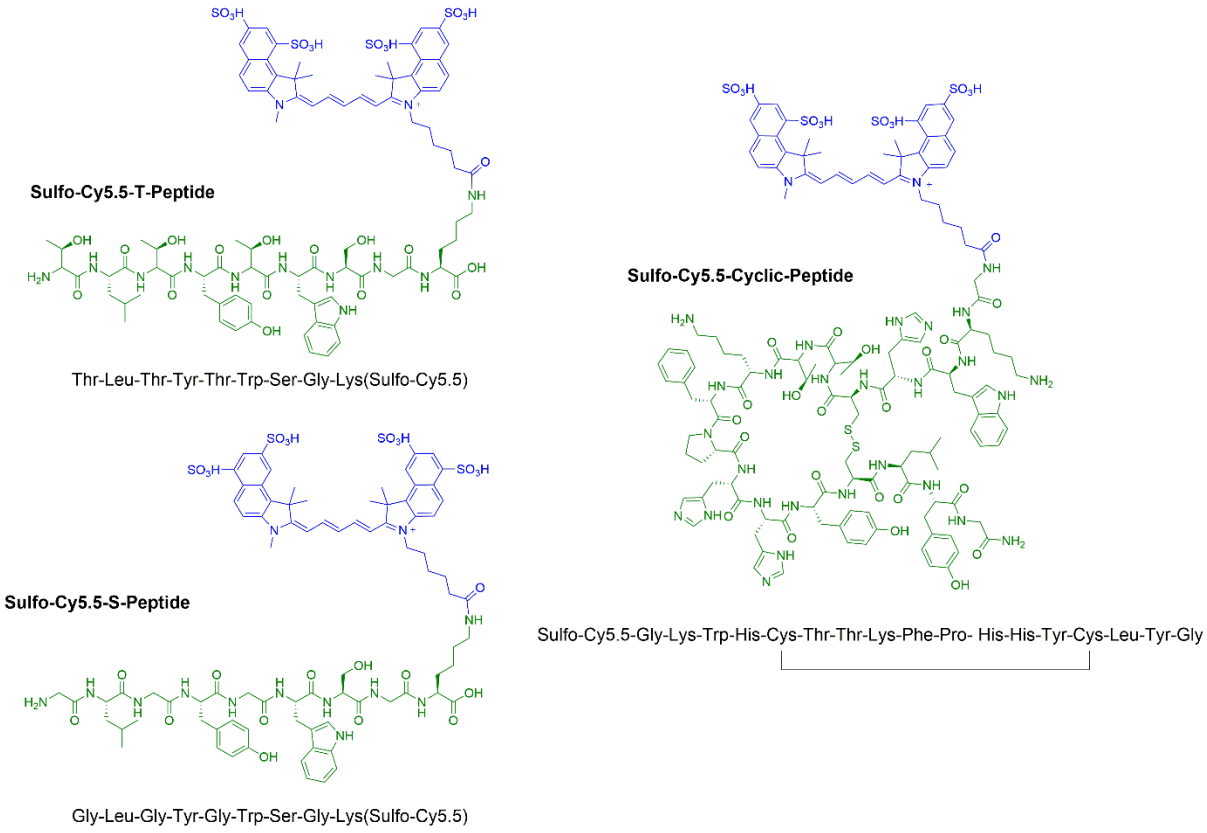
Supplemental Material

Data S1.

Supplemental Methods

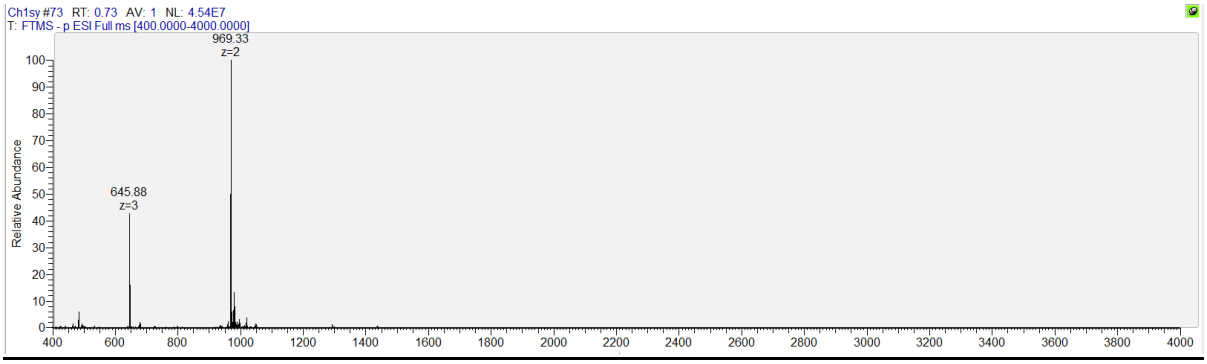
Detailed Methods for Peptides Synthesis and Purification

General Synthesis and Instrumentation. Unless otherwise stated, all reagents were purchased from commercial sources and used without further purification. ESI-MS spectra were recorded on Thermo Fisher OrbiTRAP infusion mass spectrometer. HPLC purification and analysis were performed on an Agilent 1100 series using solvent A = 0.1 % TFA in MilliQ water and solvent B = 0.1% TFA in CH_3CN. For analytical HPLC the purity of the peptides was tested by using a Hypersil BDS C18 analytical HPLC column (4.6 × 150 mm, 5 µm) at a flow rate of 1 mL/min (Method: 0-100% buffer B to A over 25 min, λ_{abs} = 254 and 280 nm). For preparative HPLC a Phenomenex Luna® 5 µm C18(2) 100 Å, LC Column 250 x 21 mm at a flow rate of 5-8 mL/min was used.

