Electronic Supplementary Material (ESI) for MedChemComm.
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Supplementary data

Reagents and materials

Rink amide AM resin (0.3 mmol/g) was purchased from Tianjin Nankai Hecheng Science &Technology (Tianjin, China). Fmoc-S_5-OH was purchased from Okenanos (Beijing, China). Fmoc amino acids, O-(6-Chlorobenzotriazol-1-yl)-N, N, N', N'-tetramethyluronium hexafluorophosphate (HCTU) and O-(7-azabenzotriazol-1-yl)-N, N, N', N'-tetramethyluronium hexafluorophosphate (HATU) were both purchased from GL Biochem (Shanghai, China). N, N-Diisopropylethylamine (DIPEA) and Triisopropylsilane (TIPS) were purchased from Ouhe Technology (Beijing, China). Acetonitrile (HPLC grade) was purchased from J.T. Baker (Phillipsburg, NJ, USA). Anhydrous diethyl ether were purchased from Sinopharm Chemical Reagent. N, N-dimethylformamide (DMF), dichloromethane (DCM), thioanisole and trifluoroacetic acid (TFA) (HPLC grade) were purchased from J&K Scientific (Beijing, China).

Ghost(3)X4/R5 cells (catalog no. 3942) were obtained from the NIH ARRRP. 293T cells were obtained from the American Type Culture Collection (catalog no. 11268).

Dulbecco's modified Eagle's medium was purchased from Gibco BRL Life Technologies. Culture dishes were purchased from Corning-Costar. pNL4-3.Luc.R-E was obtained from the NIH ARRRP. PEI transfection reagent was purchased from Sigma. Bright-Glo luciferase assay system (E2650) was purchased from Promega.

HPLC and mass spectrometry (MS)

Reversed phase HPLC was performed on Shimadzu HPLC systems. For peptide analysis, Vydac C18 column (4.6 × 250 mm) was used at a flow rate of 1.0 mL/min. For digest experiment, C4 column (4.6 × 250 mm) was used at a flow rate of 1.0 mL/min. For peptide purification, Vydac C18 (10 × 250 mm) column was used at a flow rate of 3.0 mL/min. Acetonitrile (with 0.1% TFA) and water (with 0.1% TFA) were used as the mobile phase.

ESI-MS spectra were recorded on an Agilent 1200 Series HPLC system.

General procedures for synthesis of peptides

MT-SC22EK and its stapled peptides were manually prepared by Fmoc solid phase peptide synthesis using 200 mg Rink amide AM resin (loading 0.3 mmol/g). Firstly, the resin was swollen with DCM and DMF for 1 hour to fully expose its active site. Then the Fmoc group of resin was removed by adding 20% piperidine in DMF twice (5min+10min). After a series of washing, natural amino acid was assembled to the resin by adding a mixture with 4eq Fmoc-protected amino acid, 3.8eq HCTU, 8eq DIEA and 3.5 ml DMF for reacting 1h at 36°C. For Fmoc-S_5-OH assembling, a mixture of 2eq

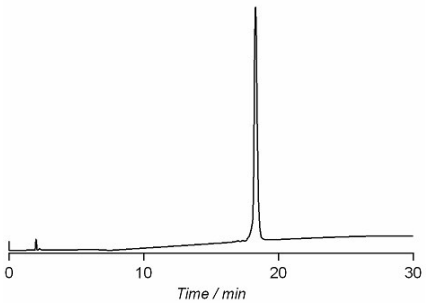
Fmoc-S_5-OH, 1.9eq HATU, 2.0eq HOAt, 4eq DIEA and 3.0 ml DMF was added to the resin for reacting 2 hour at 36°C. After coupling reaction, the resin was washed with DMF, DCM and DMF for respectively 5 times. N-terminal Fmoc group of the amino acid was removed as the same manner as that of the resin. The ring-closing olefin metathesis was carried out on resin-bound peptide with 3 mL DCE solution containing Grubbs' first-generation catalyst (32 mg). Acetylation of the peptide was accomplished by addition of acetylation solution (Ac_2O: DIEA: DMF = 1: 1: 8, v: v: v) for reacting 5 min*2. After all the amino acids fully assembled, the resin was treated with 10 mL TFA solution containing 2.5% water and 2.5% TIPS for reaction 3 hours. Followed by concentration, precipitation and centrifugation, khaki solid peptide was obtained. The crude peptide was separated and identified by HPLC and MS.

