Tuning the Affinity with Transmembrane Protein by Constructing Peptide-Conjugated *cis/trans* Isomers Based on Molecular Scaffold

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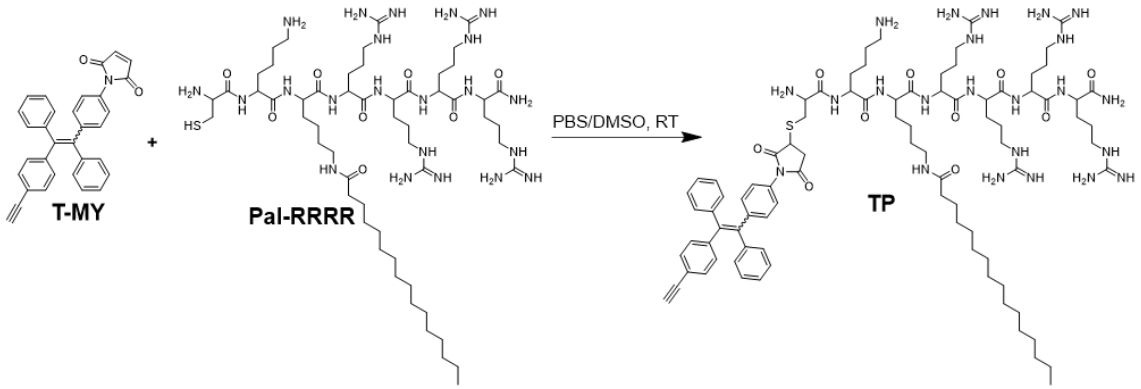
1. Materials and methods.

All peptides were purchased from GL Biochem Ltd. (Shanghai, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), cuprous bromide and sodium ascorbate (SA) were bought from Beijing InnoChem Science & Technology Co., Ltd. (Beijing, China). The Actin-Tracker Red-555 was purchased from Beyotime Institute of Biothechnology. Deionized water (18.2 MΩ·cm) was obtained with Heal Force water purification system. High resolution mass spectra (HRMS) were recorded on a Thermo Scientific Q Exactive mass spectrometer system operating in an ESI-Obitrap mode. NMR spectra were measured on a Bruker Avance III HD 400 MHz NMR spectrometer. High performance liquid chromatography (HPLC) was performed by using Shimadzu LC-20A equipped with an Ultimate C18 column (10 μm, 250 × 4.6 mm) from Welch. UV-vis absorption spectra were taken on a Shimadzu UV-2600 spectrometer. All fluorescence measurements were performed on an Edinburgh FS5 Fluorescence Spectrophotometer. Confocal laser scanning microscopy images were obtained on a Zeiss LSM 880 confocal laser scanning microscope. MTT assay was obtained on an Infinite M200 PRO Microplate Reader (Tecan, Austria).

2. Synthesis and characterization.

2.1. Synthesis of **RTP**.

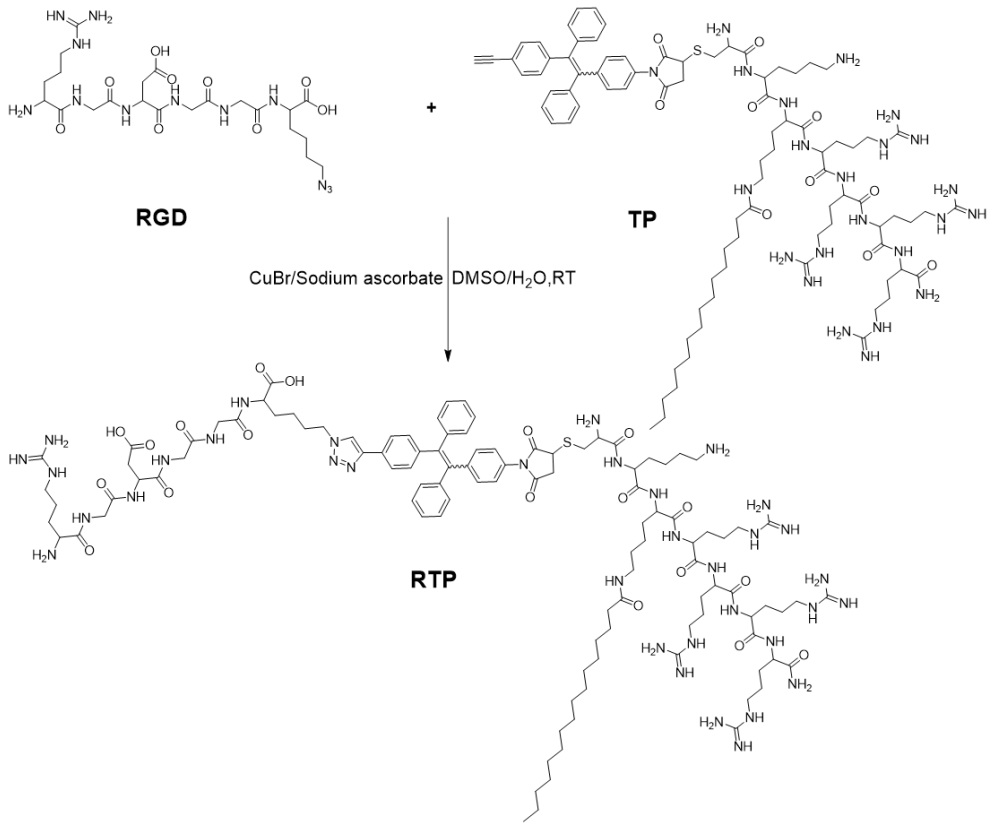
2.1.1. Synthesis of **TP**.



Scheme S1. Synthetic route of **TP**.

T-MY (9.0 mg, 20 μmol, 2 equiv.) was dissolved in DMSO and Pal-RRRR (12.4 mg, 10 μmol, 1 equiv.) was dissolved in PBS. The volume ratio of DMSO to PBS was 1:1. The reaction was stirred at room temperature under the protection of nitrogen and was monitored by HPLC. After the reaction was completed, the crude product was separated by semi-preparative HPLC. The product was freeze-dried to afford a light yellow solid (14.4 mg, yield: 85%). High-resolution mass spectra termed HRMS (ESI) m/z: $[M+3H]^{3+}$ calcd, 564.3461; found, 564.6793. $[M+4H]^{4+}$ calcd, 423.5114; found, 423.7609. $[M+5H]^{5+}$ calcd, 339.0106; found, 339.0101.

2.1.2. Synthesis of **RTP**.

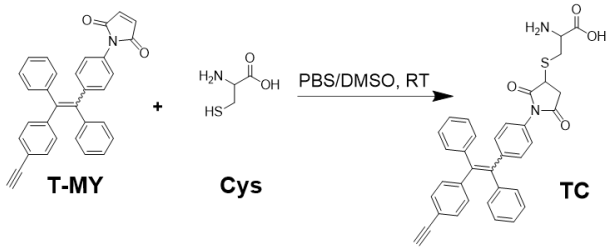


Scheme S2. Synthetic route of **RTP**.

RGD (6.1 mg, 10 μmol, 2 equiv.), TP (8.5 mg, 5 μmol, 1 equiv.), sodium ascorbate (2.0 mg, 10 μmol, 2 equiv.) and CuBr (1.5 mg, 10 μmol, 2 equiv.) were dissolved in DMSO/H_2O (v/v=1:1) and stirred at room temperature under the protection of nitrogen. The reaction was monitored by HPLC. After the reaction was completed, the crude product was separated by semi-preparative HPLC. The product was freeze-dried to afford a light yellow solid (5.5 mg, yield: 48%). HRMS (ESI) m/z: $[M+3H]^{3+}$ calcd, 769.1089; found, 769.4456. $[M+4H]^{4+}$ calcd, 577.0835; found, 577.3367. $[M+5H]^{5+}$ calcd, 461.8683; found, 462.0702. $[M+6H]^{6+}$ calcd, 385.0581; found, 385.2260.

2.2. Synthesis of **RTC**.

2.2.1. Synthesis of **TC**.

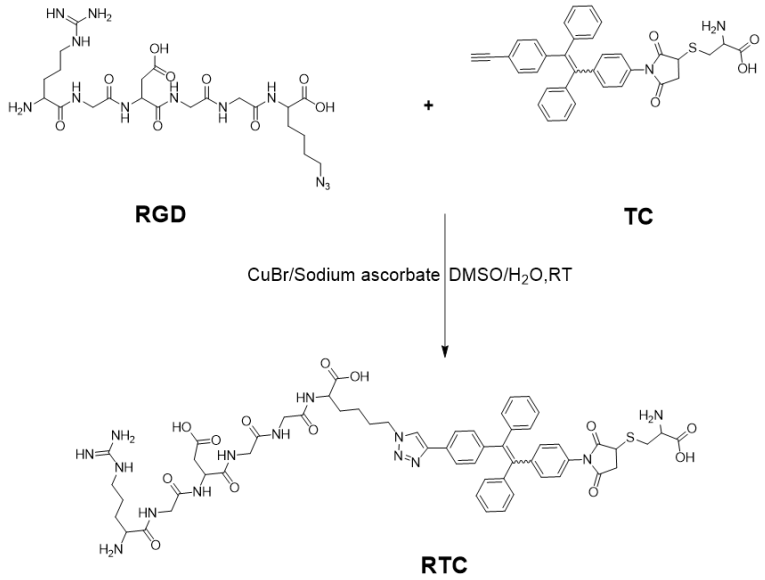


Scheme S3. Synthetic route of **TC**.

T-MY (9.0 mg, 20 μmol, 2 equiv.) was dissolved in DMSO and cysteine (Cys) (1.2 mg, 10 μmol, 1 equiv.) was dissolved in PBS. The volume ratio of DMSO to PBS was 1:1. The reaction was stirred at room temperature under the protection of nitrogen and was monitored by HPLC. After the reaction was completed, the crude product was separated by semi-preparative HPLC. The product was freeze-dried to afford a light yellow solid

(4.7 mg, yield: 83%). HRMS (ESI) m/z: $[M+H]^+$ calcd, 573.1843; found, 573.1838.

2.2.2. Synthesis of **RTC**.



Scheme S4. Synthetic route of **RTC**.

RGD (6.1 mg, 10 μmol, 2 equiv.), TC (2.9 mg, 5 μmol, 1 equiv.), sodium ascorbate (2.0 mg, 10 μmol, 2 equiv.) and CuBr (1.5 mg, 10 μmol, 2 equiv.) were dissolved in DMSO/H_2O (v/v=1:1) and stirred at room temperature under the protection of nitrogen. The reaction was monitored by HPLC. After the reaction was completed, the crude product was separated by semi-preparative HPLC. The product was freeze-dried to afford a light yellow solid (3.3 mg, yield: 56%). HRMS (ESI) m/z: $[M+H]^+$ calcd, 1187.4722; found, 1187.4722. $[M+2H]^{2+}$ calcd, 594.2397; found, 594.2397.

3. Experimental section

3.1. Theoretical calculation of binding energy

The cis-RTP was docking to the protein $\alpha_v\beta_3$ (PDBID: 1L5G) with ADT tools package provided by AutoDock 4.2, as well as the trans-RTP. The docking complex was embedded to a bilayer membrane. The bilayer membranes used was built by CHARMM-GUI input generator, and it was solvated in a TIP3P water model with 0.15 M KCl, in a size of 184.1 × 183.3 × 166.8 $Å^3$ containing about 575000 atoms. The ratio of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) to 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS) is almost 7:3 (total 892 lipids, including 624 DOPC and 268 DOPS, were used in the membranes. 6 DOPC and 2 DOPS were removed to stabilize the orientation and the depth of protein insertion in the membrane). Unbiased MD simulations by GROMACS simulation package with CHARMM36m force field were carried out to equilibrate the system for 2 ns, production simulation for 100 ns and the last 10 ns were used for analysis. The stable cis-RTP and trans-RTP structure were got from the MD simulations, to calculation the volume by Monte-Carlo method. The volumes were calculated based on the 0.001 e/bohr3 charge density envelope, which was a general rule proposed by Bader.

