Supporting information

Complexation of specific residues by Carboxylatopillar[6]arene for improving zymolytic stability of arginine-containing peptides

Longming Chen,‡[a] Zhao Meng,‡[a] Long Tian, [a] Yahan Zhang, [a] Liang Zhao, [a] Xinbei Du, [a] Mengke Ma, [a] Han Zhang, [a] Junyi Chen,*[ab] and Qingbin Meng,*[a]

[a] *State Key Laboratory of Toxicology and Medical Countermeasures, Beijing Institute of Pharmacology and Toxicology, Beijing 100850, P. R. China*

[b] *Key Laboratory of Inorganic-Organic Hybrid Functional Material Chemistry, Ministry of Education, Tianjin Key Laboratory of Structure and Performance for Functional Molecules, College of Chemistry, Tianjin Normal University, Tianjin 300387, P. R. China*

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1 General materials and methods

1.1 Materials All reagents were purchased commercially and used as received unless other specified purification. DMSO, methanol, acetonitrile and Dulbecco's modified Eagle medium (DMEM) were purchased from the Thermo Fisher Scientific. Fetal bovine serum (FBS) was purchased from Invitrogen. The Cell Counting Kit-8 (CCK-8) was purchased from Dojindo. VSMCs cell line was purchased from cell bank of Chinese Academy of Science. The Cell Counting Kit-8 (CCK-8) was purchased from Dojindo. N-Fluorenyl-9-methoxycarbonyl (Fmoc)-protected L- amino acids were purchased from GL Biochem Ltd and CS Bio Ltd (Shanghai, China). Diisopropylethylamine, trifluoroacetic acid (TFA), thioanisole, ethandithiol and anisole were purchased from J & K Scientific (Beijing, China).

1.2 Cells. L929 cells were cultured in F12K supplemented with 10% FBS, 1% penicillin and 1% streptomycin. Then cells were incubated at 37 ℃ under 5% CO_2 and 90% relative humidity, and passaged every 2 days.

1.3 Instruments. ^{1}H NMR spectrum were recorded using a JNM-ECA-400 spectrometer. Fluorescence spectroscopic studies were carried out using a FL-6500 fluorescence spectrophotometer, Perkin Elmer Co. Ltd. Peptides were synthesized by solid-phase synthesis using Fmoc chemistry on an automatic Liberty-12-Channel Automated Peptide Synthesizer with an integrated microwave system (CEM, USA). Peptides were purified by reversed-phase high-performance liquid chromatography (HPLC) using a C8 column (Waters USA). Molecular weights (MWs) of peptides were analysed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS; BrukerReex, BrukerDaltonics, Inc., CA, USA).

1.4 NMR spectroscopy. Samples for 1:1 ^{1}H NMR spectra were prepared in D_2O. All NMR spectra were acquired at 298 K in the solution state.

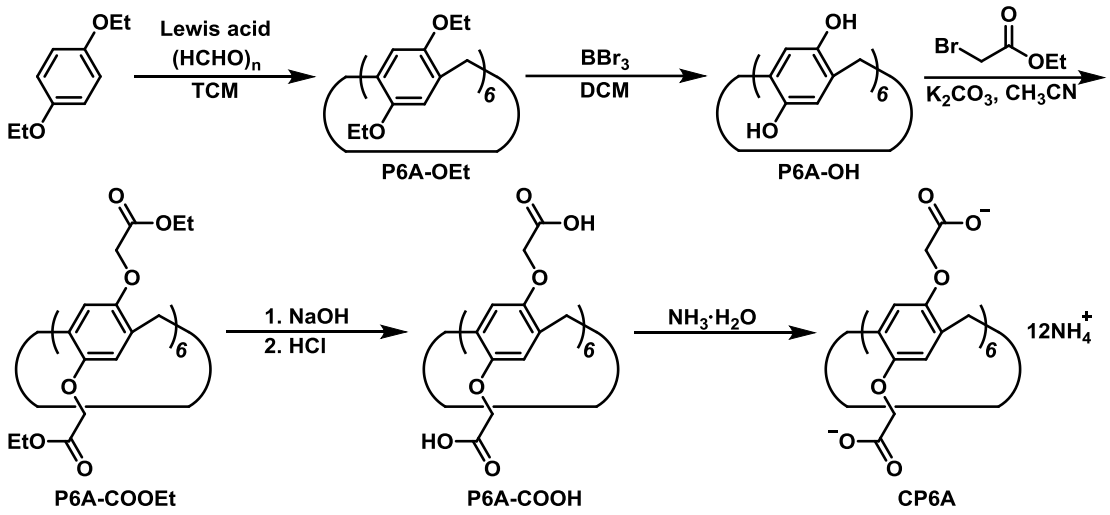
1.5 Fluorescence titration. The fluorescence titration was carried out in 10 mM phosphate buffer solution at pH 7.4. The complexations of CP6A with amino acids and peptides were performed by competitive fluorescence titrations. A mixed solution contained AO (1.0 μM) and CP6A (2.5 μM) and a competitive guest was gradually

added into 2.0 mL AO (1.0 μM)/CP6A (2.5 μM) solution in a quartz cuvette. The fluorescence intensity was measured (λ_{ex} = 493 nm, λ_{em} = 530 nm) after adding the guests. The data were well fitted by the 1:1 binding stoichiometry.[1]

1.6 Metabolic stability. All samples were precipitated with acetonitrile by direct protein precipitation method and analyzed by HPLC. Free angiotensin III (Ang III), 1:1 mixture of Ang III/CP6A (containing 1.00 mM Ang III) were dissolved in the phosphate buffer solution (PBS), and then incubated with equal amount of trypsin (1 mg·mL^{-1}) at 37 ℃. At predetermined intervals, 100 μL samples were withdrawn and quenched with 100 μL acetonitrile. After centrifugation at 4000 rpm for 10 min, the supernatant was collected and stored at 4 ℃ until HPLC analysis. HPLC analysis was performed on an LC-20AT liquid chromatography system with an SPD-20A UV-vis detector operating at 210 nm and quantified using Shimadzu LC solution Lite. The column used was a C8 reverse-phase column (SHISEDO, 4.6 * 250 nm), the mobile phase was composed of solvent A (water containing 0.1% TFA) and solvent B (70% acetonitrile and 0.1% TFA). The injection volume and flow rate were set as 20 μL and 1.00 mL·min^{-1}.[2]

2 Synthesis of CP6A and peptides.

2.1 Synthesis of CP6A



Scheme S1. Synthetic route of CP6A.

