Supporting information

A Fluorescent Arg–Gly–Asp (RGD) Peptide - Naphthalenediimide (NDI) Conjugate for Imaging Integrin $\alpha_v\beta_3$ *in vitro*

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1. Materials and method.

Synthesis of compound **2** was carried out under continuous microwave irradiation in a Biotage initiator instrument. Synthesis of compounds **2a** and **4** were carried out under nitrogen using standard Schlenk glassware and techniques. Solvents were used as purchased.

Solution multinuclear NMR spectra were recorded on a Bruker Avance 500 spectrometer. ^{1}H and ^{13}C chemical shifts are referenced to tetramethylsilane (TMS). ^{19}F chemical shift is referenced to trichloro-fluoro-methane ($CFCl_3$).

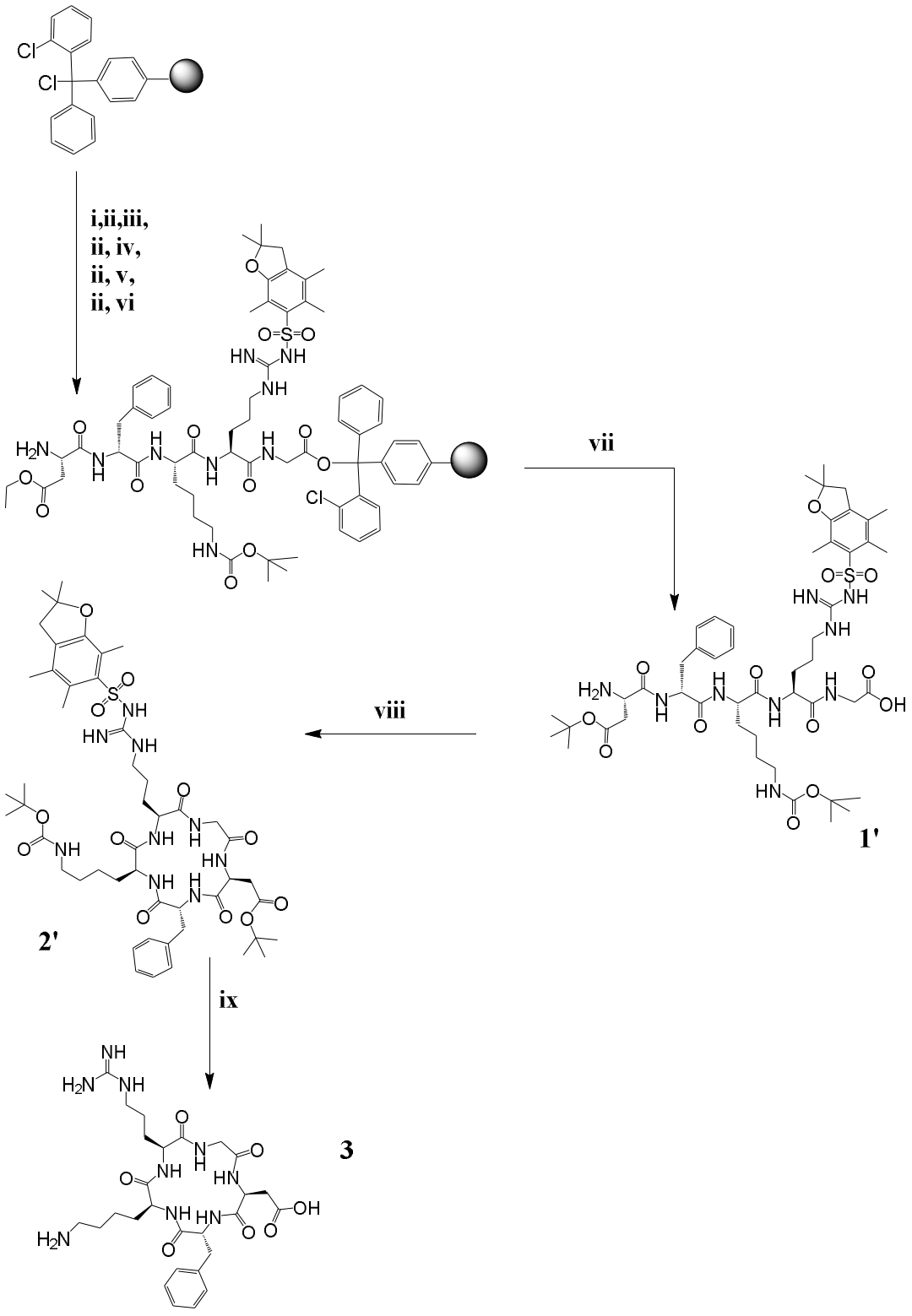
Analytical HPLC was performed on a Dionex Ultimate 3000 HPLC system equipped with a Phenomenex Gemini 5 µm C-18 (150 x 4.6 mm) column with a flow rate of 1 mL min^{-1}. Semi-preparative HPLC was performed on a Dionex Ultimate 3000 system with a FAMOS autosampler equipped with a 500 µL sample loop and a PepMap C18 3 µm (150 x 7.5 mm) column with a flow rate of 2 mL min^{-1}. Mobile phase A was 0.1 % TFA in water, mobile phase B was 0.1% TFA in acetonitrile. The gradient for analytical HPLC was T = 0 min, B = 5%; T = 10 min, B = 95%; T = 15 min, B = 95%; T = 15.1 min, B = 5%; T = 18.1 min, B = 5%. The gradient for semi-preparative HPLC was T = 0 min, B = 5%; T = 7.5 min, B = 95%; T = 16.5 min, B = 95%; T = 30 min, B = 5%.

Single crystal X-ray diffraction data was obtained for Tryptophan-tagged naphthalenediimide [TrpNDI] (**2**) using a synchrotron radiation source (SRS Daresbury). Crystals suitable for analysis were grown from DMSO. A typical crystal was mounted using the oil drop technique, in perfluoropolyether oil at 150(2) K with a Cryostream N2 open-flow cooling device. Single crystal X-ray diffraction data were collected using graphite monochromated Mo-Kα radiation (λ = 0.71073 Å) on a Nonius KappaCCD diffractometer.[S1] The structures were solved by direct methods by using the program SHELXL97.[S2] The refinement (on F2) and graphical calculations were performed with the SHELXL97.[S3] Crystallographic data (excluding structure factors) for the structure reported in this paper have been deposited with the Cambridge Crystallographic Data Centre, CCDC: 1028547. Data can be obtained free of charge via www.ccdc.cam.ac.uk/data_request/cif.

High resolution mass spectrometry (MS) analyses were performed using a mass spectrometer equipped with an electro-spray ion source (Bruker micrOTOF and MALDI-TOF with Linear and Reflectron analysers).

[S1] J. Cosier and A. M. Glazer, *J. Appl. Crystallogr.*, 1986, **19**, 105; (b) Z. Otwinowski and W. Minor, *Methods Enzymol.*, 1997, **276**, 307.
[S2] A. Altomare, G. Cascarano, C. Giacovazzo and A. Guagliardi, *J. Appl. Crystallogr.*, 1993, **26**, 343.
[S3] G. M. Sheldrick, *Acta Crystallogr., Sect. A: Found. Crystallogr.*, 2008, **A64**, 112.

2. Synthetic procedures.



Scheme S1. Overview of the synthesis of deprotected cyclic RGDfK peptide 3, as a TFA adduct: (i) Fmoc-Gly-OH, DIPEA, CH_2Cl_2 (ii) 20%(v/v) piperidine in DMF; (iii) Fmoc-Arg(Pbf)-OH, PyBOP, DIPEA,CH_2Cl_2; (iv) Fmoc-Lys(Boc)-OH, PyBOP, DIPEA, CH_2Cl_2 (v) Fmoc-D-Phe-OH PyBOP, DIPEA,CH_2Cl_2 (vi) Fmoc-Asp(OtBu)-OH, PyBop, DIPEA,CH_2Cl_2 (vii) TFA / DCM (10 mL; 1 : 99 v/v) for 10×2 min, pyridine/MeOH (10 mL, 1 : 9 v/v) (viii) PyBOP, DIPEA, 72 h rt; (ix) TFA/CH_2Cl_2 (1:1), rt, 2 h.

2.1 Synthesis of *tryptophan-tagged naphthalenediimide*, [TrpNDI], (2)

1,4,5,8-Naphthalenetetracarboxylic dianhydride (200 mg, 0.746 mmol, Compound **1** in Figure 1) and L-Tryptophan (304.5mg, 1.491 mmol) were suspended in 8 mL of DMF in a pressure-tight 15-mL microwave vial. To this suspension, 0.2 mL of dry Et_3N was added. The suspension was sonicated until the mixture became homogeneous. This reaction mixture was heated for 5 min at 140°C under microwave irradiation using a dedicated microwave system (Biotage). The solvent was removed under reduced pressure. The dark brown oil was taken up into MeOH (6 mL). This solution was added under stirring to 400 mL of 1M HCl. The resulting suspension was allowed to settle overnight and then filtered through a sintered glass funnel. The solid was then washed with 100 mL deionized water and dried under reduced pressure. The product (TrpNDI compound **2**) was obtained in the form of a brown microcrystaline solid in 88% yield. MS (ESI): *m/z*: 639.15 $[M-H]^-$.

2.2 Synthesis of tryptophan-tagged hydroxysuccinimidyl naphthalenediimide [TrpNDI-OSu], (2a)

Tryptophan-NDI (**2**) (0.2 mmol, 128.12 mg), and *N*-hydroxysuccinimide (0.8 mmol, 92 mg) were dissolved in DMF and stirred in ice bath for 15min. EDC.HCl (0.8 mmol, 153.36 mg) was dissolved in 20 mL DMF and then added to the solution which was stirred in ice bath for another 15 min. Then the ice bath was removed and the solution was kept under stirring at room temperature for 8 h. The DMF was removed by evaporating and a small amount of acetonitrile was added to ensure that the resulting product remains in solution. Subsequently, the solution was added under stirring to double-distilled H_2O, from which precipitate was formed. The precipitate was collected by filtration and fine powder was formed by adding pentane to precipitate the desired compound, which was isolated by filtration and dried under reduced pressure. Yield: 160.3 mg, 96%. MS (ESI): *m/z*: 857.19 $[M+Na]^+$.

2.3 Synthesis of Synthesis of H-Asp(OtBu)-D-Phe-Lys(Boc)-Arg(Pbf)-Gly-OH (1')

Loading of the resin was achieved by first swelling 2-chlorotrityl polystyrene resin (1.0 g) for 20 min in DCM After swelling, the resin was treated with a solution of Fmoc-Gly-OH (2 eq, 3 mmol) and DIPEA (4 eq, 1.05 mL, 6 mmol) in DCM (10 mL) by agitating for 90 min. The resin was then washed with a solution of DCM / MeOH / DIPEA (10 mL; 17: 2: 1 v/v/v) for 3 ×15 min and with DCM (3 × 10 mL), DMF (2 × 10 mL), and DCM (2 × 10 mL). The other amino acid couplings were performed using Fmoc-amino acid (2 eq, 3 mmol), PyBOP (2 eq, 3 mmol), and DIPEA (4 eq, 1.05 mL 6 mmol) in DCM (10 mL) for 90 min. Fmoc deprotection before subsequent amino acid coupling was achieved by treatment with piperidine/DMF (1:4 v/v) for 4×3 min. The coupling and deprotection reactions were monitored by the ninhydrin and chloranil tests. Washing of the resin after coupling was performed with DMF (2×8 mL), Et_2O (2×8 mL) and DCM (2×8 mL). Cleavage of the linear peptide from the resin was achieved by treatment with TFA / DCM (7 mL; 1 : 99 v/v) for 10 × 2 min. After each treatment, the cleavage solution was neutralised by aspiration into pyridine /

MeOH (10 mL; 1: 9 v/v). The cleavage solution was concentrated in vacuum, then dissolved in the minimum volume of MeOH, and the crude peptide was precipitated by adding Millipore water. The peptide was collected by centrifugation, dissolved in DCM (100 mL), and washed with water (100 mL). The DCM layer was collected and evaporated *in vacuo* to give intermediate **1' (**H2N-Asp-D-Phe-Lys-Arg-Gly-OH (D-f-K-R-G) as a white solid (1.18 g, 85% based on nominal resin loading). HPLC Retention time is 8.54 min (5-95% B over 10 min; solvent A = 0.1% TFA/H2O, solvent B = 0.1% TFA/MeCN). Negative mode ESI MS: M/z calcd for $C_{49}H_{74}N_9O_{13}S$ 1029.52; found as $[M-H]^-$ = 1028.4938; calcd for $C_{49}H_{73}N_9O_{12}S$ $[M-H_2O]$ = 1011.5099 found as $[M-H_2O-H]^-$ = 1010.5143.

2.4 Synthesis of Cyclo-(Asp(OtBu)-D-Phe-Lys(Boc)-Arg(Pbf)-Gly) (2')

Compound **1'** (139 mg, 0.13 mmol) and PyBOP (83 mg, 0.161 mmol) were dissolved in DCM (135 mL) and the solution was adjusted to pH 8 by the addition of DIPEA (0.382 mL, 2.19 mmol) and allowed to stir for 24 h. The reaction mixture was then concentrated *in vacuo*. The crude product was dissolved in DCM (20 mL) and washed with a saturated solution of $NaHCO_3$ (4 × 20 mL) and with brine (75 mL). The crude peptide was then precipitated in cold Et_2O, and centrifuged to give **2'** (fully protected cyclo(RGDfK with a HPLC trace with retention time = 10.18 min, as a white solid (127 mg, 91%). Positive mode ESI-MS: *m/z* calcd for $C_{49}H_{73}N_9O_{12}S$ as $[M+NH_4]^+$ 1029.5443 (found 1030.5311).

2.5 Synthesis of *Cyclo*-(RGDfK) (3)

The protected cyclic peptide **2'** (200 mg, 0.198 mmol) was dissolved in a TFA deprotection cocktail (TFA/CH_2Cl_2, 1: 1, 5 mL), and the solution was stirred for 2 h. The majority of the TFA was removed by purging with N_2 gas. The crude peptide was precipitated in cold Et_2O, then centrifuged to give the desired product as a TFA bis-adduct, as a light green solid (122 mg; 74%). The HPLC (5-95% B over 10 min; solvent A = 0.1% TFA/H2O, solvent B = 0.1% TFA/MeCN) led to a single peak at retention time = 4.76 min consistent with the literature data (Tetrahedron Letters 42 (2001) 2787–2790, Didier Boturyn and Pascal Dumy) Positive mode ESI-MS: *m/z* calcd for $C_{27}H_{42}N_9O_7$: 604.1521 $[M+H]^+$; found 604.3910. ^1H NMR (d^6-DMSO) showed strong similarities with the reported compounds synthesis. ^{19}F NMR showed that traces of TFA are still present, as two singlets at -76.55 and - 76.67 ppm, likely as counterions (weekly bound to the arginine protonated and/or terminal NH_2 free base) as well as free TFA.

