Self-assembly of amphipathic αaβ-tripeptide into cationic spherical particles for intracellular delivery

Raffaella Bucci, [§a] Priyadip Das, [§b,c] Filomena Iannuzzi,[d] Marco Feligioni,[d,e] Raffaella Gandolfi,[a] Maria Luisa Gelmi,[a] Meital Reches*[b] and Sara Pellegrino*[a]

[a] University of Milano, Department of Pharmaceutical Sciences, Milano, Italy
[b] The Hebrew University of Jerusalem, Institute of Chemistry, Jerusalem, Israel
[c] SRM University, SRM Nagar, Department of Chemistry, Potheri, Kattankulathur, Chennai, Tamil Nadu, India.
[d] Laboratory of Neuronal Cell Signaling, EBRI Rita Levi-Montalcini Foundation, Rome, Italy
[e] Department of Neurorehabilitation Sciences, Casa Cura Policlinico, Milan, Italy

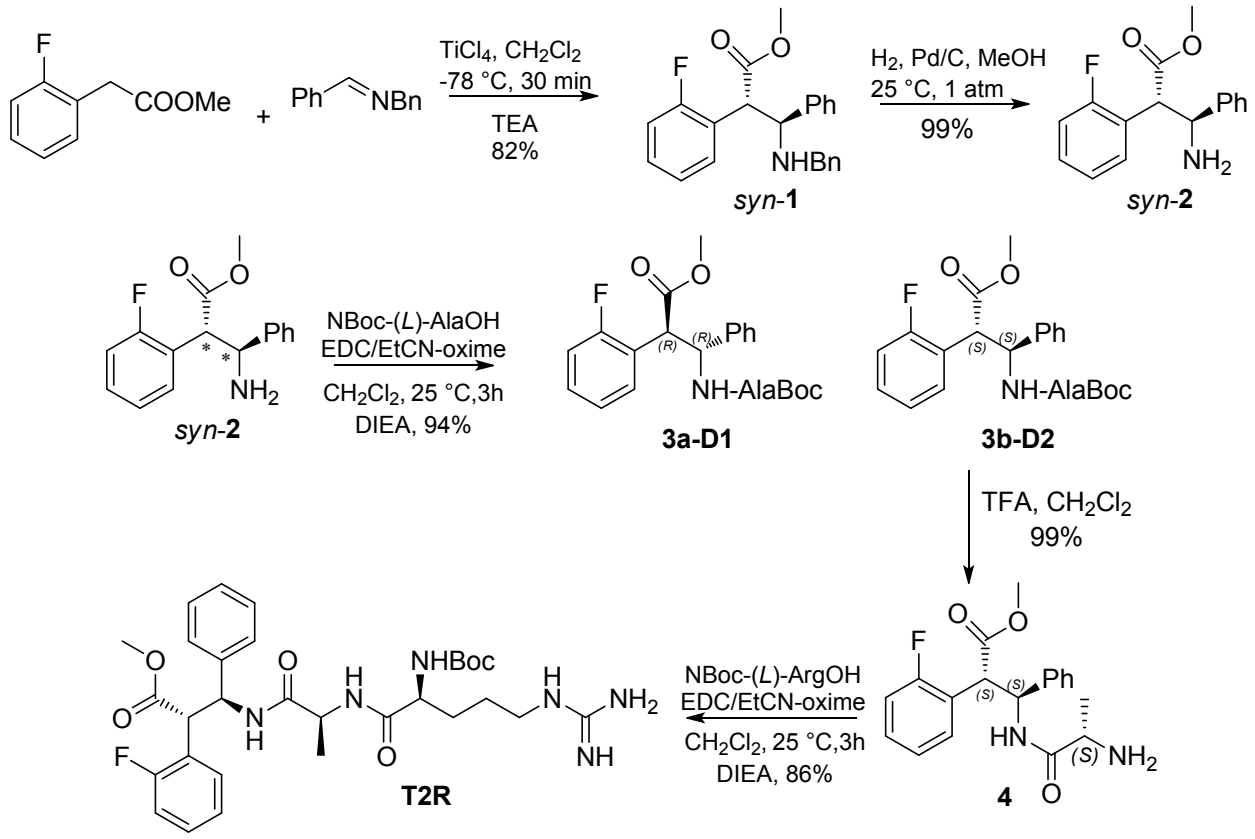
Supporting Information

Contents

General information

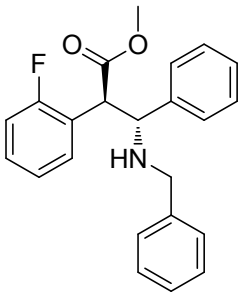
Chemicals were obtained from Sigma Aldrich and used without further purification. HPLC analyses were carried out on Jasco PU-980 pump equipped with a UV–vis detector Jasco UV-975 (wavelength: 220 nm) using Phenomenex LUNA 5µ C18 250 x 4,60 mm as column. Melting points were determined in a Stuart Scientific melting point apparatus in open capillary tubes and are uncorrected. ESI mass spectra were recorded on a LCQ Advantage spectrometer from Thermo Finningan and a LCQ Fleet spectrometer from Thermo Scientific. The NMR spectroscopic experiments were carried out either on a Varian OXFORD 300 MHz (300 and 75 MHz for ^{1}H and ^{13}C, respectively) or Bruker Avance 300 MHz spectrometers (300 and 75 MHz for ^{1}H and ^{13}C, respectively). Optical rotations were measured on a Perkin–Elmer 343 polarimeter at 25°C (concentration in g/100 mL). Chemical shifts δ are given in ppm relative to the internal standard, and the coupling constants J are reported in Hertz (Hz).

Synthetic pathway of T2R



*Scheme 1: Synthesis of **T2R***

Synthesis of amino acid *syn*-1



Operating under N_2 atmosphere, methyl (2-F-phenyl)-acetate (315 mg, 1.9 mmol) and N-benzylidene-1-phenylmethanamine (353 mg, 1.8 mmol) were dissolved in CH_2Cl_2 (17 mL) and the mixture was cooled at -78°C. $TiCl_4$ (820 mg, 0.47 mL, 4.3 mmol), dissolved in CH_2Cl_2 (4 mL), was added in 15 minutes and the mixture was stirred for 30 minutes. Then, triethylamine (280 μL, 2 mmol) was added and stirring was continued for a further 15 minutes. A saturated solution of K_2CO_3 was dropped and the temperature was raised at 25 °C.

The white solid was filtered, the organic layers were separated and the aqueous layer was extracted with CH_2Cl_2. The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated. The crude residue was purified by flesh chromatography using Hexane/EtOAc (9:1) as eluent, affording the amino acid *syn*-**1** (597 mg, 1.6 mmol, 86%).

M.p.: 78.3 °C.

MS (ESI): *m/z* calcd for $[C_{23}H_{22}FNO_2]$: 363.16; found: *m/z* 364.1 $[M+H]^+$.