Carboxylatopillar[6]arene (CP6A) was synthesized and purified according to our previously reported procedure.[3]

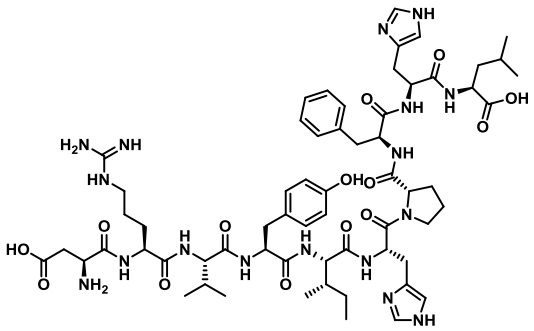
2.2 Synthesis of peptides

All peptides were synthesized by solid-phase synthesis using Fmoc chemistry on an automatic Liberty-12-Channel Automated. Peptide Synthesizer with an integrated microwave system (CEM, USA). Rink amide resin (0.44 $mmol \cdot g^{-1}$) was used as a solid phase to obtain C-terminally amidated peptides. A 20% solution of piperidine in N, N-dimethylformamide (v/v) was added to the resin for deprotection. Amino acids were coupled to rink amide resin one by one, followed by N-terminal conjugation of stearic acid. The final cleavage was performed using 10 mL of TFA (90%)/thioanisole (5%)/ethandithiol (3%)/anisole (2%) for 3 h at room temperature. Peptides were purified by reversed-phase high-performance liquid chromatography (RP-HPLC) using a C8 column (Waters, USA) and a gradient of acetonitrile and deionized water containing 0.1% (v/v) TFA. Molecular weights (MWs) of peptides were analysed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS and electron spray ionization (ESI) mass spectrometry; BrukerReex, BrukerDaltonics, Inc., CA, USA).[4]

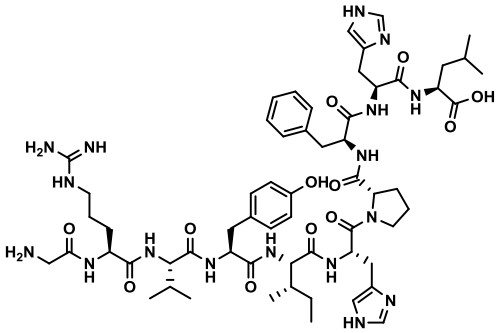
Table S1. Sequence of peptide and molecular weight of peptides

Compound	Peptide sequence	Molecular Weight	
		Calculated	Measured
Ang I	DRVYIHPFHL	1296.4990	1296.70
Ang II	DRVYIHPF	1046.1970	1046.54
Ang III	RVYIHPF	931.1090	931.48
Cpep I	GRVYIHPFHL	1238.4630	1238.316
Cpep II	GRVYIHPF	988.1610	988.53
Cpep III	GVYIHPF	831.9720	832.44

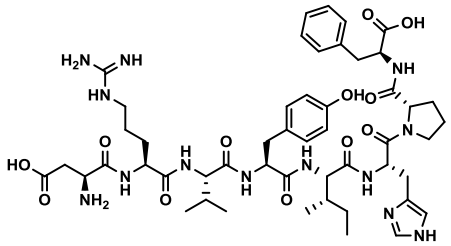
2.3 The structures of peptides



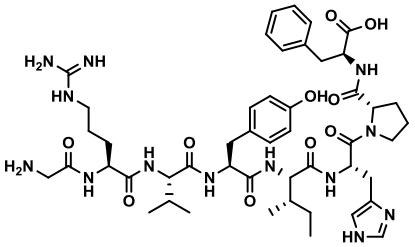
Ang I



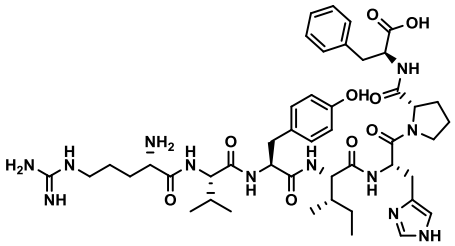
Cpep I



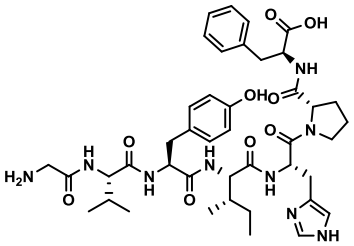
Ang II



Cpep II



Ang III



Cpep III

Scheme 2 The chemical structures of angiotensin peptides and three control peptides..

