Supporting Information

A New Synthetic Route to Human Relaxin-2

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Table of Contents

General Information

All reagents [diisopropylcarbodiimide (DIC), 6-Cl-1-hydroxybenzotriazole (6-Cl-HOBt), trifluoroacetic acid (TFA), triisopropylsilane (TIS), 2,2'-dithiobis(5-nitropyridine) (DTNP), 5,5'-dithiobis-(2-nitrobenzoic acid (DTNB, or Ellman's reagent), diethyl azodicarboxylate (DEAD), Bis(5-(2-methoxyethoxy)-2-pyrimidinyl disulfide (BMPD), triphenylphosphine, Fmoc-Cys(*t*Bu)-OH, guanidine] and solvents [*N*-methyl-2-pyrrolidone (NMP), *N,N'*-dimethylforamide (DMF), methanol (MeOH), dichloromethane (DCM), acetonitrile(ACN), diethyl ether (Et_2O) and tetrahydrofuran (THF)] were purchased and used directly as supplied unless otherwise specified. Fmoc-chemistry based amino acid cartridges were purchased from Midwest Bio-Tech Inc. and used directly. HMPB-ChemMatrix resin was purchased from PCAS BioMatrix (cat. No. 1647). NovaSyn TGA resin (cat. No. 8.55005.0005) was purchased from Novabiochem. Boc-Thr[Fmoc-Ser(*t*Bu)]-OH and Boc-Ser[Fmoc-Asp(O*t*Bu)]-OH were purchased from AAPPTec. Fmoc-Ser(Phacm)-OH (cat. No. FAA6910) and immobilized penicillin G amidase (cat. No. EZ60040) were purchased from Iris Biotech GMBH.

Analytical LCMS was carried out on an Agilent 1260 infinity LC system coupled to an Agilent 6120 quadrupole mass spectrometer. LC trace was obtained from a Phenomenex Kinetex C8 2.6 µm 100 Å (75 x 4.5 mm) column and the gradient of the eluent was 10 - 80 % B over 10 min (A: 0.05 % TFA in H_2O; B: 0.05% TFA in 90% ACN) at a flow rate of 1.0 mL/min. Preparative high-performance liquid chromatography (prep-HPLC) was performed using a Waters HPLC controller 600 through a Luna 10µ C8 100 Å AXIA packed reverse-phase column. UV absorbance was detected by Waters dual wavelength detector 2487 with 220 nm or 230 nm wavelength and the eluent was fractionized and collected by Prostar model 701 fractions collector. Flow rate varied from 8 to 12 mL/min. Buffer A: 0.1% TFA in 10% aqueous ACN. Buffer B: 0.1% TFA in pure ACN. Buffer C: 0.2% TFA in 10% aqueous ACN. Buffer D: 0.2% TFA in pure ACN. Linear gradient for peptide **1**: 90% C and 10% D to 60% C and 40% D over 120 min; .**2**: 95% A and 5% B to 65% A and 35% B over 60 min; **5**: 80% A and 20% B to 60% A and 40% B over 80 min;

Synthesis of A-chain 2: ZLYSALANKCC(tBu)HVGCTKRSLARFC(Phacm) (Z: pyroglutamic acid)

NovaSyn TGA resin (90 μm, EMD Millipore, cat. No. 855005, 2 g) was allowed to swell in 10 mL anhydrous THF for 10 min and dried. Fmoc-L-Cys(Phacm)-OH (2.45 g) was dissolved in 15 ml anhydrous THF with triphenylphosphine (1.31 g) and added to the swollen resin. DEAD solution (in toluene, 40% w/w, 2.25 mL) was added to the reaction as three equal aliquots. Agitated at room temperature for 4 h, the resin was then washed with DMF (10 mL × 4) and DCM (10 mL × 4) before dried under vacuum. The synthesis of A-chain **2** was started with assembling each residue on 0.10 mmol aforementioned, manually pre-loaded resin using ABI 433A peptide synthesizer using a standard Fmoc_HOBT_DCC 0.10 mmol(**Table S*1***) method. Pyroglutamic acid and Fmoc-Cys(*t*Bu)-OH were packed into blank cartridges and coupled under the standard conditions. The resulting resin-bound A-chain was washed with DCM (8 mL) and dried in vacuum before treated with 8 mL TFA cocktail containing 2.5% TIS and 2.5% H_2O. After agitation at r.t. for 3 h, the peptide was precipitated from the TFA solution with cold diethyl ether of 10-fold volume. After centrifugation, A-chain **2** was re-dissolved in 10% aq. ACN with 0.1% TFA and purified with prep-HPLC. Pure fractions were combined and lyophilized to afford 67 mg of **2**. Crude purity = 53% based on the AUC of LC trace. Theoretical m/z = 2858.4, Observed m/z = 2858.5. After purification, yield = 23%, purity = 98%. (**Figure S1**)

