A Perfluoroaromatic Abiotic Analog of H2 Relaxin Enabled by Rapid Flow-Based Peptide Synthesis

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1. MATERIALS

Fmoc protected amino acids, 2-(1H-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), and 2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) were purchased primarily from Chem-Impex International. Additionally, some of the Fmoc amino acids used were purchased from Advanced ChemTech and Novabiochem. 2-chlorotrityl chloride resin (200-400mesh, 1.2 mmol/g) was purchased from Chem-Impex International and used to prepare 2-chlorotrityl hydrazine resin. N,N-dimethylformamide (DMF), dichloromethane (DCM), methanol (MeOH), diethyl ether, and HPLC-grade acetonitrile were from purchased from EMD Millipore. 1,2-Ethanedithiol (EDT) was purchased from Alfa Aesar. Hexafluorobenzene was purchased from Oakwood Chemical and used as received. Maxi Clean SPE 900 mg Lrg Pore C18 desalting columns were purchased from Grace Davision Discovery Science. Solvents for LC-MS were purchased from J.T. Baker and Fluka. Recombinant H2 relaxin was purchased from PeproTech. HTRF cAMP HiRange kit was purchased from CisBio. All other reagents were purchased from Sigma-Aldrich. Common solvent mixtures used throughout these experiments include: 0.1% (v/v) TFA in water (A), 0.1% (v/v) TFA in acetonitrile (B), 0.1% (v/v) FA in water (A') and 0.1% (v/v) FA in acetonitrile (B').

2. METHODS

2.1. Preparation of 2-chlorotrityl hydrazine resin

2-chlorotrityl hydrazine resin was synthesized from 2-chlorotrityl chloride resin using a procedure adapted from Stavropoulos, George et al. *Letters in Peptide Science*. 1995, **2**, 315-318. 25 g of 2-chlorotrityl chloride resin was stirred and allowed to swell in 150 mL of anhydrous DMF under an inert atmosphere in a 500 mL, three-necked, round-bottom flask. In a separate round-bottom flask, 10 mL of anhydrous hydrazine was added to approximately 50 mL of anhydrous DMF under an inert atmosphere. To this, DIEA was added dropwise until phase separation of the mixture began. This entire hydrazine-DIEA-DMF mixture was then added dropwise to the swollen 2-chlorotrityl chloride resin and stirred for 60 min at room temperature. The reaction was quenched by adding 50 mL of methanol and then stirred for 20 min. The resin slurry was then transferred to a glass fritted funnel and washed with 500 mL aliquots of the following in order: DMF, H2O, DMF, diethyl ether. The resin was then dried and determined to have a loading of 0.7 mmol/g. A previous batch, prepared under the same conditions and used for some experiments, was found to have a loading of 0.8mmol/g.

2.2. Peptide synthesis

All peptides were synthesized on the flow-based platform with a 2nd generation vessel (2 mL volume) as previously described in Simon et al. *ChemBioChem*, 2014, **15**, 713–720. Unless noted, all reagents (coupling, wash, and deprotection) were preheated to 60°C immediately before reaching the synthesis vessel. Generally, coupling of the first amino acid to the resin was performed within the reaction vessel using 1 mmol of Fmoc-protected amino acid (at least 4-fold excess relative to resin loading) dissolved in 2.5 mL of 0.4 M HBTU or HATU and activated with 500 µL DIEA (190 µL for Cys, His, Trp). The activated coupling solution was delivered to the reaction vessel via a syringe pump injecting at 6 mL/min over approximately 40 s. Using an HPLC pump flowing at 20 mL/min, DMF was delivered to the reaction vessel for 60 sec in order to wash out the activated amino acid. 20% (v/v) piperidine in DMF was then delivered for 20 sec, followed by another 60 sec DMF wash in order to ready the resin for the subsequent coupling.

2.3. Peptide global deprotection

Unless otherwise noted, all peptides were cleaved from the resin and side chains were deprotected with a standard cleavage cocktail containing 2.5% (v/v) EDT, 2.5% (v/v) H_2O, and 1% (v/v) TIPS in TFA for 2 hours at room temperature. Afterwards, the resin was filtered out and compressed nitrogen was used for the evaporation of the cleavage solution to dryness. The resulting oils or solids were triturated and washed three times with cold diethyl ether, dissolved in 50% A / 50% B, and then lyophilized.

2.4. LC-MS and MALDI analysis of peptides

All peptides and proteins were analyzed on an Agilent 6520 Accurate Mass Q-TOF LC-MS using a narrowbore Agilent Zorbax 300SB C3 column (300 Å, 5 μm, 2.1 x 150 mm) run with the following methods at 40 °C and a flow rate of 0.8 mL/min: 5% B' in A' for 2 min, 5-65% B' in A' ramping linearly over 11 min, 65% B' in A' for 1 minute. MALDI-TOF mass spectra were taken on a PerSeptive Biosystems, Voyager-DE Pro Biospectrometry workstation. Ionizing of peptides was assisted with a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% A / 50% B, respectively.

2.5. Purification of peptides

Crude peptides were purified on a Waters 600 HPLC system with a Waters 484 or 486 UV detector using solvents A and B. The following columns were used for purification: Agilent Zorbax 300SB preparative C3 column (300Å, 7μm, 21.2 x 250mm) and Agilent Zorbax 300SB C3 semi-preparative column (300Å, 5μm, 9.4 x 250mm). Fractions were collected and screened for the desired material using LC-MS and MALDI. Flow rates and gradients for each individual peptide and ligation are detailed below.

2.6. Native chemical ligation

The general procedure for ligation of peptides using peptide hydrazides was closely followed as reported in Fang, G. M. et al. *Angew Chem Int Ed Engl* **2012**, *51*, 10347. For ligation buffer, two 0.2 M phosphate solutions (pH 3.3 and pH 7.0, respectively) containing 6 M Gn·HCl were used. Oxidative solution is defined as aqueous 200 mM $NaNO_2$. 4-mercaptophenylacetic acid (MPAA) was used to convert peptide azides into thioesters. All ligations were reduced with 0.5

M dithiothreitol (DTT) prior to LC-MS and purification. Exact ligation procedures are given for each ligation below.

2.7. Representative peptide stapling

To a sample of crude peptide (2 mM) in a plastic Falcon tube, DMF supplemented with 3M Gn·HCl and 40 mM TRIS containing 1mM of triphenylphosphine as reducing agent was added. Peptide stapling was induced by addition of hexafluorobenzene dissolved in DMF at a final concentration of 1 mM. The tube was vigorously mixed on a shaker for 30 seconds and left at room temperature for 18 hours. The reaction mixture was characterized by LC-MS. The reaction mixture was diluted with 50 mL of 0.1% TFA solution in water, centrifuged for 20 min at 4000 rpm and subjected to purification by HPLC. Fractions containing stapled peptide product were combined and lyophilized.

2.8. Oxidative folding of wild type and C6F4 linked H2 relaxin

Oxidative folding of H2 relaxin A-chain (wild type or C6F4 linked) and B-chain was performed similarly to Barlos, K. K. et al. *J Pept Sci*, **2010**, *16*, 200. and Tang, J. G. et al. *Biochemistry*, **2003**, *42*, 2731. Purified A-chain and B-chains were dissolved at a ratio of 2:1 in 6 M Gn·HCl. This solution was further diluted to 1 M Gn·HCl and a final peptide concentration of 6 mg/mL using a 0.1 M glycine buffer, pH 10.8 containing 50% (v/v) DMSO. 1 mM glutathione was also added to prevent dimerization of the B chain. The mixture was vigorously stirred for 16h at 4°C. Precipitate that formed during stirring was collected by centrifugation and redissolved in 6M Gn·HCL before combination with the main solution. The mixture was acidified with an excess of solvent A and purified on an Agilent Zorbax 300SB C3 semi-preparative column (300Å, 5µm, 9.4 x 250mm).

