Supporting Information

Bicyclic RGD-peptides with Exquisite Selectivity for the Integrin $\alpha_v\beta_3$ Receptor using a 'Random Design' Approach

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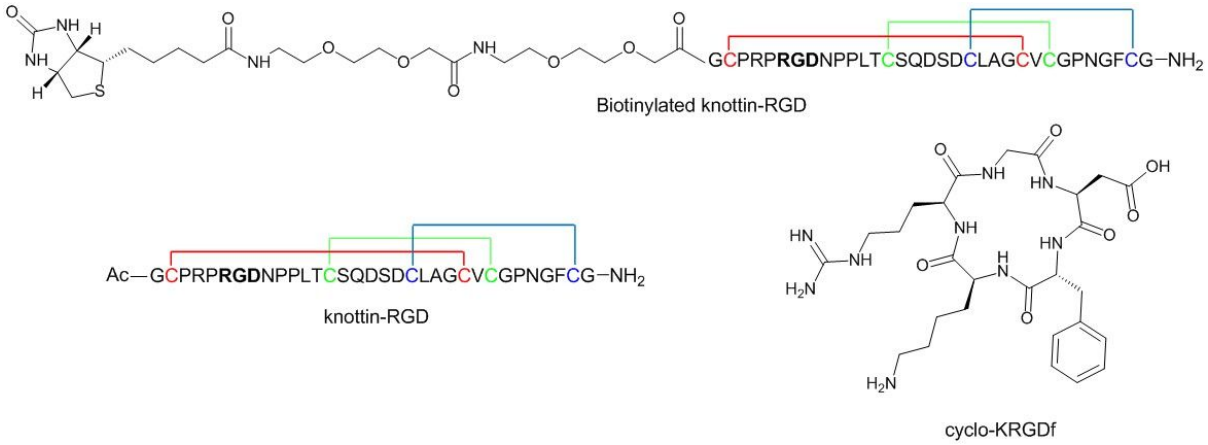


Figure S-1. Structures of biotinylated knottin-RGD, knottin-RGD and cyclo-[KRGDf].

Peptide synthesis, analysis and purification

Peptide synthesis was performed on fully automated peptide synthesizers (Multisyntech, Syro, 2-4 µmol scale for crude libraries) or Gyros Protein Technologies (Symphony, 100-200 µmol scale, for bulk production) using Fmoc-based solid-phase peptide synthesis on TentaGel® Ram resin using standard protocols. Coupling of L- and D-cysteines was performed manually using 2,4,6-trimethylpyridine as a base to maximally suppress racemization. Knottin-RGD peptide, *cyclo*-[KRGDf] and biotinylated knottin-RGD peptide (structures shown in Supporting Information, **Figure S-1**) were synthesized according to previously published protocols.[17] For IC_{50} determination and selectivity experiments, all peptides were purified by preparative HPLC on an RP-C18 column (Reprosil-Pur 120 C18-AQ 150x20 mm, Dr. Maisch GmbH, Ammerbuch, Germany) using an MeCN/H_2O gradient (5-65%) including 0.05% TFA followed by lyophilization (Christ Alpha 2-4 LDplus). Library screening and inhibition experiments with single-loop peptides were carried out using non-purified peptides.

Synthesis of crude bicyclic peptide libraries. Individual linear peptides (2 µmol) were dissolved in DMF (0.5 mL). Then, 1,3,5-tris(bromomethyl) benzene in DMF (4.1 mM, 0.5 mL) was added and the solution homogenized, followed by ammonium bicarbonate (150 mM, pH 8.0, 0.5 mL) again followed by homogenizing of the resulting solution and after 1 hour standing at room temperature the reaction mixtures were quenched with 0.5% ethanethiol (in 1:1 DMF/H_2O, 0.1 mL/peptide). Bicyclic peptide libraries were then lyophilized using a Genevac HT-4X evaporation system.