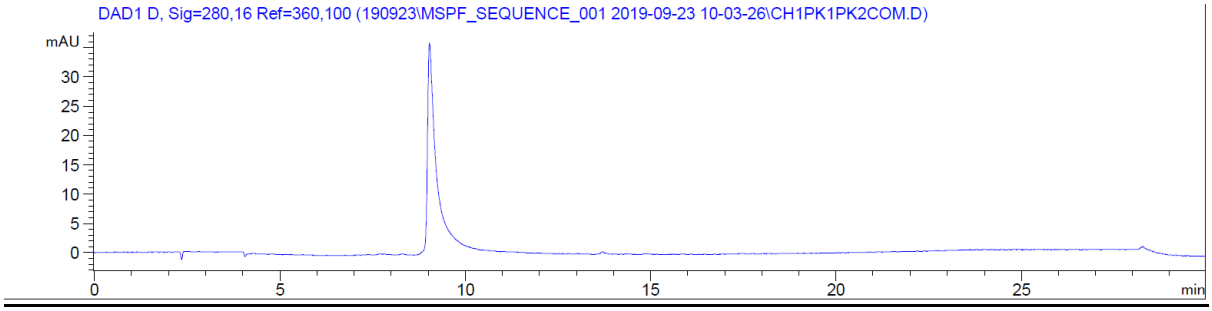


Chemical Structures of Cy5.5-conjugated peptides (main manuscript Fig. 1)

Synthesis of Cy5.5-T-Peptide. Linear Fmoc-NH-T-Peptide with sequence [Fmoc-Thr(*t*Bu)-Leu-Thr(*t*Bu)-Tyr(*t*Bu)-Thr(*t*Bu)-Trp(Boc)-Ser(tBu)-Gly-Lys(*t*Boc)-OH] were prepared using a CEM Liberty Blue™ automated microwave peptide synthesizer. Fmoc-Lys(*t*Boc)-OH was preloaded on wang resin and each coupling cycle involved HATU (1 equiv.) and DIPEA (5 equiv.), followed by deprotection of Fmoc group using 20% piperidine in DMF, but no final deprotection of the N-terminal Fmoc group. The resin cleavage was performed using a solution of triisopropylsilane (2.5%), distilled water (2.5%) and 3,6-dioxa-1,8-octanedithiol (2.5%) in TFA (10 mL) with gentle shaking for 2 h at RT. The resin was filtered, and the filtrate was concentrated under a stream of N_2, then ice-cold diethyl ether (40 mL) was added to precipitate the peptide which was collected after centrifugation. The crude peptide Fmoc-NH-T-Peptide was purified by HPLC (Phenomenex Luna® 5 µm C18(2) 100 Å, LC column 250 x 21 mm using 0.1% TFA in MilliQ water and acetonitrile) and the identity of the peptides were confirmed by ESI-MS. The peptide (6 mg, 4.69 µmol) and Sulfo-Cy5.5-NHS ester (11 mg, 9.39 µmol) were dissolved in DMF (1 mL) then DIEA (4.10 µl) was added. The solution was agitated at room temperature for 5 hrs then ice-cold diethyl ether (40 mL) was added to precipitate the peptide which was collected after centrifugation. The precipitate was treated with 20% piperidine in DMF (1 mL) for 10 min then ice-cold diethyl ether (40 mL) was added to precipitate the peptide which was collected after centrifugation and purified by HPLC (2.3 mg, yield 30%). Analytical HPLC was performed and the purity of the isolated peptides found to be >95%. The identity of the peptide was confirmed by ESI-MS. ESI-MS: Cy5.5-T-Peptide (-ve ion) $[M - 2H]^{-2}$, *m/z* = 969.3320 (experimental), calculated for $[C_{89}H_{114}N_{13}O_{28}S_4]^{-2}$: *m/z* = 969.3316