2.9. Trypsinolysis of wild type and C6F4 linked H2 relaxin

Purified, folded wild type and C6F4 linked H2 relaxin were dissolved in phosphate buffered saline, pH 7.4 at a final concentration of 100 µg/mL. 2 µg peptide were treated with a trypsin solution (0.2 µg/µL dissolved in 2 mM HCl in H_2O) which was adjusted to pH 7.5 prior before use. Approximately 1 µg trypsin was added to the corresponding peptides and the solution was incubated for 1 hr at 37°C. The reaction was stopped by adding 10 µL of 1% (v/v) TFA in B and the resulting fragments were subsequently analyzed via LC-MS.

2.10. HEK 293T Cell Culture

HEK 293T cells stably transfected with human RXFP1 (HEK293-RXFP1, Kern, A. et al. *Endocrinology*, **2008**, *149*, 1277.) constructs were a kindly provided by Dr. Gilian D. Bryant-Greenwood (University of Hawaii). HEK293-RXFP1 cells were proliferated in DMEM supplemented with 10 % fetal bovine serum, 100 U/mL penicillin and 500 µg/mL geneticin. Cells were passaged after reaching 80 % confluence at a split ratio of 1:5.

2.11. HTRF cAMP Assay

10,000 HEK293-RXFP1 cells were seeded in a 384-well format plate in a total volume of 30 µL/well and were incubated overnight at 37°C and 5 % CO_2. Prior to stimulation with our recombinant and synthetic peptides, the phophodiesterase inhibitor IBTX was added into the growth medium (4 µL/well) at a final concentration of 1 mM. Cells were then stimulated with our peptides at various concentrations for 30 min at 37°C and 5% CO_2. After incubation, of HTRF cAMP HiRange detection reagents were added to each well (20 µL/well). The HTRF cAMP HiRange detection reagents were diluted in HTRF lysis buffer as per the manufacturer's instructions (anti cAMP antibody and cAMP-d2 diluted 1:20). Plates were incubated for at least 30 min at room temperature before detection on a HTRF plate reader (Perkin Elmer). Data points were obtained in triplicate and the assay was repeated at least two times.

3. SYNTHESIS OF RELAXIN B-CHAIN

3.1 Synthesis of H2 relaxin B-chain

3.1.1. Synthesis of H2 relaxin B-chain fragment B[1]

Sequence: H_2N-DSWMEEVIKL-$NHNH_2$

H2 relaxin B-chain fragment B[1] (*Figure S1*) was synthesized using a 3-minute cycle (HATU, 50% (v/v) piperidine) at 80°C on 250 of mg 2-chlorotrityl hydrazine resin. Standard cleavage cocktails resulted in significant oxidation of Met4. Therefore, a cleavage cocktail comprised of 5% (v/v) thioanisole, 2.5% (v/v) EDT, 3% (v/v) H_2O, 2% (v/v) dimethylsulfide, and 2% ammonium iodide (1.5% in H_2O w/v) in HPLC grade TFA was used to cleave the peptide for 2 hr at room temperature as previously described in Huang, H. et al. *J Pept Res.* **1999**, *53*, 548. Approximately 95 mg of crude peptide was obtained after workup of the peptide.

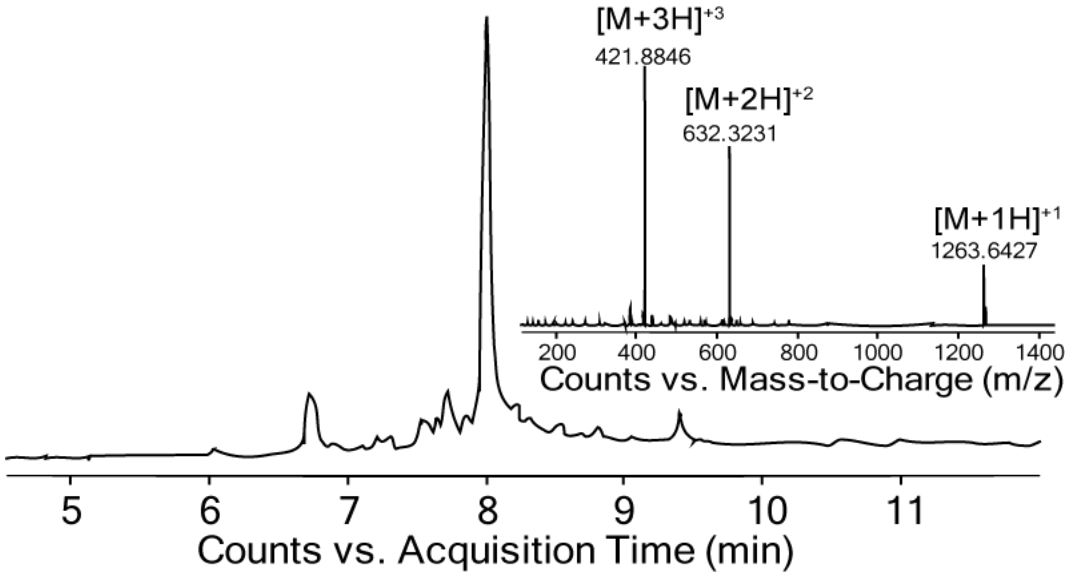


Figure S1: LC-MS (total ion current vs. time) of crude H2 relaxin B-chain fragment B[1]. Mass spectrum inset corresponds to most abundant ions at the peak apex of major product (obs. = 1262.64 Da, calc. monoisotopic mass = 1262.60 Da).

3.1.2. Synthesis of H2 relaxin B-chain fragment B[2]

Sequence: H_2N-CGRELVRAQIAI-$NHNH_2$

H2 relaxin B-chain fragment B[2] (*Figure S2*) was synthesized on a 3-minute cycle (HATU, 50% (v/v) piperidine) at 80 °C on 200 mg 2-chlorotrytyl hydrazine resin and was cleaved using a standard cleavage cocktail yielding approximately 140 mg of crude H2 relaxin B-chain fragment B[2].

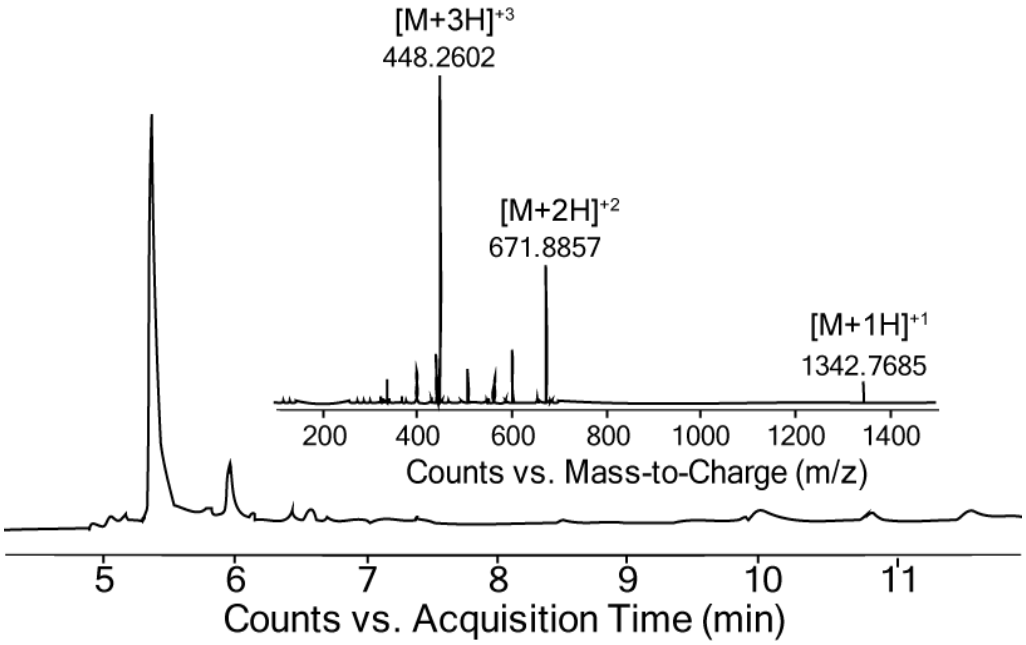


Figure S2: LC-MS (total ion current vs. time) of crude H2 relaxin B-chain fragment B[2]. Mass spectrum inset corresponds to most abundant ions at the peak apex of major product (obs. = 1341.77 Da calc. monoisotopic mass = 1341.73 Da).

