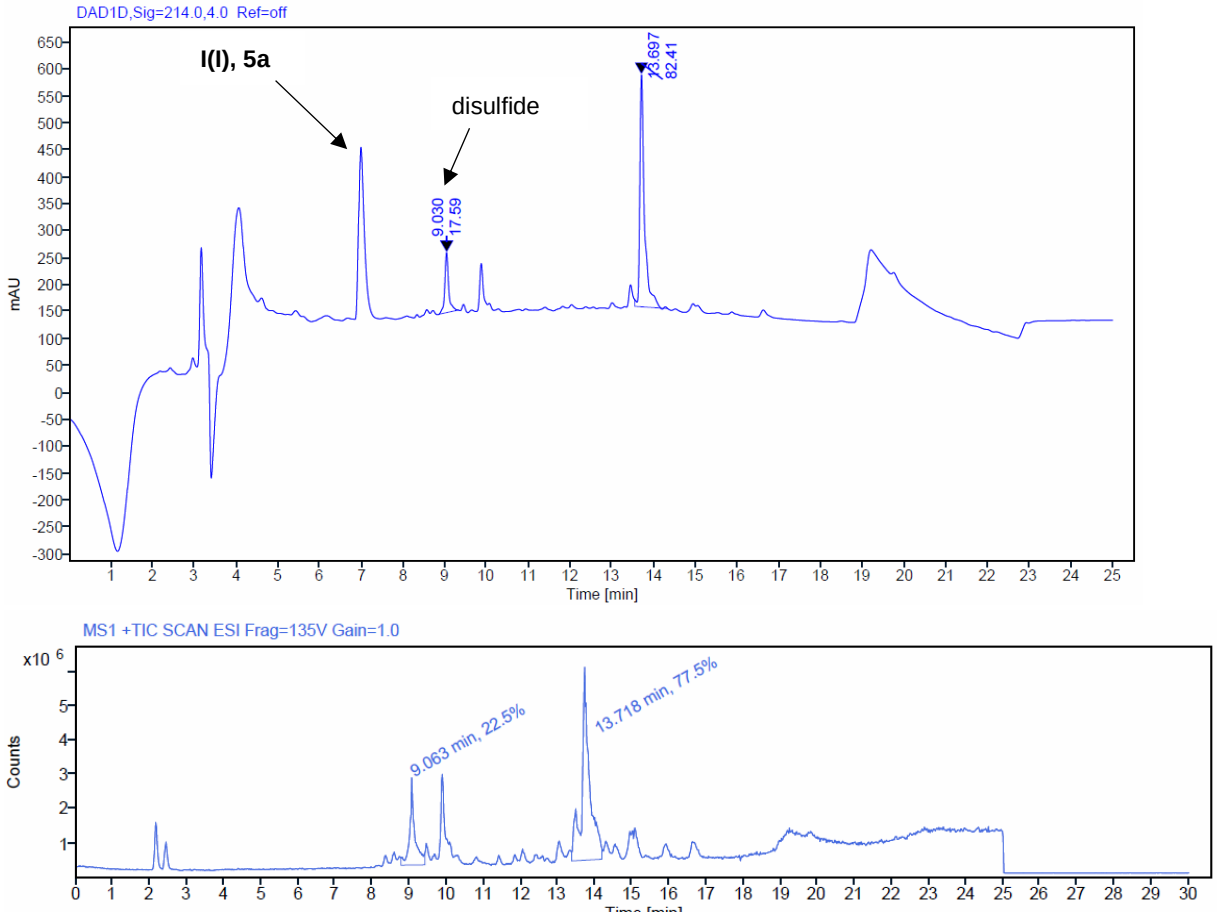
23a

23 (10.0 mg, 6.9 µmol) and **4a** (5.7 mg, 10.4 µmol) in 0.70 mL 10 mM Tris pH 7.4. Adapted general reaction procedure for **4a**. Yield for **23a** 77% (retention time = 13.7). Isolated yield 22% (2.5 mg, retention time = 15-17 min.)

HRMS (ESI/QTOF) m/z: $[M + 2H]^{+2}$ Calcd for $C_{74}H_{117}N_{17}O_{18}S_2Si^{+2}$ 811.8981; Found 811.8995.

HPLC gradient: Method 1. **Prep HPLC gradient**: Method 7.

HPLC-UV and HPLC-MS chromatogram



SUPPORTING INFORMATION

Calibration curve of **23a**

Preparation of stock solution. Pure **23a** (1.0 mg) dissolved in 90 uL of CH_3CN: H_2O (1:1). Stock solution was diluted separately and submitted for RP-HPLC. Calibrated yield, 80% (based on HPLC-UV), 83% (based on HPLC-MS).

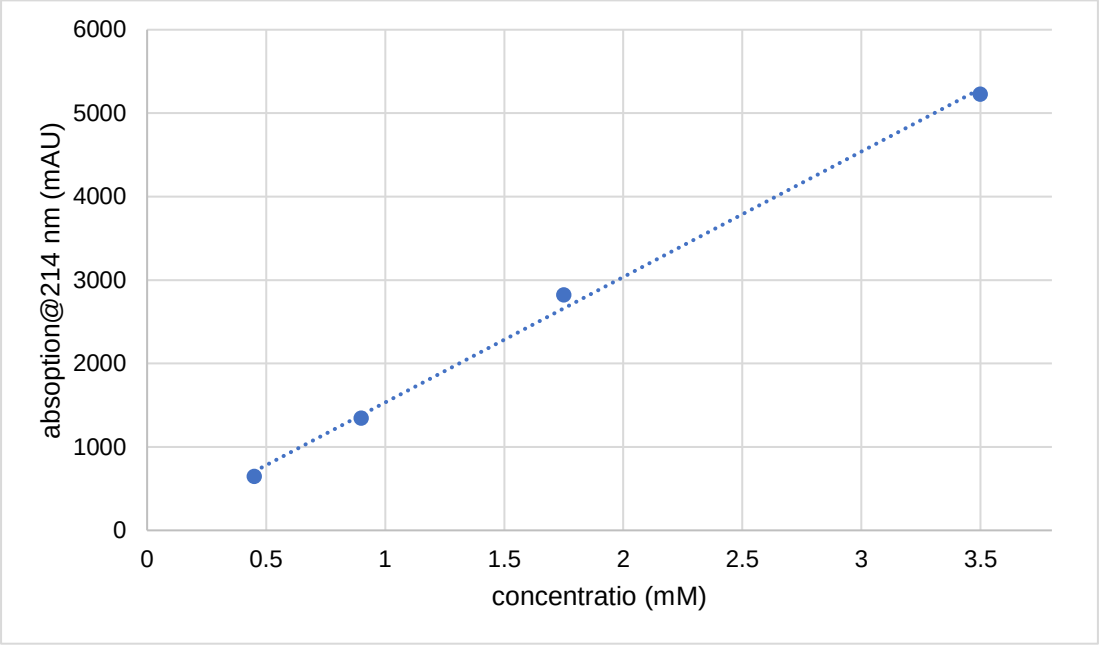


Figure S11. Calibration curve of **23a** based on HPLC-UV at 214nm.

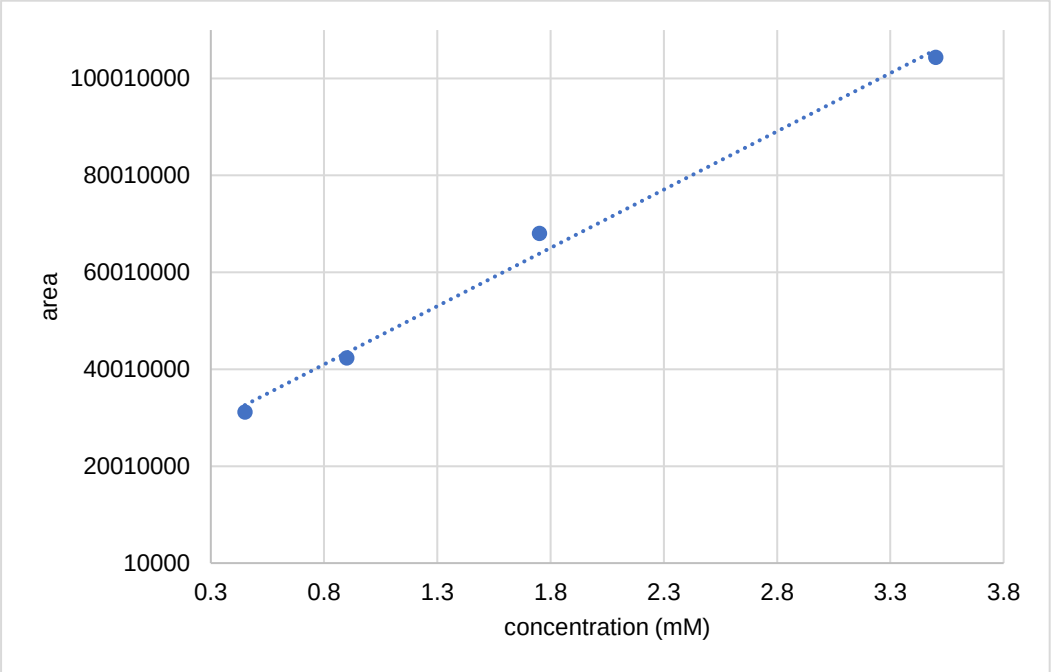
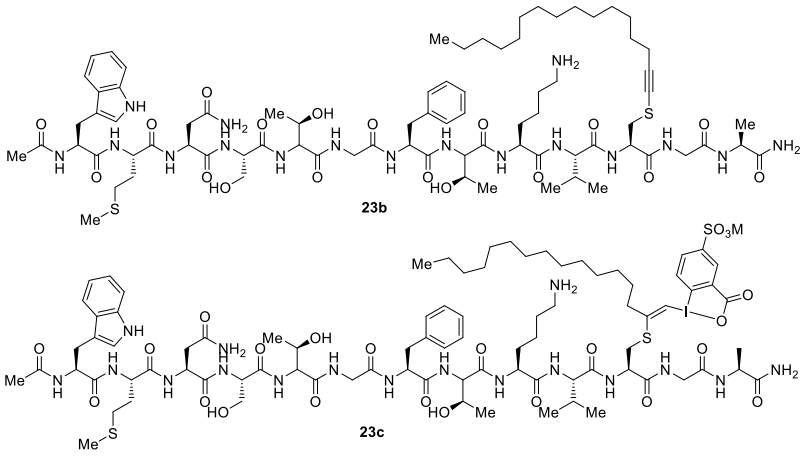


Figure S12. Calibration curve of **23a** based on HPLC-MS.

SUPPORTING INFORMATION

Alkynylated Ac-Trp-Met-Asn-Ser-Thr-Gly-Phe-Thr-Lys-Val-Cys-Gly-Ala-NH_2 (**23b)** and corresponding VBX (**23c**)



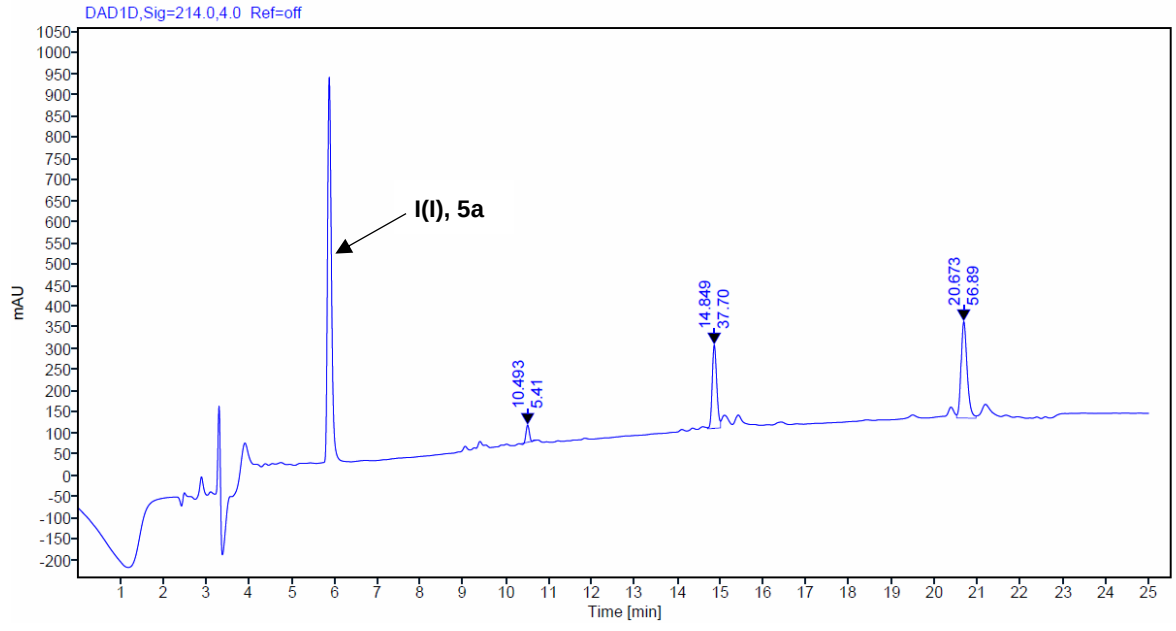
23 (1.0 mg, 0.69 µmol) and **4b** (0.48 mg, 0.83 µmol) in 86 µL 200 mM Tris pH 8.0. Adapted general reaction procedure for **4b**. Yield for **23b** 74% (retention time = 20.7), **23c** 17% (retention time = 14.8).

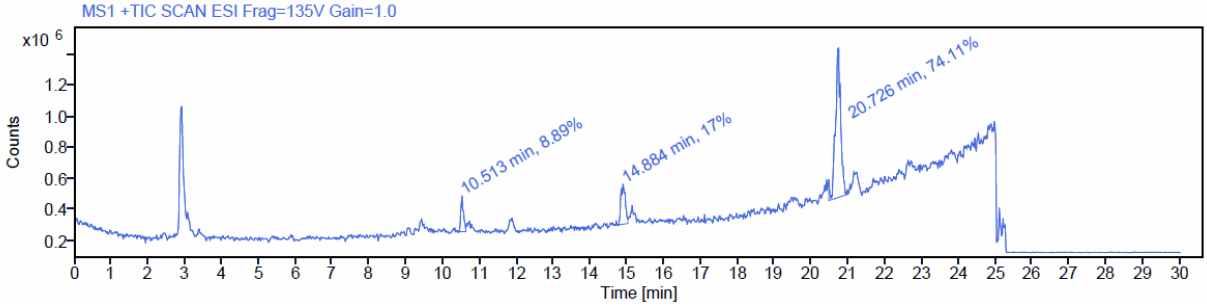
HRMS (ESI/QTOF) m/z: $[M + 2H]^{+2}$ Calcd for **23b** $C_{79}H_{125}N_{17}O_{18}S_2^{+2}$ 831.9409; Found 831.9423.

HRMS (ESI/QTOF) m/z: $[M]^-$ Calcd for **23c** $C_{86}H_{127}IN_{17}O_{23}S_3^-$ 1988.7503; Found 1988.7223.

HPLC gradient: Method 1.

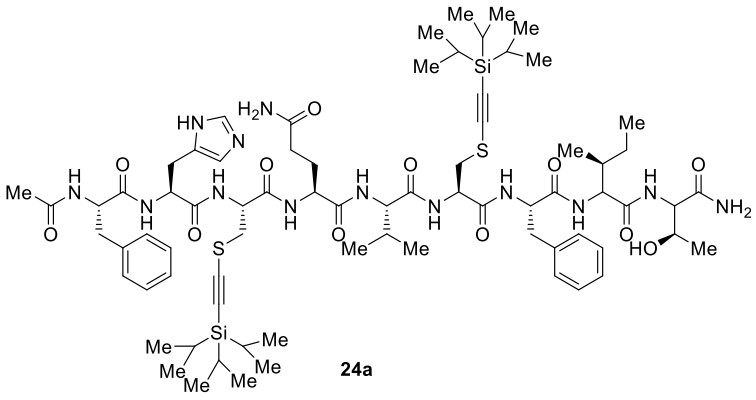
HPLC-UV and HPLC-MS chromatogram





SUPPORTING INFORMATION

Alkynylated (Phe_{32}-Thr_{40}) Ac-Phe-His-Cys-Gln-Val-Cys-Phe-Ile-Thr-NH_2 (**24a**)



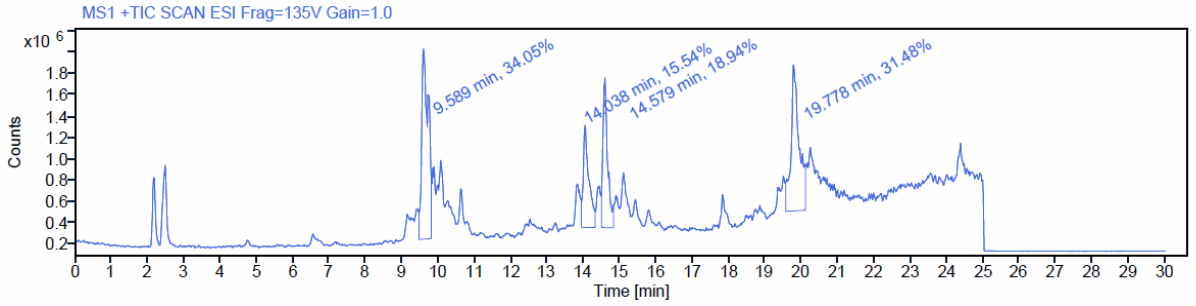
24 (0.50 mg, 0.43 µmol) and **4a** (1.5 mg, 1.0 µmol) in 175 µL 10 mM Tris pH 7.4. Adapted general reaction procedure for **4a**. With 4.0 equiv. **4a,** Yield for **24a** 31% (retention time = 19.7), with 6.0 equiv. of **4a**, 57% for **24a**.

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{74}H_{116}N_{13}O_{12}S_2Si_2^+$ 1498.7841; Found 1498.7869.

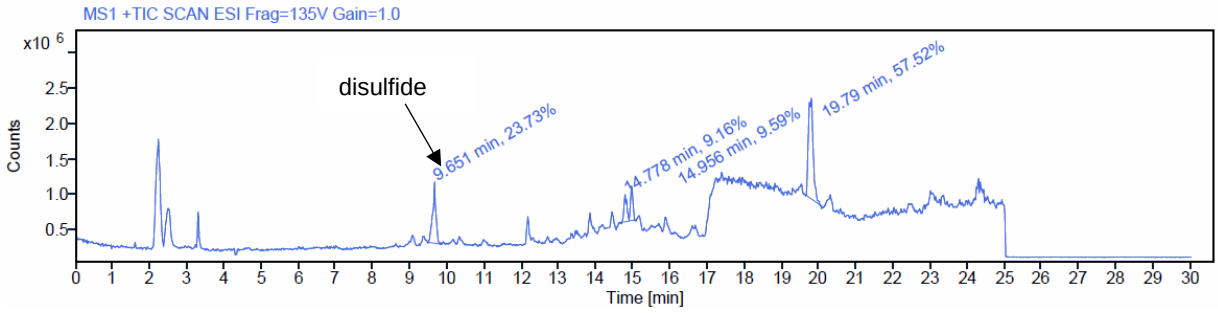
HPLC gradient: Method 1.

HPLC-MS chromatogram

After 14 h with 4.0 equivalent of **4a**



After 14 h with 6.0 equivalent of **4a**



SUPPORTING INFORMATION

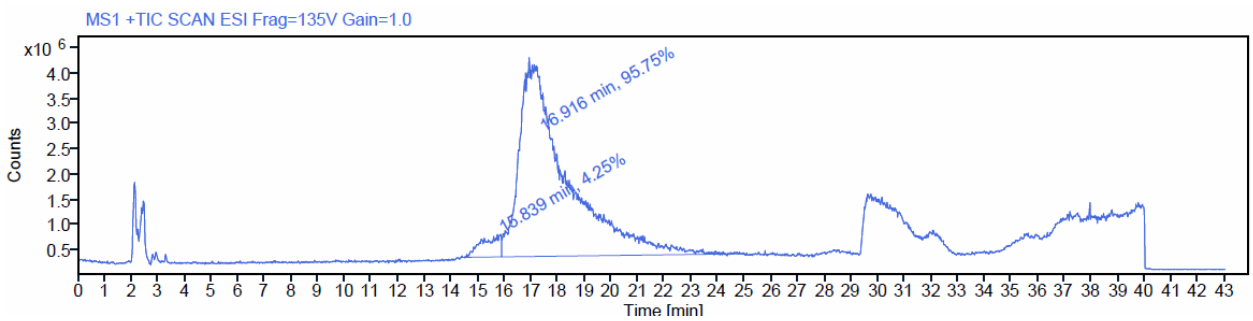
Alkynylated His_6-Cys-Ub (**25a**)

In 1.5 mL vial charged with **25** (0.4 mg, 0.04 µmol, 1.0 equiv.) and **4a** (0.20 mg, 0.36 µmol, 10 equiv.) dissolved in 200 µL 10 mM Tris pH 7.4 with small magnetic bar. Then reaction mixture was heated at 37 °C for 6 h. After 6 h an aliquot of the reaction mixture submitted for HPLC. Yield for **25a** 95% (retention time = 16.9).

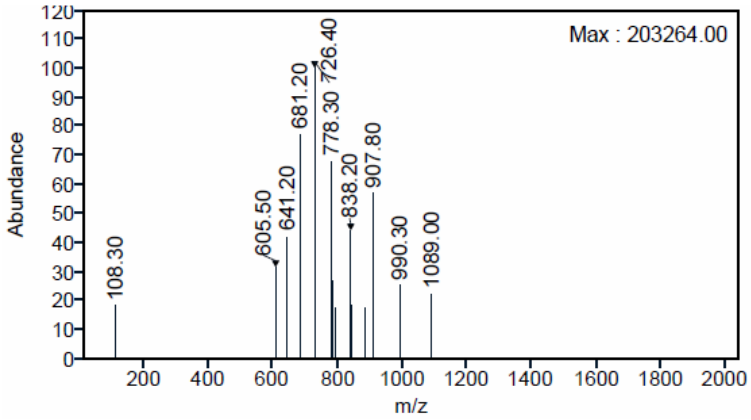
ESI-MS m/z: $[M + 15H]^{+15}$ Calcd for $C_{74}H_{117}N_{17}O_{18}S_2Si^{+15}$ 726.3; Found 726.4.