Synthesis of purified bicyclic and monocyclic peptides. To a solution of linear peptide dissolved at 0.5 mM in 1:3 MeCN/H_2O, was added 1.1 equiv. of the appropriate scaffolds (**T3** or **mT2**) dissolved in MeCN, where after the solution was homogenized. Then, 44 equiv. ammonium carbonate (0.2 M solution in H_2O) were added and shaken for 60 min. After completion (monitoring by UPLC), the reaction was quenched with 10% TFA/H_2O to pH <4, followed by lyophilization. Monocyclic peptides were further used in crude form, and bicycles were purified via preparative RP-HPLC.

For S-S-oxidation, linear peptides were dissolved at 0.5 mM in 5% MeCN/H_2O and 0.5 equiv. of DTNP (5 mg/mL in MeCN) was added. 0.2 M ammonium bicarbonate solution was added until the solution was basic (color changed to yellow). After 10 min, another 0.5 equiv. of DTNP was added. After complete disappearance of the starting material, the reaction was quenched with 10% TFA/H_2O until the solution was acidic (color change to green) and the S-S-oxidized peptide was subsequently purified preparative RP-HPLC.

Peptide labeling with Cy5. Peptides comprising an amine group were dissolved at 4 mM in DMSO, followed by adding disulfo-Cy5 NHS (1.0 equiv., 20 mg/mL in DMSO) and DIPEA (10 equiv.). After completion (30-60 min) reaction was quenched with 10% TFA/H_2O, using twice the volume of DIPEA, and subsequently purified using preparative RP-HPLC.

Peptide analysis and purification. UPLC analysis was performed on a Waters Acquity Ultra Performance LC System, equipped with a Waters Acquity UPLC BEH130 C18 1.7 µm column. A linear gradient of 5-55% MeCN (0.05% TFA) in H_2O (0.05% TFA) was used. Peptides were purified by preparative HPLC (Waters Prep LC) on an RP-C18 column (Reprosil-Pur 120 C18-AQ 150x20 mm, Dr. Maisch GmbH, Ammerbuch, Germany) using an MeCN/H_2O gradient (5-65%) including 0.05% TFA.
Freeze-drying of the peptide libraries was performed using a *GeneVac HT-4X* centrifugal vacuum evaporator.

Amino acids used in peptide libraries

The following amino acids were included in the peptide library syntheses:
Ala (A), Asp (D), Glu (E), Phe (F), Gly (G), His (H), Ile (I), Lys (K), Leu (L), Asn (N), Pro (P), Gln (Q), Arg (R), Ser (S), Thr (T), Val (V), Trp (W), Tyr (Y).
Moreover, various non-natural amino acids were included for 3rd generation $\alpha_v\beta_3$-libraries.
2-Aminobutyric acid (Z or Abu, also 2nd gen), norleucine (Nle, also 2nd gen), norvaline (NVa, also 2nd gen), 1-naphthyl alanine (1 or 1-Nal, also 2nd gen), 2-naphthyl alanine (2 or 2-Nal, also 2nd gen), 2-aminoisobutyric acid (Z or Aib), D-Ala (a), D-Phe (f), D-His (h), D-Ile (i), D-Leu (l), D-Gln (q), D-Trp (w).

Additional peptides 2nd generation libraries

C$_{T3}$1GD**C**$_{T3}$RGD**c**$_{T3}$ (1: L-1-naphthylalanine)
C$_{T3}$2GD**C**$_{T3}$RGD**c**$_{T3}$ (2: L-2-naphthylalanine)
C$_{T3}$Q1G**C**$_{T3}$RGD**c**$_{T3}$
C$_{T3}$Q2G**C**$_{T3}$RGD**c**$_{T3}$
C$_{T3}$Q(Abu)D**c**$_{T3}$RGD**c**$_{T3}$ (Abu: 2-aminobutyric acid)
C$_{T3}$Q(Aib)D**c**$_{T3}$RGD**c**$_{T3}$ (Aib: 2-aminoisobutyric acid)
C$_{T3}$H(Nle)Q**c**$_{T3}$RGD**c**$_{T3}$ (Nle: L-norleucine)
C$_{T3}$H(Nva)Q**c**$_{T3}$RGD**c**$_{T3}$ (Nva: L-norvaline)
C$_{T3}$E(Nle)G**c**$_{T3}$RGD**c**$_{T3}$
C$_{T3}$E(Aib)G**c**$_{T3}$RGD**c**$_{T3}$
C$_{T3}$S1D**c**$_{T3}$RGD**c**$_{T3}$
C$_{T3}$S2D**c**$_{T3}$RGD**c**$_{T3}$

