Supplementary Information:

Enhanced Glucose Regulation Potential of C-peptide Mimics

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Abbreviations

Fmoc	Fluorenylmethyloxycarbonyl
DIC	N,N´-diisopropylcarbodiimide
SPPS	Solid-phase peptide synthesis
TIPS	Triisopropylsilane
DMF	N,N-Dimethylformamide
TFA	Trifluoroacetic acid
DCM	Dichloromethane
MALDI-TOF-MS	Matrix-assisted laser desorption ionization-time of flight mass spectrometer
RP-HPLC	Reverse phase High-Performance liquid chromatography
Oxyma pure	Ethyl cyanohydroxyiminoacetate
DHB	2,3-dihydroxybenzoic acid
CHCA	α-Cyano-4-hydroxycinnamic acid
HRMS	High-resolution mass spectrometry
PBS	Phosphate buffer saline
aq	Aqueous
GLUT4	Glucose transporter 4
ROS	Reactive Oxygen Species
NAC	n-Acetylcysteine
PA	Palmitic acid
INS	Insulin
BSA	Bovine Serum Albumin
FBS	Fetal Bovine Serum
DMSO	Dimethyl sulfoxide
FACS	Fluorescence –activated cell sorting
DMEM	Dolbecco's Modified Eagle Medium
TMB	3,3',5,5'-Tetramethylbenzidine
IRβ	Insulin Receptor β

Materials and methods

Unless otherwise stated, all the chemicals, solvents, and reagents were obtained from Sigma Aldrich. Fmoc-L-amino acids and Rink amide MBHA resin (100-200 mesh) were sourced from Novabiochem® (Germany). DIC was obtained from TCI chemicals. Solvents used for solid phase peptide synthesis and RP-HPLC were of SPPS and HPLC grade respectively. MilliQ water was used for the purification and analysis of the compounds by RP-HPLC.

Anti-myc HRP primary antibody (ab1326) and Anti-myc dylight 650 (9E10) from Abcam; anti-AKT(C67E7), anti-phospho AKT(Ser473) 9271S, Anti-Insulin Receptor β(4B8), anti-phospho- Insulin Receptor β(Tyr1345) obtained from Cell Signalling Technology ,(14A4)Human insulin (19275), Palmitic acid (P9767-50G), 2′,7′-Dichlorofluorescin Diacetate (4091-99-0), Dimethyl Sulphoxide (317275-100ML), Fluoroshield™ with DAPI (F6057-20ML), N-Acetyl-L-cysteine (106425-5GM) were purchased from Sigma-Aldrich/Merck. BSA(TC548) Triton® X-100(TC286) was obtained from HiMedia. Synthetic human C-peptide (7750-0502) was purchased from Biorad. TMB (7004P6) was obtained from cell signalling technology and Glucose kit from Coral Clinic. Dihydroethidium (D1168) and 6-NBDG (N23106) were obtained from Thermo Fisher Scientific. GLUT4-GFP Stable CHO Cell was obtained from Applied Biological Materials Inc. (abm, Canada) (Cat.T6498).

Peptide synthesis and purification

General procedure for solid-phase peptide synthesis

All the C-peptide derivatives shown in Table S1 were prepared using a microwave-assisted automated peptide synthesizer (Liberty Blue, CEM, USA). Reactions were carried out on Rink amide MBHA resin in 0.1 mmol scale (0.36 mmol/g loading), and all the couplings were done by using Fmoc-protected amino acids in DMF (0.2 M) (Scheme 1).[79] All the microwave couplings were performed twice for 20 min each at 75 °C in the presence of Oxyma pure (0.5 M) and DIC (1 M) as an activator and activator base, respectively, in DMF. The deprotection step was performed by treating peptide-bound resin with 20% piperidine in DMF for 10 minutes. After each individual coupling and deprotection, the resin was washed again with DMF to ensure the removal of soluble impurities.

Cleavage of the synthesized peptide from solid support

After completion of the reaction, peptide attached to the resin was washed with DMF and DCM respectively (three times). Cleavage of the peptide chain from resin as well as side chain deprotection was carried out by gentle agitation of resin for 2 h using a fresh cocktail of TFA:TIS:H_2O (95:2.5:2.5, v/v/v). Thereafter, the cocktail solution along with the cleavage products was filtered and removed under reduced pressure. Then the crude peptide was finally recovered by precipitation in cold ether.

Peptide purification and characterization

Purification of crude C-peptide derivatives was performed using Waters (Hietzinger Hauptstraße, Vienna - AUSTRIA) fully automated RP-HPLC system equipped with a photo-diode array detector on a SunFire® Prep C18 OBD™ (19 mm x 250 mm, 5.0 µm) column. The collection of peptide fractions was done at 220 nm. Purity of the C-peptide mimics (> 99%) was assessed by an analytical RP-HPLC system on Waters SunFire® C18 analytical column (4.6 mm x 250 mm, 5.0 µm) using a 1 ml/min flow (ESI†, p4-p13). For both the analyses MilliQ water and acetonitrile containing 0.1% TFA were used as eluent A and B respectively. After purifications, collected fractions containing desired compounds were combined, lyophilized and stored at -20 ^{o}C for further use. Mass of all the synthesized compounds was confirmed by MALDI-TOF MS using Bruker Autoflex speed using matrix DHB or CHCA. HRMS data of one of the samples was recorded by electrospray ionization with a quadrupole time-of-flight (Q-TOF) mass analyzer (Make: Waters, Model: Xevo XS QTof mass spectrometer, Waters ACQUITY UHPLC).

Circular dichroism study

All the C-Peptide mimetic samples were dissolved in aq. potassium phosphate buffer (pH 7.0) at a concentration 2 mM. The CD spectra were recorded at JASCO J-815. All the data were acquired through 10 scans in a 1 mm path length cuvette at a scan speed of 50 scan/min. Wavelength range: 180-350 nm, Data pitch: 0.1 nm, D.I.T.: 4 sec, Accumulation: 3, Bandwidth: 2.00 nm. The CD patterns suggested an antiparallel structure for the synthetic peptides.

Methods for biological studies

Human Studies

Informed written consent was taken from all subjects, including both healthy and T2D patients according to the guidelines outlined by the Institutional Human Ethical Committee. The study was conducted on subjects who were T2D patients (n=20) and healthy volunteers in the same age group (n =14). Healthy as well as T2D patients who had any past reference of smoking, cardiovascular-related complications, sickle cell disease, uncontrolled high blood pressure, thyroid-related issues were exempted from this assay. Also, volunteers with any symptoms of liver abnormalities (liver profile significantly above the upper value of normal range (>1.5 times)) or impaired kidney function (creatinine value >1.5 mg/dL) were not included in this study. Furthermore, individuals using any vitamin supplements or herbal therapies were not included in this investigation. All consenting participants were requested to provide blood samples following an overnight fast of 8h. subsequent to the blood collection, serum tubes for chemistry profile, including fasting glucose and C-peptide) and ethylenediaminetetraacetic acid (EDTA) tubes for HbA1C were immediately delivered to the CSIR-NEIST clinical laboratory under the project No. GPP0331.

Certificate of Declaration for performing experiments with human subjects

The study protocol was approved by the Institutional Human Ethical Committee.

Statistical analysis

Data were statistically analyzed using Sigma Stat statistical software (Jandel Scientific, SanRafael, CA). Once the dataset successfully met the criteria for normality, comparisons between groups were carried out by the Student–Newman–Keuls post hoc method. A *p*-value below 0.05 was perceived as significant. The data were represented as mean ± SE.

Cell culture and treatment

Rat L6 myoblast cell line was sourced from the American Type Culture Collection (ATCC, Manassas, VA). L6-GLUT4myc myoblast cells were generously supplied by Prof. Amira Klip, The Hospital for Sick Children (Toronto, Ontario, Canada). GLUT4-GFP Stable CHO Cell Line was purchased from Applied Biological Materials Inc. (abm, Canada) (Cat.T6498). After culturing cells in high glucose DMEM with 10% FBS and 1% penicillin-streptomycin, they were kept in a humid atmosphere consisting of 5% CO_2 at a temperature of 37 °C. To distinguish myoblast cells into myotubes, 95%

confluent myoblast cells were cultured in high glucose DMEM Gibco consisting of 2% FBS and 1% penicillin-streptomycin. Differentiation media was replaced daily and multinucleated myotubes became observable following a 3-day differentiation period. One week following the initiation of differentiation, myotubes were suitable for the experiments. Myotubes were treated with palmitic acid (PA, 0.75 mM) for 24 h. Cells were supplemented with 5 nM concentration of human C-peptide (C-pep) and its mimetics for next 24 h. [80,58]

Glucose uptake assay

The glucose uptake assay was performed in differentiated L6 myotubes using 6-[N-(7-nitrobenz-2- oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (6-NBDG; Invitrogen), which is a fluorescent-based analog of 2- deoxyglucose (2-DG). After treating the cells for 24 h with human C-peptide (C-pep) and its mimetics at a concentration of 5 nM and insulin (10 nM) in presence or absence of PA (0.75 mM) followed by washing with 1X PBS. Then it was incubated with FACS buffer containing 6-NBDG (5 µM) for 45 min in 37 °C CO_2 incubator followed by washing and lysing with TritonX-100 and DMSO. Absorbance was taken at excitation/emission wavelength of 466/540 nm.[69,80]

Glucose Utilization assay

The glucose levels of the cell culture supernatant were measured at zero (0) h and again upon the completion of the experiment (24) h. The level of glucose utilization was examined by subtracting glucose values at 24 h time point from the zero (0) h glucose level. The mentioned tests were conducted in triplicate at regular time interval. The glucose assay was performed by using commercially available kits, Coral Clinical Systems.[69,81]

GLUT4 Translocation assay

L6-GLUT4myc cells were cultured and maintained following the standard protocol as mentioned earlier. After attaining confluency, cells were differentiated till day 7 to L6 myotubes using 2% HG DMEM Gibco. Then, to quantify the cell surface GLUT4-myc-GFP, the cells were incubated with an anti-mycHRP-conjugated antibody, followed by the treatment with TMB reagent and 2N H_2SO_4. The absorbance was taken at 450 nm.[58,59]

GLUT4 Exocytosis

Transfected L6 GLUT4-myc was differentiated till day 7 and incubated in serum free DMEM for 2 h. After that stimulation with insulin (100 nM) was carried out for 30 min followed by washing 2 times with 1X PBS followed by 20 min blocking with 1% BSA and 1 h incubation with anti-myc primary antibody (1:400) and 250 µL blocking solution to the control unlabelled groups. After washing 4 times with 1X chilled PBS cells were again incubated with DMEM for 2 h at 37°C proceeded by exposure with C-pep (5 nM), **CP8** (5 nM) and Insulin (10 nM) along with 0.75 mM PA with or without for 1 hr for the plates to measure exocytosis. Then the cells underwent washing and fixing with 4% paraformaldehyde. Then it was blocked with 1% BSA for 15 min and incubated with HRP conjugated primary anti-myc antibody (1:400) for 4-6 h and then washed 3-4 times with chilled 1X PBS. TMB was added after sufficient color development, 2N H_2SO_4 was used as a stop solution. Absorbance was taken at 450 nm.[82,83]