Synthesis of MT-SC22EK

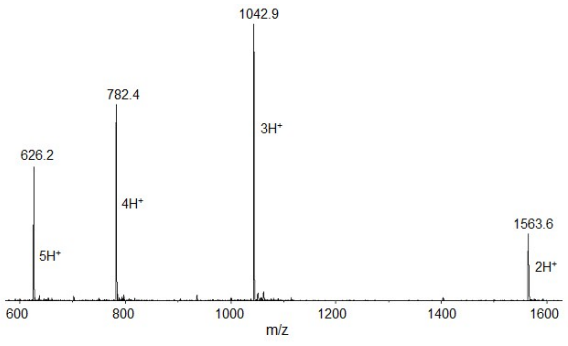
AC-MTWEEWDKKIEEYTKKIEELIKKS-NH_2

SI Figure 1. Sequence of MT-SC22EK.

MT-SC22EK was synthesized using the natural amino acid coupling method. Followed by further acetylation, cutting, concentration, precipitation and purification, a white solid (54.4) was obtained with a purity of more than 95% and an isolated yield of 29%.



SI Figure 2. HPLC chromatogram of MT-SC22EK after purification. Elution was carried out in a linear gradient from 20% to 70% acetonitrile (0.1 % trifluoroacetic acid), 30 min with C18 analytical column.

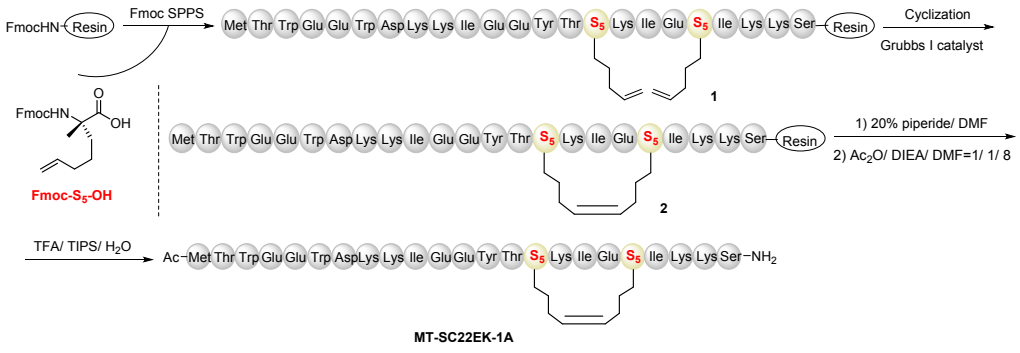


SI Figure 3. ESI-MS chart of MT-SC22EK. Calcd for $[M+H]^{+}$=3126.3 (average isotope), $[M+2H]^{2+}$=1563.7, $[M+3H]^{3+}$=1042.8, $[M+4H]^{4+}$=782.3, $[M+5H]^{5+}$=626.1. Observed for $[M+H]^{+}$=3126.2, $[M+2H]^{2+}$=1563.6, $[M+3H]^{3+}$=1042.9, $[M+4H]^{4+}$=782.4, $[M+5H]^{5+}$=626.2.

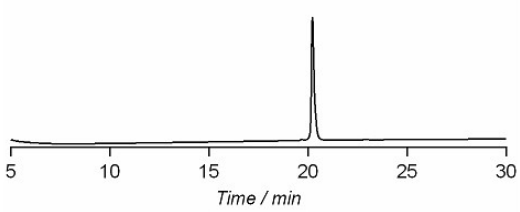
Synthesis of MT-SC22EK-1A

SI Figure 4. Sequence of MT-SC22EK -1A.

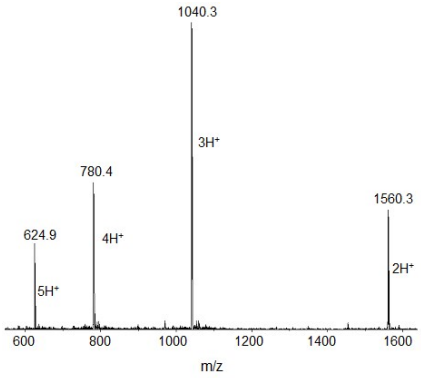
The amino acid residues at 1-14, 16-18 and 20-24 position in MT-SC22EK-1A were assembled using the natural amino acid coupling method (4eq AA, 3.8eq HCTU and 8eq DIEA). The coupling of Fmoc-S_5-OH at the positions 15 and 19 was carried out by adding a mixture with 2eq Fmoc-S5-OH, 1.9eq HATU, 2.0eq HOAt, 4eq DIPEA and 3.0 ml DMF for reaction 2 hour at 36°C. After all amino acids assembled on the resin, 3 mL Grubbs' first-generation catalyst (32 mg) DCE solution was added to carry out a side chain ring-closing olefin metathesis. After 1 hour, the same amount of Grubbs' catalyst was again added to ensure the ring-closing reaction complete. Followed by further acetylation, cutting, concentration, precipitation and purification, a white solid (37.4 mg) was obtained with a purity of greater than 95% an isolated yield of 20%. A detailed synthetic route of MT-SC22EK-2 was showed in SI Figure 5.



