**Title:** A One-Pot Chemically Cleavable bis-Linker Tether Strategy for the Synthesis of Heterodimeric Peptides

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# A One-Pot Chemically Cleavable *bis*-Linker Tether Strategy for the Synthesis of Heterodimeric Peptides

Nitin A. Patil[a,b,c] Julien Tailhades*[a], John Karas[a,b,c], Frances Separovic[b,c], John D. Wade*[a,b], and Mohammed Akhter Hossain*[a,b]

**Abstract:** Heterodimeric peptides linked by disulfide bonds are attractive drug targets. However, their chemical assembly can be tedious, time-consuming and low yielding. Inspired by the cellular synthesis of pro-insulin in which the two constituent peptide chains are expressed as a single-chain precursor separated by a connecting C-peptide, we have developed a novel chemically-cleavable *bis*-linker tether which allows the convenient assembly of two peptide chains as a single "pro"-peptide on the same solid support. Following the peptide cleavage and post synthetic modifications, this *bis*-linker tether can be removed in one-step by chemical means. This methodology was efficiently used to synthesize a drug delivery-cargo conjugate, TAT-PKCi peptide, and a two-disulfide bridged heterodimeric peptide, thionin (7-19)-(24-32R), a thionin analogue. To the best of our knowledge, this is the first report of a one-pot chemically cleavable *bis*-linker strategy for the facile synthesis of cross-bridged two-chain peptides.

Cystine-rich heterodimeric peptides or small proteins such as insulin and relaxin are attractive targets for development of next-generation therapeutics due to their pleiotropic physiological roles.[1] Structure-function relationship studies of these hetero-dimeric peptides play a crucial role in the development of new lead compounds. Their chemical synthesis can be successfully achieved by, e.g. separate assembly of the individual S-reduced chains followed by oxidative folding. However, modified analogues often cannot spontaneously fold due to loss of secondary structure within the chains. Consequently, directed, stepwise disulfide bridge formation is performed.[2] This requires orthogonal S-protection and necessitates solid phase synthesis of the two separate chains, followed by their release from the solid support and multistep solution phase reactions to control the correct disulfide pairing leading to lower yields.[3] While this method can be very effective, its complexity emphasizes the need for an improved synthetic protocol for heterodimeric peptides whereby the number of synthetic steps are reduced.

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The *in vivo* cellular production of insulin has a C-peptide that is used as a temporary link between the B- and A-chains. Once the precursor pro-peptide is folded, the C-peptide is enzymatically cleaved to generate the final native two-chain peptide. We thus developed an analogous method whereby both chains are sequentially synthesized on the same solid support separated by a chemically cleavable *bis*-linker tether (Figure 1). After isolation of the single chain and disulfide folding, the surrogate C-peptide tether is efficiently removed. Similar elegant strategies for biomimetic insulin syntheses have been developed over the years.[4] However, these approaches are limited by the need for enzyme(s) for release of the C-peptide mimic or use of specific residues (e.g. Thr and Glu) at particular positions within the individual peptide chains. In order to overcome these limitations, we have developed a *bis*-linker tether which mimics the role of a C-peptide (Figure 1). This *bis*-linker tether consists of a small peptide with a chemical spacer placed between two chemical moieties, one an N-terminal and the other a C-terminal linker. Once the entire chain assembly is accomplished, the linear two-chain peptide can be folded or conjugated by chemical means, followed by removal of the *bis*-linker tether (Figure 1). Importantly, the two chemical linkers must enable the generation of native free N- and C-termini which is often essential for the activity of some native two-chain peptides.[5] Another important advantage of having separate N- and C-terminal linkers is the flexibility of choosing the length and nature of the intermediate tether sequence that can be used to mimic the C-peptide.

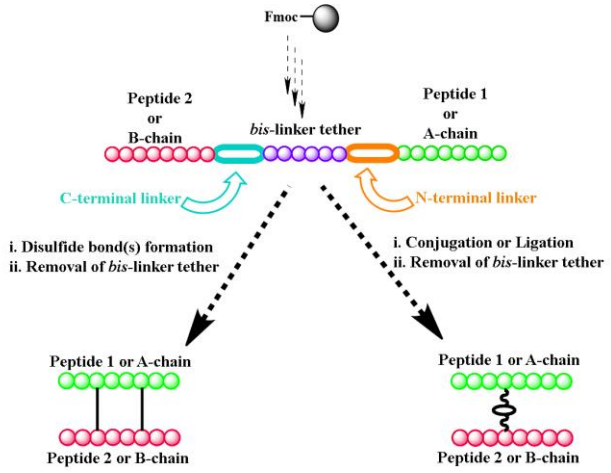
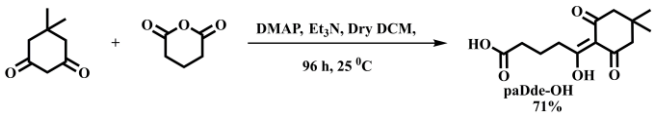



**Figure 1.** The proposed strategy for the synthesis of inter-covalently-linked two-chain peptides.

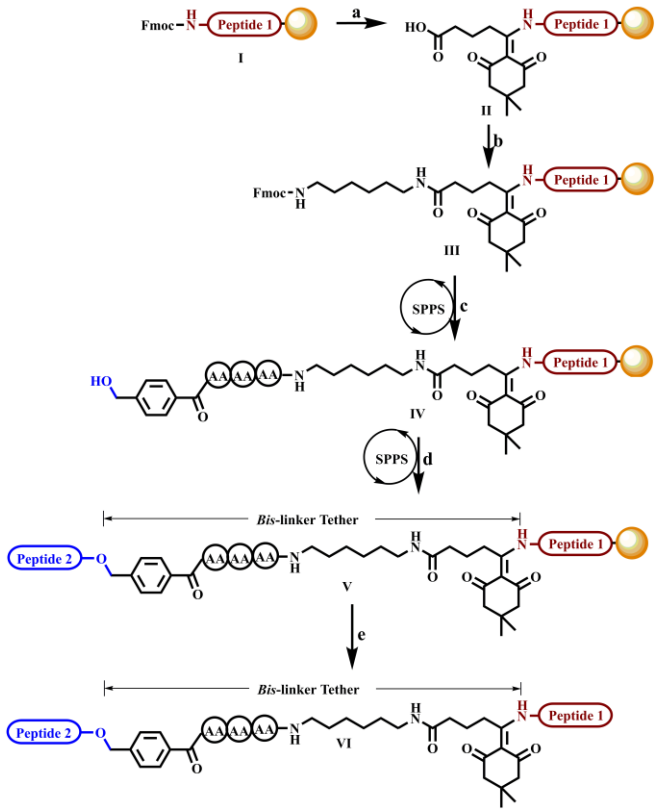
The proposed strategy (Figure 1) requires that the two chemical linkers are stable to Fmoc-solid phase peptide synthesis (SPPS) protocols, acidic peptide cleavage, the removal of the

thiol protecting groups and disulfide bond forming conditions (thiolysis). The use of hydroxymethylbenzoic acid (HMBA) as a chemically cleavable C-terminal linker has been successfully demonstrated.[6] HMBA is also compatible with various thiol protecting groups as it can be easily hydrolyzed at basic pH. An ideal N-terminal linker (e.g. on peptide 1; Figure 1) should have these characteristics and be simultaneously removable with the C-terminal HMBA linker. However, to our knowledge, no such linker has been reported to date. Several amino protecting groups have been developed[7] including the dimedone-based amino protecting groups 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl (Dde) or 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl (ivDde) which have been used in solid phase peptide chemistry due to their orthogonality to Fmoc- and Boc-SPPS[8] and, thus, provide a suitable basis for its development into an N-terminal linker. Moreover, the dimedone-based protecting group can be readily coupled onto a free amine and efficiently removed in the presence of dilute aqueous hydrazine solution. Another major advantage of hydrazine-aided removal of the dimedone protecting group is that an additional scavenger is not required as its removal results in the concomitant formation of a non-reactive indazole ring structure.[9] Existing disulfide bonds are also reported to be stable under hydrazine conditions.[10] Importantly, the basic pH of the hydrazine buffer also allows the simultaneous one-pot removal of the HMBA linker that serves as a C-terminal linker in our strategy.[7, 11] Thus, a modified form of the ivDde amino protecting group, 5-(4,4-dimethyl-2,6-dioxocyclohexylidene)-5-hydroxypentanoic acid (paDde-OH, Scheme 1), was synthesized as a novel N-terminal cleavable linker. The Knoevenagel condensation of 5,5'-dimethylcyclohexane-1,3-dione with the respective cyclic anhydride was advantageous over the reported two-step synthesis that employs a synthesis of tertiary butyl monoester of dicarboxylic acid which is then converted to the desired products.[12] Our one-step synthesis method from glutaric anhydride yielded the desired N-terminal linker 'paDde-OH' (Scheme 1) in a yield of 71% (SI Section 3).