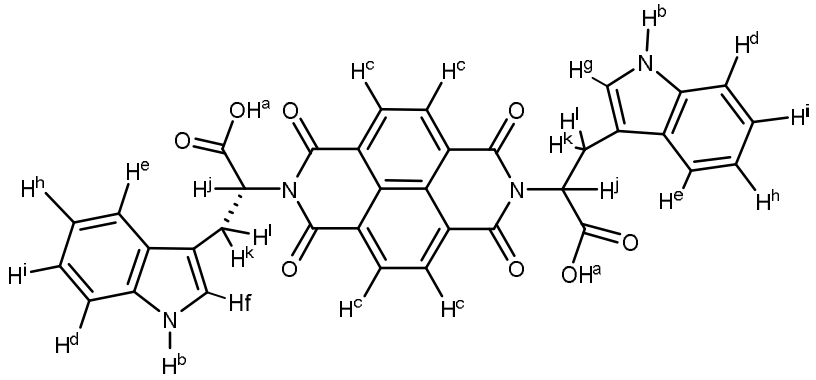
2.6 Synthesis of *Cyclo*-(RGDfK)-tryptophan-tagged naphthalenediimide [TrpNDIRGDfK], (Compound 4).

To a mixture of NHS-activated TrpNDI (**2a**) **(**10 mg, 12 μmol) and **3** (17 mg, 28 μmol) in DMF, an excess Et_3N was added (2.93 mg). The solution was stirred under N_2 for 8 h at room temperature. The solvent was removed and acetone was added to the residue. The acetone mixture was added dropwise to a (vigorously stirred) solution of 1 M HCl (200 mL). The precipitate was collected by filtration and dried under reduced pressure. MALDI mass spectrometry and analytical HPLC methods were used for analysing the resulting compound

to confirm that the product is the double-RGD substituted compound denoted NDI-RGDfK (**4**) (see sections 4.6 and 5.4). The product 4 (dissolved in DMSO) was purified by semi-preparative HPLC, in ca. 10% overall yield, whereby the peak at 8.62 min (300 nm wavelength) was collected. Note: ^{19}F NMR (in DMSO) indicated the presence of traces of TFA due to a weak resonance at -76.55 ppm. As such, it was crucial that MTT assays were carried out prior to cellular investigations to confirm biocompatibility. MALDI mass spectrometry: *m/z* calcd: $C_{90}\ H_{103}\ N_{22}O_{20}$ as $[M+H]^+$: 1811.76 and found: 1811.76.

3. NMR assignments and data.

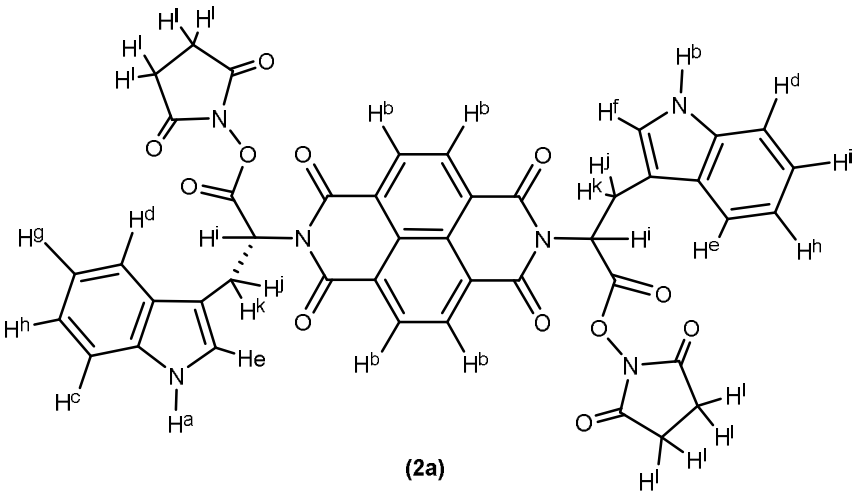
***Tryptophan-tagged naphthalenediimide*, [TrpNDI], (2)**



(2)

^{1}H NMR (500MHz, 298 K, dmso-d_6) δ = 13.00 (bs, 2H, H^a); 10.62 (s, 2H, H^b), 8.60 (s, 4H, H^c); 7.48 (d, 2H, H^d, $^{3}J_{HdHi}$ = 7.83 Hz); 7.20 (d, 2H, H^e, $^{3}J_{Hehk}$ = 7.94 Hz); 7.06 (s, 1H, H^f); 7.05 (s, 1H, H^g); 6.94 (pt, 2H, H^h ,$^{3}J_{HhHi}$ = 6.93 Hz); 6.80 (pt, 2H, H^i); 5.87 (dd, 2H, H^j , $^{3}J_{HjHk}$ = 5.5Hz, $^{3}J_{HjHl}$ = 8.9 Hz); 3.70 (dd, 2H, H^k, $^{3}J_{HkHl}$ = 14.9 Hz); 3.50 ppm (dd, 2H, H^l). **^{13}C NMR** (125MHz, 298 K, dmso-d_6) δ = 171.0 (***C***OOH); 136.4 (N***C***O), 131.5 (***C***H^c); 127.7 (124.1 (***C***H^f, ***C***H^g); 127.4 (CH^d***C***NH^b); 126.4 (CH^e***C***CCH^kH^l); 121.2 (***C***H^h); 118.46 (***C***H^i); 118.36 (***C***H^d); 111.6 (***C***H^e); 109.2 (CH^e***C***CCH^kH^l); 54.7 (***C***H^j); 24.5 (***C***H^lH^k).

***Tryptophan-tagged hydroxysuccinimidyl naphthalenediimide* [TrypNDI-OSu], (2a)**



(2a)

^{1}H NMR (500MHz, 298 K, dmso-d_6) δ = 10.80 (s, 2H, H^a), 8.70 (s, 4H, H^b); 7.55 (d, 2H, H^c, $^{3}J_{HcHh}$ = 7.76 Hz); 7.23 (d, 2H, H^d, $^{3}J_{Hdhg}$ = 7.94 Hz); 7.18 (s, 1H, H^e); 7.17 (s, 1H, H^f); 6.96 (pt, 2H, H^g ,$^{3}J_{HgHh}$ = 7.32 Hz); 6.85 (pt, 2H, H^h); 6.27 (pt, 2H, H^i , $^{3}J_{HiHk}$ = 6.9Hz, $^{3}J_{HjHl}$ = 7.3 Hz); 3.82 (dd, 2H, H^j, $^{3}J_{HkHl}$ = 14.9 Hz); 3.61 (dd, 2H, H^k); 2.76 (bs, 8H, H^l) ppm. **^{13}C NMR** (500MHz, 298 K, dmso-d_6) δ = 171.3 (***C***OOH); 165.9 (CH^l_2***C***ON) 162.4 (N***C***O) 131.9 (***C***H^b); 127.4 (CH^c***C***NH^a); 126.5 (CH^d***C***CCH^kH^l); 124.9 (***C***H^e, ***C***H^f); 121.5 (***C***H^g); 118.5 (***C***H^h); 118.4 (***C***H^c); 111.8 (***C***H^d); 109.2 (CH^e***C***CCH^kH^l); 52.7 (***C***H^i); 25.9 (***C***H^l); 24.6 (***C***H^kH^j).

3.1 ^{1}H NMR of *tryptophan-tagged naphthalenediimide*, [TrpNDI], (2)

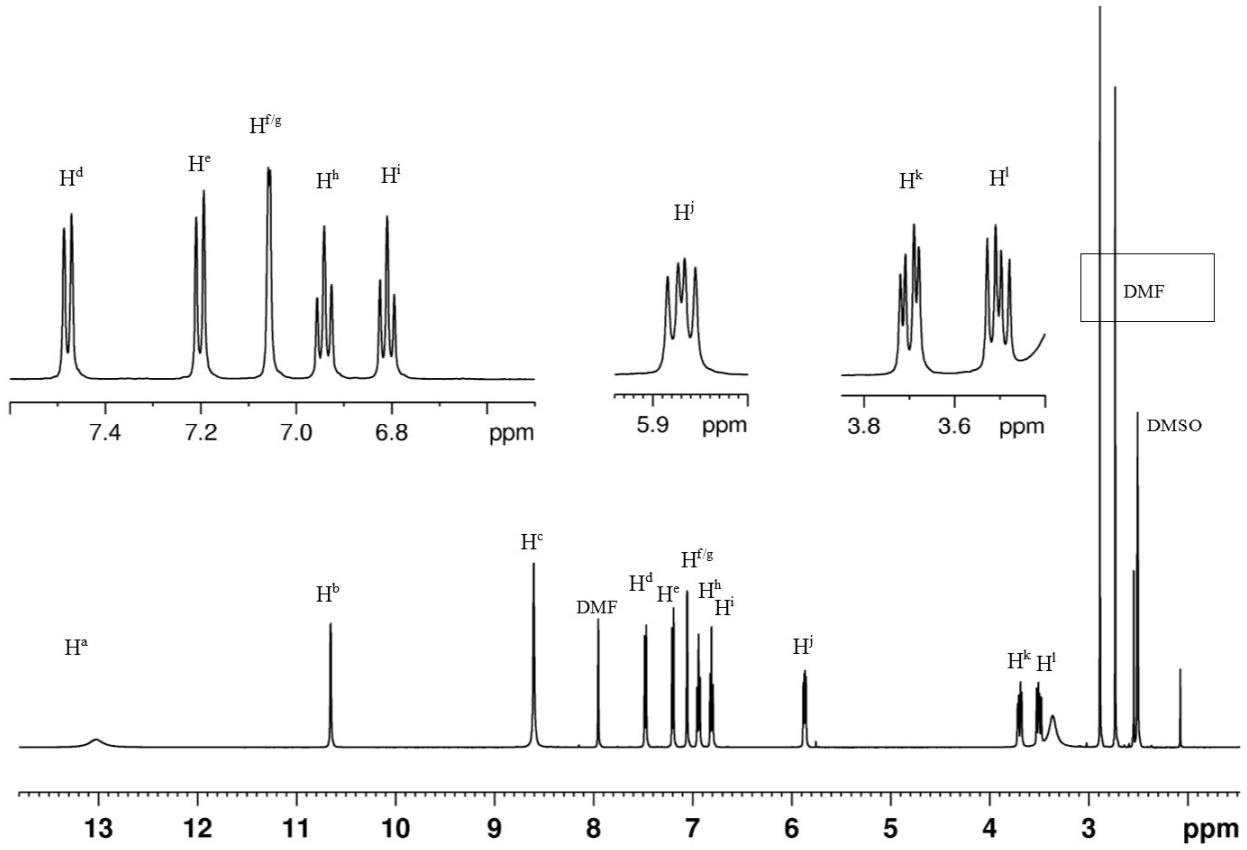


Figure S1.

3.2 ^{13}C NMR of tryptophan-tagged naphthalenediimide, [TrpNDI], (2).

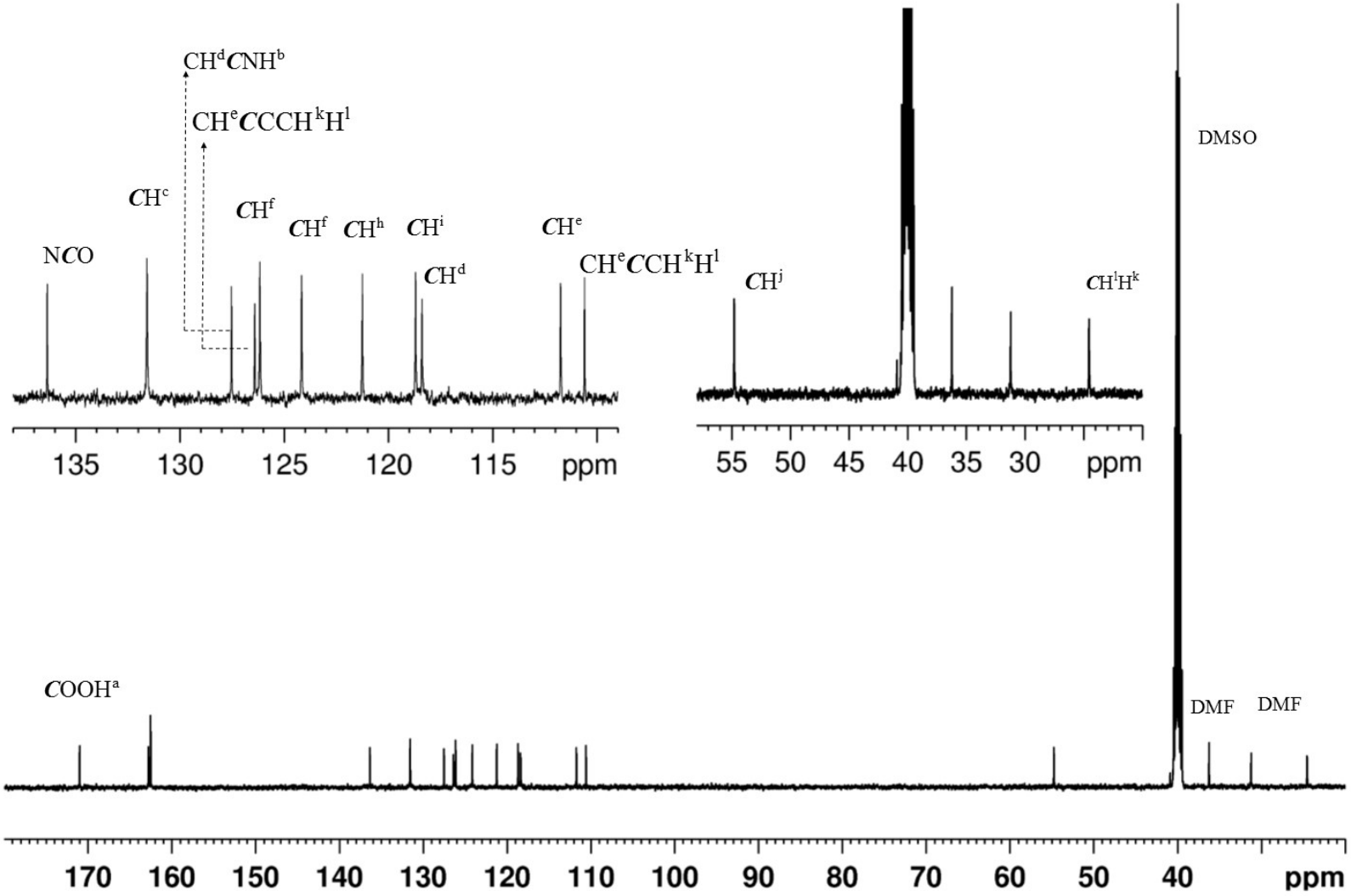


Figure S2.

3.3 ^{1}H COSY NMR of *tryptophan-tagged naphthalenediimide*, [TrpNDI], (2).