Table S-1. Parameters varied in the two different competition ELISA setups (**A**), and selected setups for library screening and IC_{50} determination (**B**).[1]

A

	Setup 1	**Setup 2**
Coating buffer	0.1 M Na_2HPO_4 pH 8 + 1 mM Ca^{2+} 0.5 mM Mg^{2+}	PBS pH 7.4 + 1 mM Ca^{2+} 0.5 mM Mn^{2+}
Concentration of integrin	0.5 µg/mL	0.25 µg/mL
Blocking buffer	0.05% Tween80/PBS + 1 mM Ca^{2+} 0.5 mM Mg^{2+}	0.05% Tween80/PBS + 1 mM Ca^{2+} 0.5 mM Mn^{2+}
Washing buffer	0.05% Tween80/PBS + 1 mM Ca^{2+} 0.5 mM Mg^{2+}	0.05% Tween80/PBS + 1 mM Ca^{2+} 0.5 mM Mn^{2+}
Incubation buffer	PBS pH 7.4 + 1 mM Ca^{2+} 0.5 mM Mg^{2+}	PBS pH 7.4 + 1 mM Ca^{2+} 0.5 mM Mn^{2+}
Concentration of biotinylated peptide	0.1 µM	
Concentration of bicyclic peptide (1st screening)	1 mM (1st generation) 5 µM (2nd generation)	5 µM
Concentration of bicyclic peptide (2nd screening)	10 µM (1st generation) 2.5 µM (2nd generation)	2.5 µM
Concentration range of IC_{50} determination	10 µM – 0.0046 µM (3rd generation) 30 µM – 0.014 µM (1st and 2nd generation)	
Strep-HRP buffer	PBS pH 7.4 + 1 mM Ca^{2+} 0.5 mM Mg^{2+}	PBS pH 7.4 + 1 mM Ca^{2+} 0.5 mM Mn^{2+}

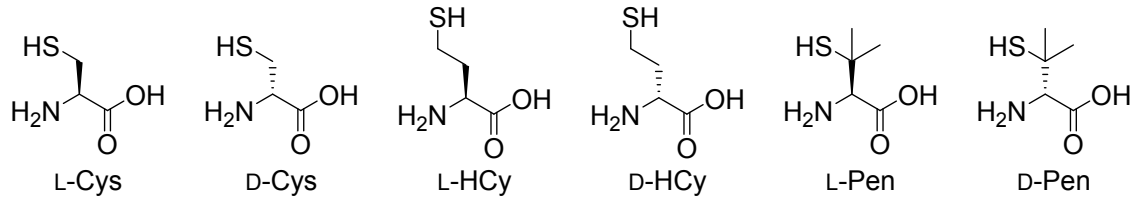
B

Library Generation	1st	2nd	3rd
Screening	1	1	2
IC_{50} determination	2	2	2

Table S-2. Inhibition values (determined using setup 1) for $\alpha_v\beta_3$ integrin for selected bicyclic peptides (three cysteines, constrained with 1,3,5-tris[bromomethyl] benzene, T3) and their monocyclic analogues (two cysteines, constrained with 1,3-bis[bromomethyl] benzene, mT2).