HPLC gradient: Method 4.

HPLC-UV and HPLC-MS chromatogram

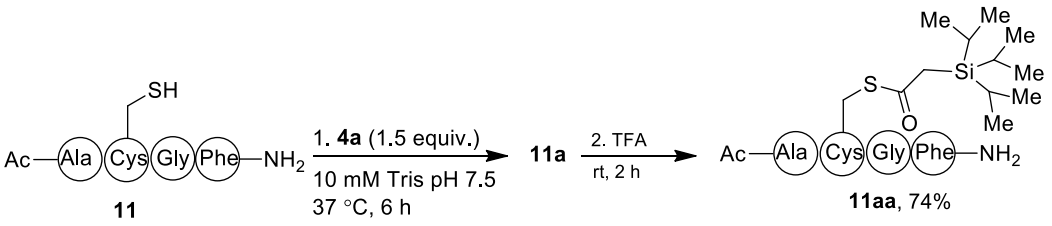


Peak RT: 16.916 min **Area %:** 95.75%



10. Synthesis of thioesters in one-pot

Reaction procedure for preparation of silylthio ester of Ac-Ala-Cys-Gly-Phe-NH_2 **11aa** in one-pot

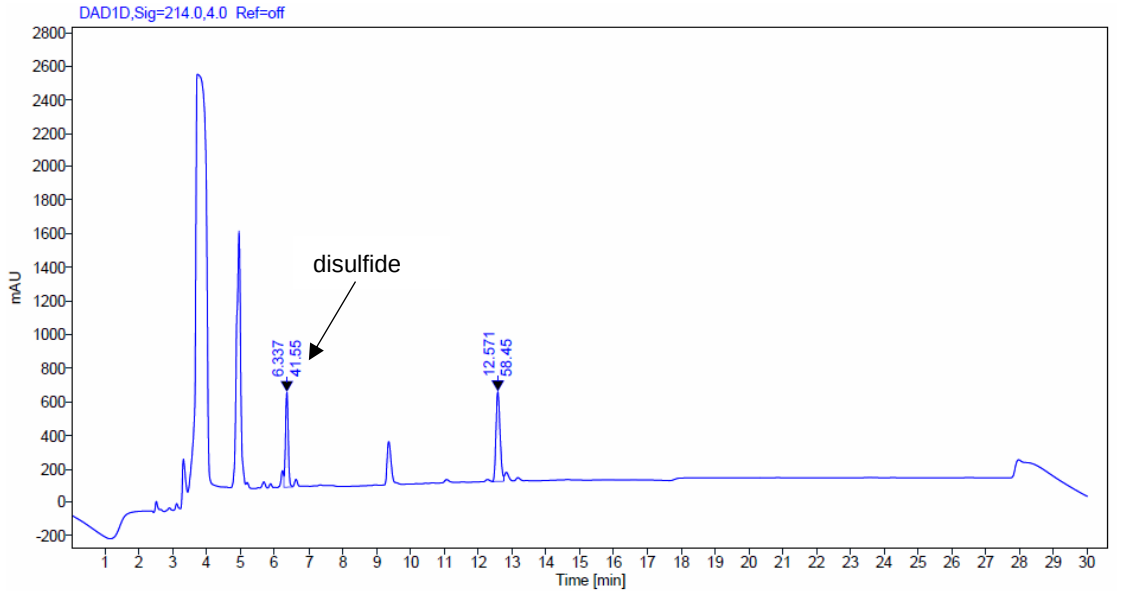


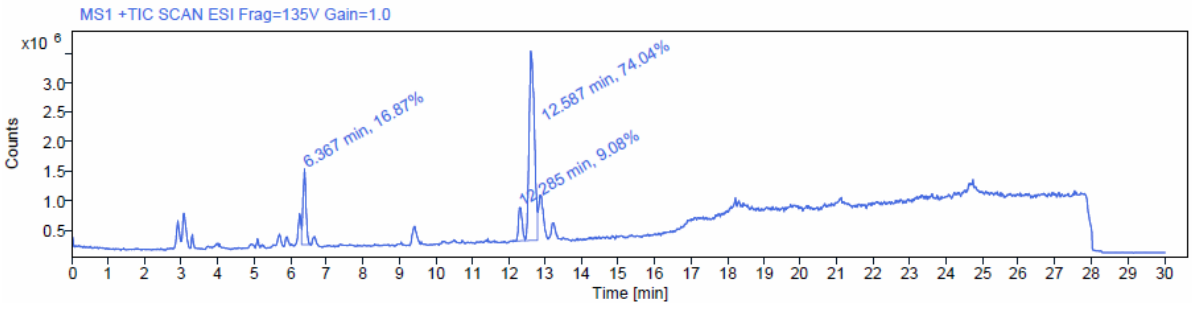
A 1.5 mL vial was charged with **11** (0.50 mg, 1.1 µmol, 1.0 equiv.) and **4a** (1.0 mg, 1.7 µmol, 1.5 equiv.) in 114 µL of 10 mM Tris buffer pH 7.4 with small magnetic stirring bar. The reaction mixture was stirred at 37 °C for 6 h. The buffer was used directly from a freshly prepared solution without degassing. After 6 h, an aliquot of 10 µL of the reaction mixture was diluted with 30 µL acetonitrile:water (1:1) to give a clear solution. The solution was submitted to HPLC. The HPLC revealed the complete consumption of **11**. TFA (50 µL, 0.65 mmol) was added and the reaction mixture was allowed to stir at rt for additional 2 h. After 2 h, 74% **11aa** (retention time = 12.5) and 17% disulfide (retention time = 6.3) were observed by HPLC-MS.

HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{30}H_{50}N_5O_6SSi^+$ 636.3246; Found 636.3258.

HPLC gradient: Method 3.

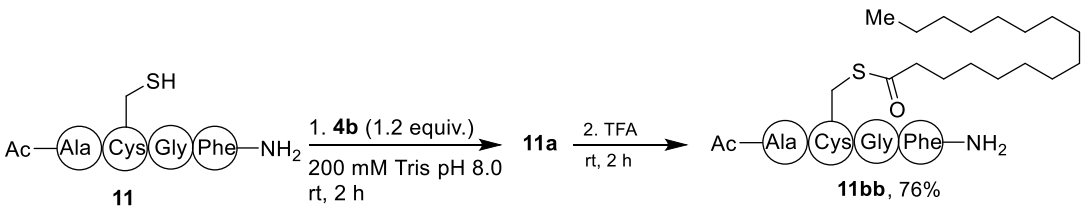
HPLC-UV and HPLC-MS chromatogram





SUPPORTING INFORMATION

Reaction procedure for the preparation of the palmitolyl-ester of Ac-Ala-Cys-Gly-Phe-NH_2 **11bb** in one -pot

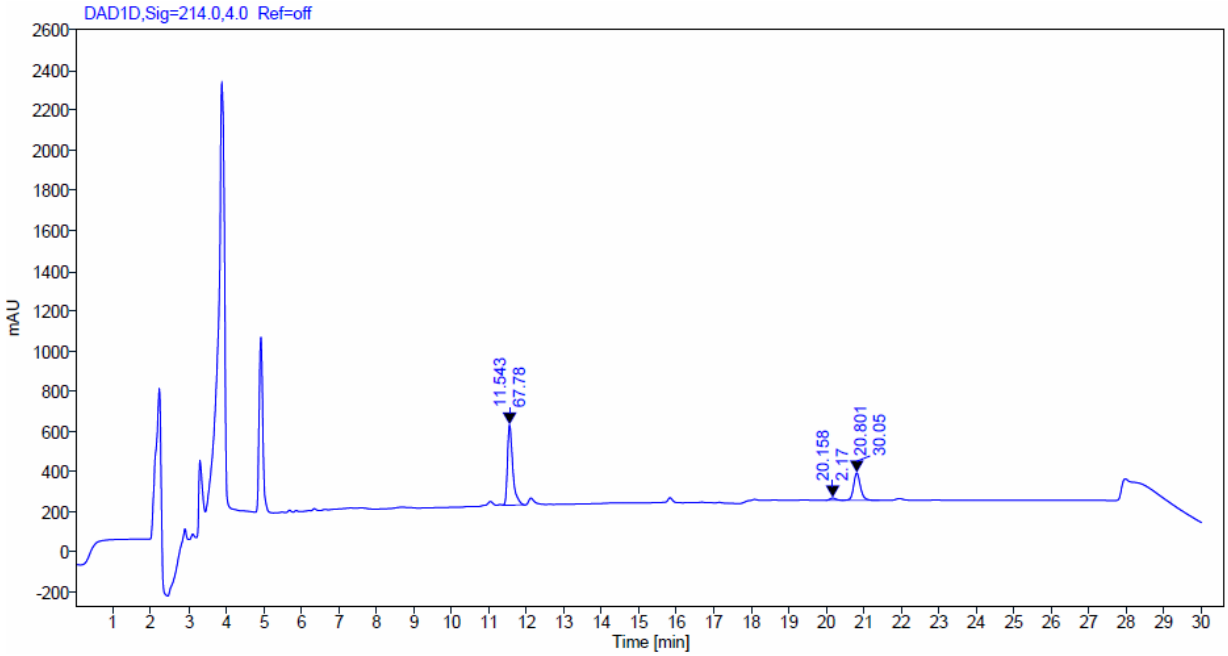


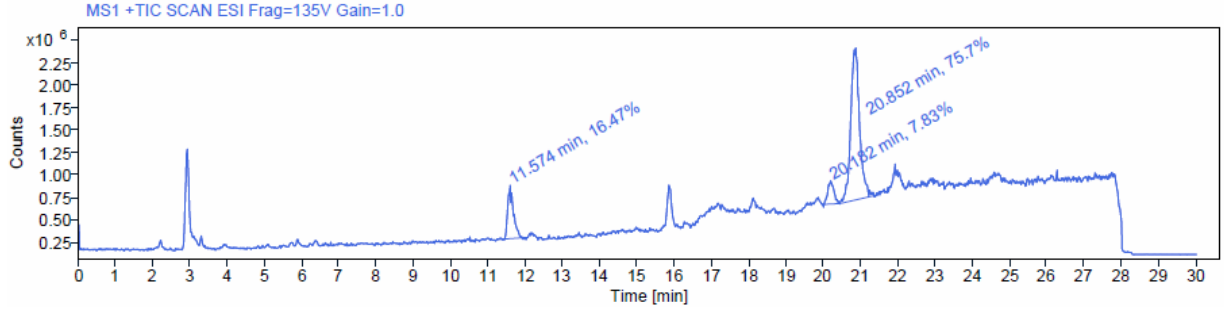
A 1.5 mL vial was charged with **11** (0.50 mg, 1.1 µmol,1.0 equiv.) and **4b** (0.80 mg, 1.4 µmol, 1.2 equiv.) in 114 µL of 200 mM Tris buffer pH 8.0 with a small magnetic stirring bar. The reaction mixture was stirred at rt for 2 h. The buffer was used directly from a freshly prepared solution without degassing. After 2 h, an aliquot of 10 µL of the reaction mixture was diluted with 30 µL acetonitrile:water (1:1) to give a clear solution. The solution was submitted to HPLC. The HPLC revealed the complete consumption of **11**. TFA (50 µL, 0.65 mmol) was added and the reaction mixture was allowed to stir at rt for additional 2 h. After 2 h, 76% **11bb** (retention time = 20.8) and 16% VBX (**11c**, retention time = 11.5) were observed by HPLC-MS.

HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{35}H_{58}N_5O_6S^+$ 676.4102; Found 676.4114.

HPLC gradient: Method 3.

HPLC-UV and HPLC-MS chromatogram





SUPPORTING INFORMATION

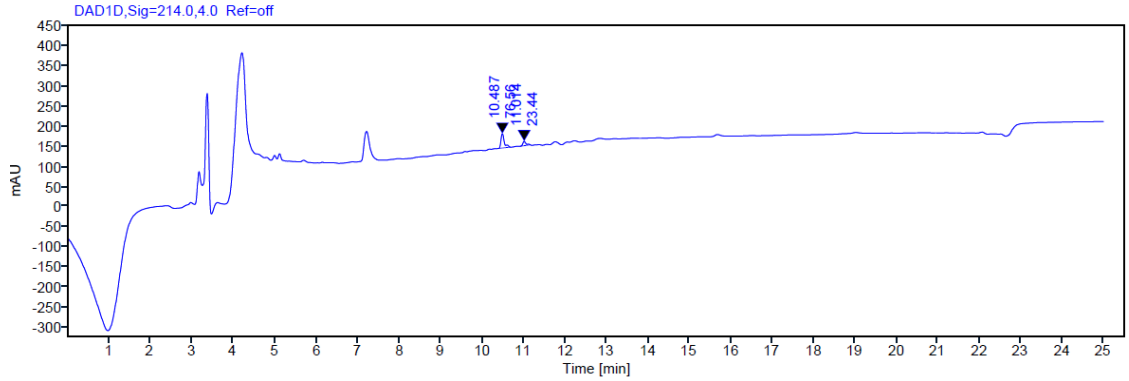
Preparation of the silylthioester of H-Gly-Cys-Ala-Phe-Lys-Thr-NH_2, **17aa**

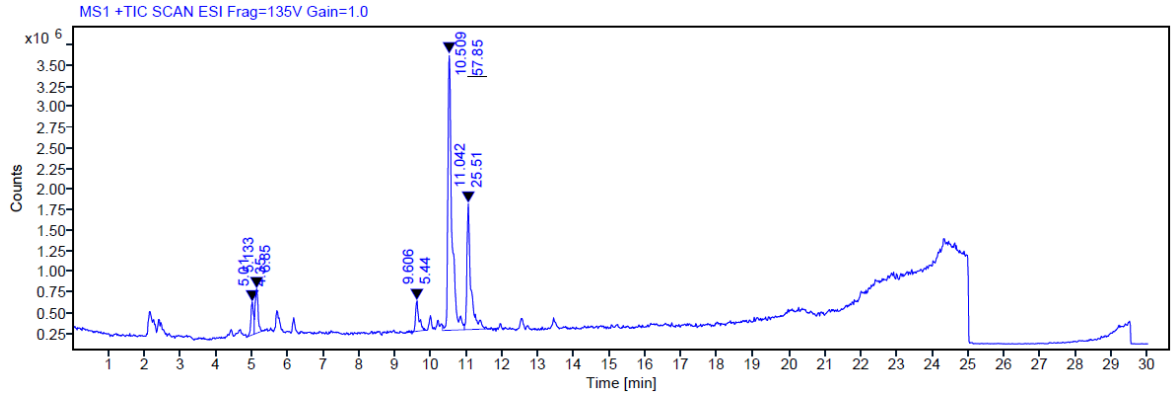
A 5 mL vial was charged with **17** (10.0 mg, 15.7 µmol,1.0 equiv.) and **4a** (13.0 mg, 23.5 µmol, 1.5 equiv.) in 1.0 mL of 10 mM Tris buffer pH 7.4 with a small magnetic stirring bar. The reaction mixture was stirred at 37°C for 5 h. After 5 h, an aliquot of 10 µL of the reaction mixture was diluted with 80 µL acetonitrile: water (1:1). The solution was submitted to HPLC. The HPLC revealed the complete consumption of **17**. TFA (1.0 mL) was added and the reaction mixture was allowed to stir at rt for additional 2 h. After 2 h, an aliquot of 10 µL of the reaction mixture was diluted with 90 µL acetonitrile: water (1:1) to give a clear solution. The solution was submitted to HPLC. 58% **17aa** (retention time = 10.4) was observed by HPLC-MS.

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{38}H_{67}N_8O_8SSi^+$ 823.4566; Found 823.4560.

HPLC gradient: Method 3.

HPLC-UV and HPLC-MS chromatogram





SUPPORTING INFORMATION

Preparation of the palmitoyl thioester of H-Gly-Cys-Ala-Phe-Lys-Thr-NH_2, **17bb**

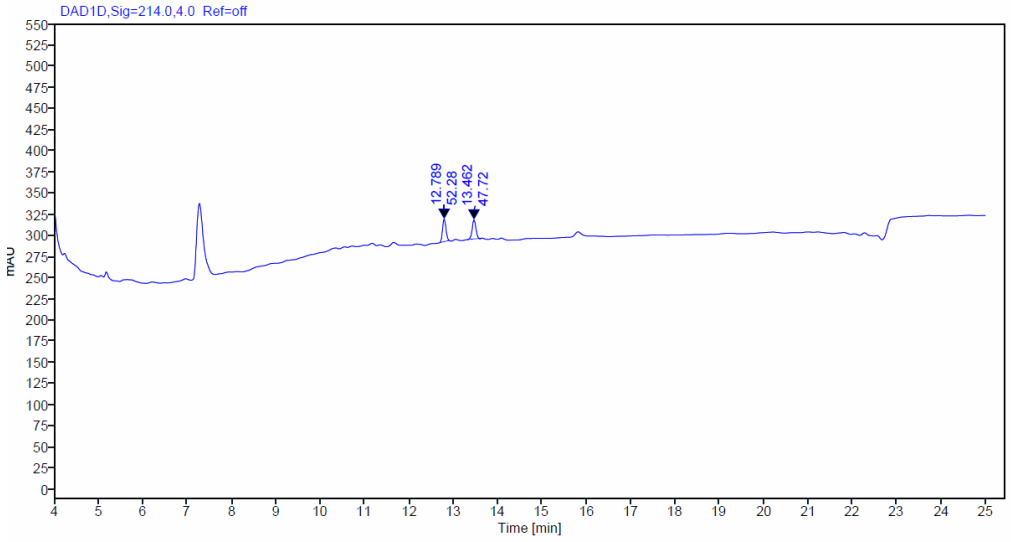
A 5 mL vial was charged with **17** (15.0 mg, 23.5 µmol,1.0 equiv.) and **4b** (17.0 mg, 28.2 µmol, 1.2 equiv.) in 1.6 mL of 200 mM Tris buffer pH 8.0 with a small magnetic stirring bar. The reaction mixture was stirred at rt for 2 h. The buffer was used directly from a freshly prepared solution without degassing. After 2 h, an aliquot of 10 µL of the reaction mixture was diluted with 80 µL acetonitrile:water (1:1) to give a clear solution. The solution was submitted to HPLC. The HPLC revealed the complete consumption of **11**. TFA (1.5 mL) was added and the reaction mixture was allowed to stir at rt for additional 2 h. After 2 h, an aliquot of 10 µL of the reaction mixture was diluted with 90 µL acetonitrile:water (1:1) to give a clear solution. The solution was submitted to HPLC. 73% **17bb** (retention time = 13.4) and 21% VBX (**17c**, retention time = 12.8) were observed by HPLC-MS. Isolated Yield of **17bb**: 34% (7.0 mg, 8.1 µmol, retention time 12-15 min).

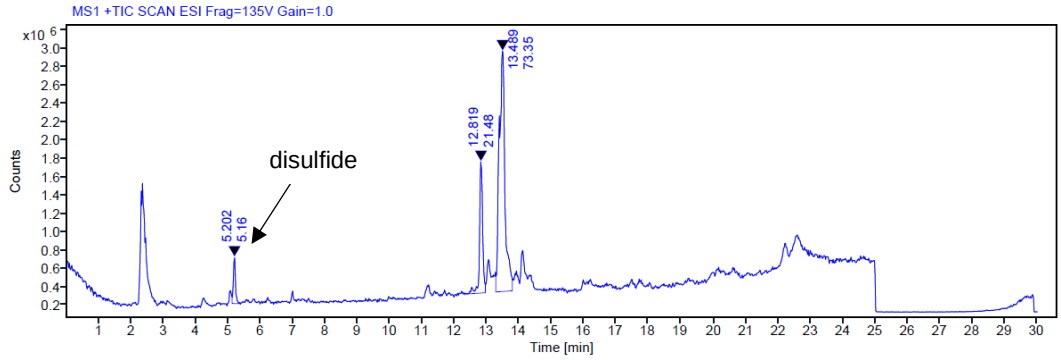
HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{43}H_{75}N_8O_8S^+$ 863.5423; Found 863.5425.

HPLC gradient: Method 1.

Prep HPLC Gradient: Method 5.

HPLC-UV and HPLC-MS chromatogram





SUPPORTING INFORMATION

Calibration curve of **17bb**

Preparation of stock solution. Pure **17bb** (0.5 mg) dissolved in 116 μL of CH_3CN: H_2O (1:1). Stock solution was diluted separately and submitted for RP-HPLC. Calibrated yield 50% (based on HPLC-UV), 40% (based on HPLC-MS).

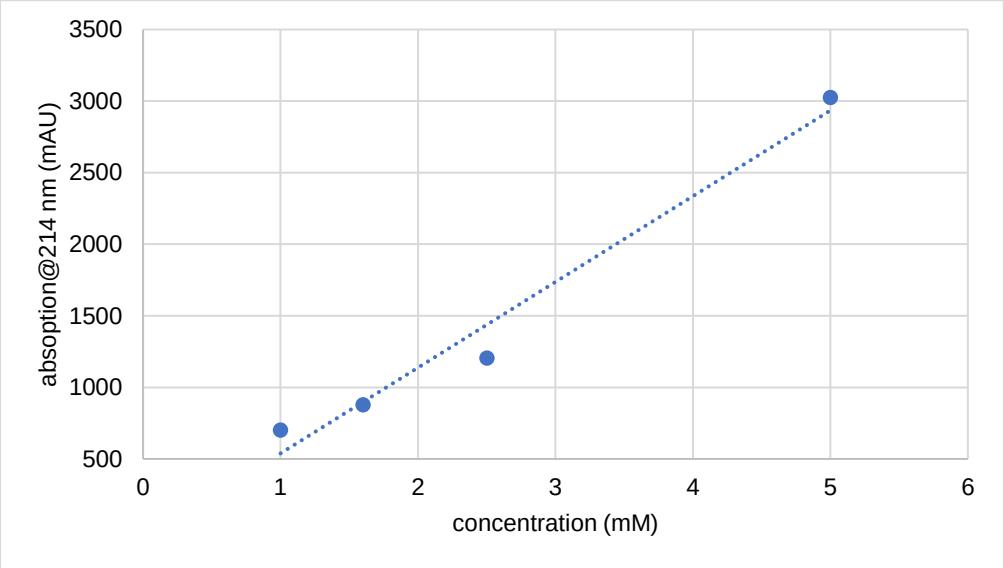


Figure S13. Calibration curve of **17bb** based on HPLC-UV absorption at 214 nm.