**Scheme 1.** Synthesis of dimedone-based N-terminal linker, paDde-OH.

The first step in the overall protocol was to sequentially assemble both peptides on a single solid support. A standard Fmoc-SPPS was employed to assemble peptide 1 (Scheme 2, **I**). The N-terminal linker, paDde-OH (obtained from Scheme 1) was coupled onto the free α-amine of the N-terminal amino acid of peptide 1 in the presence of N,N-diisopropylethylamine (DIEA) which results in a free carboxylic acid group on the resin bound peptide (**II**). This was activated with N,N,N',N'-tetramethyl-O-(6-chloro-1H-benzotriazol-1-yl)uranium hexafluorophosphate (HCTU)/DIEA followed by coupling of a Fmoc-mono-protected diamine to yield resin bound Fmoc-protected peptide (**III**). Further, standard stepwise solid phase couplings can be continued to assemble the tether including the HMBA linker to obtain a free hydroxyl group on the resin bound peptide (**IV**). In order to assemble the second peptide, the C-terminal amino acid of peptide 2 was anchored using N,N′-diisopropylcarbodiimide (DIC)/ N,N-dimethylamnopyridine (DMAP)-mediated acylation on HMBA**,** followed by standard SPPS amino acid couplings to complete the second sequence (**V**). Finally, the resin-bound tethered two-peptide product was cleaved using a TFA cleavage cocktail (**VI**).




**Scheme 2.** Strategy for solid phase peptide synthesis (SPPS) of two peptide chains tethered by *bis*-linker sequence: (a) i) 20% piperidine/DMF, ii) paDde-OH, DIEA, DMF; (b) i) HCTU (2.5 eq.), DIEA (2.5eq.), N-Fmoc-1,6-diaminohexane (2.5 eq.); (c) i) SPPS, ii) 4-hydroxymethylbenzoic acid (4 eq.), HCTU (3.8 eq.), DIEA (4 eq.), DMF; (d) i) coupling of the first amino acid of peptide 2 (Fmoc-amino acid-OH (4 eq.), DIC (2 eq.), DMAP (0.1 eq.), DMF); ii) SPPS and (e) cleavage of the linear peptide from solid support (TFA:anisole:DODT:TIPS (94:3:2:1)).

To evaluate the proposed *bis*-linker tether strategy, a suitable two-peptide system (TAT-PKCi) was selected as a proof of concept study. Transactivator of transcription (TAT) is a cell penetrating peptide sequence that has been utilized to deliver a therapeutically important phosphokinase C inhibitor (PKCi).[13] PKCi as a first peptide, TAT as second peptide and HMBA-Gly-diaminohexane-paDde as the *bis*-linker tether were assembled using the general solid phase protocol in Scheme 2 (Figure 2A). The resulting linear peptide, PKCi-*bis*-linker-TAT (**2A**, Figure 2A) was then used as a starting material for the formation of the intra-peptide-peptide conjugate. This was obtained in a one-pot fashion (either by disulfide or thioether bond) followed by removal of the *bis*-linker tether (Figure 2A). The disulfide bond was formed using 2,2'-dithiodipyridine (DPDS) oxidation that resulted in a cyclic PKCi-*bis*-linker tether-TAT peptide (**2B**).[14] In addition, the linear PKCi-*bis*-linker tether-TAT peptide (**2A**) was treated with α,α'-*bis*-bromo-m-xylene which resulted in a cyclic two-peptide product (**2E**, Figure 2A) to further investigate the compatibility of this linker with non-disulfide conjugation strategies.[15] Given the hydrophobic nature of both the *bis*-linker tether and the xylene moiety, the RP-HPLC profile of the cyclic

peptide (**2E**) was found to be broad (Figure 2C). However, the addition of 5% aqueous hydrazine for 5 min. produced the desired PKCi-TAT conjugates (**2C** and **2F**) with sharp HPLC peaks and liberated the *bis*-linker tether (**2D**) without forming acylhydrazine (Supporting Information).[16] Reported protocols for making this peptide-peptide conjugate involve two separate syntheses of individual peptide chains. Our one-pot reaction reduced the number of steps and purifications to yield the desired product (**2C**, Figure 2B) with an overall yield of 27% and the desired PKCi-TAT thioether conjugate (2F, Figure 2C) obtained in 21% overall yield (calculated from crude **2A**).

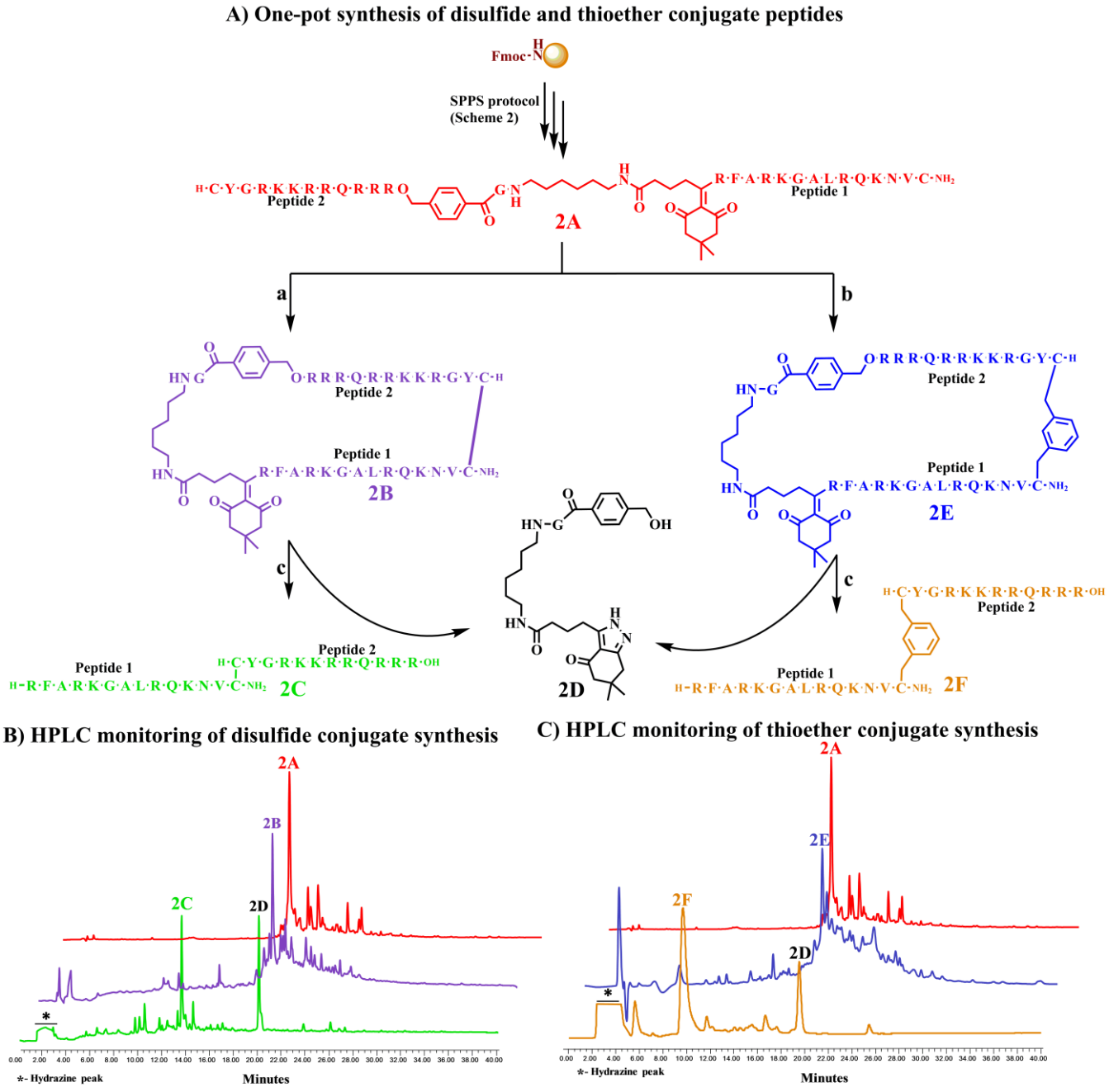



**Figure 2.** (A) One-pot conjugation and removal of *bis*-linker tether (a) 1.5 eq. DPDS, acetonitrile: water;( b) α, α'-*bis*-bromo-m-xylene, phosphate buffer, pH 7.4; (c) 5% hydrazine buffer. (B) RP-HPLC monitoring of crude peptides (gradient 10-50% buffer B, 40 min, 1%/min flow rate.): linear PKCi-*bis*-linker tether-TAT, **2A**; cyclic PKCi-*bis*-linker tether-TAT disulfide conjugate, **2B**; PKCi-TAT disulfide conjugate, **2C**; cleaved *bis*-linker tether, **2D**; and (C) RP-HPLC monitoring for crude peptides (gradient 10-50% buffer B, 40 min, 1%/min flow rate): cyclic PKCi-*bis*-linker tether-TAT thioether conjugate, **2E**; PKCi-TAT thioether conjugate, **2F**.