SI Figure 5. The synthetic route of MT-SC22EK-1A.



SI Figure 6. HPLC chromatogram of MT-SC22EK-1A after purification. Elution was carried out in a linear gradient from 20% to 70% acetonitrile (0.1 % trifluoroacetic acid), 30 min with C18 analytical column.

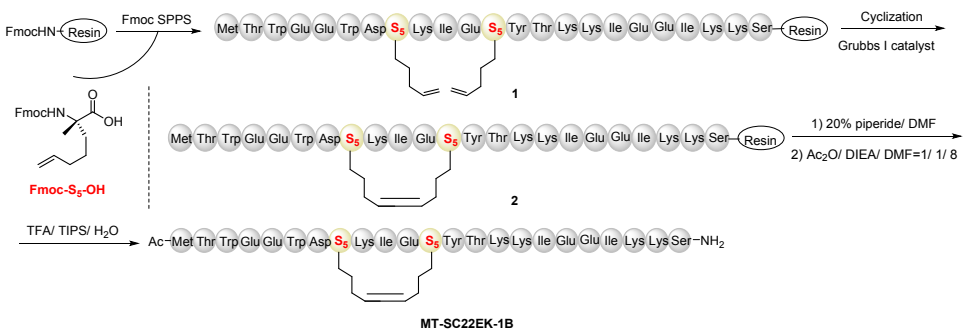


SI Figure 7. ESI-MS chart of MT-SC22EK-1A. Calcd for $[M+H]^{+}$=3119.4 (average isotope), $[M+2H]^{2+}$=1560.2, $[M+3H]^{3+}$=1040.5, $[M+4H]^{4+}$=780.6, $[M+5H]^{5+}$=624.7. Observed for $[M+H]^{+}$=3119.6, $[M+2H]^{3+}$=1560.3, $[M+4H]^{4+}$=1040.3, $[M+5H]^{5+}$=624.9.

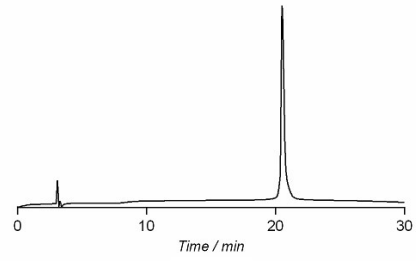
Synthesis of MT-SC22EK-1B

SI Figure 8. Sequence of MT-SC22EK-1B.

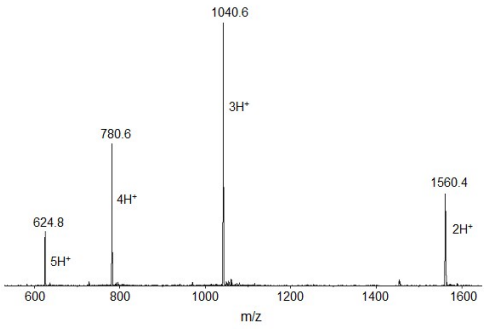
The amino acid residues at 1-7, 9-11 and 13-24 positions in MT-SC22EK-2 were assembled using 4eq AA, 3.8eq HCTU and 8eq DIEA coupling method. The amino acid residues at 8, 12positions adopted the 2eq Fmoc-S_5-OH, 1.9eq HATU, 2eq HOAt and 4eq DIEA coupling method. Until all amino acids fully assembled to the resin, the ring-closing olefin metathesis was performed in Grubbs' first-generation catalyst DCE solution. Followed by further acetylation, cutting, concentration, precipitation and purification, a white solid (33.6 mg) was obtained with a purity of greater than 95% and an isolated yield of 18%. A detailed synthetic route of MT-SC22EK-1B was showed in SI Figure 9.



SI Figure 9. The synthetic route of MT-SC22EK-1B.



SI Figure 10. HPLC chromatogram of MT-SC22EK-1B after purification. Elution was carried out in a linear gradient from 20% to 70% acetonitrile (0.1 % trifluoroacetic acid), 30 min with C18 analytical column.