3 Supporting results and experimental raw data

3.1 Characterization of CP6A and peptides

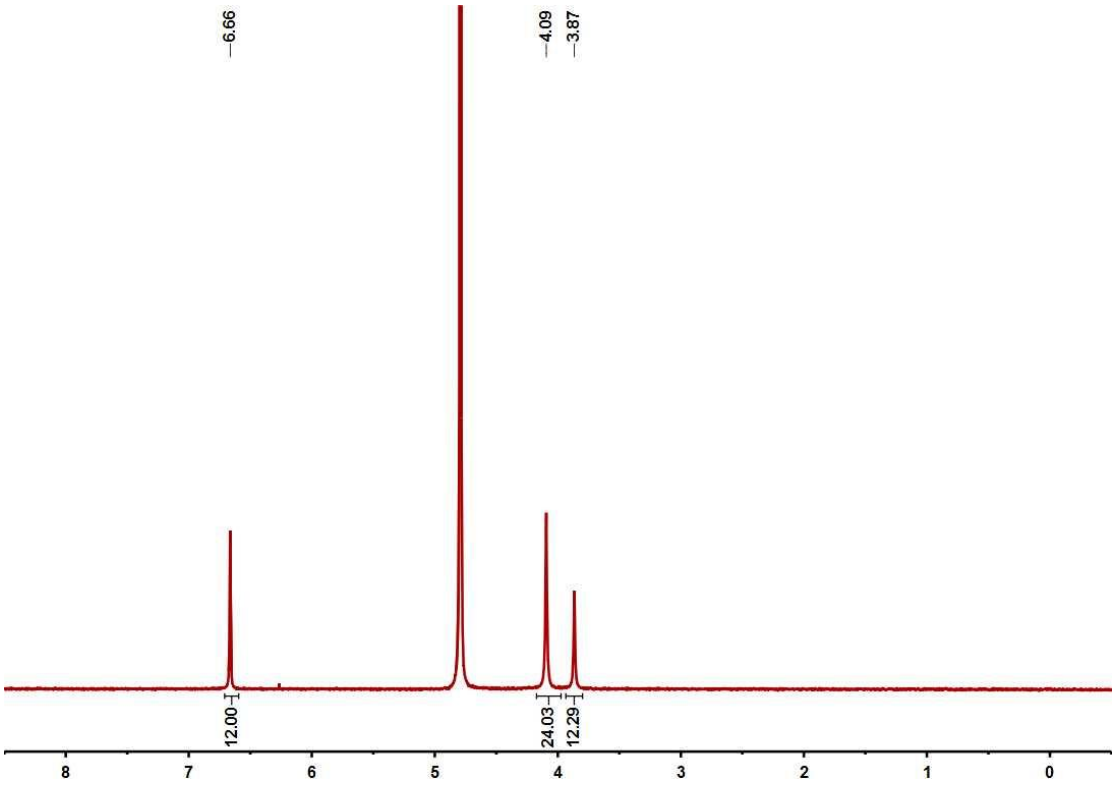


Fig. S1. ^{1}H NMR spectrum of CP6A in D_2O, 400 MHz.

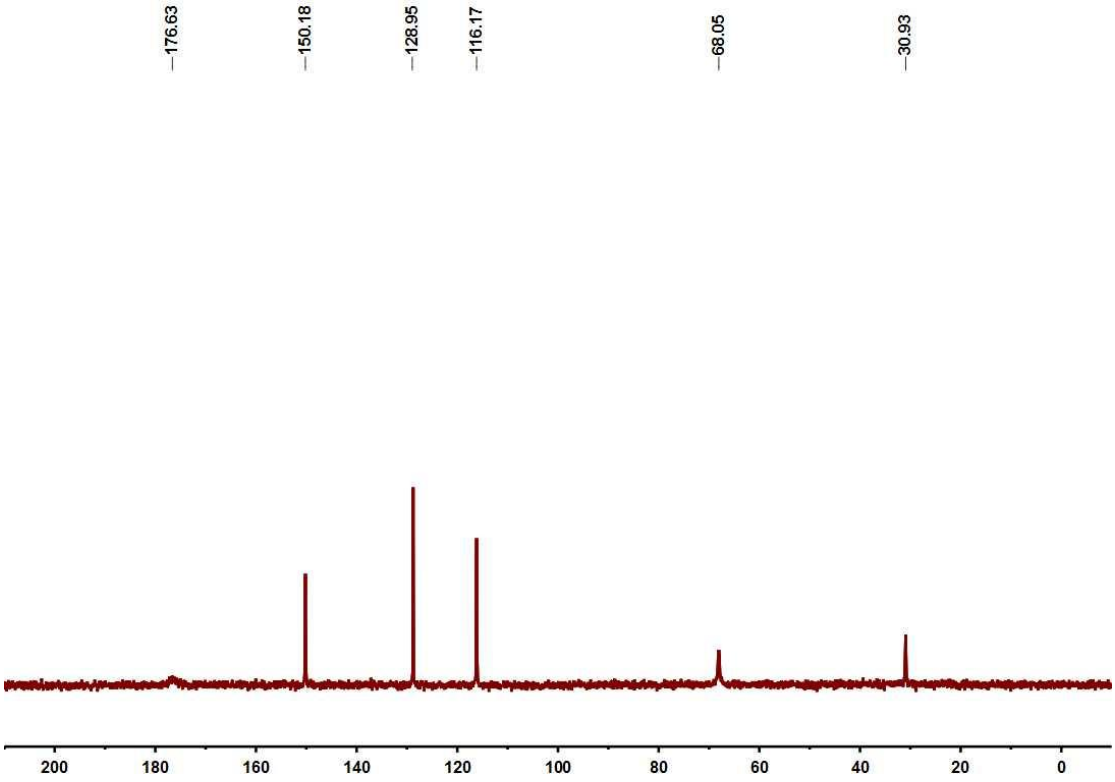


Fig. S2. ^{13}C NMR spectrum of CP6A in D_2O, 100 MHz.