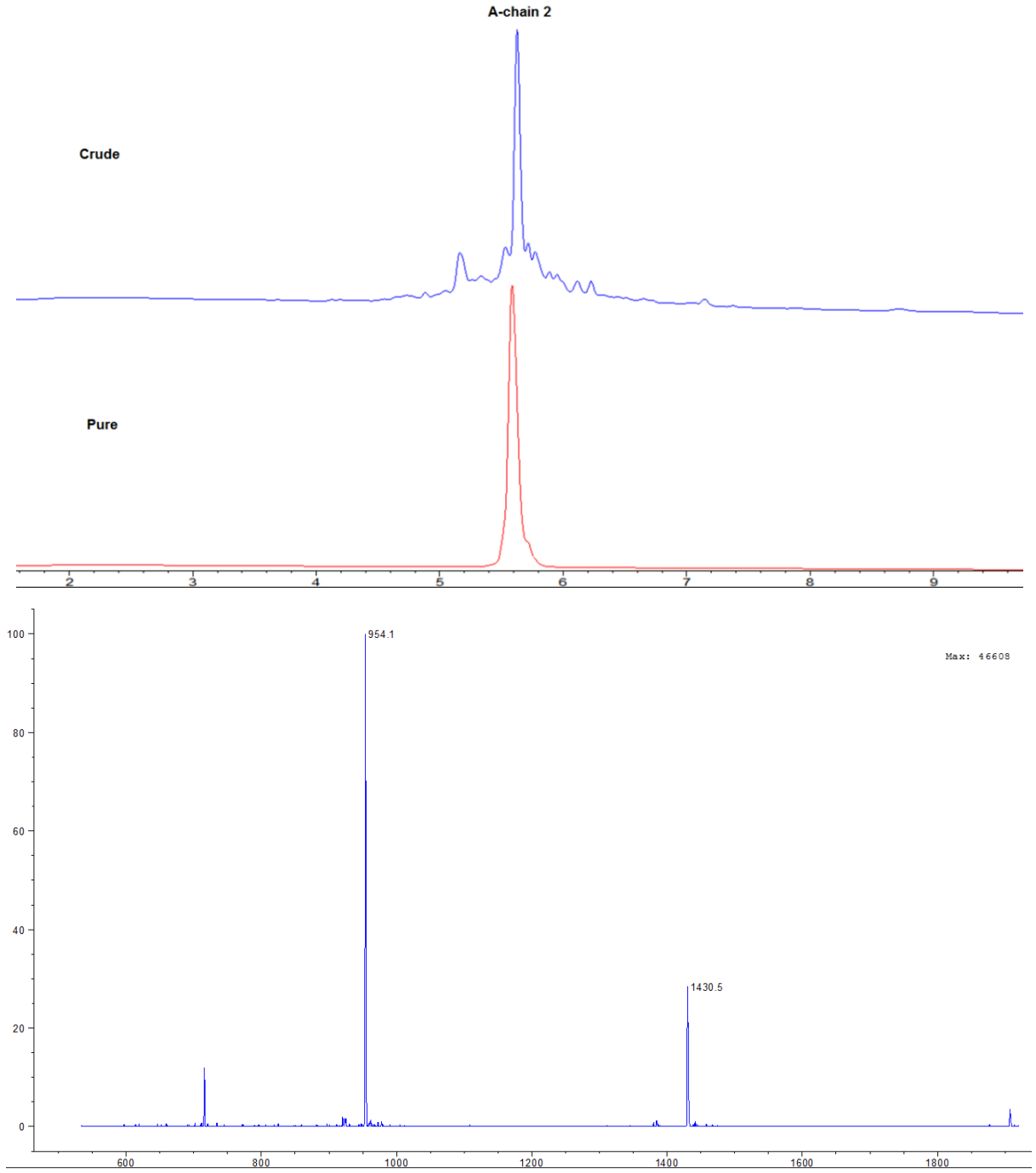

Figure S1. HPLC traces and MS of A-chain **2** (LC upper panel: crude; LC lower panel: purified)

Synthesis of A-chain 3: ZLYSALANK[CCHVGC]TKRSLARFC ([ ]: intra-molecular disulfide bond)

Clear-OX® resin (3.5 eq.) was allowed to swell in DCM (5 mL) for one hour and washed with DCM (5 mL × 2), DMF (5 mL × 3), MeOH (5 mL × 3), H_2O (5 mL × 3) and 50% aq. ACN (5 mL × 3). Purified A-chain **2** (88 mg) was dissolved in 50% aq. ACN to a concentration of 2 mg/mL. After adjusting the pH to 6.8 with ammonium hydroxide, the solution was transferred to the aforementioned resin and agitated for 1 h at r.t. After completion, the resin was filtered and washed with 50% aq. ACN (5 mL × 2). Solutions were combined and lyophilized to afford 76 mg crude peptide **3** with a crude yield of 86%. (**Figure S2**)