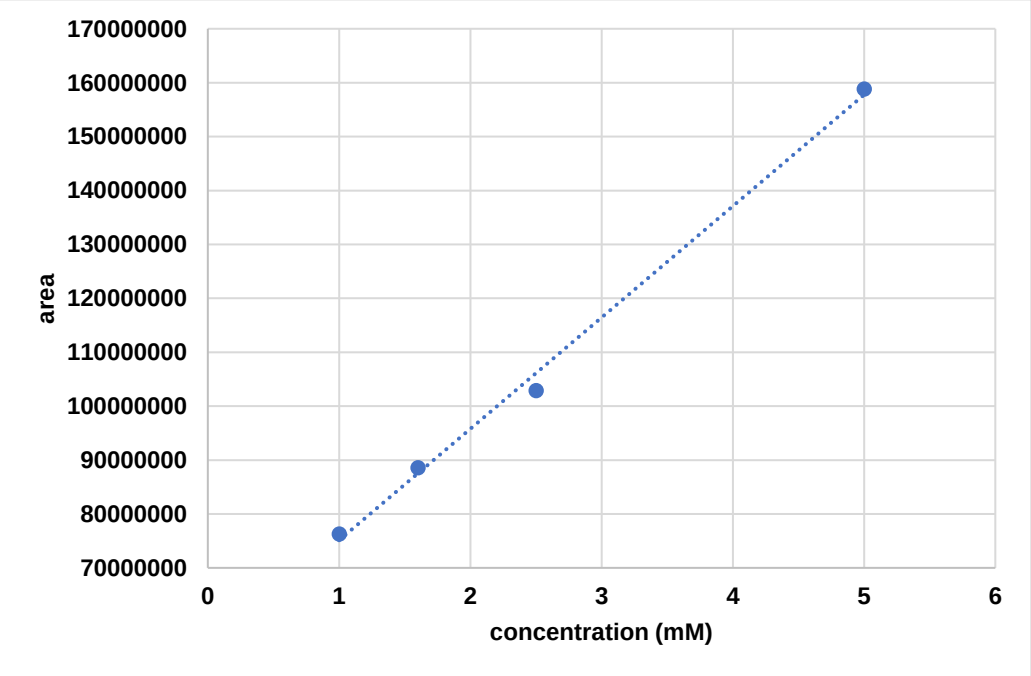


Figure S14. Calibration curve of **17bb** based on HPLC-MS.

SUPPORTING INFORMATION

Preparation of the silylthioester of H-Ala-Cys-Ala-Phe-Lys-Asp-NH_2, **18aa**

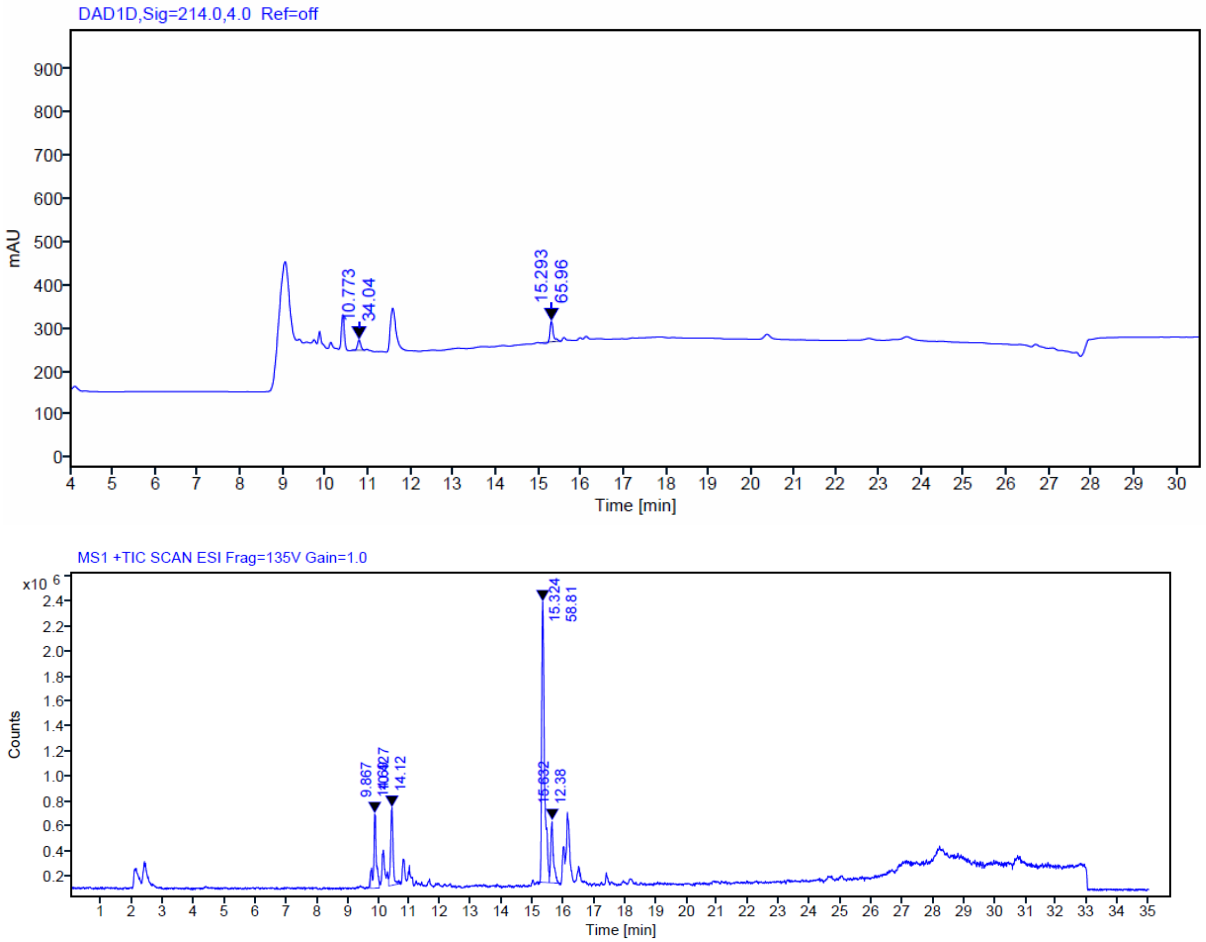
A 5 mL vial was charged with **17** (10.0 mg, 15.3 µmol,1.0 equiv.) and **4a** (9.8 mg, 22.9 µmol, 1.5 equiv.) in 1.0 mL of 10 mM Tris buffer pH 7.4 with a small magnetic stirring bar. The reaction mixture was stirred at 37°C for 5 h. The freshly prepared buffer was used without degassing. After 5 h, an aliquot of 10 µL of the reaction mixture was diluted with 80 µL acetonitrile: water (1:1). The solution was submitted to HPLC. The HPLC revealed the complete consumption of **17**. TFA (1.0 mL) was added and the reaction mixture was allowed to stir at rt for additional 2 h. After 2 h, an aliquot of 10 µL of the reaction mixture was diluted with 90 µL acetonitrile: water (1:1) to give a clear solution. The solution was submitted to HPLC. 58% **18aa** (retention time = 15.3) was observed by HPLC-MS. Isolated Yield of **18aa** 35% (4.5 mg, 5.4 µmol, retention time = 16-17 min)

HPLC gradient: Method 2.

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{39}H_{67}N_8O_9SSi^+$ 851.4515; Found 851.4521.

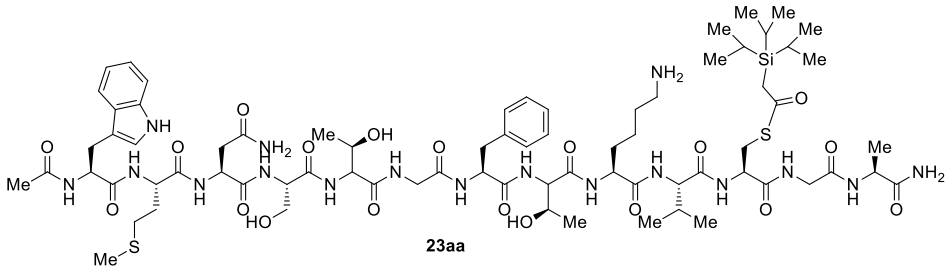
Prep-HPLC gradient: Method 5.

HPLC-UV and HPLC-MS chromatogram



SUPPORTING INFORMATION

Preparation of the silylthioester of Ac-Trp-Met-Asn-Ser-Thr-Gly-Phe-Thr-Lys-Val-Cys-Gly-Ala-NH_2, **23aa**

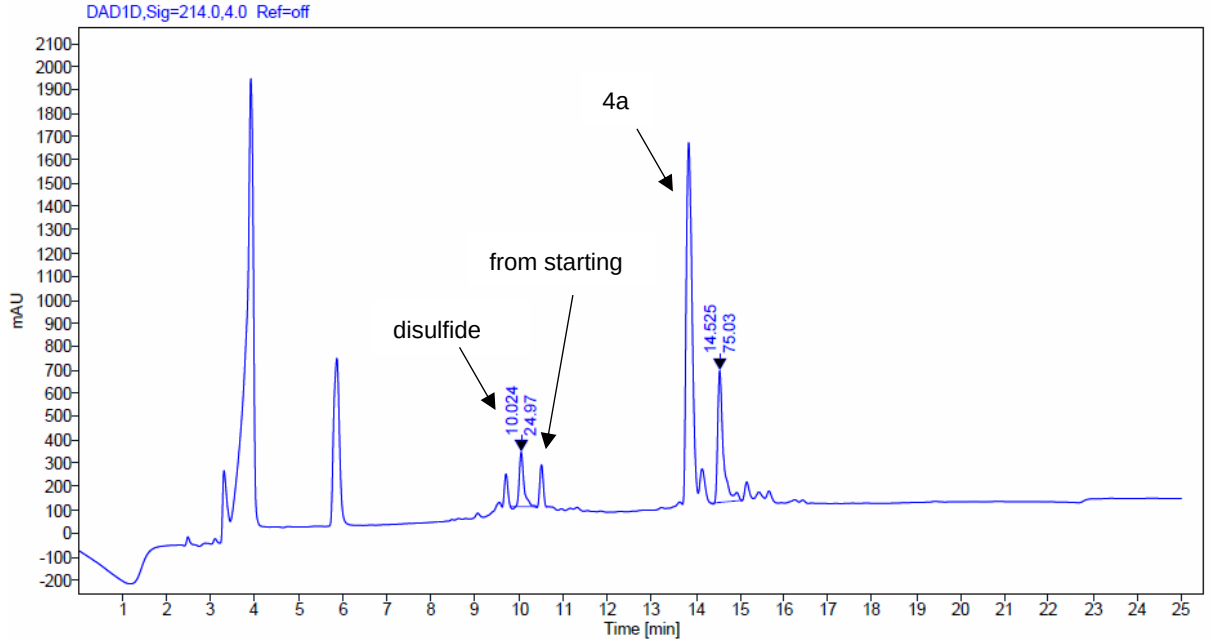


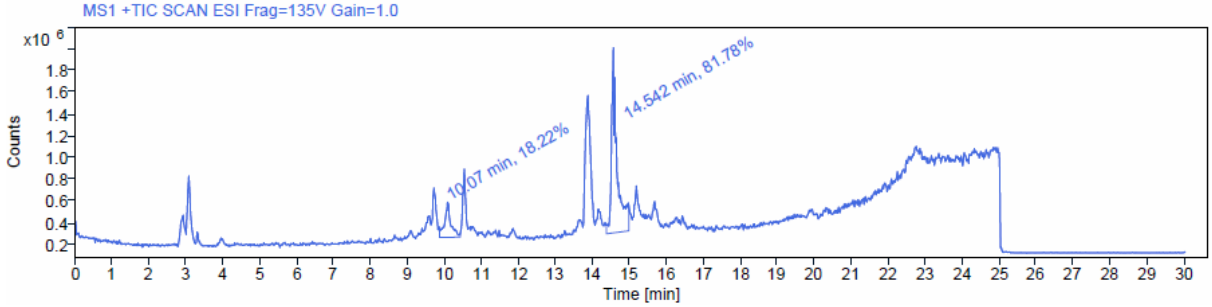
23 (1.0 mg, 0.69 µmol) and **4a** (0.60 mg, 1.0 µmol) were dissolved in 70 µL of 10 mM Tris buffer pH 7.4. Then 50 µL TFA was added. The same procedure was followed as for **11aa**. HPLC-MS yield for **23aa** was 82% (retention time = 14.5).

HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + 2H]^{+2}$ Calcd for **23aa** $C_{74}H_{119}N_{17}O_{19}S_2Si^{+2}$ 820.9034; Found 820.9073.

HPLC gradient: Method 1.

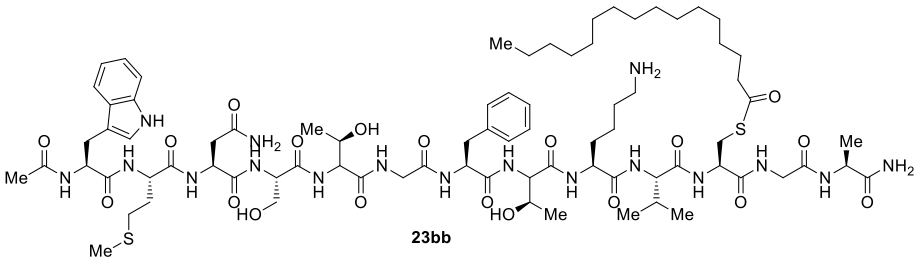
HPLC-UV and HPLC-MS chromatogram





SUPPORTING INFORMATION

Preparation of the palmitoylthioester of Ac-Trp-Met-Asn-Ser-Thr-Gly-Phe-Thr-Lys-Val-Cys-Gly-Ala-NH_2 **(23bb)**



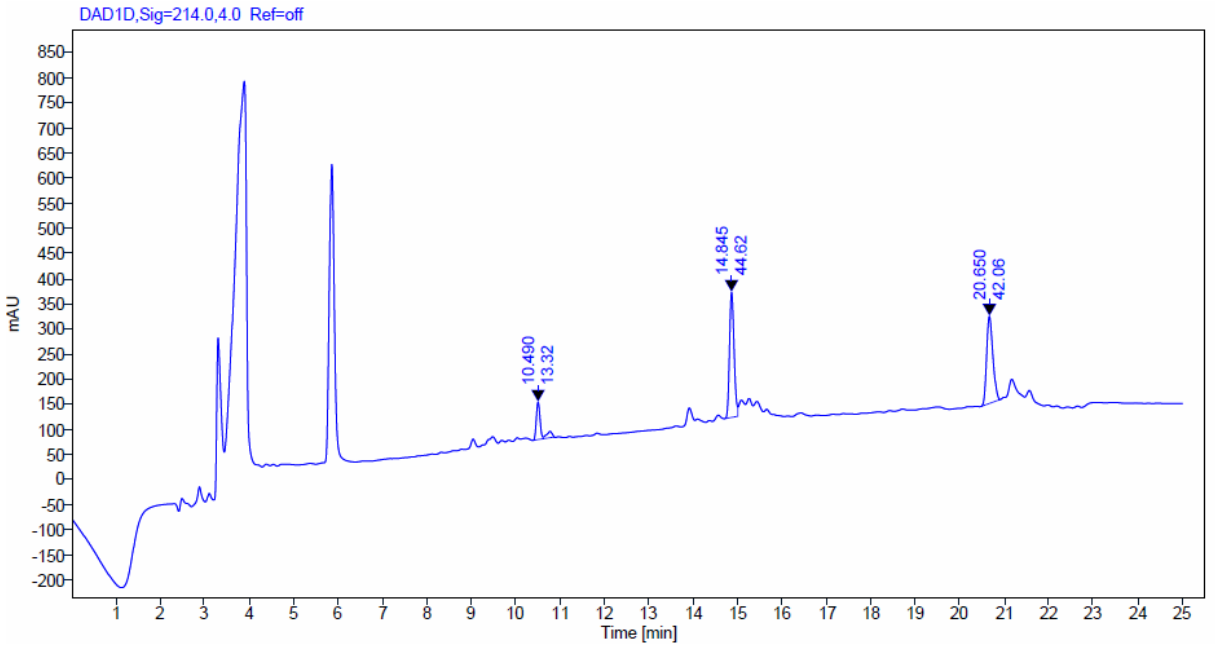
23bb

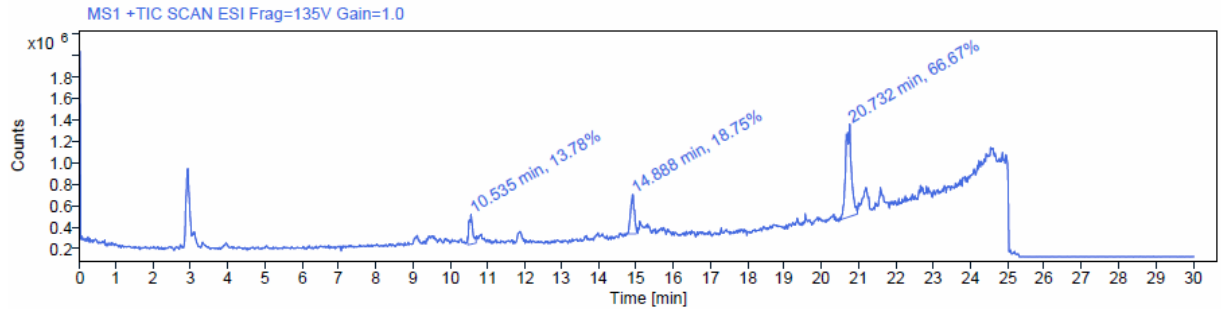
23 (1.0 mg, 0.69 µmol) and **4b** (0.48 mg, 0.83 µmol) were dissolved in 85 µL of 200 mM Tris buffer pH 8.0. The same procedure was followed as for **11bb**. Yield for **23bb** 67% (retention time = 20.6), **23c** 19% (retention time = 14.8).

HRMS (ESI/QTOF) m/z: $[M + 2H]^{+2}$ Calcd for **23b** $C_{79}H_{125}N_{17}O_{18}S_2^{+2}$ 831.9409; Found 831.9423.

HPLC gradient: Method 1.

HPLC-UV and HPLC-MS chromatogram

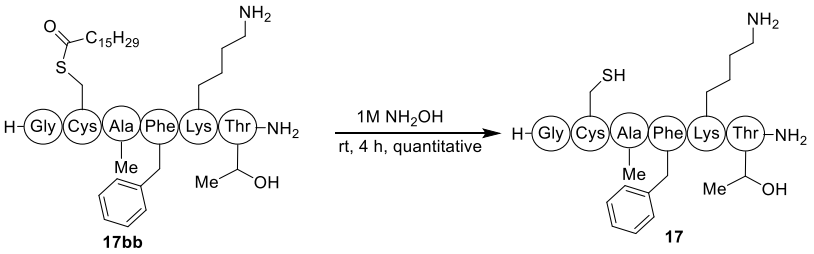




SUPPORTING INFORMATION

Cleavage of palmitoylthioester of Ac-Trp-Met-Asn-Ser-Thr-Gly-Phe-Thr-Lys-Val-Cys-Gly-Ala-NH_2 (**17bb)**

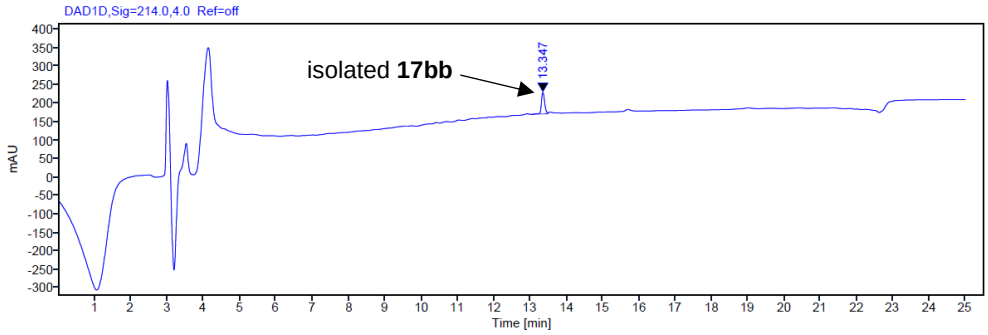
A 1.5 mL vial was charged with **17bb** (0.5 mg, 23.5 µmol,1.0 equiv.) 70 µL 1M NH_2OH with a small magnetic bar. The NH_2OH solution was freshly prepared in Milli Q water. Then the reaction mixture was stirred for 4 h at rt. After 4 h, an aliquot of 10 µL of the reaction mixture was diluted with 40 µL acetonitrile: water (1:1). The solution was submitted to HPLC. The HPLC revealed quantitative conversion. Yield for **17** and disulfide quantitative (retention time 5.0).



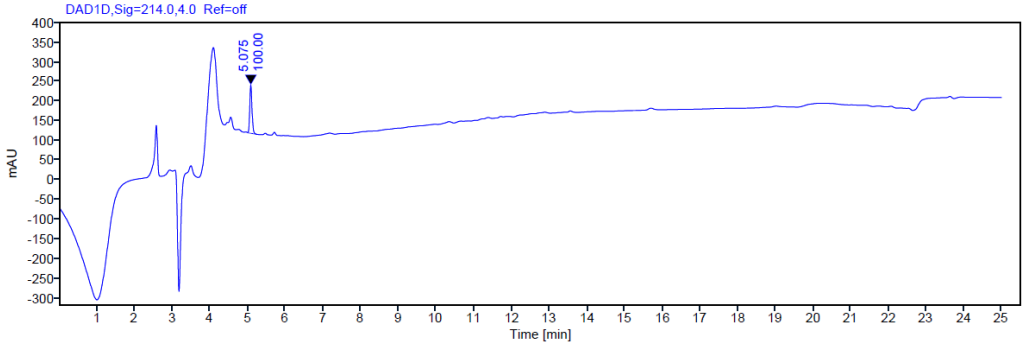
HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{27}H_{45}N_8O_7S^+$ 625.3126; Found 625.3143.