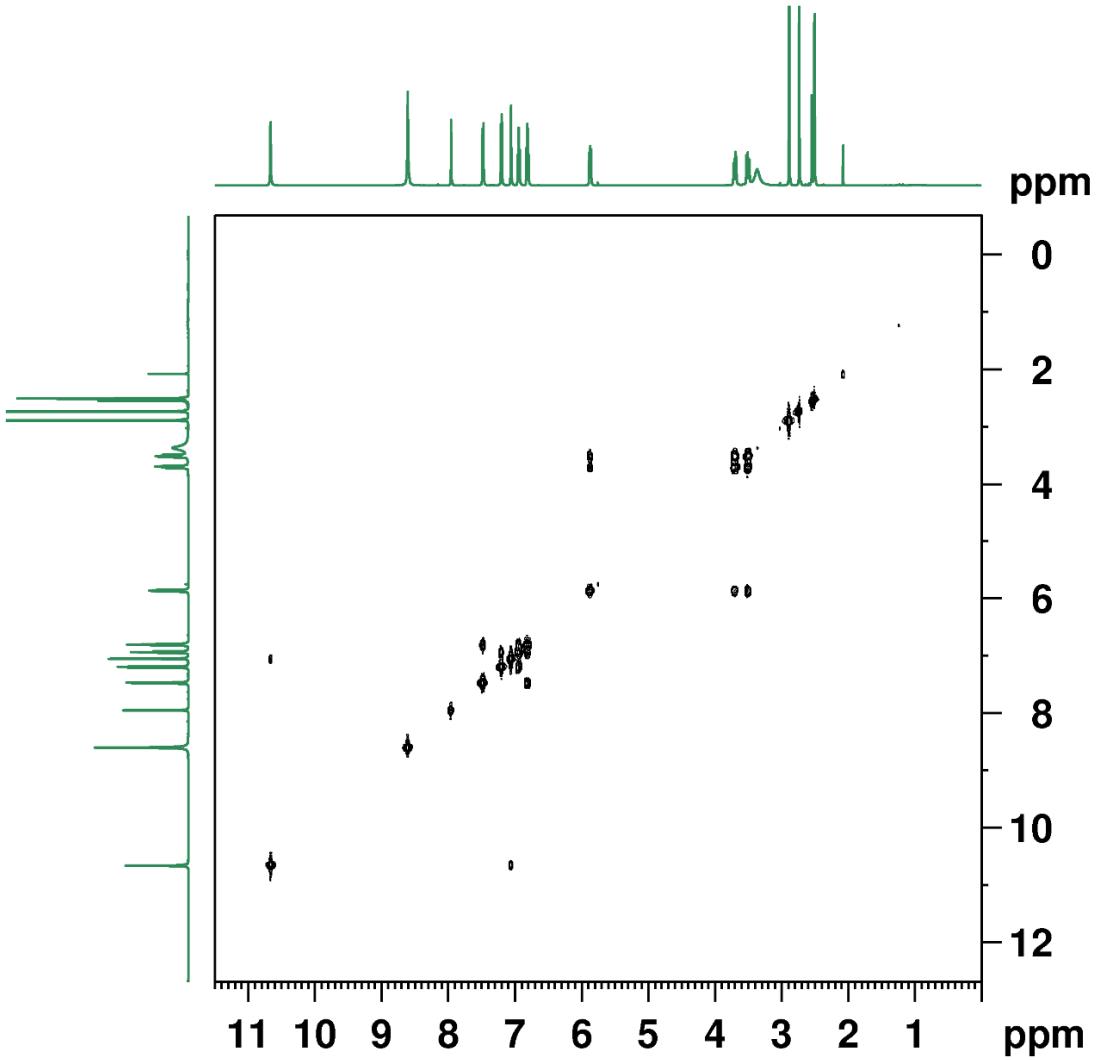


Figure S3.

3.4 ^{1}H-^{13}C HMQC NMR of *tryptophan-tagged naphthalenediimide*, [TrpNDI], (2).

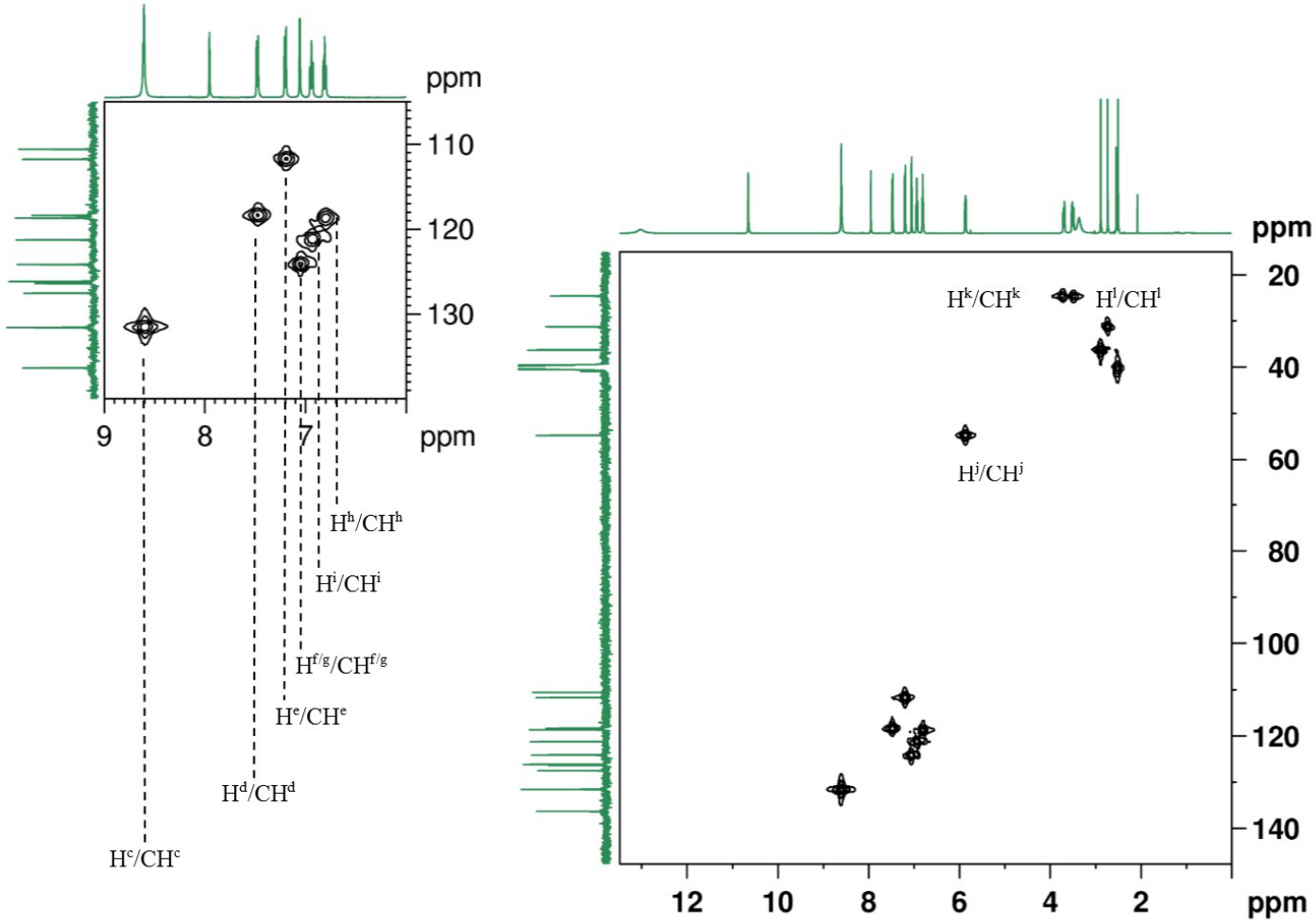


Figure S4.

3.5 ^{1}H NMR of *tryptophan-tagged hydroxysuccinimidyl naphthalenediimide* [TrypNDI-OSu], (2a)

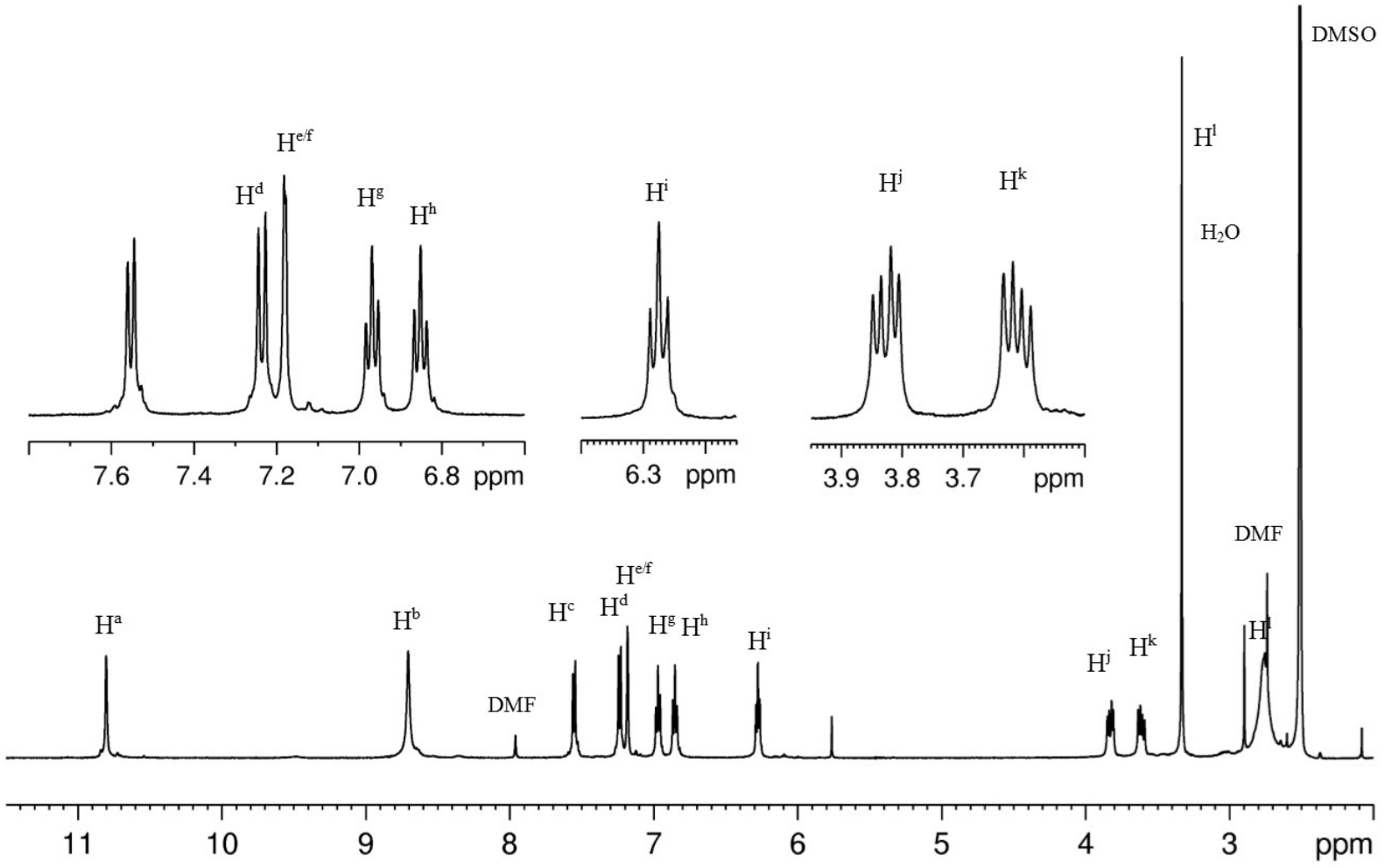


Figure S5.

3.6 ^{13}C NMR of *tryptophan-tagged hydroxysuccinimidyl naphthalenediimide* [TrypNDI-OSu], (2a)

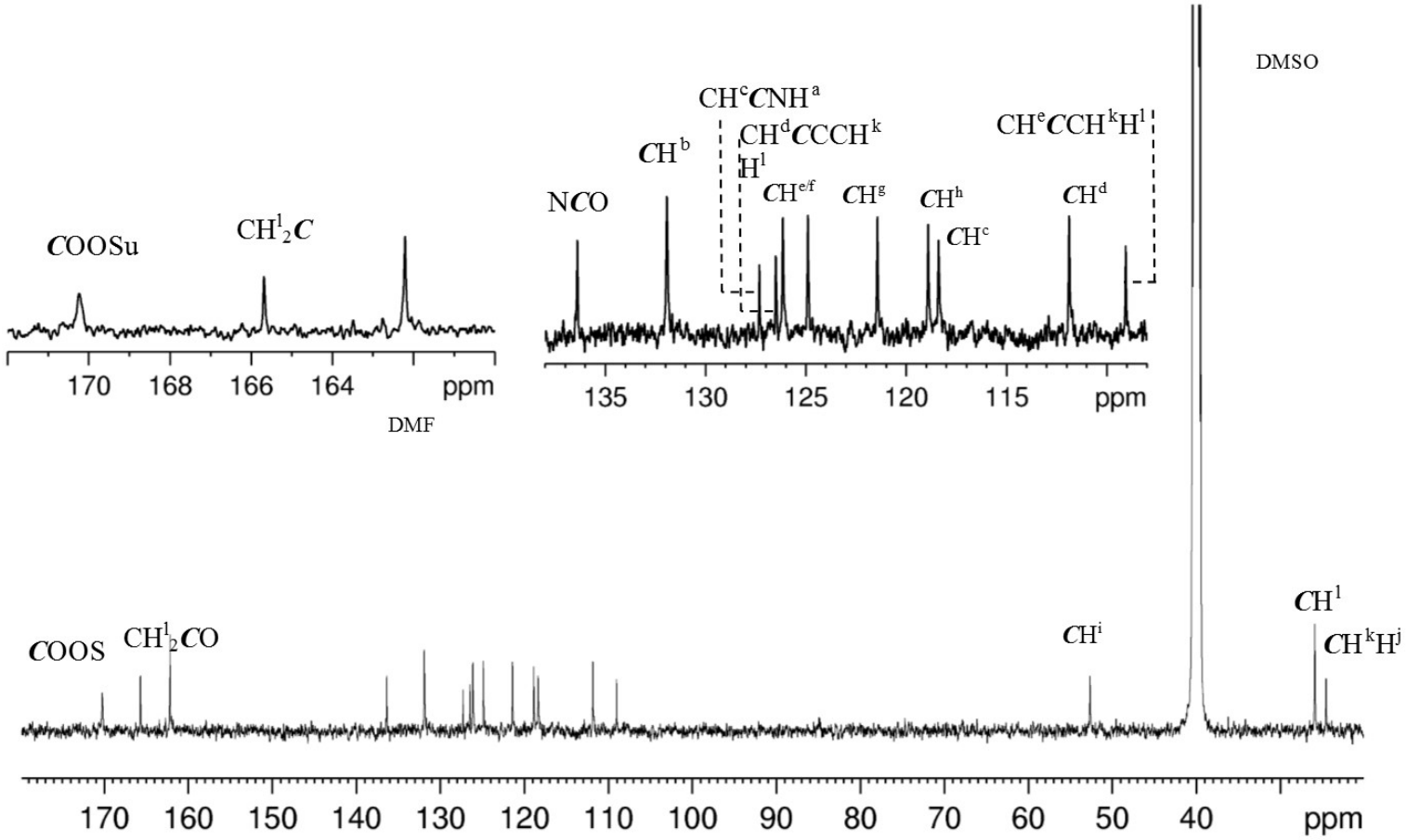


Figure S6.