G4Exo(t,s) = Abs 450 nm(t,s) – Abs 450 nm (unlabelled control) /Abs 450 nm (control without exocytosis) – Abs 450 nm (unlabelled control)

GLUT4 Endocytosis

Transfected L6 GLUT4-myc was differentiated till day 7 and incubated in serum free DMEM for 4 h followed by stimulation with 100 nM insulin for 30 min after washing with 3 times with 1X PBS followed by 20 min blocking with 1% BSA and then incubated 1 h with anti-myc primary antibody (1:400) except control unlabelled groups in ice cold condition followed by washing with ice-cold 1X PBS for 4 times, then 1 h exposure with 5 nM (C-pep, **CP8)** and Insulin (100 nM) along with 0.75 mM PA for the plates to check internalization followed by 0 min exposure of the same for control without internalization. Then fixed with 4% paraformaldehyde and blocking with 1% BSA for 15 min. The cells were then incubated with HRP conjugated primary anti-myc antibody (1:400) for 6-8 h washed 4 times with chilled 1X PBS. TMB was added after sufficient color development, 2N H_2SO_4 was used as a stop solution. Absorbance was taken at 450 nm.[82,83]

GLUT4myc not internalized (G4NI; i.e., remaining at the cell surface) at each time point (60 mins) and each stimulus condition (s):

G4NI (t,s) = Abs 450 nm(t,s) – Abs 450 nm(unlabelled control(t,s))/Abs 450 nm(0 min, s) – Abs 450 nm(unlabelled control (0 min ,s))

GLUT4myc internalized (G4I) at each time point (t) and each stimulus condition (s) is measured in the following way:

G4I (t,s) = 1 - G4NI (t,s)

Immunofluorescence of GLUT4

Differentiated L6-GLUT4myc cells and 70-90% confluent CHO-HIRc-mycGLUT4eGFP cells were first washed with 1X PBS and then treated with 5 nM concentration of **CP8**, C-Pep and insulin (10 nM) with or without Palmitic acid (0.75 mM) for 24 h. Afterwards, cells were rinsed with 1X PBS, treated with 4% paraformaldehyde for 20 min for fixation and blocked using 1% BSA for 30 min. Next, overnight incubation of cells with HRP conjugated myc tagged primary antibody was carried out at 4 °C. After washing the cells with 1X PBS (3 times), they were mounted with DAPI mounting media. Images were taken under EVOS M7000 microscope.[84]

Immunoblotting

Post-treatment, animal tissues or cell culture samples were lysed in radioimmunoprecipitation assay (RIPA) buffer consisting of 50 mM Tris, pH 8, 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, and 0.1% SDS. Additionally, this RIPA buffer was also infused with protease and phosphatase inhibitors having 1 mM PMSF, 5 µg/mL leupeptin, 2 µg/mL aprotinin, 1 mM EDTA, 10 mM NaF, and 1mMNa3VO4. Centrifugation of lysate was carried out to get clear protein supernatant, and its concentrations were measured utilizing the BCA reagent (Thermo Scientific, Rockford, IL). Each sample, containing approximately 30 µg of protein, was loaded on a 10% SDS-PAGE gel. Subsequently, it was transported to a nitrocellulose membrane which was blocked at room temperature (rt) for 2 h in blocking buffer (1% BSA) to inhibit nonspecific interaction. Incubation was carried out either with anti-AKT (1:1000), anti-phospho AKT (1:1000) (Ser 473), anti-IRβ (1:1000) or anti-phospho IRβ (Tyr 1345) (1:1000) primary antibody at 4 °C overnight. After 30 minutes wash of TBST (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.1% Tween 20), the membranes were cultured for 2 h at rt with the corresponding HRP-linked secondary antibody (1:5000 dilution). Subsequently, they were developed utilizing an ultrasensitive ECL substrate (BioRad).[52]

Assessment of intracellular reactive oxygen species (ROS)

The intracellular ROS production levels were determined with the help of fluorescent dye, H_2DCFDA (2', 7'dichlorofluorescein diacetate). Following treatment, L6 myotubes were first rinsed with PBS and subsequently loaded with 5 μM H_2DCFDA in PBS containing 4% FBS. The cells were then incubated at 37 °C for 30 min in the absence of light and followed by washing with PBS. After that, harvesting in PBS with 0.5% Triton X-100 and centrifugation at 12000Xg was carried out for 10 min at 37 °C. Thereafter, the collection of supernatants was done and its DCF fluorescence intensity was measured using a spectrofluorometer at excitation and emission wavelengths of 468 and 540 nm, respectively.[20,22]

Visualization of intracellular reactive oxygen species

Determination of the production of intracellular ROS levels was determined by using the fluorescent dye, dihydroethidium (DHE). After treatment, L6 myotubes were treated once with PBS and then charged with 0.1 μM DHE in FACS buffer. After that, the cells were taken for incubation at 37 °C for 15 min in a dark environment and thereafter washed with PBS and fixed with 4% paraformaldehyde for 15 min. After additional washing, they were mounted with mounting media containing DAPI and visualized under EVOS M7000 microscope.[20,22]

Alamar Blue assay

Alamar blue reduction bioassay was conducted to evaluate the effect of different treatments on cell viability. The method relies on the decline of Alamar blue dye by live/viable cells. Shortly after the experiment, PBS washed cells were treated with Alamar blue solution at 37 °C for 4 h. The absorbance was then recorded at the wavelengths of 560 and 600 nm. The cell viability was calculated using delta OD (570–600) and it was expressed as percentage over control.[49]

Scheme S1. General scheme illustrating standard procedure for the synthesis of C-peptide mimics using microwave-assisted solid-phase peptide synthesis

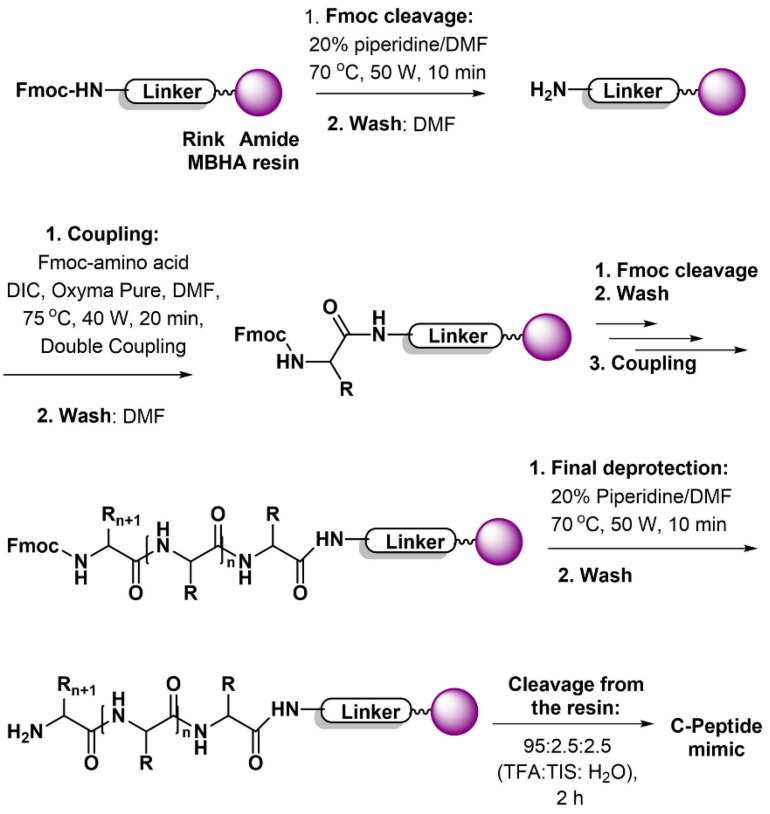

Table S1. Three letter abbreviation of synthesized C-peptide mimics (N-terminal with free amine and C-terminal with primary amide, coloured one represent mutated amino acid)

	1			5				10				15					20					25					30		
C-pep	Glu	Ala	Glu	Asp	Leu	Gln	Val	Gly	Gln	Val	Glu	Leu	Gly	Gly	Gly	Pro	Gly	Ala	Gly	Ser	Leu	Gln	Pro	Leu	Ala	Leu	Glu	Gly	Ser	Leu	Gln
CP1	Glu	Ala	Glu	Asp	Leu	Gln	Val	Gly	Gln	Val	Glu	Leu	Gly	Gly	Gly	Pro	Gly	Tyr	Gly	Ser	Leu	Gln	Pro	Leu	Ala	Leu	Glu	Gly	Ser	Leu	Gln
CP2	Glu	Ala	Glu	Asp	Leu	Gln	Val	Gly	Gln	Val	Glu	Leu	Gly	Gly	Gly	Pro	Gly	Ala	Gly	Ser	Leu	Gln	Pro	Leu	Tyr	Leu	Glu	Gly	Ser	Leu	Gln
CP3	Glu	Ala	Glu	Asp	Leu	Gln	Val	Gly	Gln	Val	Glu	Leu	Gly	Tyr	Gly	Pro	Gly	Ala	Gly	Ser	Leu	Gln	Pro	Leu	Ala	Leu	Glu	Gly	Ser	Leu	Gln
CP4	Glu	Ala	Glu	Asp	Leu	Gln	Val	Gly	Gln	Val	Glu	Leu	Gly	Gly	Gly	Pro	Gly	Ala	Tyr	Ser	Leu	Gln	Pro	Leu	Ala	Leu	Glu	Gly	Ser	Leu	Gln
CP5	Glu	Ala	Glu	Asp	Leu	Gln	Val	Gly	Gln	Val	Glu	Tyr	Gly	Gly	Gly	Pro	Gly	Ala	Gly	Ser	Leu	Gln	Pro	Leu	Ala	Leu	Glu	Gly	Ser	Leu	Gln
CP6	Glu	Ala	Glu	Asp	Leu	Gln	Val	Gly	Gln	Tyr	Glu	Leu	Gly	Gly	Gly	Pro	Gly	Ala	Gly	Ser	Leu	Gln	Pro	Leu	Ala	Leu	Glu	Gly	Ser	Leu	Gln
CP7	Glu	Ala	Glu	Asp	Leu	Gln	Val	Gly	Gln	Val	Glu	Leu	Gly	Gly	Gly	Pro	Gly	Tyr	Gly	Ser	Leu	Gln	Pro	Leu	Tyr	Leu	Glu	Gly	Ser	Leu	Gln
CP8	Glu	Ala	Glu	Asp	Leu	Gln	Val	Gly	Gln	Val	Glu	Leu	Gly	Gly	Gly	Pro	Gly	Phe	Gly	Ser	Leu	Gln	Pro	Leu	Ala	Leu	Glu	Gly	Ser	Leu	Gln
CP9	Glu	Ala	Glu	Asp	Leu	Gln	Val	Aib	Gln	Val	Glu	Leu	Gly	Gly	Gly	Pro	Gly	Phe	Gly	Ser	Leu	Gln	Pro	Leu	Aib	Leu	Glu	Gly	Ser	Leu	Gln
CP10	Glu	Ala	Glu	Asp	Leu	Gln	Val	Gly	Gln	Val	Glu	Leu	Gly	Gly	Gly	Pro	Gly	Phe	bAla	Ser	Leu	Gln	Pro	Leu	Ala	Leu	Glu	Gly	Ser	Leu	Gln