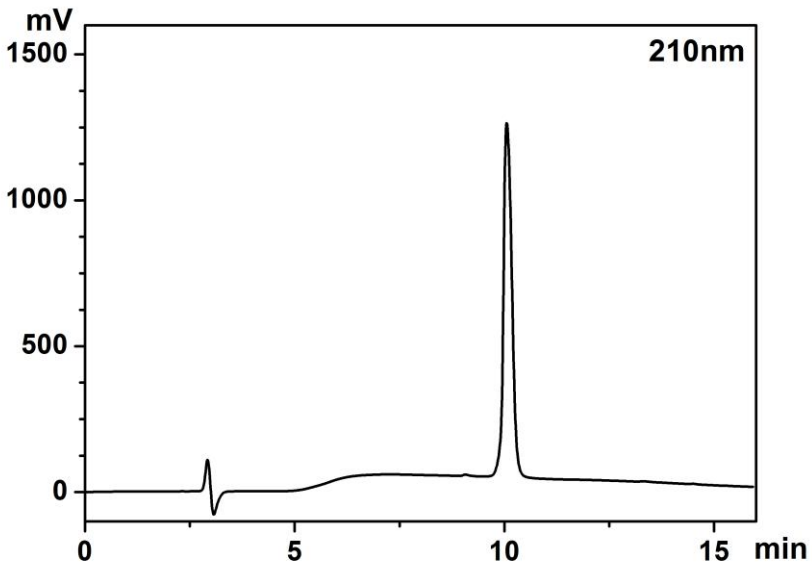


Fig. S3. RP-HPLC of Ang I.

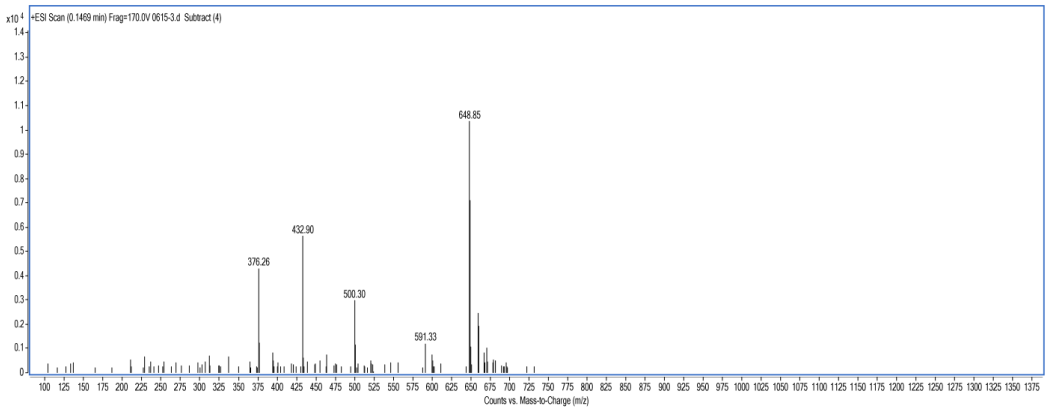


Fig. S4. ESI mass spectrum of Ang I.

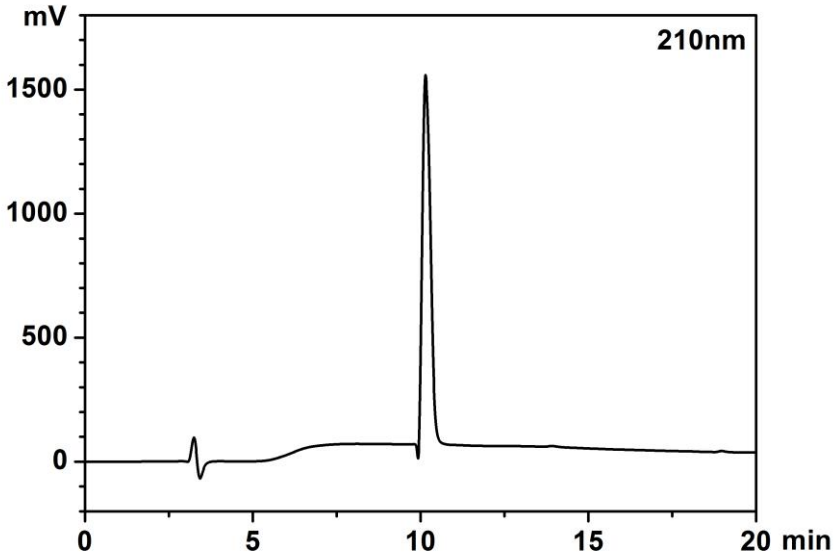


Fig. S5. RP-HPLC of Ang II.

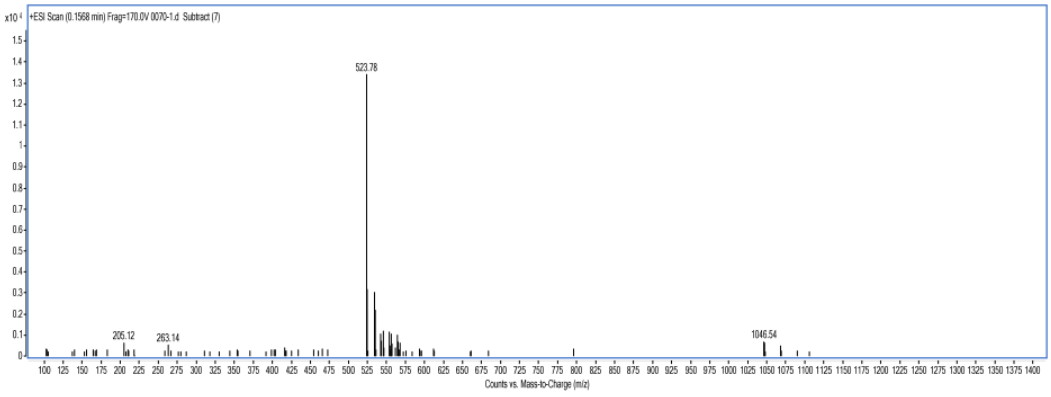


Fig. S6. ESI mass spectrum of Ang II.