3.1.3. Synthesis of H2 relaxin B-chain fragment B[3]

Sequence: H_2N-CGMSTWS-COOH

H2 relaxin B-chain fragment B[3] (*Figure S3*) was synthesized using a 3-minute-cycle (HBTU, 50% (v/v) piperidine) on 200 mg 2-chlorotrityl chloride resin and was cleaved using a standard cleavage cocktail yielding approximately 98 mg crude H2 relaxin B-chain fragment B[3].

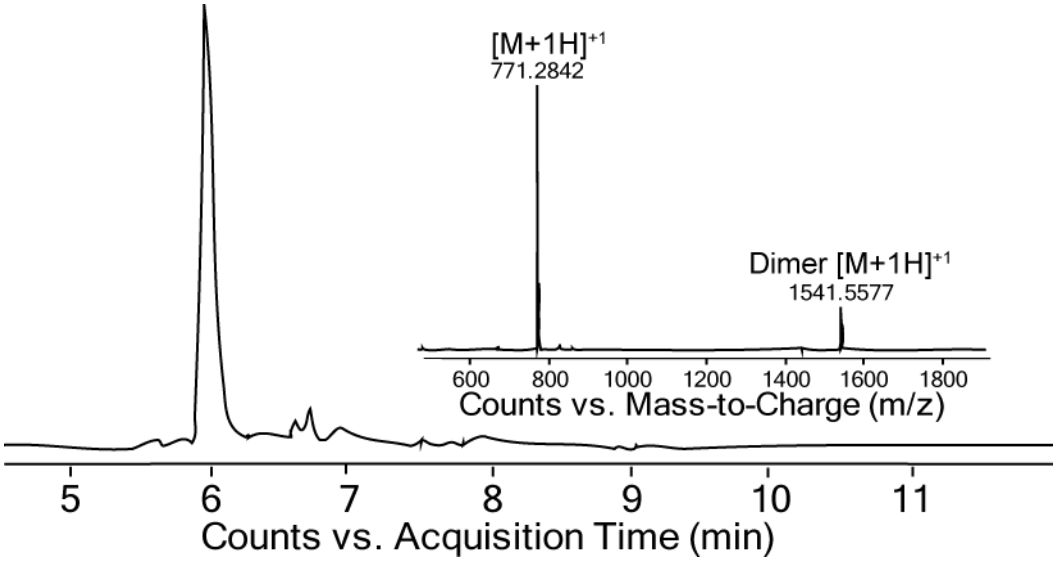


Figure S3: LC-MS (total ion current vs. time) of crude H2 relaxin B-chain fragment B[3]. Mass spectrum inset corresponds to most abundant ions at the peak apex of major product (obs. = 770.27, Da, calc. monoisotopic mass = 770.26 Da).

3.2. Purification of H2 relaxin B-chain fragment B[1] through B[3]

H2 relaxin B-chain fragments B[1] through B[3] were purified to sufficient quality for ligation (*Figure S4-S6*) in one pass over an Agilent Zorbax C3 preparative column (300 Å, 7 µm, 21.2 x 250 mm) at a flow rate of 20 mL/min. Approximately 50-150 mg of crude peptides was dissolved in volumes of 30-50 mL of solvent A containing 10% (v/v) or less of B. Samples were loaded on to the column equilibrated with 5% B in A. The purification method was performed in two steps: a fast ramp of B (1.0% B/min) and then a slow ramp (0.2% B/min) until elution of the desired product. B[1] was purified with an average yield of 22% relative to starting crude peptide, whereas purified B[2] and B[3] were obtained with a yield of 23% and 31%, respectively.

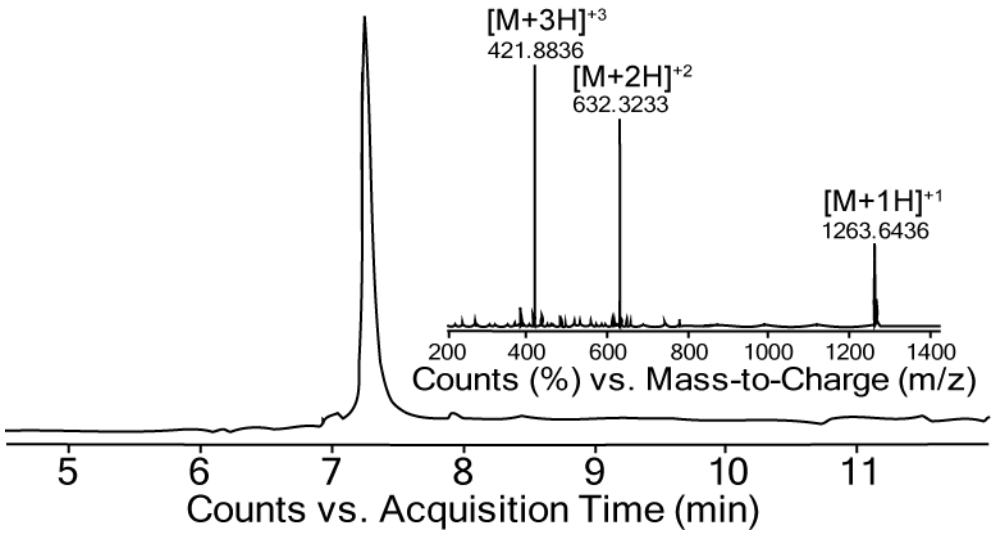


Figure S4: LC-MS (total ion current vs. time) of purified B[1]. Mass spectrum inset corresponds to most abundant ions at the peak apex of major product (obs. = 1262.65, Da, calc. monoisotopic mass = 1262.60 Da).

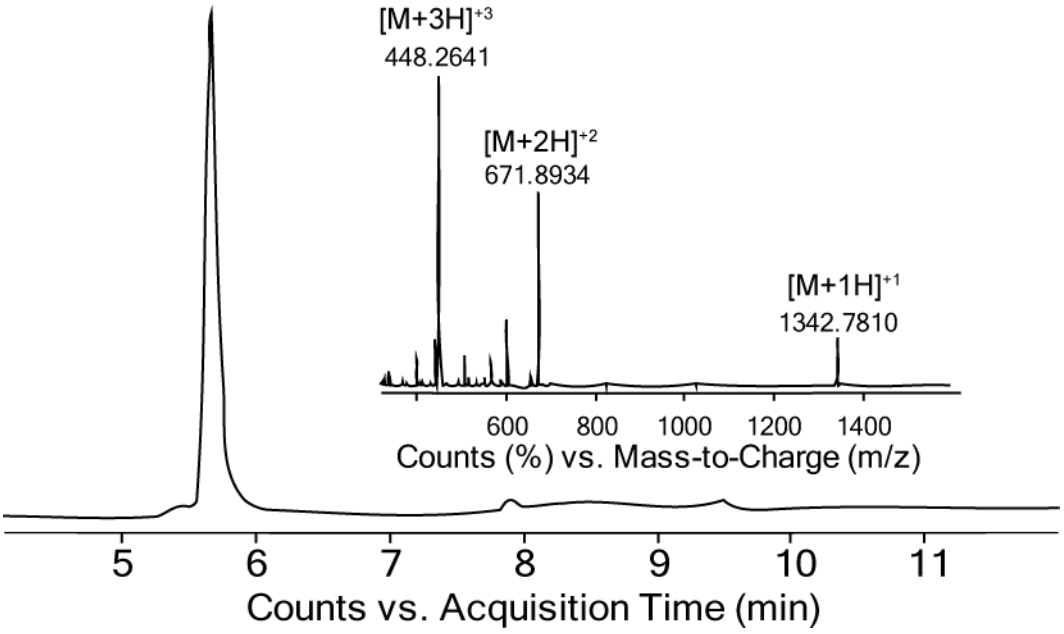


Figure S5: LC-MS (total ion current vs. time) of purified B[2]. Mass spectrum inset corresponds to most abundant ions at the peak apex of major product (obs. = 1341.79, Da, calc. monoisotopic mass = 1341.73 Da).