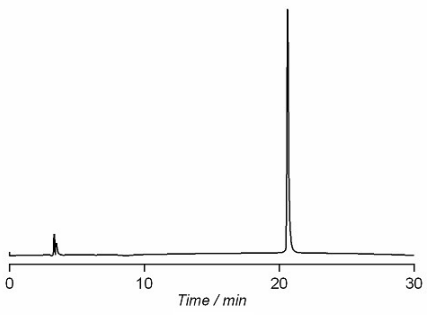


SI Figure 11. ESI-MS chart of MT-SC22EK-1B. Calcd for $[M+H]^{+}$=3119.4 (average isotope), $[M+2H]^{2+}$=1560.2， $[M+3H]^{3+}$ =1040.5, $[M+4H]^{4+}$=780.6, $[M+5H]^{5+}$=624.74. Observed for $[M+H]^{+}$=3119.8, $[M+2H]^{2+}$=1560.4，$[M+3H]^{3+}$=1040.6, $[M+4H]^{4+}$=780.6, $[M+5H]^{5+}$=624.8.

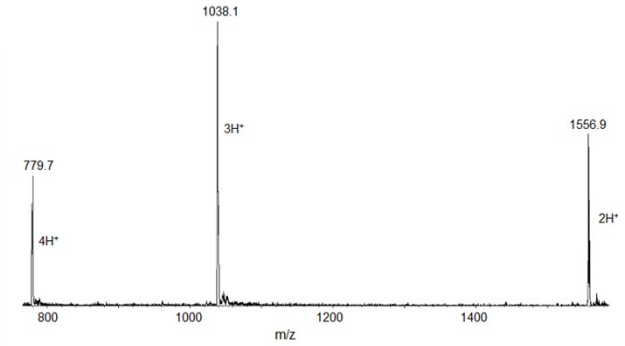
Synthesis of MT-SC22EK-2

SI Figure 12. Sequence of MT-SC22EK-2.

The amino acid residues at 1-7, 9-11, 13-14, 16-18 and 21-24 positions in MT-SC22EK-2 were assembled using 4eq AA, 3.8eq HCTU and 8eq DIEA coupling method. The amino acid residues at 8, 12, 15, 19 positions adopted the 2eq Fmoc-S_5-OH, 1.9eq HATU, 2eq HOAt and 4eq DIEA coupling method. The first ring-closing olefin metathesis needed be finished before the third Fmoc-S_5-OH assembled to the resin. Until all amino acids fully assembled to the resin, the second ring-closing olefin metathesis was performed in Grubbs' first-generation catalyst DCE solution. Followed by further acetylation, cutting, concentration, precipitation and purification, a white solid (26.1 mg) was obtained with a purity of greater than 95% and an isolated yield of 14%.



SI Figure 13. HPLC chromatogram of MT-SC22EK-2 after purification. Elution was carried out in a linear gradient from 20% to 70% acetonitrile (0.1 % trifluoroacetic acid), 30 min with C18 analytical column.



SI Figure 14. ESI-MS chart of MT-SC22EK-2. Calcd for $[M+H]^{+}$=3112.5 (average isotope), $M+2H]^{2+}$=1556.8 , $[M+3H]^{3+}$ =1038.3, $[M+4H]^{4+}$=779.0. Observed for $[M+H]^{+}$=3112.8, $[M+2H]^{2+}$=1556.9 , $[M+3H]^{3+}$=1038.1, $[M+4H]^{4+}$=779.7.

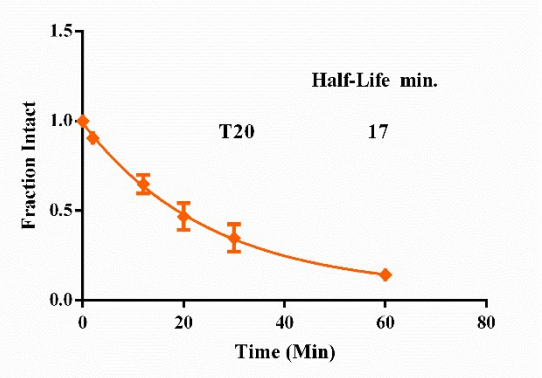
CD assay

MT-SC22EK, MT-SC22EK-1A, MT-SC22EK-1B, MT-SC22EK-2 were prepared in 1X PBS (pH 7.4) with the final concentration 50 μM, respectively. The CD data of MT-SC22EK and its stapled peptides were recorded on a Jasco-715 circular dichroism spectrometer at 25°C. The parameters of the CD spectrometer were set as follows: the wavelength was 185-260 nm, the step length was 0.5 nm, the bandwidth was 1 nm, and the data was accumulated for three times. The percent helicity (α) of MT-SC22EK and its stapled peptides were calculated using the helicity equation ($\alpha=[\theta]_{222}/[\theta]max$, where $[\theta]max=(-44000+250T)(1-k/n)$, T is the experiment temperature 25°C, k is 4.0 and n is in the number of residues 24).