3.2. Isothermal titration calorimetry (ITC) experiments

All ITC measurements were performed on a MicroCal ITC200 microisothermal titration calorimeter at 37°C. The disrupted cell membranes were dripped into the sample cell 10 μL each time, a total of 20 drips. The concentration of the probe was 40 μM.

3.3. Cell culture

The SKOV3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 1% penicillin and streptomycin (PS) and 5% carbon dioxide at 37°C in a humidified environment. Before CLSM imaging, we first seeded SKOV3 cells into glass-bottom cell culture dishes. When the cells grew to about 70%, the cells were washed for 3 times with PBS. The fresh medium containing the probes was then added to the dishes.

3.4. Confocal laser scanning microscopy (CLSM)

The fluorescence signals of cells were obtained by using the 63x oil immersion objective of an LSM 880 confocal microscope from Zeiss. The probes were excited with a 405 nm laser and fluorescent signals were collected in the 450-550 nm range. FAM and Actin-Tracker Red-555 were selected and excited with a 543 nm laser. Their fluorescence signals were collected in the 550-600 nm range.

3.5. Cell adhesion assay

The SKOV3 cells suspension was incubated with 1 mL of 40 μM probes for 1 h, then they were centrifuged and the supernatant was discarded. After 4 h of incubation with fresh medium, the cells were imaged with CLSM and the number of cells was counted.

3.6. Cell wound scratch assay

When the SKOV3 cells in the glass bottom dish had grown to 95%, a scratch was created on the dish with a 10 μL pipette tip. Subsequently, the SKOV3 cells were incubated with 40 μM probes for 1 h. Scratches were imaged at 0 h and 24 h.

3.7. Cytotoxicity assay

When the SKOV3 cells reached 80% in the 96-well plate, different concentrations of probes were added to the corresponding wells and 5 replicate wells were set for each concentration. After 24 h of probe incubation, MTT in PBS (10 μL) was added to each well. After 4 h of incubation in the dark, the supernatant was aspirated and DMSO (150 μL) was added to each well. Finally, the absorbance of MTT at 570 nm was detected with an Infinite M200 PRO microplate reader.

4. Statistical analysis.

The quantitative data were expressed as mean ± standard deviation (SD). All the experiments were repeated for at least 3 times. The statistical analysis was conducted using a two-sided Student’s t-test or one-way ANOVA. P value < 0.05 was considered statistically significant.

Table S1. Peptide sequences used in this study.

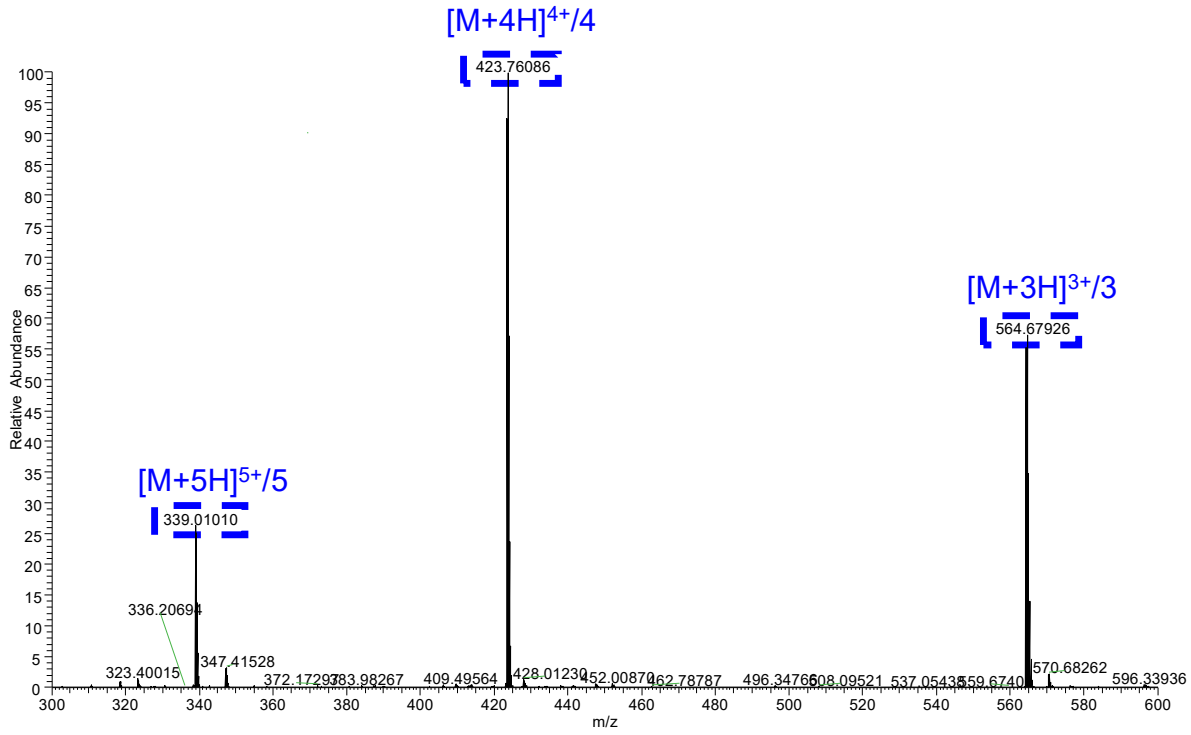
Species	Peptide sequence (N~C)
***cis*-RTP**	RGDGGK-TPE-CKK(Pal)RRRR
***trans*-RTP**	RGDGGK-TPE-CKK(Pal)RRRR
***cis*-RTC**	RGDGGK-TPE-C
***trans*-RTC**	RGDGGK-TPE-C

Table S2. The time program for separating *cis*-RTP and *trans*-RTP.

Time (min)	Solvent A (Water containing 1% trifluoroacetic acide)	Solvent B (Acetonitrile containing 1% trifluoroacetic acide)	Flow rate V
0	60%	40%	1 mL/min
20	50%	50%	1 mL/min

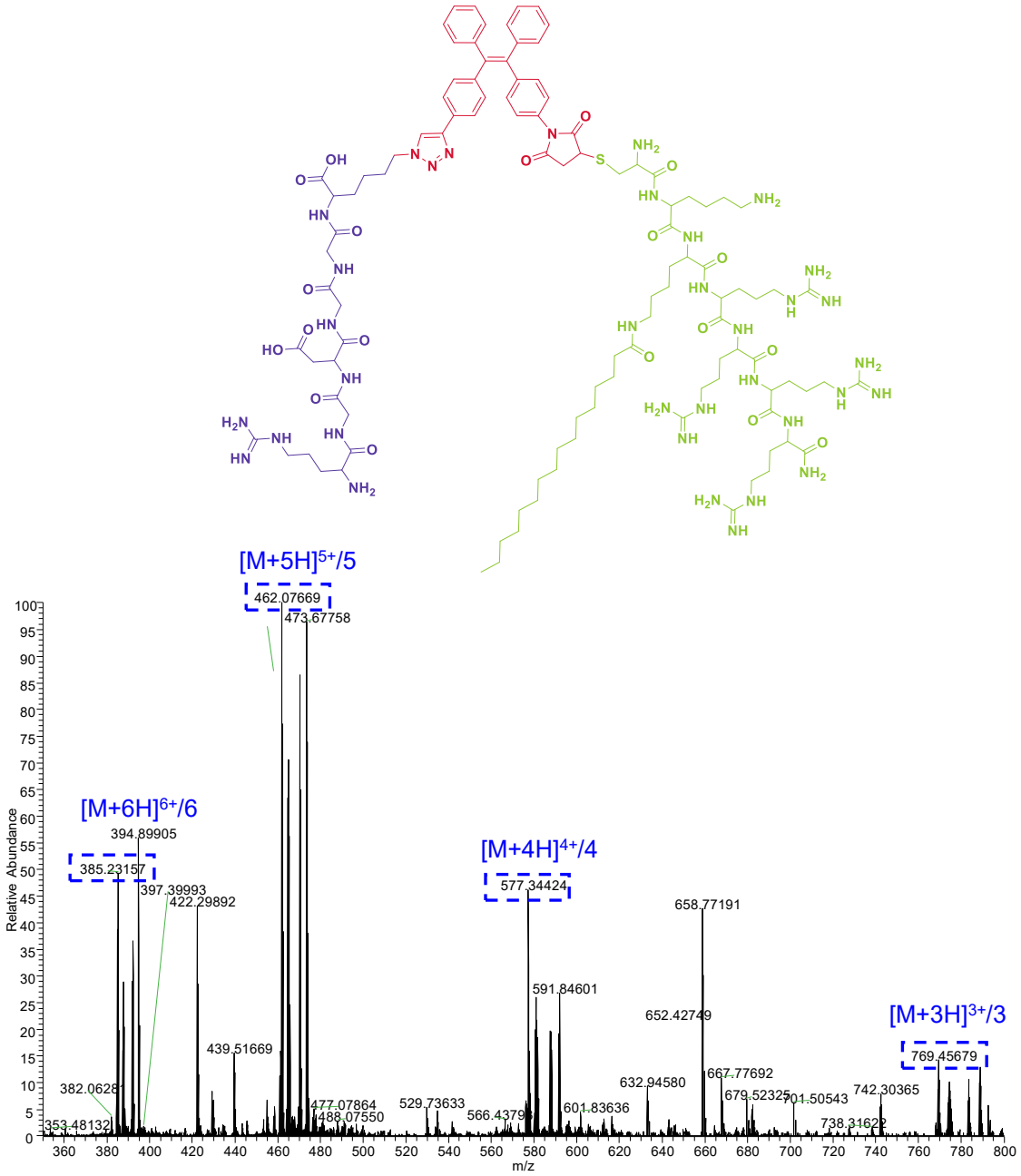
Table S3. The time program for separating *cis*-RTC and *trans*-RTC.

