Supporting Information

Chlorotoxin-derived Bicyclic Peptides for Targeted Imaging of Glioblastomas

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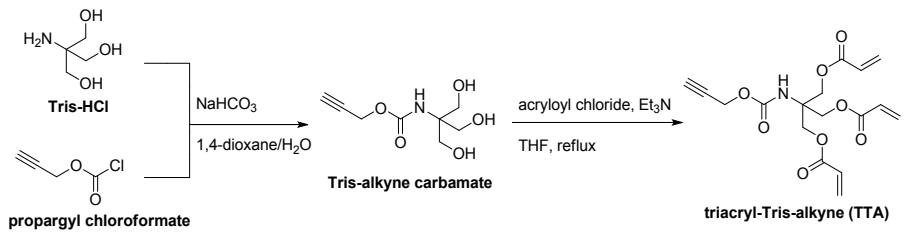
General Information

HATU (1-[bis(dimethylamino)-methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate) and the HPLC grade solvents (CH_3CN and MeOH) were purchased from J&K Scientific. DIPEA, TFA, tris(hydroxymethyl)aminomethane (Tris), propargyl chloroformate and other chemicals were acquired from Energy Chemical and were used as received.

Analytical RP-HPLC was performed on the Agilent 1260 high-performance liquid chromatography (HPLC) instrument (UV-vis detector) with Poroshell 120, EC-C18 column (4.6 x 100 mm, 2.7 μm) maintained at 30 °C. The RP-HPLC gradient was started at 10% of B (MeCN), then increased to 100% of B over 20 min (A: 0.1% TFA in water) at a flow rate of 0.5 mL/min. Semi-preparative RP-HPLC was performed on the ULTIMAT 3000 Instrument (DIONEX). UV absorbance was measured using a photodiode array detector at 220 and 254 nm. The RP-HPLC gradient was started at 10% of B (MeCN), then increased to 100% of B over 30 min (A: 0.1% TFA in water). ^1H NMR (^{13}C NMR) spectra were recorded with a Bruker AV400 at 400 (100) MHz. Chemical shifts are referenced to either tetramethylsilane as an internal standard or the signals resulting from the residual solvent. High resolution mass spectra were measured with an ABI Q-star Elite.

Experimental Procedure

1. Preparation of triacryl-Tris-alkyne (TTA)



Tris-alkyne carbamate: $NaHCO_3$ (1.68 g, 20 mmol) was added to a solution of Tris-HCl (1.21 g, 10 mmol) and propargyl chloroformate (1.427 g, 12 mmol) in 1,4-dioxane/H_2O (v/v=1/1, 10 mL). After stirred for 19 h at room temperature, the reaction mixture was extracted with EtOAc (50 ml x 3). The combined organic layer

was washed with H_2O and brine, dried over anhydrous $MgSO_4$, filtered and concentrated in vacuo. The residue was purified by column chromatography on silica gel (hexane/EtOAc) to give Tris-alkyne carbamate as an oil (1.62 g, 80%).

Triacryl-Tris-alkyne (TTA): To a solution of Tris-alkyne carbamate (0.4 g, 2 mmol) and Et_3N (1.67 mL, 12 mmol) in dry THF (10 mL) was added acryloyl chloride (0.73 mL, 9 mmol) followed by 4-dimethylaminopyridine (DMAP, 15 mg) at room temperature. Then the reaction mixture was stirred at 60 ^oC for 24 h before it was extracted with EtOAc (50 ml x 3). The combined organic layer was washed with H_2O and brine, dried over anhydrous $MgSO_4$, filtered and concentrated in vacuo. The residue was purified by column chromatography on silica gel (hexane/EtOAc) to give triacryl-Tris-alkyne (TTA) as an oil (0.55 g, 75%). ^1H NMR (400 MHz, $CDCl_3$) δ 2.50 (t, J = 2.4 Hz, 1H), 4.53 (br s, 6H), 4.67 (d, J = 2.0 Hz, 2H), 5.41 (br s, 1H), 5.91 (dd, J = 10.8, 1.6 Hz, 3H), 6.14 (dd, J = 17.2, 10.4 Hz, 3H), 6.45 (dd, J = 17.2, 1.2 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 52.7, 57.5, 62.9, 75.0, 77.7, 127.5, 132.2, 153.8, 165.5.

2. Preparation of the linear peptide P1-P5

Five linear peptides (P1-P5) were synthesized using Fmoc-based solid-phase peptide synthesis (SPPS) on Rink Amide AM resin. Fmoc 9-fluorenylmethoxycarbonyl) was deprotected with 20% piperidine in DMF. The resin was washed with DMF (5 x 30 s) and dry DMF (1 x 30 s). For peptide elongation, the protected amino acid (4 eq.) was activated using DIC (diisopropylcarbodimide) (4 eq.) and HOAt (1-hydroxy-7-azabenzotriazole) (4 eq.) in dry DMF. The extent of coupling was assessed by the Kaiser test. Peptides were cleaved with concomitant removal of the side-chain protecting groups using TFA (trifluoroacetic acid), thioanisole, EDT (1,2-ethanedithiol), phenol and H_2O (87.5:5:2.5:2.5:2.5). After cleavage of the peptides, the solvent was evaporated applying a stream of N_2. The residue was washed 3 times by suspension in tert-butyl methyl ether and subsequent centrifugation. The cleaved peptides were then dissolved in MeCN/H_2O (1:1) with 0.1% TFA and freeze-dried. The sequences of the synthetic fragments were listed as follows:

P1: MCMPAFTTDHQMARKADDCGGKGRGKAYGPQALCR-NH_2

P2: MCMPAFTTDHQCARKADDACG-NH_2

P3: AcCMPAFTTDHQCARKADDAC-NH_2

P4: ACGGKGRGKCYGPQALCR-NH_2

P5: CQMARKADDCAGGKGRGKC-NH_2

3. Preparation of the bicyclic peptide P1-P5

Cyclization of linear peptide P1-P5 (5 μmol) with triacryl-Tris-alkyne (TTA, 6 μmol) was conducted in aqueous NH_4HCO_3 (100 mM, pH 8.0, 0.7 mL) acetonitrile (0.3 mL) at 4 °C. The absence of the thiol groups was accessed by Ellman's test. The crude bicyclic peptide was purified by semi-preparative RP-HPLC and lyophilized to provide bicyclic peptide P1-P5 as a white solid (~70%).

Bicycle-P1: HRMS (ESI-TOF) m/z: calcd. for $C_{172}H_{274}N_{53}O_{54}S_6$ $[M+3H]^{3+}$ 1379.2877, found 1379.2873.

Bicycle-P2: HRMS (ESI-TOF) m/z: calcd. for $C_{107}H_{164}N_{30}O_{38}S_5$ $[M+2H]^{2+}$ 1318.5208, found 1318.5120.

Bicycle-P3: HRMS (ESI-TOF) m/z: calcd. for $C_{102}H_{154}N_{28}O_{37}S_4$ $[M+2H]^{2+}$ 1245.4951, found 1245.4947.

Bicycle-P4: HRMS (ESI-TOF) m/z: calcd. for $C_{91}H_{147}N_{29}O_{28}S_3$ $[M+2H]^{2+}$ 1095.0061, found 1095.0139.

Bicycle-P5: HRMS (ESI-TOF) m/z: calcd. for $C_{91}H_{153}N_{31}O_{32}S_4$ $[M+2H]^{2+}$ 1160.0085, found 1159.9848.

4. Preparation of the FITC-labelled bicyclic peptide P1-P5

To a solution of bicyclic peptide P1-P5 (2 μmol) and FITC-N_3 (2.4 μmol) in *t*BuOH and H_2O (v/v=1:1, 200 μL) were added sodium ascorbate (4 μmol) and $CuSO_4{\cdot}5H_2O$ (1.2 μmol). The reaction mixture was stirred at room temperature for 12 h. Then the crude product was purified by semi-preparative RP-HPLC and lyophilized to provide FITC-labelled bicyclic peptide P1-P5 as a yellow solid (~75%).

FITC-bicycle-P1: HRMS (ESI-TOF) m/z: calcd. for $C_{196}H_{295}O_{60}N_{57}S_7$ $[M+7H]^{7+}$ 661.5684, found 661.5705.

FITC-bicycl-P2: HRMS (ESI-TOF) m/z: calcd. for $C_{131}H_{183}N_{35}O_{43}S_6$ $[M+2H]^{2+}$ 1563.0761, found 1563.0746.

FITC-bicycle-P3: HRMS (ESI-TOF) m/z: calcd. for $C_{126}H_{173}N_{33}O_{42}S_5$ $[M+2H]^{2+}$

1490.0504, found 1490.0522.

FITC-bicycle-P4: HRMS (ESI-TOF) m/z: calcd. for $C_{115}H_{165}N_{34}O_{33}S_4$ $[M+H]^+$ 2678.1156, found 2677.0997.

FITC-bicycle-P5: HRMS (ESI-TOF) m/z: calcd. for $C_{115}H_{172}N_{36}O_{37}S_5$ $[M+2H]^{2+}$ 1404.5638, found 1404.5548.

5. Preparation of the Cy5.5-labelled bicyclic peptide P1-P5

To a solution of bicyclic peptide P1-P5 (2 μmol) and Cy5.5-N_3 (2.4 μmol) in *t*BuOH and H_2O (v/v=1:1, 200 μL) were added sodium ascorbate (4 μmol) and $CuSO_4{\cdot}5H_2O$ (1.2 μmol). The reaction mixture was stirred at room temperature for 12 h. Then the crude product was purified by semi-preparative RP-HPLC and lyophilized to provide Cy5.5-labelled bicyclic peptide P1-P5 as a blue solid (~75%).

Cy-bicycle-P1: HRMS (ESI-TOF) m/z:calcd. for $C_{215}H_{323}N_{59}O_{55}S_6$ $[M+3H]^{4+}$ 1200.8148, found 1200.8390.

Cy-bicycle-P2: HRMS (ESI-TOF) m/z: calcd. for $C_{150}H_{212}N_{36}O_{39}S_5$ $[M+H]^{2+}$ 1650.7152, found 1650.6996.