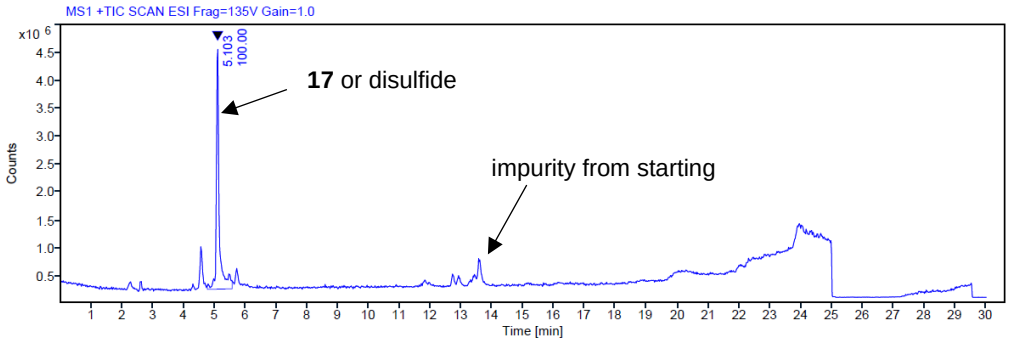
HPLC gradient: Method 1.

HPLC-UV chromatogram of starting material **17bb**



HPLC-UV and HPLC-MS chromatogram of reaction mixture





SUPPORTING INFORMATION

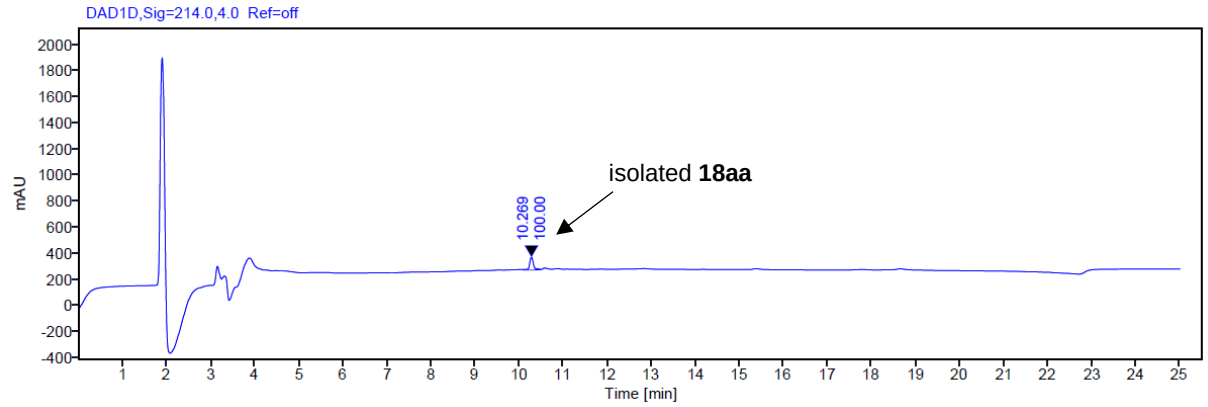
Cleavage of TIPS-thioester of Ac-Trp-Met-Asn-Ser-Thr-Gly-Phe-Thr-Lys-Val-Cys-Gly-Ala-NH_2 (**18aa)**

A 1.5 mL vial was charged with isolated **18aa** (0.5 mg, 0.6 µmol,1.0 equiv.) and KF (1.0 mg, 17.2 µmol, 28.7 equiv.) and 70 µL 1M NH_2OH with a small magnetic bar. The NH_2OH solution was freshly prepared in Milli Q water. Then the reaction mixture was stirred for 14 h at 37 °C. After 14 h, an aliquot of 10 µL of the reaction mixture was diluted with 40 µL water. The solution was submitted to HPLC. The HPLC revealed 90% conversion after 14 h.

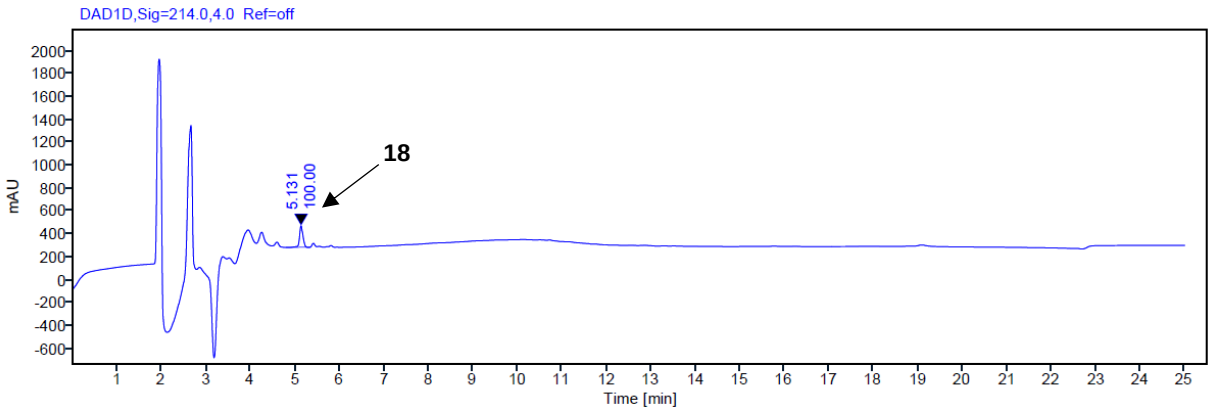
HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{28}H_{45}N_8O_8S^+$ 653.3076; Found 653.309.

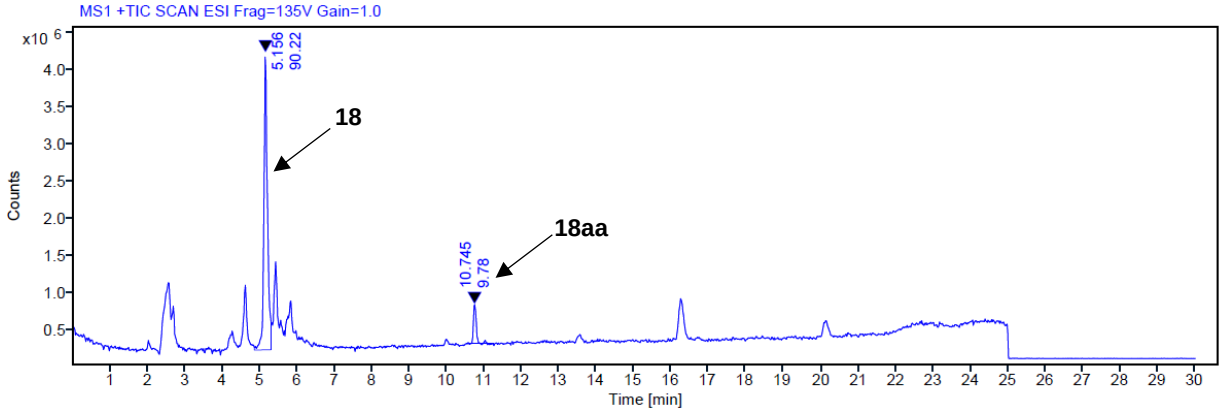
HPLC gradient: Method 1.

HPLC-UV chromatogram of starting material **18aa**

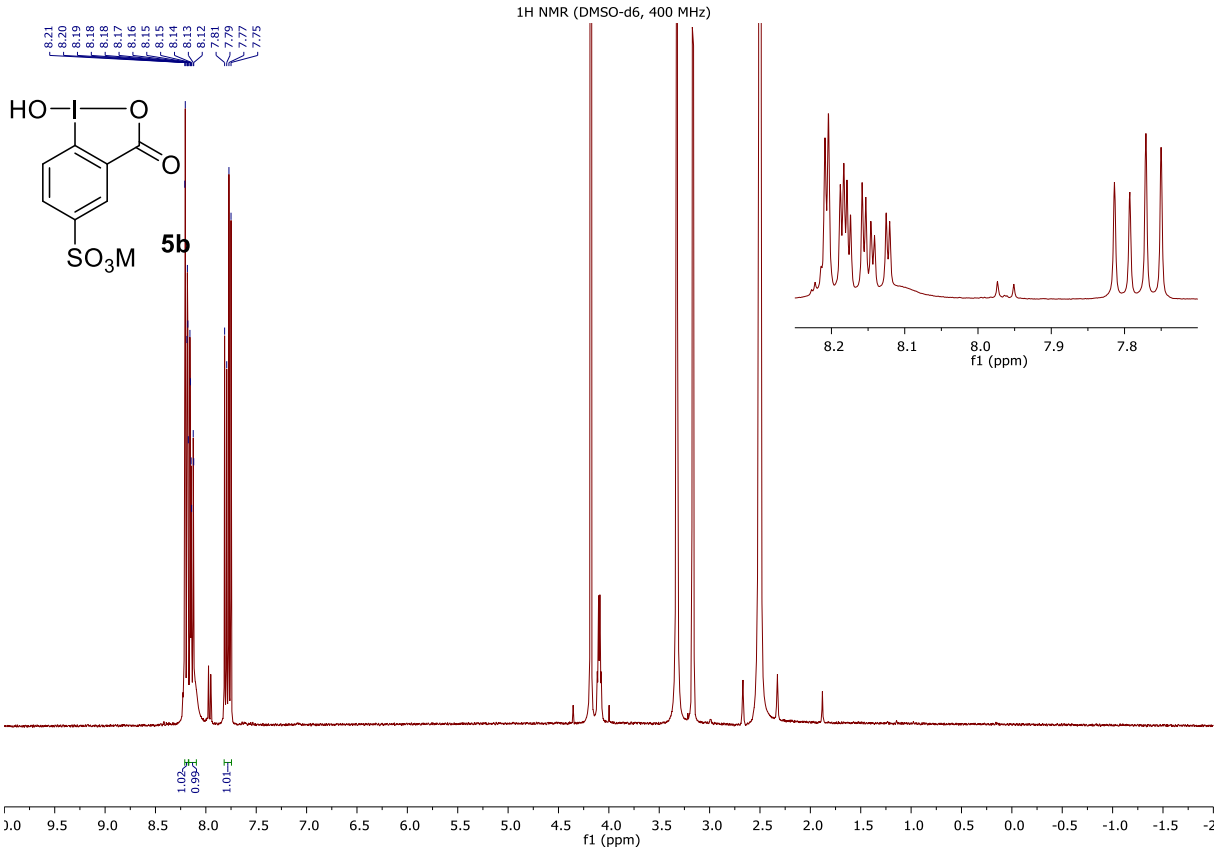


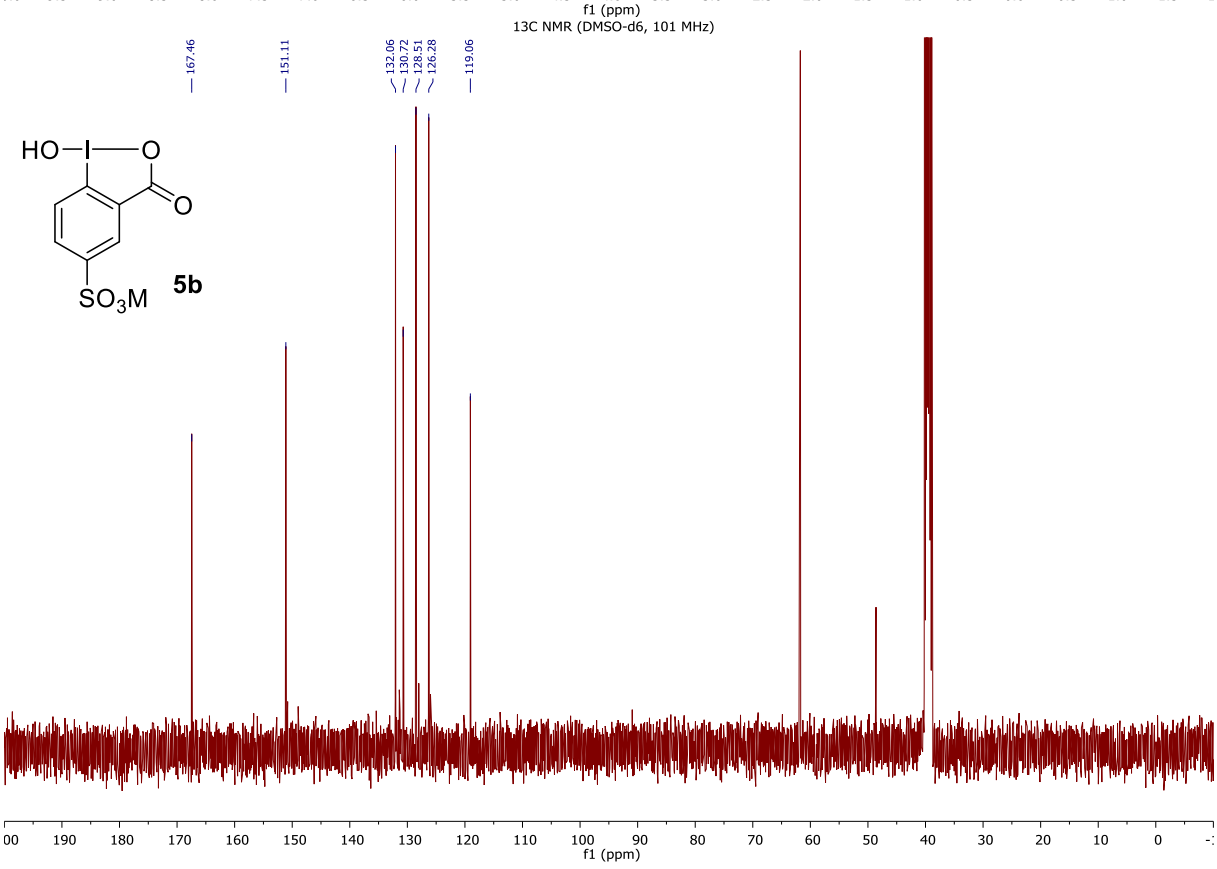
HPLC-UV and HPLC-MS chromatogram of reaction mixture