3.7 ^{1}H COSY NMR of *tryptophan-tagged hydroxysuccinimidyl naphthalenediimide* [TrypNDI-OSu], (2a)

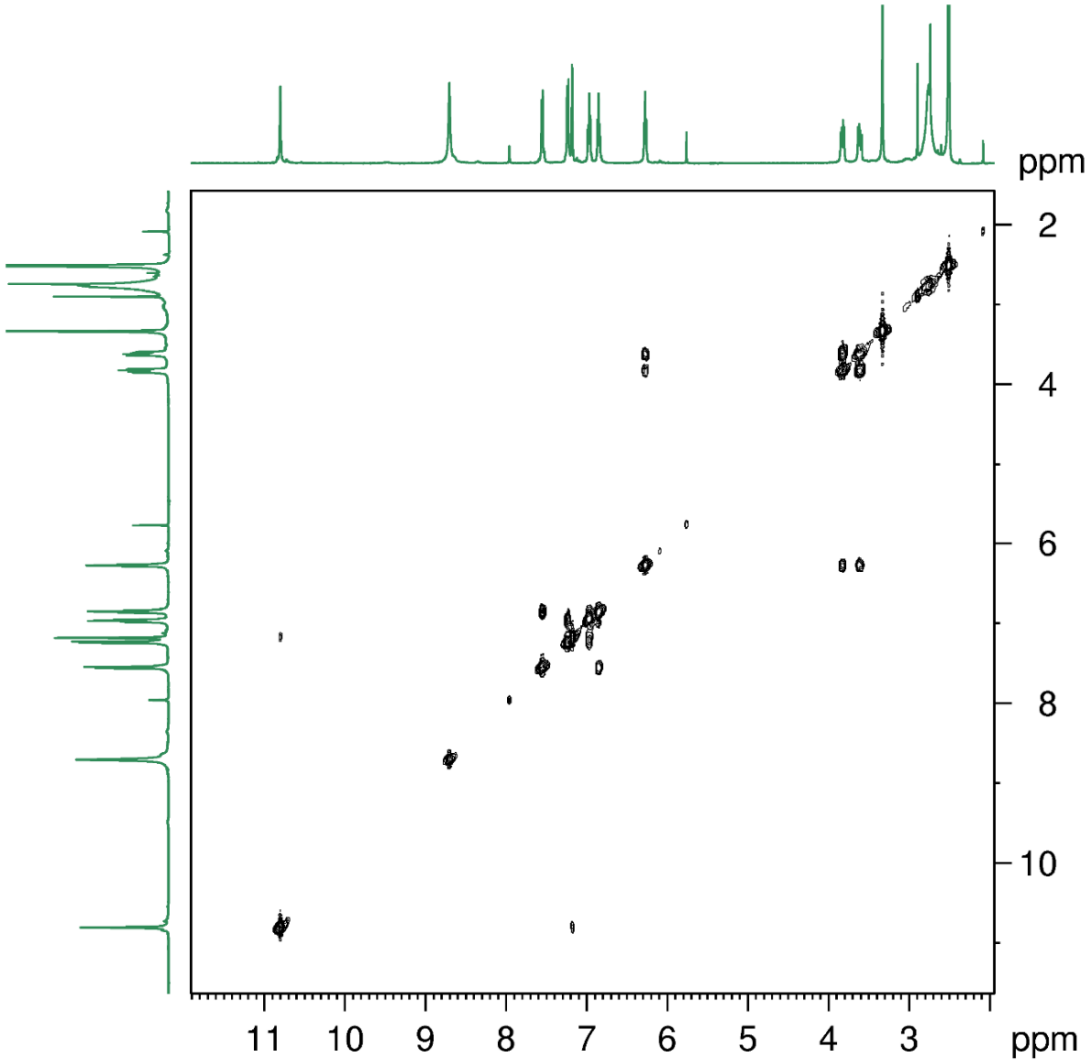


Figure S7.

3.8 ^{1}H-^{13}C HMQC NMR of *tryptophan-tagged hydroxysuccinimidyl naphthalenediimide* [TrypNDI-OSu], (2a)

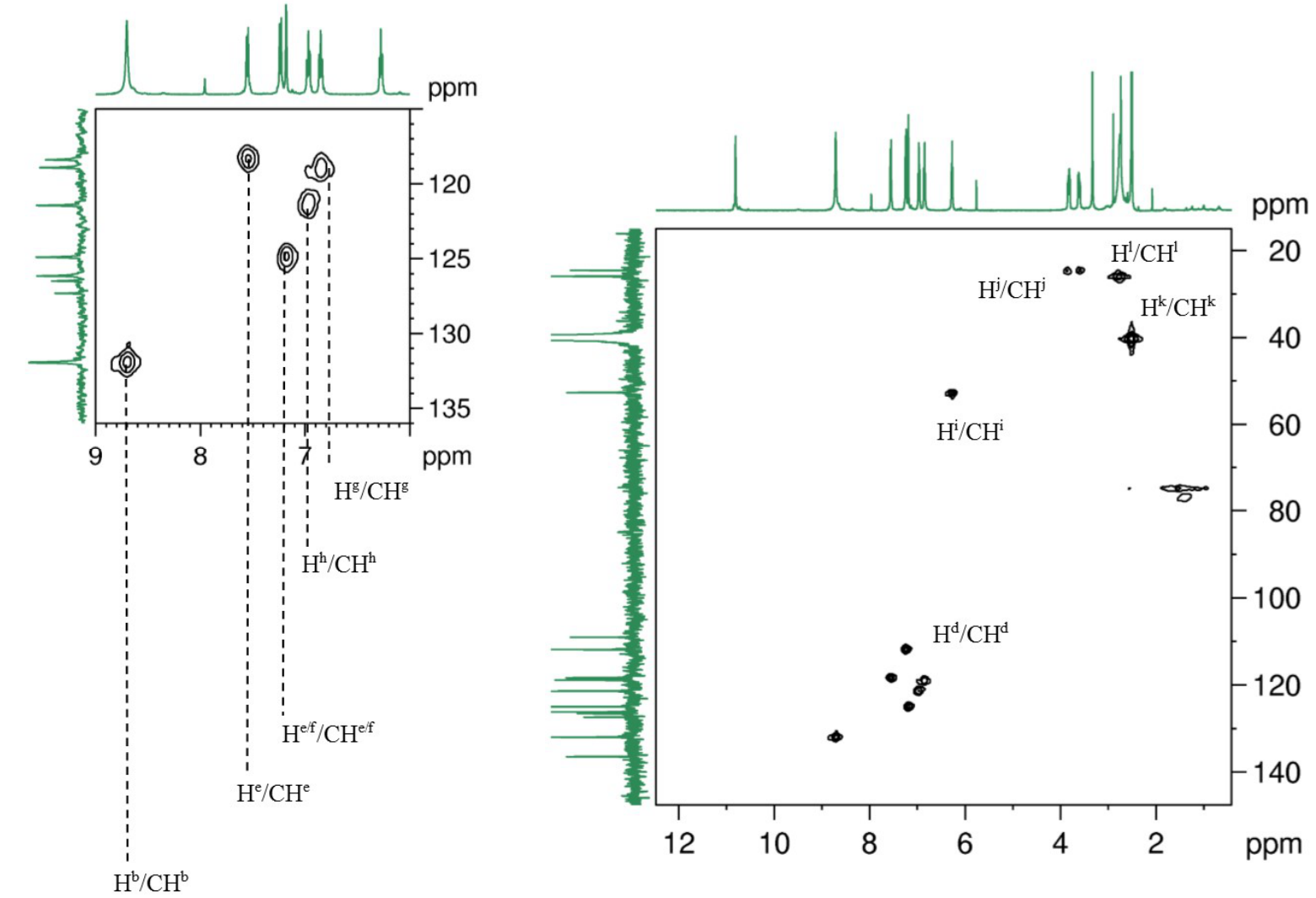


Figure S8.

3.9 ^{1}H NMR of *Cyclo*-(RGDfK), (3).

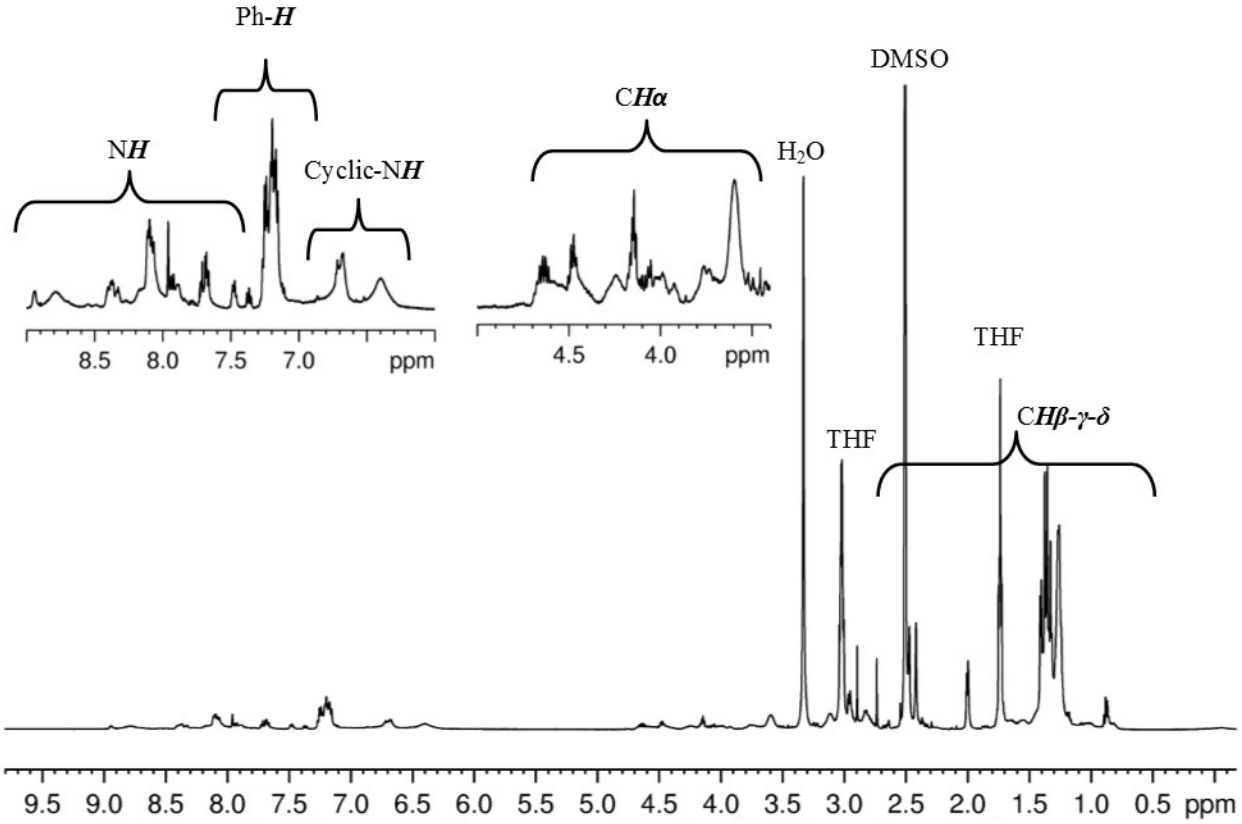


Figure S9. Inset shows expanded region of relevant proton nuclei in the high and low field of the spectrum.

3.10 ^{1}H NOESY NMR of *Cyclo*-(RGDfK), (3).

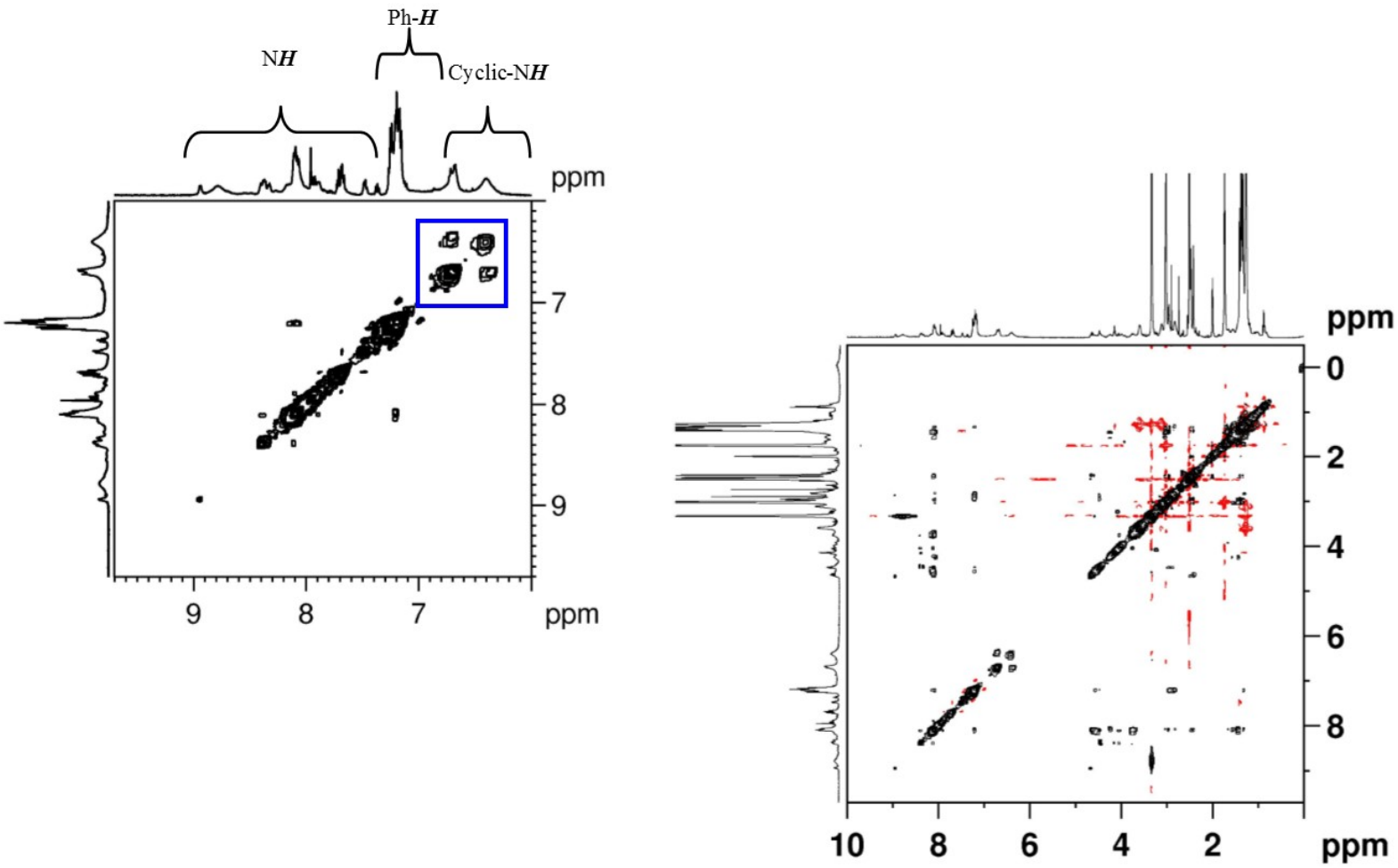


Figure S10. Exchange peaks in the cyclic-NH region (blue square) suggests that a bent cyclized backbone is present.