^{1}H NMR (300 MHz, $CDCl_3$): δ 7.48-7.00 (m, 9H), 4.30 (AB system, J = 9.5, 2H), 3.55 (AB system, J = 13.9, 2H), 3.38 (s, 3H), 1.65 (br, 1H).

^{13}C NMR (50 MHz, $CDCl_3$): δ = 50.7, 50.9, 52.0, 63.6, 115.7 (d, J = 22.9), 123.5, 124.5, 124.6, 127.1, 127.9, 128.2, 128.4, 128.6, 129.4, 129.5, 129.7, 143.4, 161.5 (d, J = 246), 171.98.

^{19}F NMR (282 MHz, $CDCl_3$): δ = -117.4

IR (KBr) ν_{max} = 1738.0, 1490.5, 1155.1 cm^{-1}

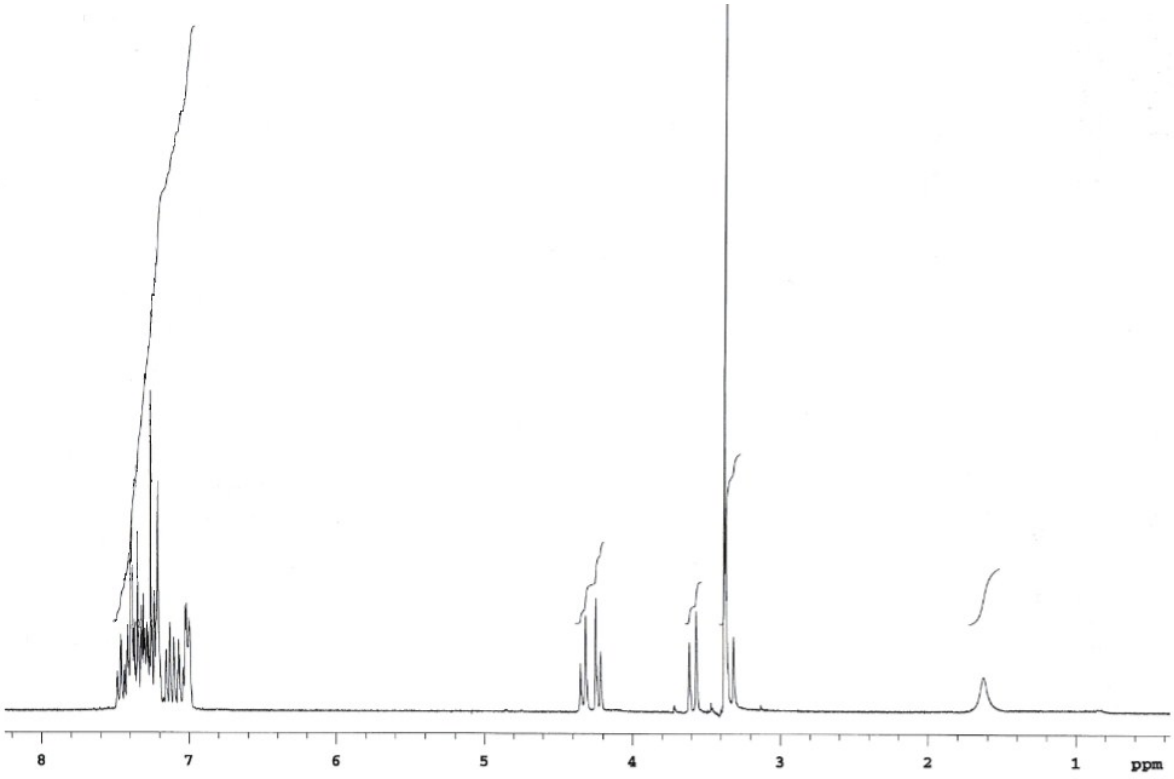


Figure 1: ^{1}H *NMR of compound syn-**1** (300 Mz,* $CDCl_3$*)*

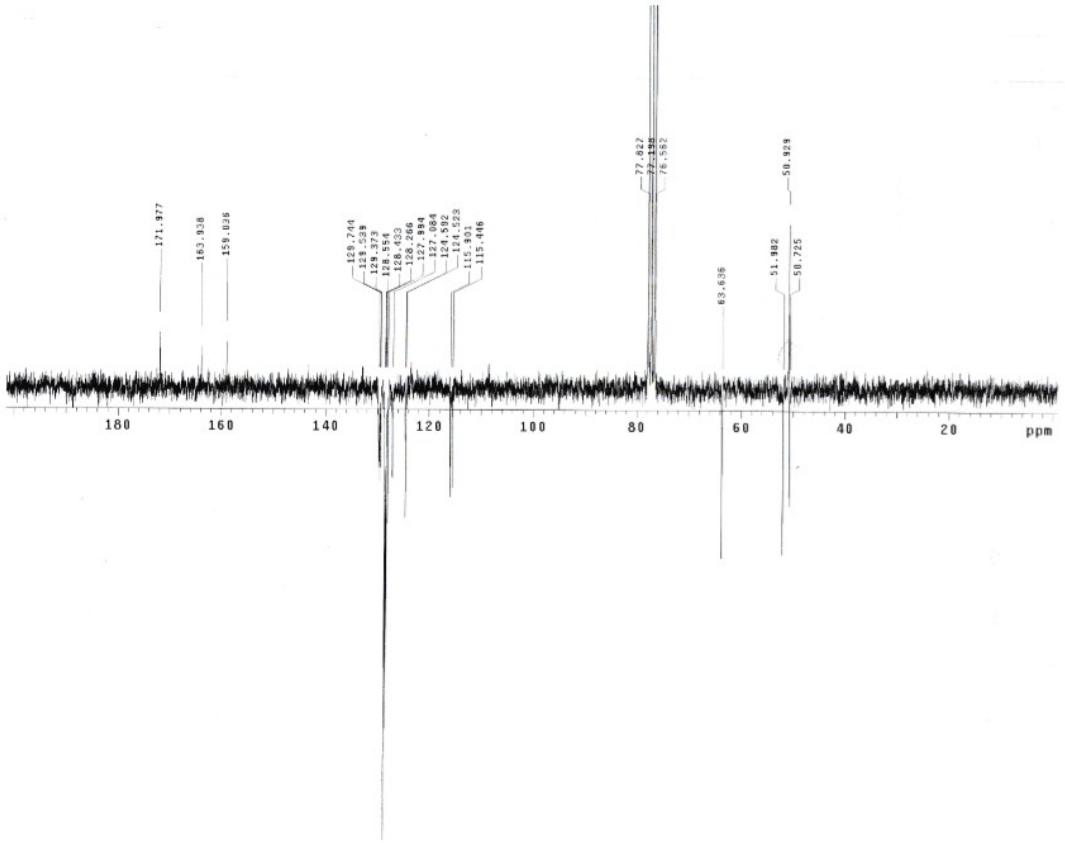


*Figure 2: ^{13}C NMR of compound syn-**1** (50 MHz, CDCl3)*

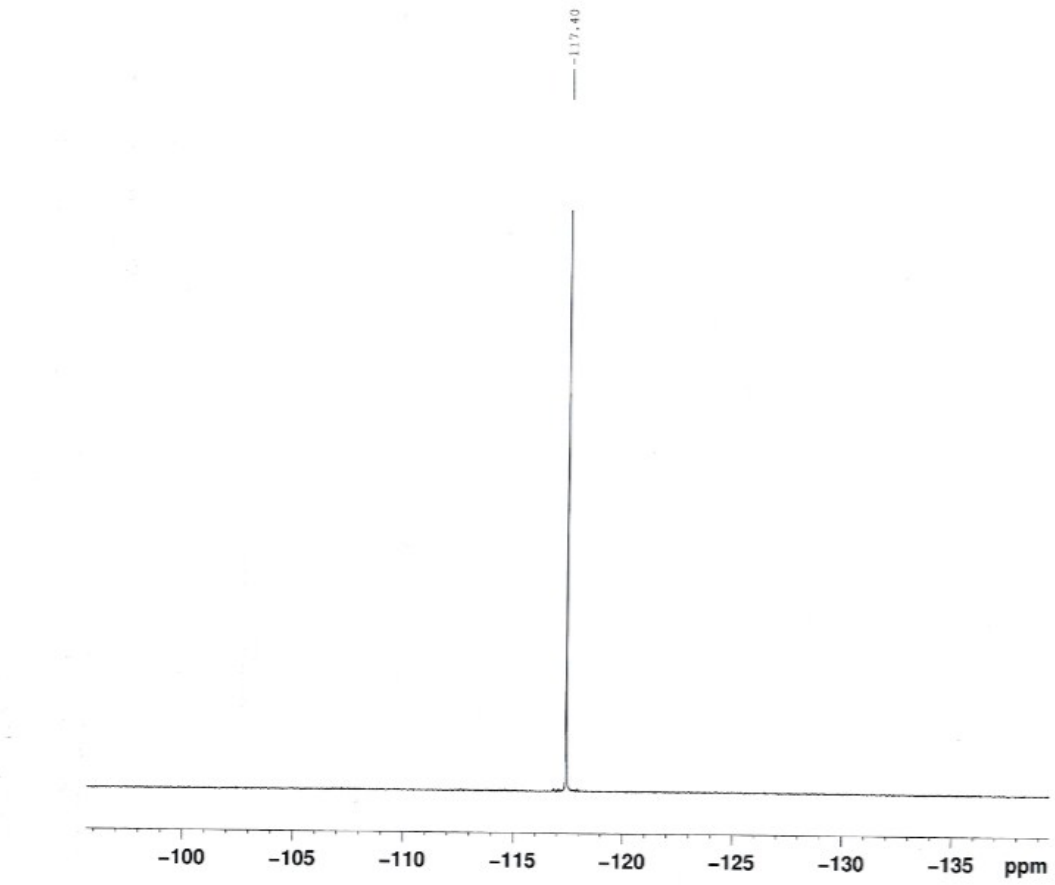
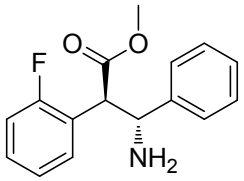


*Figure 3: ^{19}F NMR of compound syn-**1** (282 MHz, CDCl3)*

Synthesis of amino acid *syn*-2

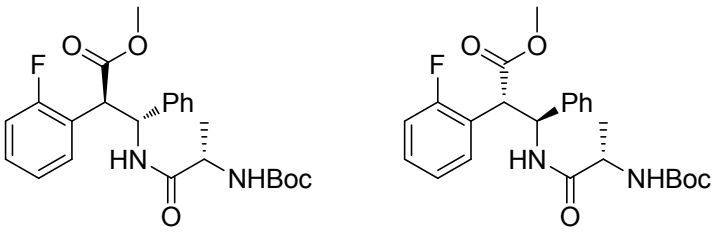


Amino acid *syn*-**2** was obtained by deprotection of amino group on amino acid *syn*-**1**, by catalytic reduction. Amino acid *syn*-**1** (6.0 g, 16.6 mmol) was dissolved in MeOH (400 mL). Pd/C (3.7 g, 3.5 mmol) was added and the mixture was stirred under H_2 atmosphere at room temperature for 24 h.

The mixture was filtered on celite pad, the filtrated solution was concentrated under reduced pressure and the crude mixture was recristallyzed from methanol, affording pure product *syn*-**2** (4.5 g, 16.4 mmol, quantitative yield)[1].

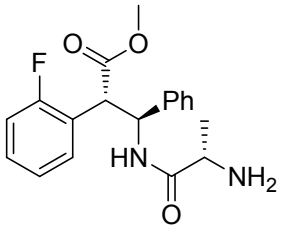
[1] For complete characterization: A. Bonetti, S. Pellegrino, P. Das, S. Yuran, R. Bucci, N. Ferri, F. Meneghetti, C. Castellano, M. Reches, M. L. Gelmi *Org. Lett*., **2015**, *17*, 4468–4471.