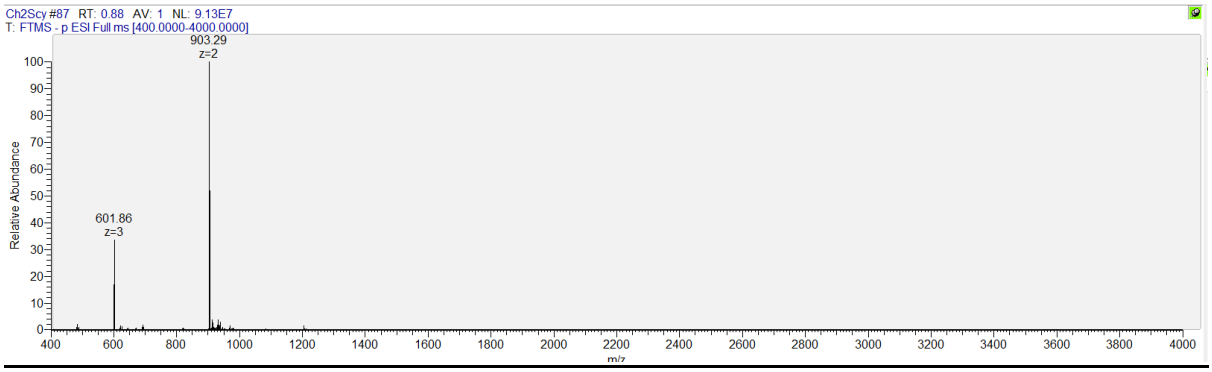


ESI-MS spectrum for Cy5.5-T-peptide

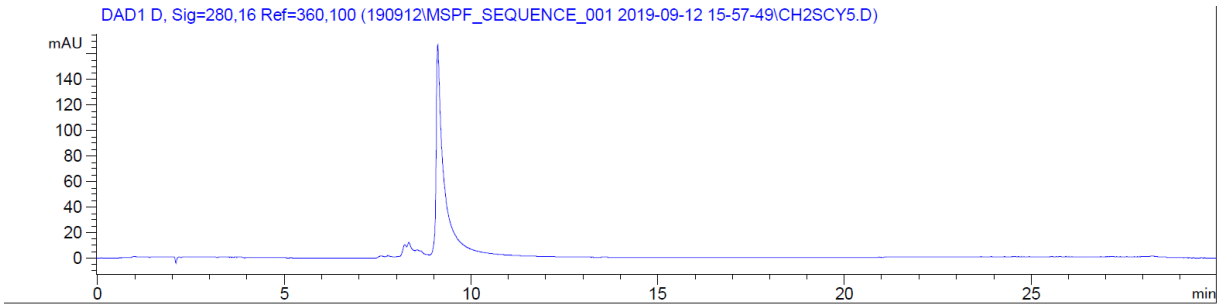


Analytical HPLC chromatogram for Cy5.5-T-peptide (Method: UV Abs at 280 nm, gradient 0% B in A – 100% over 20 min (A = 0.1 % TFA in MIlliQ, B = 0.1 % TFA in MeCN)

Synthesis of Cy5.5-S-Peptide. Linear Fmoc-NH-S-Peptide with the sequence [Fmoc-Gly-Leu-Gly-Tyr(*t*Bu)-Gly-Trp(Boc)-Ser(tBu)-Gly-Lys(*t*Boc)-OH] was prepared using the same method described above for the Fmoc-NH-T-Peptide, followed by the same procedure for Cy5.5 conjugation and purification (3 mg, yield 38%). Analytical HPLC was performed and the purity of the isolated peptides found to be >95%. The identity of the peptide was confirmed by ESI-MS. ESI-MS: Cy5.5-S-Peptide (-ve ion) $[M - 2H]^{-2}$, *m/z* = 903.2908 (experimental), calculated for $[C_{83}H_{102}N_{13}O_{25}S_4]^{-2}$: *m/z* = 903.2923