Finally, to evaluate the proposed *bis*-linker tether strategy, the synthesis of a more complex model peptide, thionin (7-19)-(24-32R), a thionin analogue, with two chains and two disulfide bonds was attempted (Figure 3).[17] Both cystines can be formed regioselectively using trityl (Trt) and acetamidomethyl (Acm) thiol protecting groups (Figure 3A). Compared with peptides such as insulin, this thionin analogue is a relatively small and hydrophilic sequence and thus an ideal model system to study the compatibility of the *bis*-linker tether approach together with regioselective disulfide bond formation. Using the same general protocol of synthesizing two peptides on a single solid support (Scheme 2), both the A- and B-chains were assembled, with an

additional Lys and Arg in the tether in order to provide enough length to form the disulfide bonds and also to avoid the aggregation that we observed in the synthesis of peptide **2E**. The linear A-chain-*bis*-linker-B-chain peptide (**3A**) was subjected to simple DPDS-mediated oxidation to form the first disulfide bond (**3B**). After iodine-mediated removal of 'Acm' groups and simultaneous second disulfide bond formation to obtain **3C**, the desired peptide (**3D**) was obtained by the addition of aqueous hydrazine in an overall yield of 24% as calculated from crude **3A**. Notably, epimerization of Fmoc-AA-OH may take place, especially in case of esterification involving Cys and His.[18] We did not observe such epimerization as Arg is the first amino acid of peptide 2, in both PKCi-TAT (Figure 2) and thionin analogue (Figure 3). Epimerization of Cys or His can also be avoided as reported by Sandhya *et al*.[18]

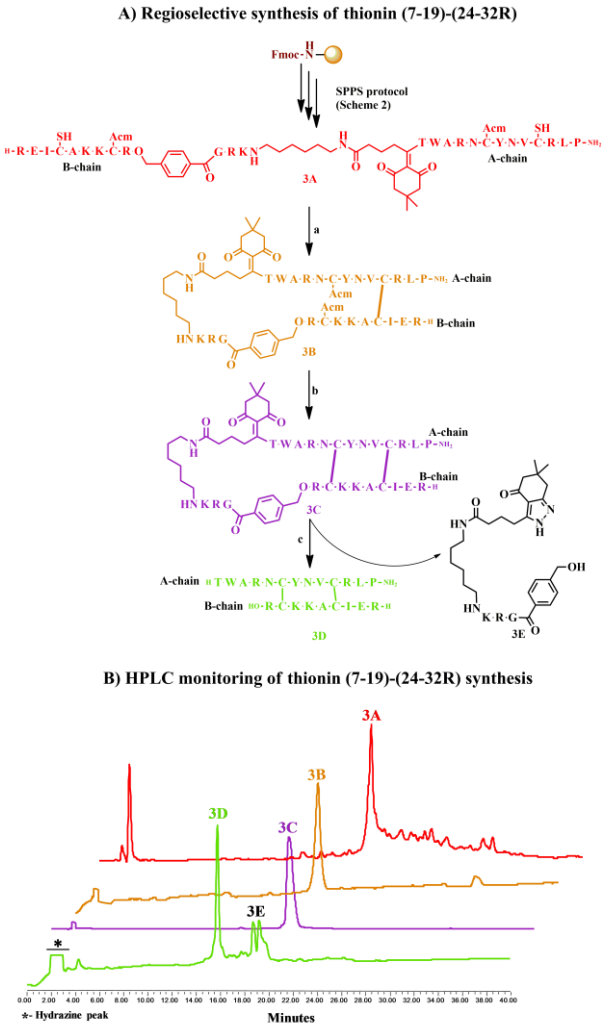



**Figure 3.** (A) Regioselective approach for the synthesis of thionin analogue: a) 1.5 eq. DPDS, acetonitrile:water, b) 60 mM iodine solution in acetic acid, c) 5% hydrazine buffer. (B) RP-HPLC monitoring of peptides (gradient 10-50% buffer B, 40 min, 1%/min flow rate.): crude reduced thionin analogue (A-*bis*-linker tether-B), **3A**; purified oxidized thionin analogue (A-*bis*-linker tether-B)-one disulfide bond, **3B**; purified oxidized thionin analogue (A-*bis*-linker tether-B) with two disulfide bonds, **3C**; crude thionin analogue (A-B), **3D**; and cleaved *bis*-linker tether, **3E**.

In conclusion, a novel dimedone-based linker was developed and successfully used together with an HMBA-based linker to synthesize a heterodimeric peptide (thionin analogue) as well as peptide-peptide conjugates (PKCi-TAT). Importantly, this *bis*-linker tether method is compatible with both Fmoc- or Boc-SPPS. Since this strategy reduced the number of intermediate and purification steps, the peptides were obtained in good overall yields. This preliminary study on the stability of the *bis*-linker and its removal followed by regioselective disulfide bridge formation will enable the preparation of two chain cysteine-rich peptides and proteins such as insulin and insulin-like peptides (e.g. relaxin). Since dimedone-based protecting groups are widely used in solid phase synthesis including of peptide nucleic acids and in carbohydrate chemistry, this linker could also be applicable to the synthesis of multiple polynucleotide or polysaccharide chains.

## Acknowledgements

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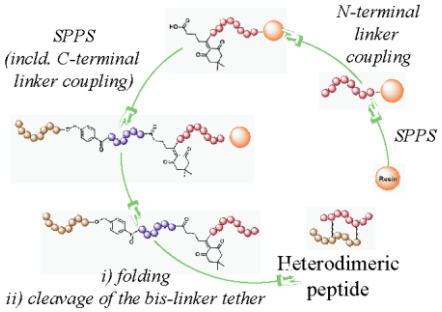
## Table of Contents

## COMMUNICATION

A dimedone-based *N*-terminal linker was developed for use in a *bis*-linker tether strategy for efficient synthesis of heterodimeric peptides. Sequential solid phase synthesis of two peptide chains on same solid support separated by the tether followed by its folding and chemical cleavage of the *bis*-linker tether yielded the heterodimeric peptide.

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**A One-Pot Chemically Cleavable *bis*-Linker Tether Strategy for the Synthesis of Heterodimeric Peptides**

**Keywords:** peptides • solid phase synthesis • heterodimeric peptides • N-terminal linker • conjugation

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**SI1. Reagents and materials**

H-PAL-ChemMatrix (0.42 mmol/g) amide resin, hydrazine monohydrate, triisopropylsilane (TIPS), anisole, 2,2'-(ethylenedioxy)diethanethiol (DODT), 2,2'-dithiodipyridine (DPDS) were purchased from Sigma-Aldrich. Fmoc-L-amino acids, N, N, N′, N′-tetramethyl-O-(6-chloro-1H-benzotriazol-1-yl) uronium hexafluorophosphate (HCTU) were purchased from GL Biochem. acetonitrile (AcN), acetic acid (AcOH), dichloromethane (DCM), diisopropylethylamine (DIEA), N, N-dimethylformamide (DMF), diethyl ether, piperidine, methanol (MeOH) were purchased from Merck. Trifluoroacetic acid (TFA) was purchased from AusPep, Iodine was purchased from Ajax Chemicals.

**SI2. General methods for SPPS and characterization**

***SI2. 1. Solid Phase Peptide Synthesis:***

Peptide elongation and TFA cleavage: Peptide chain assembly was carried out on a Liberty$^{TM}$ microwave peptide synthesizer. Fmoc removal was carried out with a 20% piperidine solution in DMF at 75°C for 3 minutes. The coupling was done through an activation of Fmoc-protected amino acids (4 eq) in the presence of HCTU (3.8 eq) and DIEA (4 eq) at 75°C for 5 minutes; except for arginine and cysteine that are coupled at 50°C for 5 minutes. Side chain protecting groups and peptide were cleaved from the solid support with a solution of TFA/TIS/DODT/$H_2O$ (96/3/2/1, v/v'/v''/v''') for 2 hrs at room temperature. After cleavage, the resin was removed by filtration, the filtrate was concentrated under a stream of nitrogen and the peptide products were precipitated in ice-cold $Et_2O$ and washed by centrifugation three times. The peptides were analysed and purified by RP-HPLC using water and acetonitrile with 0.1% TFA gradients. The final products were characterized by RP-HPLC and MALDI-TOF MS and/or ESI-MS.

Coupling of paDde-OH (5-(4,4-dimethyl-2,6-dioxocyclohexylidene)-5-hydroxypentanoic acid): A solution of paDde-OH (2 eq), DIEA (2 eq) in DMF was added onto the resin and allowed to react overnight at room temperature. The completion of the reaction was confirmed with TNBS test as well as a small scale cleavage followed by MALDI-TOF MS analysis.

Coupling of Fmoc-N'-hexane diamine: The resin bound carboxylic group was activated by addition of a solution containing HCTU (2.5 eq), DIEA (4 eq) in DMF (1 ml), The activation was continued for 15 min followed by addition of Fmoc-N'-hexane diamine (3.0 eq) in DMF. The completion of the reaction was confirmed with a small scale cleavage followed by MALDI-TOF MS analysis.

Coupling of C-terminal amino acid of the second peptide (HMBA acylation): A solution of Fmoc-amino acid (6 eq), DIC (3 eq), DMAP (0.3 eq) in DMF was added onto the resin and stirred overnight at room temperature. The completion of the reaction was confirmed with a small scale cleavage followed by MALDI-TOF MS analysis.

DPDS mediated disulfide bond formation: All disulfide bond formation reactions were performed at a concentration of 0.2 mM. For each pair of cysteine, 1 eq. of DPDS was dissolved in the minimum quantity of MeOH and added to the peptide to oxidise.

Iodine-mediated removal of acetamidomethyl group and simultaneous disulfide bond formation: The acetamidomethyl (Acm) thiol protecting group was cleaved and simultaneous disulfide bond formation was achieved by previously mentioned protocol.[1]

***SI2. 2. Characterization:***

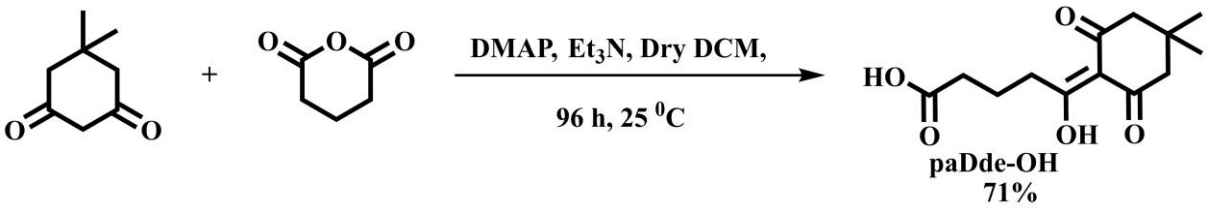
*Analytical and preparative RP-HPLC*: All the RP-HPLC analysis as well as purifications, were carried out using buffer A (0.1% TFA in MilliQ water) and buffer B (0.1% TFA in acetonitrile). The peptides were analyzed using 5 µm C18 analytical column (250 × 4.6 mm, Gemini® Phenomenex) and purified using 5 µm XB-C18 preparative column (150 × 21.2 mm, Phenomenex), using a 600-pump controller and 996-diode array detector (Waters).