Digest assay

T20, MT-SC22EK, MT-SC22EK-1A, MT-SC22EK-1B and MT-SC22EK-2 was dissolved in DMSO to prepare a stock solution with a concentration of 1 mM. Protease digestion was performed by adding SC34EK stock solution (50 μL) to PBS (1950 μL, 50 mM, pH 7.4) containing

chymotrypsin (0.5 ng/μL) and $CaCl_2$ (50 mM) at 30 °C. Reaction was quenched by adding 20 μL HCl (1M) to 100 μL reaction solution. The residual amount of T20, MT-SC22EK MT-SC22EK-1A, MT-SC22EK-1B and MT-SC22EK-2 was analyzed by HPLC at different time points, respectively.



SI Figure 15. Degradation curves of T20 in the chymotrypsin digestion

Cell culture

Both cell lines were cultured using Dulbecco's modified Eagle's medium (containing 10% heat-inactivated fetal bovine serum) at 37°C in a humidified 5% CO_2-95% air environment. Ghost (3)X4/R5 cells (catalog no. 3942) obtained from the NIH ARRRP, were used to express CD4, CXCR4, and CCR5. HEK293T cells were obtained from the American Type Culture Collection (catalog no. 11268). Cultures were incubated.

Pseudovirus preparation and infection assay

HIV pseudovirus was obtained by transfecting exponentially dividing HEK293T cells (5 × 106 cells in 10 ml growth medium in a 100 mm culture dish) with 6 μg rev/env expression plasmid, 24 μg env-deficient HIV-1 backbone vector (pNL4-3.Luc.R-E, catalog no. 3418), and PEI as transfection reagent. Culture supernatants containing pseudovirus were harvested 2 days after transfection, filtration (0.45 μm), then stored at -80°C in 1ml aliquots.

To measure the activity of peptide fusion inhibitors against diverse HIV-1 subtypes, peptides with 3-fold dilutions, were mixed with 100 TCID50 of viruses, and then incubated for 1 h at room temperature. The mixture was added to Ghost (3)X4/R5 cells (104 cells/ well), then incubation for 48 h at 37°C. Antiviral activity of fusion inhibitors was measured by analyzing the reduction of luciferase activity compared with controls (Bright-Glo luciferase assay system, E2650). Half-maximal inhibitory concentrations (IC_{50}) was the concentration of peptides required to inhibit 50% of the infection. IC_{50} valueof each fusion inhibitor was calculated by using the dose-response inhibition model with a variable slope in GraphPad Prism, version 5.0.

The activity of inhibitors against T20-resistant variants was determined by the similar method as

that of pseudovirus.

Assembly and crystallization

The MT-SC22EK-2/T21 complex was prepared by adding MT-SC22EK-2 (3.1 mg, ~1 μmol) and T21 (4.5 mg, ~1 μmol) to 2.0 mL denaturing buffer (100 nM NaH_2PO_4, 10 mM Tris, pH=8.0, 8 M Urea). After transferring MT-SC22EK-2/T21 mixture to dialysis bag, the renaturation was performed in refolding buffer containing 50 mM Tris (pH 7.5) and 100 mM NaCl at 4 °C overnight. Further the refolded MT-SC22EK-2/T21 complex was purified by size-exclusion chromatography. The purified sample was concentrated to a concentration of 12 mg/mL for further crystallization.

Crystallization of MT-SC22EK-2/T21 complex was carried out by the hanging drop vapor diffusion technique at 18°C. The drop was prepared by mixing equal volumes of the buffered complex solution and the reservoir solution (0.1 M magnesium chloride，0.1 M HEPES, pH 7.5, 30% PEG 400). Crystals suitable for X-ray diffraction were sent to the PXIII beamline (Swiss Light Source, Paul Scherrer Institute) to collect crystal data. The resolution limit of X-ray was 2.2 Å. The crystal structure of MT-SC22EK-2/T21 complex was determined using the molecular replacement method and taking the MT-SC22EK/T21 crystal structure (PDB 3VU6) as a search model.

SI Table 1. Data collection and refinement statistics

Crystal	Calciseptine
Data collection	
Space group	I4
Unit Cell	84.737, 84.737, 66.144
	90.00, 90.00, 90.00
Resolution(Å)	21.184~2.199
Completeness (%)	98.14
No. reflections	117450
Refinement	
R_{work} / R_{free}	0.2378/0.2377
No. atoms	
Protein	1546
Water	25
R.M.S. deviations	

Bond lengths (Å)	0.016
Bond angles ( )	1.077
Ramachandran plot statistics (%)	
Most favourable	99.39%
Allowed	0.00%