Synthesis of compounds D1 and D2



Boc-Alanine-OH (3.1 g, 16.3 mmol) was dissolved in CH_2Cl_2 (150 mL), the solution was cooled to 0°C and then EDC (3.1 g, 16.1 mmol) and EtCN-oxime (2.3 g, 16.1 mmol) were added. The mixture was stirred at 0°C for 1h. After this time, racemic amino acid *syn*-**2** (4 g, 14.7 mmol), dissolved in DCM (100 mL), and DIEA (2.56 mL, 14.7 mmol) were added and the mixture was stirred at room temperature for 3 h. A saturated solution of $NaHCO_3$ (200 mL) was then added. The aqueous layer was separated and the organic one was washed first with a saturated solution of NH_4Cl (200 mL) and then with a saturated solution of NaCl (200 mL). The organic layer was dried over Na_2SO_4 and the solvent was removed under reduced pressure. Dipeptides **3a-D1** and **3b-D2** were obtained as a mixture of diastereoisomers (6.1 g, 94%) and were separated by column chromatography on silica gel using n-hexane/AcOEt (5:1) as eluent (**3a-D1**: 45%, **3a-D2**: 43%)[1].

Synthesis of compound 4



Compound **3b-D2** (100 mg, 0.23 mmol) was dissolved in CH_2Cl_2 (4 mL) and cooled to 0°C. After 5 minutes, TFA (4 mL) was slowly dropped to the solution. The mixture was stirred at room temperature for 1 h. The solvent was removed under reduced pressure affording dipeptide **4** as trifluoroacetic salt.

The product was washed first with saturated solution of $NaHCO_3$ and then with saturated solution of NaCl. After the separation of the aqueous layer, the organic layer was dried over Na_2SO_4 and the solvent was removed under reduced pressure, affording product **4** (80 mg, quantitative yield).