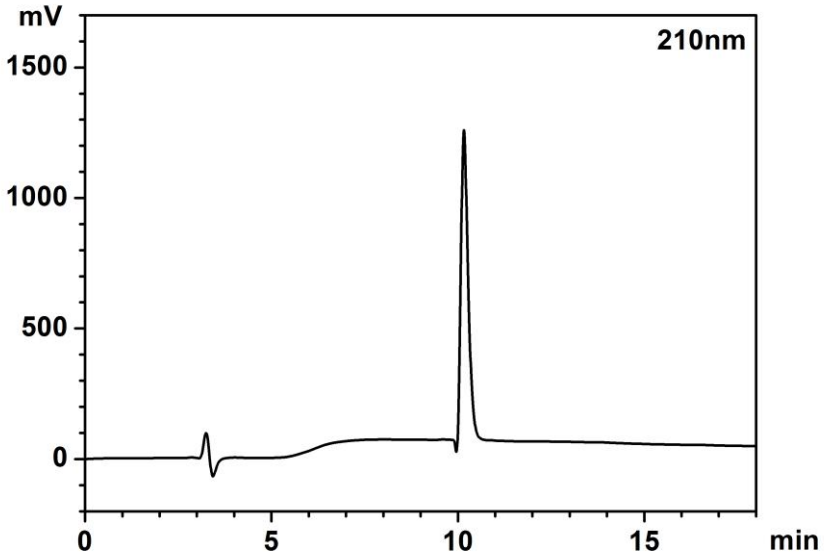


Fig. S7. RP-HPLC of Ang III.

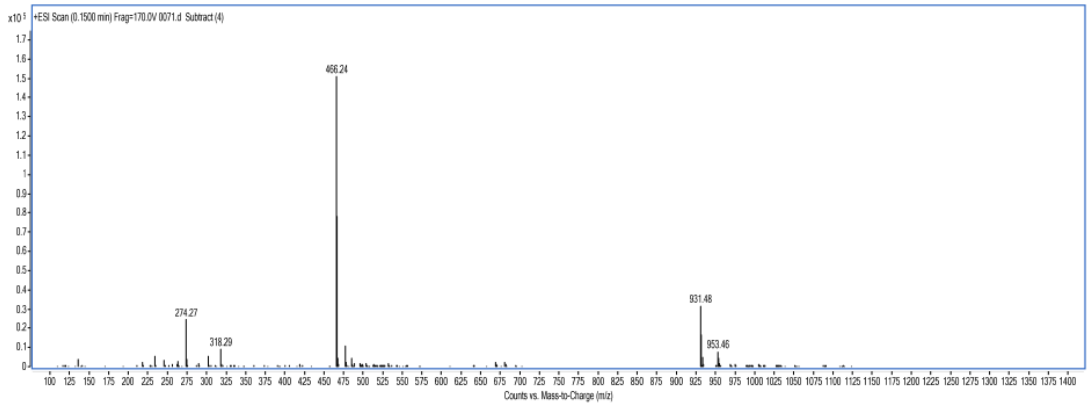


Fig. S8. ESI mass spectrum of Ang III.

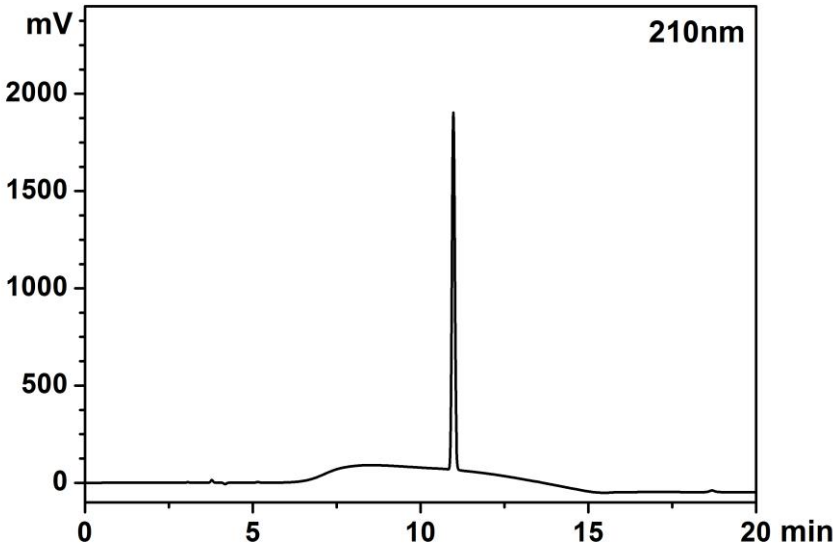


Fig. S9. RP-HPLC of Cpep I.

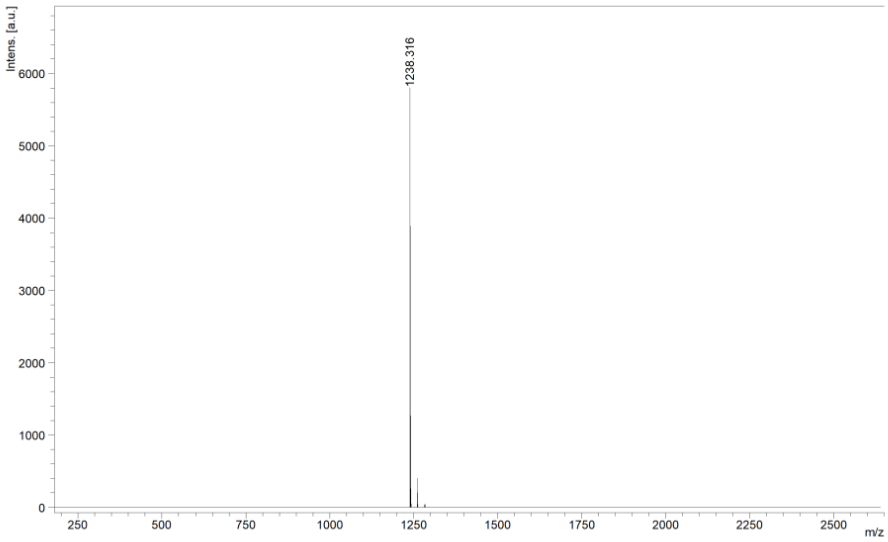


Fig. S10. MALDI-TOF mass spectrum of Cpep I.