CP1

C-peptide analog **CP1** has been synthesized using the general procedure starting from Rink amide MBHA resin (278 mg, 0.1 mmol). The final product, **CP1** was purified via RP-HPLC. 10.2 mg was obtained (yield 3.2%). **MALDI-TOF MS**: For M = $C_{135}H_{215}N_{36}O_{48}$, Calculated [M + Na]$^+$ (*m/z*)= 3131.539, Found = 3131.716

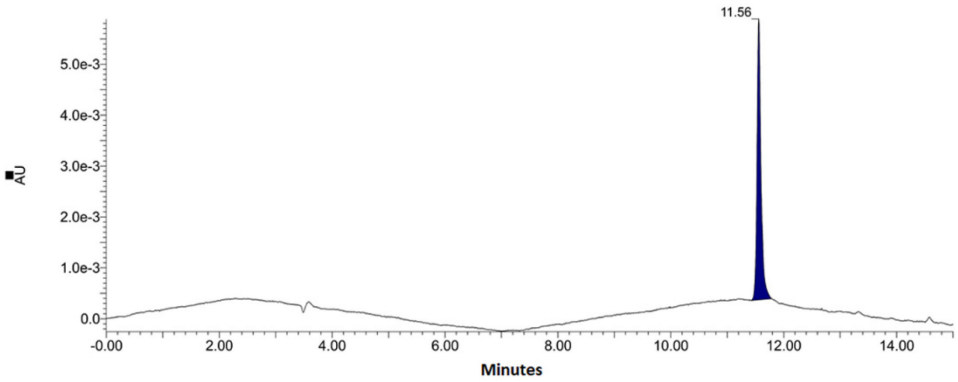

Figure S1. Analytical HPLC at 220 nm

Table S2. HPLC gradient, retention time and purity of **CP1**

C-peptide mimetic	Time (min)	% A	% B	Retention time (min)	Purity%
CP1	0	85	15	11.56	100
	3	85	15		
	12	50	50		
	13	50	50		
	14	85	15		
	15	85	15		

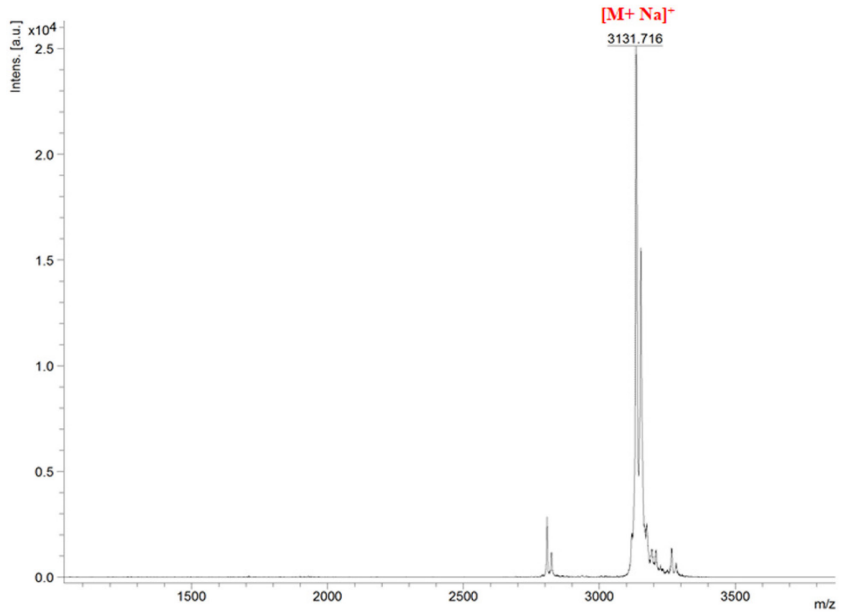

Figure S2. MALDI-TOF mass spectrum of **CP1** using matrix CHCA

CP2

C-peptide analog **CP2** has been synthesized using the general procedure starting from Rink amide MBHA resin (278 mg, 0.1 mmol). The final product, **CP2** was purified via RP-HPLC. 27.1 mg was obtained (yield 8.7%). **MALDI-TOF MS**: For M = $C_{135}H_{216}N_{36}O_{48}$, Calculated $[M + Na]^+$ (*m/z*)= 3132.546, Found = 3132.512

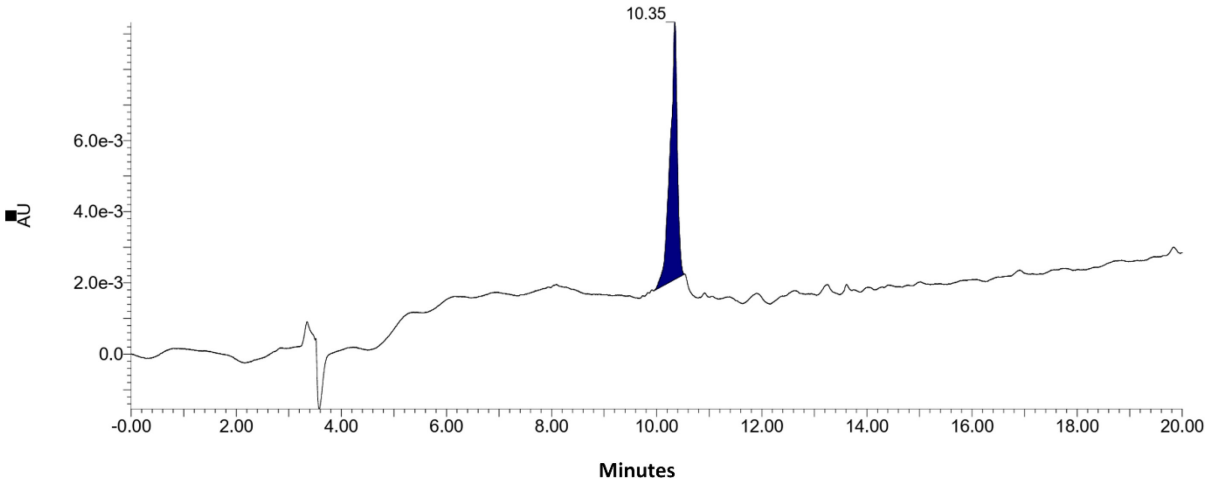

Figure S3. Analytical HPLC at 220 nm

Table S3. HPLC gradient, retention time and purity of **CP2**

C-peptide mimetic	Time (min)	% A	% B	Retention time (min)	Purity%
CP2	0	90	10	10.35	100
	15	20	80		

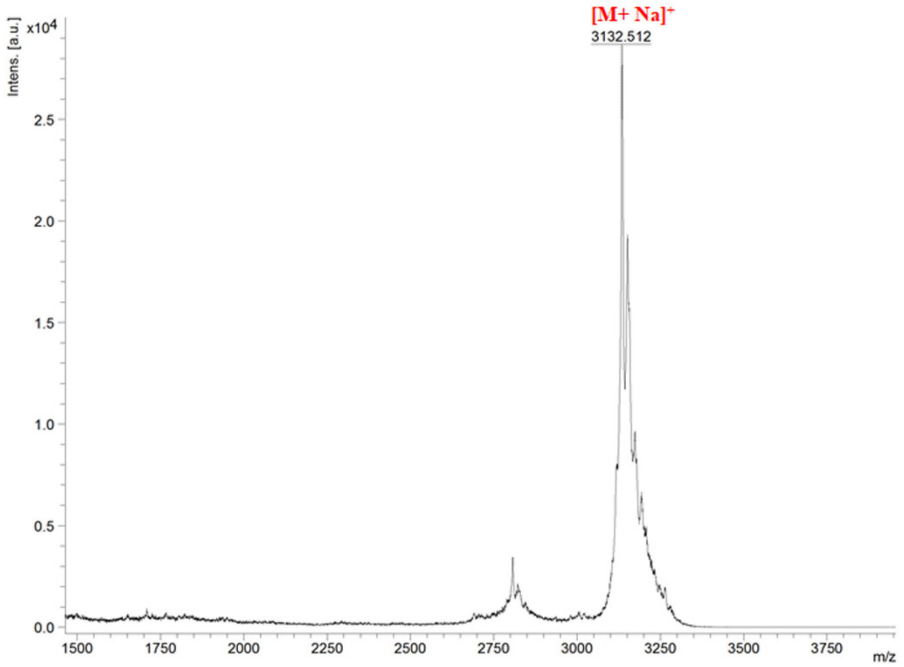

Figure S4. MALDI-TOF mass spectrum of **CP2** using matrix CHCA

CP3

C-peptide analog **CP3** has been synthesized using the general procedure starting from Rink amide MBHA resin (278 mg, 0.1 mmol). The final product, **CP3** was purified via RP-HPLC. 40.7 mg was obtained (yield 13%). **MALDI-TOF MS**: For M = $C_{136}H_{218}N_{36}O_{48}$, Calculated $[M + Na]^+$ (*m/z*)= 3146.562, Found = 3146.703