*MALDI-TOF MS analysis*: All peptides were analysed on a Bruker Daltonics Autoflex II TOF/TOF using DHB or SA matrices.

*ESI-MS analysis:* Compound **1** was analysed on an Agilent 6220 LC/ESI-TOF mass spectrometer.

**SI3. Synthesis and characterization of N-terminal linkers**

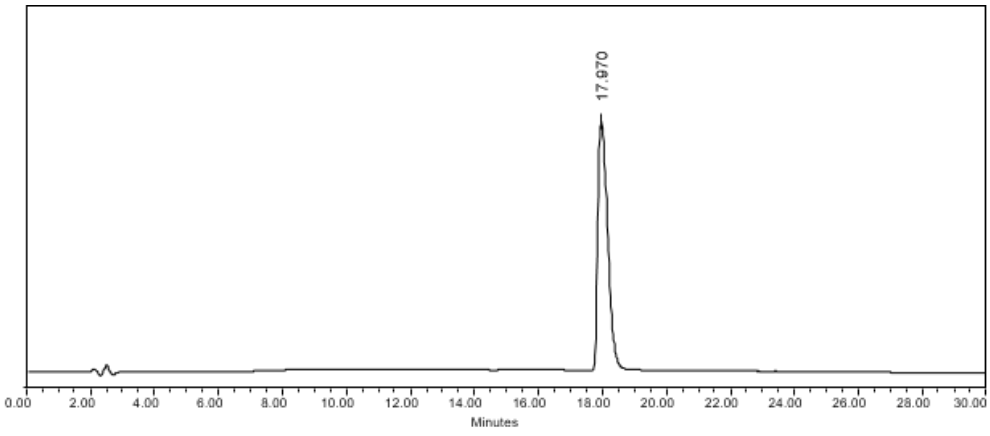
***SI3.1 Synthesis* of "5-(4,4-dimethyl-2,6-dioxocyclohexylidene)-5-hydroxypentanoic acid", (paDde-OH):**



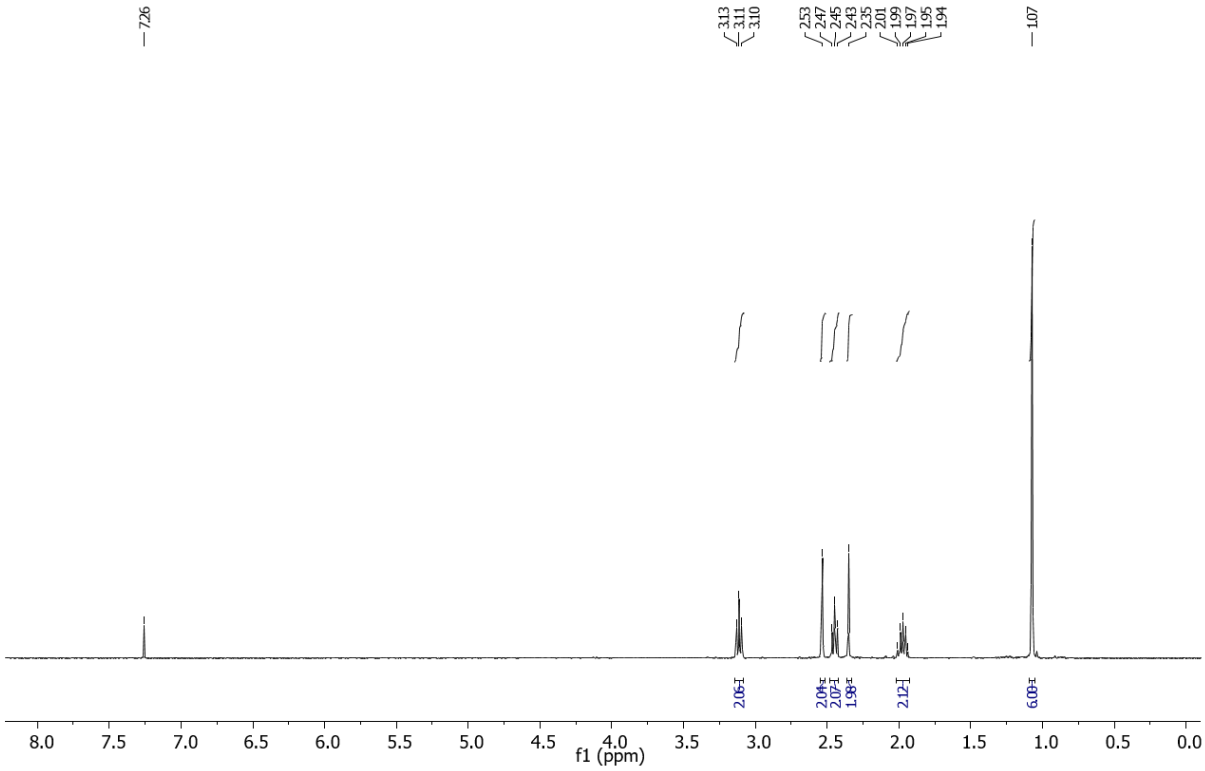

A solution of dihydro-2H-pyran-2,6(3H)-dione (34.2 mmol) and DMAP (28.5 mmol) in dry DCM was added dropwise to the solution of 5,5-dimethylcyclohexane-1,3-dione (28.5 mmol) in dry DCM and stirred for 96 hrs under nitrogen atmosphere. The reaction was quenched by the addition of 0.5 M $KHSO_4$ solution and followed by successive extraction with ethyl acetate. The combined organic ethyl acetate layer was dried over sodium sulphate, filtrated and evaporated under reduced pressure. The crude was adsorbed on silica gel, purified using ethyl acetate/n-hexane mobile phase. The desired product was eluted out at 20% ethyl acetate in n-hexane with 71% yield.

Characterization of paDde-OH:

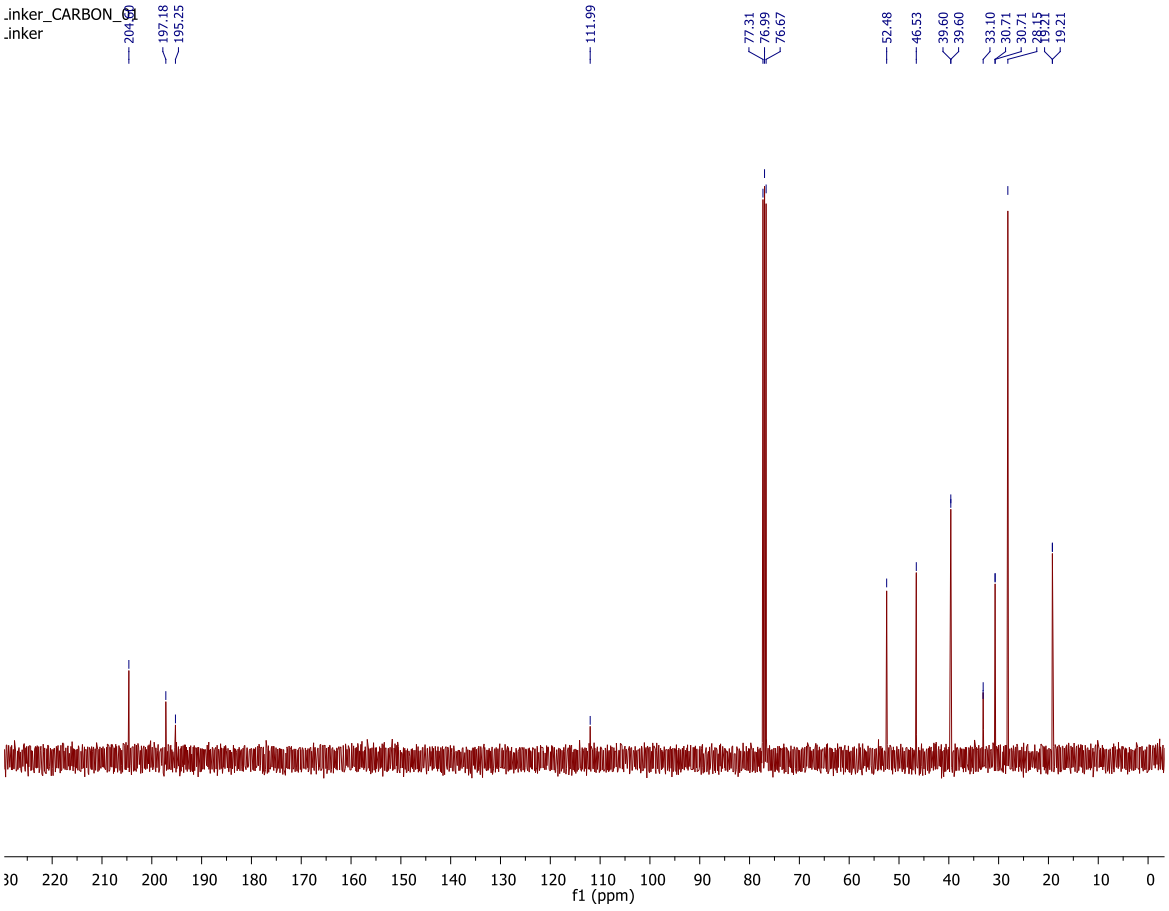
RP-HPLC (214 nm): 17.97 min (99%) in a gradient of 15% to 75% buffer B in 30 min.