Time (min)	Solvent A (Water containing 1% trifluoroacetic acide)	Solvent B (Acetonitrile containing 1% trifluoroacetic acide)	Flow rate V
0	80%	20%	2 mL/min
20	60%	40%	2 mL/min
25	40%	60%	2 mL/min
30	10%	90%	2 mL/min
45	0%	100%	2 mL/min
50	0%	100%	2 mL/min



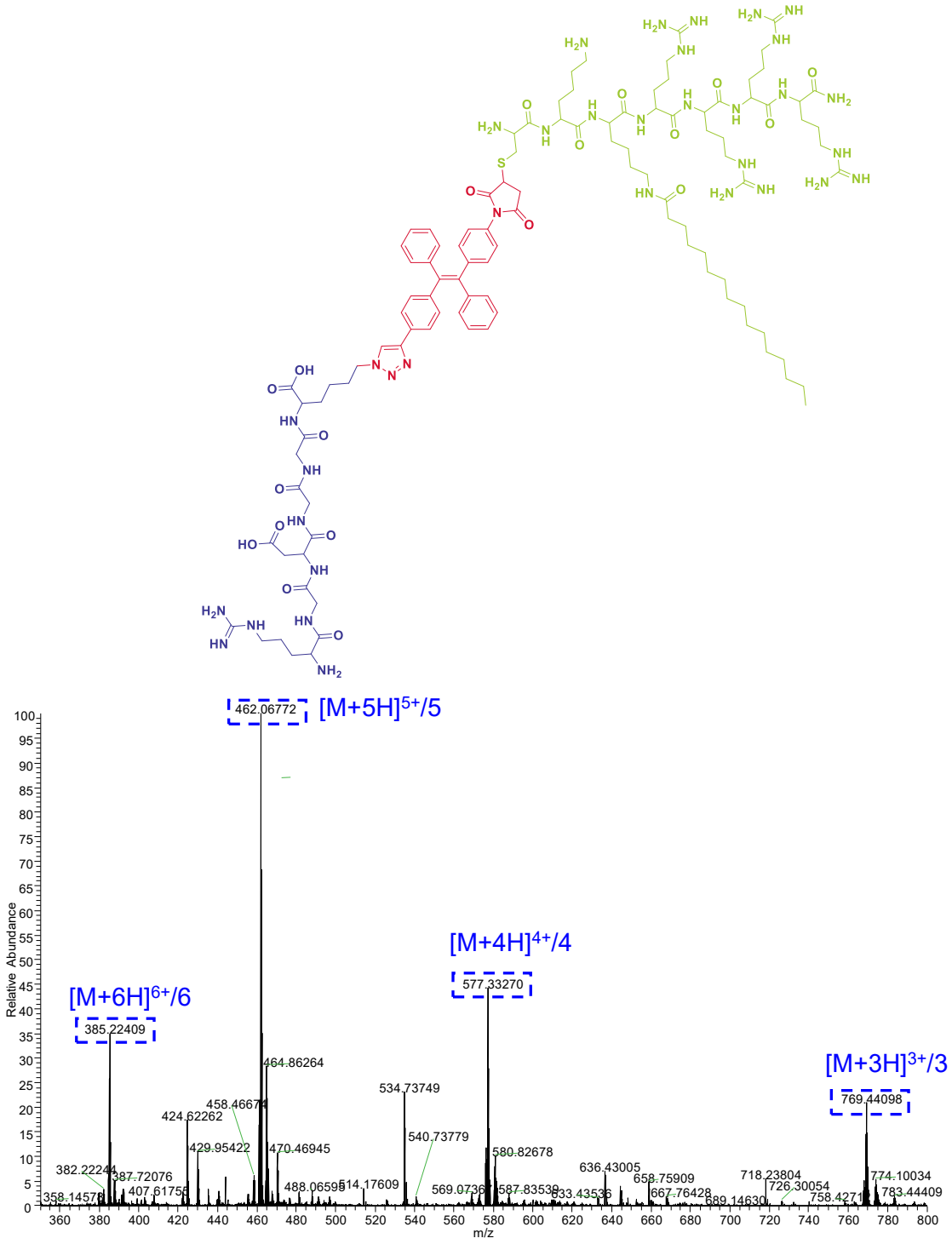
TP	[M+3H]3+/3	[M+4H]4+/4	[M+5H]5+/5
m/z	564.3461	423.5114	339.0106

Figure S1. HRMS of **TP**. The ***cis*-TP** and ***trans*-TP** have the same molecule weight.



***cis*-RTP**	[M+3H]$^{3+}$/3	[M+4H]$^{4+}$/4	[M+5H]$^{5+}$/5	[M+6H]$^{6+}$/6
m/z	769.1089	577.0835	461.8683	385.0581

Figure S2. HRMS spectrum of ***cis*-RTP**.



***trans*-RTP**	[M+3H]$^{3+}$/3	[M+4H]$^{4+}$/4	[M+5H]$^{5+}$/5	[M+6H]$^{6+}$/6
m/z	769.1089	577.0835	461.8683	385.0581

Figure S3. HRMS spectrum of ***trans*-RTP**.

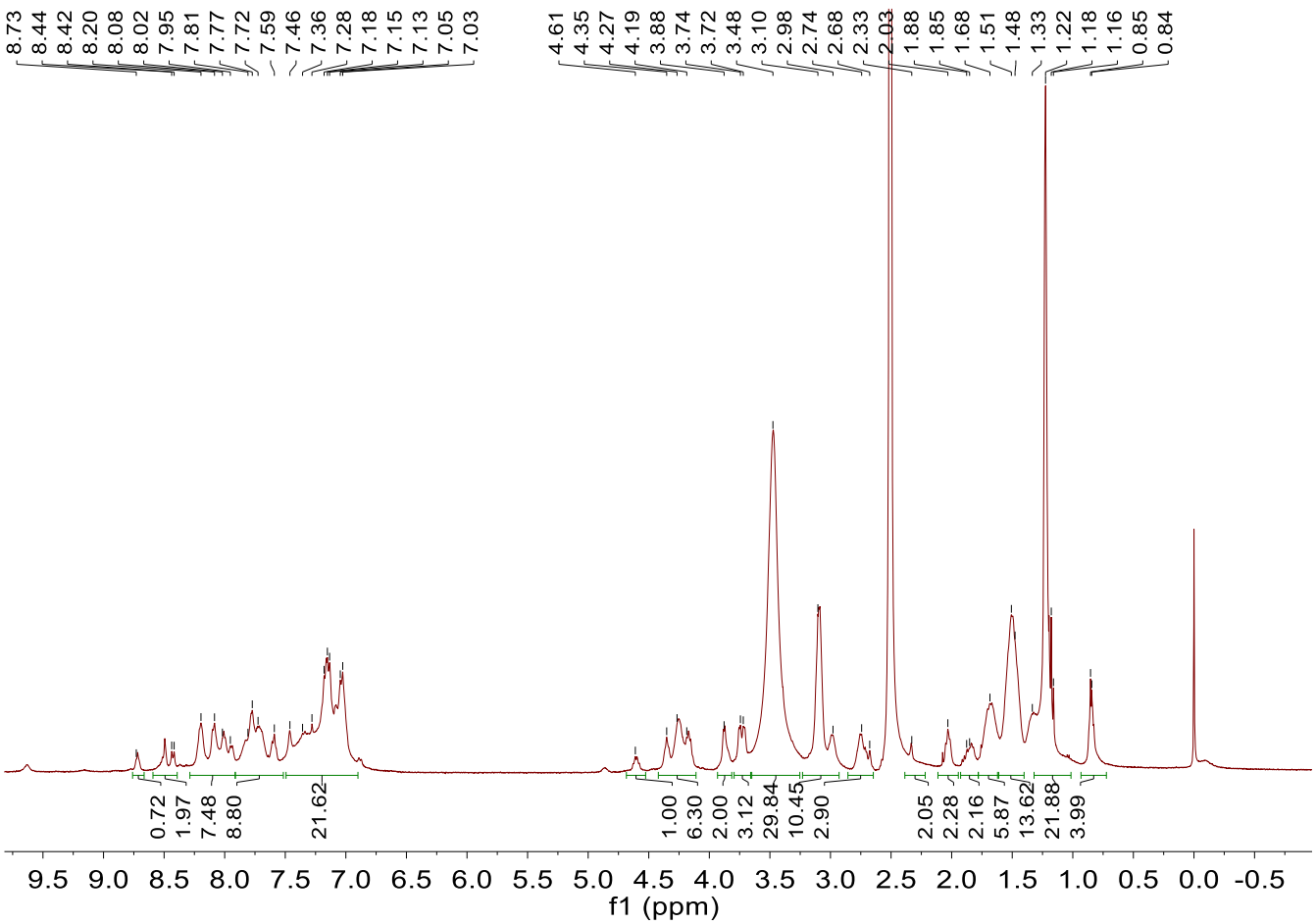


Figure S4. ^{1}H NMR spectrum of ***cis*-RTP** in DMSO-d_6.

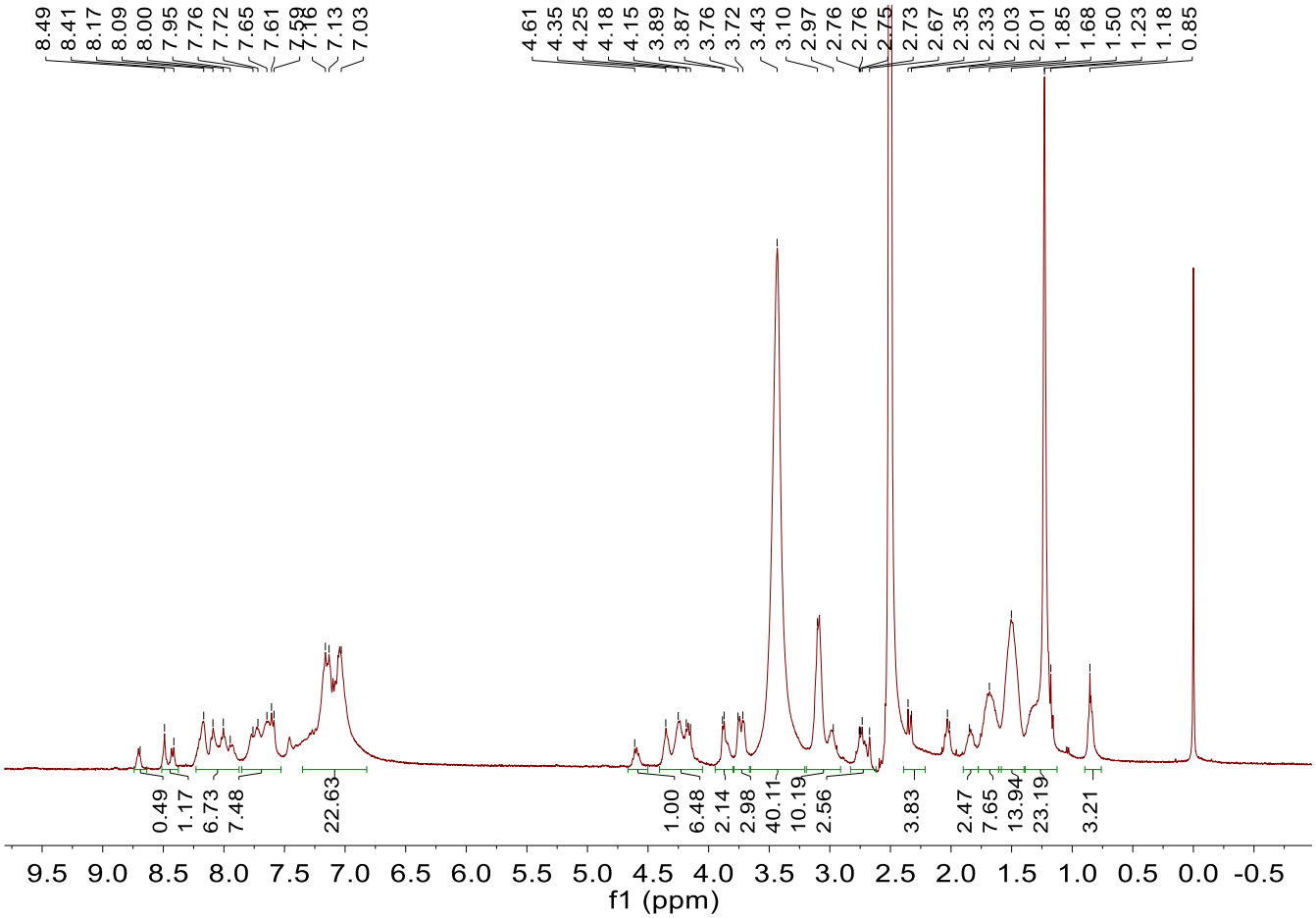


Figure S5. ^{1}H NMR spectrum of ***trans*-RTP** in DMSO-d_6.