	Inhibition at 10 µM [%]
C Q A D c R G D c	79
C Q A D a R G D c	32
A Q A D c R G D c	7
C Q A D c R G D a	0
C H E Q c R G D c	92
C H E Q a R G D c	16
A H E Q c R G D c	22
C H E Q c R G D a	28
C W G D C R G D c	75
C W G D A R G D c	23
A W G D C R G D c	52
C W G D C R G D a	8
C R G D C	23
C R G D c	57
C R G D c	41

A



B

$X^1{}_{T3}$HWQ$X^2{}_{T3}$RGDc$_{T3}$		Inhibition [%]		$X^1{}_{T3}$HWQ$X^2{}_{T3}$RGD**1**$_{T3}$		Inhibition [%]		$X^1{}_{T3}$HWQ$X^2{}_{T3}$RGD**2**$_{T3}$		Inhibition [%]	
X^1	X^2	10 µM	1 µM	X^1	X^2	10 µM	1 µM	X^1	X^2	10 µM	1 µM
Cys	Cys	88	55	Cys	Cys	80	37	Cys	Cys	<0	5
Cys	HCy	85	53	Cys	HCy	20	12	Cys	HCy	<0	<0
Cys	Pen	<0	<0	Cys	Pen	2	15	Cys	Pen	<0	<0
HCy	Cys	30	<0	HCy	Cys	62	33	HCy	Cys	<0	<0
Pen	Cys	31	8	Pen	Cys	<0	24	Pen	Cys	<0	<0
HCy	HCy	8	<0	HCy	HCy	58	31	HCy	HCy	<0	10
HCy	Pen	<0	<0	HCy	Pen	20	18	HCy	Pen	<0	<0
Pen	HCy	49	22	Pen	HCy	18	33	Pen	HCy	<0	6
Pen	Pen	<0	<0	Pen	Pen	<0	6	Pen	Pen	<0	<0

c: D-Cys **1**: D-HCy **2**: D-Pen

Figure S-2. A) Molecular drawings of L/D-cysteine (Cys) and non-natural amino acids L-homocysteine (HCy), L-penicillamine (Pen), D-homocysteine (**1**) and D-penicillamine (**2**); B) Inhibition values (using setup 2, Table S-1) for various derivatives of 2nd generation $\alpha_v\beta_3$-inhibitor **C$_{T3}$**HWQ**C$_{T3}$**RGD**c$_{T3}$**-T3.

Binding ELISA protocol for testing HPQ-streptavidin binding

Greiner cell culture plates were coated with 100 µL 0.2% glutardialdehyde in PBS pH 7.4 coating buffer (see setup 2, **Table S-1**) at r.t. for 4 h, followed by 3x washing with 400 µL washing buffer. Aoa-functionalized peptides were incubated overnight at 4 °C, followed by 3x washing and blocking with 150 µL 1% I-Block™ in blocking buffer (1 h, r.t). After another 3x washing plates were incubated with 100 µL 1:1000 Strep-HRP in strep-HRP buffer (1 h, r.t.), followed by 4x washing and incubation with 150 µL ABTS-substrate buffer at r.t. Absorbance was measured at 405 nm after 45 min using a Molecular Devices Spectramax M2 plate reader and corrected for background absorbance (background refers to absorbance without non-labeled peptide present). Experiments were carried out in triplicate.

Table S-3. Binding ELISA studies to measure streptavidin-affinity of selected 2nd generation $\alpha_v\beta_3$-binding bicyclic peptides and control sequences (monocyclic HPQ and RGD, and biotinylated linear GRGDS). Absorbance values shown as mean of triplicate including standard deviation.

Peptide	Absorbance (405 nm) 100 µM	10 µM	1 µM
Aoa [PEG] C H P Q c R G D c	-0.01 ± 0.07	0.01 ± 0.03	0.02 ± 0.06
C H P Q c R G D c [PEG] K(Aoa)	0.00 ± 0.04	0.00 ± 0.04	0.02 ± 0.03
Aoa [PEG] C H N Q c R G D c	0.03 ± 0.03	0.02 ± 0.04	0.01 ± 0.04
C H N Q c R G D c [PEG] K(Aoa)	-0.02 ± 0.04	0.01 ± 0.04	0.00 ± 0.04
Aoa [PEG] C H F Q c R G D c	0.01 ± 0.04	0.01 ± 0.04	0.03 ± 0.03
C H F Q c R G D c [PEG] K(Aoa)	0.13 ± 0.08	0.00 ± 0.03	0.03 ± 0.05
Aoa [PEG] C H P Q c	0.39 ± 0.08	-0.02 ± 0.06	0.03 ± 0.04
Aoa [PEG] c R G D c	0.04 ± 0.05	-0.01 ± 0.06	0.02 ± 0.03
Biotin [PEG] G R G D S P [PEG] K(Aoa)	3.80 ± 0.03	3.14 ± 0.09	0.65 ± 0.07