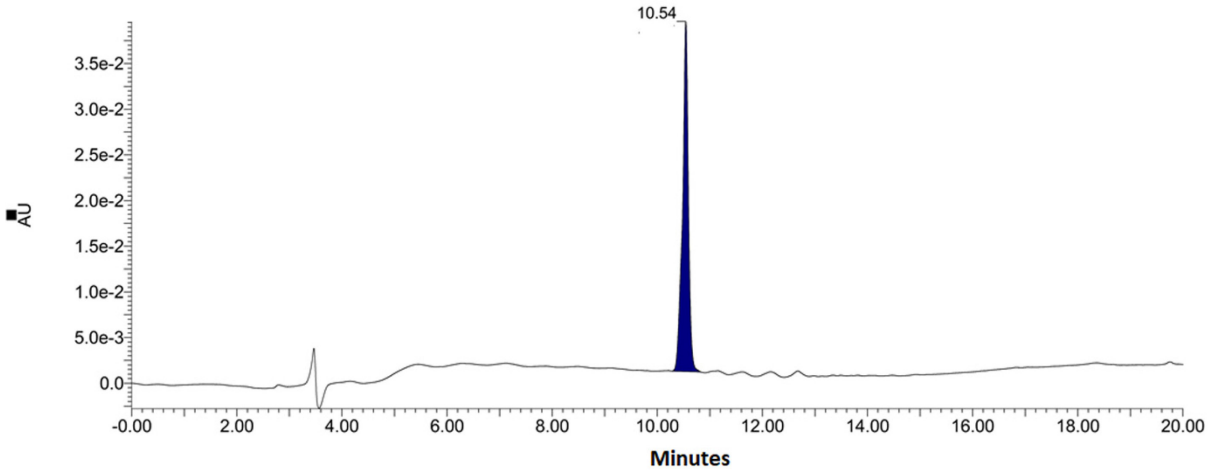

Figure S5. Analytical HPLC at 220 nm

Table S4. HPLC gradient, retention time and purity of **CP3**

C-peptide mimetic	Time (min)	% A	% B	Retention time (min)	Purity%
CP3	0	90	10	10.54	100
	20	20	80		

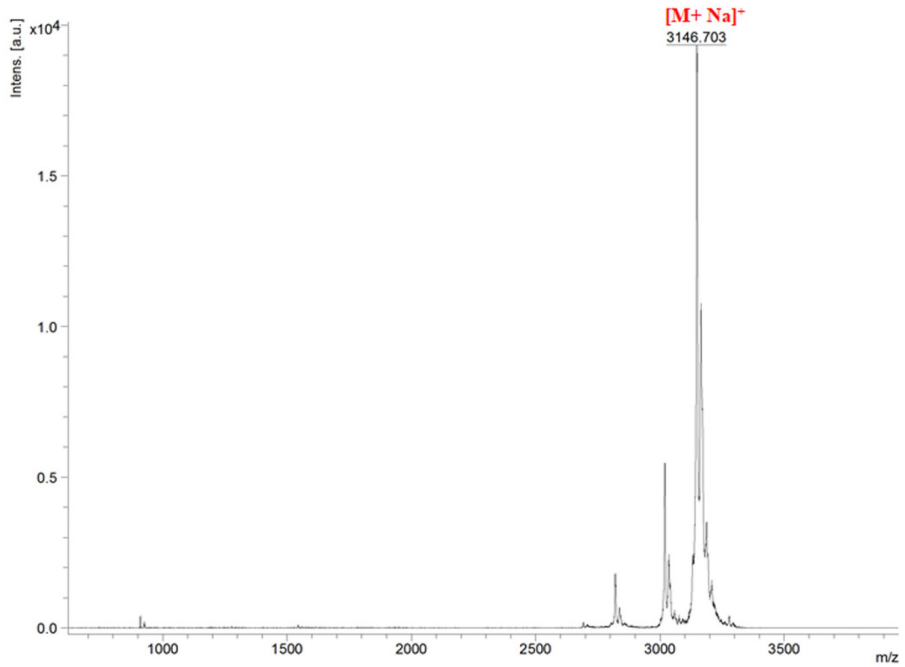

Figure S6. MALDI-TOF mass spectrum of **CP3** using matrix DHB

CP4

C-peptide analog **CP4** has been synthesized using the general procedure starting from Rink amide MBHA resin (278 mg, 0.1 mmol). The final product, **CP4** was purified via RP-HPLC. 28.5 mg was obtained (yield 9%). **MALDI-TOF MS**: For M = $C_{136}H_{218}N_{36}O_{48}$, Calculated $[M + Na]^+$ (*m/z*)= 3146.562, Found = 3146.591

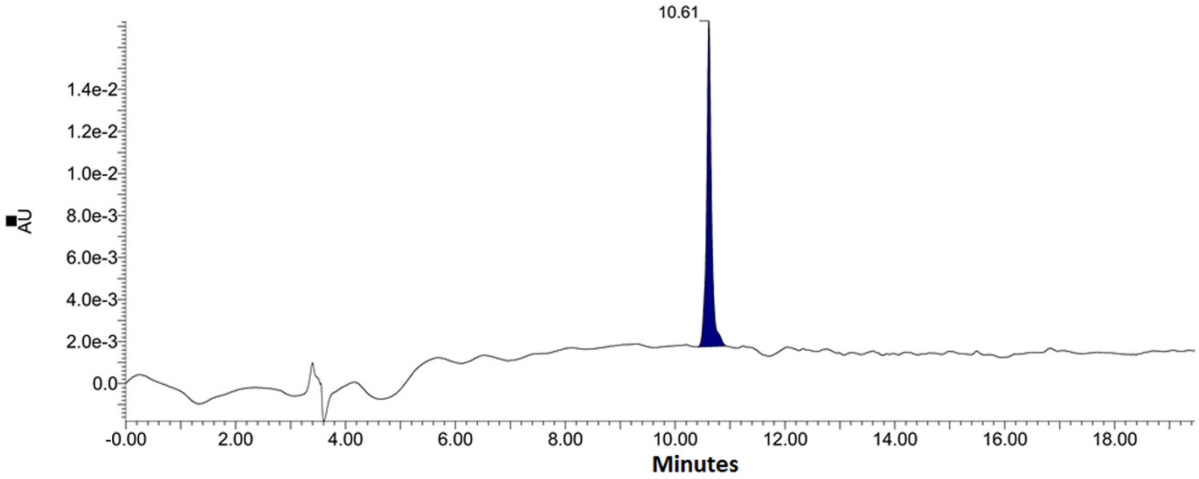

Figure S7. Analytical HPLC at 220 nm

Table S5. HPLC gradient, retention time and purity of **CP4**

C-peptide mimetic	Time (min)	% A	% B	Retention time (min)	Purity%
CP4	0	90	10	10.61	100
	20	20	80		

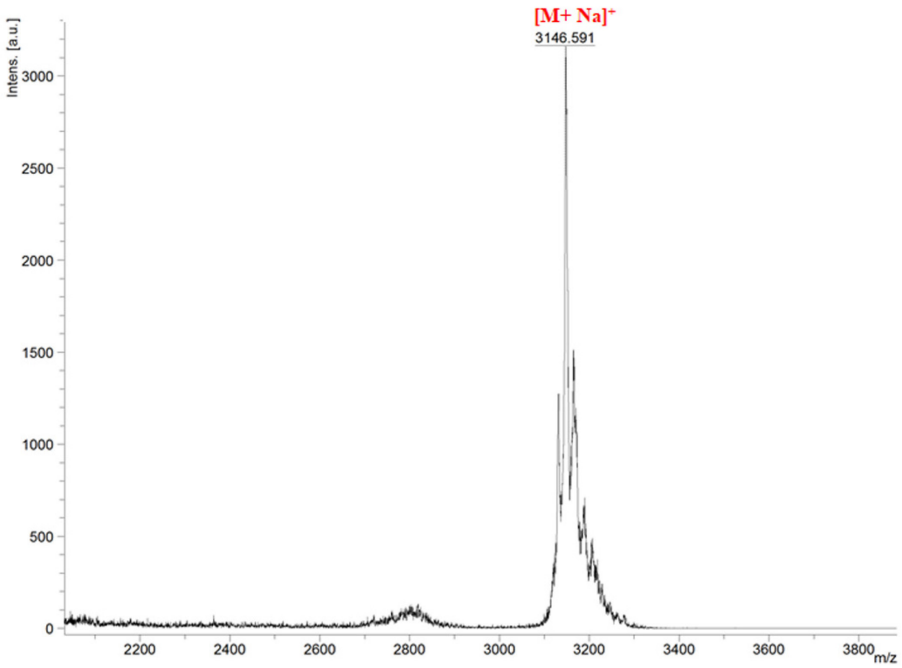

Figure S8. MALDI-TOF mass spectrum of **CP4** using matrix CHCA

CP5

C-peptide analog **CP5** has been synthesized using the general procedure starting from Rink amide MBHA resin (278 mg, 0.1 mmol). The final product, **CP5** was purified via RP-HPLC. 25.3 mg was obtained (yield 8%). **MALDI-TOF MS**: For M = $C_{132}H_{210}N_{36}O_{48}$, Calculated $[M + Na]^+$ (*m/z*)= 3090.499, Found = 3090.455

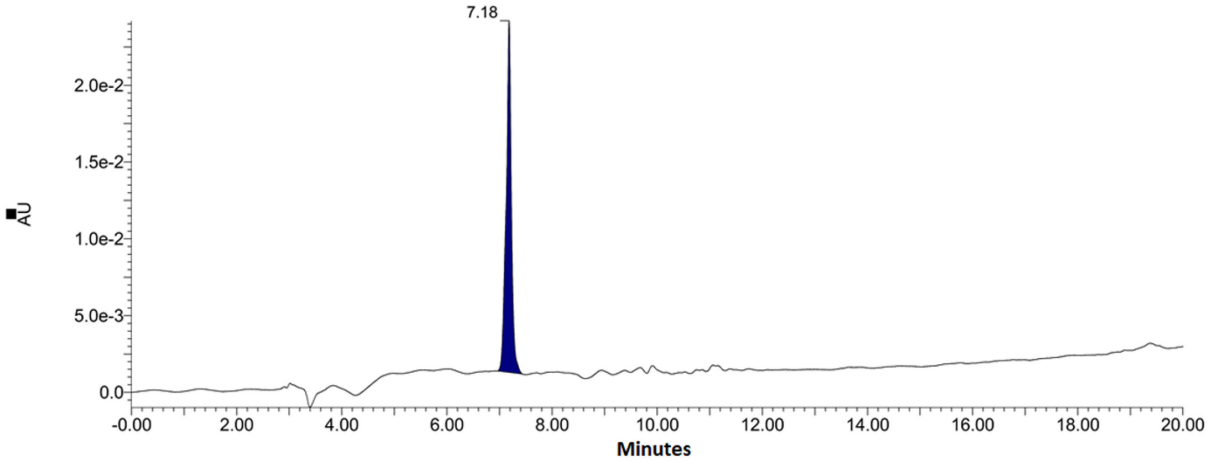

Figure S9. Analytical HPLC at 220 nm

Table S6. HPLC gradient, retention time and purity of **CP5**

C-peptide mimetic	Time (min)	% A	% B	Retention time (min)	Purity%
CP5	0	90	10	7.18	100
	20	20	80		