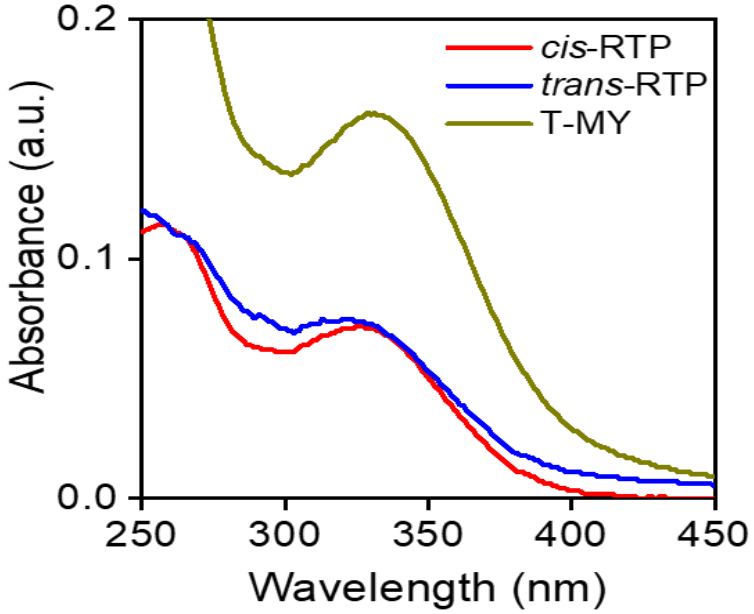


Figure S6. UV−vis absorption spectra of probes (10 μM) in DMSO/water mixture (v/v=1/99).

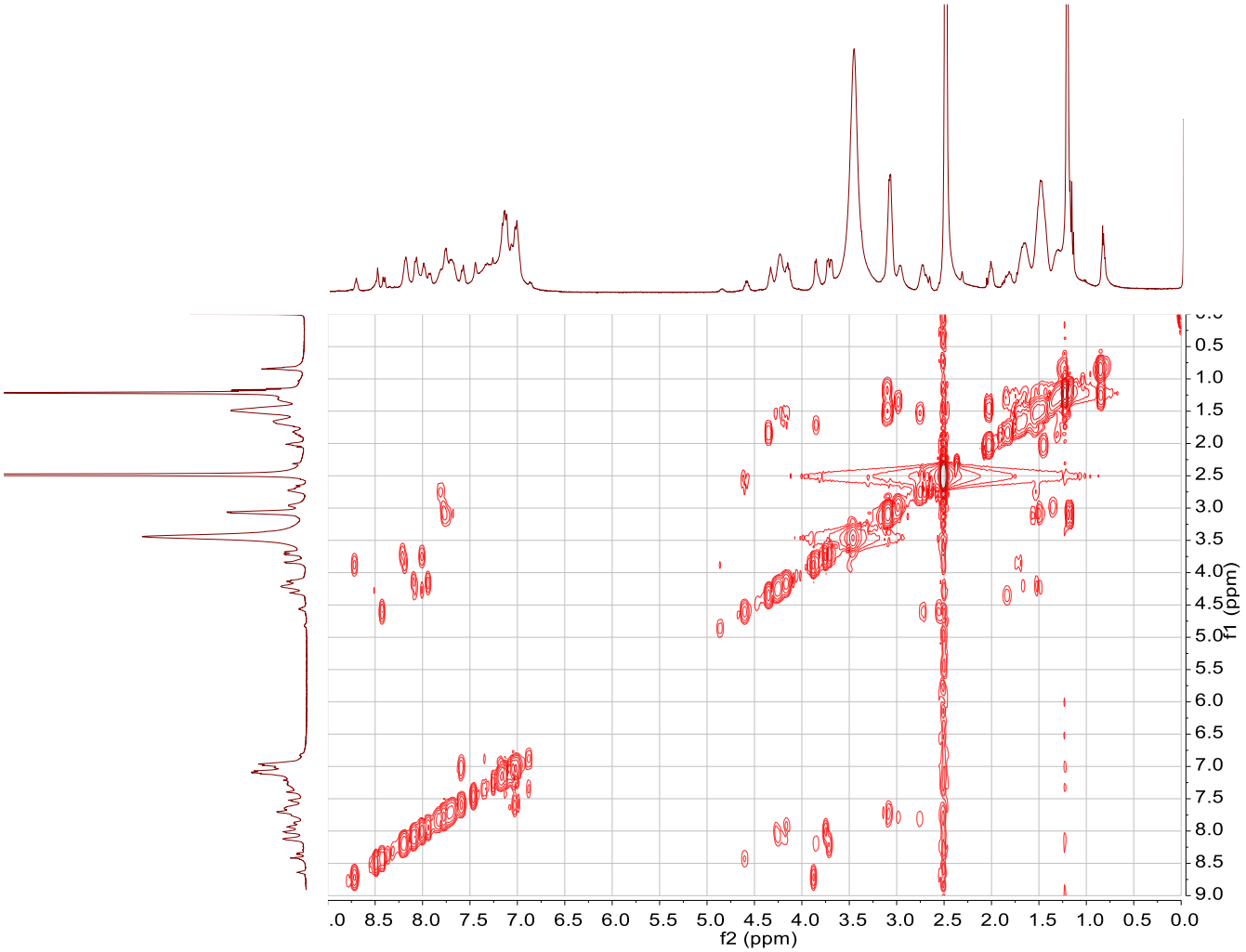


Figure S7. $^{1}H-^{1}H$ COSY NMR spectrum of ***cis*-RTP** in DMSO-d_6.

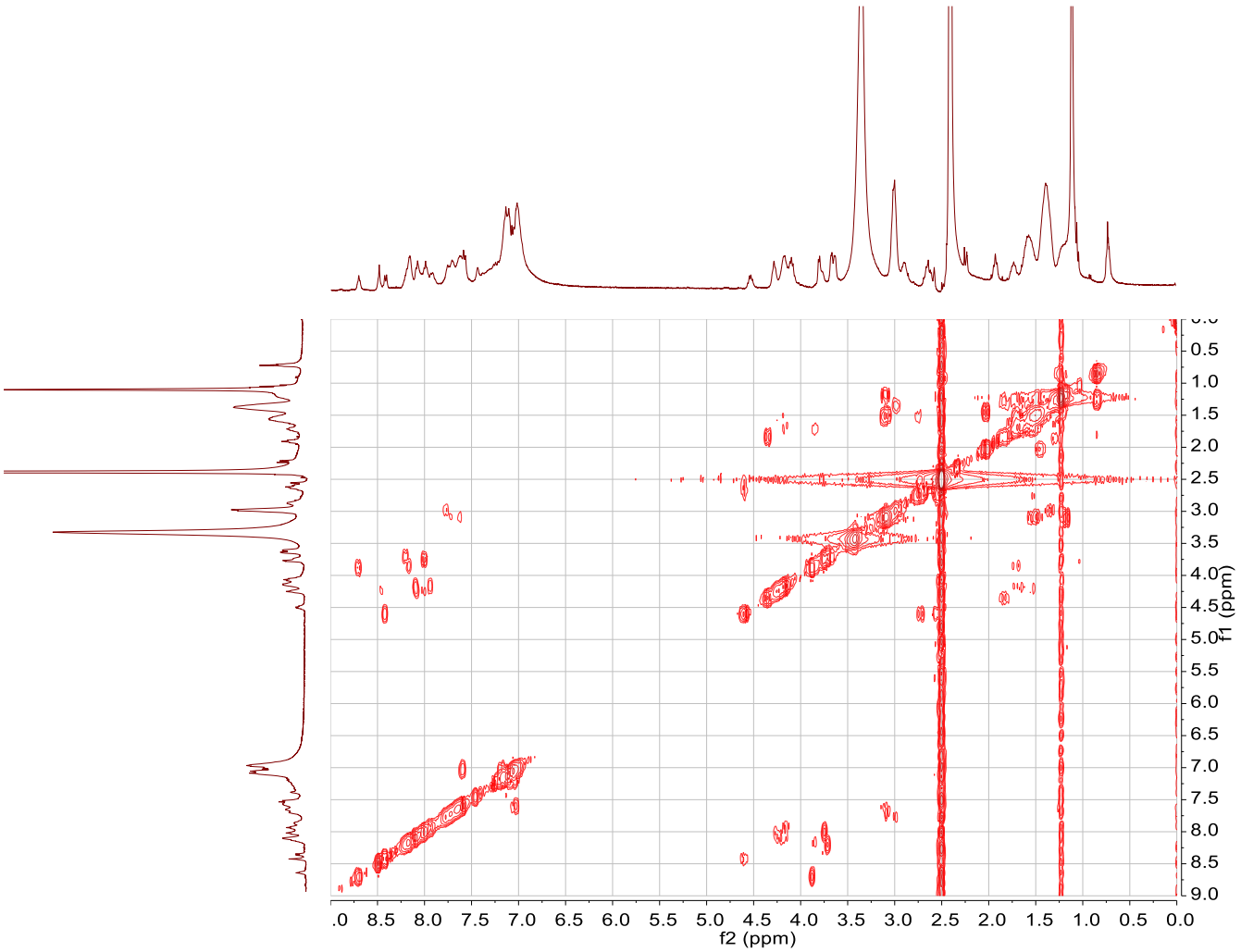
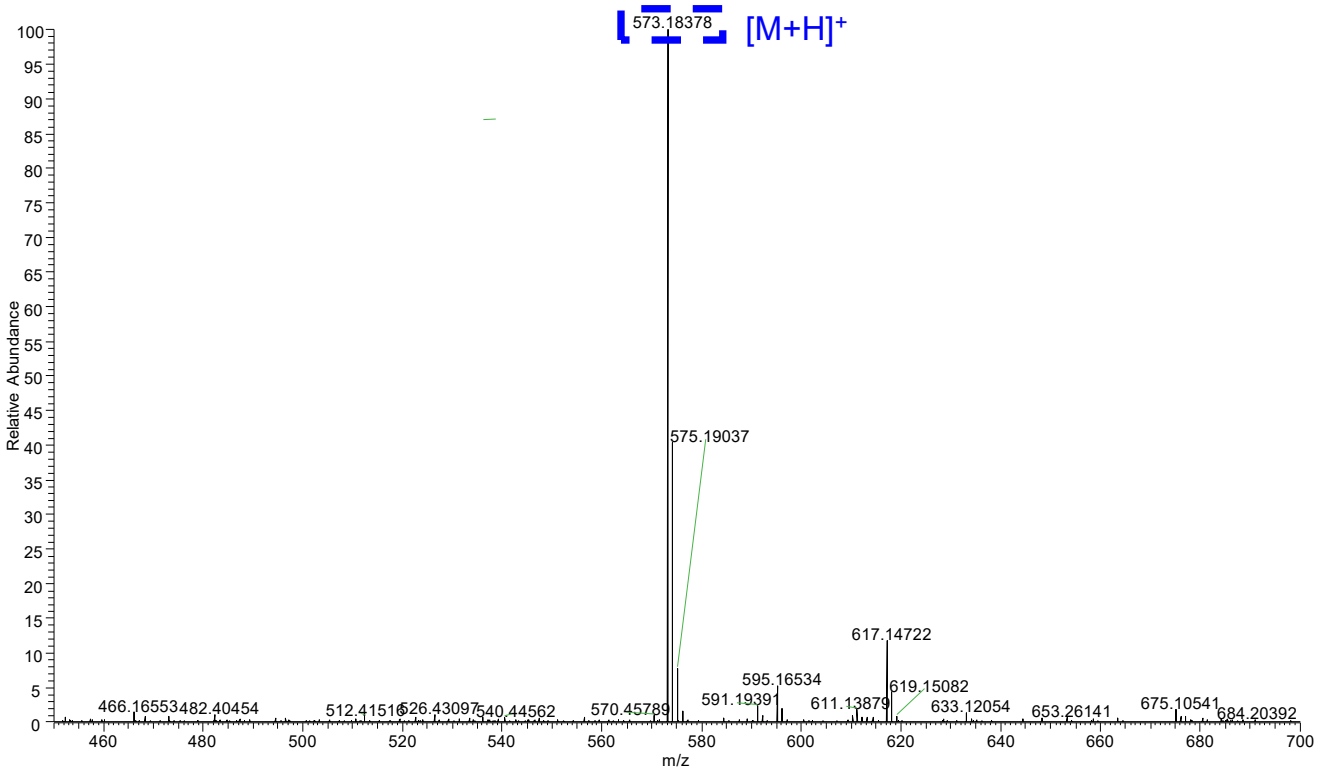
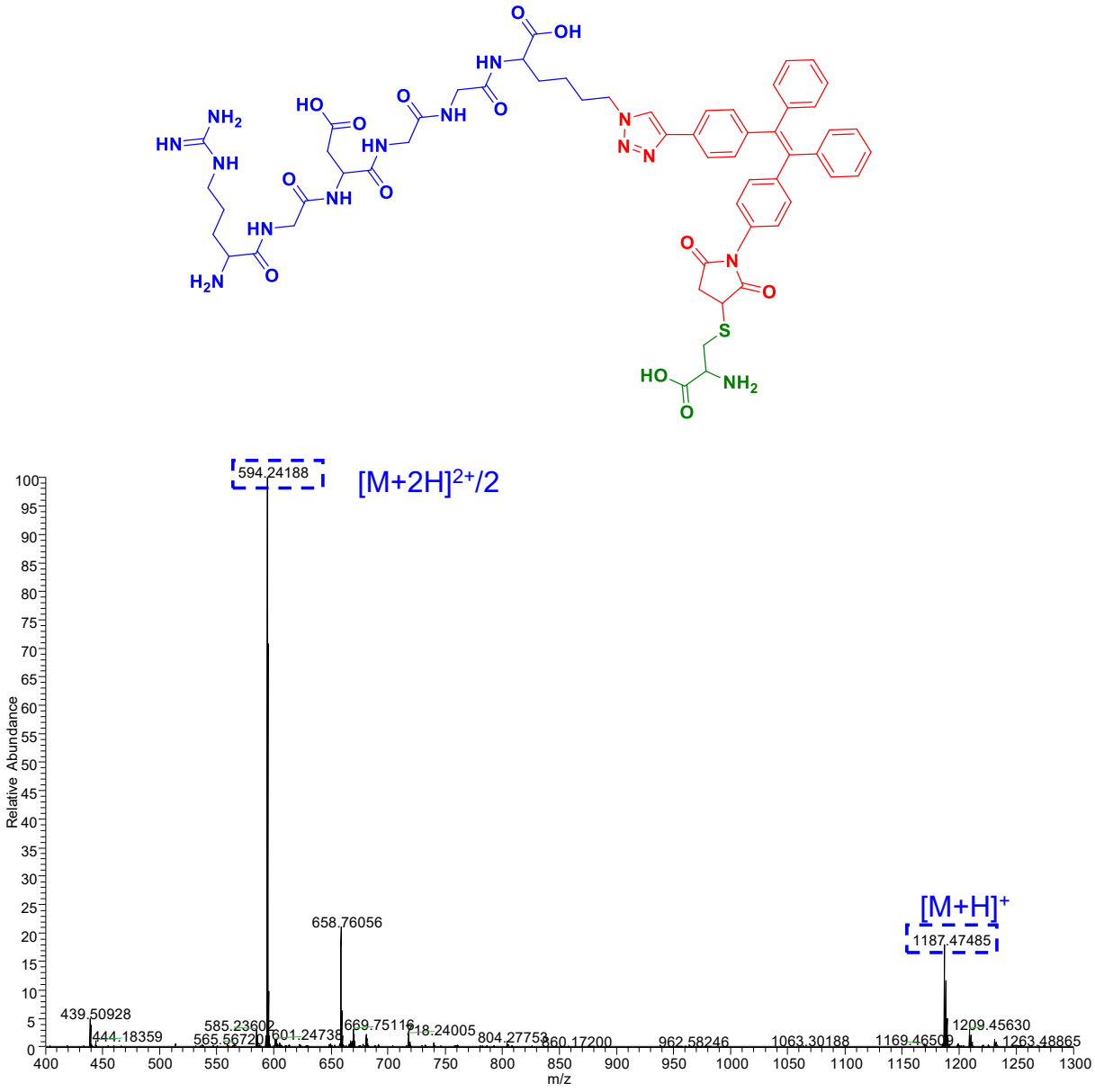