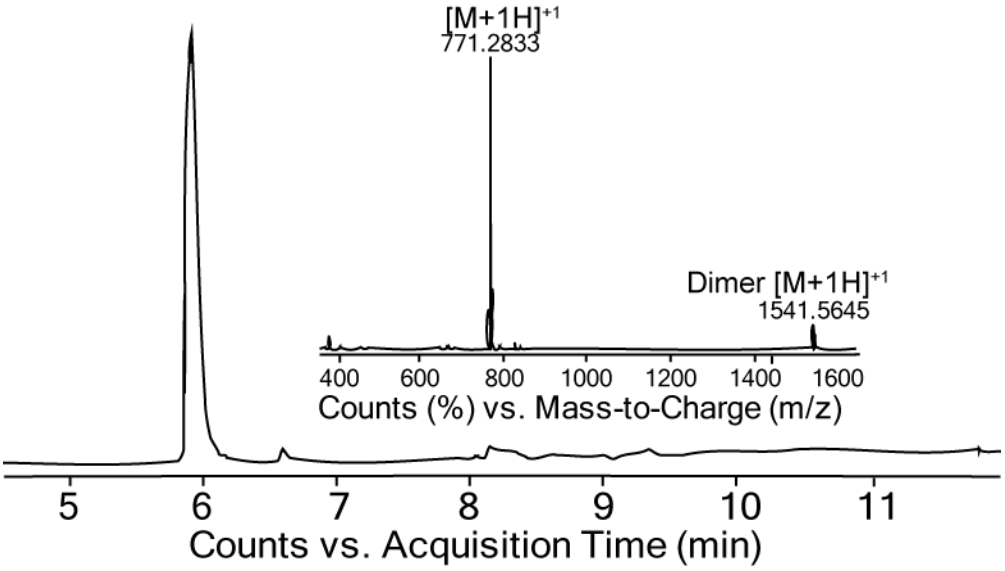


Figure S6: LC-MS (total ion current vs. time) of purified B[3]. Mass spectrum inset corresponds to most abundant ions at the peak apex of major product (obs. = 770.28, Da, calc. monoisotopic mass = 770.26 Da).

3.3. Ligation of H2 relaxin B-chain fragments

3.3.1 Ligation of B[1] and B[2]

5.50 mg B[1] (4.3 µmol) was dissolved in 1.4 mL ligation buffer at pH 3.3. The solution was stirred in an ethanol bath at –15°C prior to oxidation of the hydrazide by dropwise addition of 140 µL of 200 mM $NaNO_2$ dissolved in H_2O. After 30 min, 1.4 mL of 400 mM MPAA in ligation buffer at pH 7.0 was added dropwise to the solution in order to quench the $NaNO_2$ and convert the acyl azide into the corresponding MPAA thioester. Thioesterfication was allowed to proceed for 30 min before 7.68 mg of B[2] (5.72 µmol) was added. The pH of the resulting solution was slowly adjusted to pH 7.0 with 2 M NaOH as required for native chemical ligation. Two separate ligations were performed in parallel. The ligation reaction was completed in less than 3 hours (*Figure S7*) and was purified over a semi-preparative Zorbax C3, 5 µm column to yield 16.1 mg of the purified ligation product (*Figure S8*).

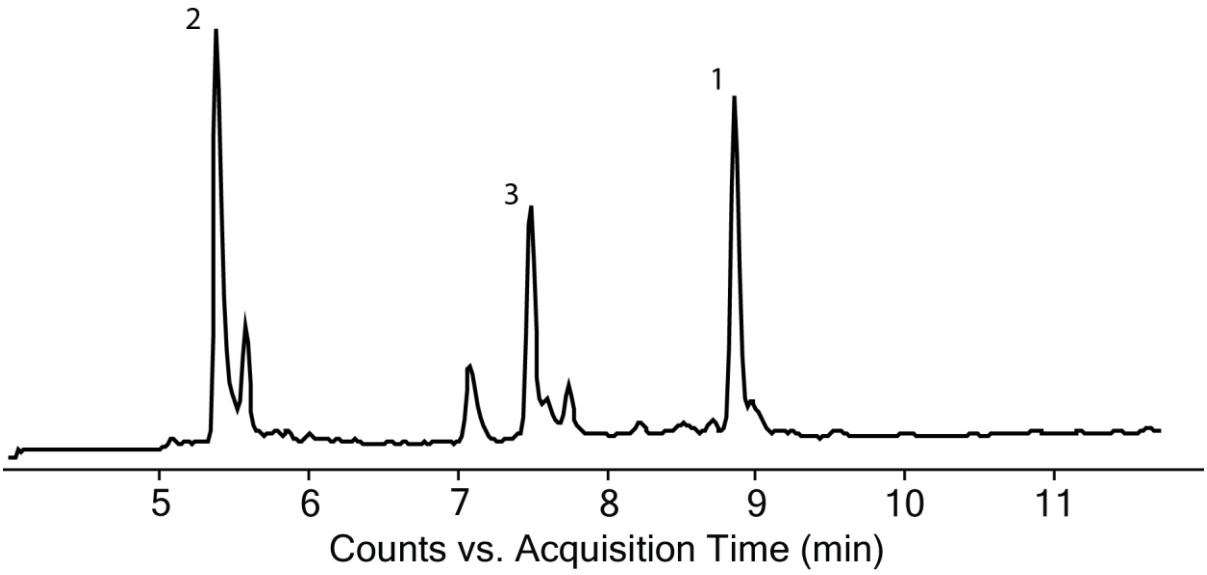


Figure S7: LC-MS (total ion current vs. time) of representative NCL between B[1] and B[2] after 14 hrs. **1** – B[1]-B[2] ligation product, obs. monoisotopic mass = 2572.34, **2** – B[2] starting material, obs. monoisotopic mass = 1341.7, **3** – Unoxidized B[1] starting material, obs. monoisotopic mass = 1262.62.