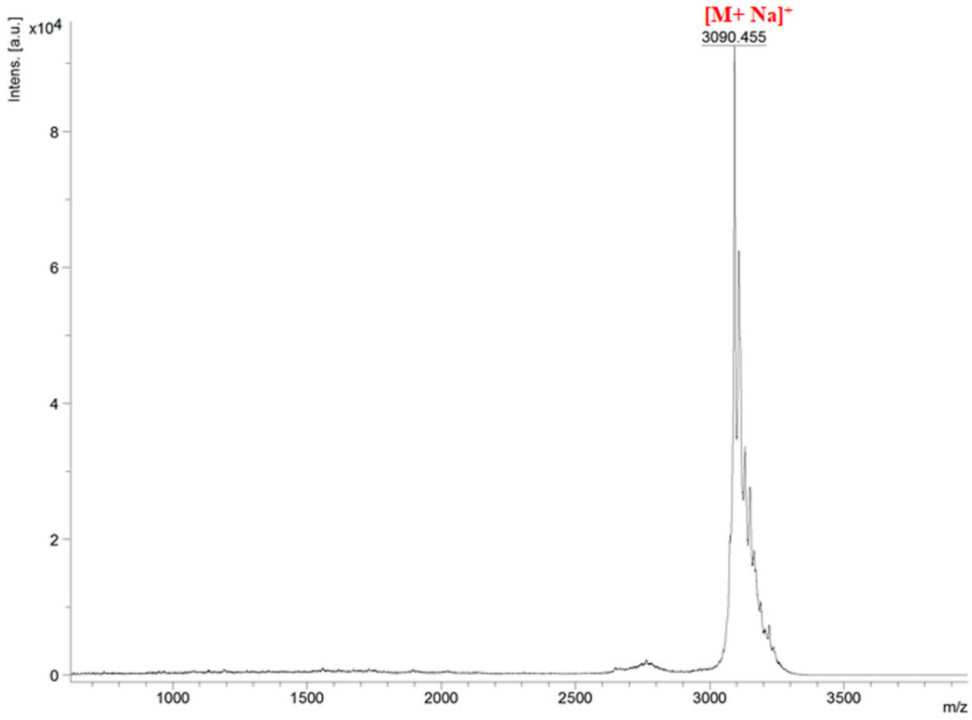

Figure S10. MALDI-TOF mass spectrum of **CP5** using matrix CHCA

CP6

C-peptide analog **CP6** has been synthesized using the general procedure starting from Rink amide MBHA resin (278 mg, 0.1 mmol). The final product, **CP6** was purified via RP-HPLC. 24.2 mg was obtained (yield 7.8%). **MALDI-TOF MS**: For M = $C_{133}H_{212}N_{36}O_{48}$, Calculated [M + Na]$^+$ (*m/z*)= 3104.515, Found = 3104.631

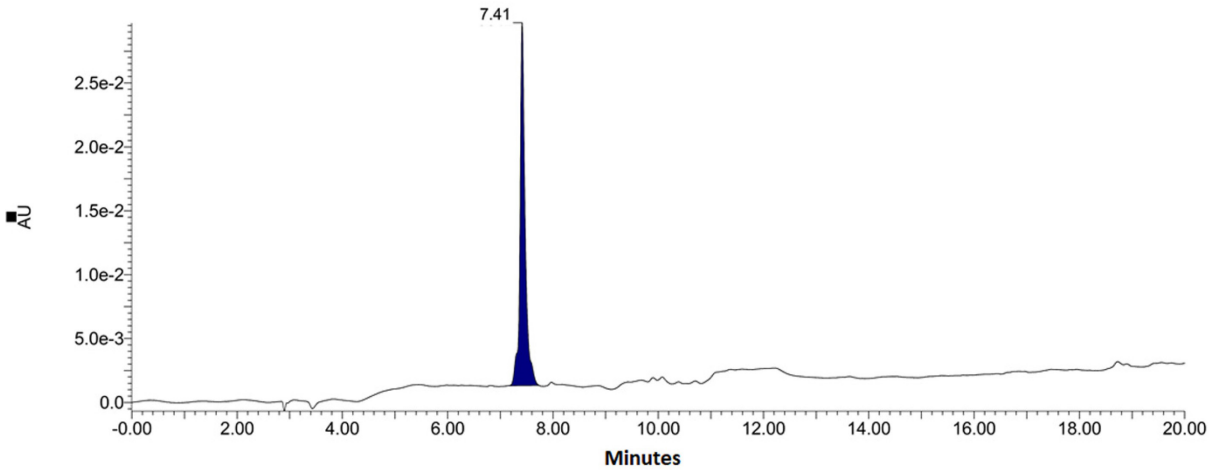

Figure S11. Analytical HPLC at 220 nm

Table S7. HPLC gradient, retention time and purity of **CP6**

C-peptide mimetic	Time (min)	% A	% B	Retention time (min)	Purity%
CP6	0	90	10	7.41	100
	20	20	80		

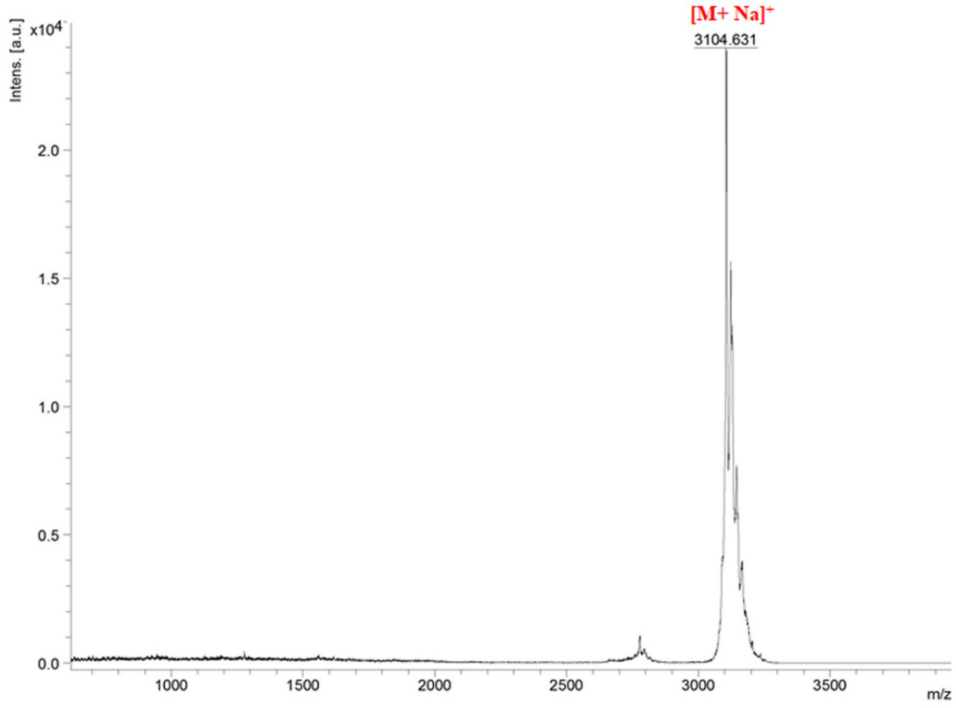

Figure S12. MALDI-TOF mass spectrum of **CP6** using matrix CHCA

CP7

C-peptide analog **CP7** has been synthesized using the general procedure starting from Rink amide MBHA resin (278 mg, 0.1 mmol). The final product, **CP7** was purified via RP-HPLC. 47.5 mg was obtained (yield 15%). **MALDI-TOF MS**: For M = $C_{141}H_{220}N_{36}O_{48}$, Calculated $[M + Na]^+$ (*m/z*)= 3224.573, Found = 3224.506

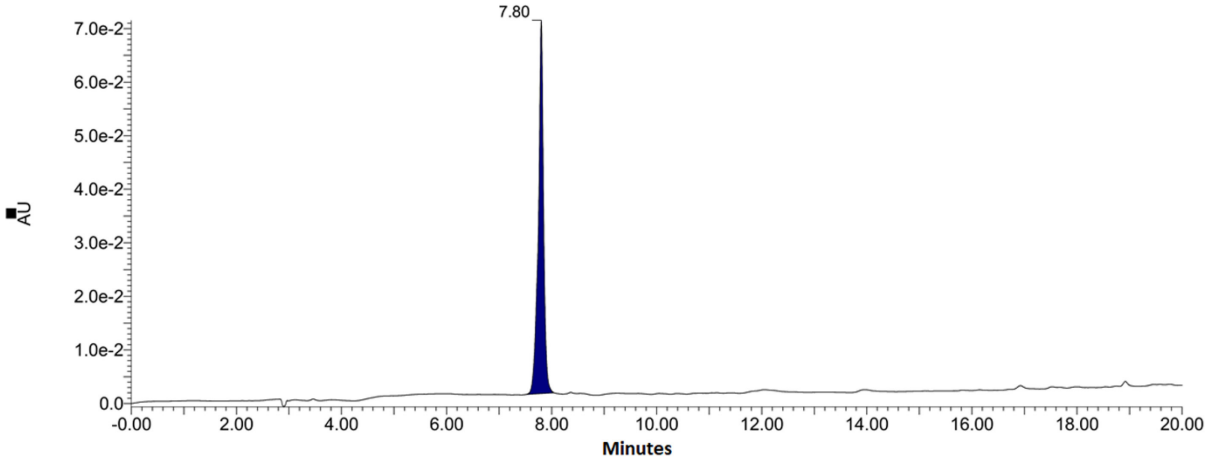

Figure S13. Analytical HPLC at 220 nm

Table S8. HPLC gradient, retention time and purity of **CP7**

C-peptide mimetic	Time (min)	% A	% B	Retention time (min)	Purity%
CP7	0	90	10	7.41	100
	20	20	80		