Figure S8. $^{1}H-^{1}H$ COSY NMR spectrum of ***trans*-RTP** in DMSO-d_6.



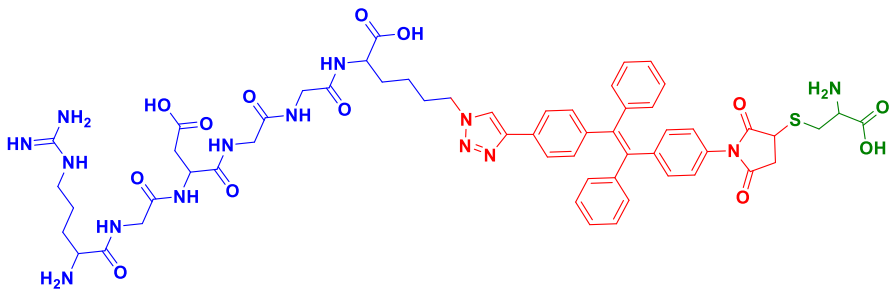
TC	[M+H]+
m/z	573.1843

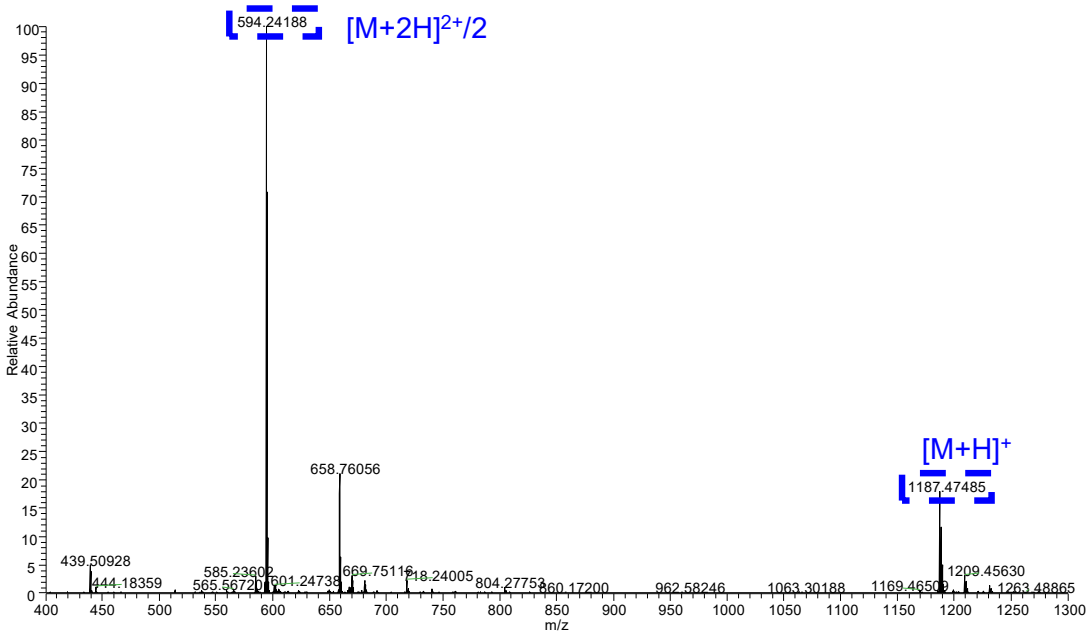
Figure S9. HRMS spectrum of TC. The *cis*-TP and *trans*-TP have the same molecule weight.



***cis*-RTC**	$[M+H]^+$	$[M+2H]^{2+}/2$
m/z	1187.4722	594.2397

Figure S10. HRMS spectrum of ***cis*-RTC**.





***trans*-RTC**	$[M+H]^+$	$[M+2H]^{2+}/2$
m/z	1187.4722	594.2397

Figure S11. HRMS spectrum of ***trans*-RTC**.

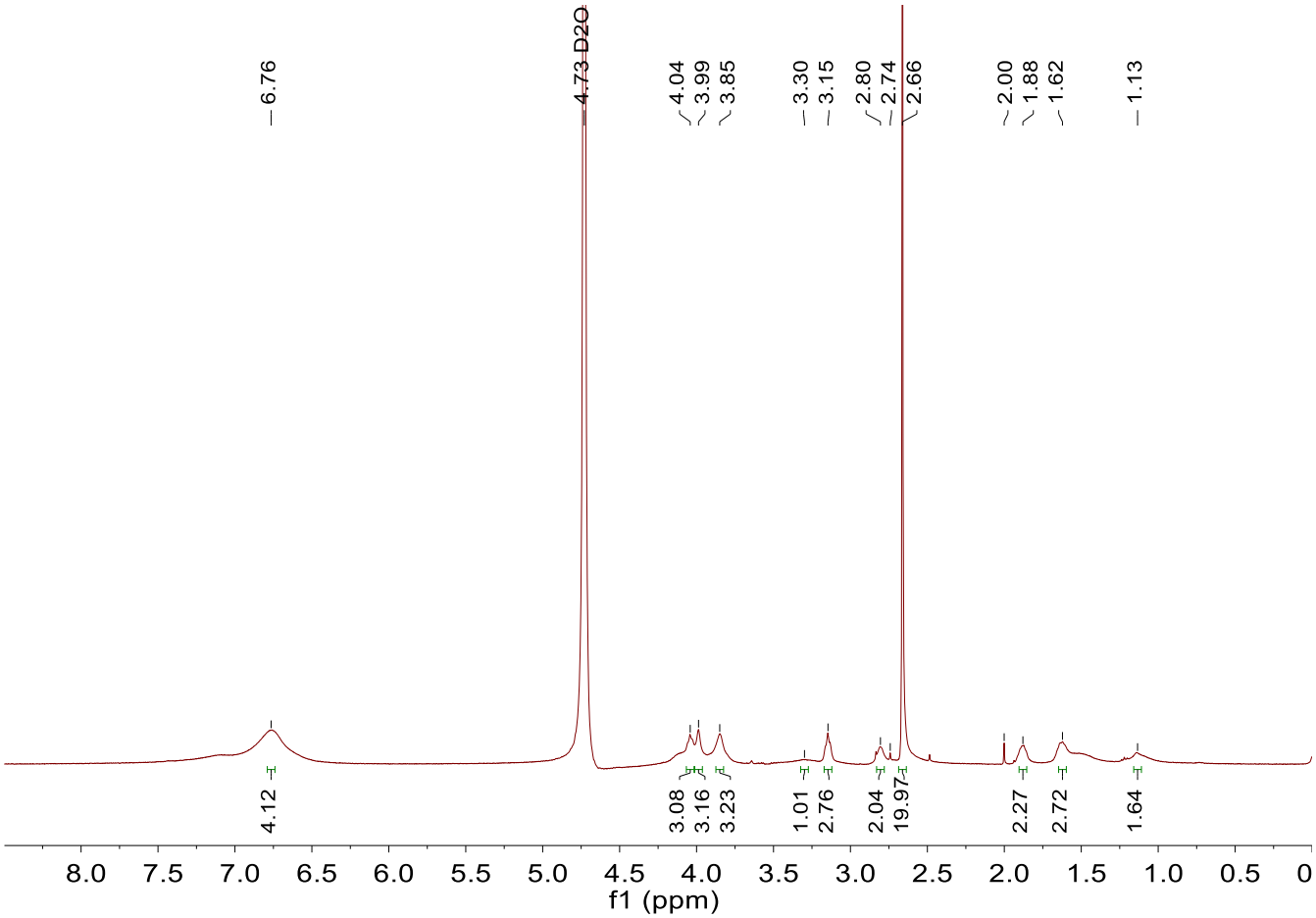


Figure S12. ^{1}H NMR spectrum of ***cis*-RTC** in D_2O.