Cy-bicycle-P3: HRMS (ESI-TOF) m/z: calcd. for $C_{145}H_{202}N_{34}O_{38}S_4$ $[M+H]^{2+}$ 1577.6896, found 1577.6872.

Cy-bicycle-P4: HRMS (ESI-TOF) m/z: calcd. for $C_{134}H_{195}N_{35}O_{29}S_3$ $[M+H]^{2+}$ 1427.2006, found 1427.2017.

Cy-bicycle-P5: HRMS (ESI-TOF) m/z: calcd. for $C_{134}H_{202}N_{37}O_{33}S_4$ $[M+3H]^{4+}$ 746.3533, found 746.3541.

6. Preparation of the FITC- and Cy5.5-labelled CTX

CTX was purchased from NJPeptide, which was prepared using Fmoc-based SPPS with the four disulfide bonds formed on resin one by one. Bioconjugation of CTX was performed using a mixture of CTX [2 mg/mL in bicarbonate buffer (pH 8.5)] and Fluorescein isothiocyanate (FITC-NCS, purchased from Energy Chemical) or Cy5.5-NHS-ester (purchased from Lumiprobe) at the molar ratio of 3:1 (dye/CTX). Conjugation was done in the dark at room temperature for 1 h. Unconjugated dye was removed by dialysis against PBS using Slide-A-Lyzer (Pierce Biotechnology) membrane (M_r cutoff, 3500) up to 18 h at 4 °C. Samples were diluted with PBS to produce 40 μM of FITC- or Cy5.5-labelled CTX solution and filtered with a 0.2 μm syringe filter before use, which were demonstrated by HRMS to be mixtures of mono-, di- and trilabelled peptides

7. Circular dichroism (CD) spectra of the designed bicyclic peptides and CTX.

CD spectra were collected on a chirascan spectropolarimeter (Applied Photophysics, England) at 25°C using a 1 mm path length, scanning between 260 and 190 nm at 1 nm/s with a bandwidth of 1.0 nm and response time of 1 s. CTX and the bicycle peptides were dissolved in PB (50 mM, pH=7.4) with a appropriate concentration. And each spectrum is the average of three scans. The observed ellipticity (θ_{obs}) obtained is converted to mean residual ellipticity (MRE) in deg.cm^2.dmol^{-1} by using the following equation by Beer-Lambert law:

$$MRE = \theta_{obs}/(l.c)$$

Where MRE is the mean residual ellipticity in deg.cm^2.dmol^{-1}, θ_{obs} is the observed ellipticity in mdeg, l is the path length in mm, c is the molar concentration of the peptides.

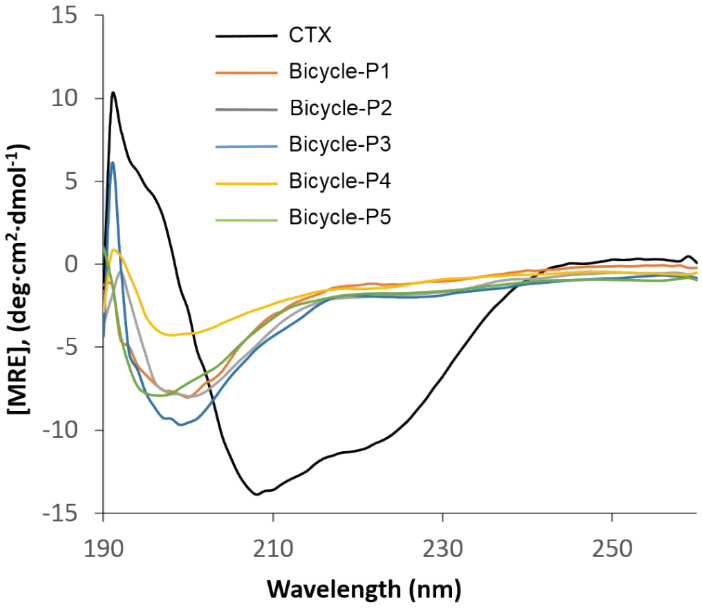


Fig. S1 CD spectra of the designed bicyclic peptides and CTX.

8. The stability of bicyclic peptide P1-P5 in rat serum

The blood was collected from the abdominal artery of male rats with syringes and was centrifuged at 14000 g for 10 min to remove the lipid component. Then the serum was incubated at 37 °C with bicyclic peptide P1-P5 at a final concentration of 180 μM for 24 h. The 40 μL triplicate aliquots were extracted at 0, 0.5, 1, 2, 4, 5, 6, 8, 12, and

24 h and quenched with 6 M urea (40 μL) at 4 °C for 10 min. Then 20% trichloroacetic acid (40 μL) was added and the mixture was incubated at 4 °C for another 10 min. After 10 min centrifugation at 14000 g and 4 ºC, the supernatant was filtered and analyzed on RP-HPLC. The RP-HPLC gradient was started at 10% of B (MeCN), then increased to 100% of B over 20 min (A: 0.1% TFA in water) at a flow rate of 0.5 mL/min. The amount of the intact peptide at different time points assessed by HPLC was used to determine the extent of degradation caused by peptidases in serum.

As expected, cyclization of the linear peptides can dramatically improve the protease resistance, although still less stable than cyclic CTX, which has been shown to be highly stable in human serum. As shown in Fig. S1, about 50% of bicycle-P2 and bicycle-P3 remained intact after 10 h, whereas their linear counterparts P2 and P3 were decomposed in 10 minutes. The different degradation rates of bicyclic P1, P2, P4 and P5 suggest that the serum stability is highly related to the peptide sequences. Mass spectrometry analysis of the degradation intermediates revealed that both N- and C- terminal residues of bicyclic peptides in the format of XCXmCXnCX-NH2 were susceptible to proteolytic cleavage. Therefore, we envisioned that removal of these two terminal amino acids would give rise to an increased serum activity and N-terminal acetylation could further improve resistance to serum degradation. As a result, bicycle-P3 with the format of AcCXmCXnC-NH2 was found to be the most proteolytically stable peptide with a half-life of 12.5 h.

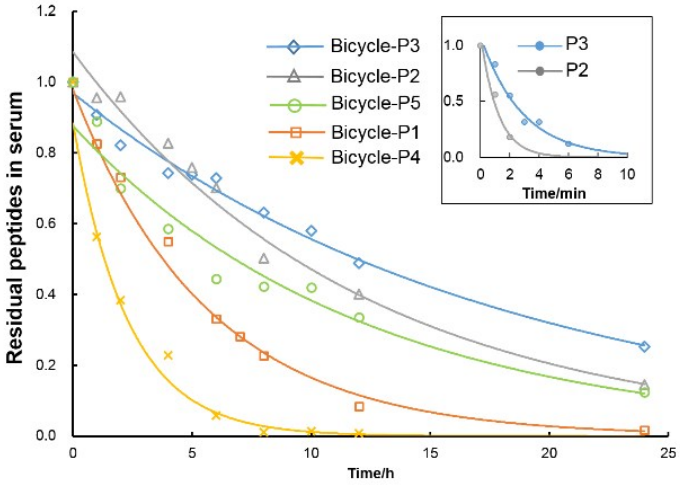


Fig. S2 Stability of the bicyclic peptide P1-P5 and linear P2, P3 in rat serum. The stability of peptides in rat serum was determined over 24 h. The amount of the residual peptide incubated in rat serum at different times was detected by HPLC.

9. Specific uptake of bicyclic peptide P1-P5 by glioma cells

Cell Culture: The glioblastoma cell lines (U-87 MG and U-87 LUC) and the human breast adenocarcinoma cell line (MCF7) were kindly provided by Prof. Lintao Cai (Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences). All cell lines were maintained in DMEM (Hyclone) supplemented with 10% FBS (Gibco), 100 U/mL penicillin, and 0.1 mg/mL streptomycin.

Isolation of Mouse Primary Neuronal Cells: The tissue culture dishes were coated for 2 h with Poly-D-Lysine (BD cat no. 354210, 0.05 mg/ml) one day prior to the isolation of mouse primary neuronal cells, washed three times with UltraPure Distilled water, and subsequently air dried for ~4 h. A 16.5 days pregnant female mouse was sacrificed. The abdominal cavity was opened and uterus containing embryos was removed. Under dissecting microscope, the immature mice were decapitated carefully with clean sterile scalpel. The thin skin layer was carefully removed by pinching skin at center and peeling away to obtain the brain from skull. Two hemispheres were separated from the midbrain and the meninges were removed to acquire the whole hippocampus. The hippocampus was incubated with trypsin in 37 °C for 15 min to digest tissue pieces and disperse the neuronal cells. Finally, the neuronal cells were cultured in B-27 Plus Neuronal Culture Medium (Gibco).

Cellular Uptake: U-87 MG cells and primary neuronal cells were incubated in a 4-well chambered coverglass slips (Thermo Fisher Scientific) to approximately 50% to 60% confluency. FITC-labelled bicyclic peptide P1-P5 or FITC-labelled CTX (Ex/Em: 488/520 nm) was diluted with media to 2 μM. The resulting mixture was added to the well and cultured at 37 ^{o}C for 2 h. After incubation, the cell nuclei were stained with Hoechst 33258 (Ex/Em: 405/460 nm) for 30 min in the dark. Then the sample-containing medium was replaced with equal volume of cold medium for three times. Following this step, the cells were fixed with 4% formaldehyde solution for 15 min before they were rinsed by PBS three times. The fluorescence images were taken by

the Leica SD AF confocal microscope.

Flow Cytometry Analysis: The primary neuronal cells and U-87 MG were incubated in a 12-well plate as 10^5 cells/well one day before the assay. FITC-labelled peptides were diluted with media to 2 μM and disposed with cells for 2 h at 37℃. Media were rinsed three times with cold PBS. The washing steps were followed by trypsinizing and resuspending in 500 μL cold PBS. The fluorescence intensities in cells were quantified by fluorescence activated cell sorting (FACS) and analysed using FlowJo software (Tree Star, Inc.). 10,000 cells were examined to acquire the mean fluorescence emission intensity for 488 nm excitation laser and 530/30 band-pass filter.