4. Mass spectroscopy.

4.1 Mass spectrum of *tryptophan-tagged naphthalenediimide*, [TrpNDI], (2).

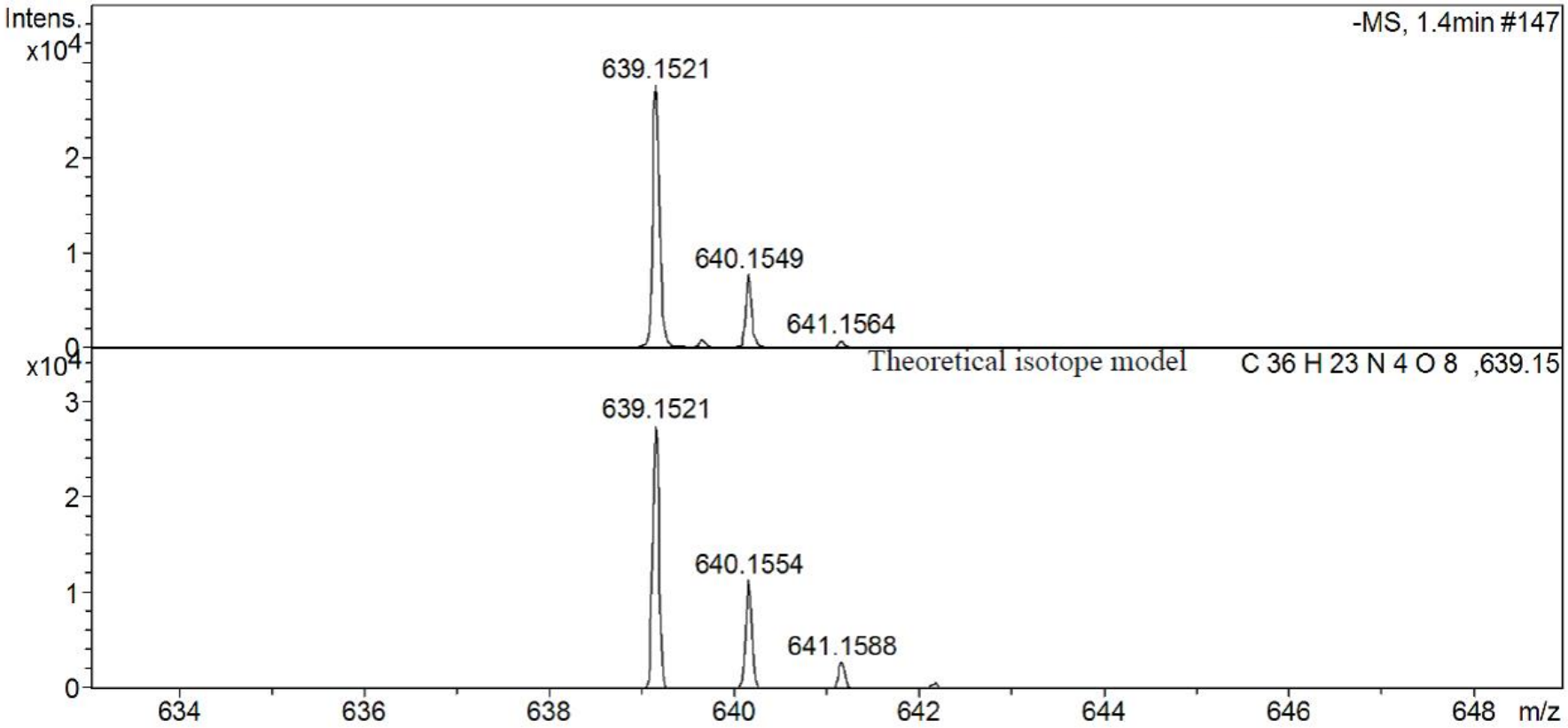


Figure S11. Observed (above) and calculated (below) isotopic pattern of **(2)** for $[M-H]^-$.

4.2 Mass spectrum of *tryptophan-tagged hydroxysuccinimidyl naphthalenediimide* [TrypNDI-OSu], (2a).

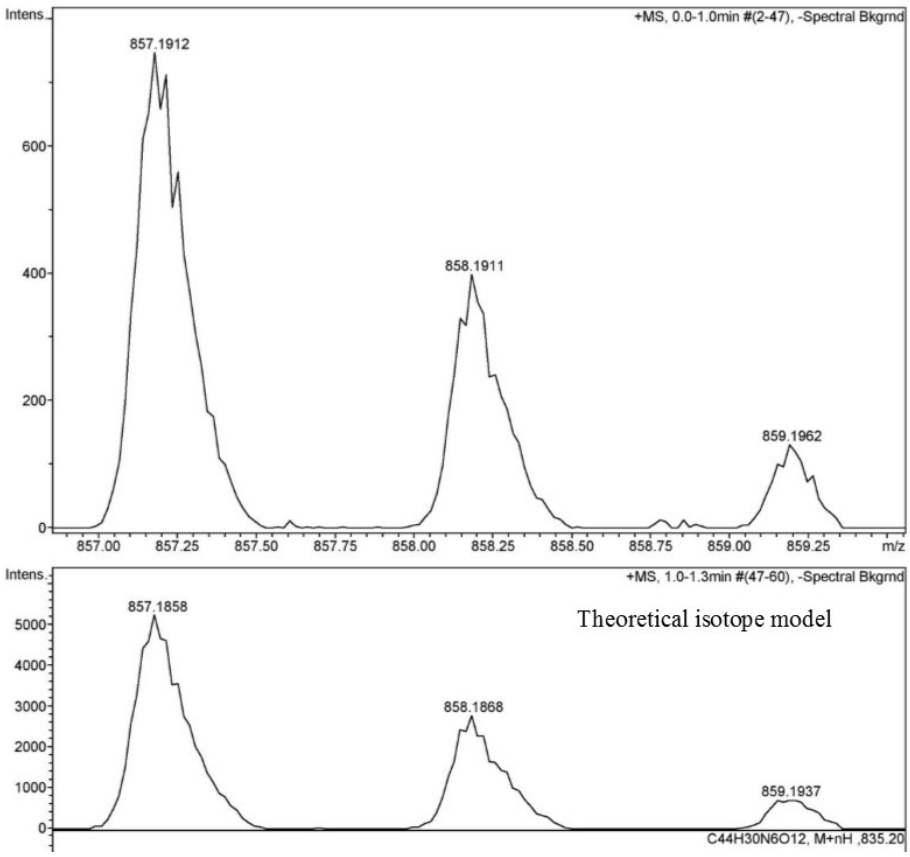


Figure S12 Observed (above) and calculated (below) isotopic pattern of (**2a)** for $[M+Na]^+$.

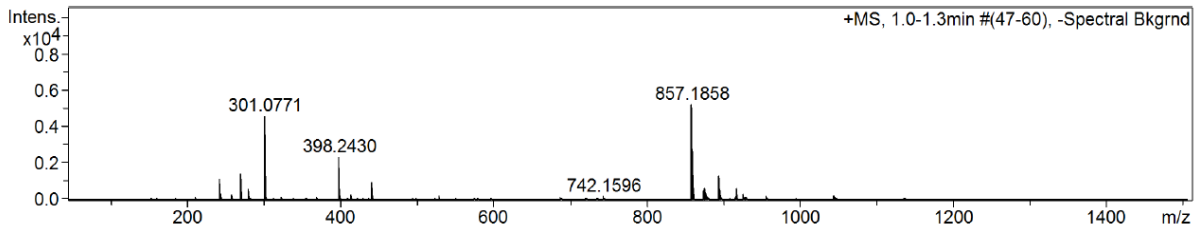


Figure S13. Positive mode ESI spectrum of (**2a**): m/z: 857.1858 (100%) [M+Na]+.

4.3 Mass spectrum of Linear-(RGDfK), (1')

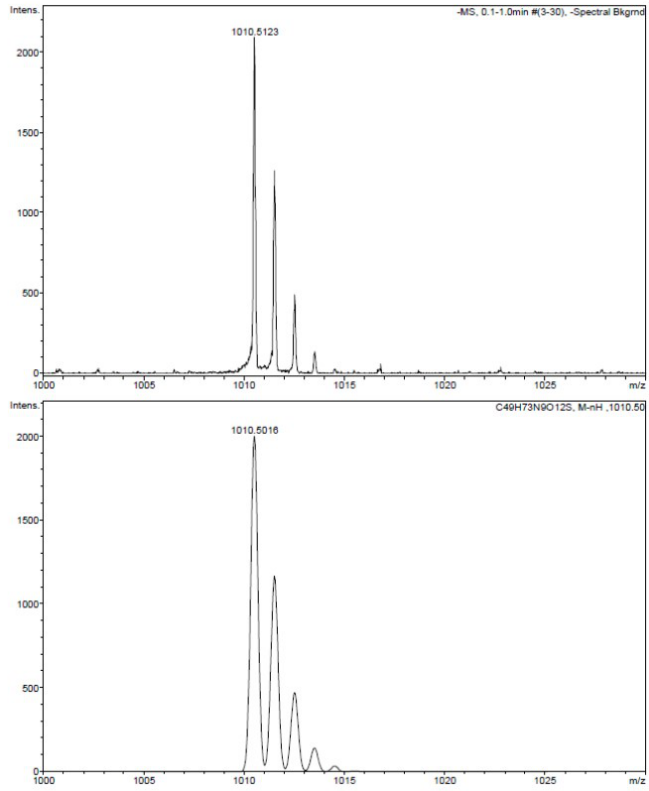


Figure S14. Observed (above) and calculated (below) isotopic pattern of (**1'**) for $[M\text{-}H_2O\text{-}H]^-$.

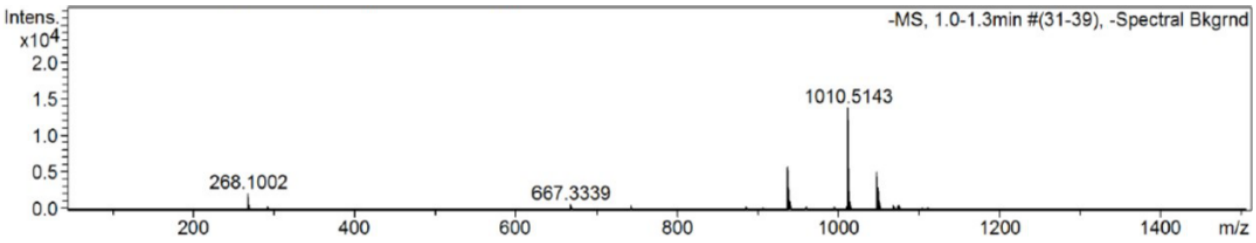


Figure S15. Negative mode MS ESI spectrum of (1'): m/z: found as $[M\text{-}H_2O\text{-}H]^-$ = 1010.5143.

4.4 Mass spectrum of *Cyclo-(Asp(OtBu)-D-Phe-Lys(Boc)-Arg(Pbf)-Gly*) (**2'**)

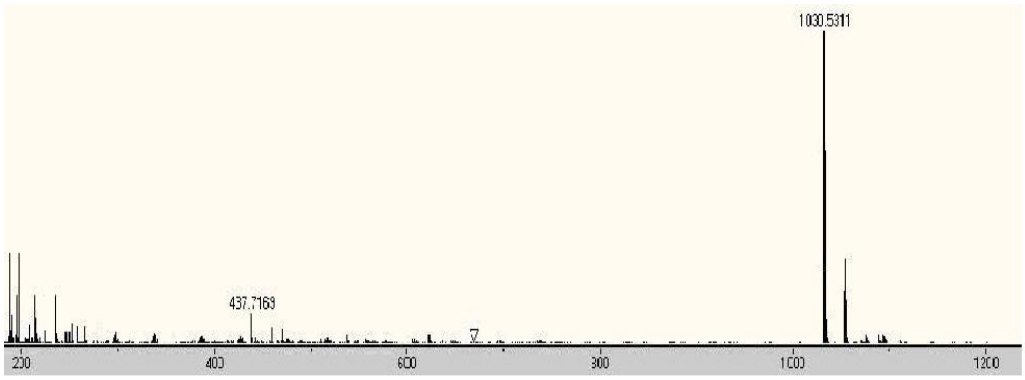


Figure S16. Positive mode ESI MS spectrum of (**2'**): *m/z* 1030.5311 $[M+NH_4]^+$.

4.5 Mass spectrum of Cyclo-(RGDfK), (3)

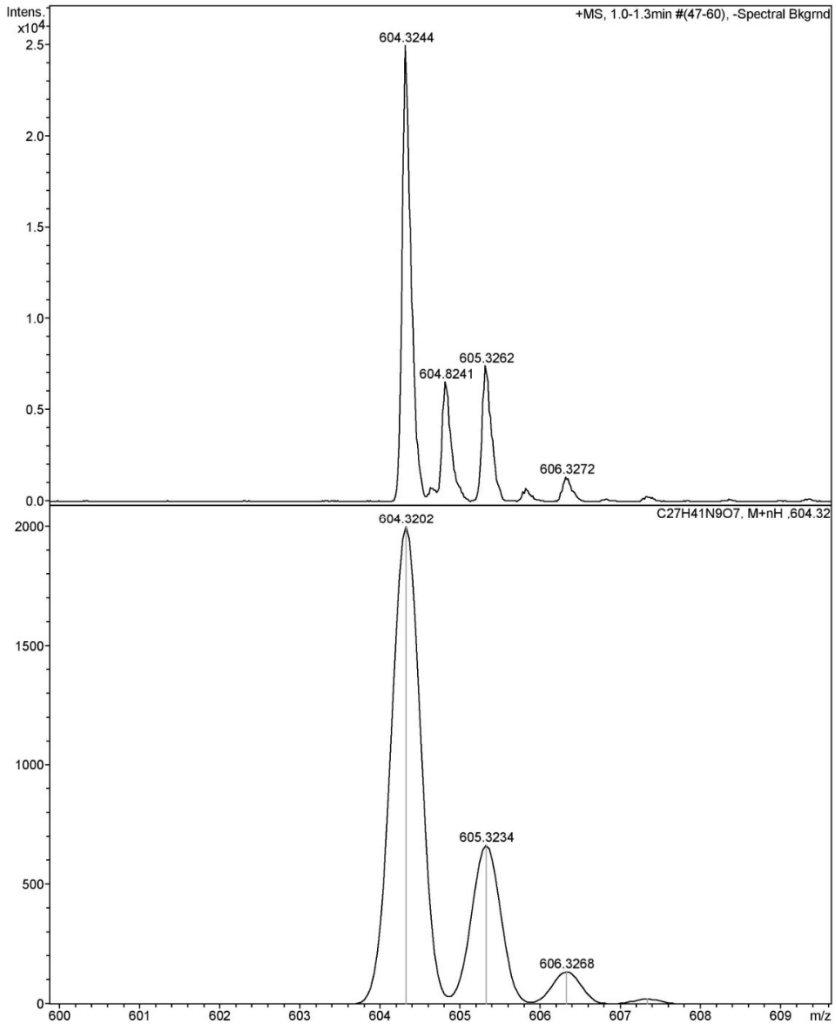


Figure S17. Observed (above) and calculated (below) isotopic pattern of (**3)** for $[M+H]^+$.