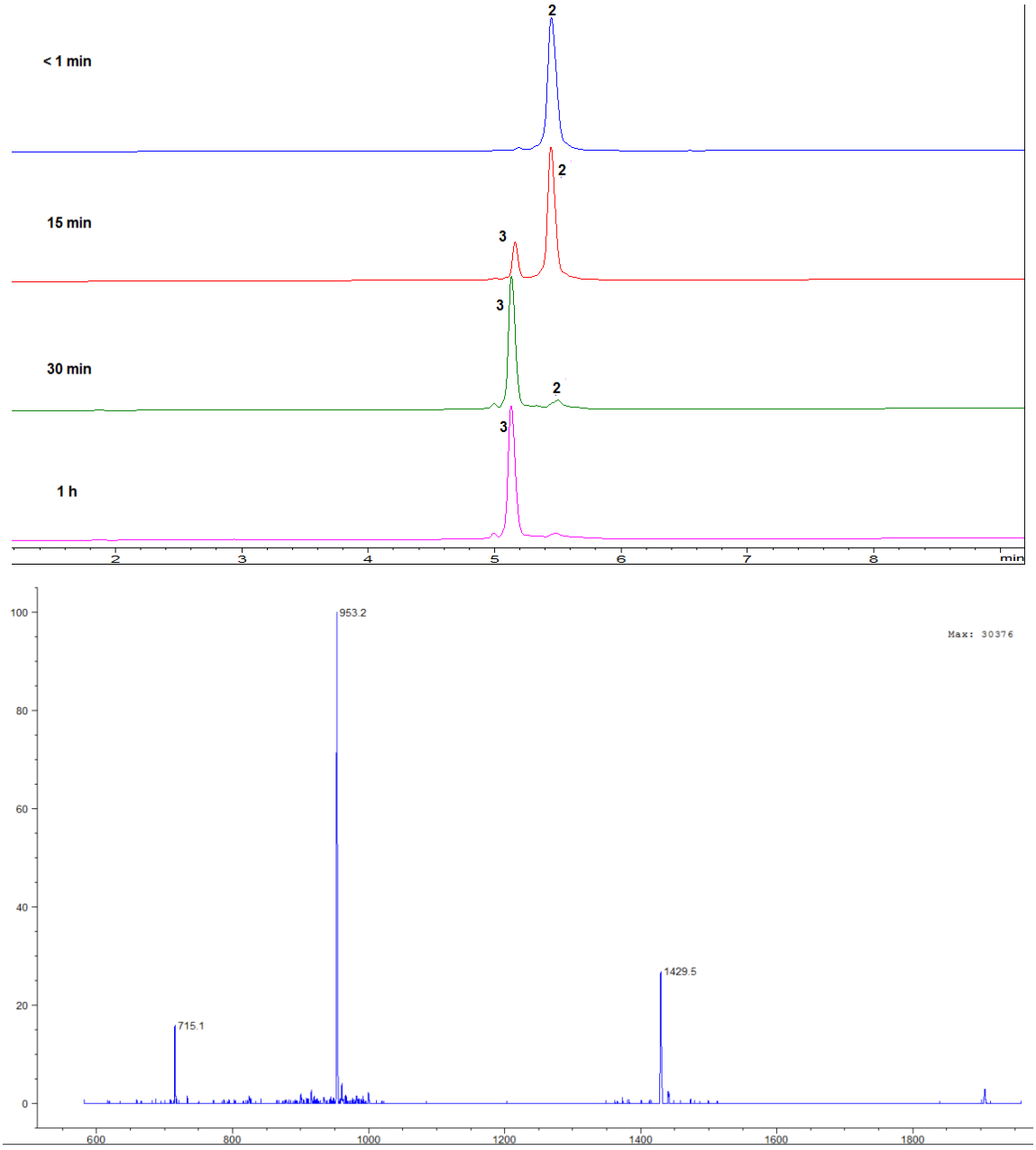

Figure S2. HPLC traces of the intra-A-chain disulfide bond formation and MS of **3**

Synthesis of A-chain 4: ZLYSALANK[CC(SNpy)HVGC]TKRSLARFC(Phacm)

Crude A-chain **3** (76 mg) and DTNP (82 mg, 10 eq.) was dissolved in 7.6 mL TFA and constantly stirred at r.t. for 4 h. The resulting peptide was then precipitated with 50 mL of cold ether and washed with 5% DMF in ether (50 mL × 3) and pure ether (50 mL × 3). The peptide was centrifuged and re-dissolved in 0.1% TFA-containing 10% aq. ACN for purification using prep-HPLC. After combining the pure fractions and lyophilization, 46 mg of A-chain **4** was obtained with a yield of 59% based on the crude starting material **3**. (**Figure S3**)