$[\alpha]_D^{25}$ = +76.8 ($CHCl_3$, *c* 1.0)

M.p.: 87.7 °C.

MS (ESI): *m/z* calcd for $[C_{19}H_{21}FN_2O_3]$: 344.15; found: *m/z* 345.3 $[M+H]^+$.

^{1}H NMR (300 MHz, $CDCl_3$): δ 8.02 (d, *J* = 9.5, 1H), 7.57 (ddd, *J* = 1.5, *J* = 7.3, *J* = 15.1 1H), 7.41-6.98 (m, 9H), 5.67 (t, *J* = 10.1, 1H), 4.52 (d, *J* = 10.5, 1H), 3.49 (s, 3H), 3.40-3.80 (m, 2H), 0.84 (d, *J* = 6.9, 3H)

^{13}C NMR (75 MHz, $CDCl_3$): δ = 19.6, 49.0 (d, *J* = 3.0), 50.0, 52.2, 54.3, 115.2 (d, *J* = 21.8), 122.5 (d, *J* = 13.8), 124.5 (d, *J* = 3.5), 127.4, 127.9, 128.6, 129.5 (d, *J* = 8.1), 129.8 (d, *J* = 3.4), 139.6, 161.1 (d, *J* = 245.3), 171.0, 172.1.

IR (KBr) ν_{max} = 1736.54, 1670.79, 1591.76, 1494.62 cm^{-1}

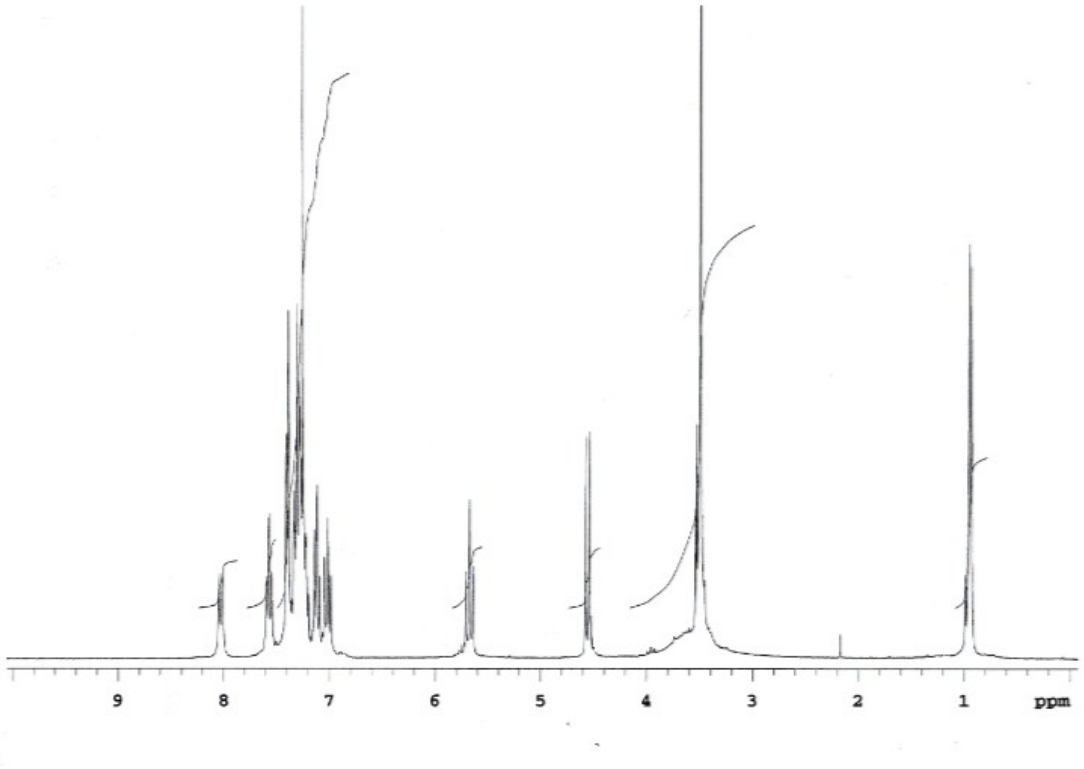


*Figure 4: ^{1}H NMR of compound **4** (300 MHz, CDCl3)*

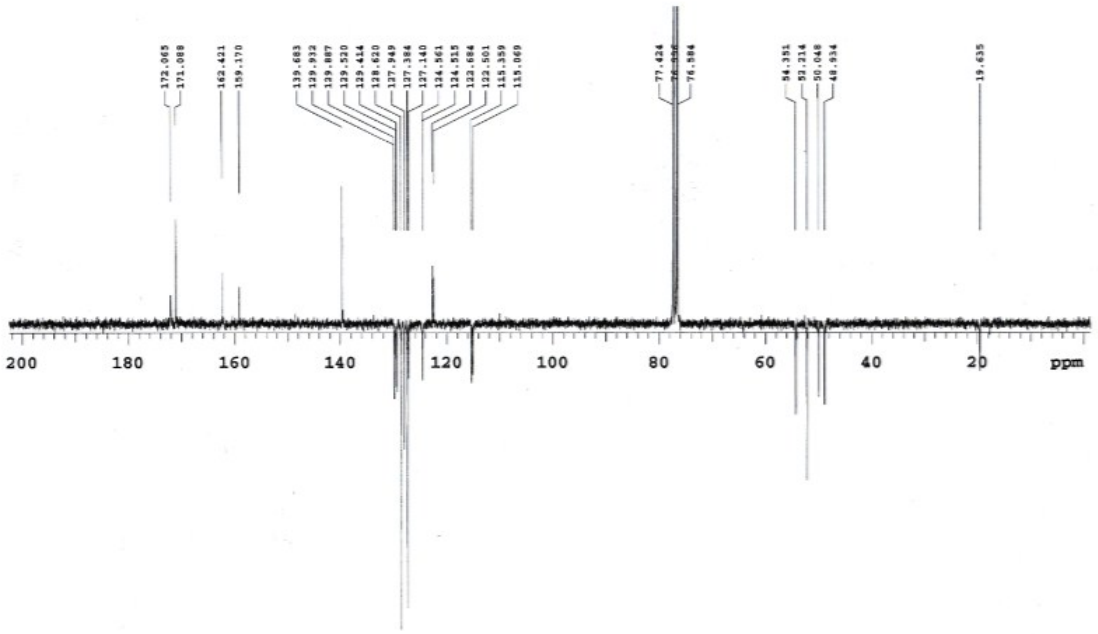
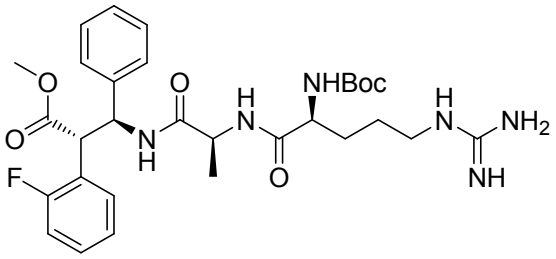


*Figure 5: ^{13}C NMR of compound **4** (50 MHz, CDCl3)*

Synthesis of compound T2R



Boc-NH-Arginine (82 mg, 0.25 mmol) was dissolved in CH_2Cl_2 (3 mL), the solution was cooled to 0° C and then EDC (52 mg, 0.27 mmol) and EtCN-oxime (40 mg, 0.27 mmol) were added. The mixture was stirred at 0 °C for 1 h. Then dipeptide **4** (85 mg, 0.25 mmol), dissolved in DCM (1 mL), and DIEA (1 eq., 43 μL, 0.247 mmol) were added and the mixture was stirred at room temperature overnight.