4.6 Mass spectrum of Cyclo-(RGDfK)-tryptophan-tagged naphthalenediimide [TrpNDIRGDfK], (4).

Maldi mass spectrometry and analytical HPLC methods were used for analysing the resulting compound to confirm that the product is the double-RGD substituted compound denoted NDI-RGDfK (**4**). The product (**4**) was dissolved in DMSO and purified by semi-preparative HPLC, in ca. 10% overall yield, whereby the peak at 8.62 min (300 nm wavelength) was collected. MALDI mass spectrometry: *m/z* calcd: C_{90} H_{103} $N_{22}O_{20}$ as $[M+H]^+$: 1811.76 and found: 1811.76.

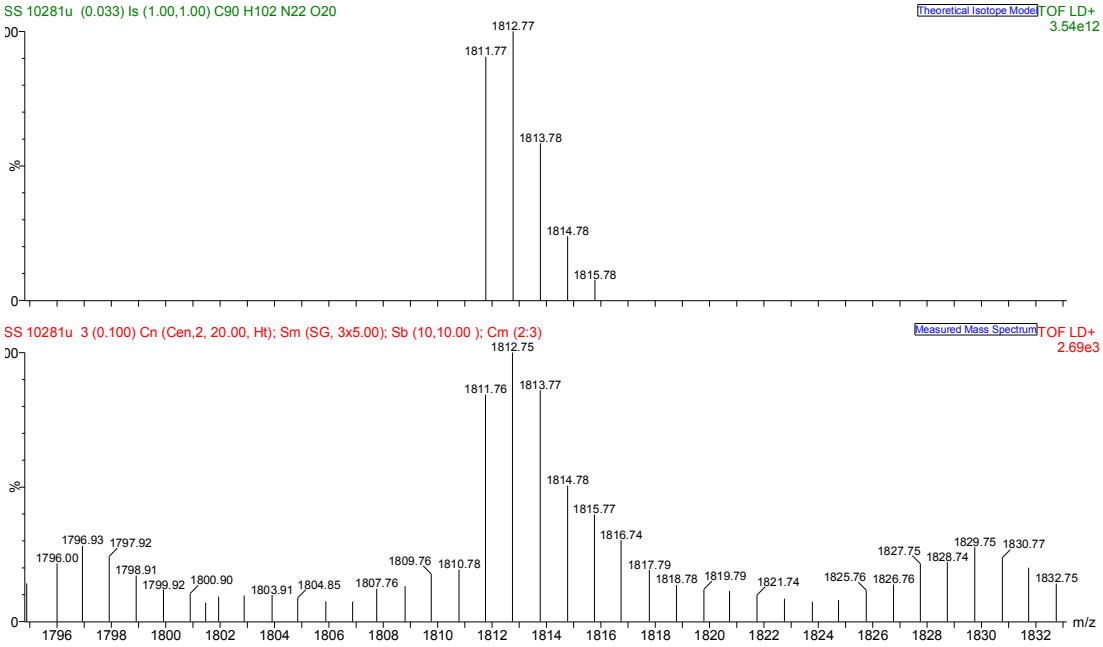


Figure S19. MS MALDI spectrum of TrpNDI-RGDfK (**4**). Observed (above) and calculated (below) isotopic pattern of (**4**) for $[M+H]^+$.

5. HPLC data

5.1 HPLC of *tryptophan-tagged naphthalenediimide*, [TrpNDI], (2)

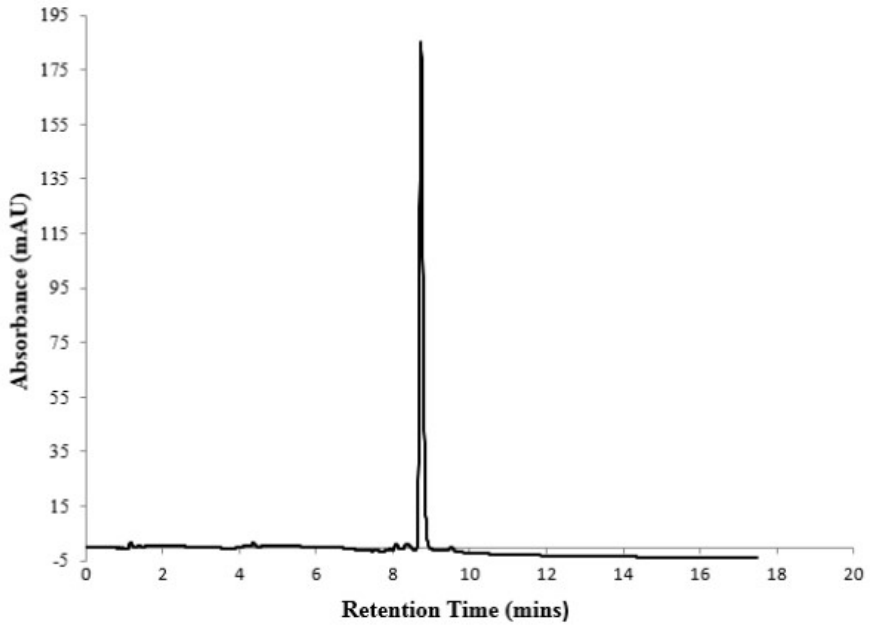


Figure S20

5.2 HPLC of *tryptophan-tagged hydroxysuccinimidyl naphthalenediimide* [TrypNDI-OSu], (2-A).

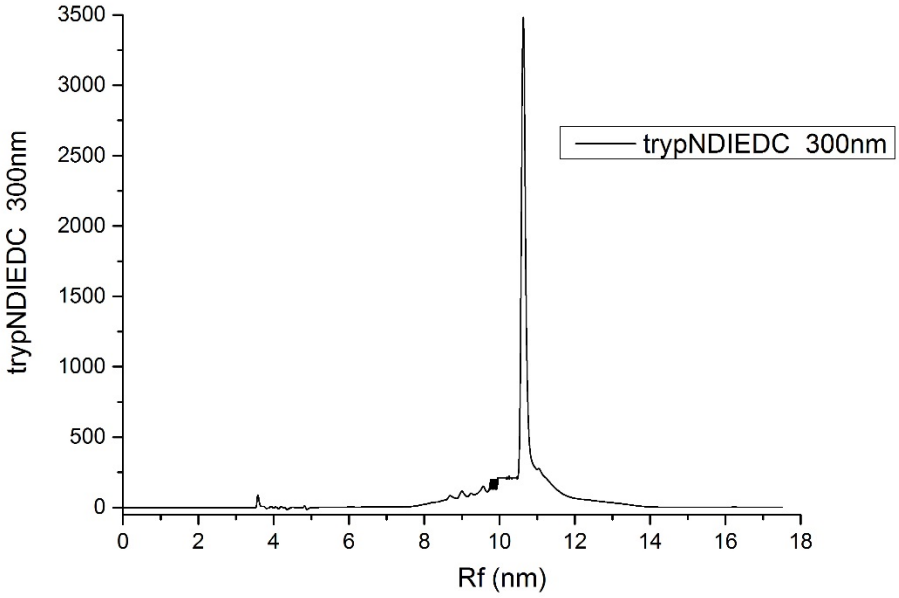


Figure S21

5.3. HPLC of Cyclo-(RGDfK)-tryptophan-tagged naphthalenediimide [TrpNDIRGDfK], (4).

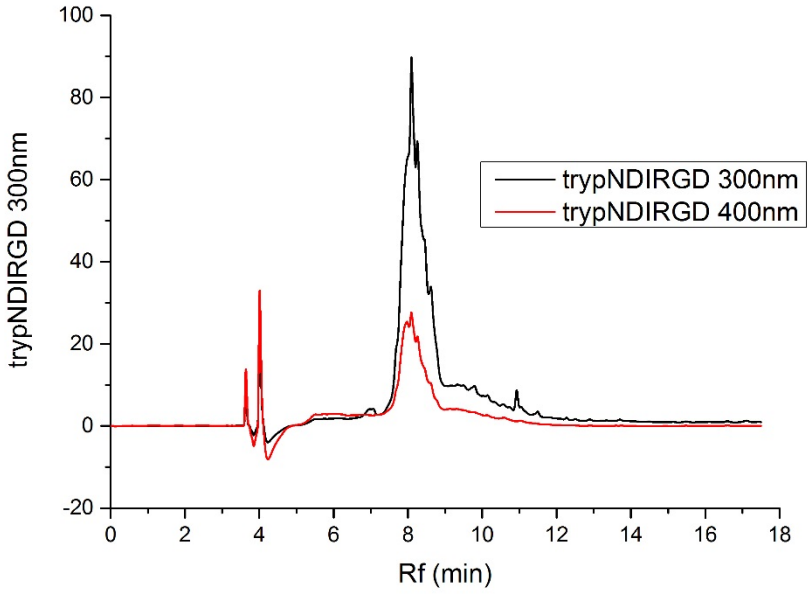


Figure S23. Overlay of HPLC retention times of (**4**) prior to semi-prep separation.

Table S1. Summary of mass spectrometry and reverse phase HPLC analysis of TrpNDI-RGDfK (Compound **4**) and relevant starting materials (detector wavelength: 300 nm).

Molecule	Molecular formula	MS calculated	MS observed *m/z*	RP-HPLC retention time(min)
3	$C_{27}H_{41}N_9O_7$	604.32 $[M+H]^+$	604.39	4.76
2-A	$C_{44}H_{30}N_6O_{12}$	857.18 $[M+Na]^+$ 873.16 $[M+K]^+$	857.45 873.46	10.63
4	$C_{90}H_{102}N_{22}O_{20}$	1811.77 $[M+H]^+$	1811.76	8.62

6. X-ray diffraction

Crystal data and structure refinement for $C_{27}H_{41}N_9O_7$•3DMSO•$2H_2O$ Tryptophan-NDI•3DMSO•$2H_2O$: $C_{42}H_{46}N_4O_{13}S_3$, M = 911.02, Z = 2, monoclinic P21 space group a = 8.6884(8) Å, b =19.6749(18) Å, c = 25.149(2) Å, β = 99º, U = 4299.0(7) $Å^3$, T = 150 (2) K, μ = 0.243mm–1, λ = 0.69040Å. Of 35763 reflections measured, 13220 were independent (Rint = 0.065). Final R = 0.0529 (4973 reflections with I > 3.00 (I)) and wR = 0.0569.

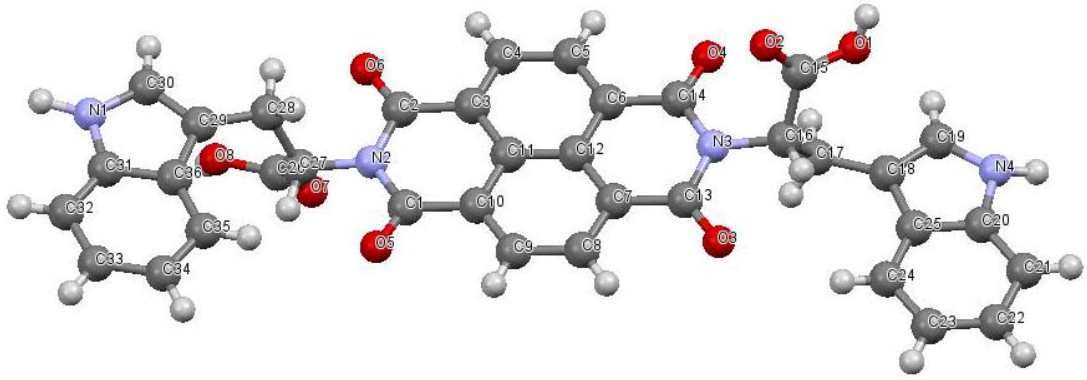


Figure S24. Top view of the molecular structure of **2** showing the atom labeling scheme single crystals obtained from DMSO solution. Displacement ellipsoids are scaled to 50% probability. Disordered DMSO and H_2O molecules have been removed for clarity.

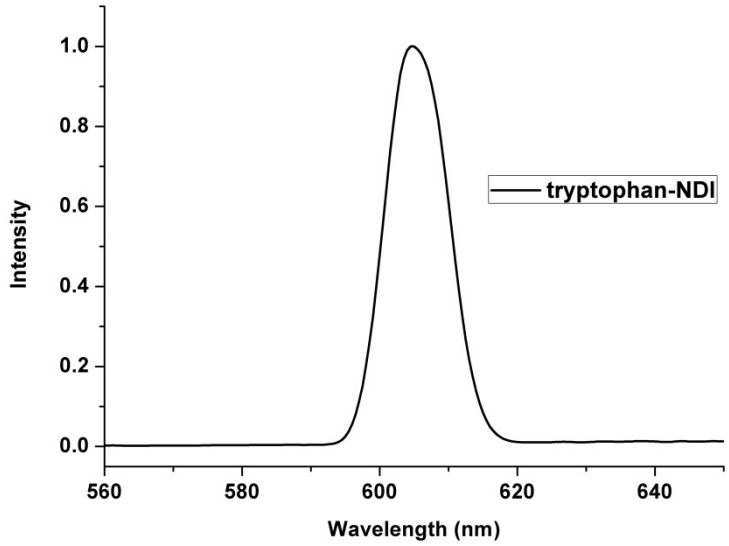


Figure S34. Steady-state normalised single photon fluorescence emission spectrum of Compound **2** in DMSO (recorded at 405 nm excitation, 10 mM). Quantum yield (measured with respect to fluorescein): 0.002.

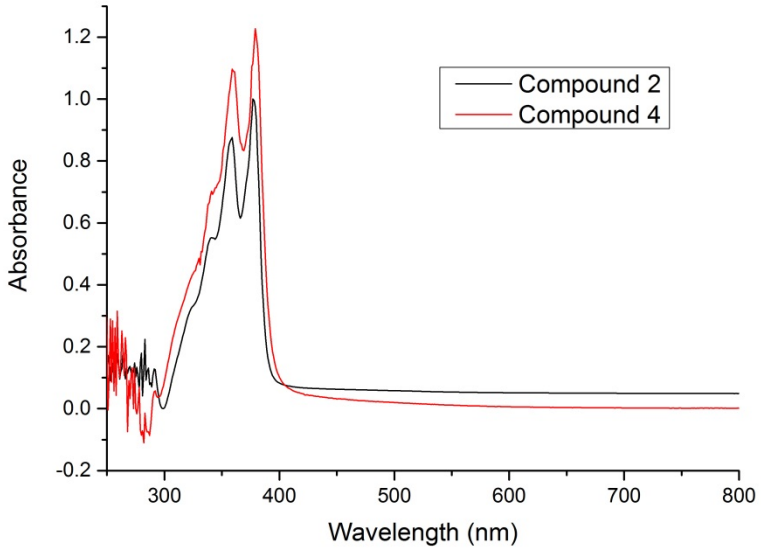


Figure S25. UV Vis spectroscopy (10 mM in DMSO) of Compound **2** (TrypNDI) and Compound **4** (TrypNDIRGDfK).