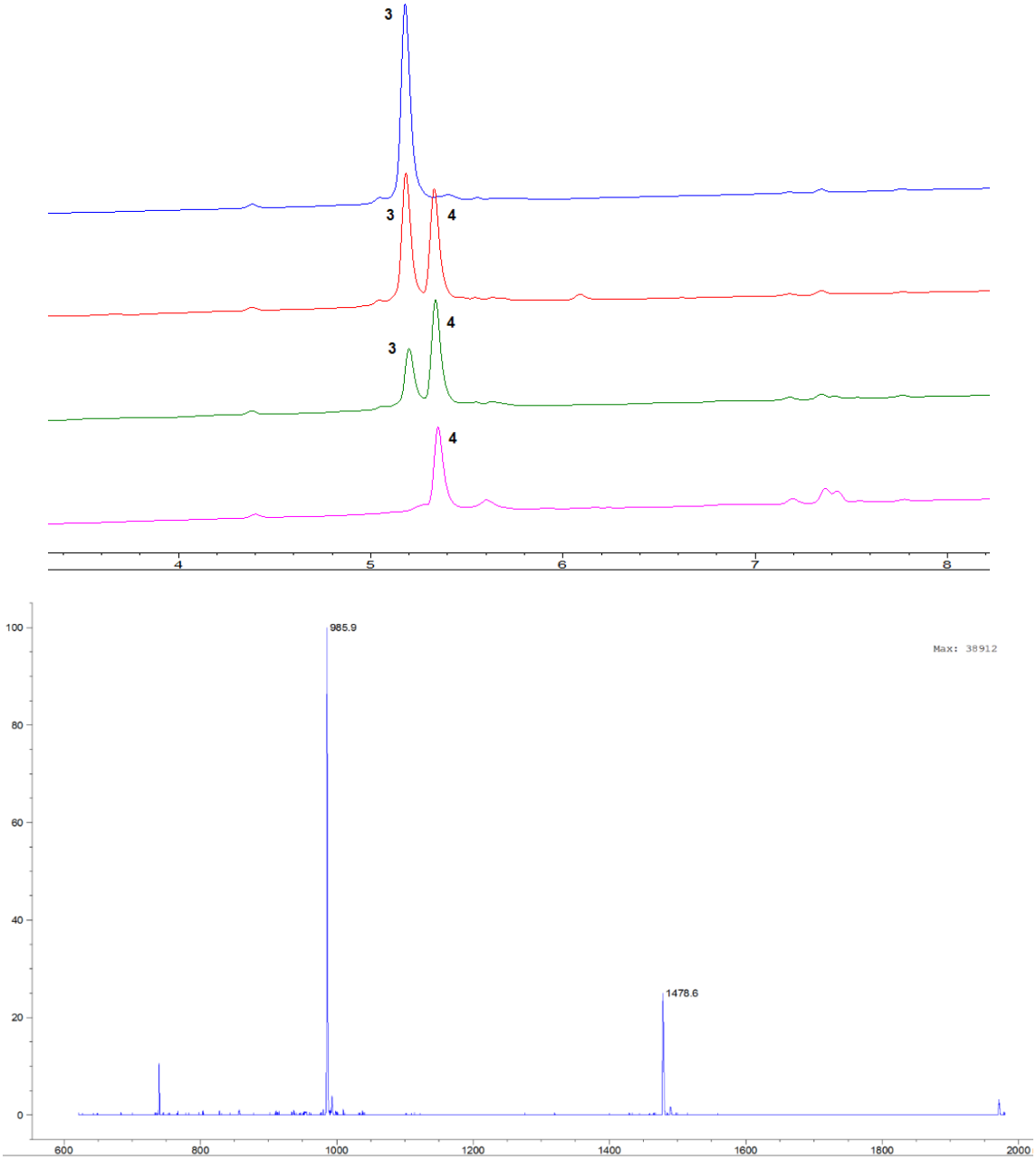

Figure S3. HPLC traces of activating A-chain **3** to **4** by coverting Cys-*t*Bu to Cys-SNpy and MS of **4**

Synthesis of B-chains

B-chain **5**: DSWMEEVIKLCGRELVRAQIAIC(Phacm)GMSTWS (__: isoacyl dipeptide)