10. *In vivo* biodistribution and imaging of bicyclic peptide P1-P5

Mouse orthotopic xenografts: All experiments with animals were performed under the guideline of Institutional Ethical Committee of Animal Experimentation of Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences (IACUC). Luciferase gene was transfected into U-87 MG to constitute U-87 LUC cells, a powerful tool for locating orthotopic xenografts precisely by detecting photon emission derived from luciferase. When the cells were full of petri dishes, suspended them with trypsase followed by re-suspended them in a volume of fresh HBSS at a dose of 2×10^5 cells/μL. The BALB/c nude male mice, 5-8 weeks of age were anesthetized by intraperitoneal injection of pentobarbital. Then the anesthetized mouse was transferred to the Mouse Stereotactic platform to make an incision in the mid-scalp with a sterile scissor, confirming the visualization of the bregma. The bone at a position 1 mm anterior and 1.6 mm lateral to the bregma in the right cerebral hemisphere was pierced with a 0.45 mm syringe needle. 3~5 μL (10^6 cells) cell suspension was injected through the burr hole at a rate of 0.5 μL/min using the Nanomite injector syringe pump. Then the burr hole in the skull was sealed with sterile bone wax, and the wound was stitched up. Animals were monitored regularly for any potential symptoms.

***In Vivo* Optical Imaging and Quantification:** On day 5–7, at which point the tumor

reached an average diameter of 4–5 mm, animals were injected via tail vein with Cy5.5-labelled bicyclic peptide P1-P5 or Cy5.5-labelled CTX at a concentration of 20 μM. After 4h, the tumor-bearing mice were anesthetized with 2% isoflurane and oxygen in a chamber. In the meantime, they were injected intraperitoneally with 100 μL of D-Luciferin and imaged by using an IVIS Imaging System (Life Sciences). Luminescent and fluorescent images were taken with settings at an open filter and an excitation filter at 640 nm, respectively. For luminescent images, the Exposure time was fixed at 1 min, and binning was set at 8. For fluorescent images, the Exposure time was set at "Auto". All images were acquired and analyzed with Living Image software. The tumor sites were determined by the luminescence signals on the head. ROIs (Region of Interest) were located at the same position to quantify the accumulation of Cy5.5-labelled bicyclic peptides. Fluorescence from other parts of the head without tumor were considered as background signals. Signals for each bicyclic peptide were taken from ≥3 mice for statistical analysis.

Pharmacokinetic Properties of Cy5.5-labelled Bicycle-P3: Given that bicycle-P3 is more stable in serum than bicycle-P2, Cy5.5-labelled bicycle-P3 was thus selected to explore the pharmacokinetic properties. As shown in Fig. S2, bicycle-P3 accumulated mostly in kidney at 4 h post-injection, indicating that the CTX derivative was mainly eliminated by renal excretion pathways. Consistent with this *ex vivo* observation, the fluorescence imaging revealed rapid renal clearance of bicycle-P3 within two days in both tumor-bearing mice and normal mice (Fig S3a). Moreover, the fluorescence signal in the tumor region maintained its maximum at 4 h post-injection and then decreased gradually. The remained signal in the tumor region was still detectable as long as 12 h following injection, but almost disappeared at 24 h post-injection (Fig. S3b). These results suggest that there is a time window for *in vivo* tumor imaging by using bicycle-P3.

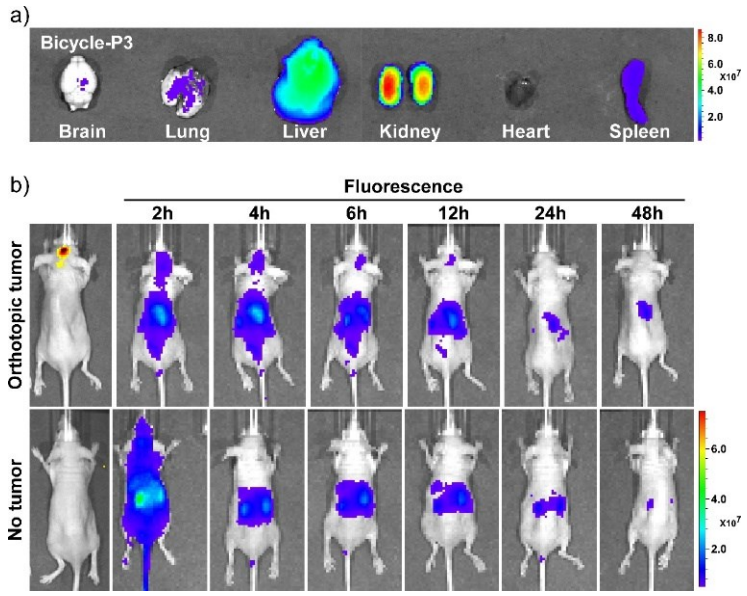


Fig. S3 Pharmacokinetic properties of Cy5.5-labelled bicycle-P3. a) *Ex vivo* fluorescence images of major organs from mice with brain tumor at 4 h post-injection of Cy-bicycle-P3. b) Real-time biodistribution of Cy-bicycle-P3 in mice with or without brain tumor.

NIRF Imaging of Cy5.5-labelled Peptides in Brain Sections: To further determine the precise distribution of these Cy5.5-labelled bicyclic peptides, glioma-bearing mice were sacrificed after whole-body imaging and the brains from the mice were sliced coronally to expose the tumor for additional analysis. The tumor regions were identified by DAPI staining. As shown by NIRF imaging, Cy5.5-labelled bicycle-P2 and bicycle-P3 could specifically accumulated in glioma tissues just like Cy5.5-labelled CTX. In contrast, although bicycle-P1, P4 and P5 could also target tumor tissue, the fluorescence signal was very weak and was mainly detected at the periphery region of the tumor tissue (Fig. S4a). Further quantitation result showed that the fluorescence intensity of bicycle-P3 in tumor tissues was nearly threefold higher than that of bicycle-P1, P4, and P5 (Fig. S4b).

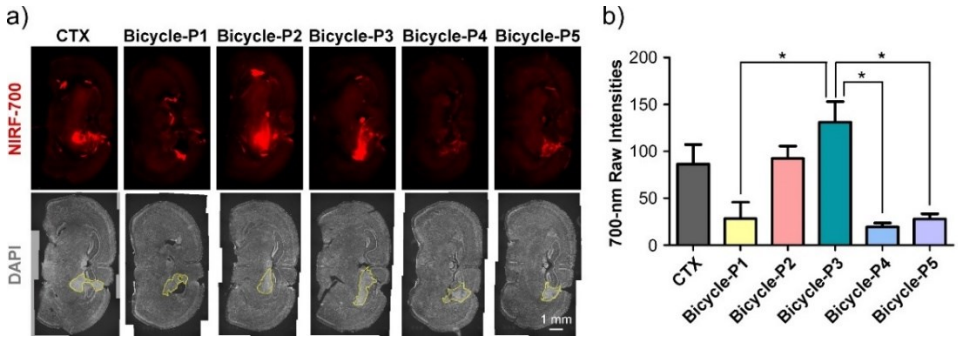


Fig. S4 The precise localization of Cy5.5-labelled peptides in brains. a) Near infrared fluorescence images of brain sections taken from animals at 4 h post-injection of Cy5.5-labelled peptides. The signals of Cy5.5 are shown in red. Cell nuclei stained by DAPI are shown in white. Xenografts are encircled by yellow lines. b) Quantification in brain sections. Statistical analyses were performed using Student's t-test. All experiments were performed as a minimum of 3 independent repeats. Data are presented as mean ± SEM. *p<0.05.

Brain Section Immunostaining and Imaging: 50 μm brain sections were fixed with 4% formaldehyde overnight, rinsed by PBS and then incubated with 3% bovine serum albumin (BSA) and 0.2% Triton X-100 for 1h to block non-specific binding. Primary antibodies against mouse-nestin (Millipore, MAB353, 1:400) and against rabbit-TUJ1 (Covance, PRB-435P, 1:1000) were employed to brain sections for 2 h at room temperature. After washing with PBS three times (5 min for each time) at room temperature, the brain sections were incubated for 1 h with the anti-mouse secondary antibody (Invitrogen, A-21202, 1:500, Ex/Em: 488/520 nm), anti-rabbit secondary antibody (Invitrogen, A-31572, 1:500, Ex/Em: 561/590 nm) and DAPI (1:500, Ex/Em: 405/460 nm). Again, the brain sections were washed with PBS three times (5 min for each time) at room temperature and imaged on the Leica SD AF confocal microscope and Licor Odyssey CLx dual band IR imaging system.

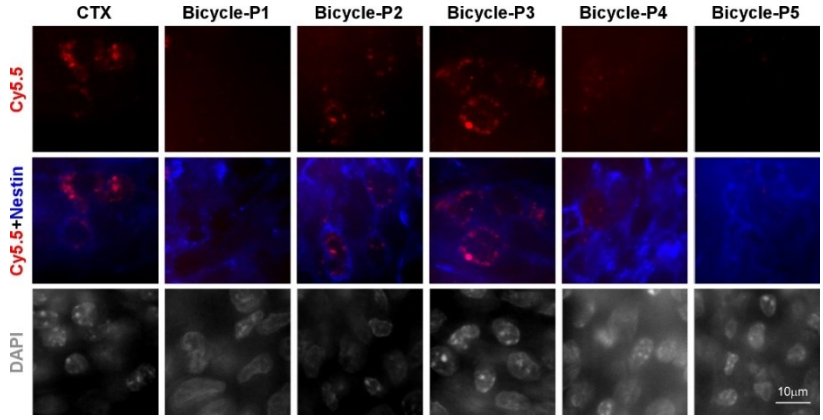


Fig. S5 *In vivo* internalization of Cy-bicycle-P3 by glioblastoma cells. The signals of Cy5.5 are shown in red. The glioma cells marked with anti-nestin antibody are shown in blue. Cell nuclei stained with DAPI is shown in white.

11. The glioma cell targeting of bicycle-P3 *via* MMP2

Competitive Binding Assay: U-87 MG cells were pre-incubated with 100 μM CTX for 1h at 4 °C. Then 4 μM FITC-bicycle-P3 was added and the cells were incubated for another 1h at 4 °C. U-87 MG cells incubated with 4 μM FITC-bicycle-P3 alone were used as control. The cells were washed and fixed just as described above. Then

the fluorescence images were taken by the Leica SD AF confocal microscope.