SPFS observation of affinity binding kinetics

Optical system. An optical system combining surface plasmon resonance (SPR) and surface plasmon enhanced fluorescence spectroscopy (SPFS) was used for direct investigation of affinity interaction of selected peptides and integrins. The Kretschmann configuration of attenuated total reflection method (**Figure S-3**) was used for the resonant excitation and interrogation of surface plasmons as described before in more detail.[2] Briefly, a laser beam (633 nm) was coupled to a high refractive index glass prism and glass substrate coated with SPR-active thin gold film, and optically matched to its base. The beam was made incident at the angle of incidence that was tuned close to θ_{SPR} where surface plasmons are resonantly excited at the outer interface of gold surface. A flow cell was clamped against the gold sensor surface in order to flow liquid samples with a flow rate of 40 µL/min. The reflected beam intensity was measured with a lockin amplifier (EG&G, USA) in order to track changes in SPR signal. The fluorescence signal excited via surface plasmons that was propagating from the sensor surface through the flow cell was collected by a lens with a numerical aperture about NA=2, and detected by a photomultiplier (H6240-01, Hamamatsu, Japan) connected to a counter (53131A, Agilent, USA). The intensity of the excitation beam irradiating area on the sensor chip of about 1 mm^2 was reduced to 30-60 µW in order to reduce bleaching of Cy5 excited by the enhanced field intensity of surface plasmons. The fluorescence light emitted by Cy5 at wavelength of about 670 nm was spectrally separated from the excitation light (633 nm) by using a set of laser notch filters (XNF-632.8-25.0M, CVI Melles Griot, USA) and fluorescence band pass filters (670FS10-25, Andover Corporation Optical Filter, USA).

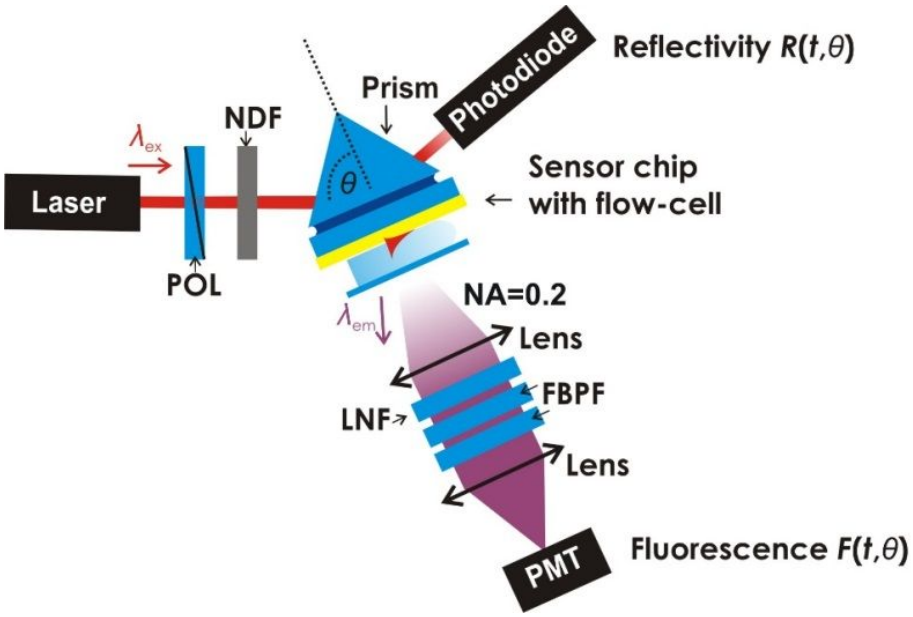


Figure S-3. Schematics of the optical instrument used for the surface plasmon resonance (SPR) and surface plasmon-enhanced fluorescence spectroscopy (SPFS) measurements with polarizer (POL), neutral density filter (NDF), laser notch filter (LFN), fluorescence bandpass filter (FBPF).