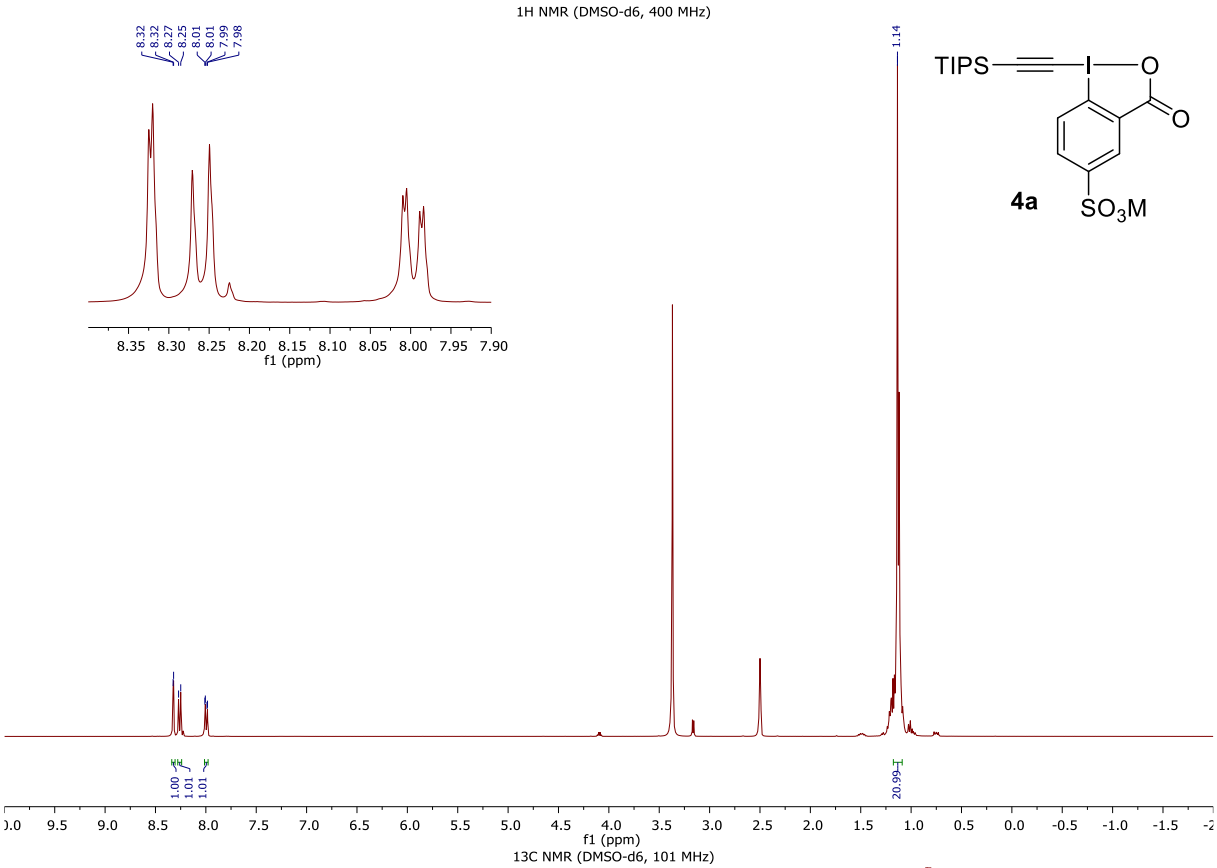


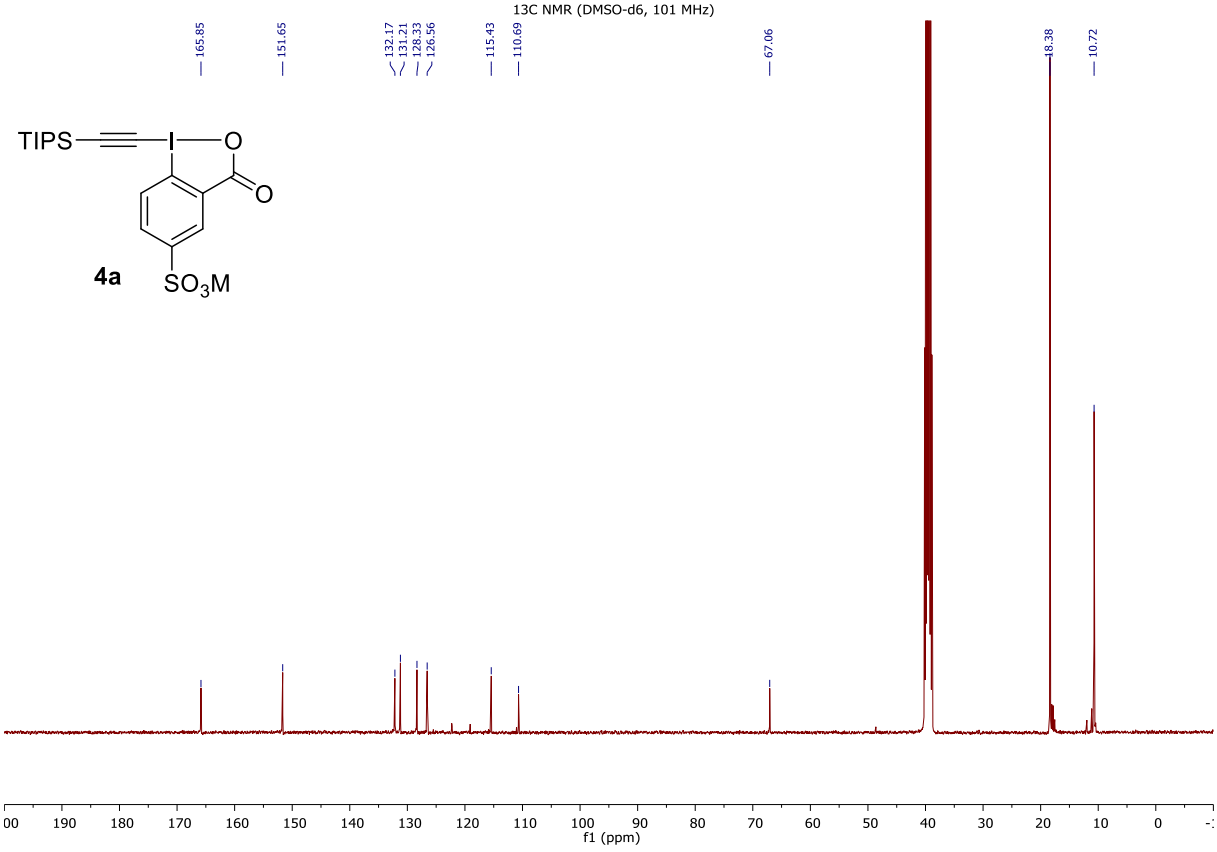
11. NMR spectra of isolated products



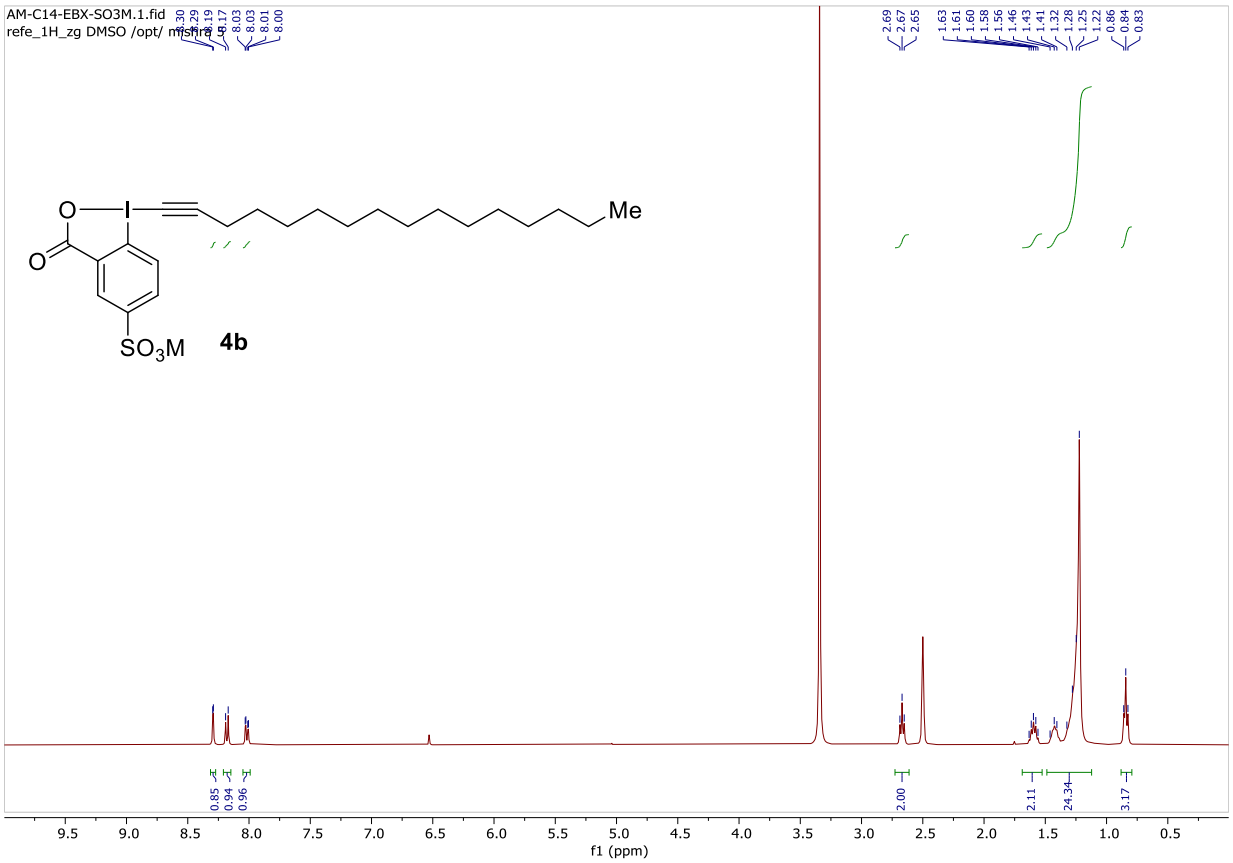


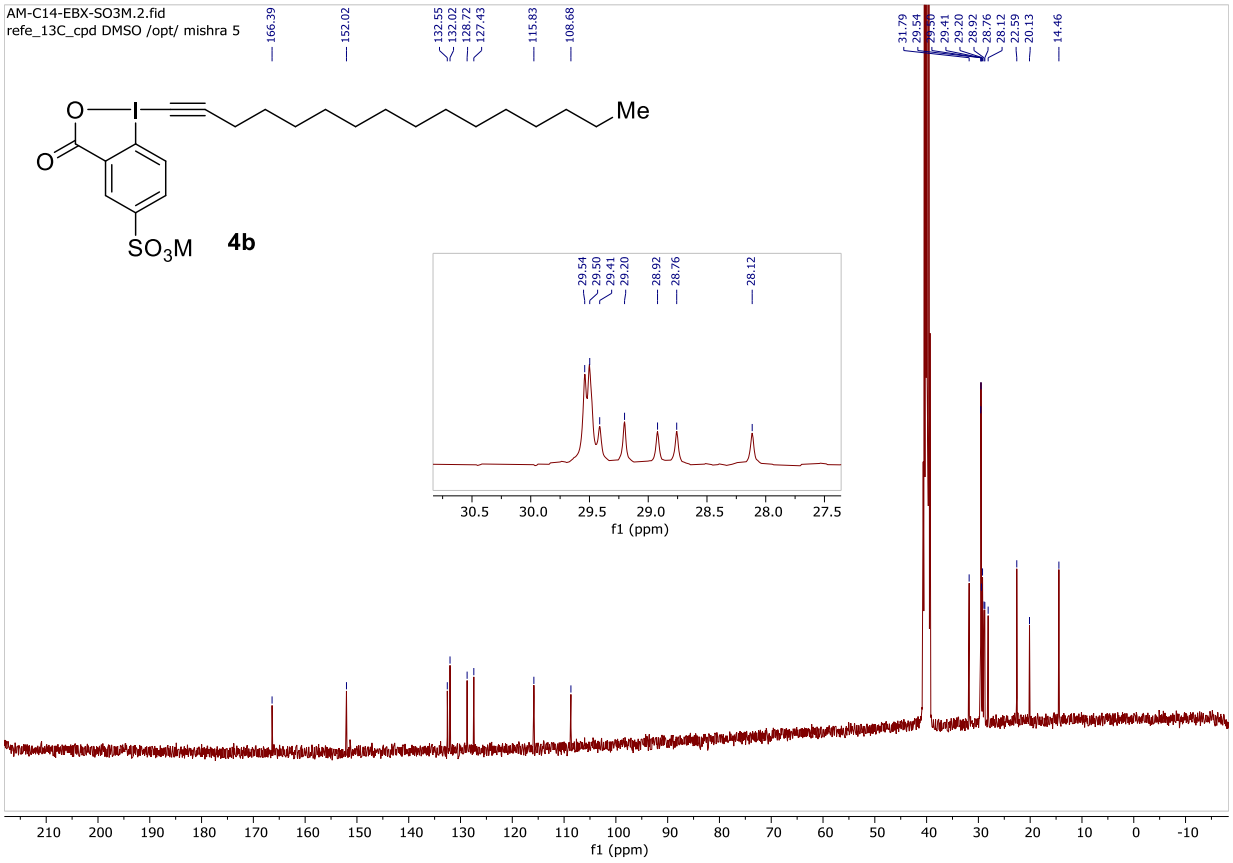
SUPPORTING INFORMATION





SUPPORTING INFORMATION





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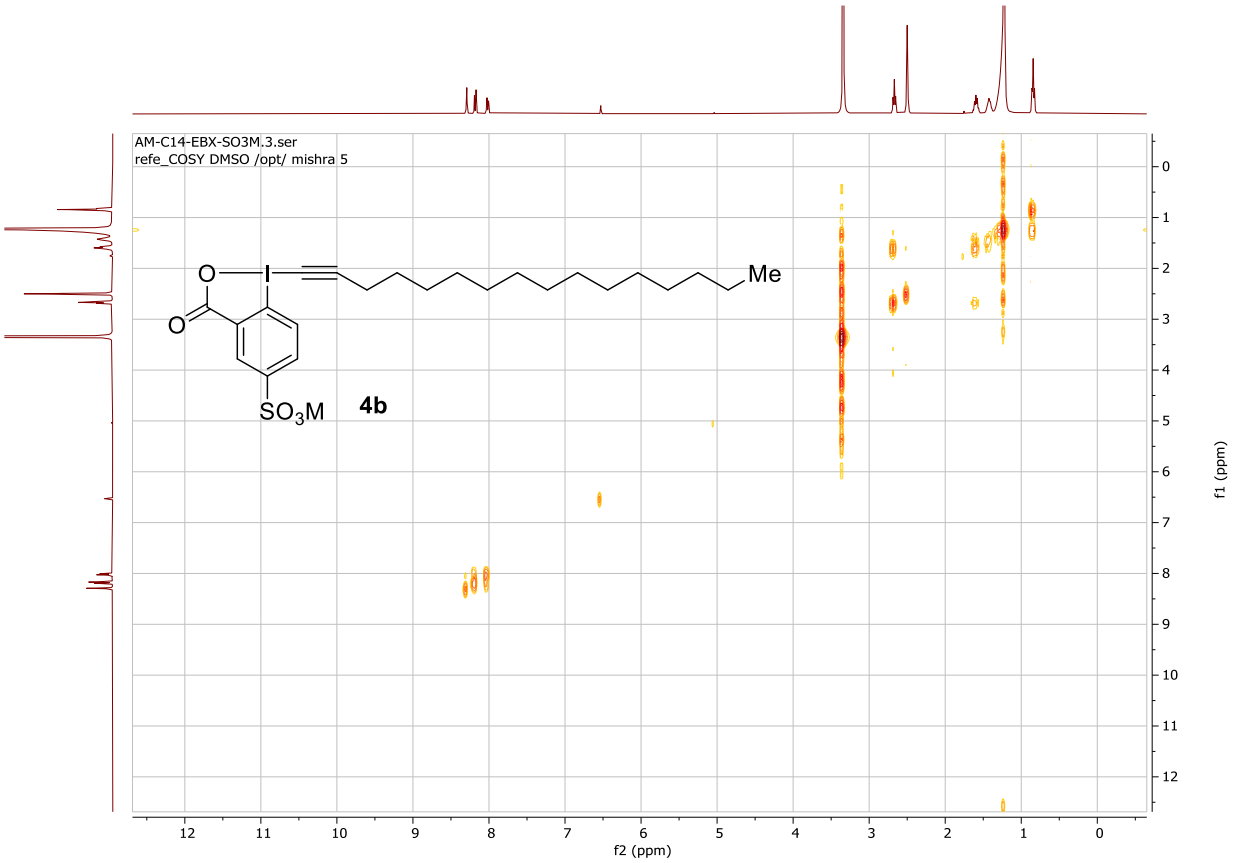

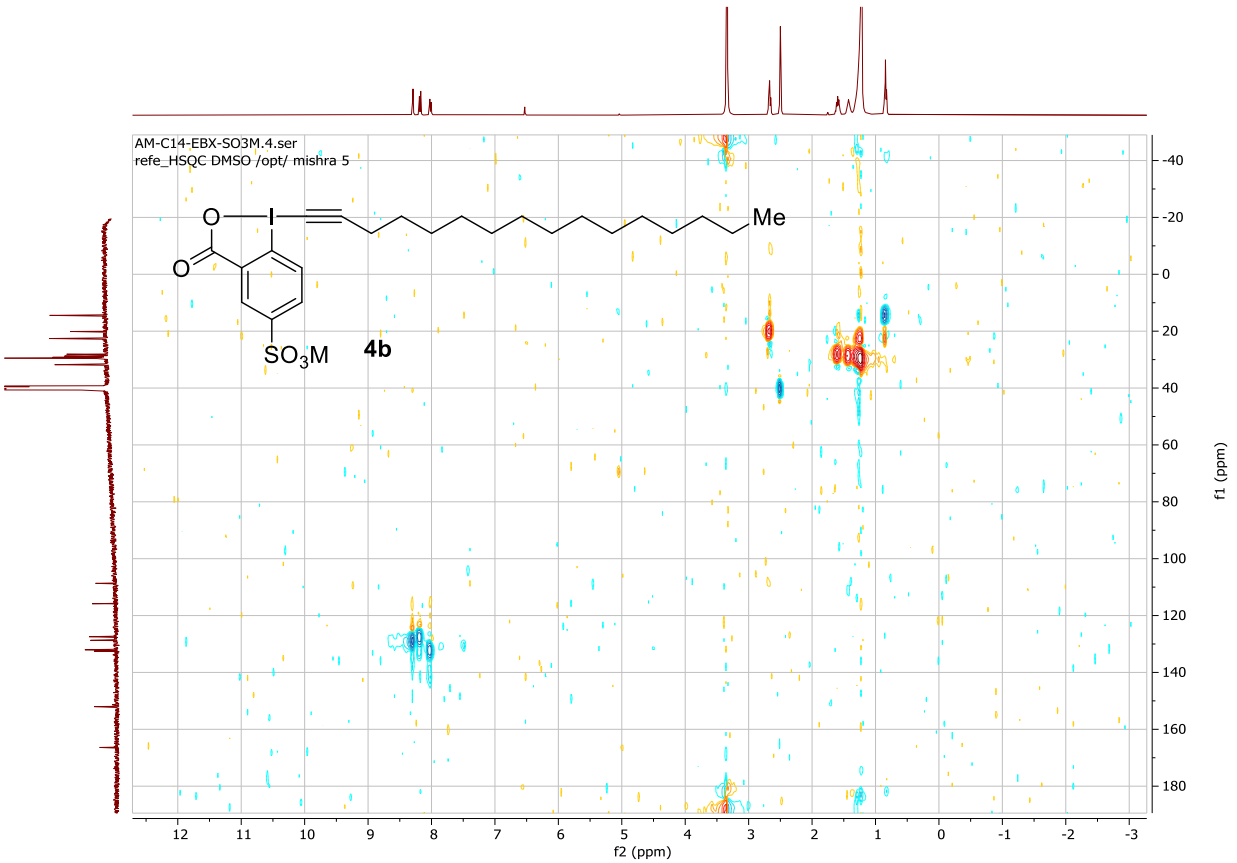

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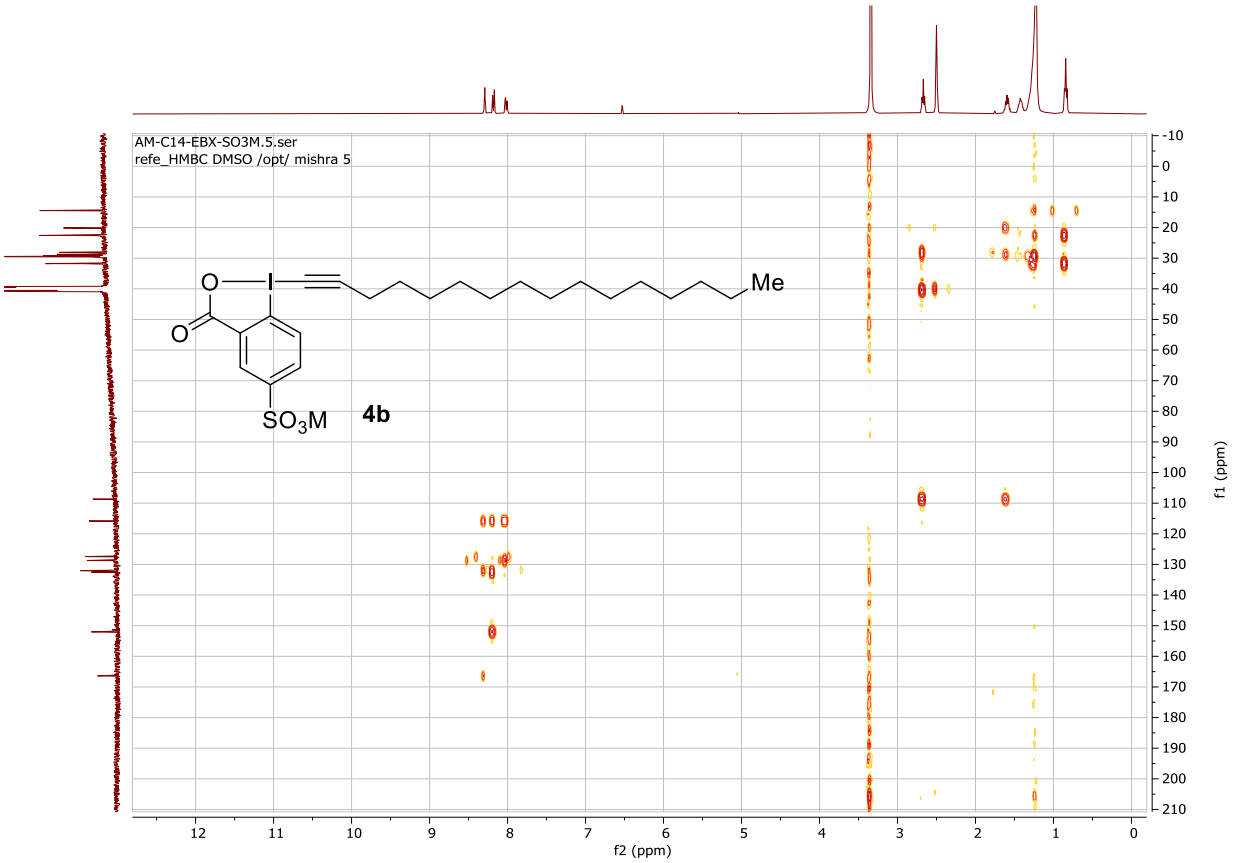
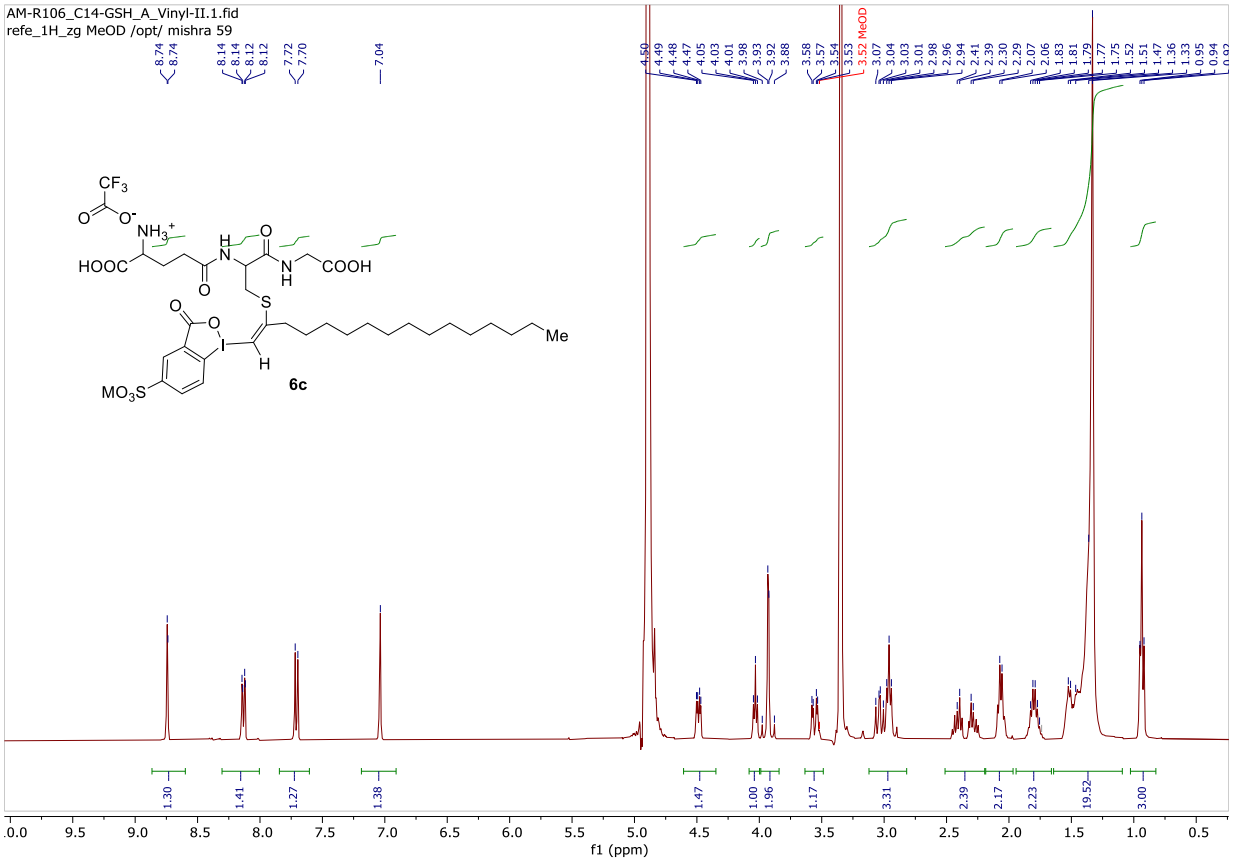
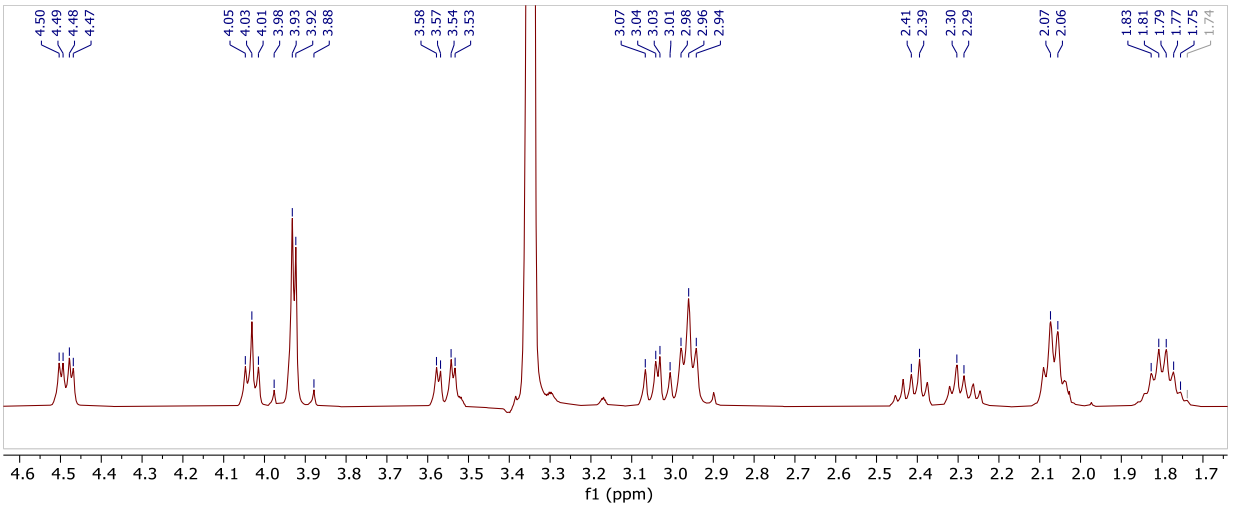
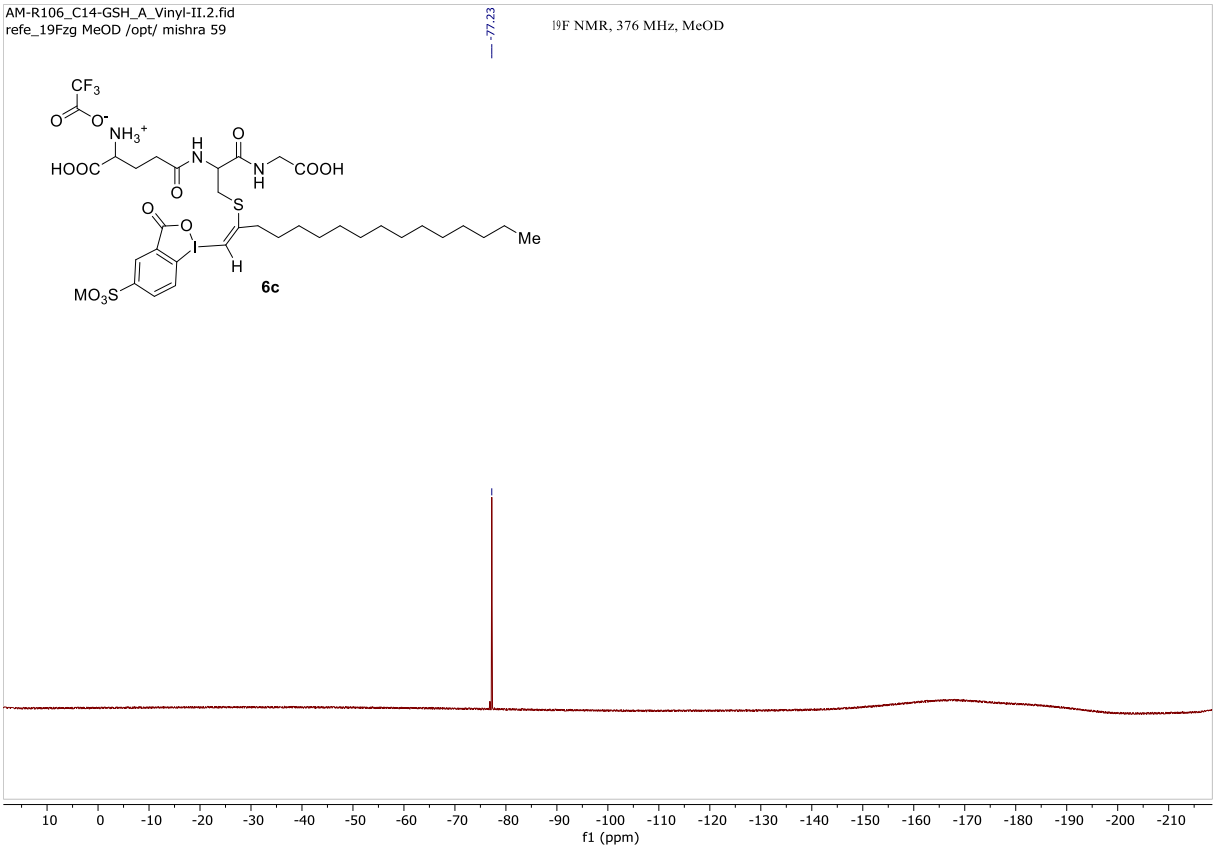
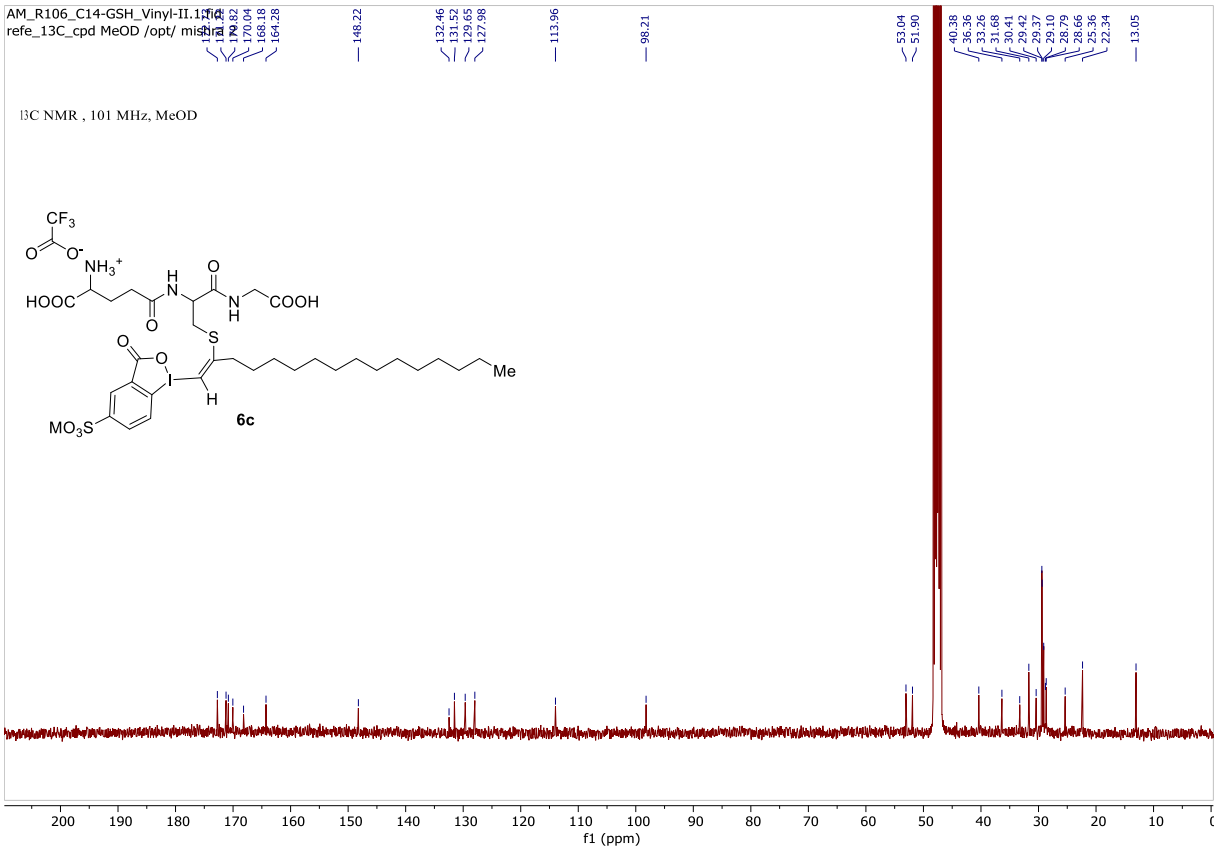