Affinity Pulldowns: 50 μl Dynabeads™ Myone™ Streptavidin T1(Invitrogen) were incubated overnight with 5 μM biotinylated bicycle-P3 at 4 °C, then washed five times to remove the unconnected peptide. 0.5 μg MMP2 protein (Sino Biological) was pre-incubated with either PBS or 50 μM unmodified bicycle-P3 at 4 °C for 1h. Mix the MMP2 protein with the washed Magnetic Beads at 4 °C overnight. Equal amounts of protein were loaded on a 10% SDS/PAGE gel, transferred to a PVDF membrane (Merck Millipore), and detected using an anti-MMP2 antibody (Cell Signaling). Goat anti-rabbit IgG-HRP was used as the secondary antibody (Abcam).

Overexpression of MMP-2 Protein in MCF7 Cells: MCF7 cells were seeded in a 24-well plate on coverslips and transfected with 0.3 μg pCMV3-C-FLAG-MMP2 plasmid DNA (Sino Biological) using Lipofectamine™ 3000 transfection reagent (Invitrogen). After 24 h, cells were incubated with 2 μM FITC-bicycle-P3 for 1h in 37 °C. The washing and fixing steps were conducted just as described above. Exogenous MMP2 protein was labelled with anti-FLAG antibody (Cell Signaling). Anti-mouse antibody was used as the secondary antibody (Invitrogen). The fluorescence images were taken by the Leica SD AF confocal microscope.

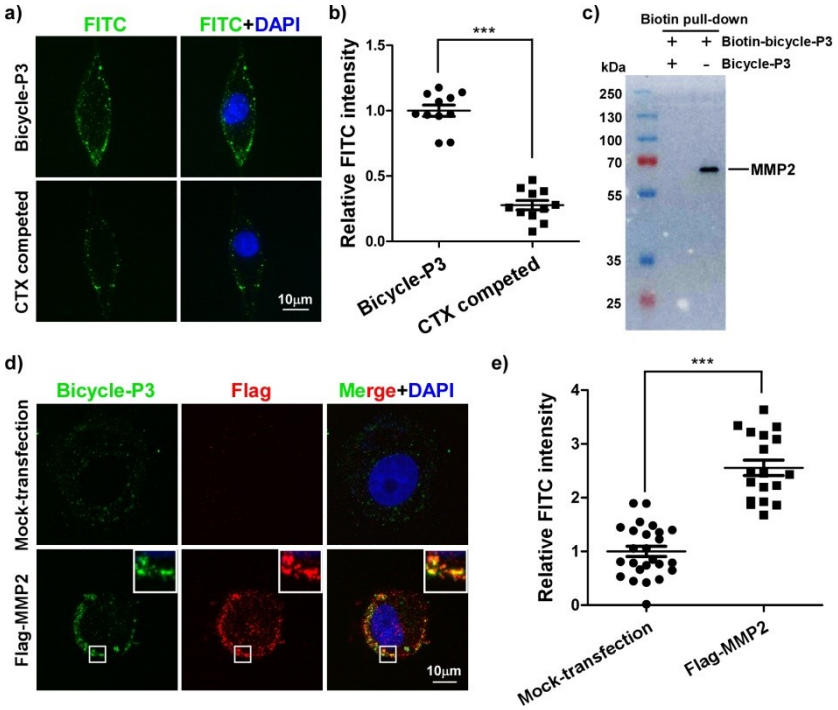
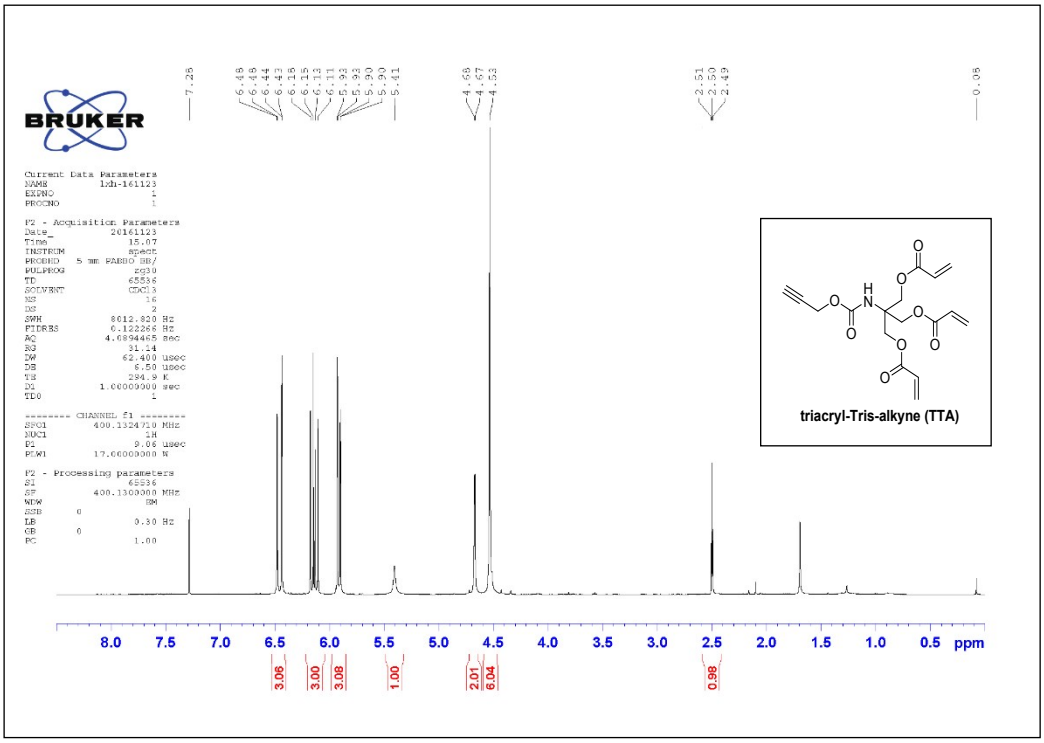


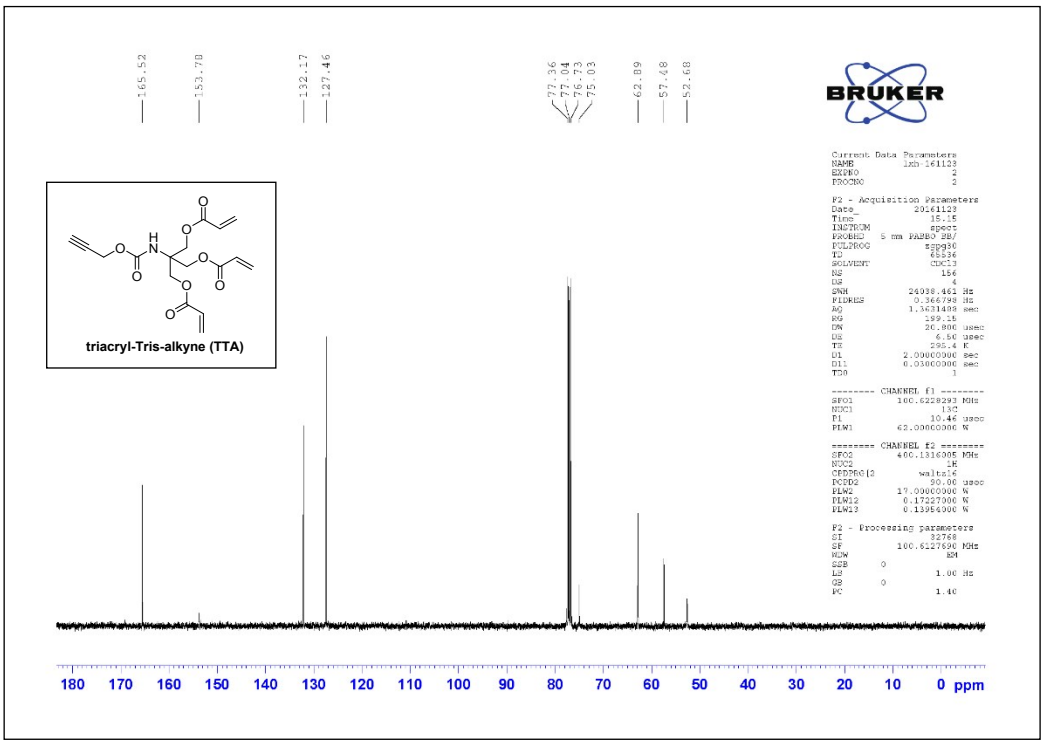
Fig. S6 The glioblastoma cell targeting of bicycle-P3 *via* MMP2. a) Confocal images of FITC-labeled bicycle-P3

(green) binding to U-87 MG cells in the presence of 25-fold excess amount of unmodified CTX. Cell nuclei stained with DAPI is shown in blue. b) Quantification of mean fluorescence intensity of FITC signal within U-87 MG cells. Data are presented as mean ± SEM. n=11, ***p<0.001. c) Interaction of bicycle-P3 with MMP2 protein.