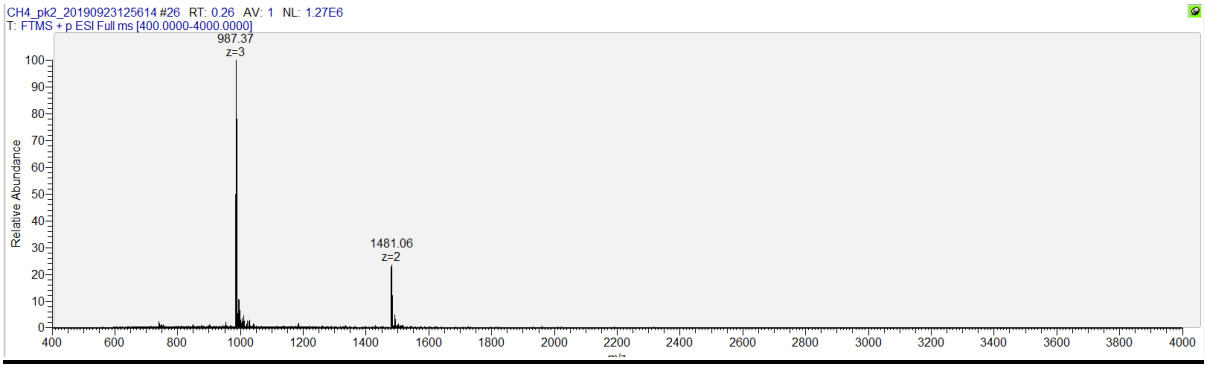


ESI-MS spectrum for Cy5.5-S-peptide

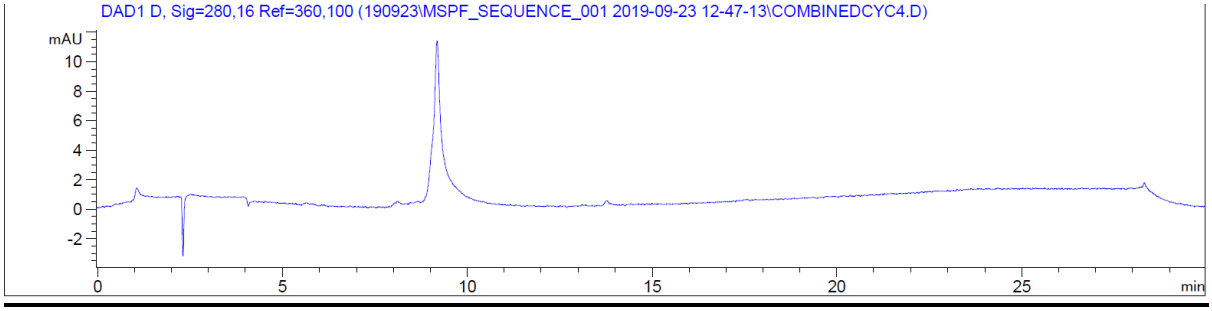


Analytical HPLC chromatogram for Cy5.5-S-peptide (Method: UV Abs at 280 nm, gradient 0% B in A – 100% over 20 min (A = 0.1 % TFA in MIlliQ, B = 0.1 % TFA in MeCN)

Synthesis of Cy5.5-Cyclic-Peptide. Fmoc-Gly-OH was preloaded on Rink amide resin, then the linear sequence [Fmoc-Gly-Lys(Boc)-Trp(Boc)-His(trt)-Cys(Acm)-Thr(*t*Bu)-Thr(*t*Bu)-Lys(Boc)-Phe-Pro- His(trt)-His(trt)-Tyr(*t*Bu)-Cys(Acm)-Leu-Tyr(*t*Bu)-Gly-OH] was prepared using the same method described for Fmoc-NH-T-Peptide. The resin-bound linear peptide was then cyclized using iodine (1 mg/mg of resin) in DMF (20 mL) for 4 hours. After cyclization, the N-terminus Fmoc group was removed by 20% piperidine in DMF (1 mL) for 10 min. Ice-cold diethyl ether (40 mL) was then added to precipitate the peptide, which was collected after centrifugation. Resin cleavage was then performed using a solution of triisopropylsilane (2.5%), distilled water (2.5%), Phenol (2.5%), DMB (2.5%) and 3,6-dioxa-1,8-octanedithiol (2.5%) in TFA (10 mL) with gentle shaking for 2 h at RT. The resin was filtered and the filtrate concentrated under a stream of N_2. Ice-cold diethyl ether (40 mL) was then added to precipitate the peptide, which was collected by centrifugation. The crude peptide Fmoc-NH-Cyclic-Lys(ivDDe)-Peptide was purified by HPLC (Phenomenex Luna® 5 µm C18(2) 100 Å, LC column 250 x 21 mm using 0.1% TFA in MilliQ water and acetonitrile) and its identity confirmed by ESI-MS. The peptide (4 mg, 1.9 µmol) and Sulfo-Cy5.5-NHS ester (4.4 mg, 3.85 µmol) were dissolved in DMF (1 mL) and DIEA (4.0 µl) was added. The solution was agitated at room temperature for 5 hrs. Ice-cold diethyl ether (40 mL) was then added to precipitate the peptide, which was centrifuged and collected. The precipitate was treated with 20% piperidine in DMF (1 mL) for 10 min, followed by addition of ice-cold diethyl ether (40 mL) to induce peptide precipitation. After centrifugation, the peptide pellet was treated with 2% hydrazine in DMF to remove the ivDDe protecting groups from Lys residues, followed by final HPLC purification (1.2 mg, yield 20%). ESI-MS: Cy5.5-Cyclic-Peptide (+ve ion) $[M + 2H]^{+2}$, *m/z* = 1480.0527 (experimental), calculated for $[C_{137}H_{173}N_{29}O_{34}S_6]^{+2}$: *m/z* = 1481.0505