A saturated solution of NH_4Cl was added. The aqueous layer was separated and organic layer was washed first with a saturated solution of $NaHCO_3$ and then with saturated solution of NaCl.

The organic layer was dried over Na_2SO_4 and the solvent was removed under reduced pressure. The crude mixture was recrystallized from DCM/Et_2O (1:5), affording pure compound **T2R** (127.5 mg, 0.21 mmol, 86%).

$[\alpha]_D^{25}$ = +35.0 (CH_3OH, *c* 0.4)

M.p.: 141.7 °C.

MS (ESI): *m/z* calcd for $[C_{30}H_{41}FN_6O_6]$: 600.31; found: *m/z* 601.2 $[M+H]^+$.

^{1}H NMR (300 MHz, CD_3CN): δ = 8.31 (br, 1H), 7.74 (d, *J* = 8.8, 1H), 7.61(ddd, *J* = 1.59, *J* = 7.6), 7.50-7.12 (m, 9H), 6.62 (br, 3H), 5.66-5.69 (m, 2H), 4.58 (d, *J* = 11.3, 1H), 4.02-3.98 (m, 2H), 3.42 (s, 3H), 3.12 (m, 2H), 1.71 (br, 1H), 1.51 (m, 2H), 1.41 (s, 9H), 1.33 (m, 1H), 0.91 (d, *J* = 7.3, 3H).

^{13}C NMR (75 MHz, CD_3CN): δ = 16.9, 23.8, 27.5, 28.6, 39.9, 49.5, 50.1, 51.7, 53.1, 53.3, 79.1, 115.3 (d, *J* = 14.7), 122.9 (d, *J* =14.4), 124.2 (d, *J* = 3.2), 127.6, 127.7, 128.4, 129.6 (d, *J* = 8.4), 130.0 (d, *J* = 2.88), 140.7, 156.0, 157.5, 161.0 (d, *J* = 245.8), 171.0, 171.6, 171.9.