B-chain **6**: DSWMEEVIKLCGRELVRAQIAIC(Phacm)GMSTWS

HMPB-ChemMatrix resin (2 g) was allowed to swell in 15 mL anhydrous DCM for 15 min and washed with anhydrous DCM (15 mL × 2) before dried. Fmoc-Ser(*t*Bu)-OH (7.7g) was dissolved in 20 mL DCM. After the addition of 1.5 mL DIC, the mixture was agitated at r.t. for 15 min and then transferred to the swollen resin along with catalytic amount of DMAP (5 mg). The reaction was filtered and the resin was washed with DMF (10 mL × 4) and DCM (10 mL × 4) after agitated at r.t. for 4 hours. Acetic anhydride (4.7 mL) in DMF (15 mL) was added and the resin was agitated for 60 min to cap any possible unreacted linkers. The resin was then washed with DMF (10 mL × 4) and DCM (10 mL × 4) and dried under vacuum. Standard Fmoc_HOBt_DCC_0.1 mmol SPPS was performed on an ABI 433A synthesizer to assemble B-chain **1** by coupling commercially available amino acids to this manually pre-loaded Fmoc-Ser(*t*Bu)-HMPB-ChemMatrix resin on a scale of 0.1 mmol. Fmoc-Cys(Phacm)-OH and isoacyl dipeptides (ST, DS) were packed into empty cartridges and coupled under the standard conditions. The resulting resin-bound B-chain was washed with DCM (8 mL) and dried in vacuum before treated with 8 mL TFA cocktail containing 2.5% TIS and 2.5% H_2O. After agitation at r.t. for 3 h, the peptide was precipitated from the TFA solution with cold diethyl ether of 10-fold volume. After centrifugation, the pellet was re-dissolved in 10% aq. ACN with 0.1% TFA and purified by prep-HPLC. Pure fractions were combined and lyophilized to afford B-chain **5** of 49 mg. Crude purity = 51% based on the AUC of LC trace. Theoretical m/z = 2858.4. Observed m/z = 2858.5. After purification, yield = 23%, purity = 98%. Lyophilized B-chain was dissolved in 50% ACN with 50 mM ammonium carbonate (pH = 8.5) to trigger the spontaneous O-N acyl shift and B-chain **6** was lyophilized after 10 min of reaction at r.t. (**Figure S4**)