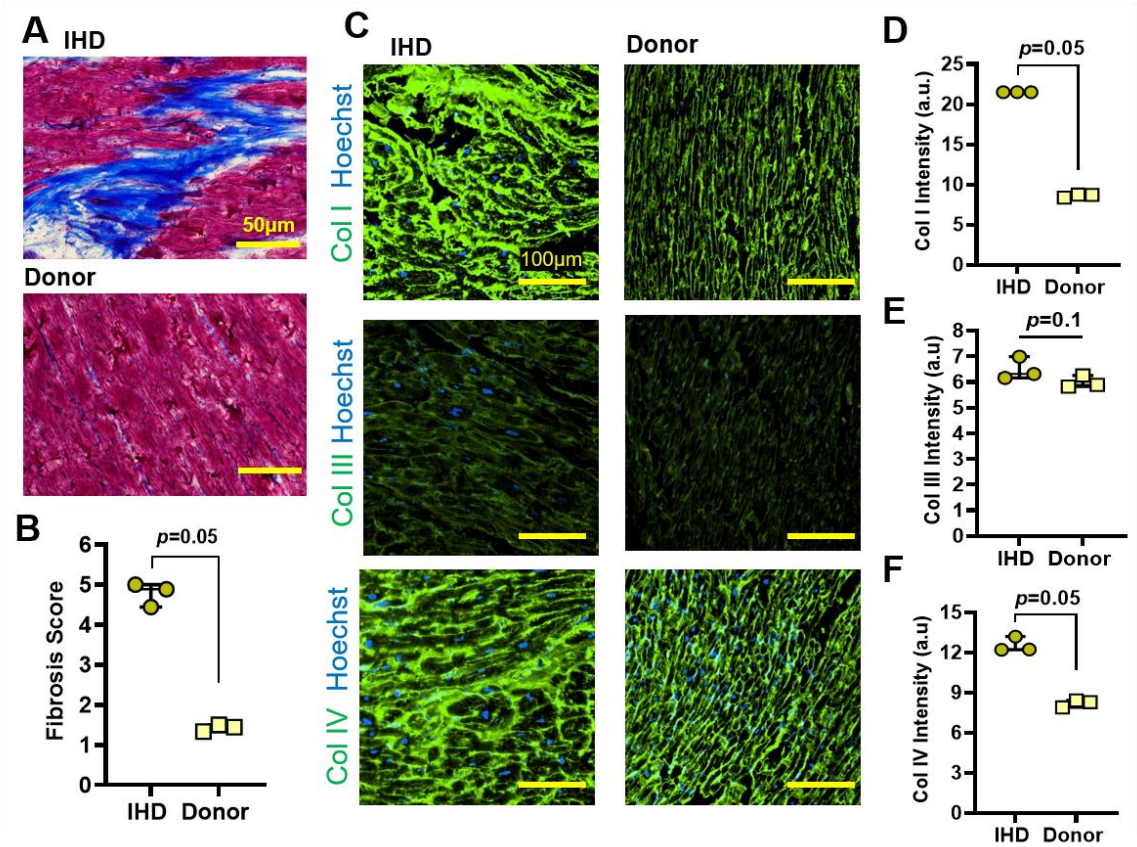


ESI-MS spectrum for Cy5.5-Cyclic-peptide



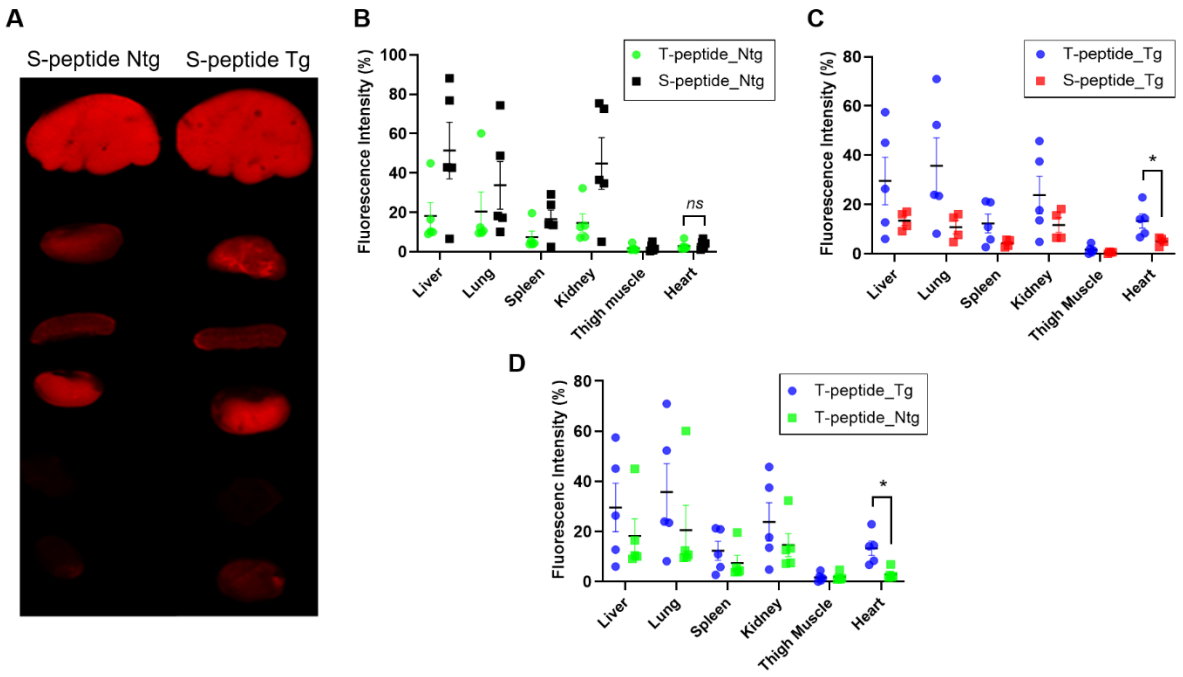
Analytical HPLC chromatogram for Cy5.5-Cyclic-peptide (Method: UV Abs at 280 nm, gradient 0% B in A – 100% over 20 min (A = 0.1 % TFA in MIlliQ, B = 0.1 % TFA in MeCN)

Figure S1. Characterisation of fibrosis and collagen types in human cardiac specimens from an ischaemic heart disease (IHD) patient and a healthy donor.