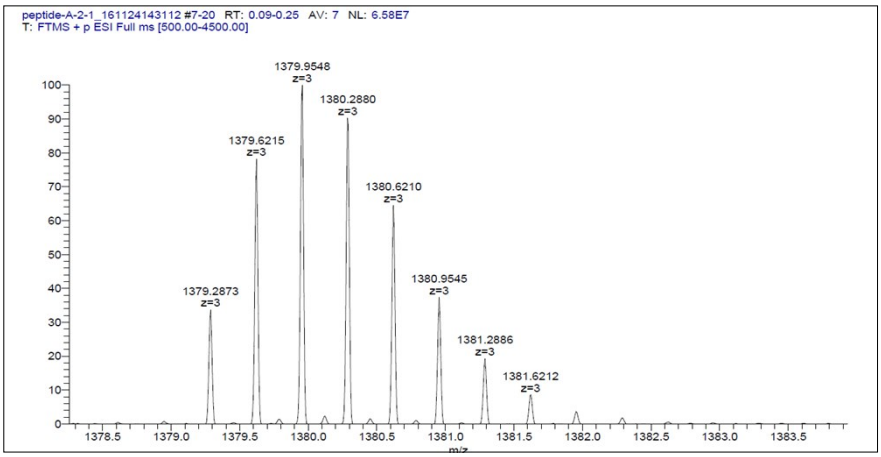
^{1}H, ^{13}C NMR and HRMS Spectra



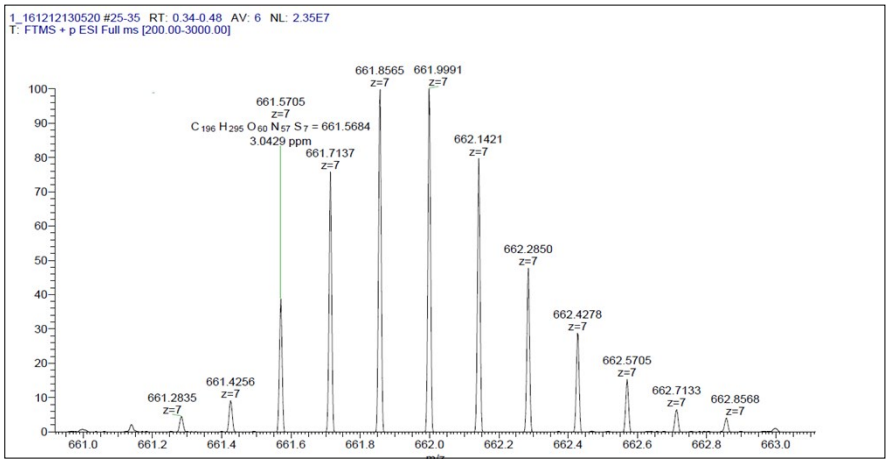
^{1}H NMR spectrum of triacryl-Tris-alkyne (TTA)



^{13}C NMR spectrum of triacryl-Tris-alkyne (TTA)

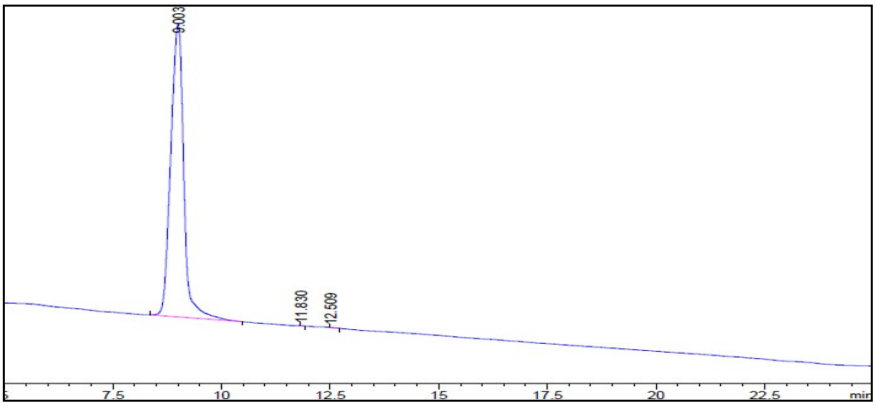


HRMS spectrum of bicycle-P1 {HRMS (ESI-TOF) m/z: calcd. for $C_{172}H_{274}N_{53}O_{54}S_6$ $[M+3H]^{3+}$ 1379.2877, found 1379.2873.}



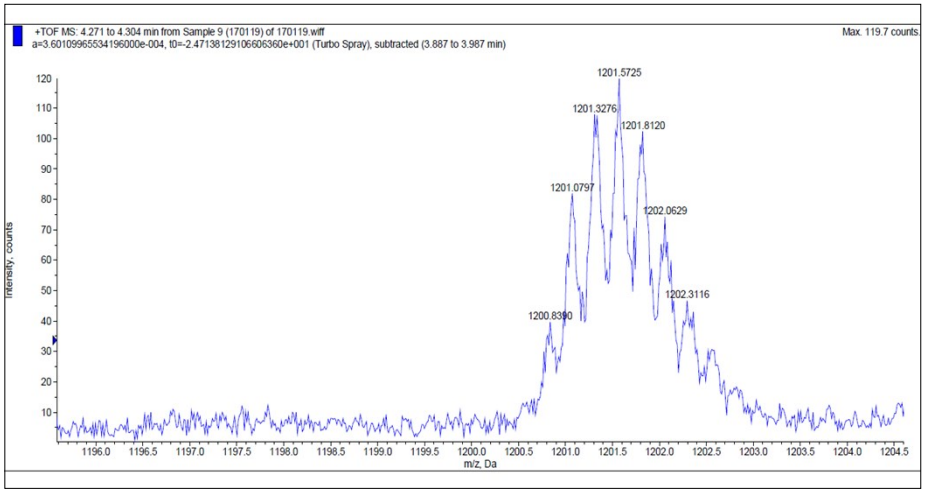
HRMS spectrum of FITC-bicycle-P1 {HRMS (ESI-TOF) m/z: calcd. for $C_{196}H_{295}O_{60}N_{57}S_7$ $[M+7H]^{7+}$ 661.5684, found 661.5705.}

HPLC analysis of FITC-bicycle-P1：



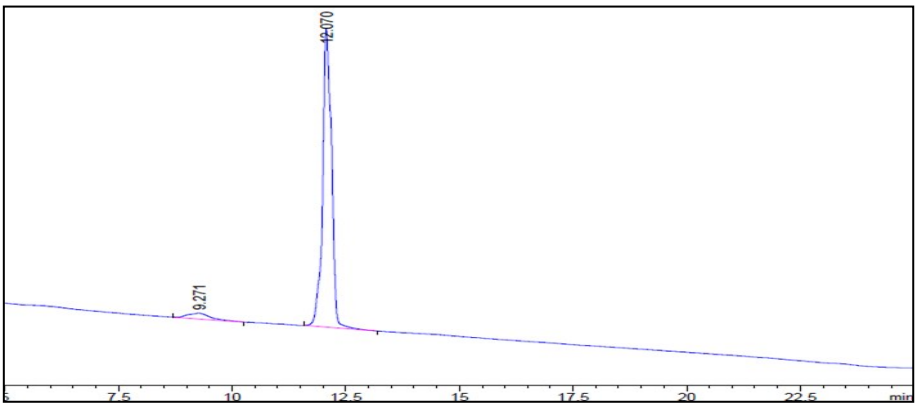
220 nm Result

Peak #	Retention Time	Height (mAU)	Area (mAU*s)	Area%
1	9.003	916.69891	1.90757e4	99.9094
2	11.830	1.35791	5.31117	0.0278
3	12.509	1.31487	11.98951	0.0628
Total		919.37170	1.90930e4	



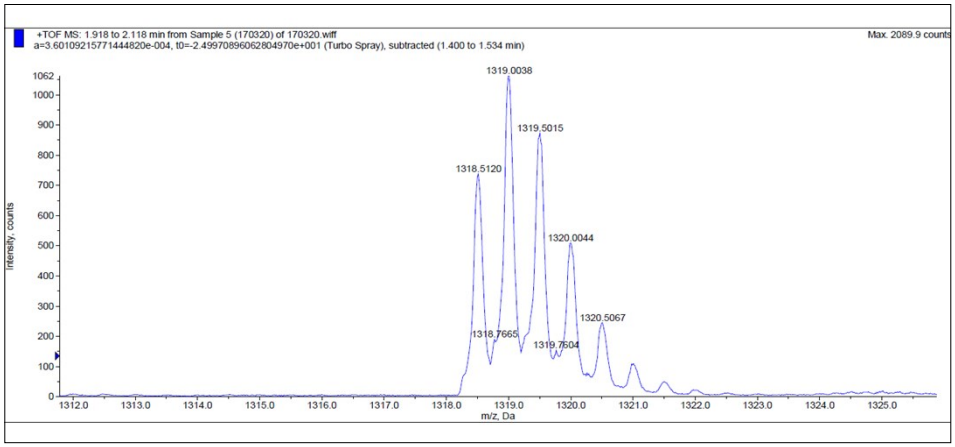
HRMS spectrum of Cy5.5-bicycle-P1 {HRMS (ESI-TOF) m/z: calcd. for $C_{215}H_{323}N_{59}O_{55}S_6$ $[M+3H]^{4+}$ 1200.8148, found 1200.8390.}

HPLC analysis of Cy5.5-bicycle-P1：

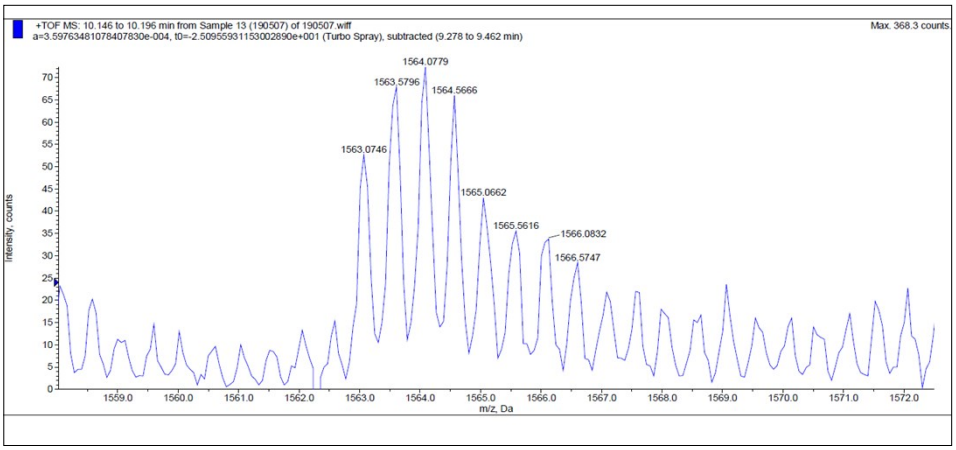


220 nm Result

Peak #	Retention Time	Height (mAU)	Area (mAU*s)	Area%
1	9.271	17.60874	587.58276	4.4727
2	12.070	919.58234	1.25495e4	95.5273
Total		937.19107	1.31371e4	