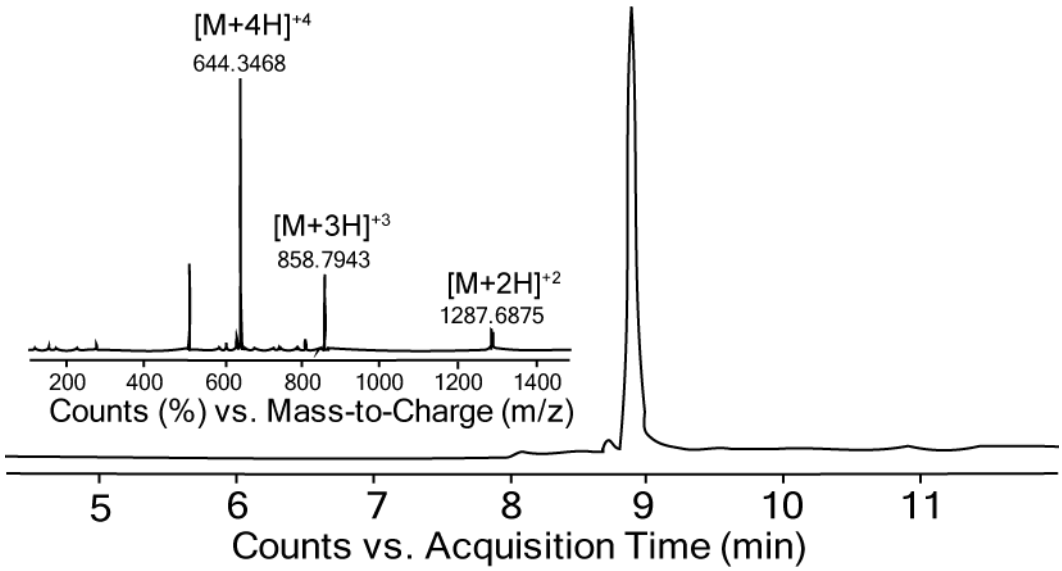


Figure S8: LC-MS (total ion current vs. time) for NCL product of B[1] and B[2]. Mass spectrum inset corresponds to most abundant ions at the peak apex of major product (obs. = 2572.38, Da, calc. monoisotopic mass = 2572.32 Da).

3.3.2. Ligation and synthesis of full length H2 relaxin B-chain

7.5 mg of B[1]-B[2] ligation product (2.15 µmol) were dissolved in 700 µL ligation buffer at pH 3.3. While the solution was stirred in an ethanol bath at -20°C, 70 µL of a 200 mM $NaNO_2$ aqueous solution was added dropwise in order to oxidize the hydrazide. After 30 min, 700 µL of 400mM MPAA was added dropwise in order to quench the oxidation and to convert the acyl azide into the corresponding MPAA thioester. Thioesterfication was allowed to proceed for 30 min before 1.5 mg of B[3] (5.72 µmol) was added. The pH of the resulting solution was slowly adjusted to pH 7.0 with 2 M NaOH as required for native chemical ligation. Two separate ligations were performed in parallel. The ligation reaction was completed after 24 hr (*Figure S9*) and was purified over a semi-preparative Zorbax C3, 5 µm column to yield 6.1 mg of full length H2 relaxin B-chain.

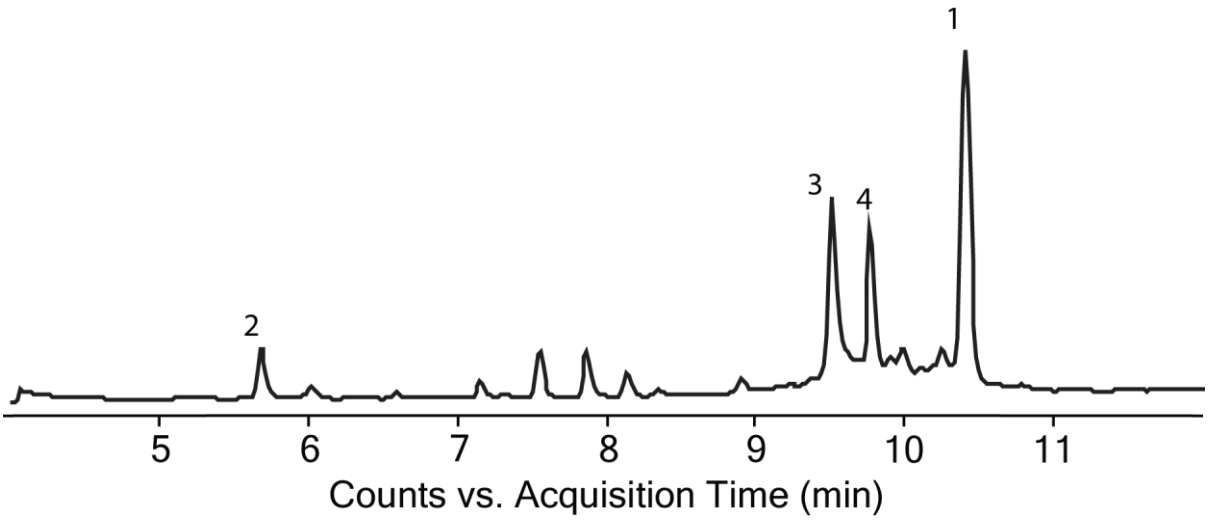


Figure S9: **Figure S7**: LC-MS (total ion current vs. time) of representative NCL between B[1]-B[2] ligation product and B[3] after 14 hrs. **1** – full length B-chain ligation product, obs. monoisotopic mass = 3310.56, **2** – B[3] starting material, obs. monoisotopic mass = 770.27, **3** – B[1]-B[2] ligation product, obs. monoisotopic mass = 2572.36, **4** – Undetermined oxidation side product, obs. monoisotopic mass = 2694.02.

4. SYNTHESIS OF RELAXIN A-CHAINS

4.1. Synthesis of H2 relaxin A-chain

Sequence: H_2N-ZLYSALANKCCHVGCTKRSLARFC-COOH

H2 relaxin A-chain was synthesized using a standard 3-minute cycle (HATU, 50 % (v/v) piperidine) and 200 mg 2-chlorotrityl chloride resin. In order to avoid multiple coupling of pyroglutamic acid (Z), 1 mmol of this amino acid was manually coupled (HBTU) using 190 µL DIEA for 20 min at room temperature. The peptide was isolated after cleavage from the resin using a standard cleavage cocktail with an approximate yield of 140 mg peptide (*Figure S10)*.

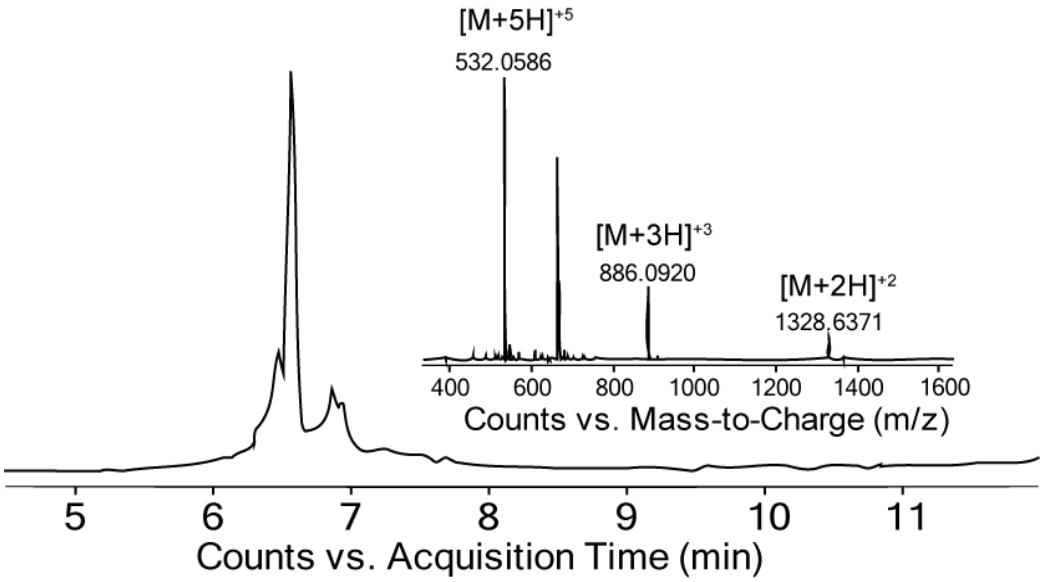


Figure S10: LC-MS (total ion current vs. time) of crude H2 relaxin A-chain. Mass spectrum inset corresponds to most abundant ions at the peak apex of major product (obs. = 2654.28 Da, calc. monoisotopic mass = 2654.34 Da). The shoulder left of the main peak corresponds to an isomer of the main peak.