(A, B) Representative images **(A)** and quantification of cardiac fibrosis **(**percentage of the blue areas; **B)** based on Masson's trichrome staining of IHD and healthy donor heart tissues. **(C-F)** Representative immunohistochemical images of collagen types **(C)**, as well as quantification of the mean fluorescence intensities of collagen I **(D)**, collagen III **(E)** and collagen IV **(F)** in IHD and healthy donor cardiac specimens. Median ± Interquartile range. n=1 sample per condition, averaging 3 tissue planes per sample. Mann-Whitney test (one-tailed). Scale bars = 100µm.

Figure S2. Characterisation of T- and S-peptide uptake in whole organs of transgenic (Tg) and non-transgenic (Ntg) β2-AR mice.

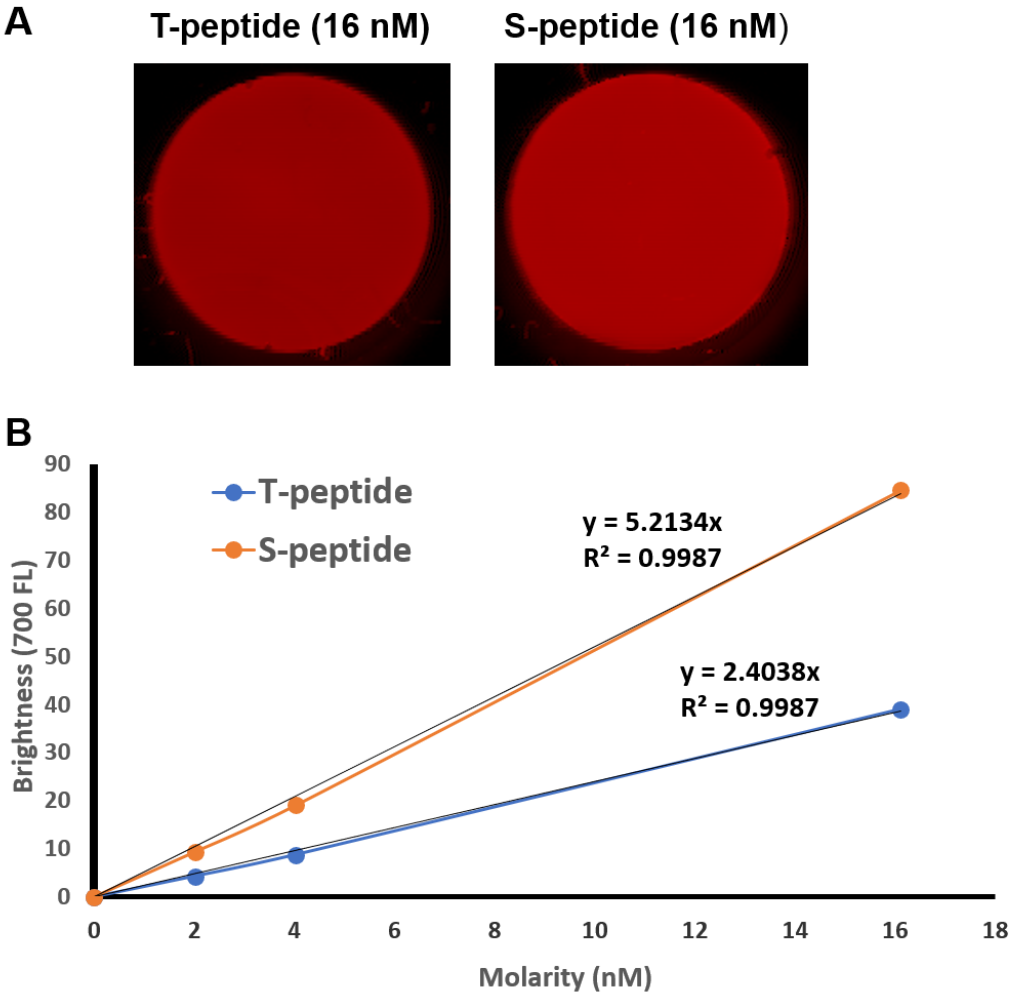


Representative images **(A)** and quantitative comparisons between the accumulation profiles of Cy5.5-T- and S-peptides in Ntg **(B)** and Tg animals **(C)**, highlighting a trend for specific accumulation of the T-peptide in several organs, including the heart, in the latter. **(D)** Comparison of T-peptide uptake in fibrotic (Tg) *vs.* healthy (Ntg) mice demonstrating a significant difference in T-peptide uptake only in the heart, in line with the cardiac-specific fibrotic phenotype of the β2-AR model. Detailed analyses of the cardiac uptake are described in **Fig. 2**. Mean±SEM. n=5 per group. Multiple Mann-Whitney tests with Holm-Sidak post-hoc (α=0.1).

Figure S3. T-peptide enhancement of β2-AR transgenic mouse hearts at 7-months of age.