IR (KBr) ν_{max} = 3401.72, 1737.96, 1660.48, 1529.72, 1455.85 cm^{-1}

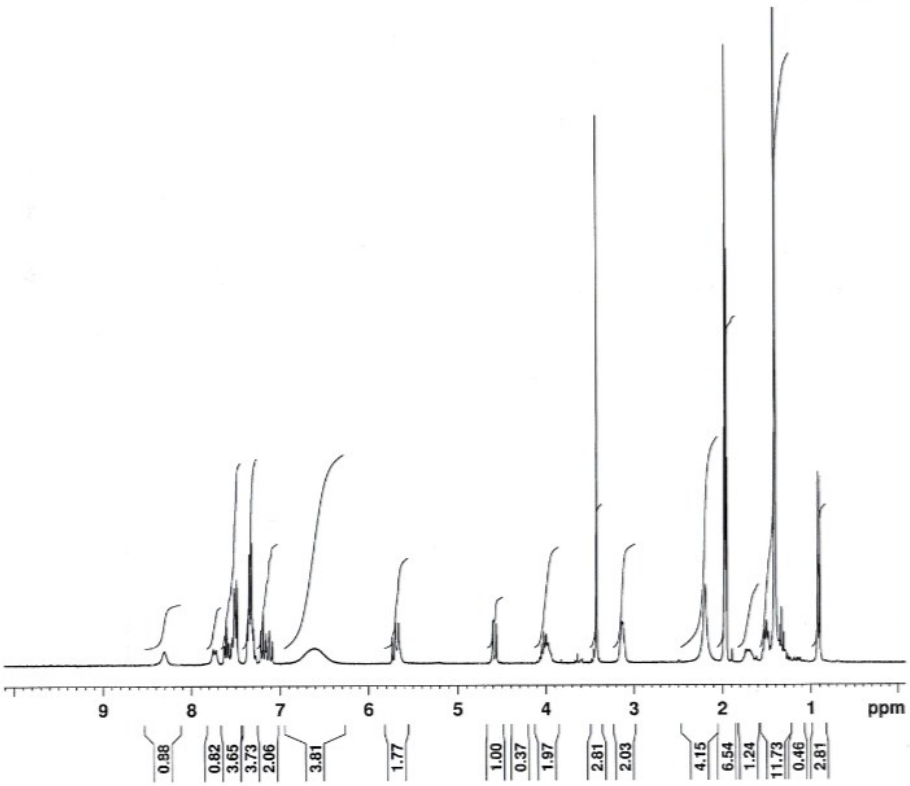


*Figure 6: [1]H NMR of compound **T2R** (300 MHz, CD3CN)*

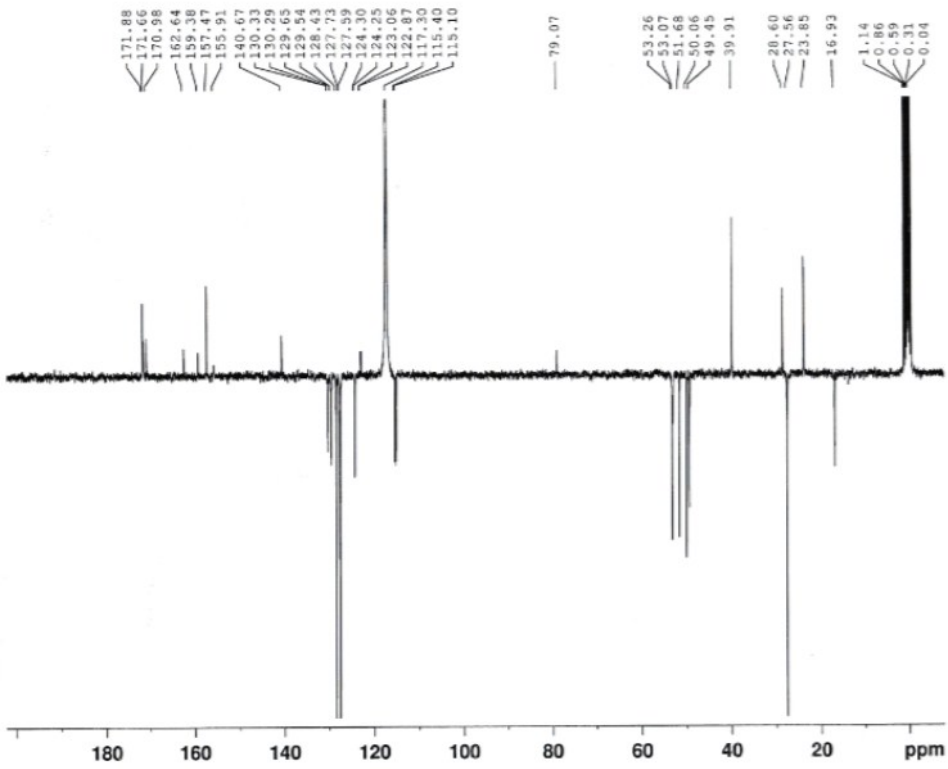


*Figure 7: [1]H NMR of compound **T2R** (7 MHz, CD_3CN)*

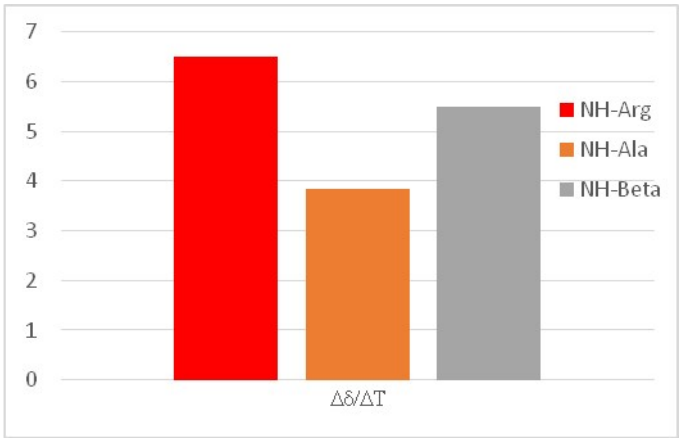
NMR analysis of compound T2R

^{1}H, ^{13}C, bidimensional and dynamic-NMR experiments were carried out in CD_3CN (17 mM solution, 293 K) using a 300 and 75 MHz instrument, respectively.

AA	atom		^{1}H NMR δ (CD_3CN, 300 MHz)	Molteplicity J (Hz)	^{13}C NMR δ (CD_3CN, 75 MHz)	Noesy tmix=300ms
Arg-1	CO				171.88	
	CH		3.98	overlapped	53.07	NH_{Ala} (s); $CH_2\alpha$ (s); $CH_2\beta$ (s); $CH_2\gamma$ (m); NH_{Arg} (m); Boc (s)
	CH_2 α		1.71 1.33		39.91	CH_{Arg} (s); $CH_2\gamma$ (w)
	CH_2 β		1.51	overlapped	23.85	$CH_2\gamma$ (s); CH_{Arg} (s); NH_{Arg} (m)
	CH_2 γ		3.13		28.60	CH_{Arg} (m); $CH_2\alpha$ (w);
	NH $_{Guanidinium}$		8.31	br		$CNH_{Guanidinium}$ (s); NH_2 $_{Guanidinium}$ (s)
	CNH $_{Guanidinium}$		6.62	br	155.88	NH_2 $_{Guanidinium}$ (s); NH $_{Guanidinium}$ (s)
	NH_2 $_{Guanidinium}$		6.62	br		
	NH		5.66	overlapped		CH_{Arg} (m); $CH_2\beta$ (m)
	Boc	CO			157.5	
		C			79.07	
		CH_3	1.41	s	27.56	$CH_2\alpha$ (s)
Ala-2	CO				171.66	
	CH		4.02	overlapped	50.06	CH_{3Ala} (s); NH_{Ala} (s); NH_{Beta3} (s)
	Me		0.91	d, J = 7.3	16.93	NH_{Beta3} (s); NH_{Ala} (s); CH_{Ala} (s)

	NH	7.53	overlapped	7.53	CH_{3Ala} (s); CH_{Ala} (s);
Beta-3	CO			170.98	
	2	4.58	d, J = 11.3	49.45	CH_{3Beta} (s); NH_{Beta} (s); CH_{F-6} (m); Ph (m); OMe (vw)
	3	5.69	overlapped	53.96	CH_{2Beta} (s); NH_{Beta} (m); CH_{F-6} (m); Ph (m); OMe (vw)
	Arom	7.61 $_{F-6}$ 7.50-7.30 7.33 $_{F-4}$ 7.19 $_{F-5}$ 7.12 $_{F-3}$	ddd, J = 1.6, J = 7.6 overlapped overlapped overlapped overlapped	C_{F-6}130.0 (d, J = 2.9) 140-128.4-127.7-127.6 C_{F-4}129.6 (d, J = 8.4) C_{F-5}124.2 (d, J = 3.2) C_{F-3}115.3 (d, J = 14.7) C_{F-1} 122.9 (d, J =14.4) C_{F-2} 161.0 (d, J = 245.8)	CH_{3Beta} (s); CH_{2Beta} (s); NH_{Beta} (s)
	NH	7.74	d, J = 8.8		CH_{Ala} (w); CH_{3Beta} (m); CH_{2Beta} (s); Ph (s)
	OMe	3.42	s	51.68	CH_{3Beta} (vw); CH_{2Beta} (vw)

The ^{1}H-NMR experiments at variable temperatures excluded the presence of any intramolecular hydrogen bonds, as it is shown in the graph below.