4.2. Purification of wild type H2 relaxin A-chain

H2 relaxin A-chain was purified to sufficient quality for oxidative folding in one pass on an Agilent Zorbax C3 preparative column (300 Å, 7 µm, 21.2 x 250 mm) at a flow rate of 20 mL/min. Approximately 100-150 mg of crude peptide was dissolved in volumes of 30-50 mL of solvent A containing 5 % (v/v) of B. Samples were loaded on to the column equilibrated with 5% B in A. The purification method was performed in two steps: a fast ramp of B (1.0% B/min) and then a slow ramp (0.2% B/min) until elution of the desired product with an average yield of approximately 30 mg, or ~20% relative to starting crude peptide.

4.3. Synthesis of Cys(Acm)11,24 H2 relaxin A-chain

Sequence: H-ZLYSALANKCC^{ACM}HVGCTKRSLARFC^{ACM}-COOH

Cys(Acm)11,24 H2 relaxin A-chain was synthesized using a standard 3-minute cycle (HATU, 50% (v/v) piperidine) and 200 mg 2-chlorotrityl chloride resin. In order to avoid multiple coupling of pyroglutamic acid (Z), 1 mmol of this amino acid was manually coupled (HBTU) using 190 µL DIEA for 20 min at room temperature. The peptide was isolated after cleavage from the resin using a standard cleavage cocktail with an approximate yield of 130 mg peptide (*Figure S11).*

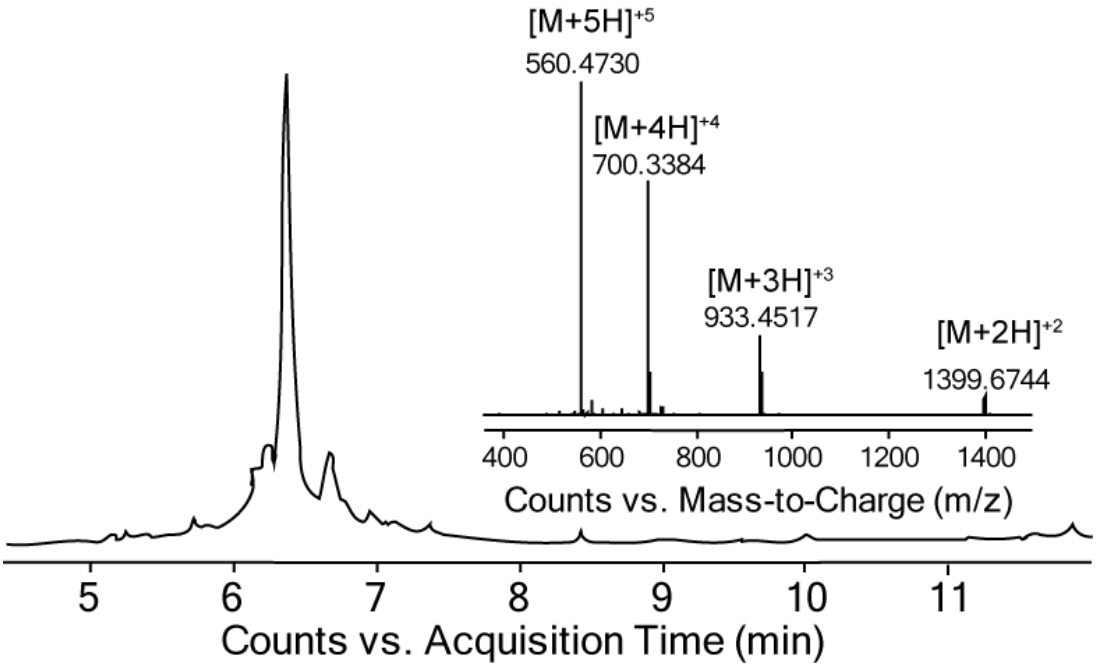


Figure S11: LC-MS (total ion current vs. time) of crude Cys(Acm)11,24 H2 relaxin A-chain. Mass spectrum inset corresponds to most abundant ions at the peak apex of major product (obs. = 2796.35 Da, calc. monoisotopic mass = 2796.33 Da). The shoulder left of the main peak corresponds to an isomer of the main peak.

4.4. Synthesis of C6F4 linked, Cys(Acm)11,24 H2 relaxin A-chain

Approximately 21 mg crude Cys(Acm)11,24 A-chain was dissolved in 1.88 mL DMF, containing 40 mM TRIS. To this, 1.88 mL DMF containing 40 mM TRIS and 6 M Gn·HCl was added. 37.5 µL of a 1 M triphenylphosphine solution dissolved in DMF was added and then 37.5 µL of a 1 M hexafluorobenzene solution dissolved in DMF was added. The reaction was incubated at room temperature overnight and analyzed by LC-MS (*Figure S12).*

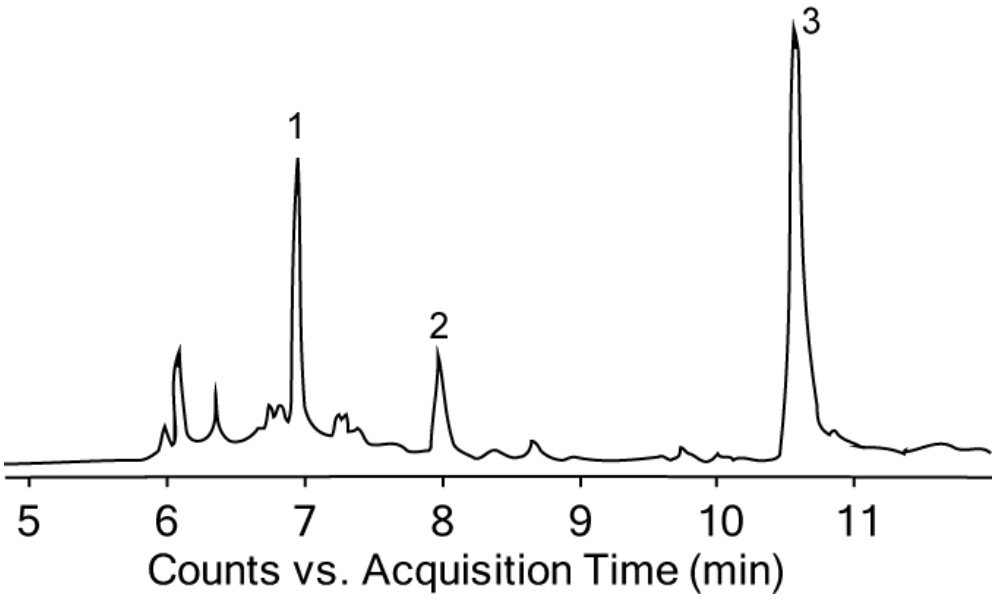


Figure S12: LC-MS (total ion current vs. time) of reaction between hexafluorobenzene and Cys(Acm)11,24 A-chain after 24 hr. (1) desired disubstituted peptide product (calc. monoisotopic mass = 2942.41 Da, obs. = 2942.34 Da). (2) oxidized triphenylphosphine; (3) reduced triphenylphosphine.