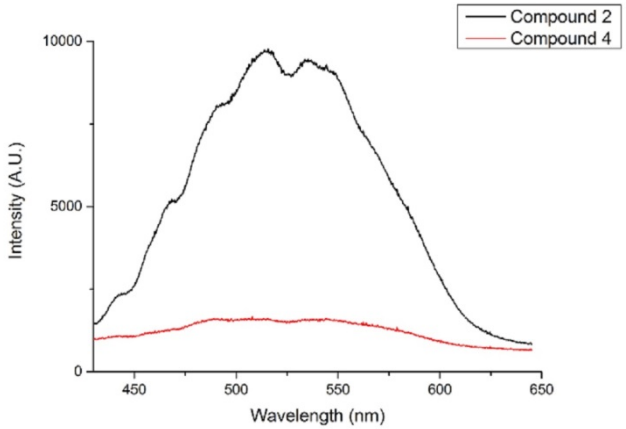


Figure S26. Two photon fluorescence emission spectroscopy (10 mM in DMSO) of Compound **2** (TrypNDI) and Compound **4** (TrypNDIRGDfK) at 810 nm excitation (5.7 mW, 10 s).

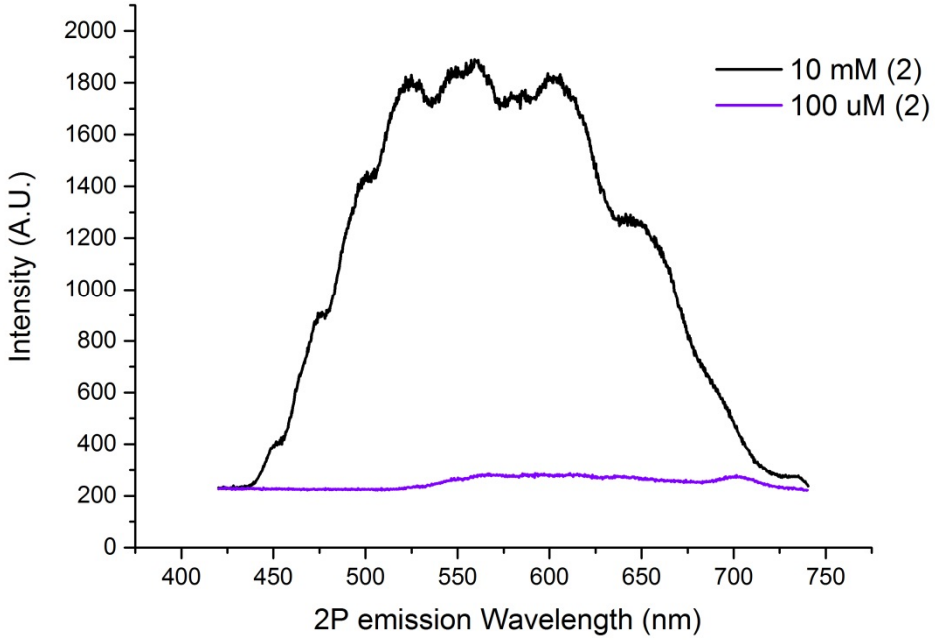


Figure S27. Two photon fluorescence emission spectroscopy of Compound **2** (TrypNDI) at 10 mM and at 100 μM concentrations in DMSO recorded at 810 nm excitation (8.7 mW, 60 s).

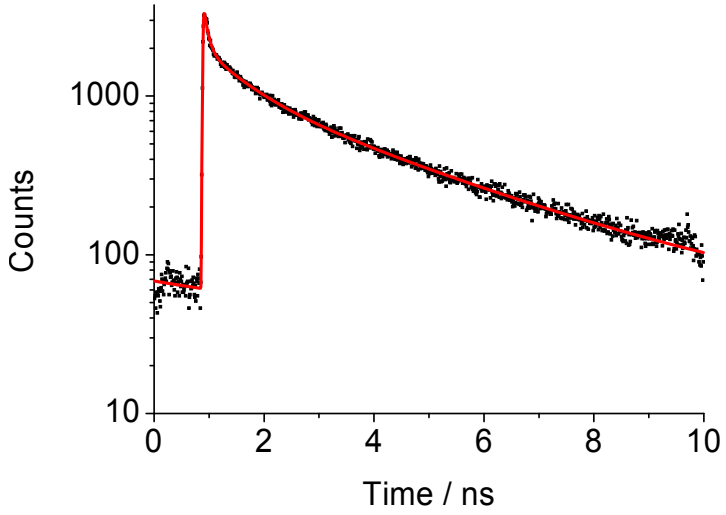


Figure S28. Two-photon time-correlated single photon counting: fluorescence decay trace and corresponding fitted curve for the lifetime determinations (λ_{ex} = 810 nm, compound **2**, 100 μM, pure DMSO, 5.7 mW), χ^2 = 1.18, τ_1 = 0.1 ns (50%), τ_2 = 0.7 ns (22.8%), τ_3 = 3.2 ns (27.2%).

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7. Cell imaging – single and two-photon fluorescence spectroscopy

Two photon imaging experiments were carried out at the Rutherford Appleton Laboratory. Laser light at a wavelength of 580-630 nm was obtained from an optical parametric oscillator pumped by a mode locked Mira titanium sapphire laser (Coherent Lasers Ltd) producing 180 fs pulses at 75 MHz. This laser was pumped by a solid state continuous wave 532 nm laser (Verdi V18, Coherent Laser Ltd). The oscillator fundamental output of 810 ± 2 nm was used. The laser beam was focused to a diffraction limited spot through a water immersion ultraviolet corrected objective (Nikon VC x60, NA1.2) and specimens illuminated at the microscope stage of a modified Nikon TE2000-U. The focused laser spot was raster scanned using an X-Y galvanometer (GSI Lumonics). Fluorescence emission was collected without de-scanning, bypassing the scanning system and passed through a colored glass (BG39) filter. The scan was operated in normal mode and line, frame and pixel clock signals were generated and synchronised with an external fast microchannel plate photomultiplier tube used as the detector (R3809-U, Hamamatsu, Japan). These were linked *via* a Time-Correlated Single Photon Counting (TCSPC) PC module SPC830. Emission spectral detection was carried out using an Acton Research Component 275 spectrometer and an Andor iDus 740-BU CCD camera.

7.1 PC-3 cells culture and fluorescence imaging

PC-3 cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in air and split once confluence had been reached, using DMEM medium with 10% foetal calf serum (FCS), 200 U mL^{-1} L-glutamine and 100 U mL^{-1} penicillin. The medium contained no fluorescent indicator dyes such as phenol red and was therefore suitable for use in fluorescence imaging studies.

Samples for fluorescence imaging were prepared in the following way: surplus supernatant after culturing (containing dead cell matter and excess proteins) was discarded. The live adherent cells were then washed with two 5 mL aliquots of phosphate buffer saline solution to remove any remaining medium containing FCS since this contains protease inhibitors which inactivate trypsin, thus inhibiting the resuspension of the cells. For re-suspension in solution, the cells were incubated in 3 mL of trypsin–EDTA (500 mg L-1 trypsin, 200 mg L-1 EDTA) solution for 5 min at 37 °C. After trypsinizing, fresh DMEM (10% FCS) was added to the suspended cells to give a sufficient concentration of cells (ca. 50000 cells/mL). The cells were plated in a Petri dish with a glass cover slip (MaTek) and left for 24 h to adhere before fluorescence imaging measurements were made.

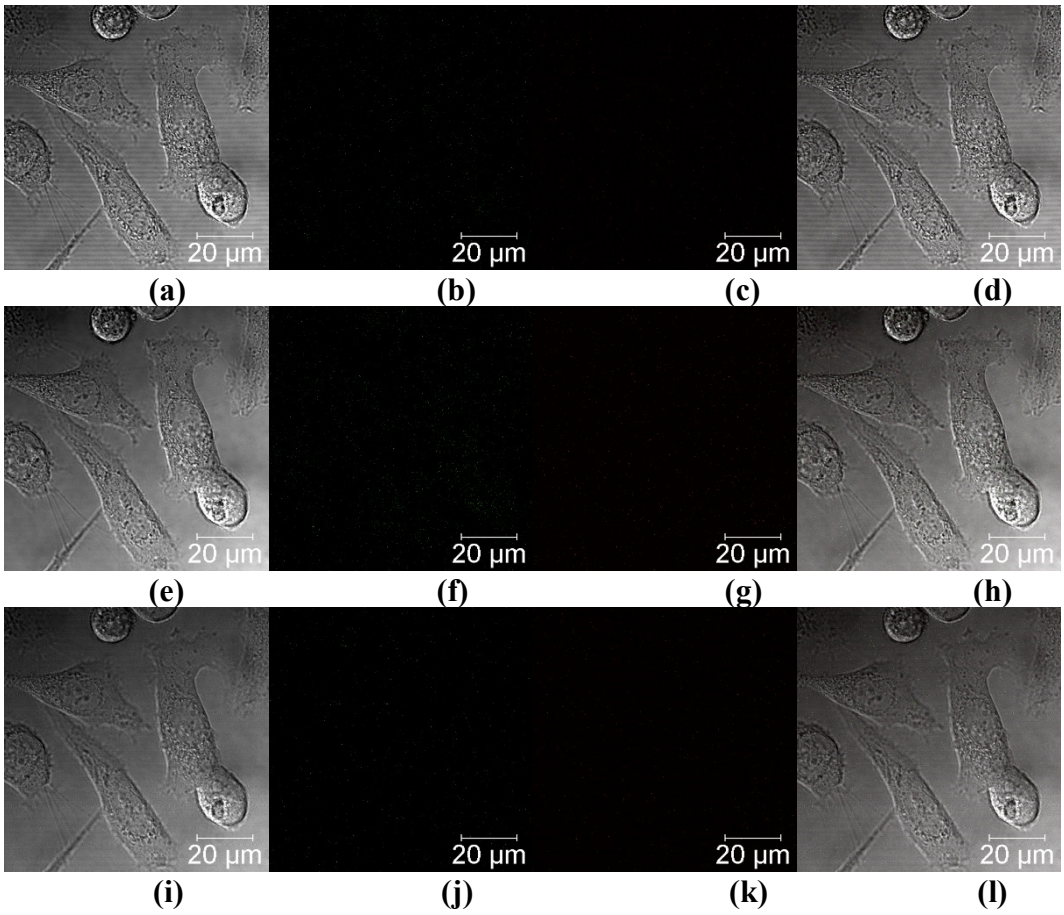


Figure S29. Single-photon laser-scanning confocal microscopy of PC-3 cells incubated for 25 mins at 37 ^{o}C (control at 5: 95 % DMSO: serum free medium): (**a-d**) (λ_{ex} = 405 nm); (**a-d**) (λ_{ex} = 488 nm), (**ι–λ**) (λ_{ex} = 543 nm). (**a**, **e**, **i**) DIC channel, (**b**, **f**, **j**) green channel, (**c**, **g**, **k**) red channel, (**d**, **h**, **l**) overlay image. Scalebar, 20 µm.

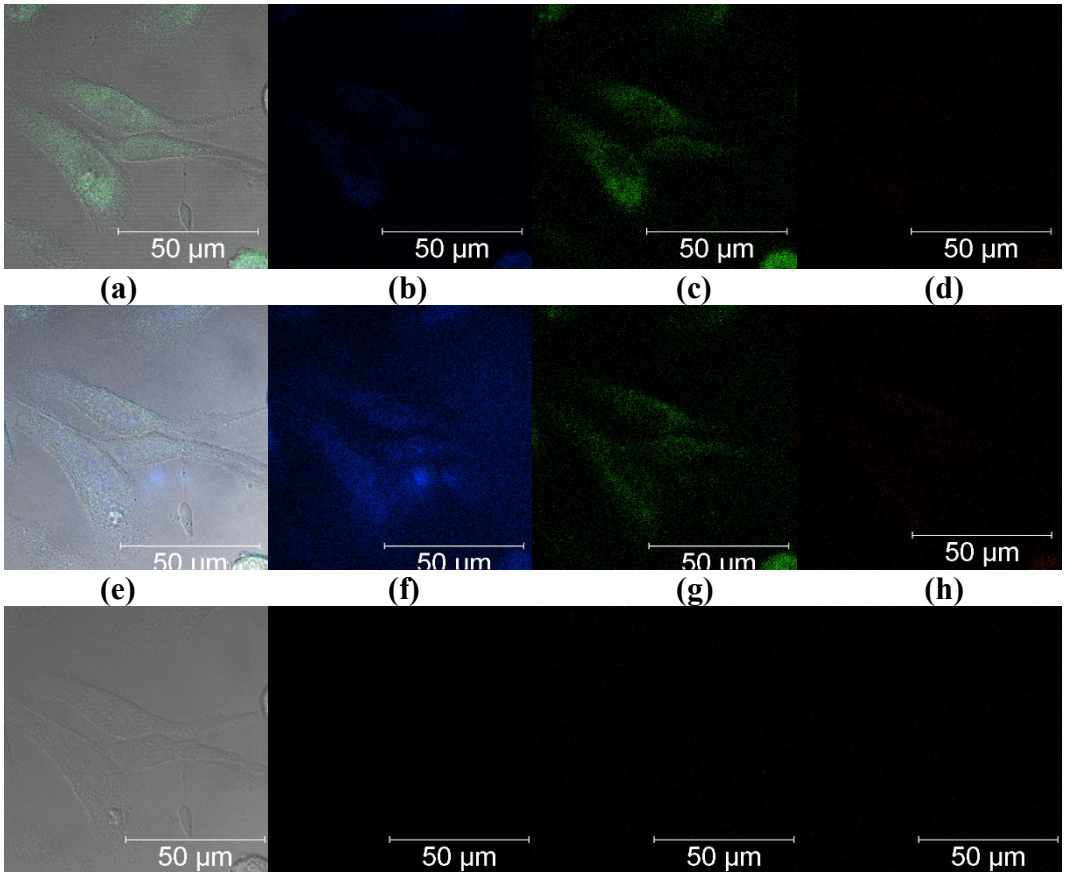
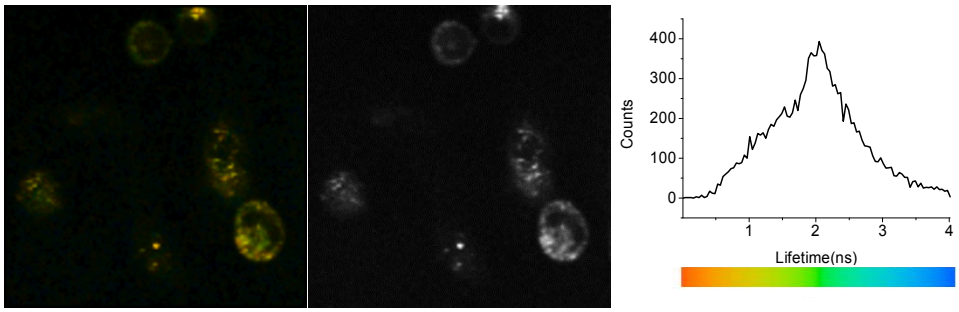


Figure S30. Single-photon laser-scanning confocal microscopy of PC-3 cells incubated for 25 mins at 37 ºC with Compound **2** (100 µM in 1: 99, DMSO: serum free medium): (**a-d**) (λ_{ex} =405 nm); (**e-h**) (λ_{ex} =488 nm), (**i-l**) (λ_{ex} = 543 nm). (**a**, **e**, **i**) overlay image, (**b**, **f**, **j**), blue channel, (**c**, **g**, **k**) green channel, (**d**, **h**, **l**) red channel. Scalebar: 50 µm.