Sensor chip preparation. Sensor chips were prepared on BK7 glass substrates which were subsequently coated with 2 nm chromium and 50 nm gold films by thermal vacuum evaporation (Model HHV FL400, HHV Ltd, UK). Onto the gold surface integrin $\alpha_v\beta_3$ was attached by using two surface architectures (**Figure S4**). The 2D architecture relied on a mixed self-assembled monolayer (SAM) that was formed by immersing the gold surface in a 1 mM ethanolic solution with a dithiol-PEG6-COOH and dithiol-PEG3, mixed at molar ratio of 1:9. After overnight incubation, the gold surface was rinsed with ethanol, dried in a stream of air, and stored under argon atmosphere.

Immobilization of ligand. The immobilization of integrin $\alpha_v\beta_3$ was performed *in situ* by amine coupling according to standard protocols. The surface reactions were monitored by SPR (Supporting Information, **Figure S-4A**). First, PBS and acetate buffer (pH=4) were flowed over the gold surface to reach a stable baseline SPR signal. Then, the sensor surface carrying carboxylic acid groups on mixed thiol SAM layer was reacted with 75 mg/mL EDC and 21 mg/mL NHS dissolved in water for 15 minutes. Recombinant human integrins (c: 10 µg/mL), dissolved in acetate buffer (pH=4), was flowed over the activated sensor surface for 90 minutes to immobilize the integrin molecules via their amine groups to activated carboxylic groups. Finally, the remaining active ester groups were inactivated with 1M ethanolamine/H_2O for 15 minutes.

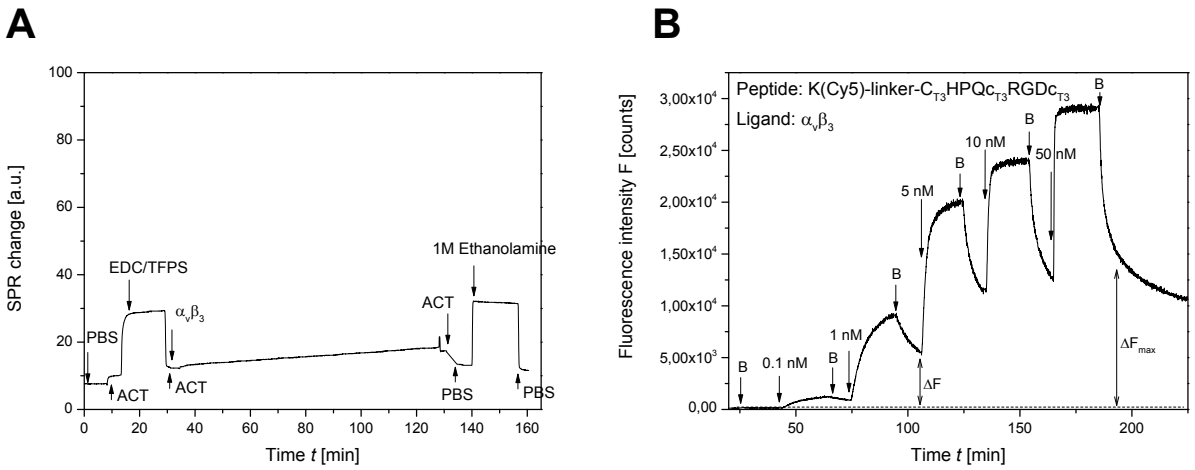


Figure S-4. Example of A) SPR sensorgram showing covalent immobilization of integrin $\alpha_v\beta_3$ ligand into a 3D hydrogel binding matrix, and B) fluorescence signal kinetics acquired upon titration of peptide Cy5-functionalized $\mathbf{C}_{T3}$HPQ$\mathbf{c}_{T3}$RGD$\mathbf{c}_{T3}$-**T3**.