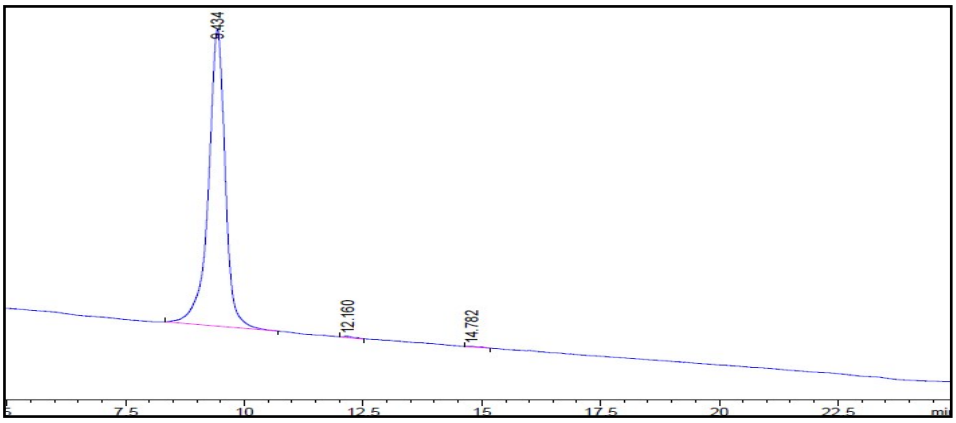


HRMS spectrum of bicycle-P2 {HRMS (ESI-TOF) m/z: calcd. for $C_{107}H_{164}N_{30}O_{38}S_5$ $[M+2H]^{2+}$ 1318.5208, found 1318.5120.}



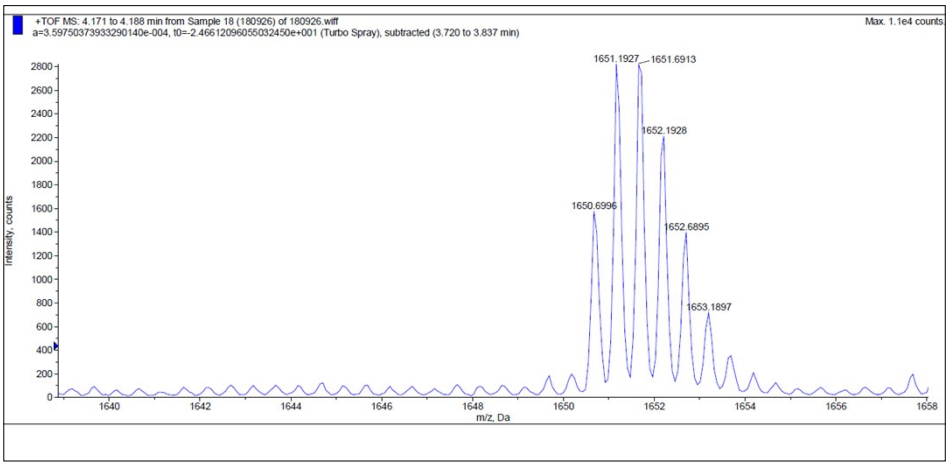
HRMS spectrum of FITC-bicycle-P2 {HRMS (ESI-TOF) m/z: calcd. for $C_{131}H_{183}N_{35}O_{43}S_6$ $[M+2H]^{2+}$ 1563.0761, found 1563.0746.}

HPLC analysis of FITC-bicycle-P2：



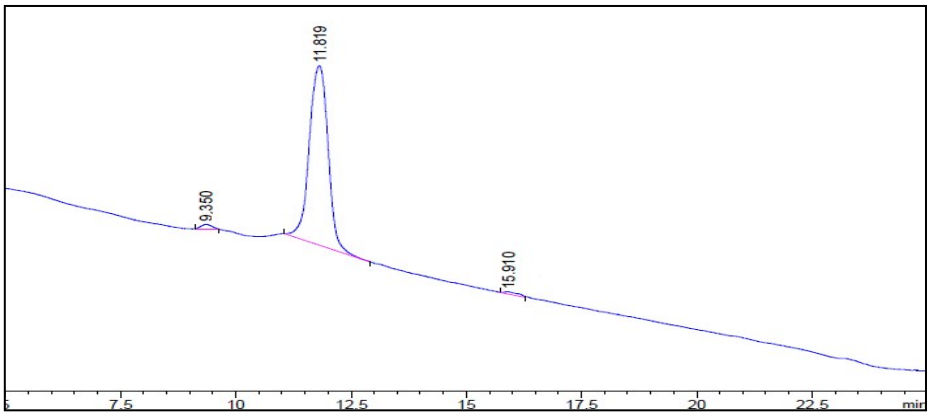
220 nm Result

Peak #	Retention Time	Height (mAU)	Area (mAU*s)	Area%
1	9.434	785.73950	1.93837e4	99.4575
2	12.160	3.91249	61.69202	0.3165
3	14.782	2.27498	44.03445	0.2259
Total		791.92697	1.94894e4	



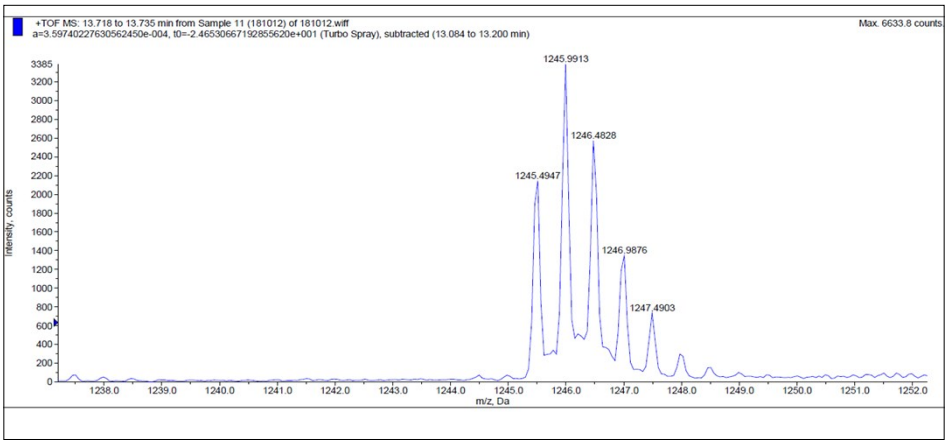
HRMS spectrum of Cy5.5-bicycle-P2 {HRMS (ESI-TOF) m/z: calcd. for $C_{150}H_{212}N_{36}O_{39}S_5$ $[M+H]^{2+}$ 1650.7152, found 1650.6996.}

HPLC analysis of Cy5.5-bicycle-P2：

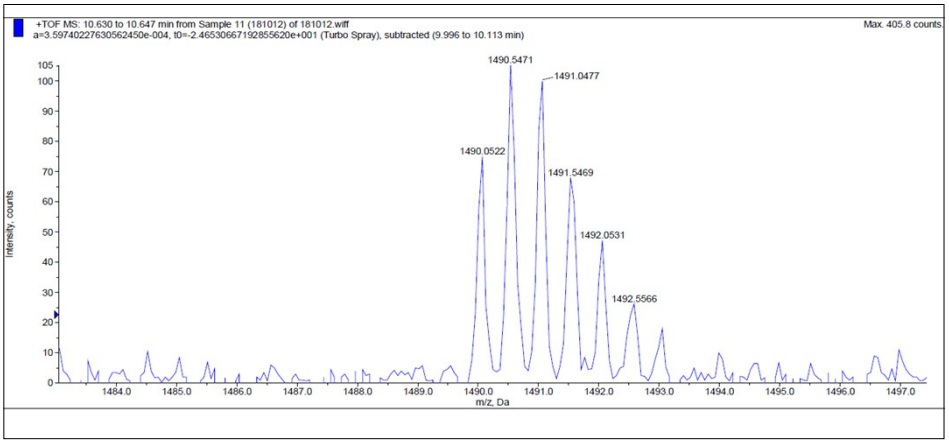


220 nm Result

Peak #	Retention Time	Height (mAU)	Area (mAU*s)	Area%
1	9.350	5.37915	92.24747	1.6164
2	11.819	196.40581	5552.61572	97.2947
3	15.910	2.68131	62.14547	1.0889
Total		204.46627	5707.00866	

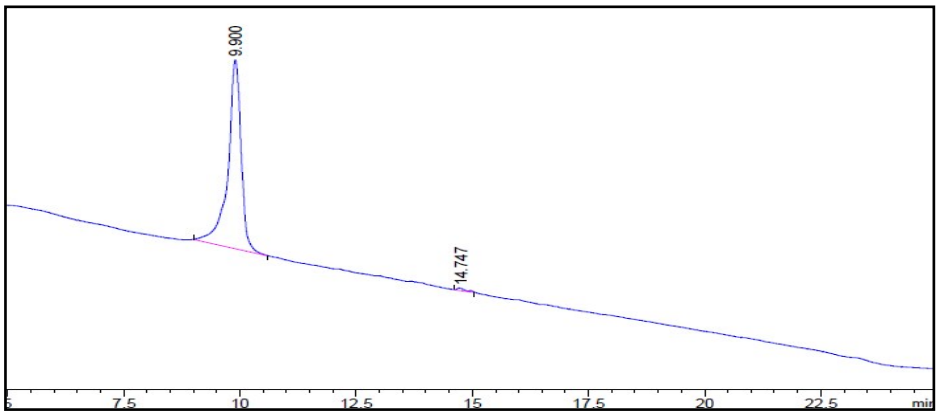


HRMS spectrum of bicycle-P3 {HRMS (ESI-TOF) m/z: calcd. for $C_{102}H_{154}N_{28}O_{37}S_4$ $[M+2H]^{2+}$ 1245.4951, found 1245.4947.}