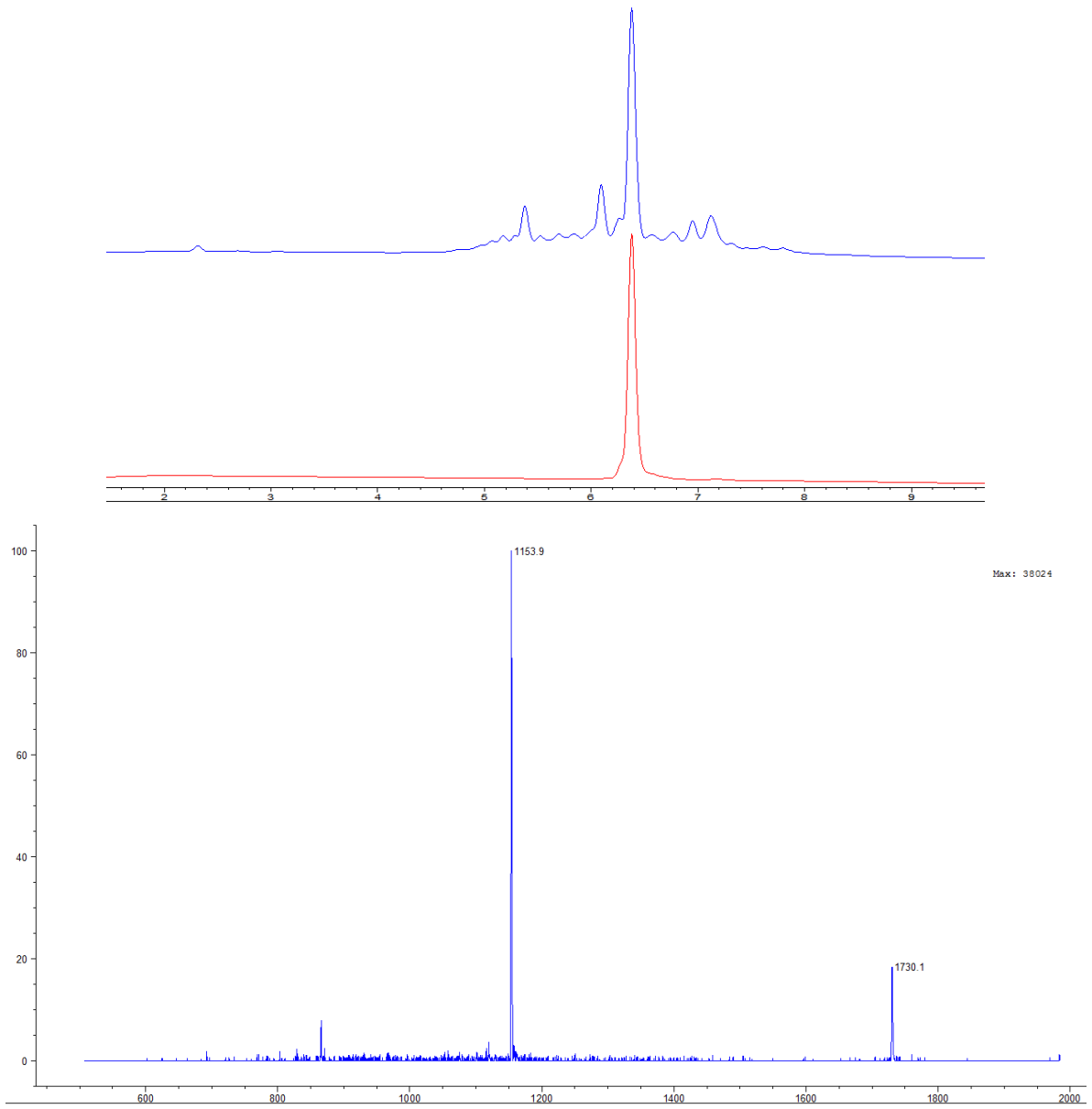

Figure S4. HPLC traces and MS of B-chain **5** (LC upper panel: crude; LC lower panel: purified)

Synthesis of A-B heterodimer 7

A-chain **4** (30 mg, 10.2 μmol) and B-chain **6** (33 mg, 9.5 μmol) were dissolved in 5 mL DMF. DIEA (0.050 mL) was added to trigger the chain-ligation. Reaction was completed within 30 min. The resulting A-B dimer, peptide **7**, was precipitated by the addition of cold diethyl ether (45 mL), and the pellet was washed with 5% DMF-containing ether (50 mL × 2) and pure ether (50 mL × 3). After freeze-drying, crude A-B heterodimer **7** (52 mg) was obtained with a crude yield of 87%, which was subject to next step without purification. (**Figure S5**)

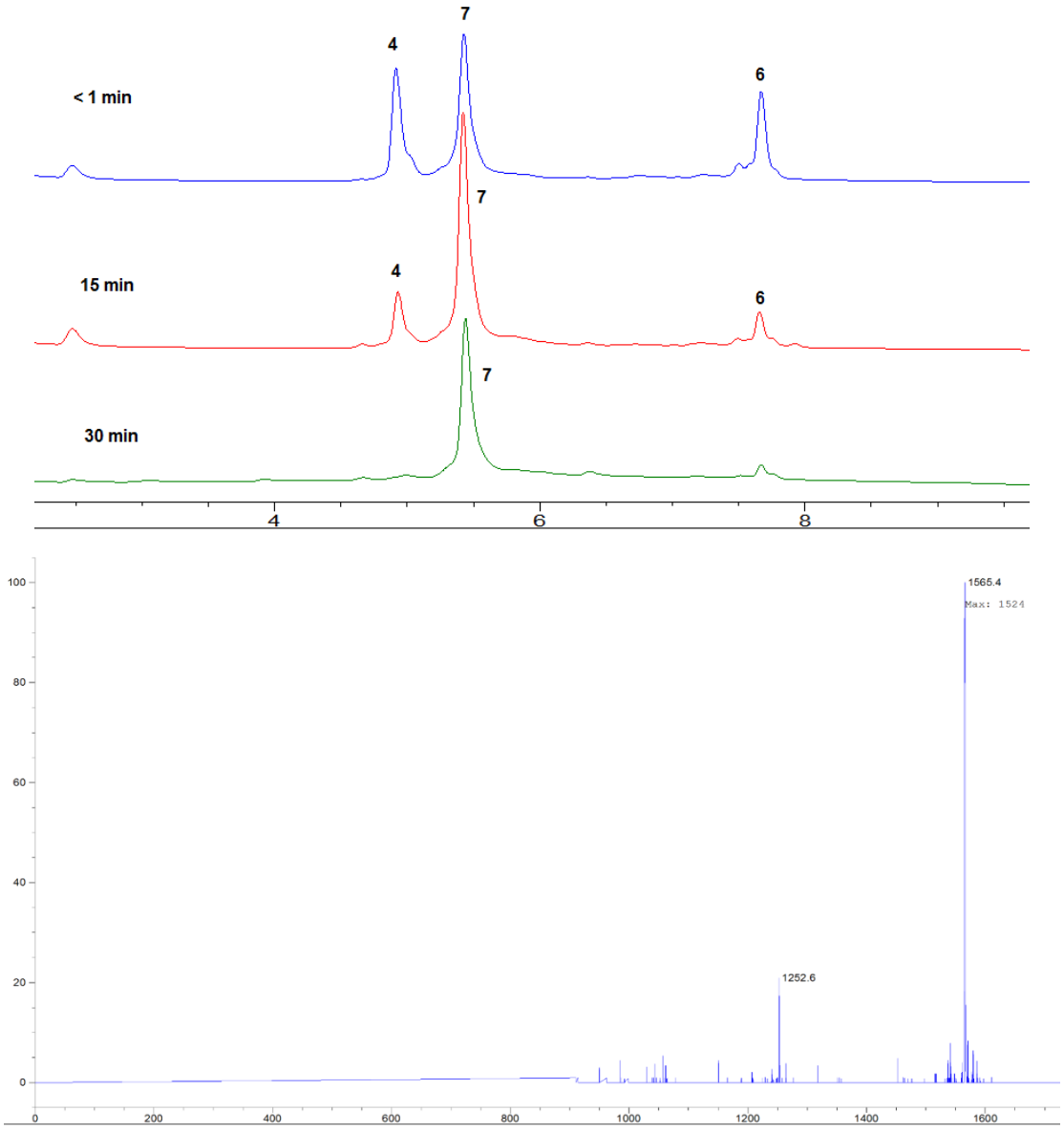

Figure S5. HPLC traces of the ligation of A-chain **4** and B-chain **6** and MS of crude **7**