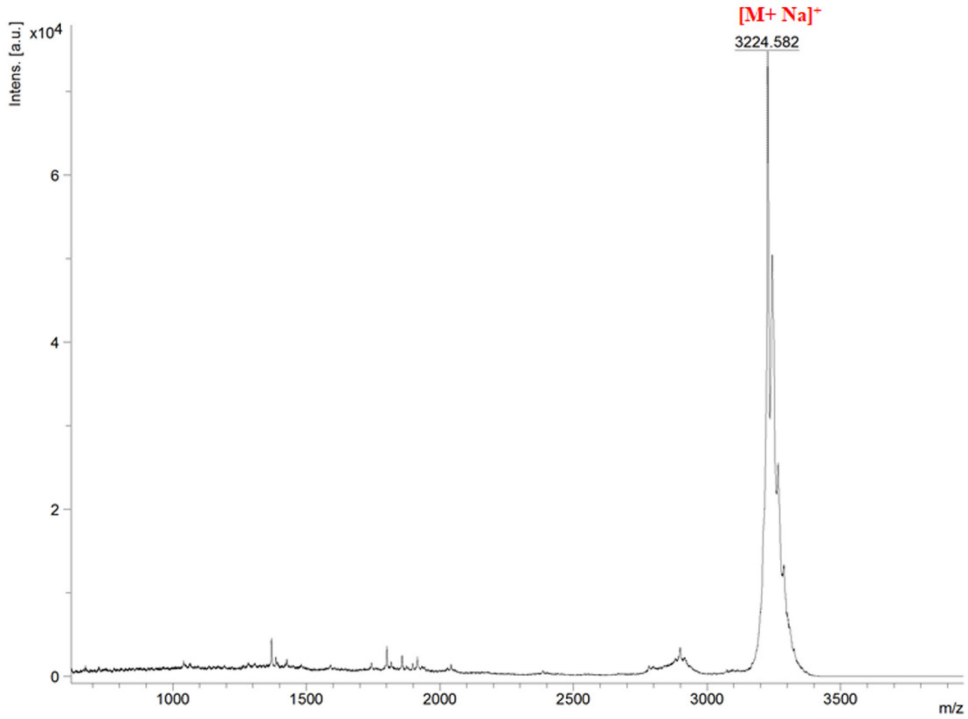

Figure S14. MALDI-TOF mass spectrum of **CP7** using matrix CHCA

CP8

C-peptide analog **CP8** has been synthesized using the general procedure starting from Rink amide MBHA resin (278 mg, 0.1 mmol). The final product, **CP8** was purified via RP-HPLC. 42.5 mg was obtained (yield 14%). **MALDI-TOF MS**: For M = $C_{135}H_{216}N_{36}O_{47}$, Calculated $[M + Na]^+$ (*m/z*) = 3116.552, Found = 3116.575

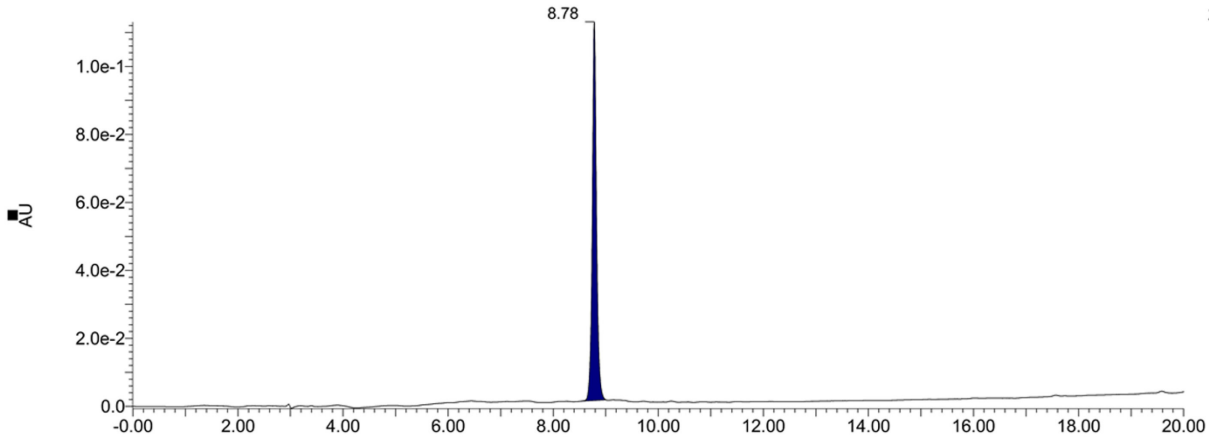

Figure S15. Analytical HPLC at 220 nm

Table S9. HPLC gradient, retention time and purity of **CP8**

C-peptide mimetic	Time (min)	% A	% B	Retention time (min)	Purity%
CP8	0	80	20	8.78	100
	1	80	20		
	18	10	90		
	20	80	20		

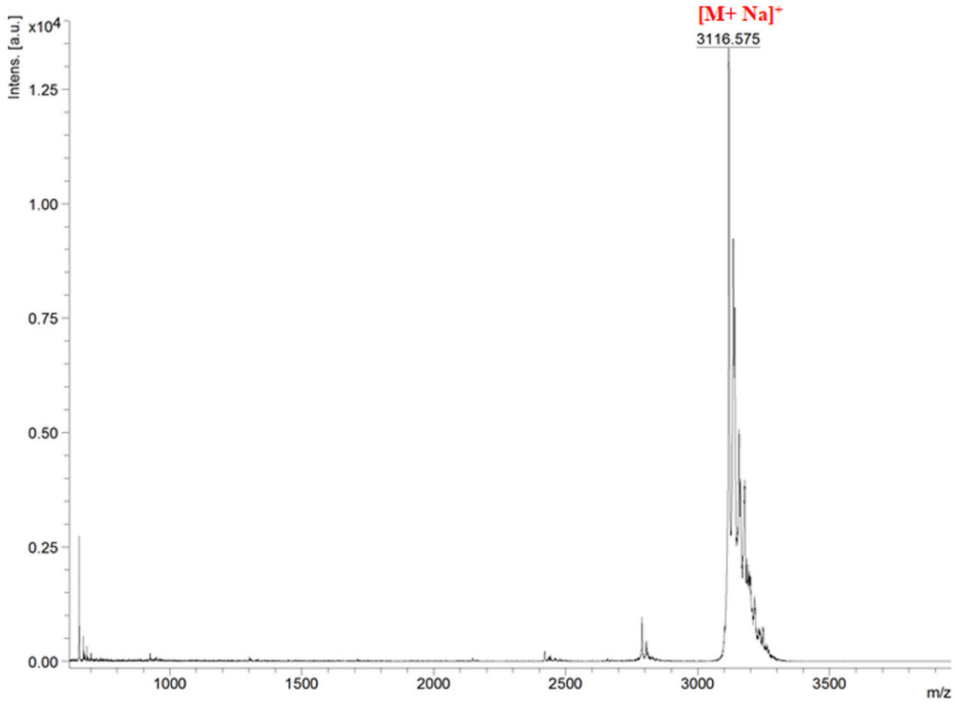

Figure S16. MALDI-TOF mass spectrum of **CP8** using matrix CHCA

CP9

C-peptide analog **CP9** has been synthesized using the general procedure starting from Rink amide MBHA resin (278 mg, 0.1 mmol). The final product, **CP9** was purified via RP-HPLC. 9.2 mg was obtained (yield 3%). **HRMS (ESI)$^+$**: For M = $C_{138}H_{222}N_{36}O_{47}$, Calculated monoisotopic $[M+H]^+$ (*m/z*) =3136.6, Found = 3136.8, calculated isotopic $[M + 3H]^{3+}$ (*m/z*)= 1046.5387, Found = 1046.6757

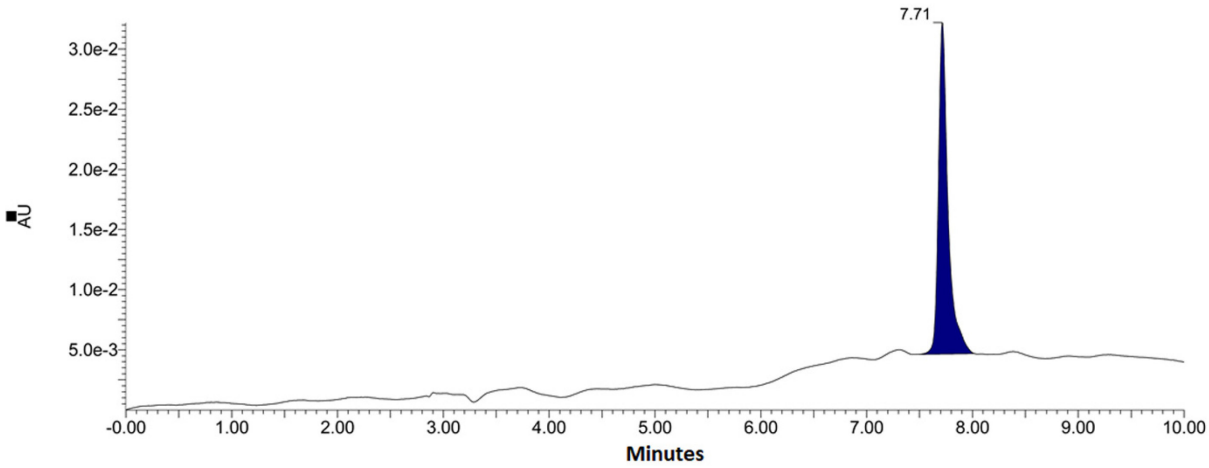

Figure S17. Analytical HPLC at 220 nm

Table S10. HPLC gradient, retention time and purity of **CP9**

C-peptide mimetic	Time (min)	% A	% B	Retention time (min)	Purity%
CP9	0	75	25	7.71	100
	2	75	25		
	5	50	50		
	6	50	50		
	8	75	25		
	10	75	25		

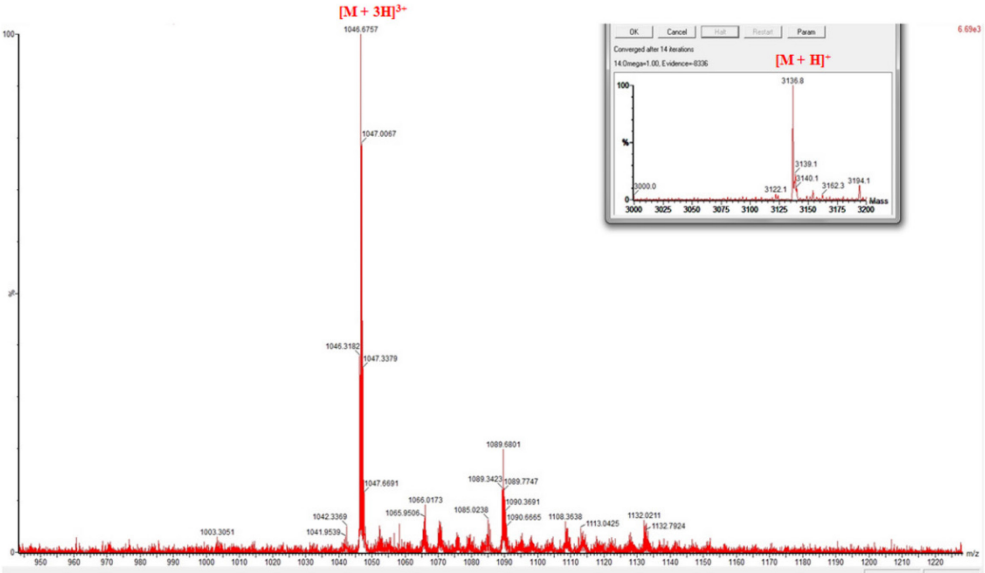

Figure S18. HRMS spectrum of **CP9**

CP10

C-peptide analog **CP10** has been synthesized using the general procedure starting from Rink amide MBHA resin (278 mg, 0.1 mmol). The final product, **CP10** was purified via RP-HPLC. 6.8 mg was obtained (yield 2.2%). **MALDI-TOF MS**: For M = $C_{136}H_{218}N_{36}O_{47}$, Calculated $[M + Na]^+$ (*m/z*)= 3130.567, Found = 3130.751