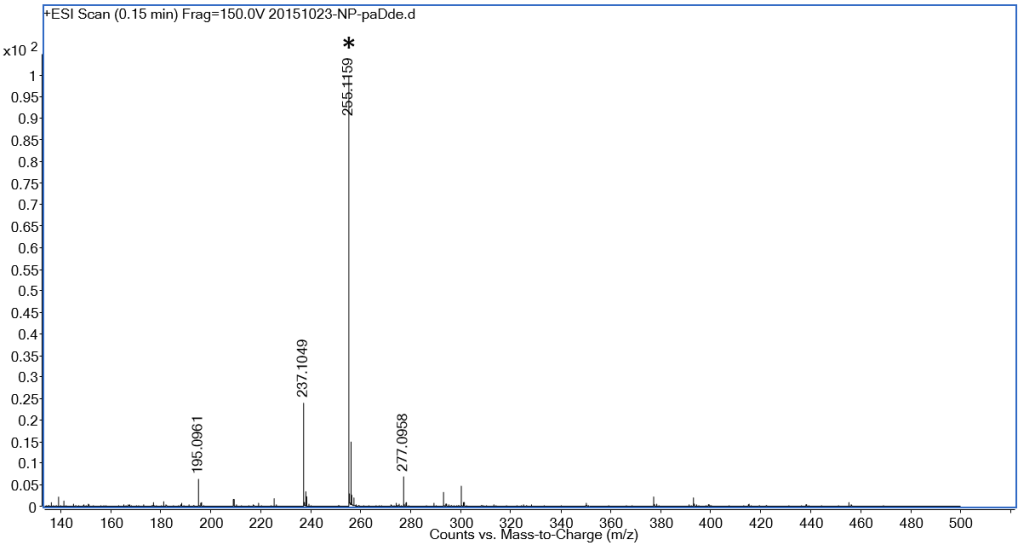
$^{1}$H NMR (400 MHz $CDCl_3$): 3.13-3.10 (2H, t, *J* = 2., 3.6), 2.53 (2H, s), 2.47-2.43 (2H, t, *J* = 1.9, 3.7), 2.35 (2H, s), 2.01-1.94 (2H, m *J* = 1.8, 3.6, 3.7), 1.07 (6H, s)



$^{13}$C NMR (100 MHz $CDCl_3$): 204.60, 197.18, 195.25, 111.99, 52.48, 46.53, 39.60, 33.12, 30.71, 28.15, 19.21.

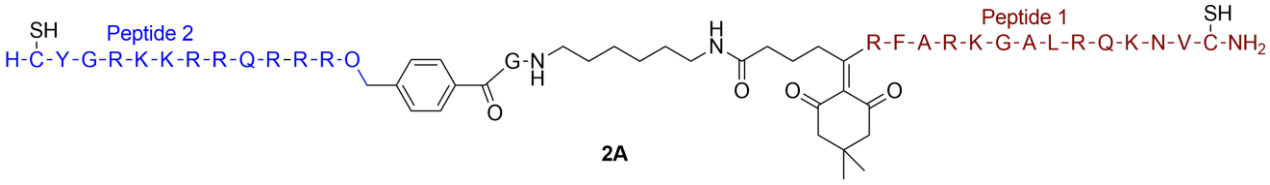



ESI-MS analysis: Molecular Weight: 254.28; Exact Mass: 254.12 Da; Observed: 255.116 (*****desired), 277.0958 (M+Na) Da.



## SI4. Development of one-pot strategy for conjugation and linker removal

### *SI4.1. Synthesis and characterization of 2A*



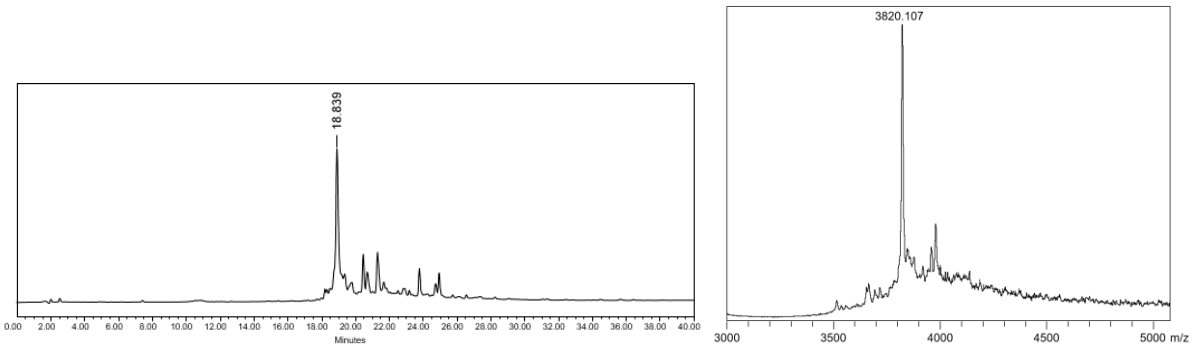

Chemical formula: $C_{166}H_{284}N_{64}O_{36}S_2$

Molecular weight: 3816.57; Exact Mass: 3814.18 Da; Observed: 3820.107 Da.

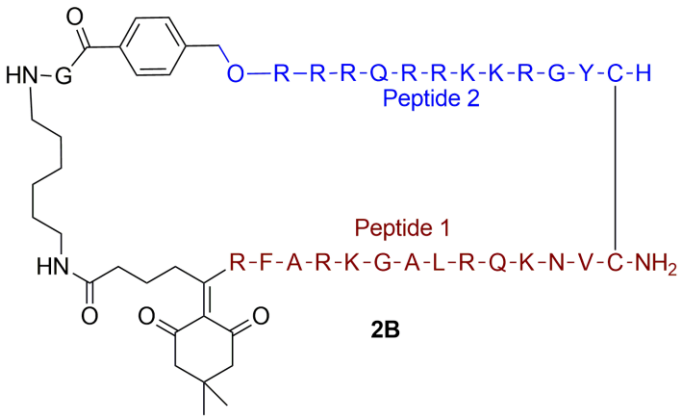
The observed masses did not match with that of calculated exact mass due to a calibration error (+6 Da) in our MALDI MS.

Crude yield: 67.6%

RP-HPLC (214 nm): 18.84 min in a gradient of 10% to 50% buffer B in 40 min.



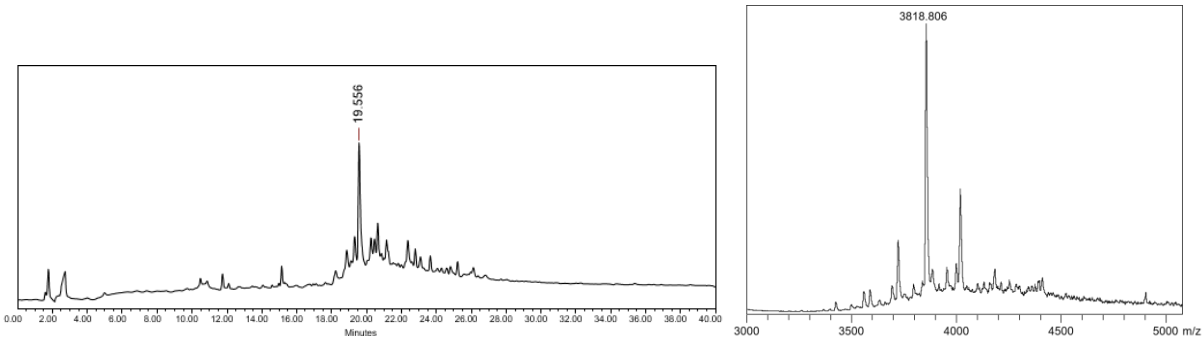

### *SI4.2. Synthesis and characterization of 2B:*




Chemical formula: $C_{166}H_{282}N_{64}O_{36}S_2$

Molecular weight: 3814.55; Exact Mass: 3812.16 Da; Observed: 3818.806 Da

RP-HPLC (214 nm): 19.56 min in a gradient of 10% to 50% buffer B in 40 min.




### *SI4.3. Synthesis and characterization of 2C:*

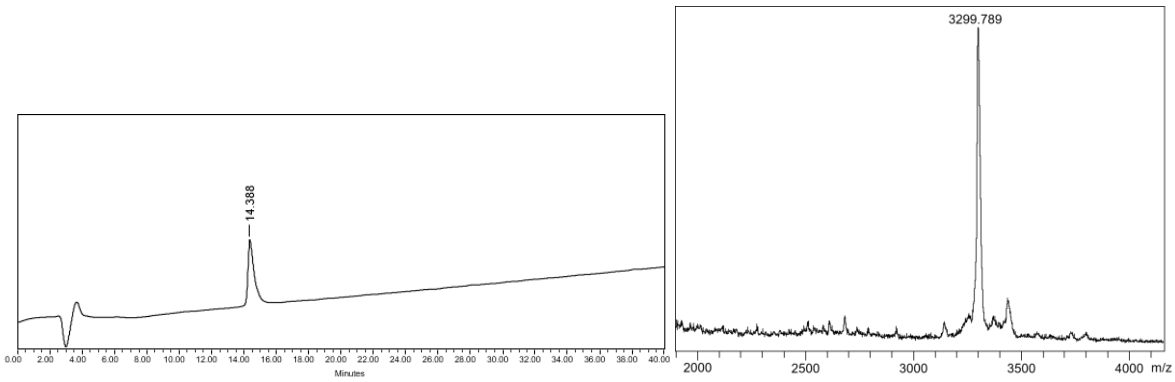

**2C**

Chemical formula: $C_{137}H_{246}N_{61}O_{31}S_2$

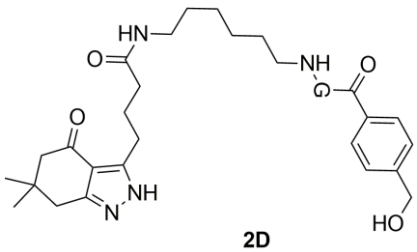
Molecular weight: 3307.94; Exact Mass: 3305.90 Da; Observed: 3299.789 Da

Overall yield: 27%

RP-HPLC (214 nm): 13.55 min in a gradient of 10% to 50% buffer B in 40 min.