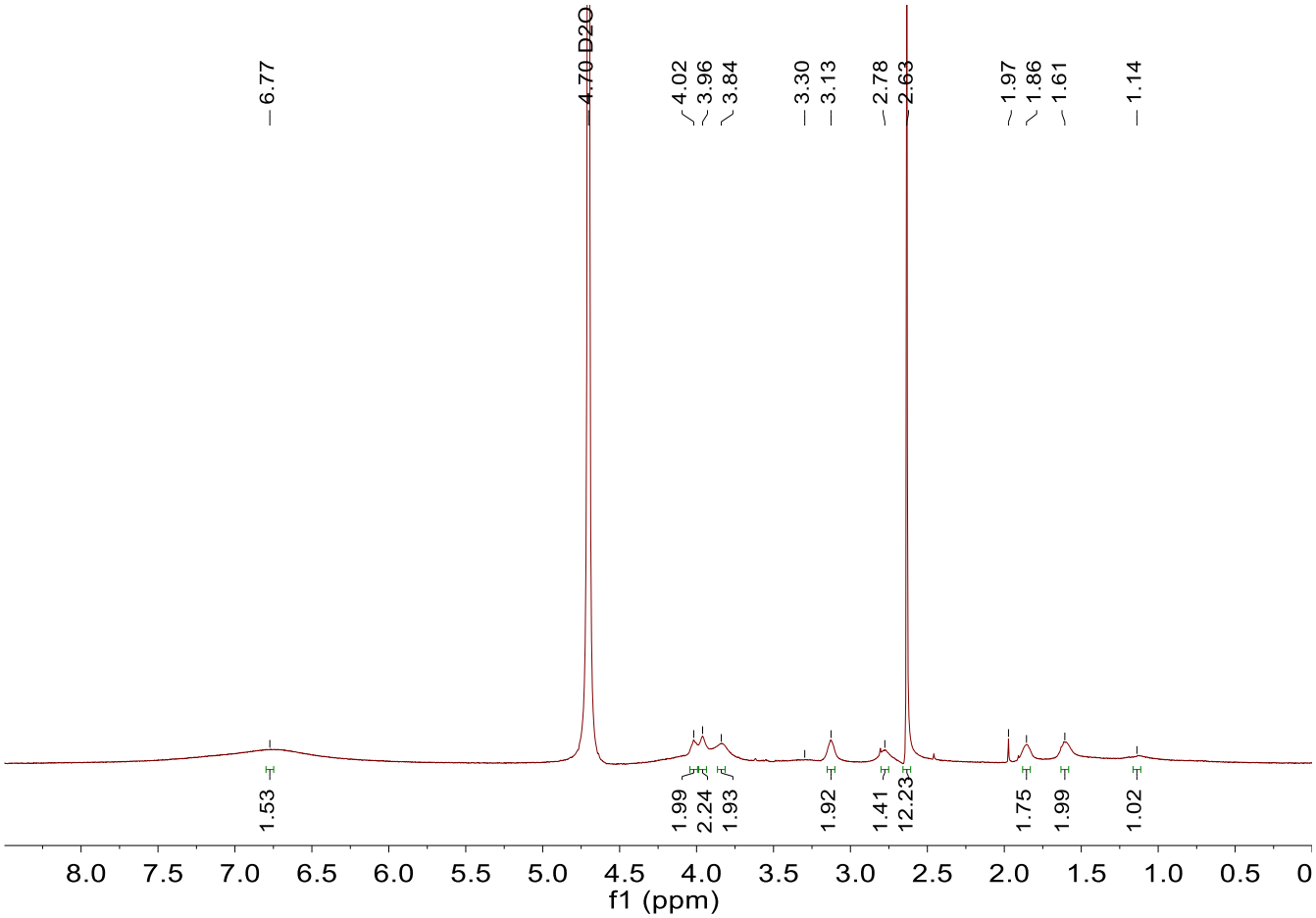


Figure S13. ^{1}H NMR spectrum of ***trans*-RTC** in D_2O.

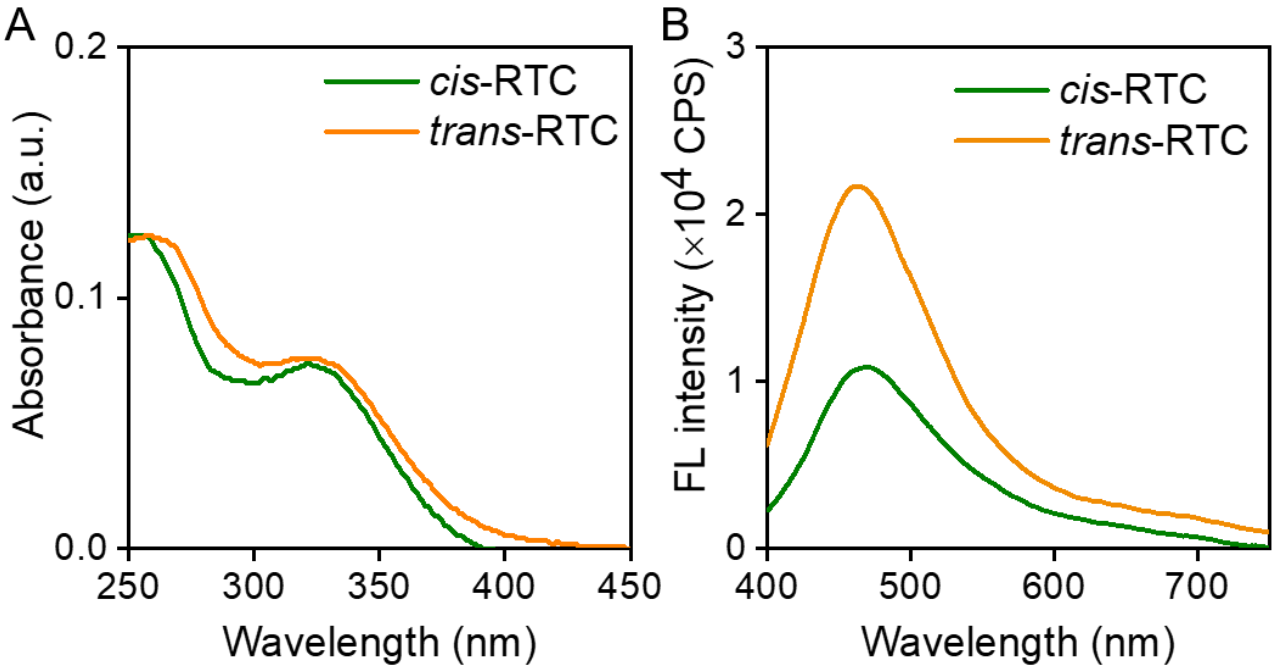


Figure S14. UV−vis absorption spectra and FL spectra of *cis*-RTC/*trans*-RTC (10 μM) in DMSO/water mixture (v/v=1/99). λ_{ex} = 330 nm.

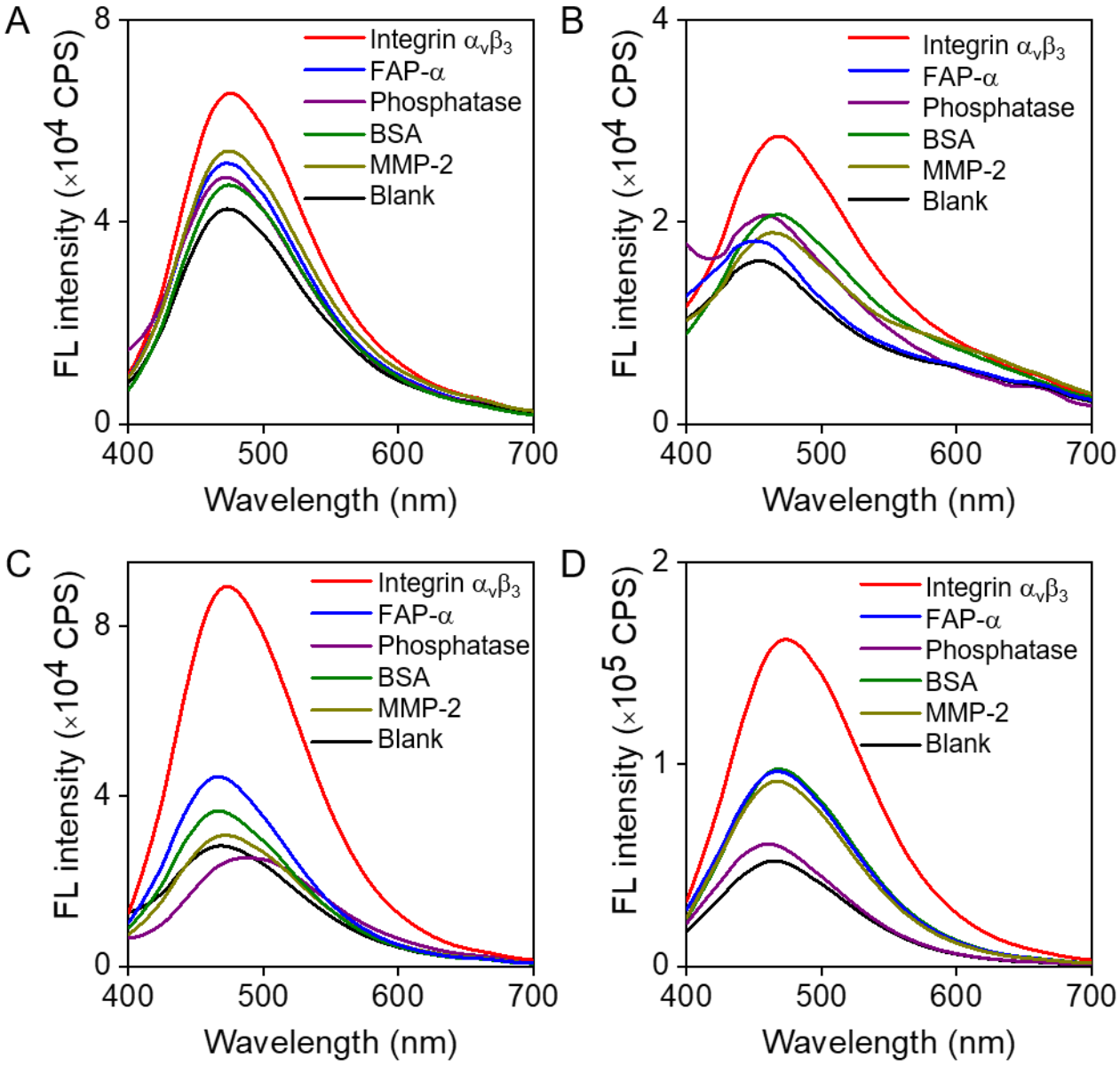


Figure S15. FL spectra of (A) *cis*-RTP, (B) *trans*-RTP, (C) *cis*-RTC and (D) *trans*-RTC versus different proteins. The proteins include integrin $\alpha_v\beta_3$, fibroblast activation protein-α (FAP-α), phosphatase, bovine serum albumin (BSA) and matrix metalloproteinase 2 (MMP-2). The concentration of the probes was 40 μM, λ_{ex} = 330 nm.