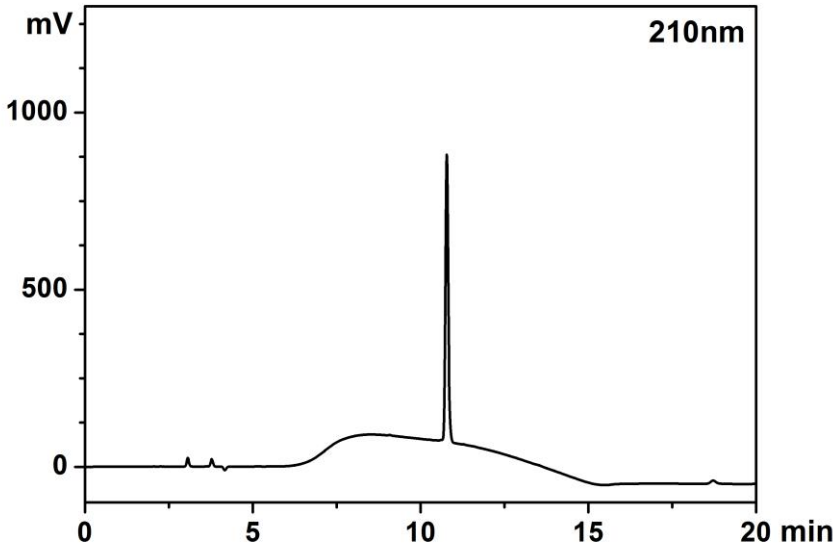


Fig. S11. RP-HPLC of Cpep II.

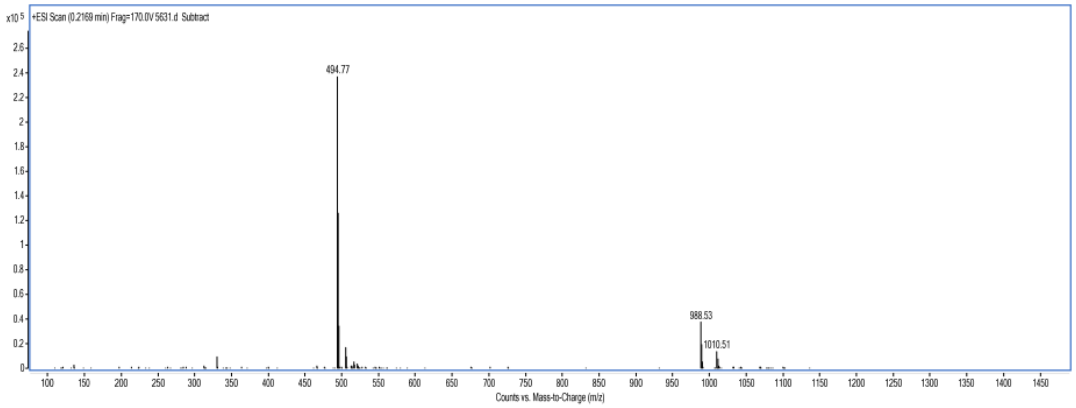


Fig. S12. ESI mass spectrum of Cpep II.

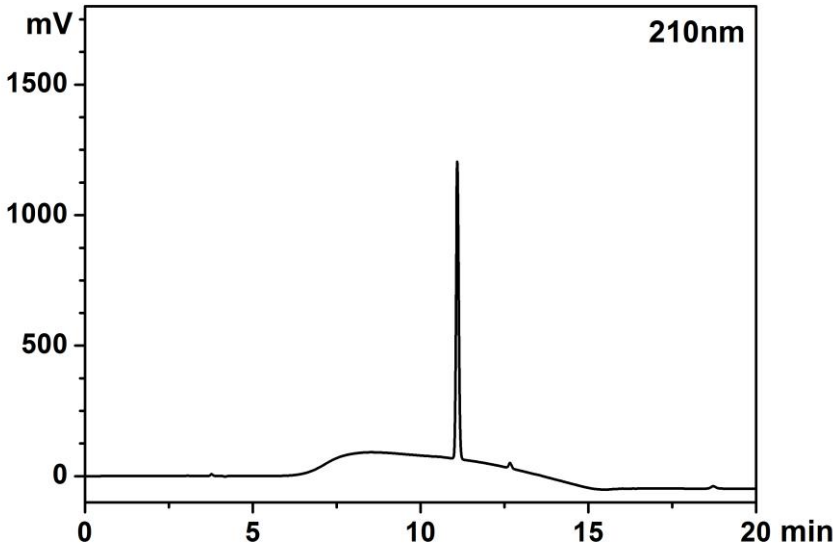


Fig. S13. RP-HPLC of Cpep III.