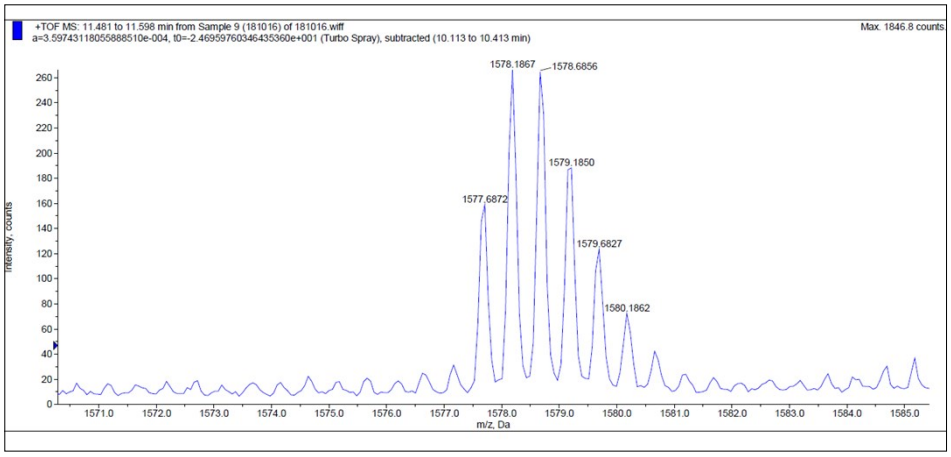
HRMS spectrum of FITC-bicycle-P3 { HRMS (ESI-TOF) m/z: calcd. for $C_{126}H_{173}N_{33}O_{42}S_5$ $[M+2H]^{2+}$ 1490.0504, found 1490.0522.}

HPLC analysis of FITC-bicycle-P3：



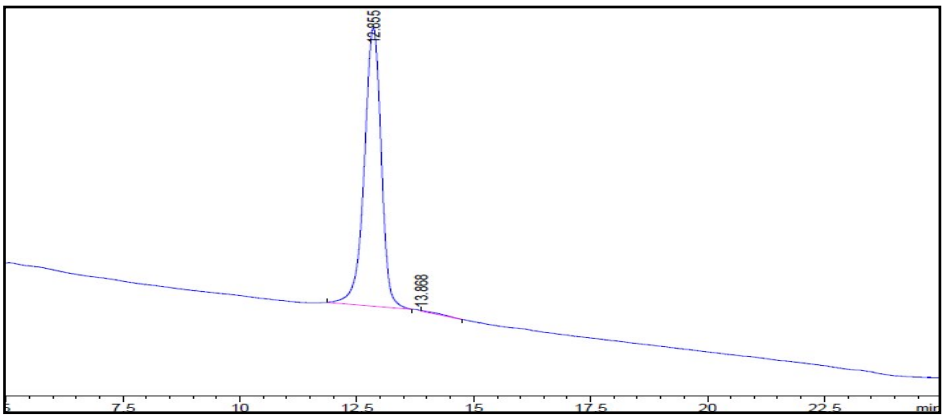
220 nm Result

Peak #	Retention Time	Height (mAU)	Area (mAU*s)	Area%
1	9.900	223.67113	4446.65674	99.1990
2	14.747	2.86370	35.90422	0.8010
Total		226.53483	4482.56096	



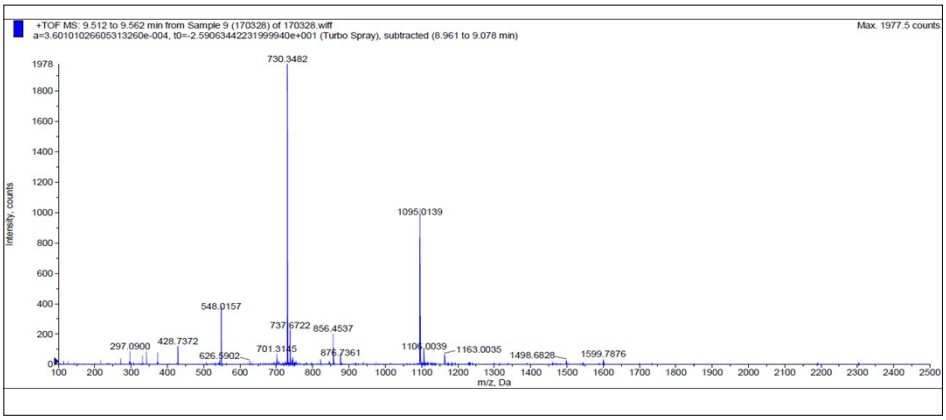
HRMS spectrum of Cy5.5-bicycle-P3 {HRMS (ESI-TOF) m/z: calcd. for $C_{145}H_{202}N_{34}O_{38}S_4$ $[M+H]^{2+}$ 1577.6896, found 1577.6872.}

HPLC analysis of Cy5.5-bicycle-P3：

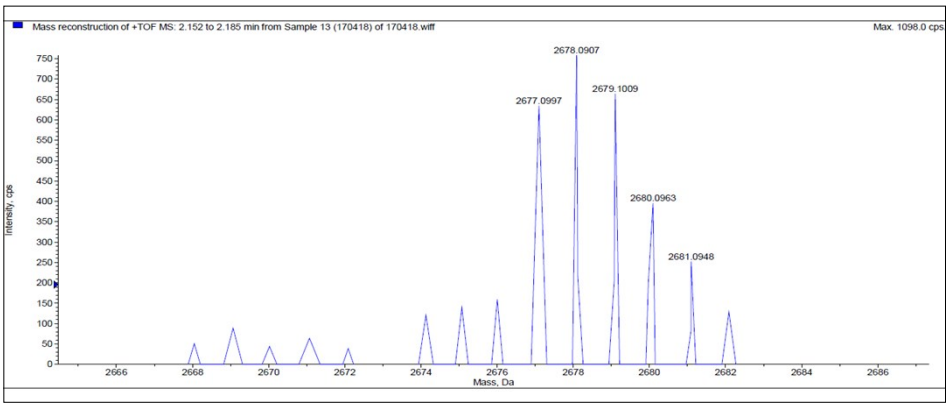


220 nm Result

Peak #	Retention Time	Height (mAU)	Area (mAU*s)	Area%
1	12.855	492.02820	1.23972e4	99.4982
2	13.868	1.40806	62.51948	0.5018
Total		493.43625	1.24597e4	

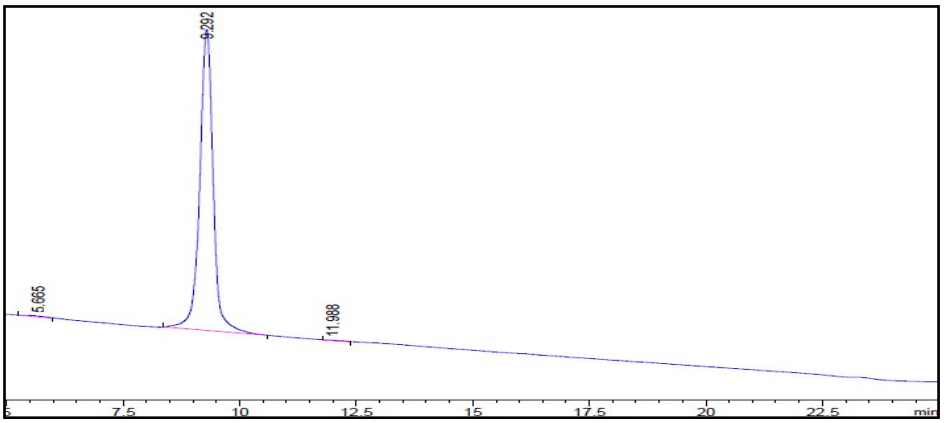


HRMS spectrum of bicycle-P4 {HRMS (ESI-TOF) m/z: calcd. for $C_{91}H_{147}N_{29}O_{28}S_3$ $[M+2H]^{2+}$ 1095.0061, found 1095.0139.}



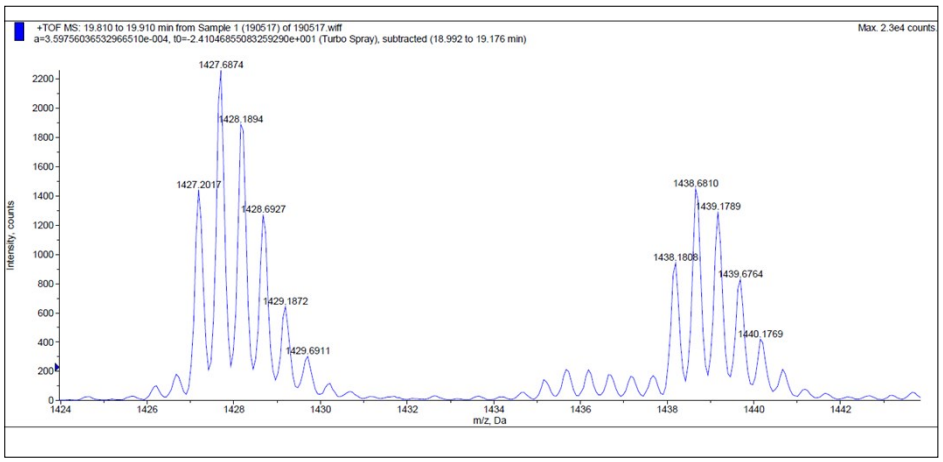
HRMS spectrum of FITC-bicycle-P4 {HRMS (ESI-TOF) m/z: calcd. for $C_{115}H_{165}N_{34}O_{33}S_4$ $[M+H]^+$ 2678.1156, found 2677.0997.}

HPLC analysis of FITC-bicycle-P4：



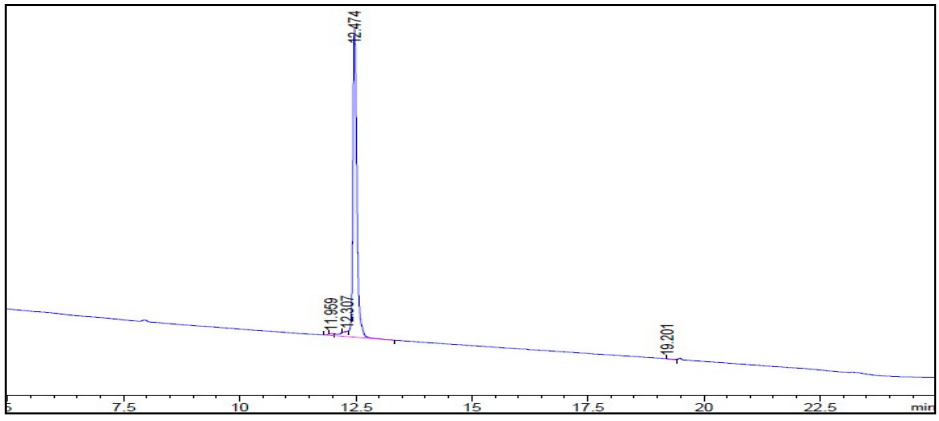
220 nm Result

Peak #	Retention Time	Height (mAU)	Area (mAU*s)	Area%
1	5.665	3.01176	82.71459	0.4606
2	9.292	889.85242	1.78194e4	99.2224
3	11.988	2.31162	56.92682	0.3170
Total		895.17579	1.79590e4	