### *SI4.4. Synthesis and characterization of 2D:*

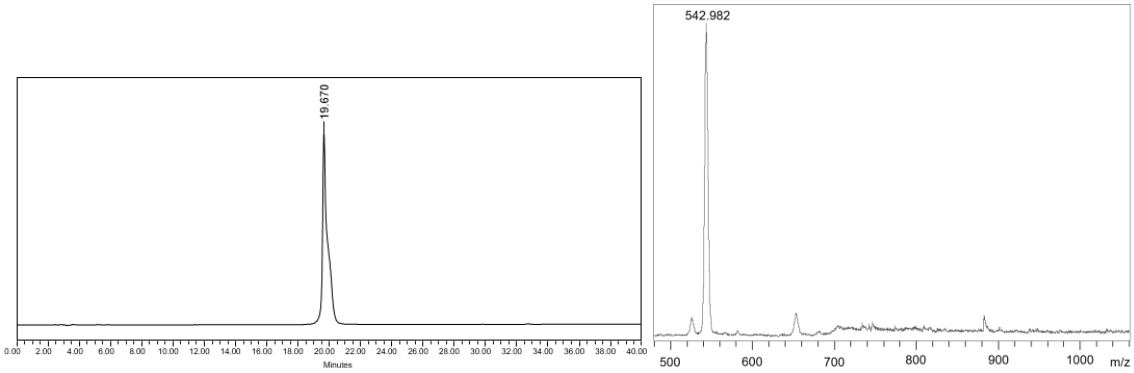



**2D**

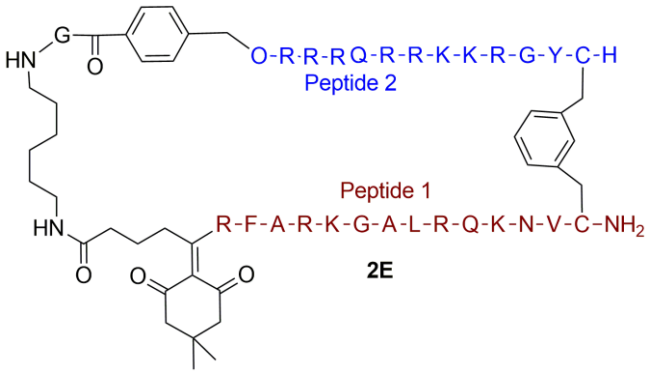
Chemical formula: $C_{29}H_{41}N_5O_5$

Molecular weight: 539.67; Exact Mass: 539.39 Da; Observed: 542.982 Da

RP-HPLC (214 nm): 19.67 min in a gradient of 10% to 50% buffer B in 40 min.



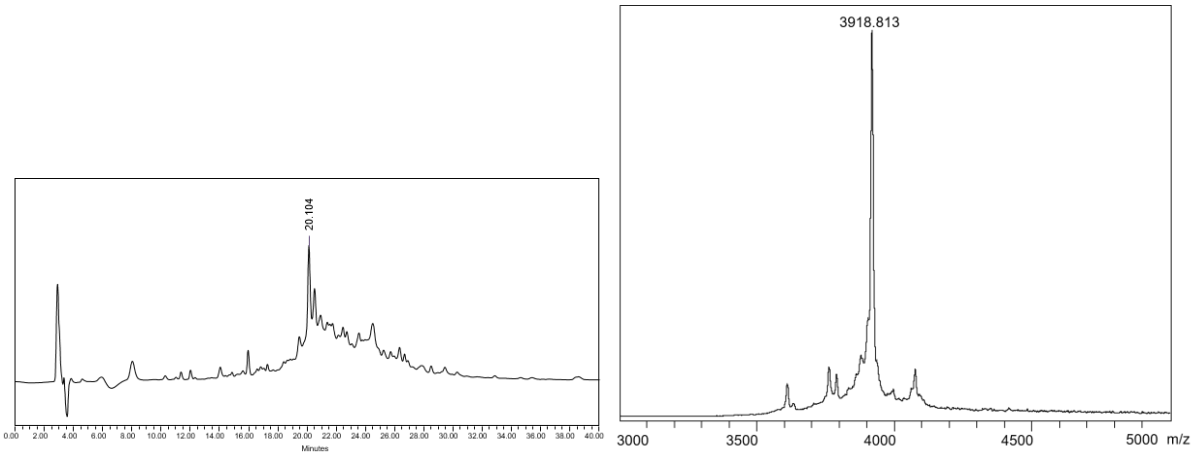

***SI4.5. Synthesis and characterization of 2E:***



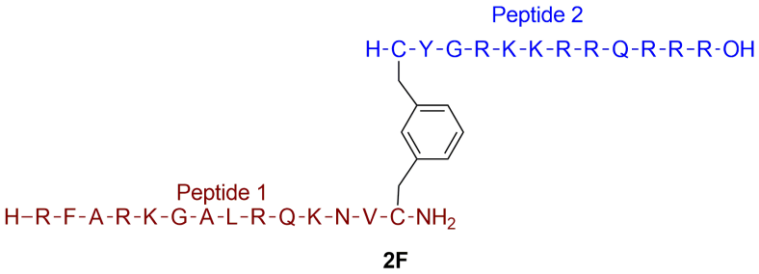

Chemical formula: $C_{176}H_{294}N_{64}O_{37}S_2$

Molecular weight: 3920.72; Exact Mass: 3918.24 Da; Observed: 3918.813 Da

RP-HPLC (214 nm): 20.104 min in gradient of 10% to 50% buffer B in 40 min.



***SI4.6. Synthesis and characterization of 2F:***



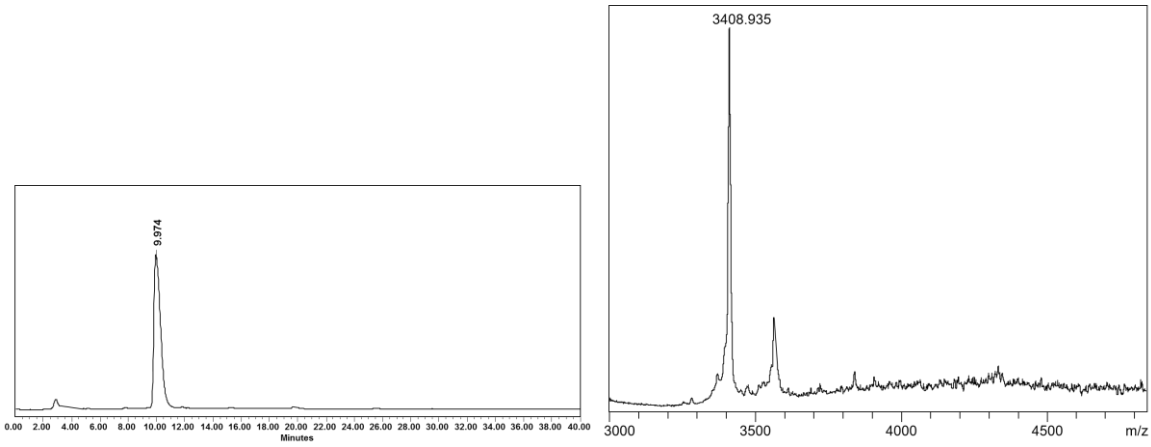

**2F**

Chemical formula: $C_{145}H_{253}N_{61}O_{31}S_2$

Molecular weight: 3411.08; Exact Mass: 3408.95 Da; Observed: 3408.935 Da

Overall yield: 21%

RP-HPLC (214 nm): 13.77 min in a gradient of 10% to 50% buffer B in 40 min.



### *SI5. Bis-linker tether cleavage and stability with conjugation buffers*

#### *SI5. 1 Bis-linker removal kinetics study:*

Disulfide bond shuffling is a commonly observed phenomenon in cysteine-rich peptides.[2] To minimize this during the removal of the bis-linker tether, the cleavage should be rapid. Earlier reports suggest the use of hydrazine solution for removal of dimedone-based protecting groups.[3] As the pH is basic, the hydrazine buffer is also suitable for the simultaneous hydrolysis of the HMBA ester at the C-terminus of the second peptide. To determine suitable conditions for the removal of bis-linker tether, two concentrations of hydrazine hydrate, 1% and 5%, in aqueous buffer were evaluated. The cyclic PKCi-bis-linker-TAT peptide (2E) was used for determining the linker removal kinetics.

Linker cleavage buffers were prepared with 1% and 5% aqueous hydrazine hydrate solutions.

Quenching solution: 1% TFA in water (This TFA concentration was chosen as 0.1% TFA is not sufficient to neutralize 5% hydrazine at given volume).

Peptide solutions:1) 1 µg/µL solution in 1% TFA as a control; 2) Stock solution: 6 µg/µL peptide in water.