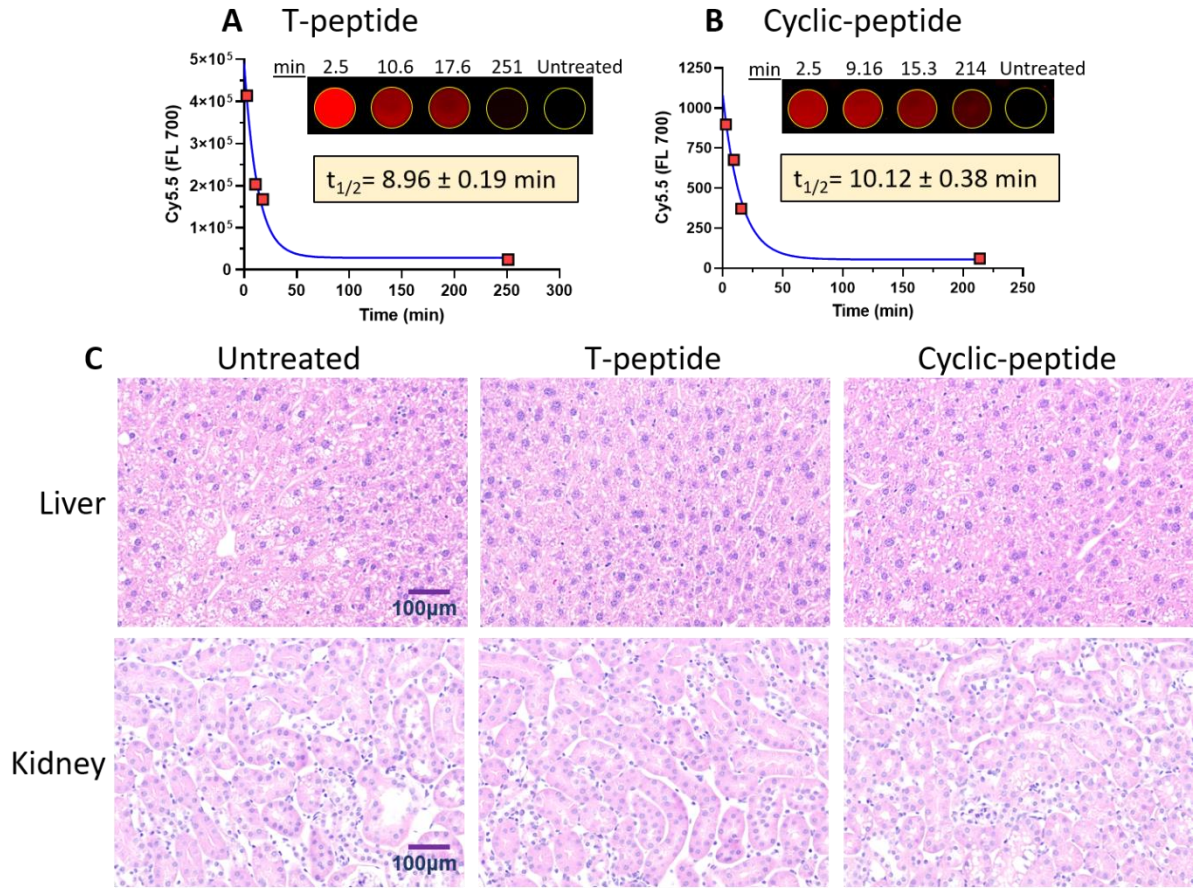
(A, B) *Ex vivo* Odyssey images (all hearts in **A** and higher resolution scans of the framed hearts in **B**) showing a Cy5.5-T-peptide ventricular enhancement of transgenic *vs*. non-transgenic β2-AR mouse hearts at 7-months of age, when they develop an intermediate cardiac fibrosis. **(C)** Quantification of the ventricular T-peptide uptake. Median + Interquartile range. n=3 per group. Mann-Whitney test (one-tailed).

Figure S4. Standard curves of Cy5.5-T- and S-peptides comparing their brightness on a molar basis.