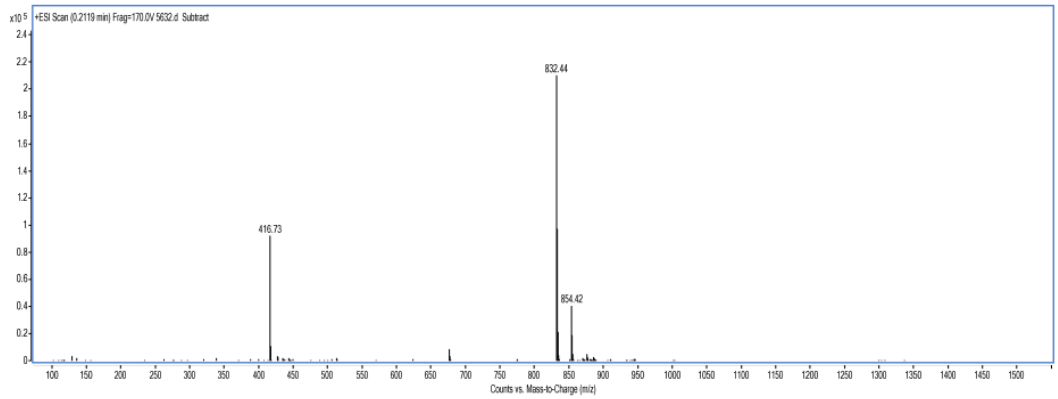


Fig. S14. ESI mass spectrum of Cpep III.

3.2 Binding of CP6A with other 19 naturally occurring amino acids.

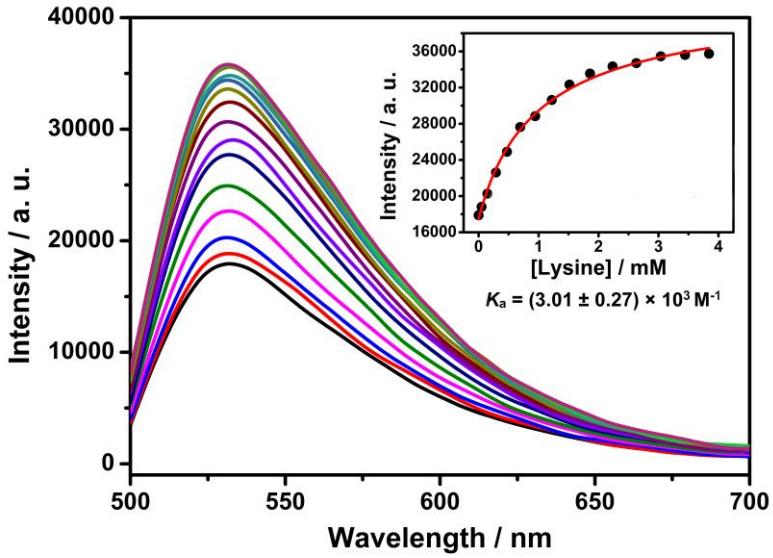


Fig. S15. Competitive fluorescence titration of lysine in the presence of AO (1.0 μM)/CP6A (2.5 μM) in 10 mM PBS buffer at pH 7.4, λ_{ex} = 493 nm. Insert: the associated competitive titration curve at λ_{em} = 530 nm and fit according a 1:1 competitive binding model.

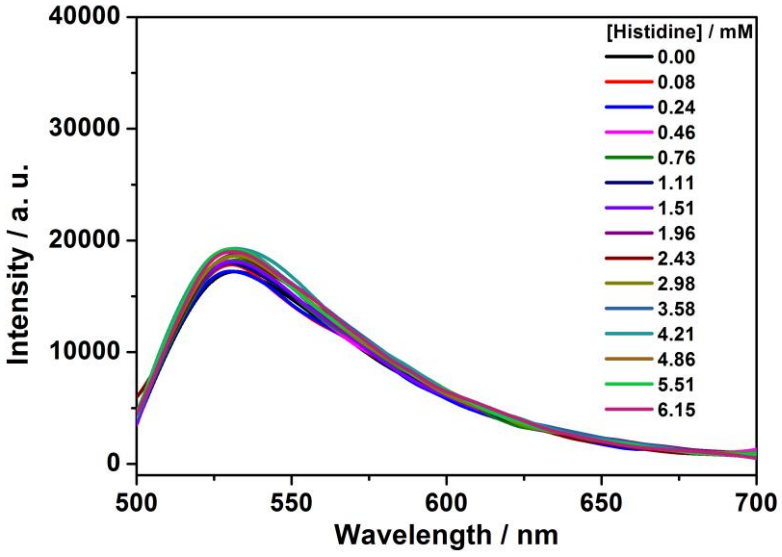


Fig. S16. Competitive fluorescence titration of histidine in the presence of AO (1.0 μM)/CP6A (2.5 μM) in 10 mM PBS buffer at pH 7.4, λ_{ex} = 493 nm. The recovery of fluorescence is too small relative to range of quenching, so no reasonable association constant can be obtained.

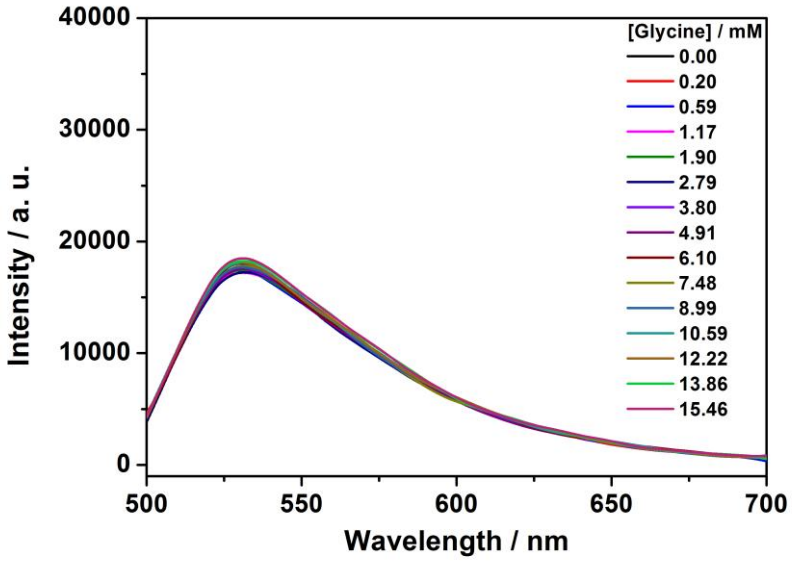


Fig. S17. Competitive fluorescence titration of glycine in the presence of AO (1.0 μM)/CP6A (2.5 μM) in 10 mM PBS buffer at pH 7.4, λ_{ex} = 493 nm. The recovery of fluorescence is too small relative to range of quenching, so no reasonable association constant can be obtained.