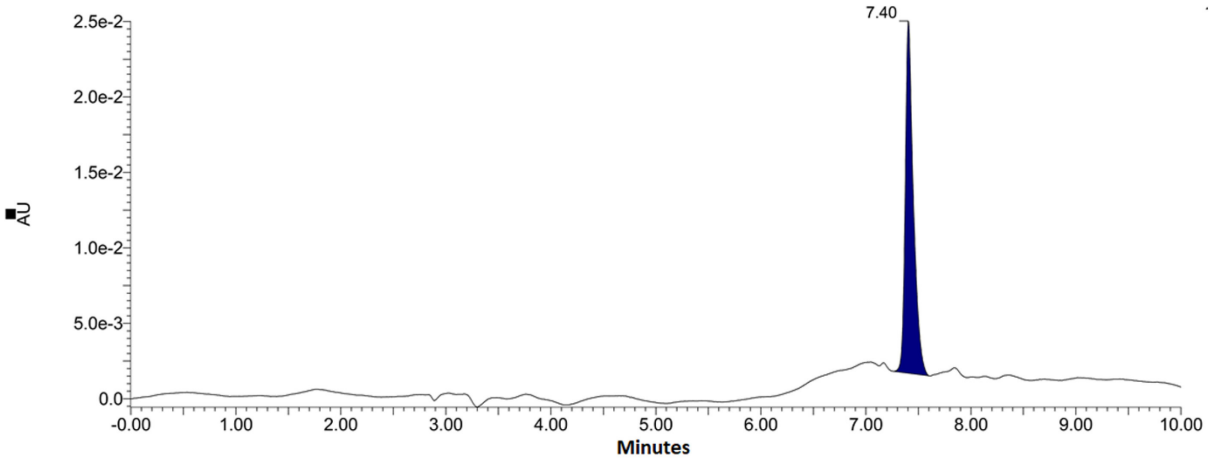

Figure S19. Analytical HPLC at 220 nm

Table S11. HPLC gradient, retention time and purity of **CP10**

C-peptide mimetic	Time (min)	% A	% B	Retention time (min)	Purity%
CP10	0	75	25	7.40	100
	2	75	25		
	5	50	50		
	6	50	50		
	8	75	25		
	10	75	25		

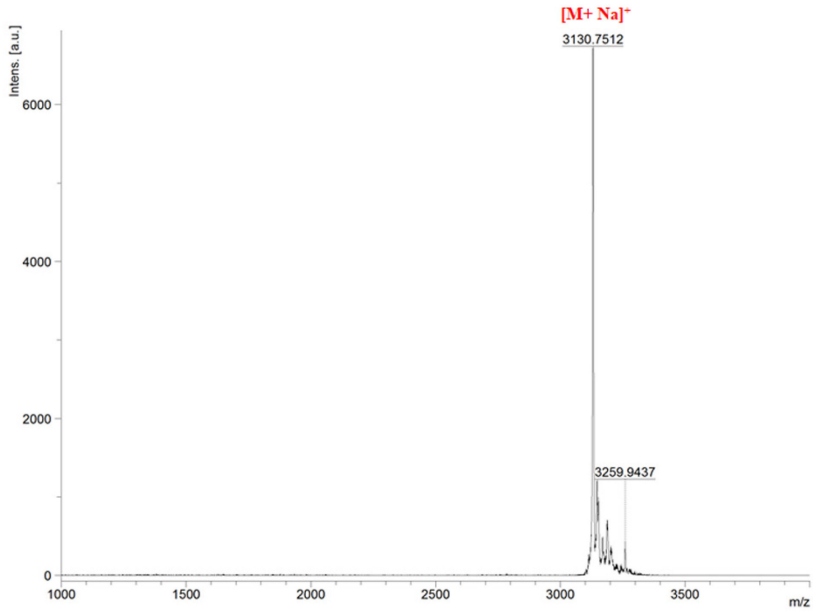

Figure S20. MALDI-TOF mass spectrum of **CP10** using matrix DHB

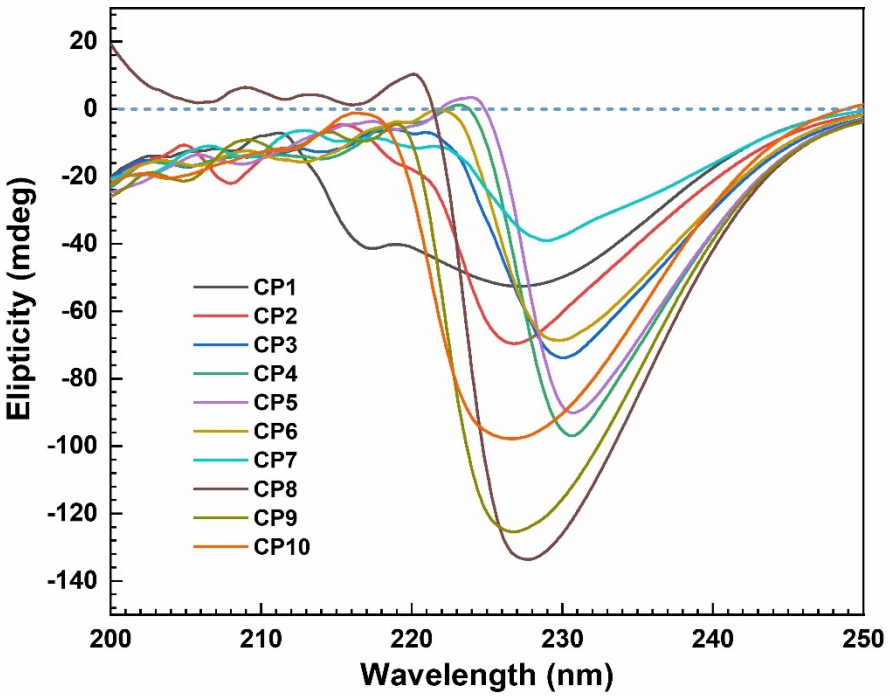

Figure S21. Circular dichroism study of C-peptide mimics **CP1-10**

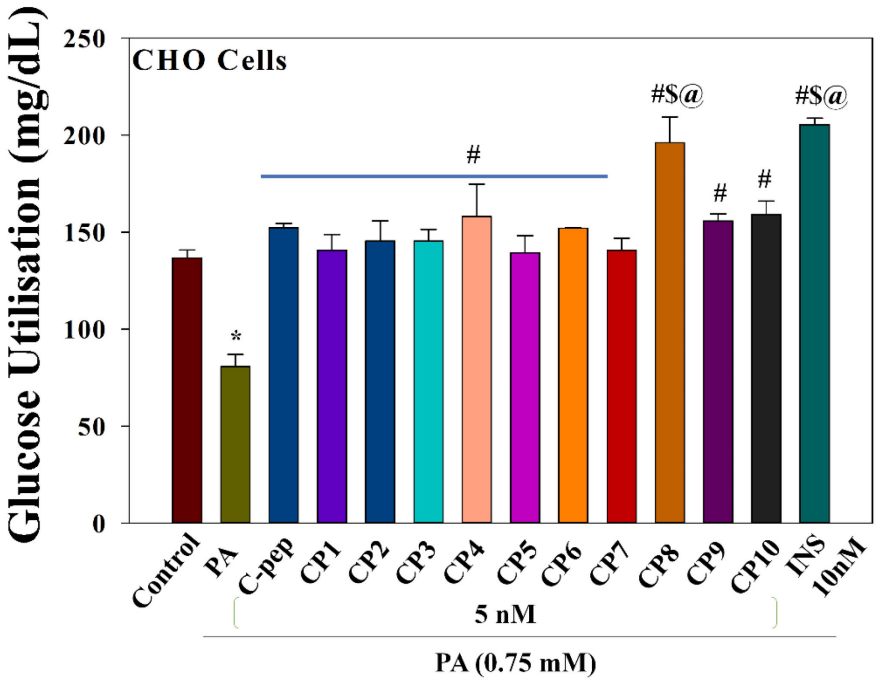

Figure S22. The effect of various C-peptide mimics on glucose utilisation in CHO cells with or without PA(0.75mM). Values are mean ± SE (n = 3). "*" denotes significant difference from control group, "#" from PA treated groups, "$" from human C-peptide treated group (*#$ P<0.005) and "@" from **CP1**.

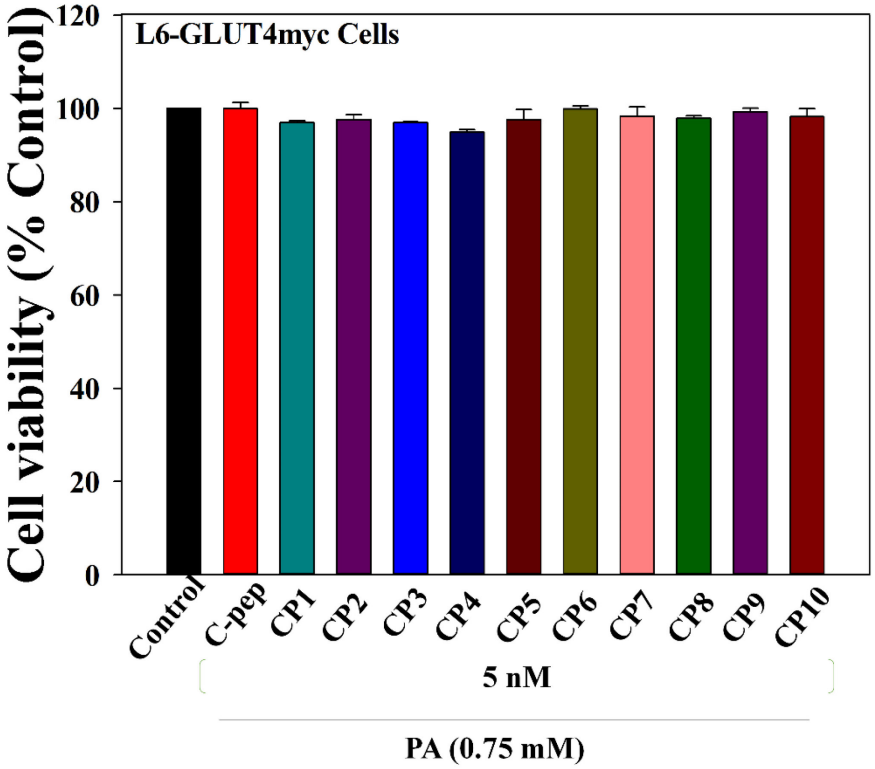

Figure S23. Cell viability of C-peptide mimics in L6-GLUT4myc cell using Alamar blue assay