Synthesis of human relaxin-2, 1

Crude A-B dimer **7** (31 mg, 4.9 μmol) was dissolved in 5 mL 6 M guanidine buffer with 200 mM sodium phosphate. Resulting solution was further diluted with 10 mL distilled water, to which BMPD (18.2 mg) was then added. Immobilized PGA (120 u/g, 408 mg) was added after adjusting the pH to 6.8. After agitated at r.t for 2 h, the immobilized PGA was filtered off and washed with 50% aq.ACN (10 mL × 3). The washing solutions were combined with the filtrate and diluted with distilled water such that the content of ACN was lower than 15% prior to purification by prep-HPLC. Pure fractions were combined and lyophilized to afford 8.6 mg of relaxin with a yield of 28% based on crude dimer **7**. The overall yield calculated from the limiting B-chain **6** was 25%.

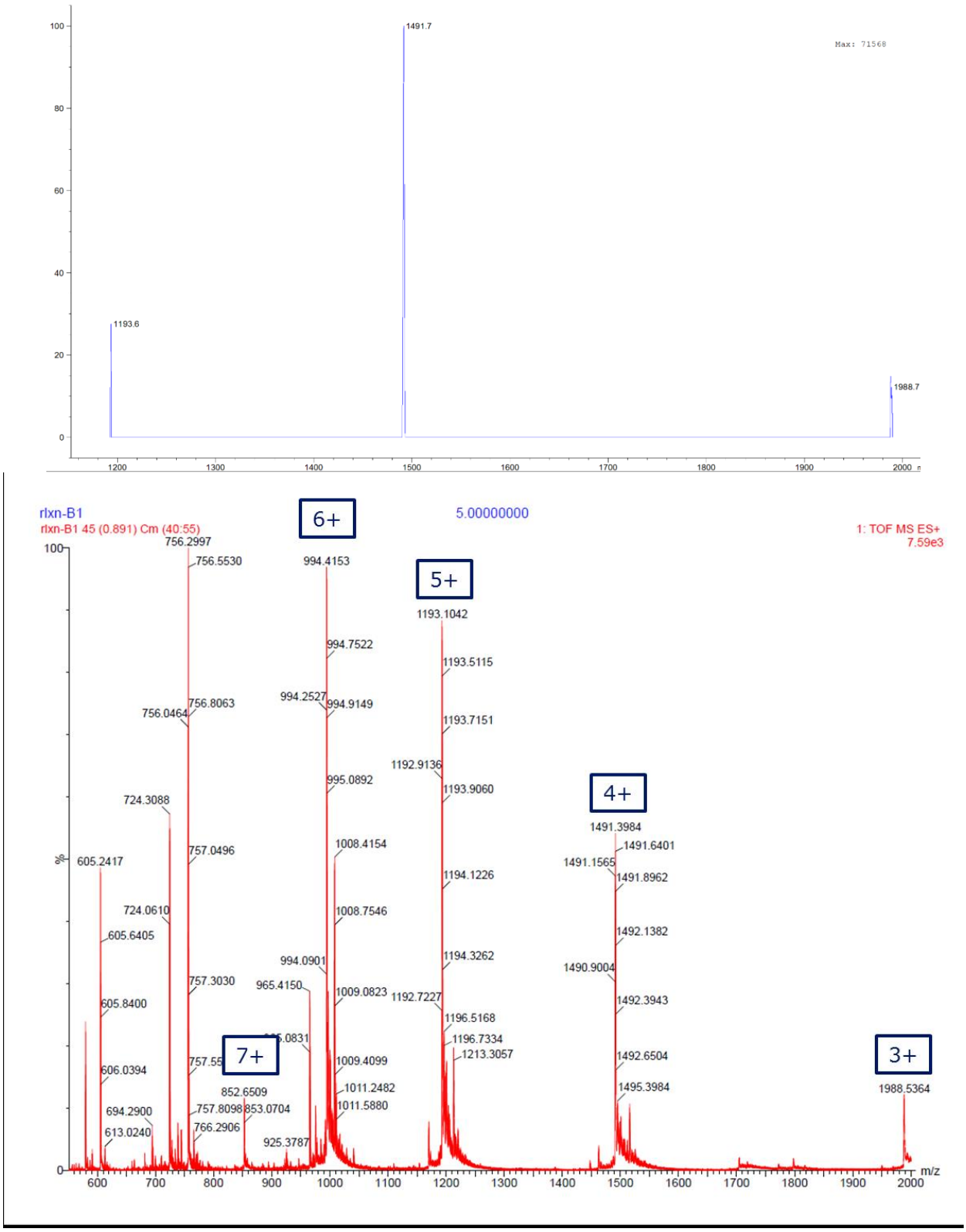

Figure S6. MS of human relaxin-2 (**1**)