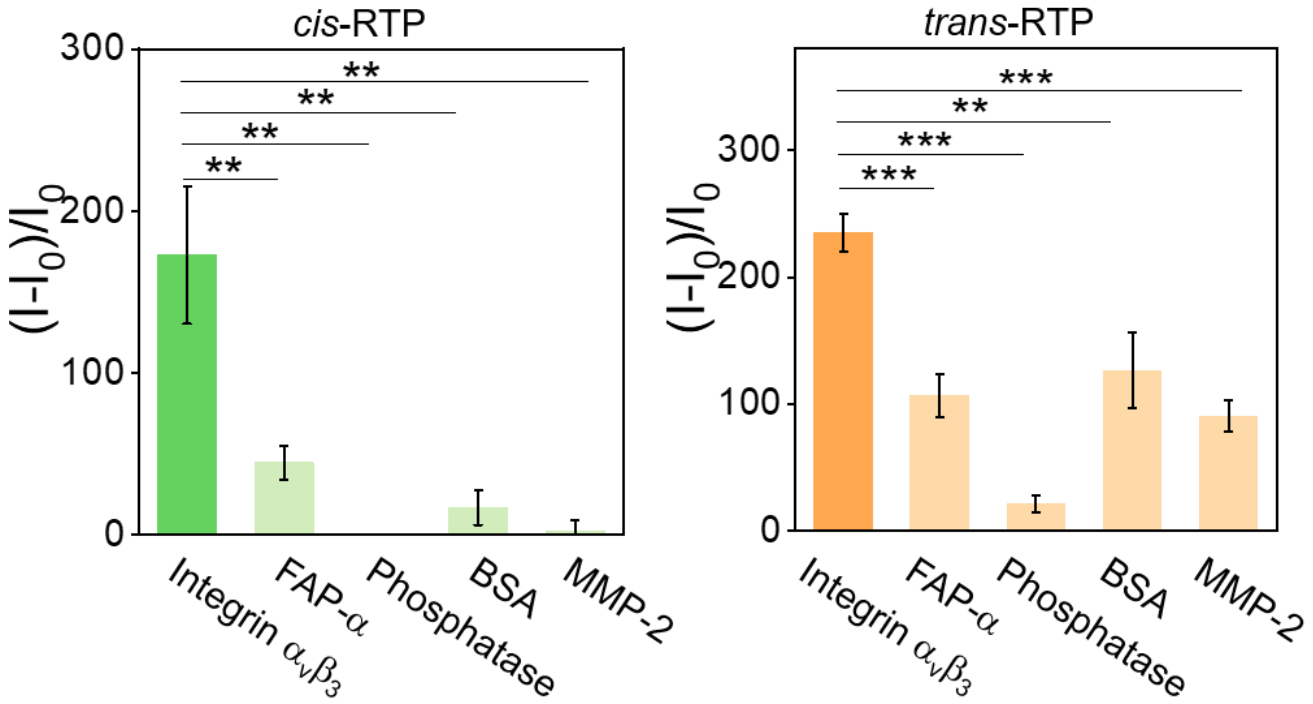


Figure S16. Plot of $(I- I_0)/I_0$ versus different proteins, where I and I_0 were the FL intensities at analyte concentrations of 100 and 0 μg mL^{-1}. The proteins include $\alpha_v\beta_3$, FAP-α, phosphatase, BSA and MMP-2. The concentration of the probes was 40 μM, λ_{ex} = 330 nm.

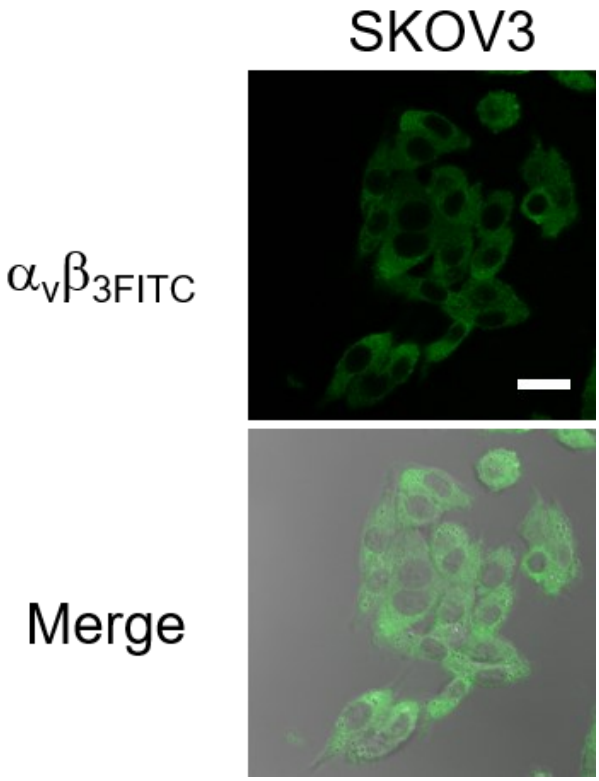


Figure S17. The CLSM images of SKOV3 cells. The cells were incubated with 10 μg/mL FITC-labelled $\alpha_v\beta_3$-antibody for 1 h in cell culturing condition, respectively. Scale bar: 20 μm.

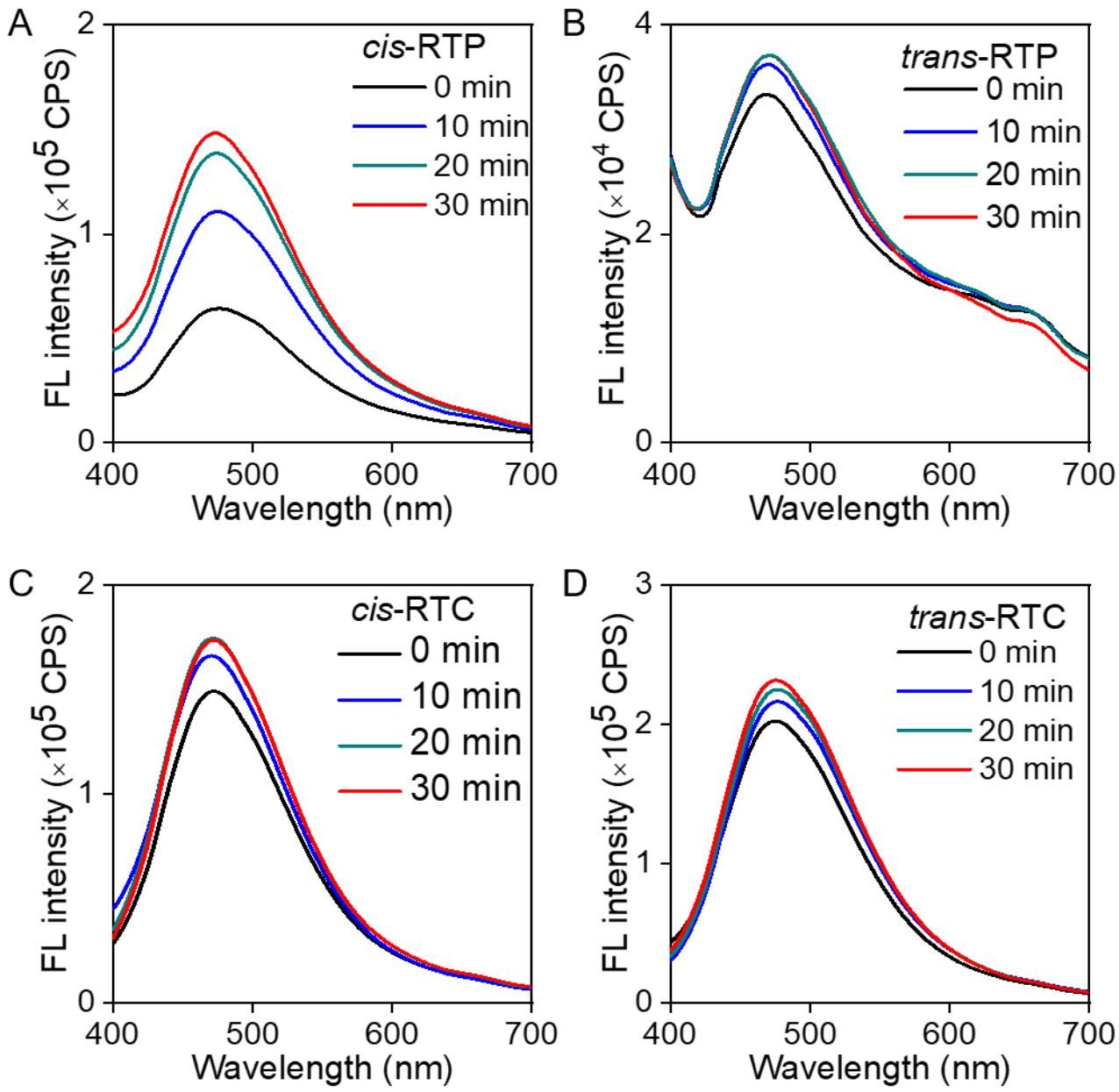


Figure S18. FL spectra of (A) *cis*-RTP, (B) *trans*-RTP, (C) *cis*-RTC and (D) *trans*-RTC with extracted cell membrane for different times. The concentration of the probes was 40 μM and the concentration of integrin $\alpha_v\beta_3$ was 100 μg mL^{-1}, λ_{ex} = 330 nm

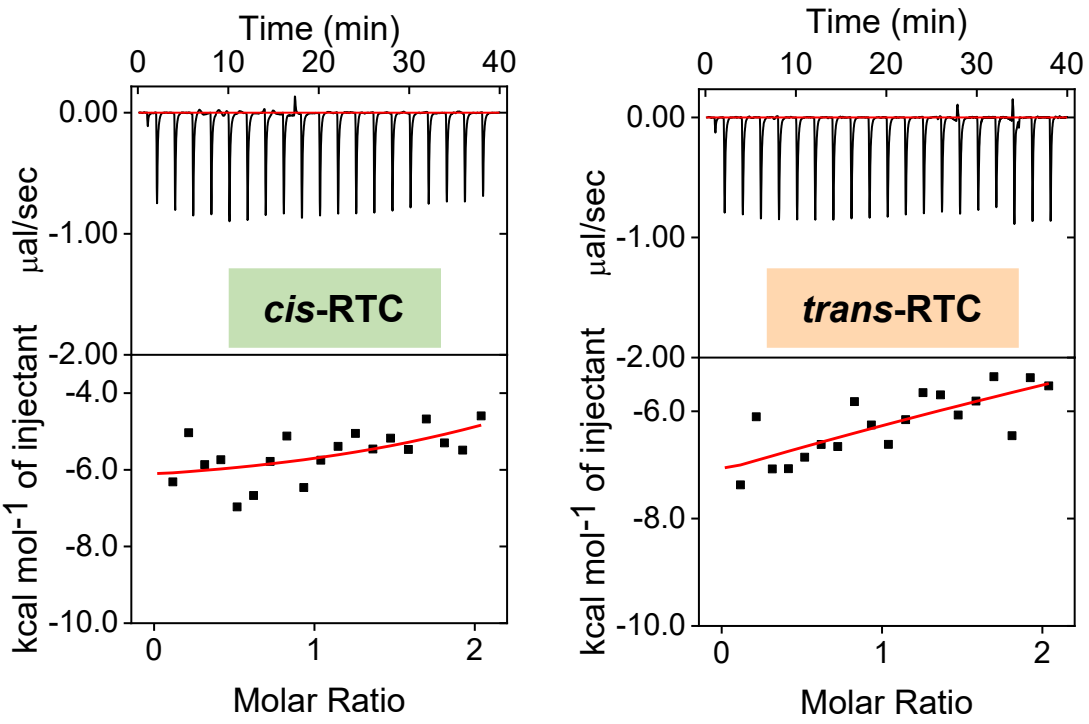


Figure S19. ITC results of *cis*-RTC/*trans*-RTC in PBS with cell membrane at 37 °C. The concentration of the probes was 40 μM.