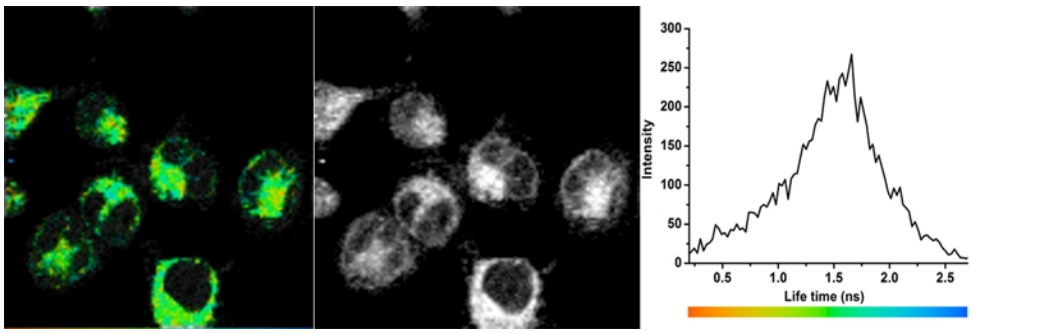
7.2 PC-3 cells culture and fluorescence imaging and control experiments, including TCSPC solution experiments

PC-3 cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in air and split once confluence had been reached, using DMEM/F-12 with 10% foetal calf serum (FCS), 200 U mL-1 L-glutamine and 100 U mL-1 penicillin. The medium contained no fluorescent indicator dyes such as phenol red and was therefore suitable for use in fluorescence imaging studies. Samples for fluorescence imaging were prepared in the following way: surplus supernatant after culturing (containing dead cell matter and excess proteins) was discarded. The live adherent cells were then washed with 5 mL aliquots of phosphate buffer saline solution to remove any remaining medium containing FCS two times. For re-suspension in solution, the cells were incubated in 3 mL of trypsin–EDTA (500 mg L^{-1} trypsin, 200 mg L^{-1} EDTA) solution for 5 min at 37 °C. After trypsinising, fresh DMEM (10% FCS) was added to the suspended cells to give a sufficient concentration of cells (ca. 50,000 cells/mL). The cells were plated into a glass bottom Petri dish (MatTek) and left for 24 h to adhere before fluorescence imaging measurements were made.



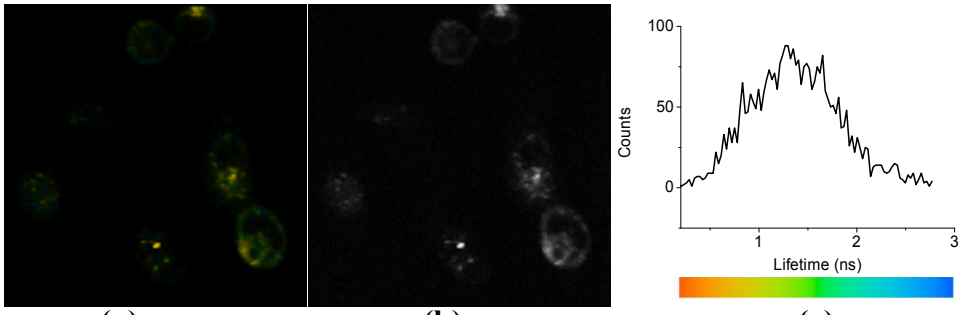
(a) **(b)** **(c)**

Figure S31. Control experiment: Two-photon laser confocal fluorescence (λ_{ex} = 810 nm):(a, b and c) Typical micrograph of PC-3 cells incubated for 15 min at 37 ºC as a control (1: 99 % DMSO: Serum Free Medium showing lifetime mapping of τ_m (**a**),two photon intensity image (**b**), and corresponding lifetime distribution curve and scale-bar (**c**). Power was increased to 8.9 mW to allow autofluorescence emission (if any) to be detected.



(a) **(b)** **(c)**

Figure S32. Two-photon laser confocal fluorescence (λ_{ex} = 810 nm):(**a**, **b**, **c**) Typical micrograph of PC-3 cells incubated for 20 min at 37 ºC with **2** (500 µM in 5: 95% DMSO: EMEM) showing: lifetime mapping (**a**), intensity image (**b**), and corresponding lifetime distribution curve and scalebar (**c**).



(a) **(b)** **(c)**

Figure S33. Two-photon laser confocal fluorescence (λ_{ex} = 810 nm): Typical micrograph of PC-3 cells incubated for 15 min at 37 ºC (1:99 DMSO: Serum Free Medium) used as a control showing lifetime mapping of τ_m (**a**), intensity image (**b**) and corresponding lifetime distribution curve and scale-bar (**c**). Power was 5.7 mW for both experiments.

Table 2. Lifetime decay constants for compounds **2** in PC-3 cancer cells:

Compound	τ_1 / ns	FHHM / ns	A_1 %	τ_2 / ns	FHHM / ns	A_2 %	χ^2
2 (100 uM)	1.0	0.5	80.1	4.0	2.3	20.1	1.16
2 (500 uM)	0.6	0.1	62.4	2.5	0.7	37.6	1.21

8. MTT assays

Method 1 - 24 h assay:

Within the limitations of the compounds' solubility with the low percentage of DMSO required (maximum 1% for extended time incubations, over 24h observations) they appear to be non-toxic and the MI50 is > 100 µM against either PC-3 (prostate cancer cells) or FEK-4 (non-cancerous cell line). The cytotoxicity of compounds was measured by MTT cell viability test. The FEK-4 cells were seeded in 96-well plates at a density of 5000 cells per well with a total medium volume of 100 µL and incubated for 24 h. Then, the solution was removed, 100 µL of EMEM medium (Eagle's Modified Essential Medium) was added, containing 200 µM solutions of the compounds 2 and 4 respectively (TrypNDI: 200 µM made from 12.8µg solid/(1 µL DMSO + 99 µLDMEM) and TrypNDI-RGDfK: 200 µM made from 36.2 µg solid/(1µLDMSO+99 µLDMEM). Twenty-four hours later, the medium was replaced with fresh medium supplemented with 15 µL of MTT reagent (5 mg/mL).

Four hours later, the medium containing MTT was removed, and DMSO (100 µL/well) was added to dissolve the formazan crystals. The optical density of the solution was measured at 570 nm using a microplate reader. Cells treated with EMEM medium and DMSO (without

compound) were used as the control, in addition to the EMEM control experiment. The experiments were repeated three times.

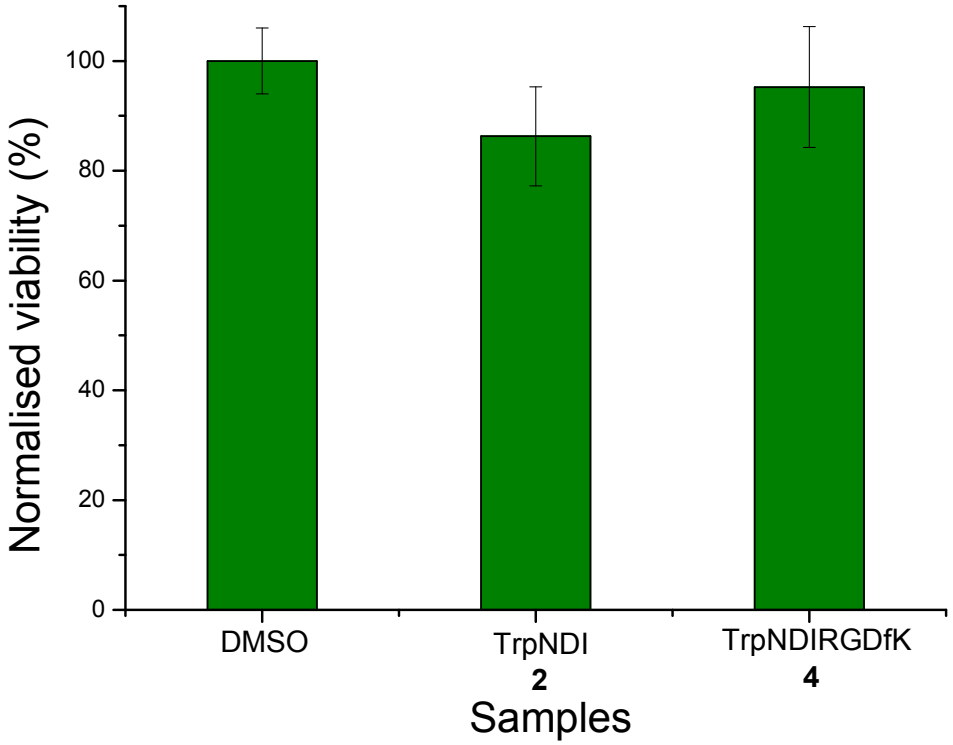


Figure S34. MTT assays of compound 2 and 4 in FEK4 cell line, at 200 μM concentration for 24 hours at 37ºC, showing 24 h cytotoxicity (expressed as % normalised viability)

Method 2 - 48 h assay:

After cell splitting, cells (3×10^3 cells per well) were seeded on a T75 sterile 96 well plate and incubated for 48 hours to adhere. Varieties of compound were subsequently loaded at different concentration into wells and cultured for another 48 hours. The Concentration used was ranged between 250 μM (1% DMSO, 99% Eagle's Modified Essential Medium (10% FCS) and 100nM as they were shown in table 6-1. Subsequently, cells were washed three times with PBS and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was added (0.5 mg/ mL, 10% PBS: SFM) followed by three hours incubation. After aspiration, 100 μl of DMSO was added and 96 well plates were read at an ELISA plate reader, Molecular Devices Versa Max (BN02877). The absorption wavelength was at 570 nm and 630 nm wavelength was used as a reference.

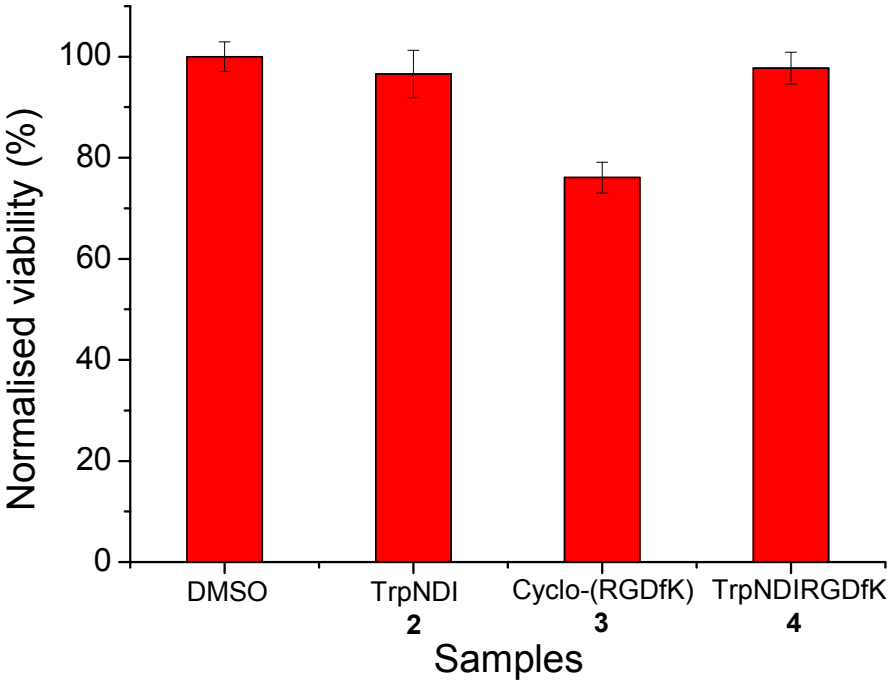


Figure S35. MTT assays of compound 2, 3 and 4 in FEK4 cell line, at 100 µM concentration for 48 hours at 37ºC, showing 48 h cytotoxicity expressed as % normalized viability.

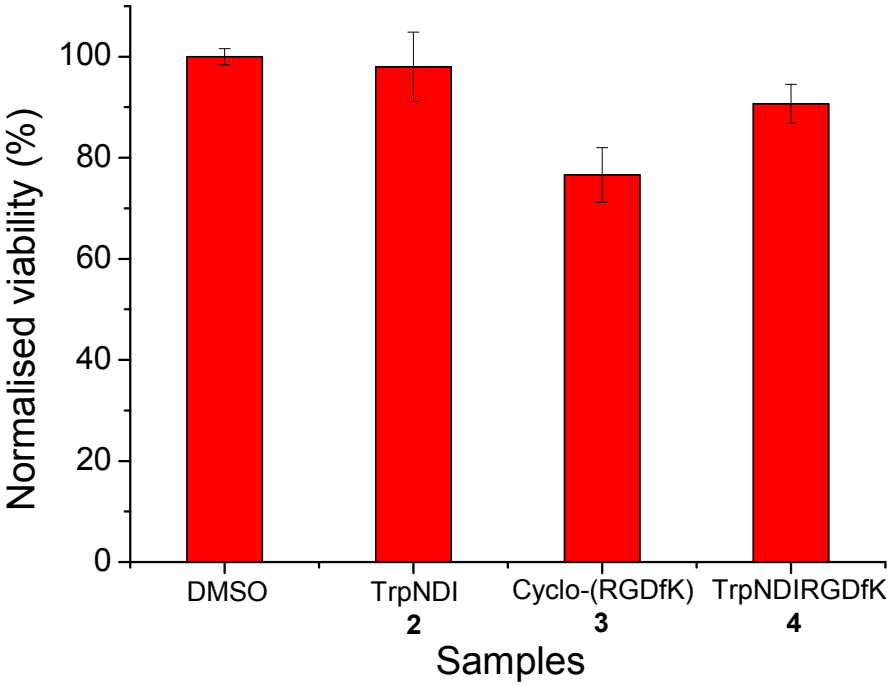


Figure S32. MTT assays of compound 2, 3 and 4 in PC-3 cell line, at 100µM concentration for 48 hours at 37ºC, showing 48 h cytotoxicity expressed as % normalized viability.