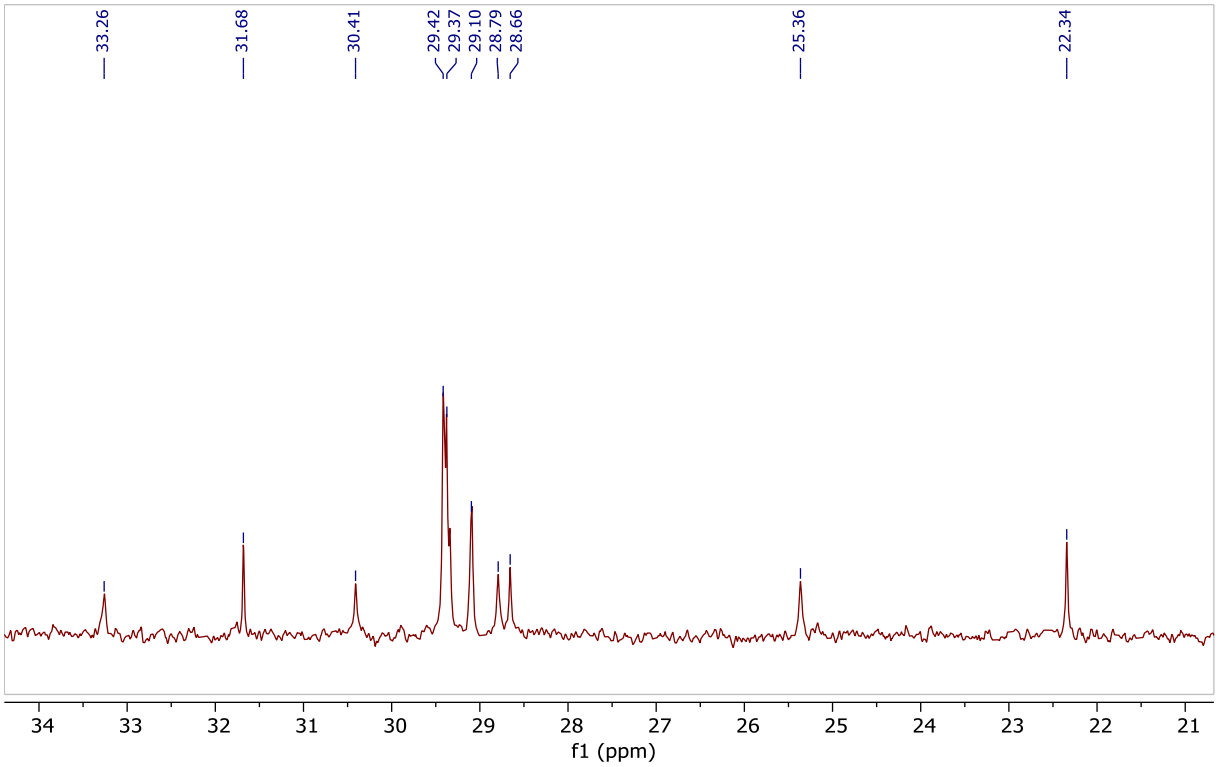



4.50
4.49
4.48
4.47
4.05
4.03
4.01
3.98
3.93
3.92
3.88
3.58
3.57
3.54
3.53
3.07
3.04
3.03
3.01
2.98
2.96
2.94
2.41
2.39
2.30
2.29
2.07
2.06
1.83
1.81
1.79
1.77
1.75
1.74
4.6 4.5 4.4 4.3 4.2 4.1 4.0 3.9 3.8 3.7 3.6 3.5 3.4 3.3 3.2 3.1 3.0 2.9 2.8 2.7 2.6 2.5 2.4 2.3 2.2 2.1 2.0 1.9 1.8 1.7
f1 (ppm)


AM-R106_C14-GSH_A_Vinyl-II.2.fid
refe_19Fzg MeOD /opt/ mishra 59
-77.23
19F NMR, 376 MHz, MeOD
CF3
NH3+
HOOC
COOH
Me
MO3S
6c
10 0 -10 -20 -30 -40 -50 -60 -70 -80 -90 -100 -110 -120 -130 -140 -150 -160 -170 -180 -190 -200 -210
f1 (ppm)

SUPPORTING INFORMATION





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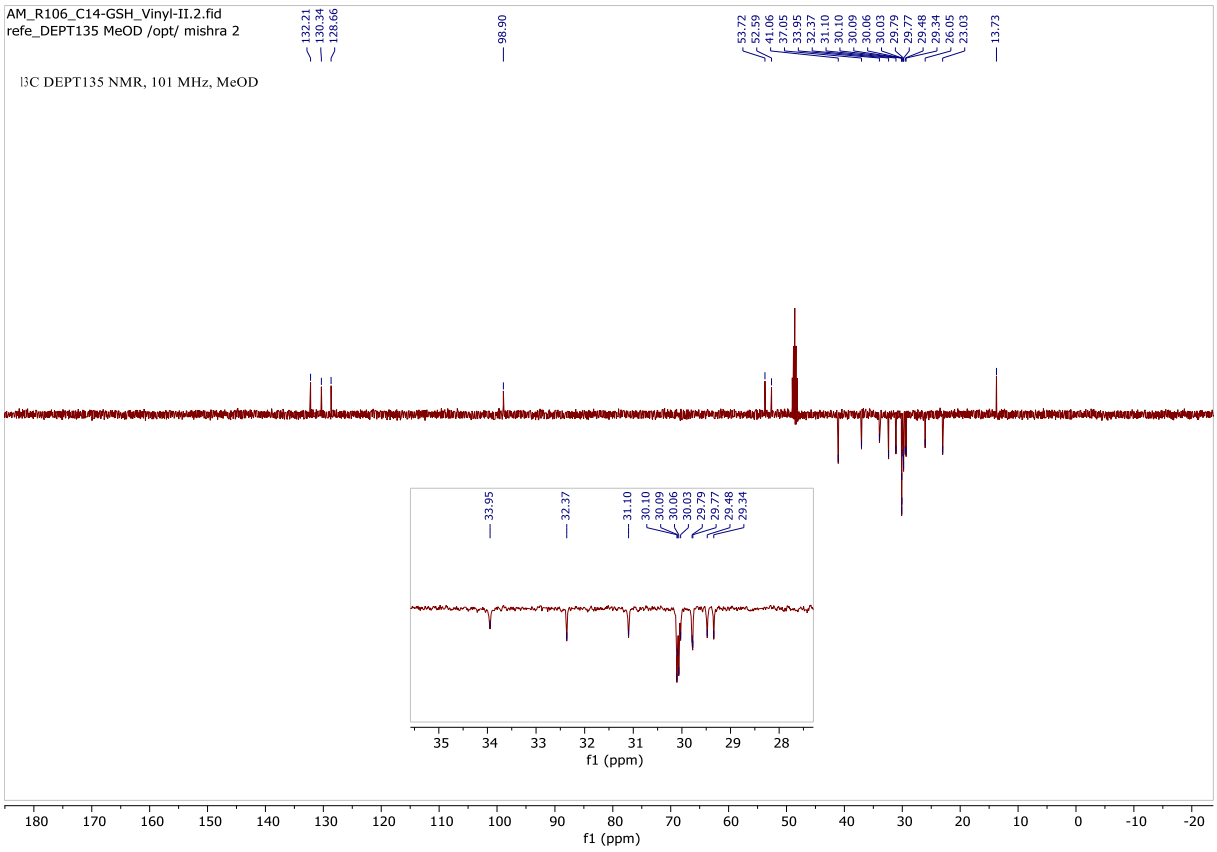