4.5. Purification of C6F4 linked, Cys(Acm)11,24 H2 relaxin A-chain

Crude C6F4 linked, Cys(Acm)11,24 A-chain was purified to sufficient quality in one pass over an Agilent Zorbax C3 preparative column (300 Å, 7 µm, 21.2 x 250 mm) at a flow rate of 20 mL/min. Approximately 20 mg of crude stapled peptide in DMF was dissolved in a volume of at least 75 mL of solvent A. Samples were loaded on to the column equilibrated at 5 % B in A. The purification method was performed in two steps: a fast ramp of B (1.0% B/min) and then a slow ramp (0.2% B/min) until elution of the desired product with an yield of approximately 2.6 mg (13 %) (*Figure S13).*

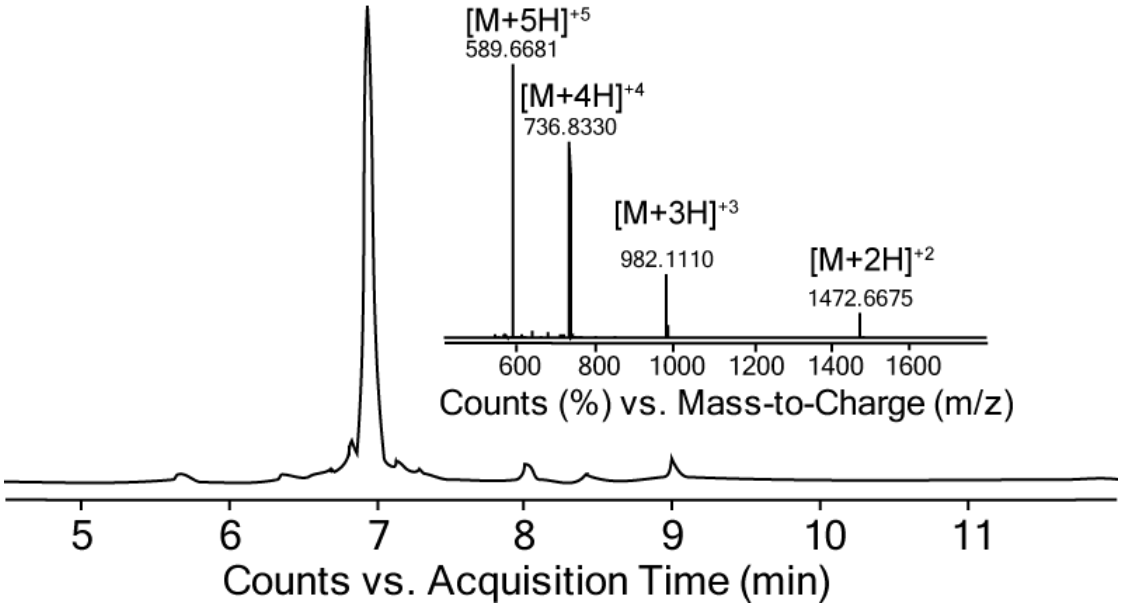


Figure S13: LC-MS (total ion current vs. time) of purified Cys(Acm)11,24 A-chain. Mass spectrum inset corresponds to most abundant ions at the peak apex of major product (obs. = 2942.33 Da, calc. monoisotopic mass = 2942.41 Da).

4.6. Synthesis of C6F4 linked H2 relaxin A-chain

C6F4 linked, Cys(Acm)11,24 A-chain was deprotected with 200 eq. silver acetate in 50% aqueous acetic acid at room temperature. Rate of deprotection for protected cysteines was closely monitored by LC-MS and stopped after 90 min by quenched by adding 2 volumes of 1 M DTT in 6 M Gn-HCl. The mixture was stirred for 20 min at room temperature and the peptide and then desalted by use of a C18 reverse-phase desalting columns. Lyophilizaiton of the eluent yielded 2.4 mg stapled RLX-A chain (12%).

5. MISCELANEOUS

5.1. Oxidative folding of wild type and C_6F_4-linked H2 relaxin

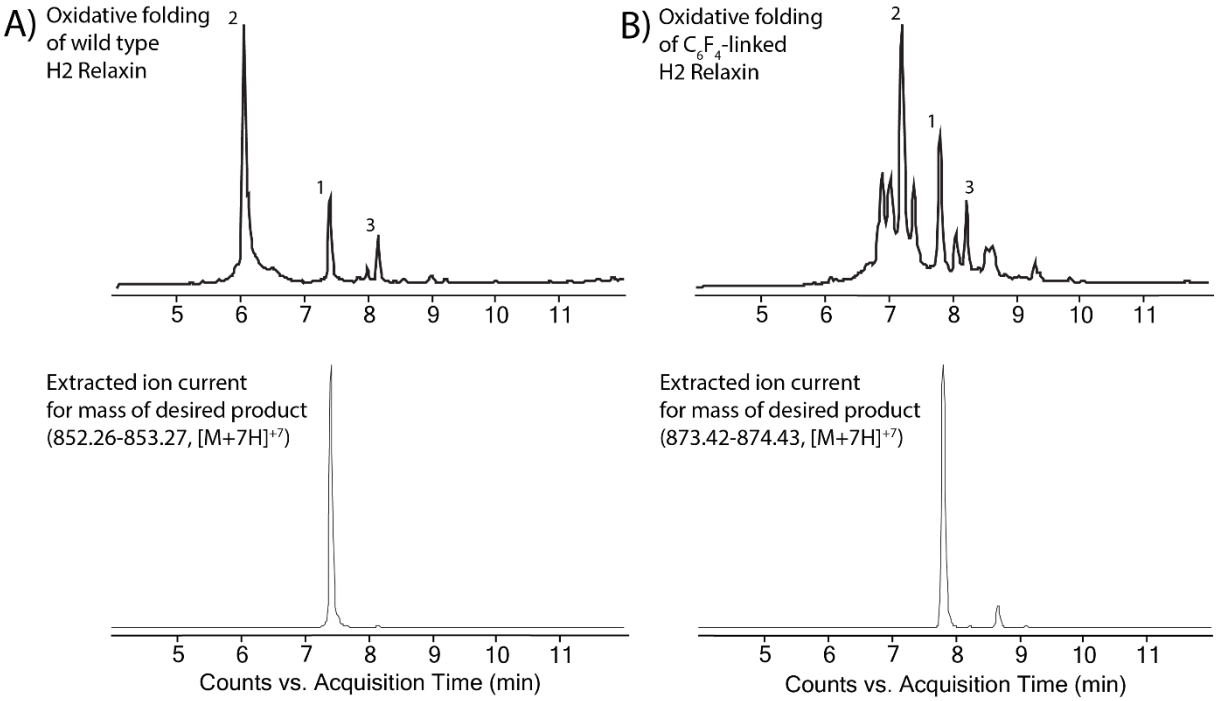


Figure S14: Oxidative folding overnight (greater than 16 hrs) of A) wild type and B) C_6F_4-linked H2 relaxin under conditions outlined in SI section 2.8. **1** – Folded, desired products, **2** – wild type / C_6F_4-linked A-chain, **3** – B-chain. Corresponding extracted ion currents for masses corresponding to the desired, folded products reveal a single predominant folding product possessing the expected mass of wild type or C_6F_4-linked H2 relaxin.