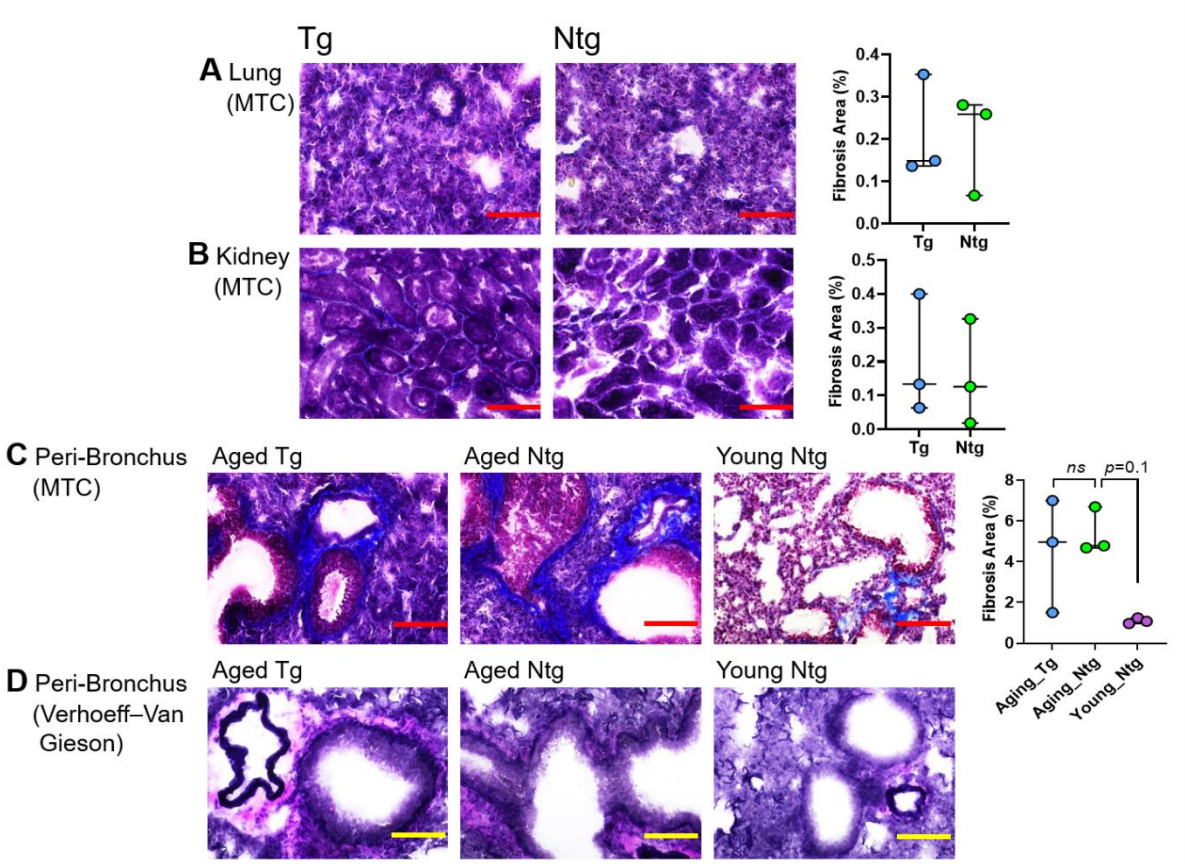
(A) Representative Odyssey scans of 96-wells containing equimolar concentrations (16 nM) of sulfo-Cy5.5-conjugated T- and S-peptides. An increased brightness of the S-peptide is observed. **(B)** Standard curves and linear regressions comparing the two peptides on an equimolar basis. The slope ratio S/T is 5.2134/2.4038 = 2.169, meaning that the S-peptide is 2.169-fold brighter than the T-peptide.

Figure S5. Blood clearance and toxicity profiles of T- and Cyclic-peptides.



(A, B) Representative Odyssey blood scans and the corresponding blood clearance curves, as well as the calculated half-lives ($t_{1/2}$) of Cy5.5-conjugated T-peptide **(A)** and cyclic-peptide **(B)** within 4 h after intravenous peptide administration (0.5 mg/kg) to non-transgenic β2-AR mice at 8-9 months of age. **(C)** Representative images of H&E-stained livers (top panels) and kidneys (bottom panels) from untreated and peptide-treated mice (the same animals as in A & B) 24 h post-peptide administration. No cell death, abnormal morphology or tissue destruction are observed following peptide injections, despite the high exposure of these organs to the peptides. n= 2. $t_{1/2}$ in A & B is calculated by one-phase exponential decay regression. Scale bars = 100µm.

Figure S6. Elastin and fibrosis staining in the lungs and kidneys of aged β2-AR and young wild-type mice.



(A, B) Representative Masson's trichrome (MTC) staining of lungs **(A)** and kidneys **(B)** of fibrotic (transgenic; Tg; left) and normal (non-transgenic; Ntg; right) β2-AR mice. Quantification of the renal and lung fibrotic areas demonstrates no differences between the genotypes **(A, B**; right panels**)**. **(C, D)** Representative MTC **(C)** and Verhoeff–Van Gieson elastin staining **(D)** of peri-bronchial fibrosis in lungs from fibrotic (Tg) and non-fibrotic (Ntg) β2-AR mice, as well as young wild-type C57Bl/6 mice at 5 weeks of age. No quantitative differences in peri-bronchial fibrosis between the β2-AR genotypes is observed **(C**; right**)**. Median ± Interquartile range. n=3. In A & B Mann-Whitney test (one-tailed); In C Kruskal-Wallis test with Dunn's post-hoc of preselected pairs of columns (α=0.1). Scale bars = 100µm.

Supplemental Video Legends:

Confocal microscope reconstruction videos of Cy5.5-T-peptide binding to collagen type IV in mouse and human fibrotic heart tissues.

Video S1. 3-D confocal microscope reconstruction video of Cy5.5-T-peptide binding (red) to collagen type IV (green) in fibrotic β2-AR mouse heart. The T-peptide was administered intravenously, while collagen IV was revealed by immunohistochemistry *ex vivo*. Nuclei are depicted in blue (Hoechst). Best viewed with Windows Media Player.

Video S2. 3-D confocal microscope reconstruction video of Cy5.5-T-peptide binding (red) to collagen type IV (green) in cardiac human sample from patient with an ischaemic heart disease (IHD). Nuclei are depicted in blue (Hoechst)**.** Best viewed with Windows Media Player.