*Graph 1: $\Delta\delta/\Delta T$ values for **T2R** in CD_3CN*

DLS analysis of T2R aggregates

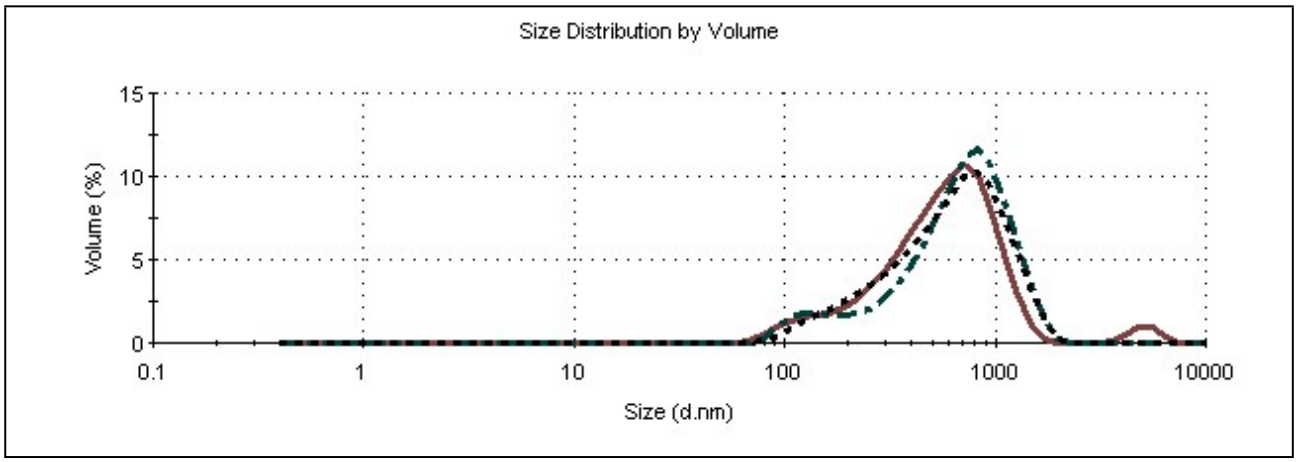
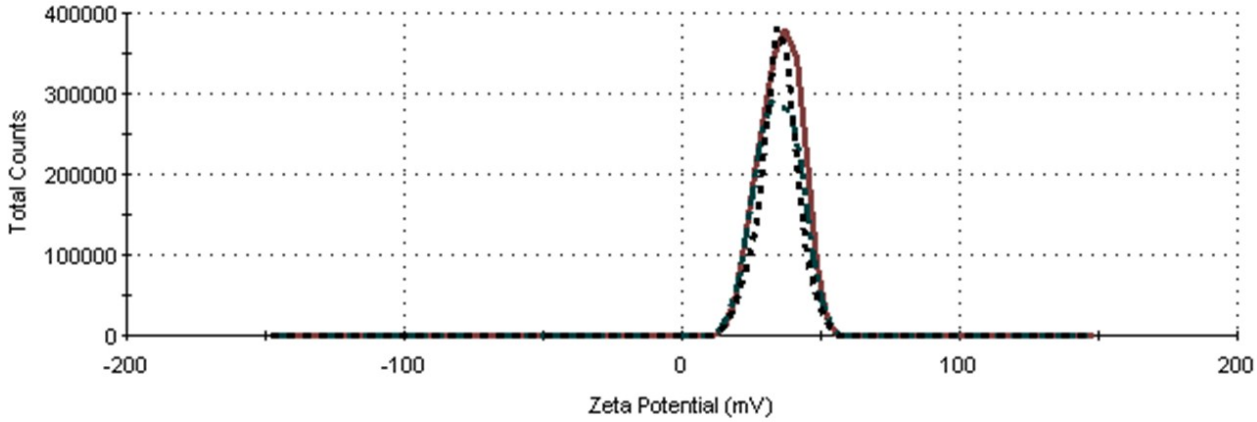


Figure 8

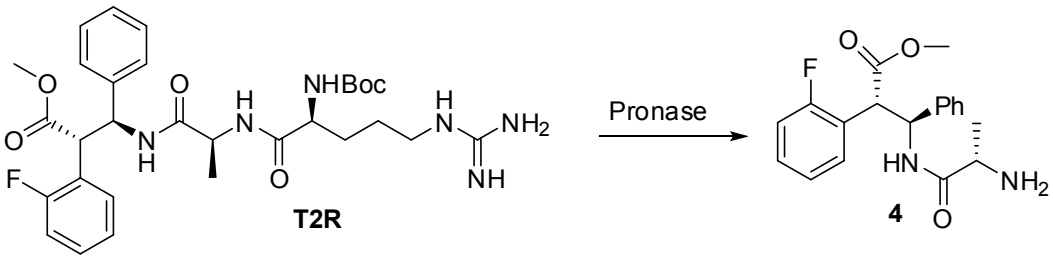
Zeta potential analysis of T2R aggregates



Zeta Potential (mV):	34.7
Zeta Deviation (mV):	6.81
Conductivity (mS/cm):	0.298
Result quality :	Good
Temperature (°C):	25.0
Count Rate (kcps):	127.6
Cell Description:	Clear disposable zeta cell
Zeta Runs:	12
Measurement Position (mm):	2.00
Attenuator:	6

Figure 9

Protease stability of T2R



Enzymatic degradation studies were carried out in PBS buffer (0.01M, pH 7) in presence of $CaCl_2$ (10 mM). **T2R** (5 m g/ml) was incubated at 25°C under magnetic stirring in absence or in presence of Pronase from *Streptomyces griseus* (0-0.5-1-10 mg/ml). Aliquots (50 µl) of the sample were analyzed at different times from 0 h to 24 h. The reaction was quenched adding 10 µl of acetic acid (25% v/v) and 150 µl of a mixture H_2O: MeCN (60:40). The degradation of **T2R** was monitored by RP- HPLC (Phenomenex LUNA 5µ C18 250 x 4,60 mm) using as eluents 60% water with 0,1% TFA and 40% MeCN (flow rate of 0.8 ml/min). Detection was performed by UV measurement at 220 nm. To evaluate the stability of the enzyme, 1mg/ml of protease was stirred at 25°C. After 24 h, 5 mg/ml of **T2R** was added and aliquots were analyzed as previously described. The partial thermal inactivation of Pronase (1mg/ml) was observed after 24 h at 25°C, in fact adding **T2R** to the reaction system, the degradation carried out with a lower rate (t= 3h molar conversion= 46%) .