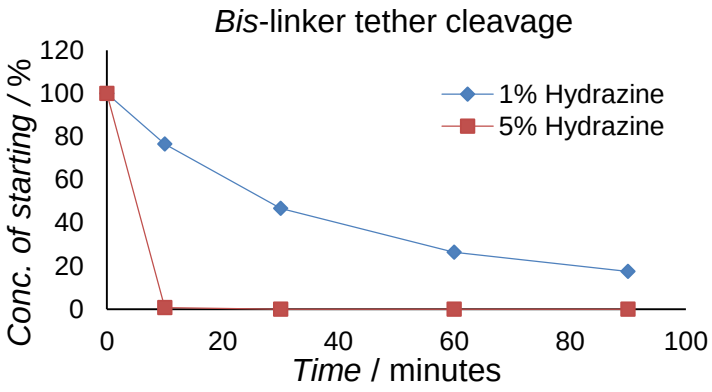
Procedure: A stock solution of peptide in MilliQ water (6 µg/µL solution) was prepared. 30 µL of the stock solution was added to 60 µL of each hydrazine buffer and allowed to shake. At each time point, 10 µL of this reaction mixture was taken out and quenched with 10 µL of 1% aq. TFA. The quenched solution was subjected to analytical HPLC. As a control, only peptide stock solution (1 µg/µl) in 1% TFA was subjected to RP-HPLC. Similarly, a blank (without the peptide) was performed using each buffer solution.

Table S1: Normalized HPLC data at each time point for 1% and 5% hydrazine buffers

| min | 1% hydrazine | 5% Hydrazine |
|---|---|---|
| 01 | 100 | 100 |
| 10 | 76.60 | 0.83 |
| 30 | 46.81 | 0.01 |
| 60 | 26.40 | ND |
| 90 | 17.54 | ND |

At each time point percent relative concentrations were calculated based on peak area, considering initial concentration as 100%, which was obtained from the control. The graph

suggest, 5% aqueous hydrazine was more efficient without promoting any side reactions and therefore preferred for removal of bis-linker tether throughout the study.




***SI5. 2 Bis-linker tether stability in various conjugation buffers:***

The challenge of synthesizing peptides with two-chains (e.g. insulin-like peptides) has recently been minimized by using native chemical ligation or oxime ligation. Moreover, click chemistry, thioether bridges, thiol-maleimide conjugation and native chemical ligation are some modern-day peptide chemistry tools used to obtain conjugated peptide products.[4] Consequently, the stability and the removal kinetics of this bis-linker tether, specifically the dimedone linker, were examined in different buffers. As the disulfide bonds are susceptible to reduction under native chemical ligation conditions, the thioether-linked cyclic peptide (**2E**) was used to investigate the stability of the linker in guanidium hydrochloride buffer (pH 8.5), phosphate buffer (pH 7.4), click reaction buffer (pH 7.4), native chemical ligation buffer (pH 6.8) and folding buffer (pH 7.4). The peptide concentration in each reaction buffer was monitored by RP-HPLC over a 24 h period (Figure 3B). The bis-linker tether was sufficiently stable and able to be used effectively in combination with specific reactions, which may be useful for the synthesis of multi-conjugated peptide systems.

Five buffers were used for evaluating the stability of linker namely 6 M GnHCl buffer (pH 8.5), phosphate buffer (pH 7.4), click buffer (20 mM CuSO4 and 20 mM ascorbic acid in phosphate buffer, pH 7.4), native chemical ligation buffer (1 M mercaptophenylacetic acid and 0.1 M TCEP in 6 M GnHCl buffer, pH 7.5) and folding buffer (GSH+ GSSG, pH 7.4)

Quenching solution: 1% TFA in water

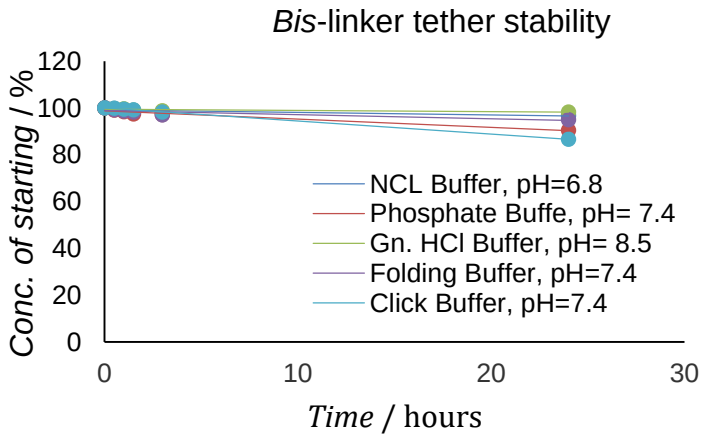
Peptide solutions: 1) 1 µg/µL solution in 1% TFA as a control; 2) Stock solution: 6 µg/µL peptide in water.

Procedure: A stock solution of peptide in MilliQ water (6 µg/µL solution) was prepared. 30 µL of the stock solution was added to 60 µL of each reaction buffer and allowed to shake. At each time point, 10 µL of this reaction mixture was taken out and quenched with 10 µL of 1% aq. TFA. The quenched solution was subjected to analytical HPLC. As a control, only peptide stock solution (1 µg/µl) in 1% TFA was subjected to RP-HPLC. Similarly, a blank (without the peptide) was performed using each buffer solution.

Table S2: Normalized HPLC data at each time point in various conjugation buffers

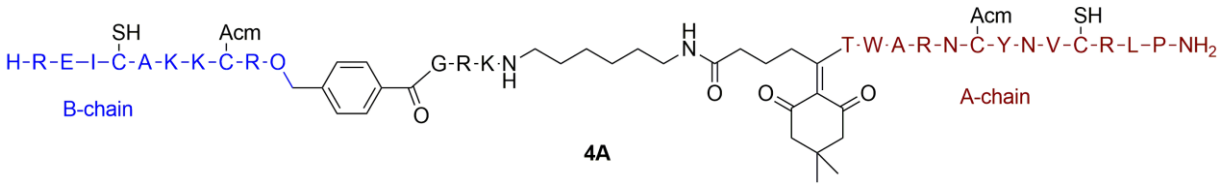
| Hrs | NCL Buffer (pH = 6.8) | Phosphate Buffer(pH=7.4) | Gn. HCl Buffer (pH =8.5) | Folding Buffer (pH =7.4) | Click Buffer (pH =7.4) |
|---|---|---|---|---|---|
| 0.02 | 100 | 100 | 100 | 100 | 100 |
| 0.5 | 99.70 | 99.09 | 99.71 | 99.19 | 99.83 |
| 1 | 99.21 | 98.34 | 99.64 | 98.72 | 99.47 |
| 1.5 | 98.46 | 97.44 | 99.09 | 98.29 | 99.20 |
| 3 | 97.84 | 97.14 | 98.78 | 96.97 | 98.30 |
| 24 | 96.79 | 90.48 | 98.27 | 94.92 | 86.61 |

At each time point percent relative concentrations were calculated based on peak area, considering initial concentration as 100 %. Bis-linker was found to be stable in in different bio-conjugation reaction conditions over 24 hrs.



## SI6. Development of "regioselective" strategy for forming 2 disulfide bonds

### *SI6. 1. Synthesis and characterization of 4A*



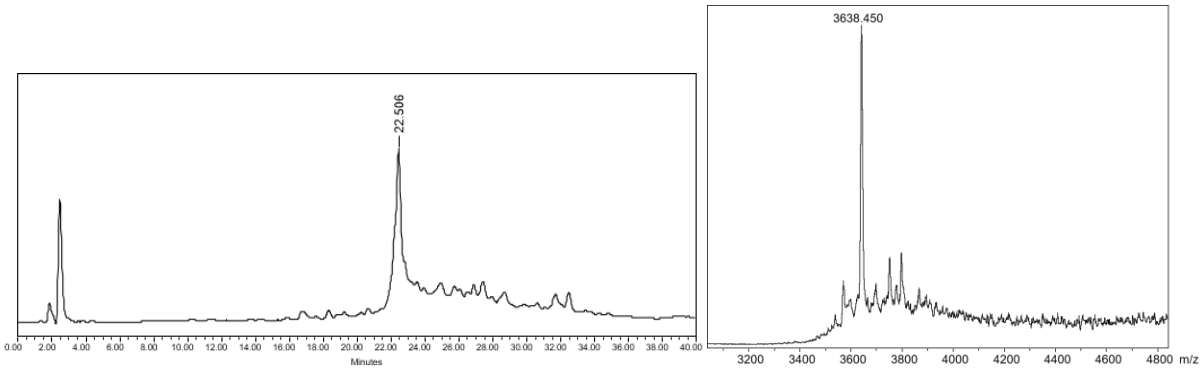

**4A**

Chemical formula: $C_{160}H_{261}N_{51}O_{36}S_4$

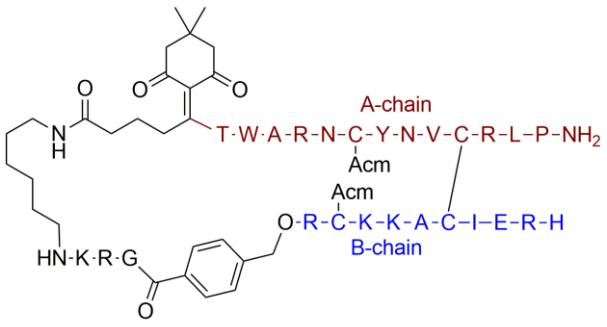
Molecular weight: 3635.36; Exact Mass: 3632.89 Da; Observed: 3638.450 Da

Crude yield: 57.8%

RP-HPLC (214 nm): 22.51min in a gradient of 10% to 50% buffer B in 40 min.