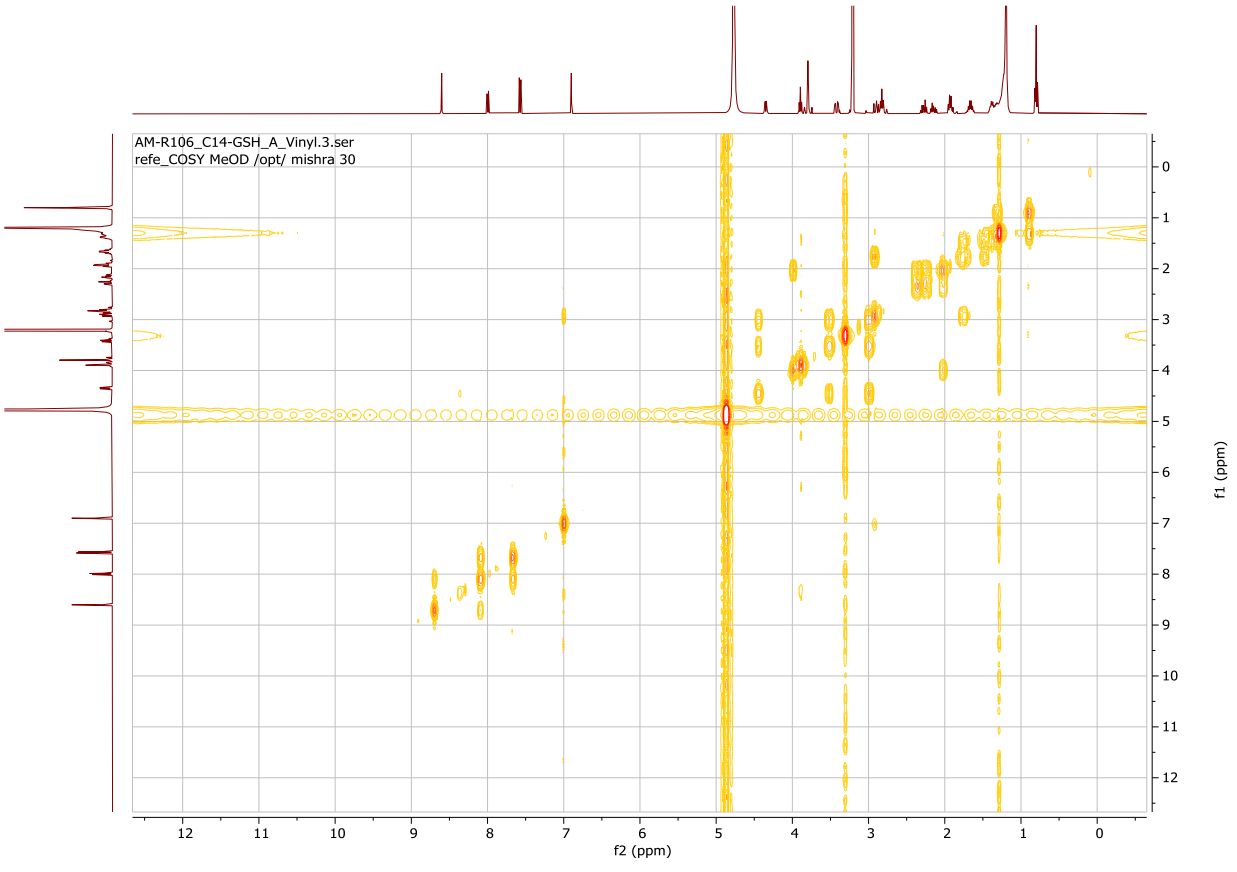

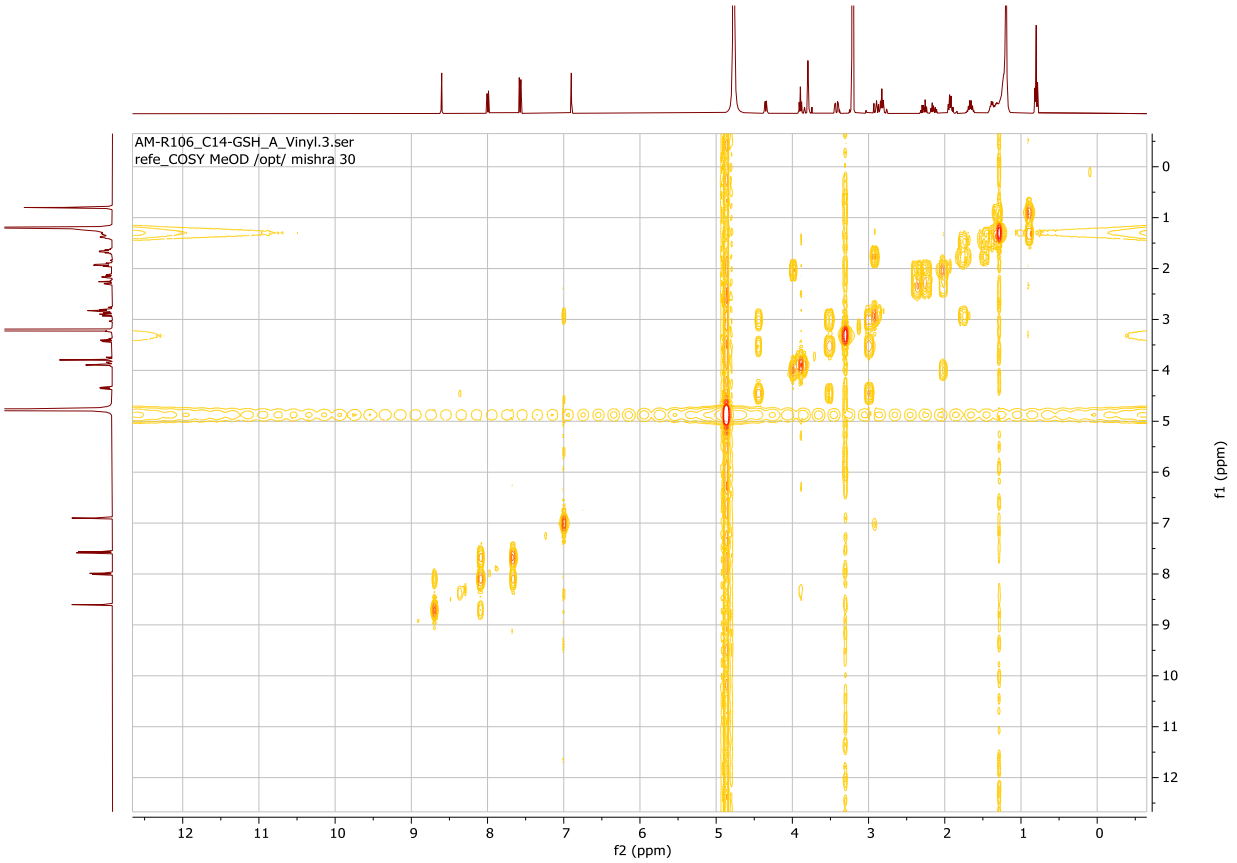
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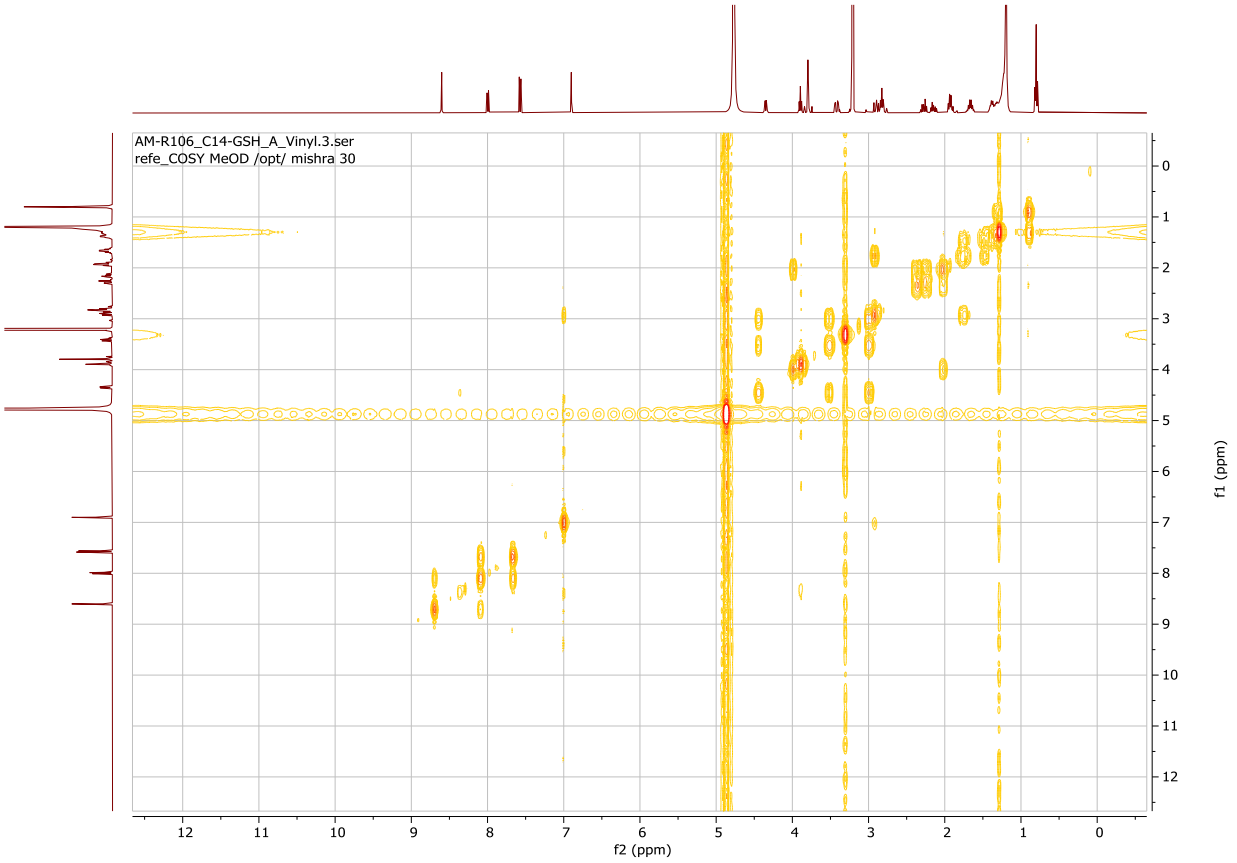
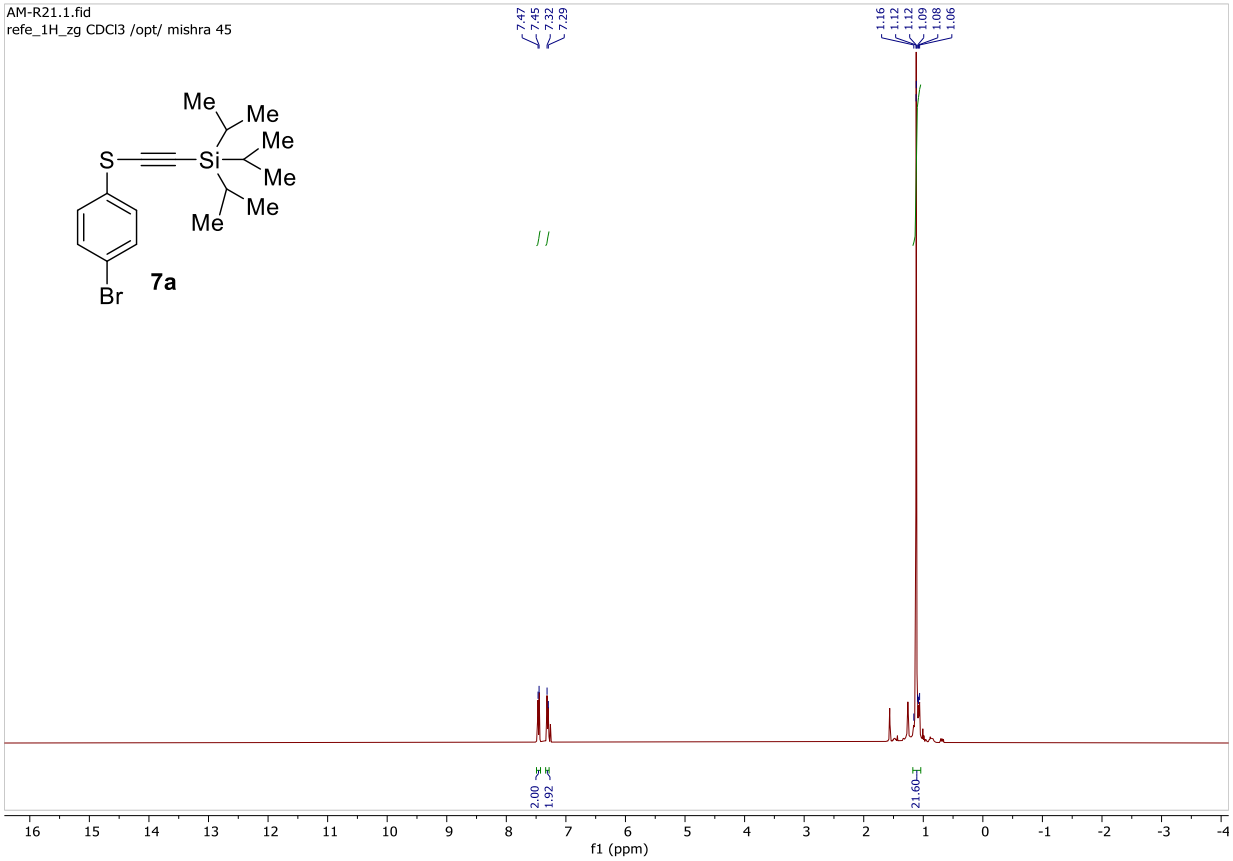
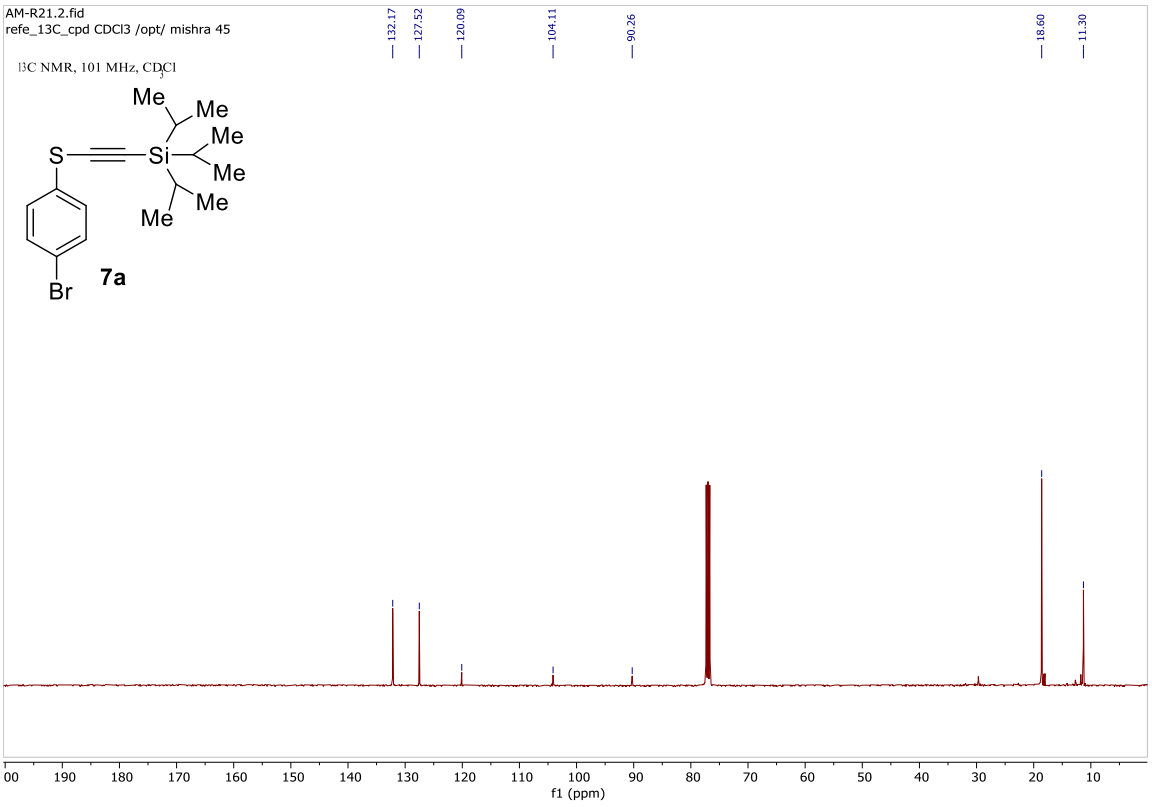
refe_COSY MeOD /opt/ mishra 30
f2 (ppm)
f1 (ppm)


AM-R106_C14-GSH_A_Vinyl.3.ser
refe_COSY MeOD /opt/ mishra 30
f2 (ppm)
f1 (ppm)

SUPPORTING INFORMATION





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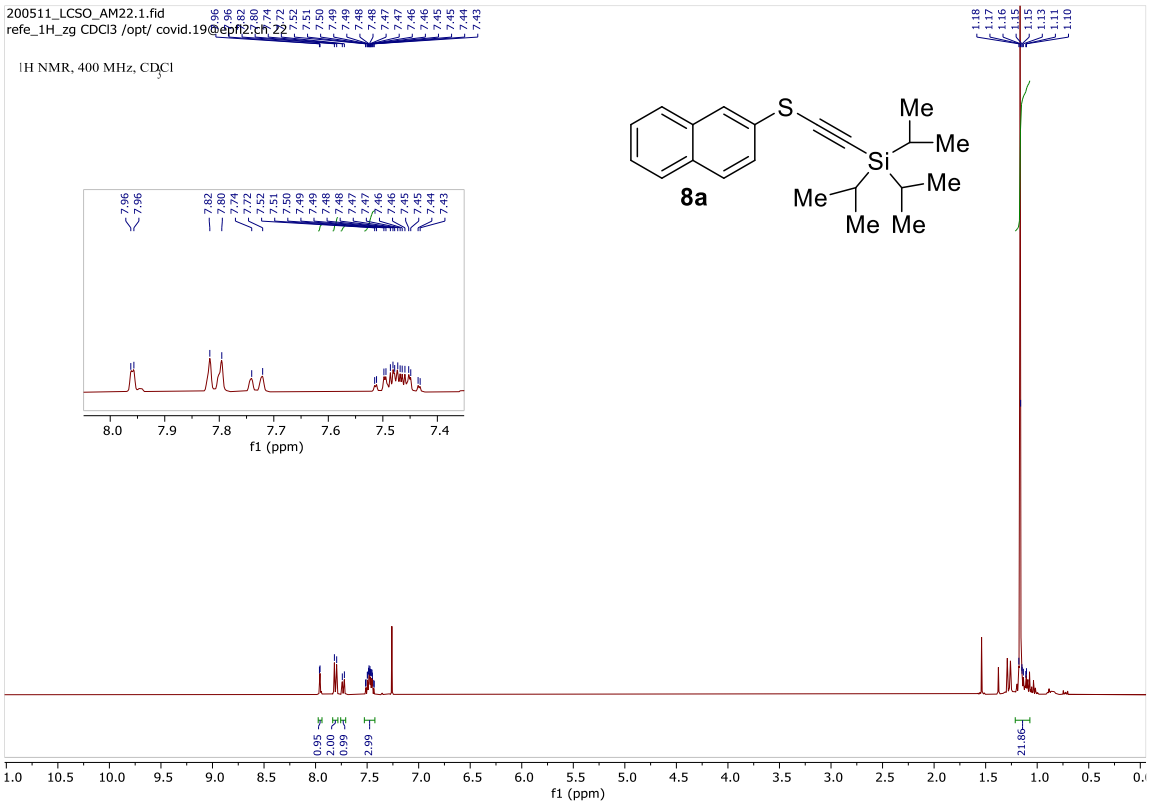

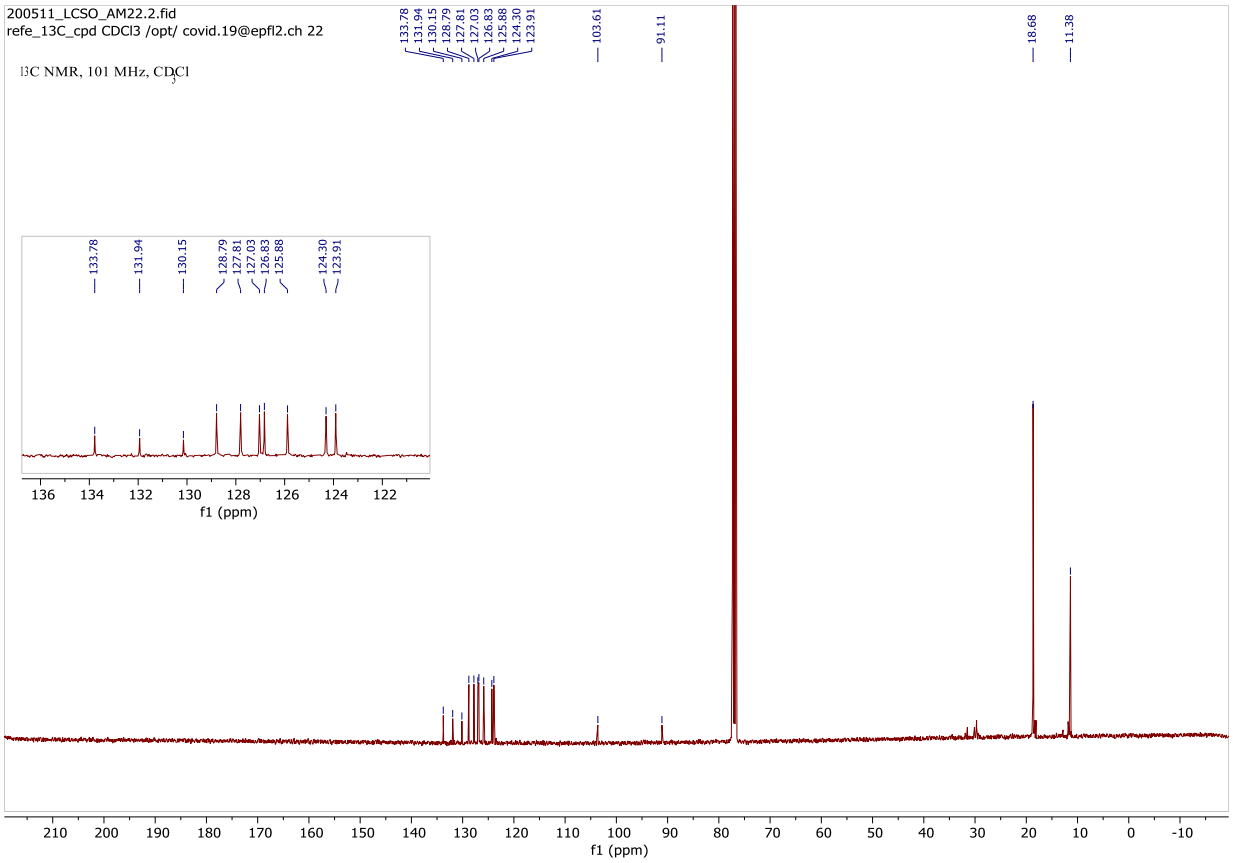

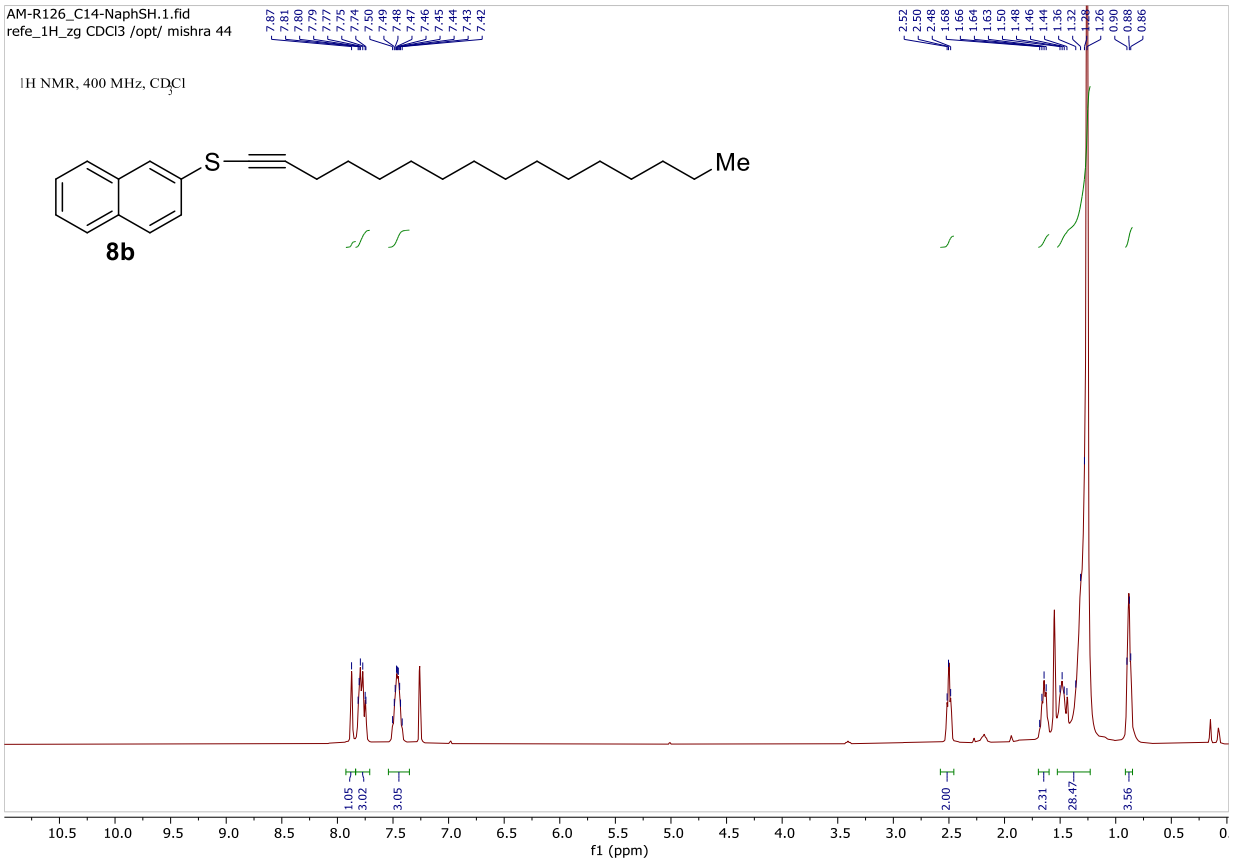
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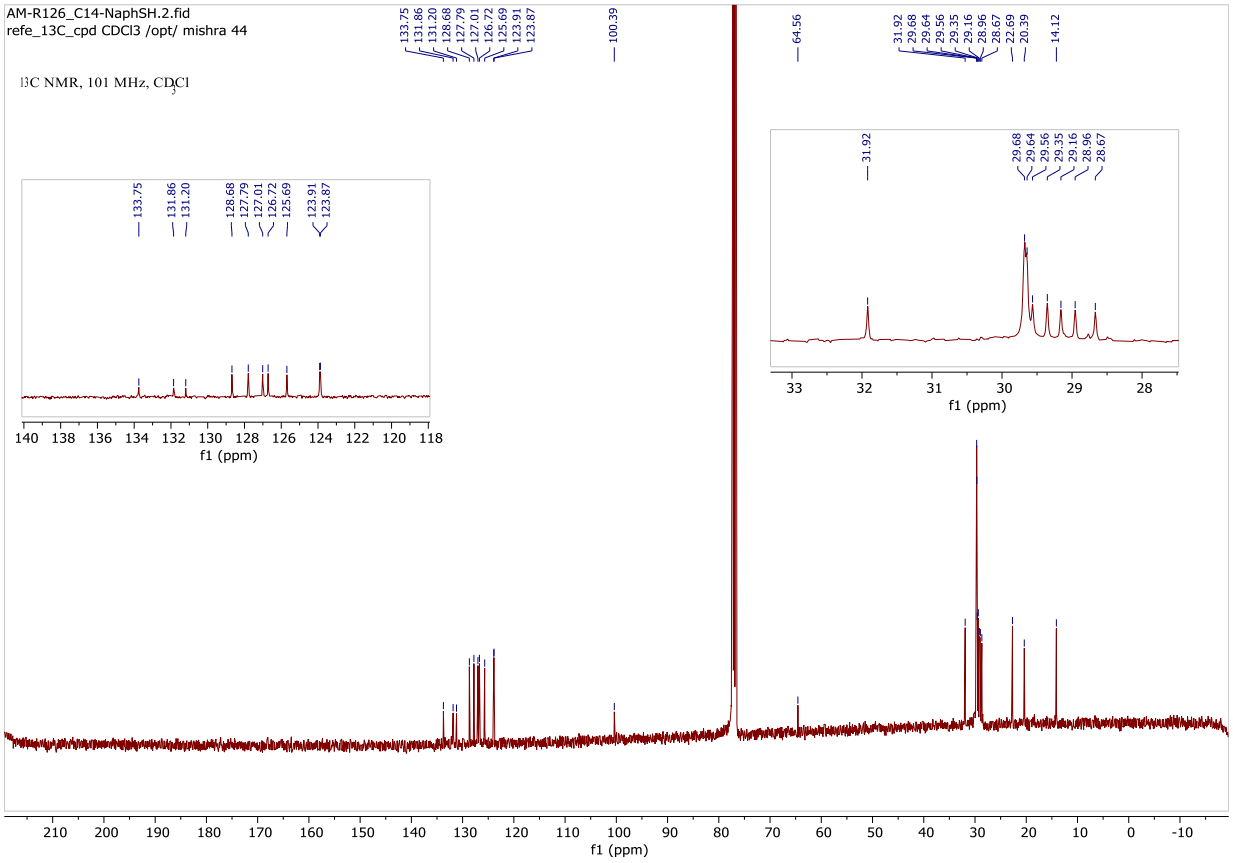
refe_1H_zg CDCl3 /opt/ mishra 44
1H NMR, 400 MHz, CDCl3
S
Me
8b
f1 (ppm)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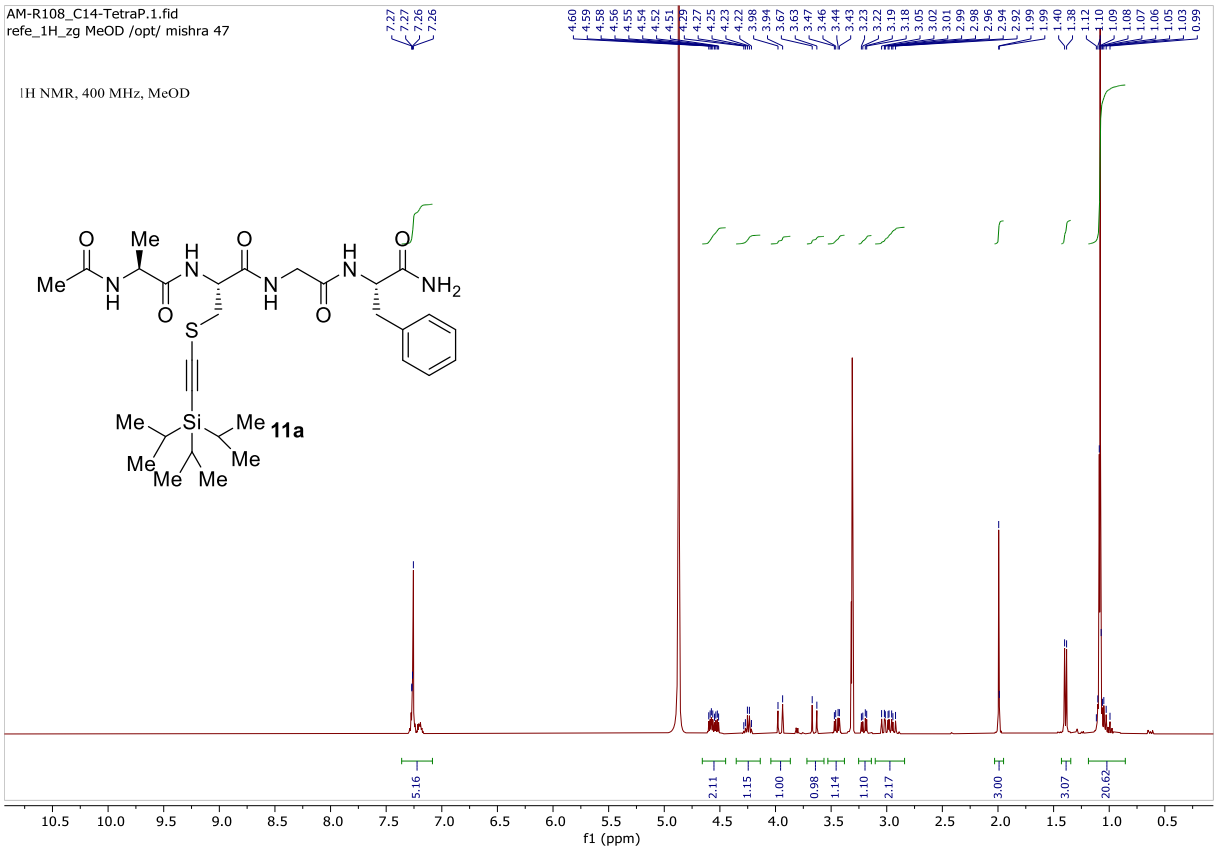
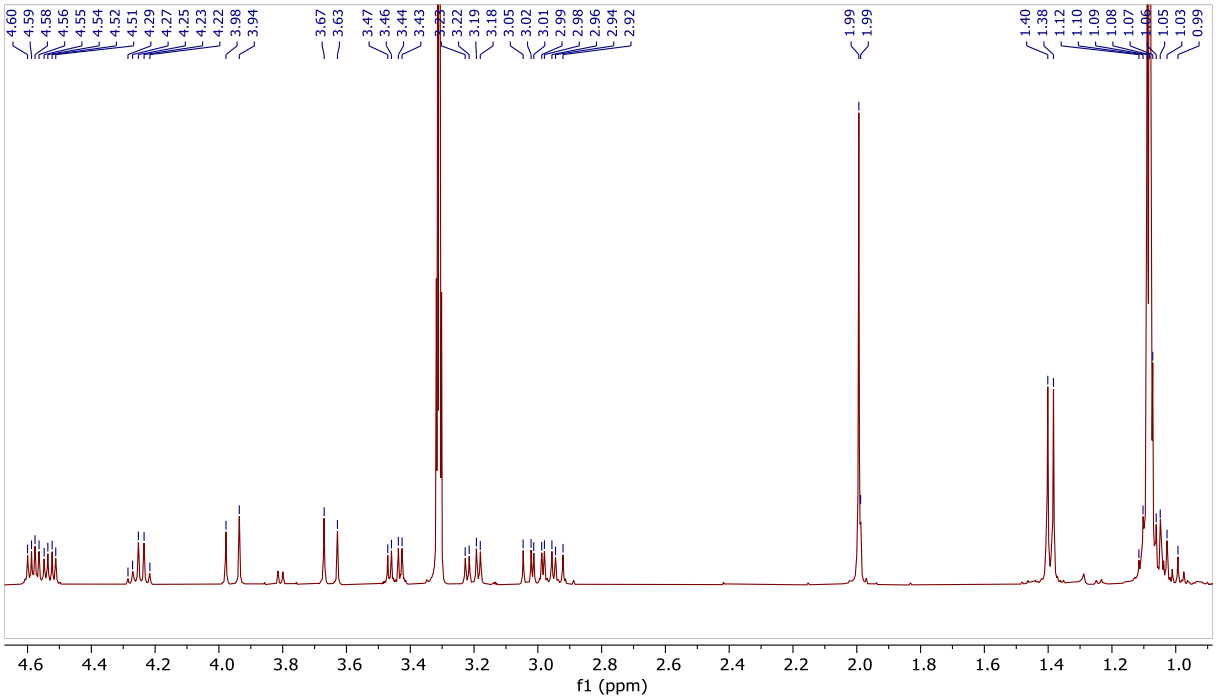
refe_13C_cpd CDCl3 /opt/ mishra 44
13C NMR, 101 MHz, CDCl3
f1 (ppm)