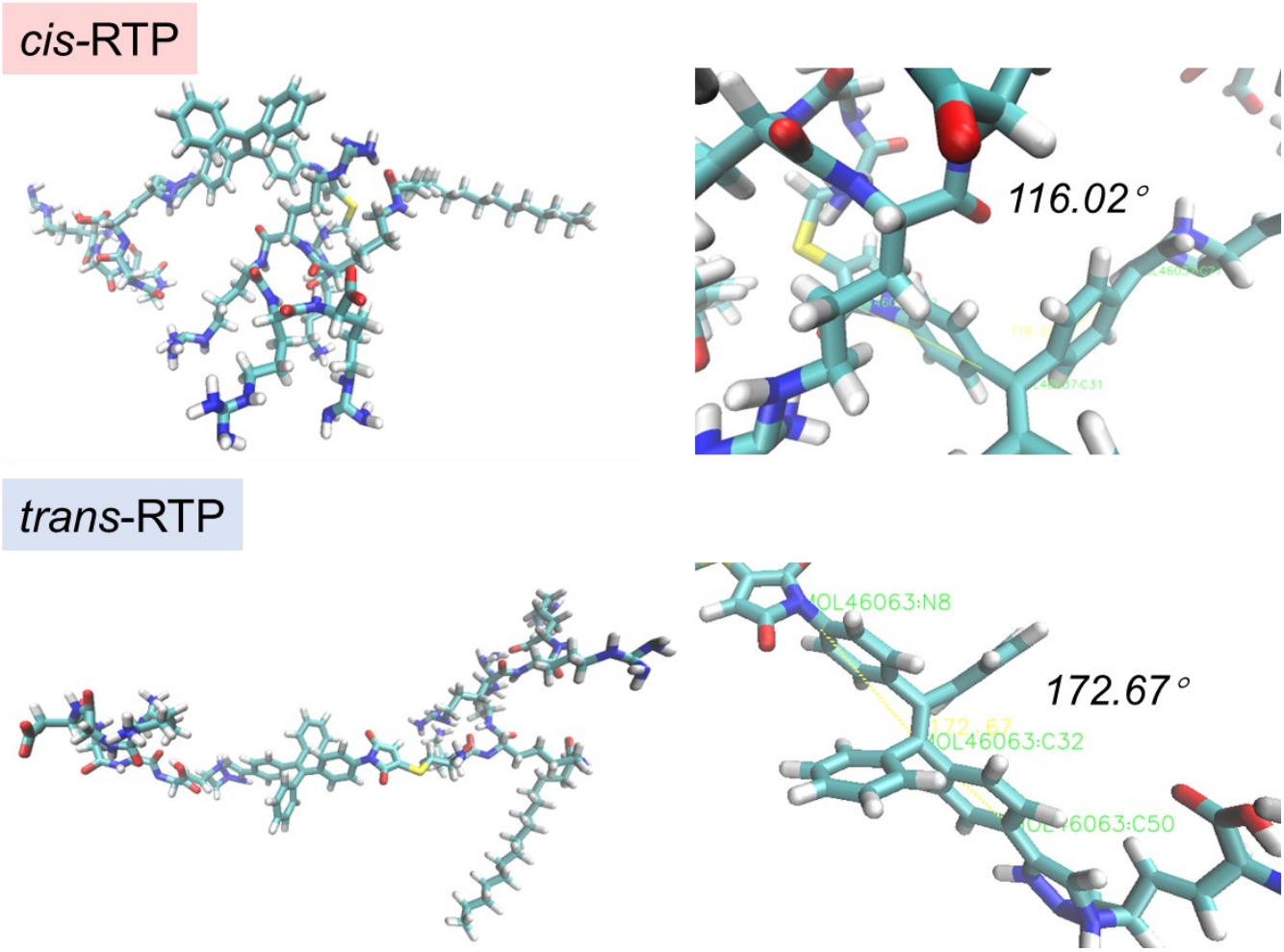


Figure S20. The angles between the linkages of RGD and Pal-RRRR to benzene rings in *cis*-RTP and *trans*-RTP.

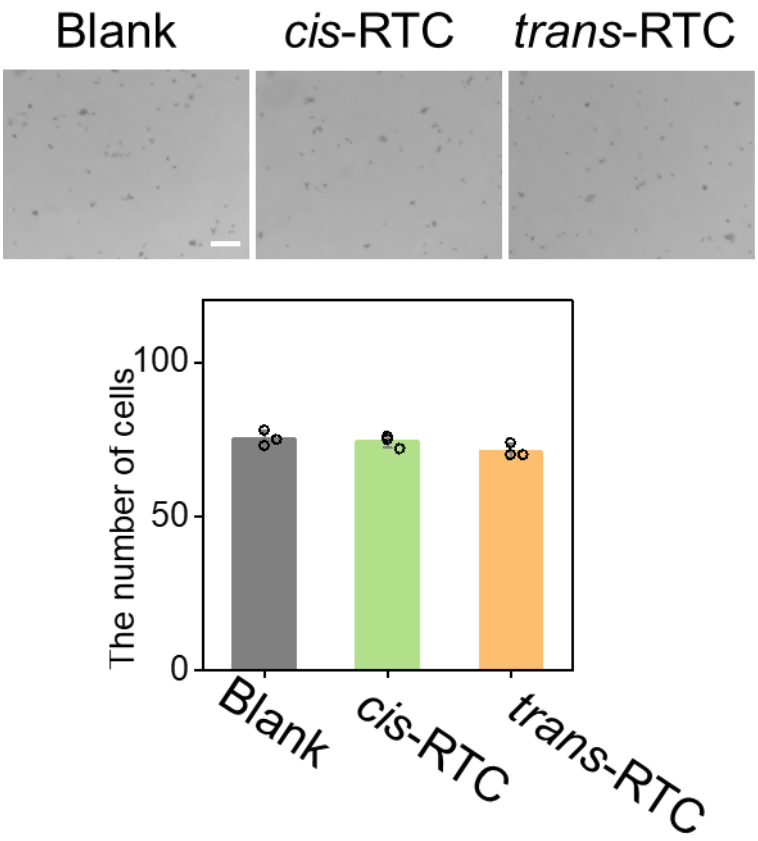


Figure S21. The adhesion result of suspended SKOV3 cells incubated with *cis*-RTC/*trans*-RTC (40 μM) for 1 h and then incubated with fresh medium for 4 h and correspondingly quantitative data of adherent SKOV3 cells in three separate fields. Scale bars: 200 μm.

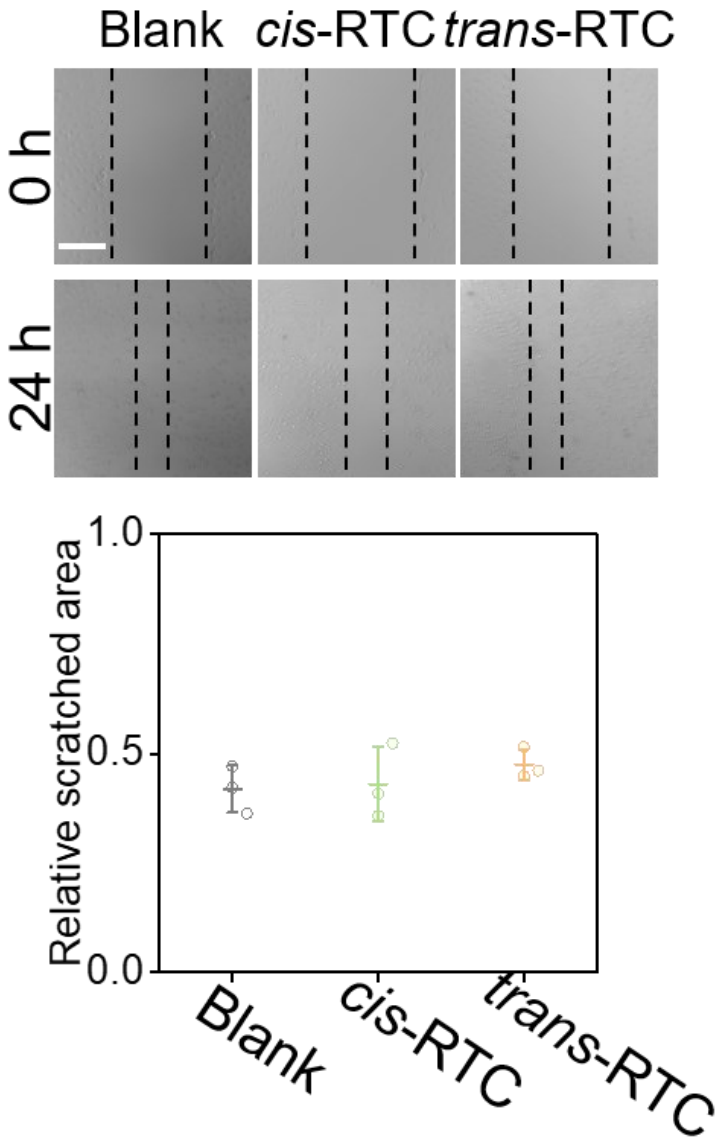


Figure S22. Cell migration of SKOV3 cells incubated with *cis*-RTC/*trans*-RTC (40 μM) for 1 h and then incubated with fresh medium and closure degree of the scratched area was expressed as a percentage of the initial scratched area. Scale bar: 100 μm.

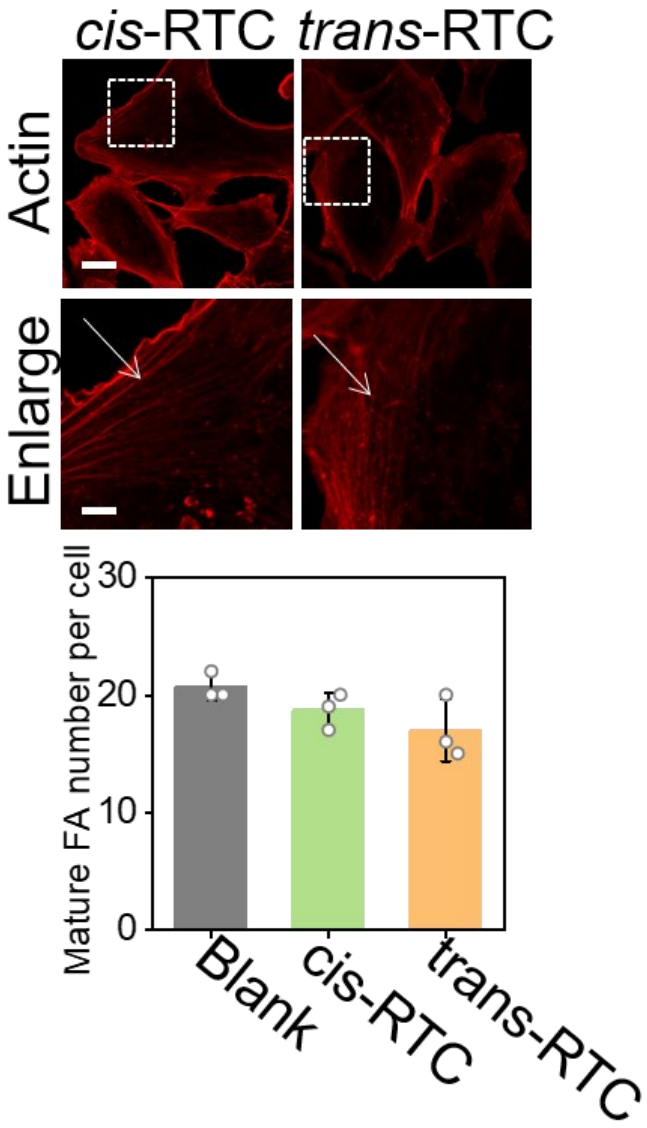


Figure S23. CLSM images of the actin in SKOV3 cells incubated with *cis*-RTP/*trans*-RTP (40 μM) for 1 h, then stained with Actin-Tracker Red-555 and quantification of dorsal bundle number. The scale bar of actin images was 20 μm and scale bar of enlarged images was 5 μm.