5.2. Evaluation of synthetic wild type and C_6F_4-linked H2 relaxin purity

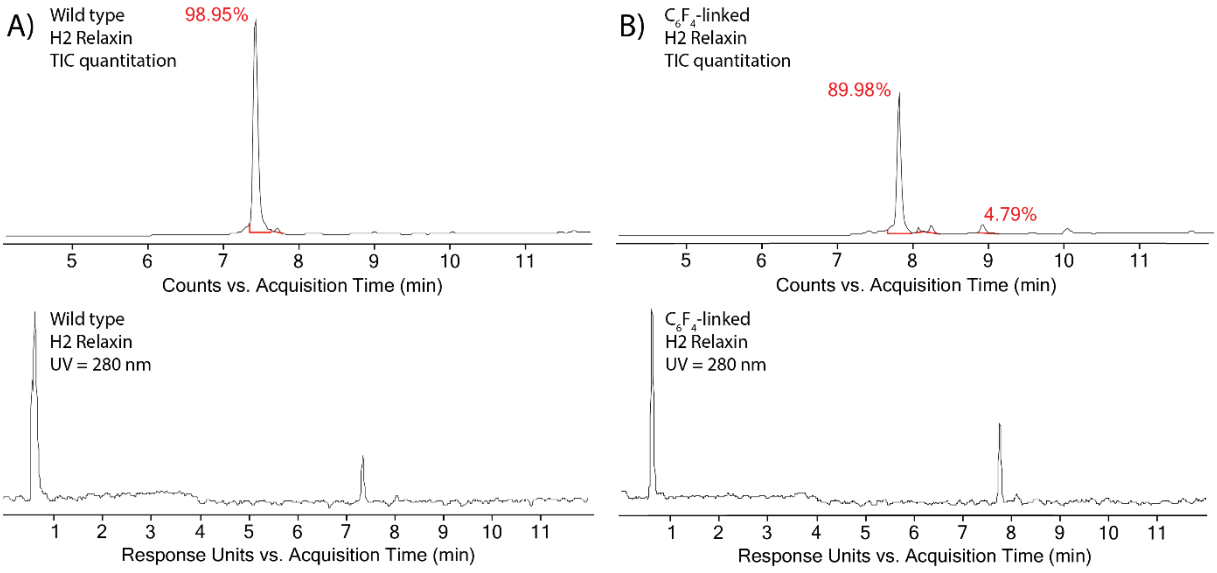


Figure S15: Evaluation and quantitation of A) wild type and B) C_6F_4-linked H2 relaxin purity by HPLC-UV and TIC peak integration from LCMS of samples.

5.3. Trypsinolysis

Synthetic wild-type H2 relaxin and C6F4 linked H2 relaxin were analyzed for correct disulfide orientation by trypsin digest and peptide mapping. *Figure S14* displays the theoretical fragments and observed trypsinolysis fragments of (A) synthetic wild-type H2 relaxin synthetic and (B) C6F4 linked H2 relaxin as observed by LC-MS.

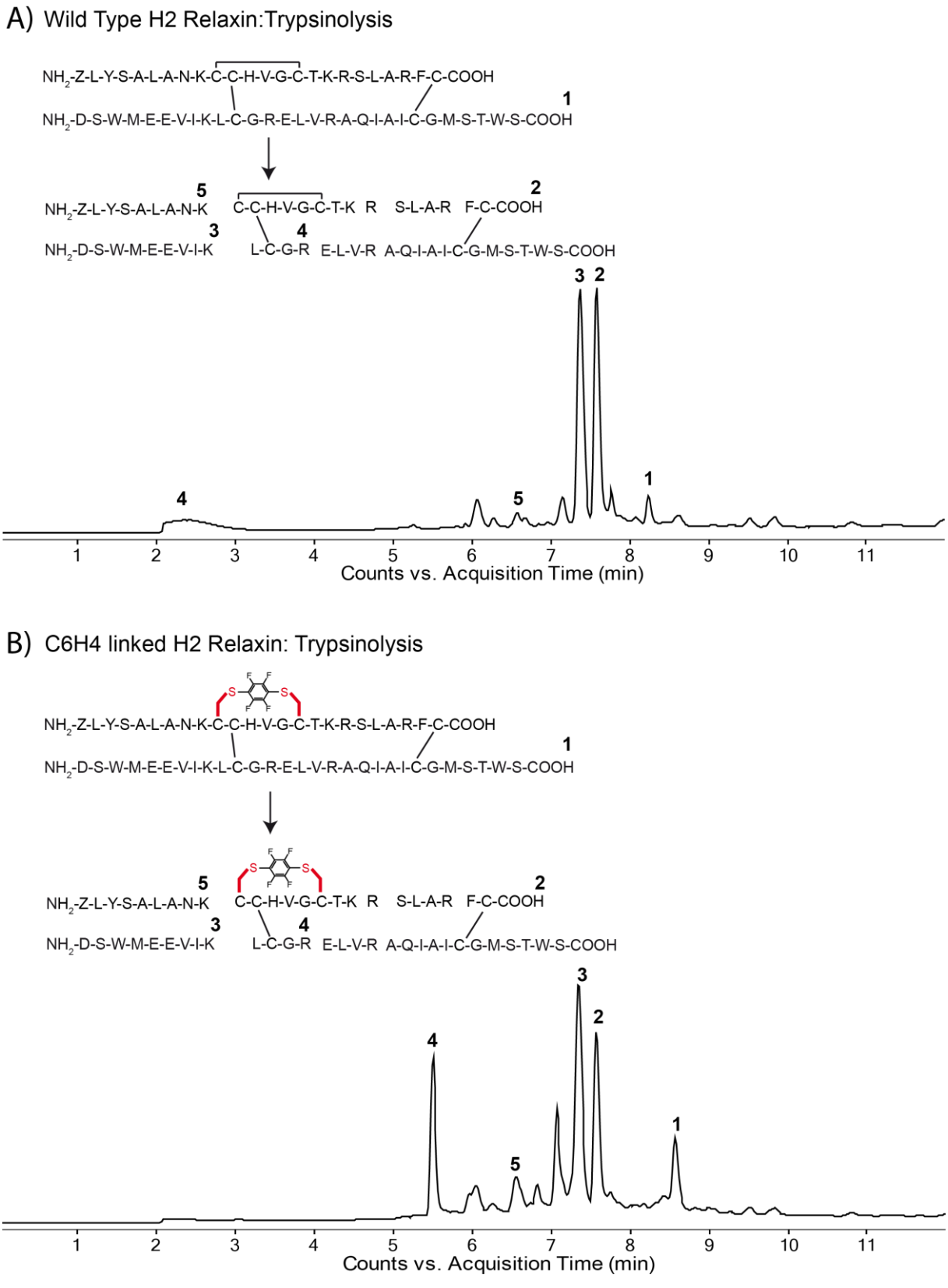
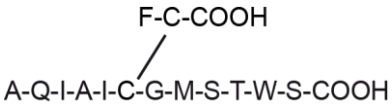
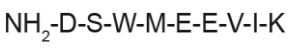
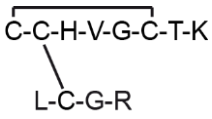
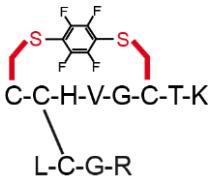
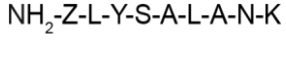


Figure S16: LC-MS (total ion current vs. time) of peptide fragments after trypsin digest of (A) synthetic wild-type H2 relaxin and (B) C6F4 linked H2 relaxin. Theoretical fragments for both starting peptides are shown and indexed here and correspondingly in *Table S1*.

Table S1: Observed and calculated monoisotopic masses for peptide fragments resulting from trypsin digest of synthetic wild-type H2 relaxin and C6F4 linked H2 relaxin peptides that were observed by LC-MS in *Figure S15*.

Fragment [#] Sequence	**Synthetic WT H2 relaxin** Observed monoisotopic mass (Da)	**Synthetic WT H2 relaxin** Calculated monoisotopic mass (Da)	**C6F4 linked H2 relaxin** Observed monoisotopic mass (Da)	**C6F4 linked H2 relaxin** Calculated monoisotopic mass (Da)
2  	1532.67	1532.66	1532.65	1532.66
3  	1135.53	1135.51	1135.52	1135.51
4 A  	1292.52	1293.57	-	-
4 B  	-	-	1440.53	1441.57
5  	989.53	989.59	989.53	989.59