SUPPORTING INFORMATION





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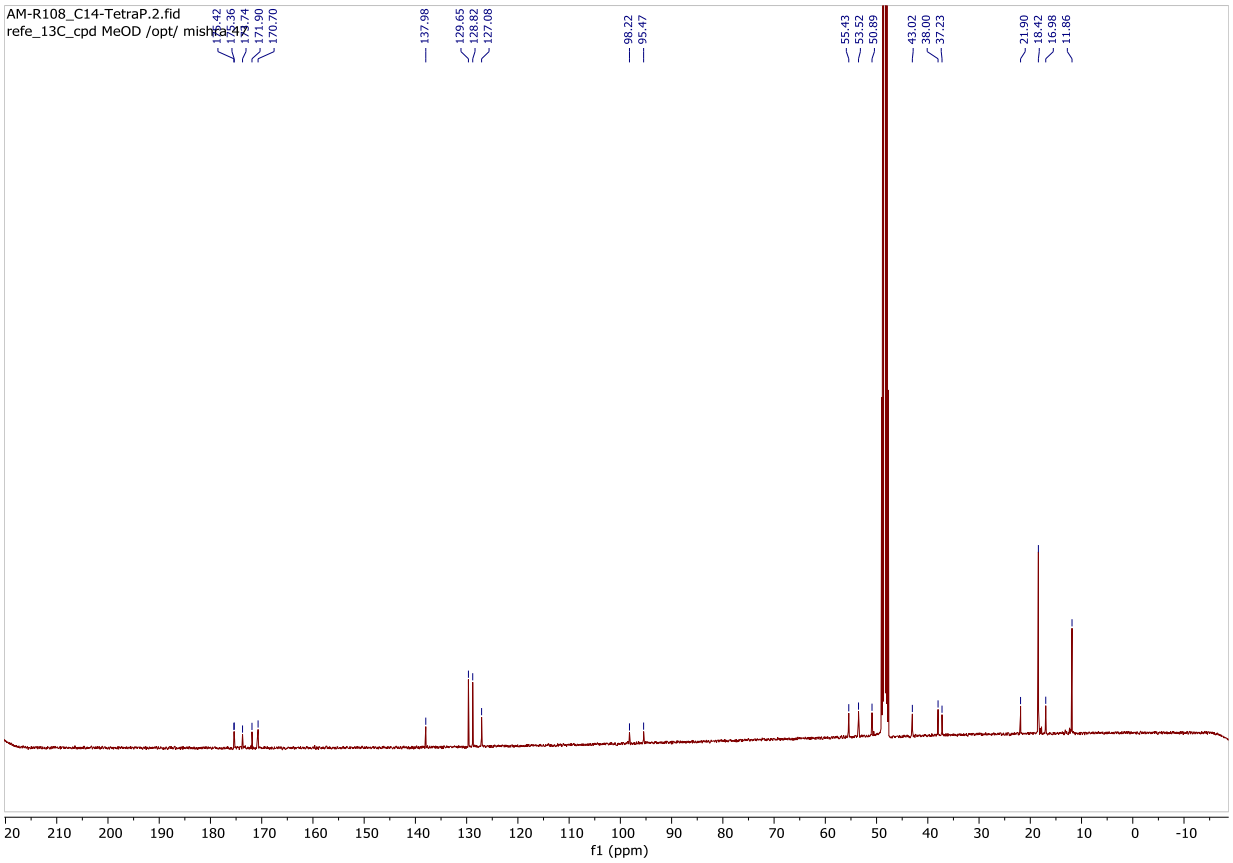

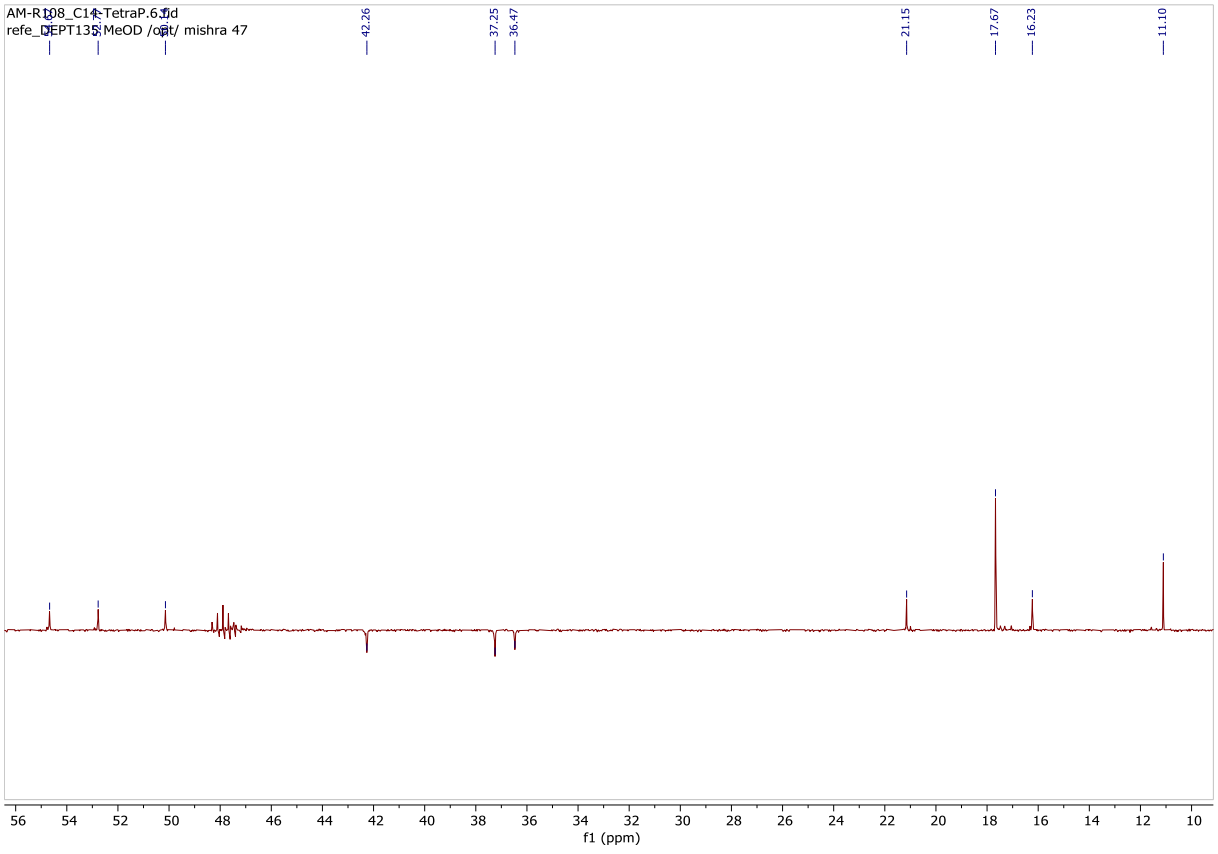

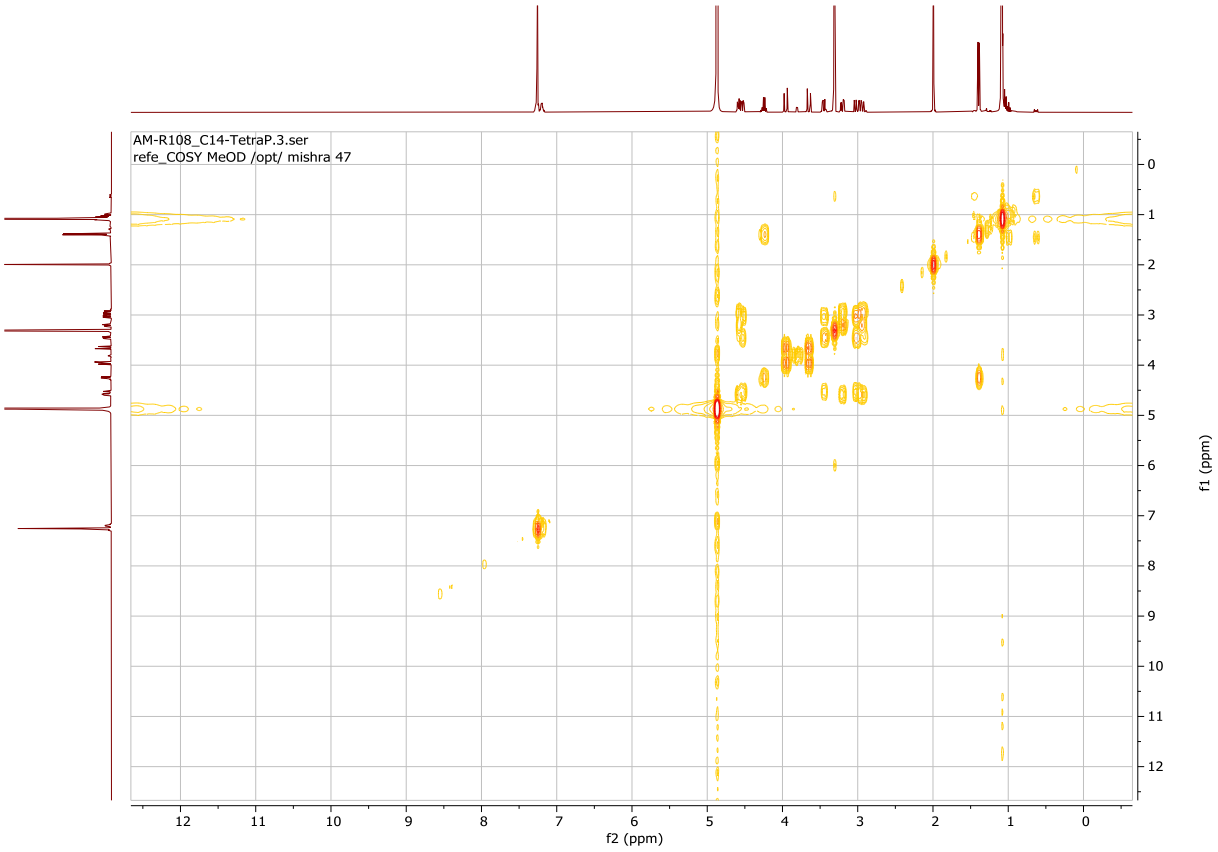
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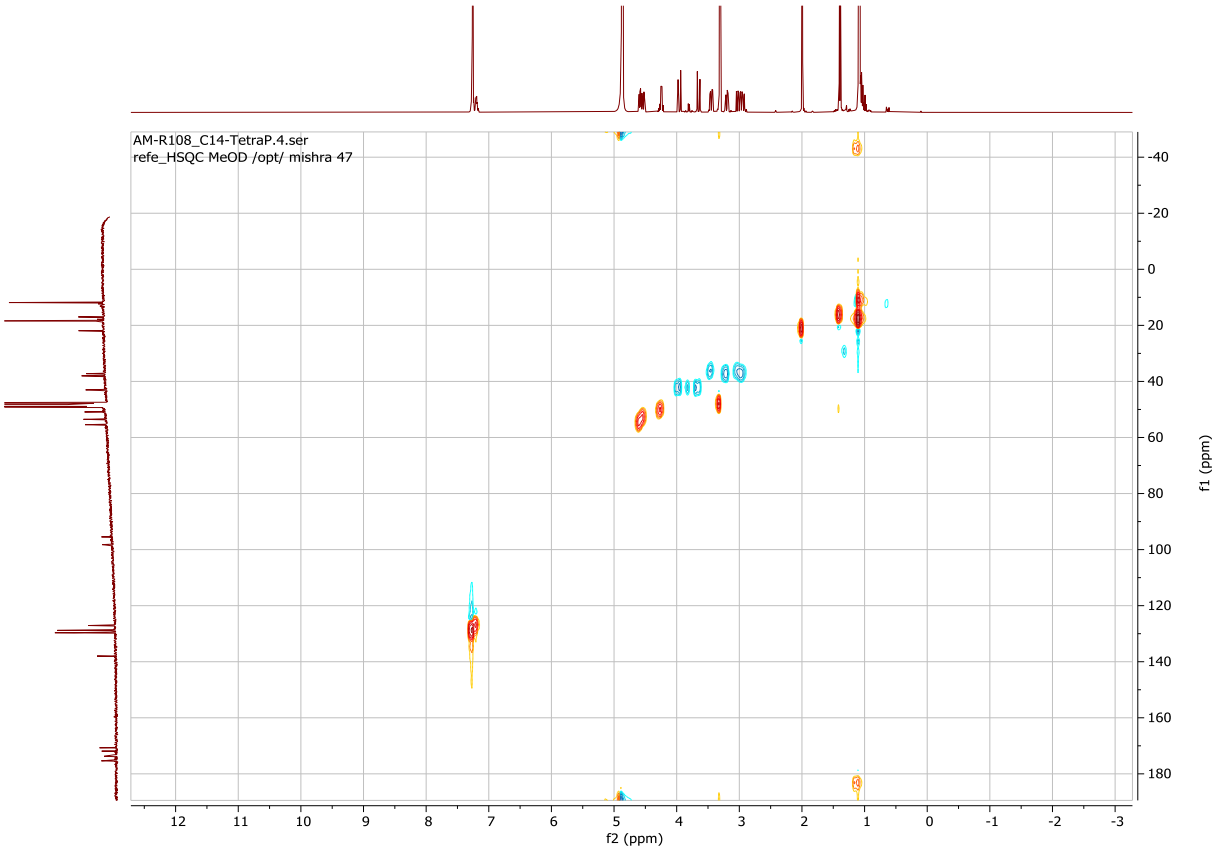
refe_COSY MeOD /opt/ mishra 47
f2 (ppm)
f1 (ppm)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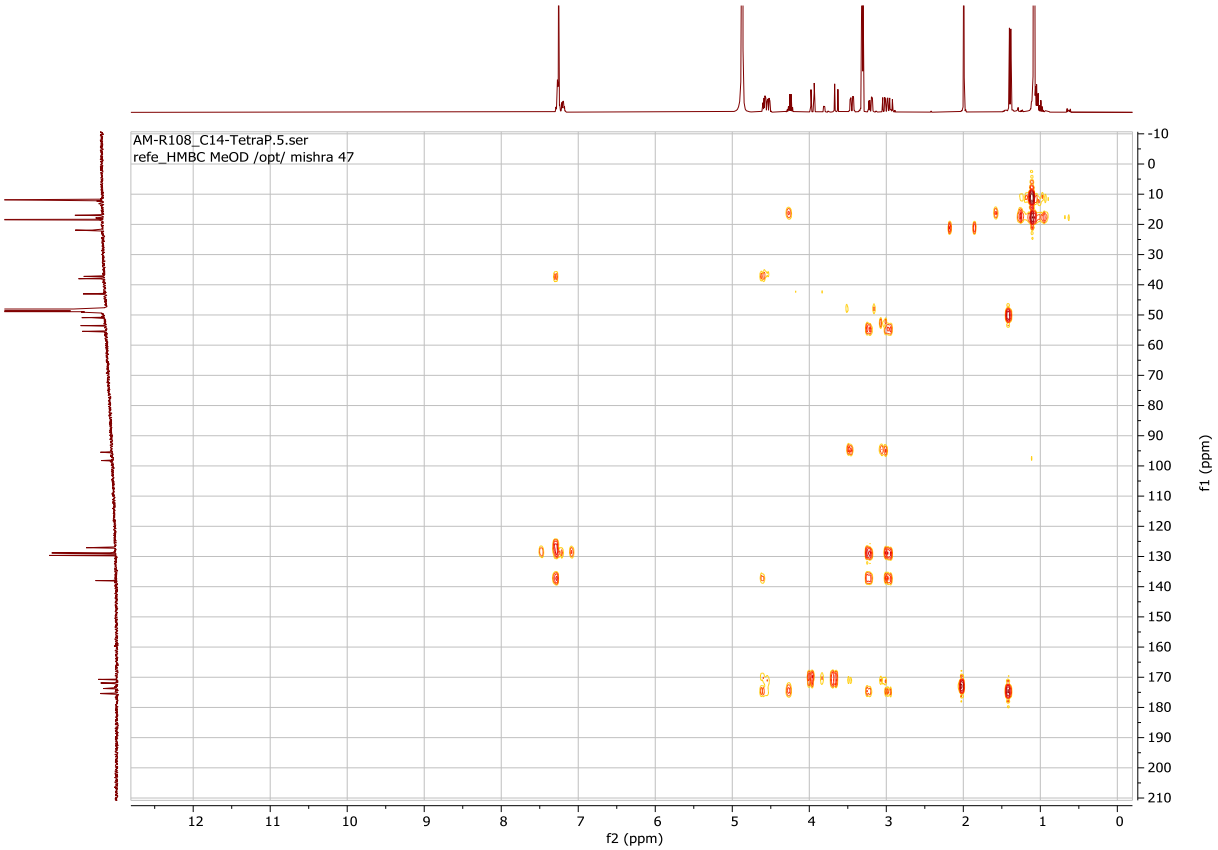
f2 (ppm)
f1 (ppm)


AM-R108_C14-TetraP.5.ser
refe_HMBC MeOD /opt/ mishra 47
f2 (ppm)
f1 (ppm)