### *SI6. 2. Synthesis and characterization of 4B*:



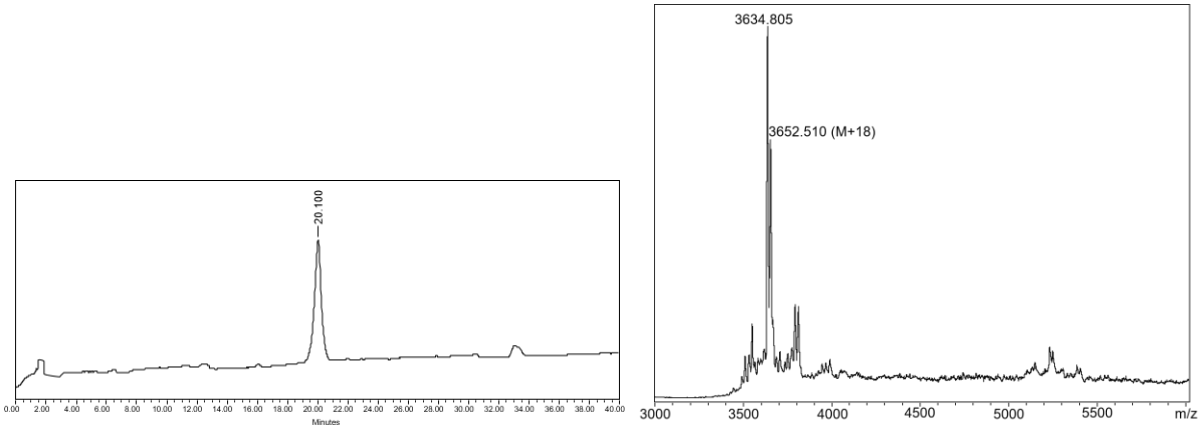

**4B**

Chemical formula: $C_{160}H_{259}N_{51}O_{36}S_4$

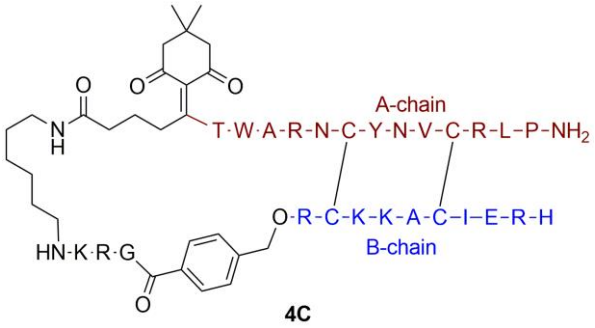
Molecular weight: 3633.35; Exact Mass: 3630.88 Da; Observed: 3634.805 Da

MALDI-MS average: $[M + H]^+$ ion (DHB) = 3634.805 Da; 3652.510 Da (+17.105: addition of $H_2O$ from Hydrolysis of ester bond).

RP-HPLC (214 nm): 20.10 min in gradient of 10% to 50% buffer B in 40 min.




***SI6. 3. Synthesis and characterization of 4C:***



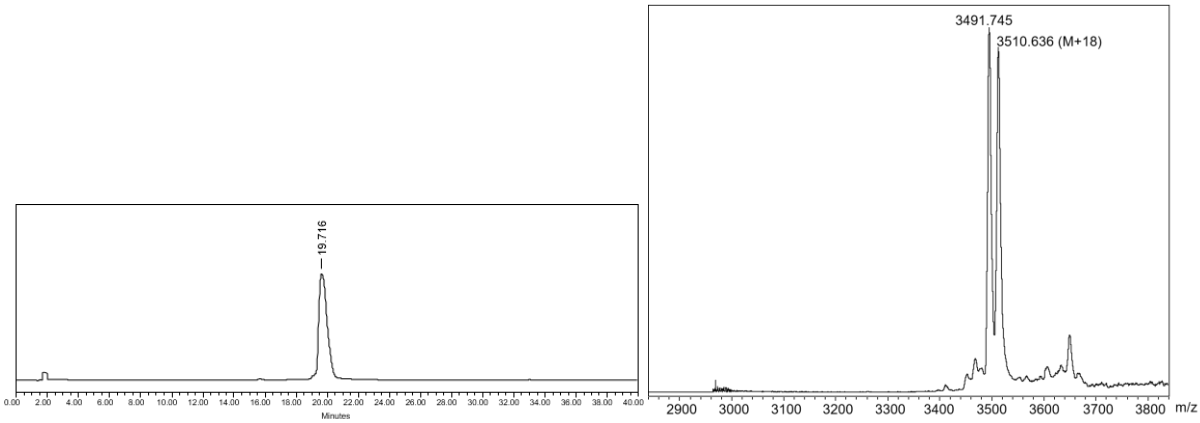

**4C**

Chemical formula: $C_{165}H_{283}N_{63}O_{35}S_2$

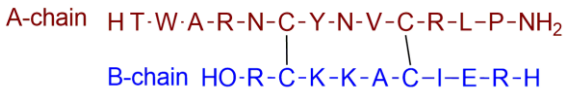
Molecular weight: 3489.18; Exact Mass: 3486.79 Da; Observed: 3491.745 Da

MALDI-MS average: $[M + H]^+$ ion (DHB) = 3491.745 Da; 3510.636 Da (+18.291: addition of $H_2O$ by hydrolysis of ester bond).

RP-HPLC (214 nm): 19.72min in gradient of 10% to 50% buffer B in 40 min.



***SI6. 4. Synthesis and characterization of 4D:***



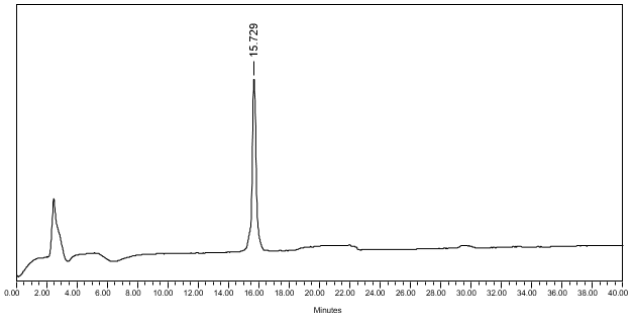

**4D**

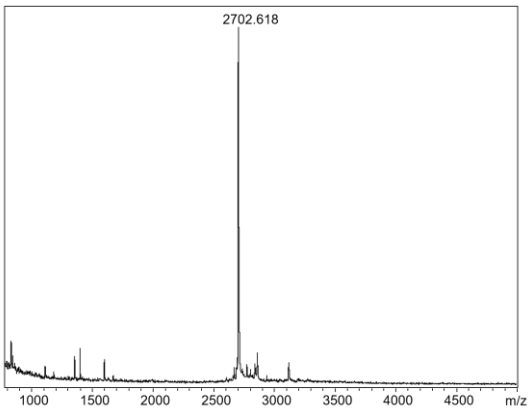
Chemical formula: $C_{165}H_{283}N_{63}O_{35}S_2$

Molecular weight: 2697.20; Exact Mass: 2695.32 Da; Observed: 2702.618 Da

Pure Yield: 24%

RP-HPLC (214 nm): 15.73min in a gradient of 10% to 50% buffer B in 40 min.



**SI7. Abbreviations**

AcOH: Acetic acid

AcN: Acetonitrile

Acm: Acetamidomethyl

DBU: 1, 8-Diazabicyclo[5.4.0]undec-7-ene

DHB: Dihydroxybenzoic acid

DIC: *N,N'*-Diisopropylcarbodiimide

DIEA: *N, N*-Diisopropylethylamine

DMAP: N, N-Dimethylamnopyridine

DMF: Dimethylformamide

DODT: 2,2'-(Ethylenedioxy)diethanethiol

DCM: Dichloromethane

DPDS: 2,2'-Dithiodipyridine

ESI-MS: Electron-spray ionization mass spectrometry

$Et_2O$: Diethyl ether

GnHCl: Guanidine hydrochloride

HCTU: N,N,N',N'-Tetramethyl-O-(6-chloro-1H-benzotriazol-1-yl)uranium hexafluorophosphate

HMBA: 4-hydroxymethylbenzoic acid

$KHSO_4$: Potassium hydrogen sulfate

MALDI-TOF MS: Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

MeOH: Methanol

NMR: Nuclear magnetic resonance spectroscopy

paDde-OH: 5-(4,4-dimethyl-2,6-dioxocyclohexylidene)-5-hydroxypentanoic acid

RP-HPLC: Reversed-phase high-performance liquid chromatography

SPPS: Solid phase peptide synthesis

TFA: Trifluoroacetic acid

TIPS: Triisopropylsilane

Trt: Trityl

SA: Sinapinic acid

**SI8. References:**

[1] S. Zhang, F. Lin, M. A. Hossain, F. Shabanpoor, G. W. Tregear, J. D. Wade, *Intl. J. Pept. Res. Ther.* **2008**, *14*, 301.
[2] E. De Bernardez Clark, E. Schwarz, R. Rudolph, in *Methods in enzymology, Vol. Volume 309*, Academic Press, **1999**, pp. 217-236.
[3] B. W. Bycroft, W. C. Chan, S. R. Chhabra, N. D. Hone, *J. Chem. Soc., Chem. Commun.* **1993**, 778.
[4] M. Galibert, V. Piller, F. Piller, V. Aucagne, A. F. Delmas, *Chem. Sci.* **2015**, *6*, 3617.