Time (h)	Molar conversion (%)
0	0
2	39
4	46
7	65
24	76

*Table **2**: Hydrolysis of **T2R** (5 mg/mL) with Pronase (1 mg/mL) at 25°C*

HPLC chromatogram of **T2R.**

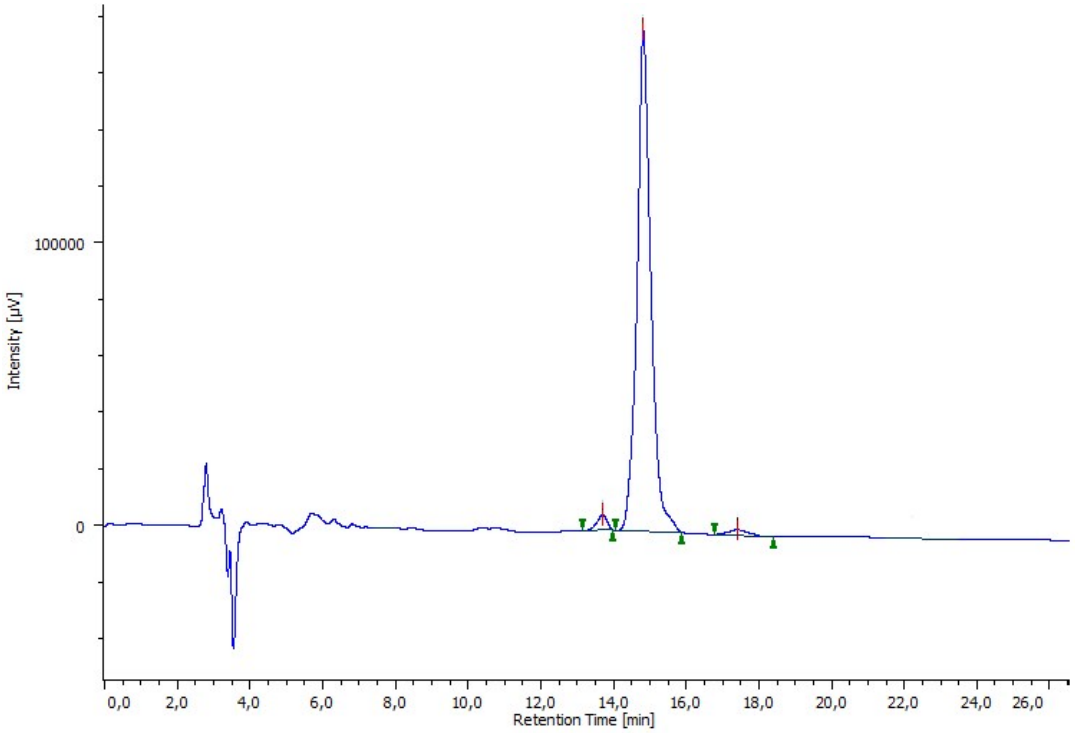


Figure 9

HPLC chromatogram of product **4**

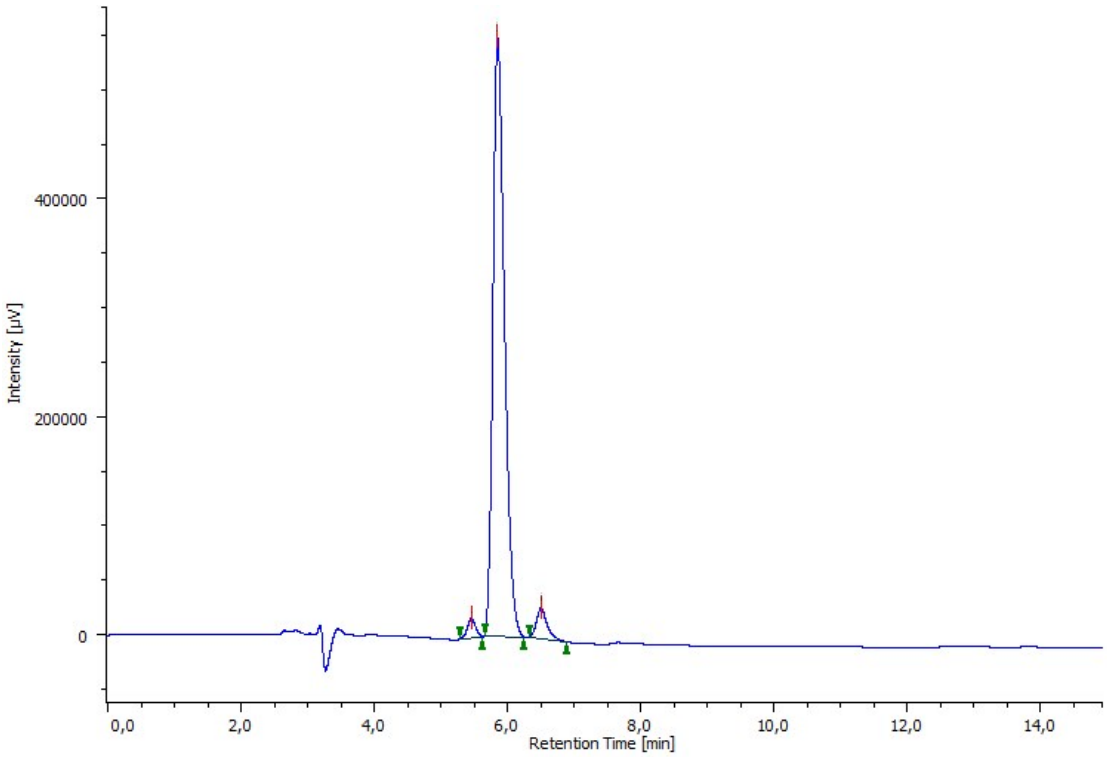


Figure 10

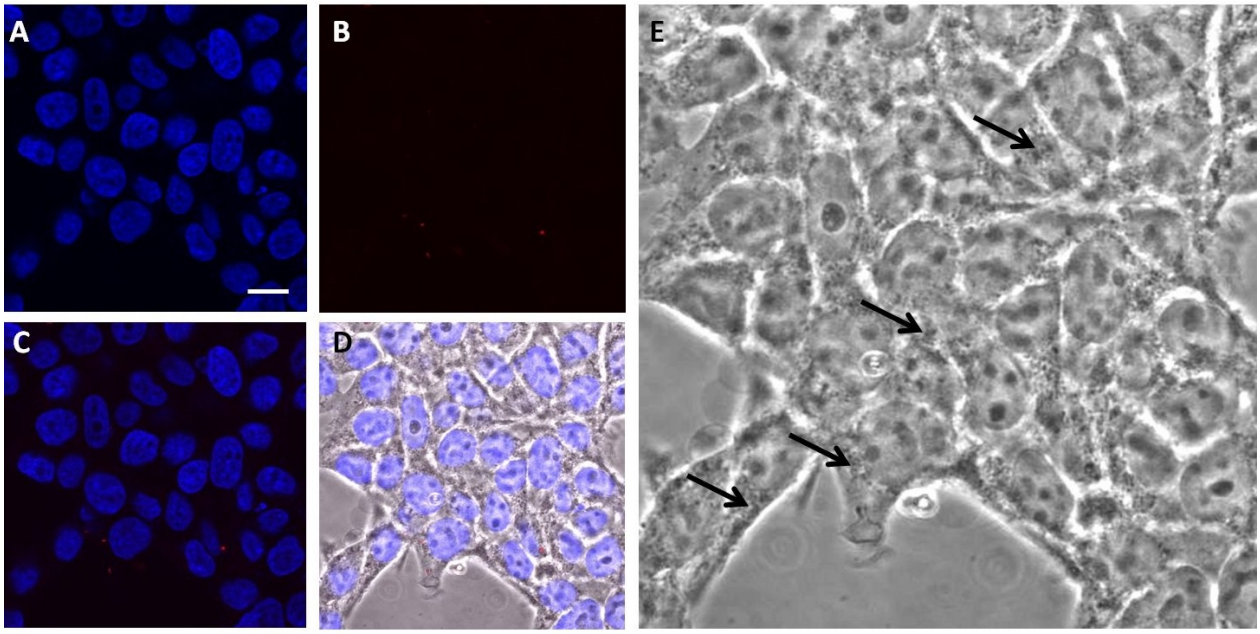


Figure 11. Confocal microscopy images of HEK cells treated for 2 h with **RhB-T2R**. A) Representative image of HEK cell culture nuclei stained with DAPI. B) Representative image of **RhB-T2R** in the HEK cells. C) Merged image of DAPI and **RhB-T2R** images. D) Merged image of DAPI, **RhB-T2R** and bright field images. E) Representative bright field image of HEK cells treated with **RhB-T2R**. Particles in the intracellular compartments are indicated by black arrows. Scale bar = 10 µm