NMR

All NMR spectra were collected on a Bruker Avance III 500 MHz spectrometer equipped with a Prodigy BB cryoprobe at 298 K. Samples were prepared by dissolving the compounds in D_2O and adding a small amount of DSS for internal referencing. ^{1}H-NMR spectra were acquired using 32 scans and a relaxation delay of 3 s. 2D COSYDQF spectra with presaturation were acquired with a 6000 Hz spectral width in both dimensions using 2048 x 512 points and processed using 2048 x 512 points, 4 scans per increment and a relaxation delay of 1.5 seconds. 2D gradient TOCSY spectra with presaturation were acquired with a 5000 Hz spectral width in both dimensions using 1024 x 512 points and processed using 1024 x 1024 points, 8 scans per increment, a relaxation delay of 2 seconds and a TOCSY mix time of 100 ms. A TOCSY spinlock field of 8.3 kHz was applied. 2D gradient ROESY spectra were acquired with a 6000 Hz spectral width in both dimensions using 4096 x 512 points and processed using 4096 x 512 points, 24 scans per increment, a relaxation delay of 1.5 seconds and a ROESY mix time of 0.3 s. A ROESY spinlock field of 5 kHz was applied. Multiplicity-edited ^{1}H-^{13}C HSQC spectra were acquired using a 6010 Hz spectral width in F2 and 18868 Hz spectral width in F1 using 1024 x 512 points and processed to 1024 x 1024 points, 2 scans per increment, relaxation delay of 1.5 seconds and 1-bond J_{CH} = 145 Hz. ^{1}H-^{13}C HMBC spectra were acquired using a 5319.1 Hz spectral width in F2 and 22321.4 Hz spectral width in F1 using 2048 x 512 points and processed to 2048 x 2048 points, 4 scans per increment, relaxation delay of 1.5 seconds and a long-range J_{CH} = 8 Hz.

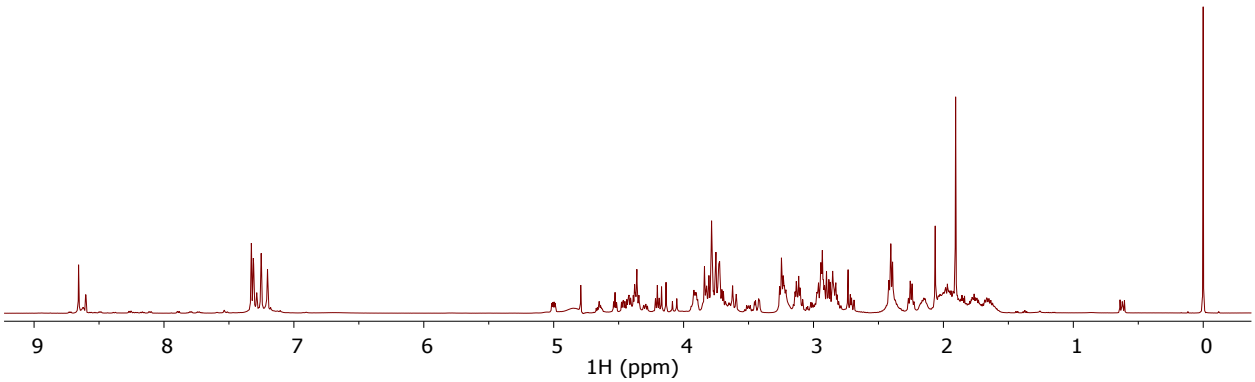


Figure S-5. ^{1}H spectrum of $\mathbf{C_{T3}}$HPQ$\mathbf{c_{T3}}$RGD$\mathbf{c_{T3}}$ acquired with composite pulse presaturation. The spectrum was referenced to the trimethylsilyl peaks of the internal standard of DSS at 0.0 ppm. NS = 32, D1 = 3 s, AQ = 3.3 s.

References

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(3) Wang, Y.; Huang, C. J.; Jonas, U.; Wei, T.; Dostalek, J.; Knoll, W. Biosensor based on Hydrogel Optical Waveguide Spectroscopy. *Biosens. Bioelectron.* **2010**, *25*, 1663–1668.