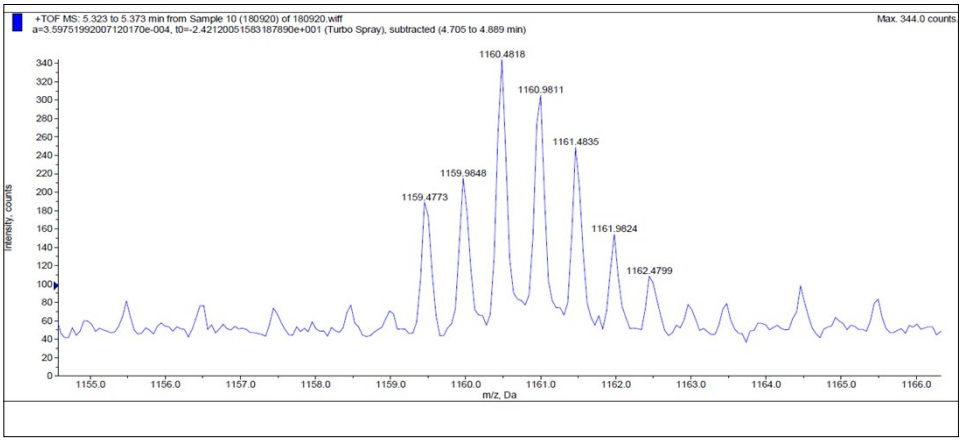
HRMS spectrum of Cy5.5-bicycle-P4 {HRMS (ESI-TOF) m/z: calcd. for $C_{134}H_{195}N_{35}O_{29}S_3$ $[M+H]^{2+}$ 1427.2006, found 1427.2017; HRMS (ESI-TOF) m/z: calcd. for $C_{134}H_{194}N_{35}NaO_{29}S_3$ $[M+Na]^{2+}$ 1438.1916, found 1438.1808.}

HPLC analysis of Cy5.5-bicycle-P4：

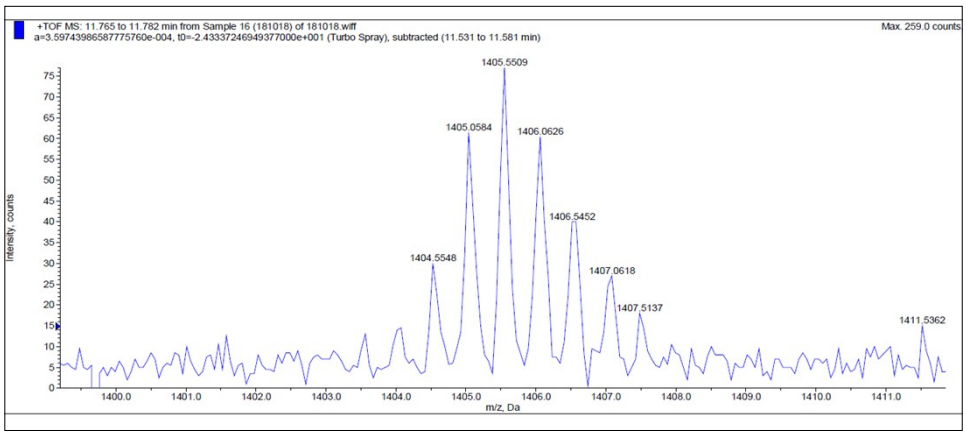


220 nm Result

Peak #	Retention Time	Height (mAU)	Area (mAU*s)	Area%
1	11.959	1.40794	6.12693	0.1030
2	12.307	1.21440	6.89408	0.1159
3	12.474	964.64417	5918.92090	99.5429
4	19.201	1.27942	14.15864	0.2381
Total		968.54592	5946.10055	

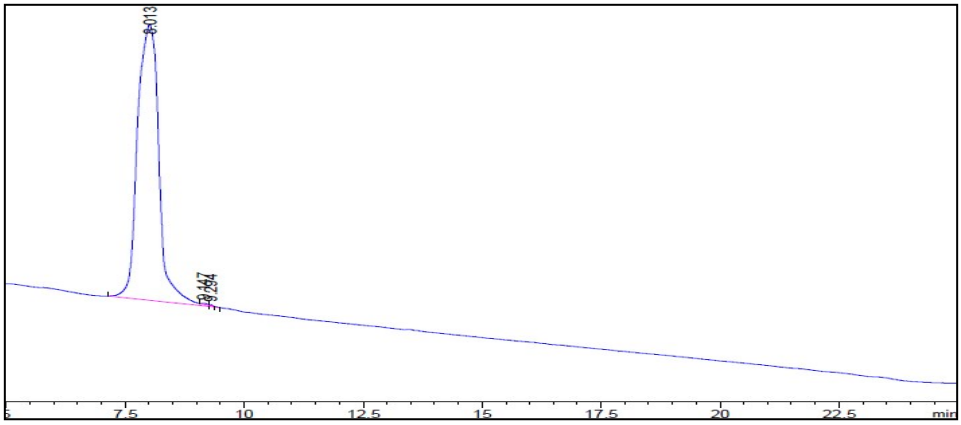


HRMS spectrum of bicycle-P5 { HRMS (ESI-TOF) m/z: calcd. for $C_{91}H_{153}N_{31}O_{32}S_4$ $[M+2H]^{2+}$ 1160.0085, found 1159.9848.}



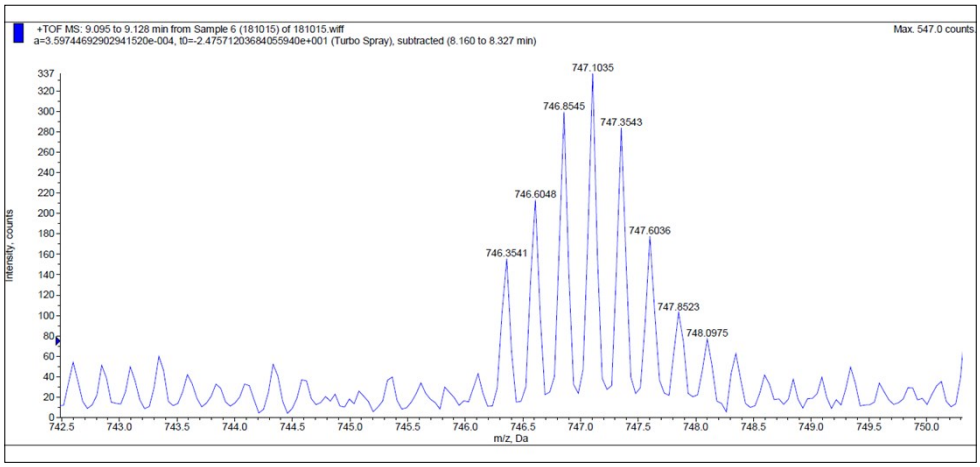
HRMS spectrum of FITC-bicycle-P5 {HRMS (ESI-TOF) m/z: calcd. for $C_{115}H_{172}N_{36}O_{37}S_5$ $[M+2H]^{2+}$ 1404.5638, found 1404.5548.}

HPLC analysis of FITC-bicycle-P5：



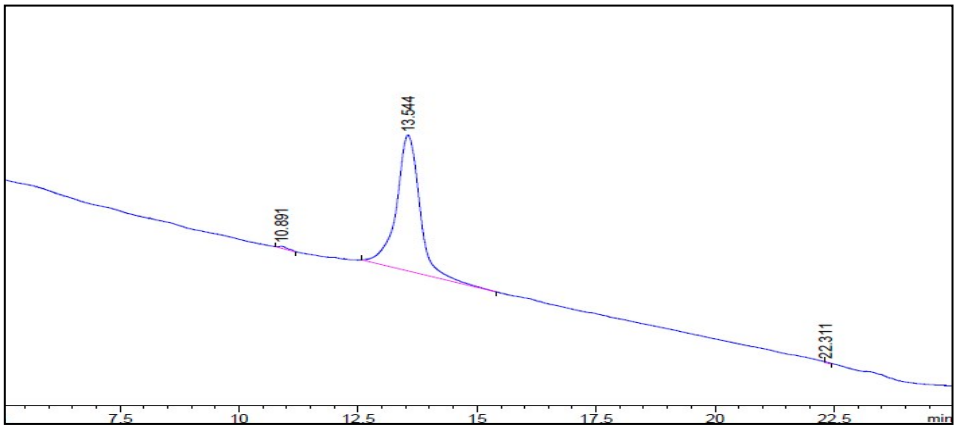
220 nm Result

Peak #	Retention Time	Height (mAU)	Area (mAU*s)	Area%
1	8.013	562.55084	1.74577e4	99.8080
2	9.147	2.91541	23.36457	0.1336
3	9.294	2.65586	10.22426	0.0585
Total		568.12211	1.74913e4	



HRMS spectrum of Cy5.5-bicycle-P5 {HRMS (ESI-TOF) m/z: calcd. for $C_{134}H_{202}N_{37}O_{33}S_4$ $[M+3H]^{4+}$ 746.3533, found 746.3541.}

HPLC analysis of Cy5.5-bicycle-P5：



220 nm Result

Peak #	Retention Time	Height (mAU)	Area (mAU*s)	Area%
1	10.891	2.12591	30.36716	0.6645
2	13.544	133.01094	4531.50977	99.1639
3	22.311	1.02932	7.84097	0.1716
Total		136.16617	4569.71790	