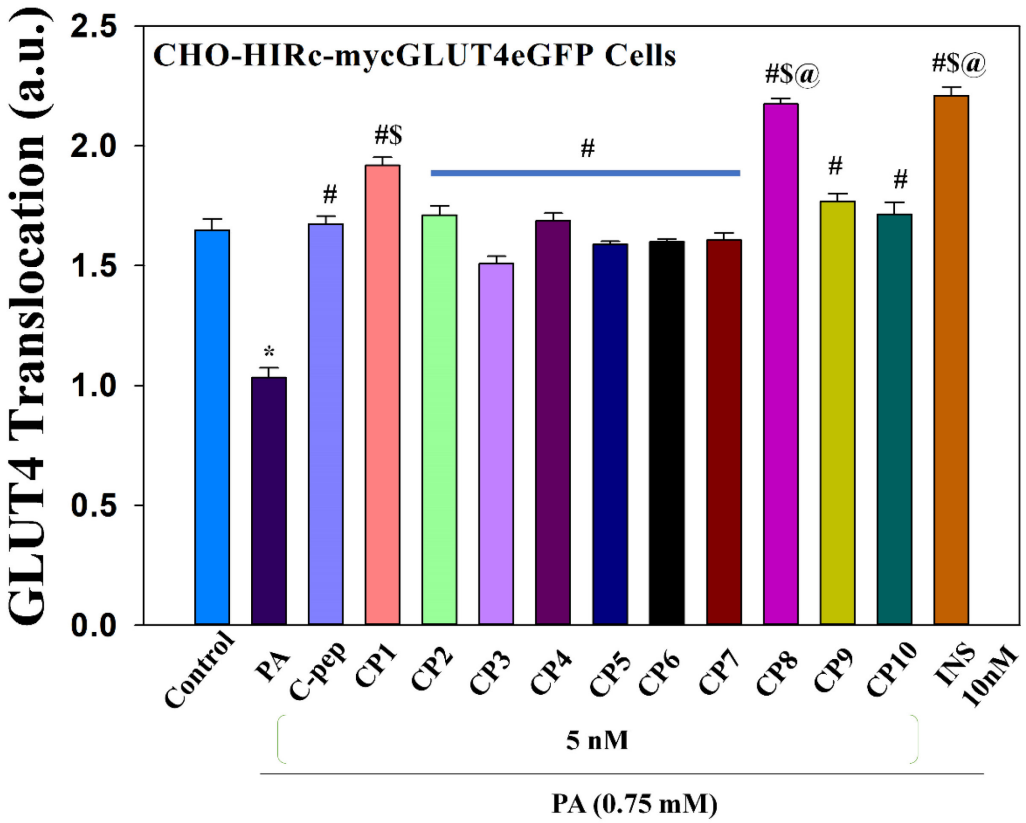

Figure S24. The effect of various C-peptides mimics on GLUT4 translocation in CHO-HIRc-mycGLUT4eGFP cells with or without PA(0.75mM). Values are mean ± SE (n = 3). "*" denotes significant difference from untreated group ($p<0.05$), "#" denotes significant difference from PA treated group ($p<0.05$), "$" denotes significant difference from human C-peptide treated group ($p<0.05$) and "@" denotes significant difference from **CP1**.

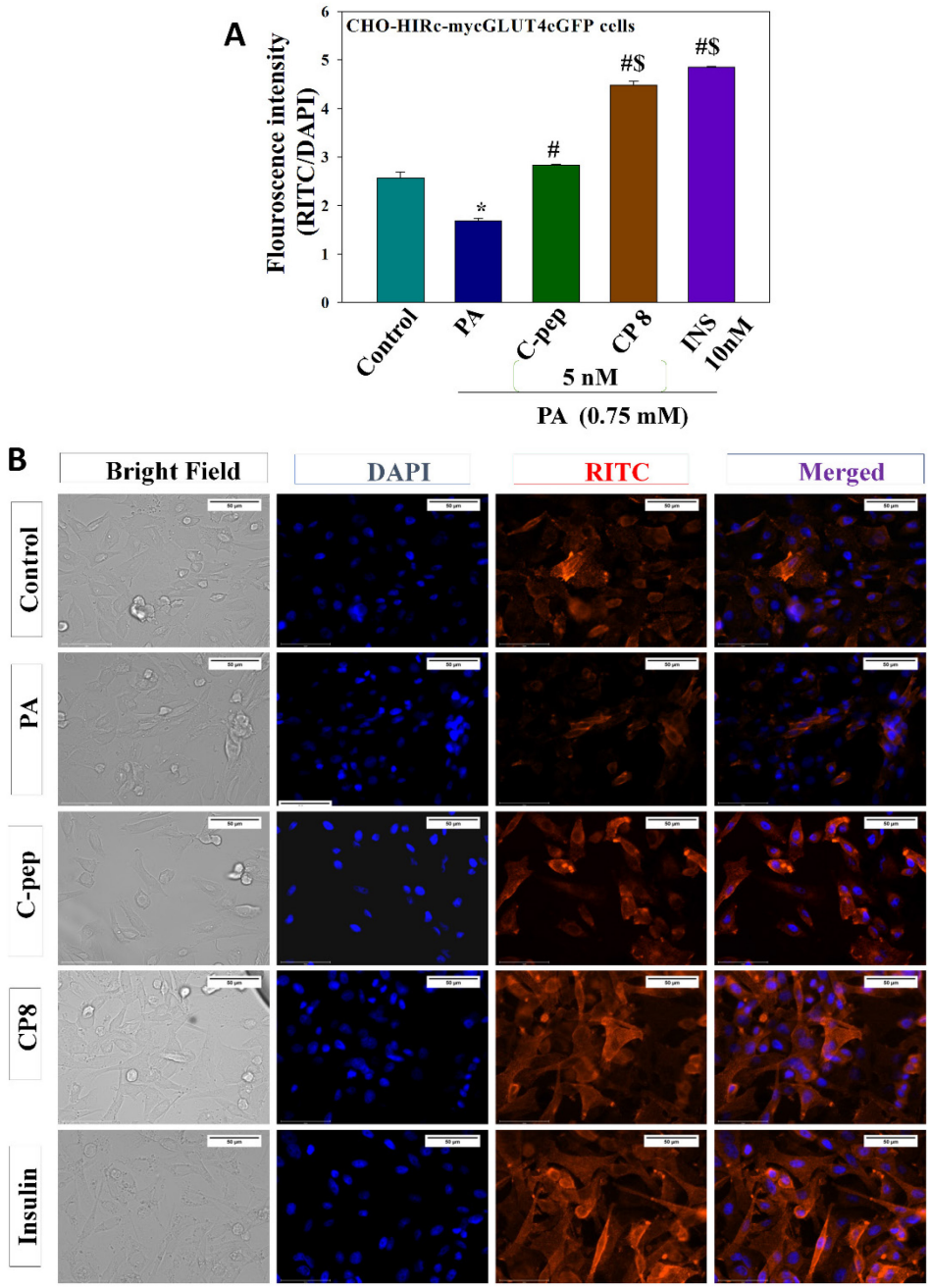

Figure S25. (A) The quantitative fluorescence analysis of C-peptide mimetic **CP8.** Values are mean ± SE (n = 3). "*" denotes significant difference from control groups, "#" from PA treated groups and "$" from human C-peptide treated groups (*#$ P<0.005). (B) The propitious effect of C-peptide mimics **CP8** on GLUT4 immunofluorescence in CHO-HIRc-mycGLUT4eGFP cells with or without PA (0.75 mM). Scale bar: 50 µm.

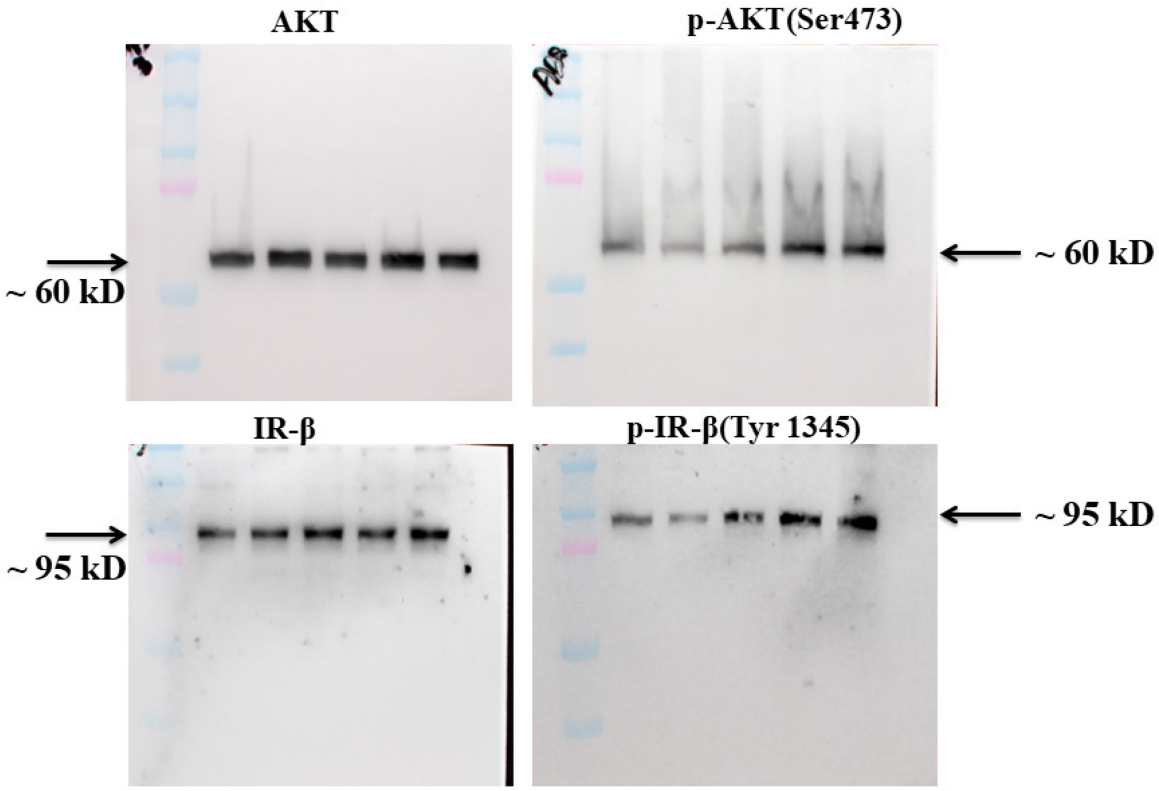

Figure S26. Immunoblot assay: Full length blots corresponding to **Figure 4** in main text

Supplementary References (in continuation with main paper)

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