Human relaxin receptor 1 activation assays

The ability of peptides to activate human relaxin-1 receptor was studied *in vitro* using firefly luciferase reporter gene assay designed to indirectly measure cAMP production. Human embryonic kidney cells (HEK-293, ATCC CRL-1573) overexpressing human relaxin-1 receptor and firefly luciferase under control of cAMP-response element were grown in Dulbecco's modified Eagle medium (DMEM, HyClone SH30022, Logan, UT) supplemented with 10% Bovine Growth Serum (HyClone SH30541, Logan, UT), 100 IU/ml penicillin, 100 µg/mL streptomycin, and 10 mM HEPES at 37 °C, 5% CO_2 and 90% humidity. Sixteen to twenty hours prior to the experiment the cells were plated at $2x10^4$ cells per well in 96 well Isoplate (Perkin-Elmer 6005040, Waltham, MA) in DMEM supplemented with 0.25% Bovine Growth Serum. Serial dilutions of peptides were prepared in sterile DMEM containing 0.5% Bovine Serum Albumin (Roche Applied Sciences #100350, Indianapolis, IN) and added to the wells with adherent cells. The plates were incubated 5h at 37°C and 5% CO_2 in humidified atmosphere. At the end of the incubation 100 ul/well of Steady Lite HTS luminescence substrate reagent (Perkin-Elmer, Waltham, MA) was added to each well. The plate was sealed and shaken 10 min at 800 rpm. Luminescence signal was measured in Beckman-Coulter DTX-880 Multimode Reader. The luminescence data were plotted against peptide concentrations and EC_{50} values were calculated by using logistic nonlinear 3 parameter regression in GraphPad Prism 6 (GraphPad Software, La Jolla, CA).

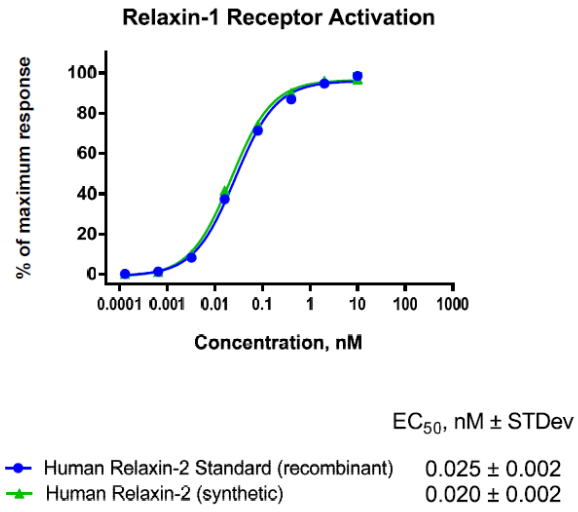

Figure S7. In vitro activity of synthetic human relaxin-2 (**1**)

Table S1. Details of Fmoc_HOBt_DCC_0.1 mmol method used on ABI 433A synthesizer.

Coupling Reagents	1.0 M DIC in NMP (1.0 mL) and 1.0 M 6-Cl-HOBt in NMP (1.0 mL)
Amino Acid	1.0 mmol cartridge
Solvents	NMP and DCM
Activation time	15 min
Coupling time	37 min
Deprotection of Fmoc groups	20% piperidine in NMP, 11 min

Table S2. LC-MS characterization of synthetic peptides.

Peptide #	Peptide Name	m/z		Purity by HPLC (214 nm)
		Theoretical	Observed	
1	Relaxin-2	5960.8	5962.7	96%
2	[Cys(*t*Bu)A11, Cys(Phacm)A24] A-chain	2858.4	2858.5	98%
3	[Disulfide A10-A15, Cys(*t*Bu)A11, Cys(Phacm)A24] A-chain	2856.4	2856.3	crude
4	[Disulfide A10-A15, Cys(SNPy)A11, Cys(Phacm)A24] A-chain	2954.3	2954.8	88%
5	[Isoacyl (B1-B2, B26-B27), Cys(Phacm)B23] B-chain	3458.7	3459.7	93%
6	Cys(Phacm)B23 B-chain	3458.7	3460.4	crude
7	[Disulfide (A10-A16, A11-B11), Cys(Phacm)A24, Cys(Phacm) B23] A-B dimer	6256.0	6258.7	crude
8	[Isoacyl B26-B27, Cys(Phacm)B23] B-chain	3458.7	3459.7	91%