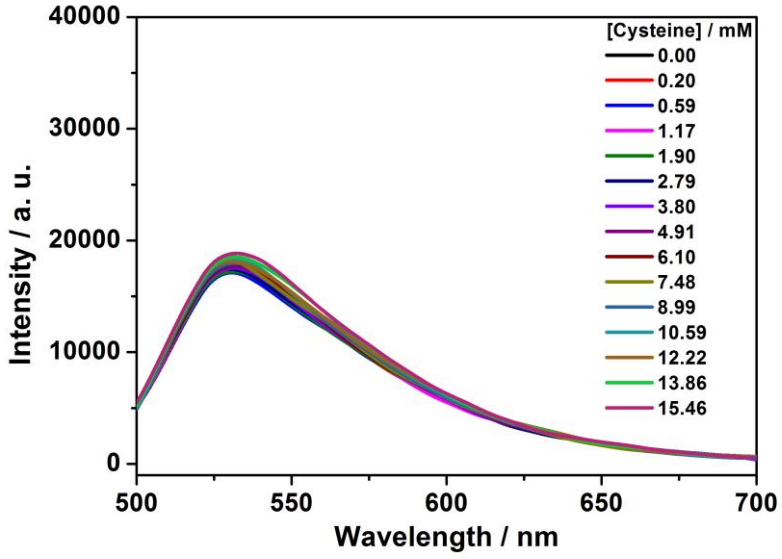


Fig. S18. Competitive fluorescence titration of cysteine in the presence of AO (1.0 μM)/CP6A (2.5 μM) in 10 mM PBS buffer at pH 7.4, λ_{ex} = 493 nm. The recovery of fluorescence is too small relative to range of quenching, so no reasonable association constant can be obtained.

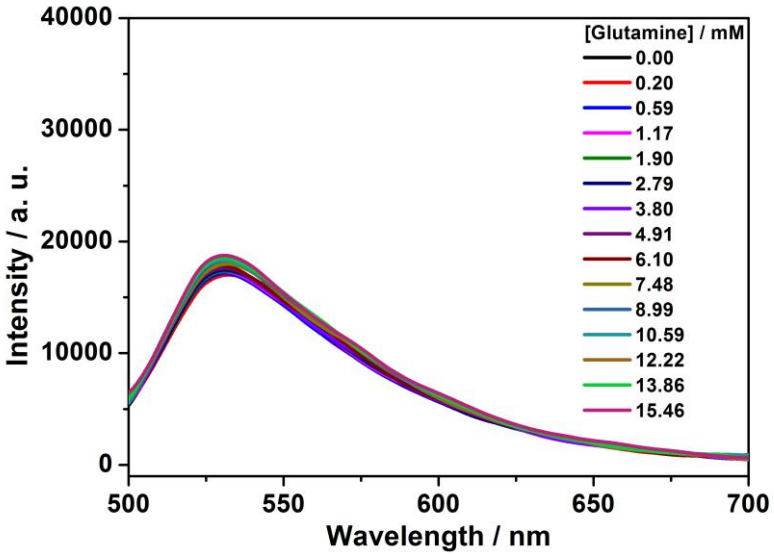


Fig. S19. Competitive fluorescence titration of glutamine in the presence of AO (1.0 μM)/CP6A (2.5 μM) in 10 mM PBS buffer at pH 7.4, λ_{ex} = 493 nm. The recovery of fluorescence is too small relative to range of quenching, so no reasonable association constant can be obtained.

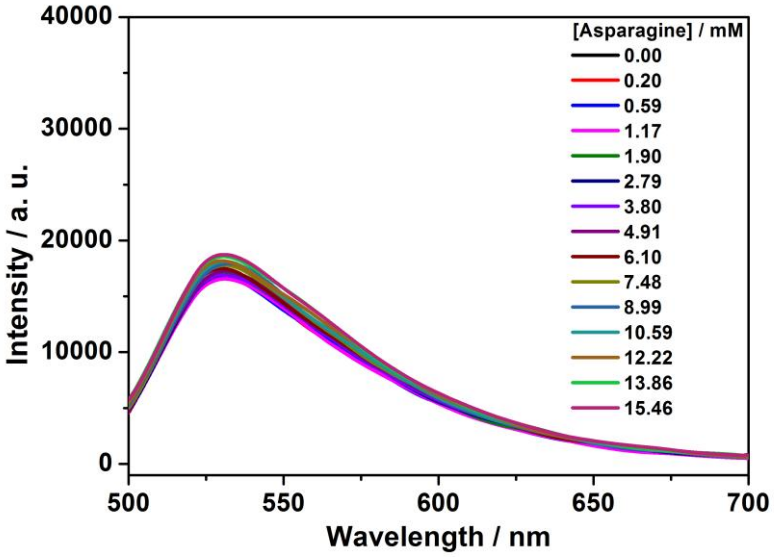


Fig. S20. Competitive fluorescence titration of asparagine in the presence of AO (1.0 μM)/CP6A (2.5 μM) in 10 mM PBS buffer at pH 7.4, λ_{ex} = 493 nm. The recovery of fluorescence is too small relative to range of quenching, so no reasonable association constant can be obtained.

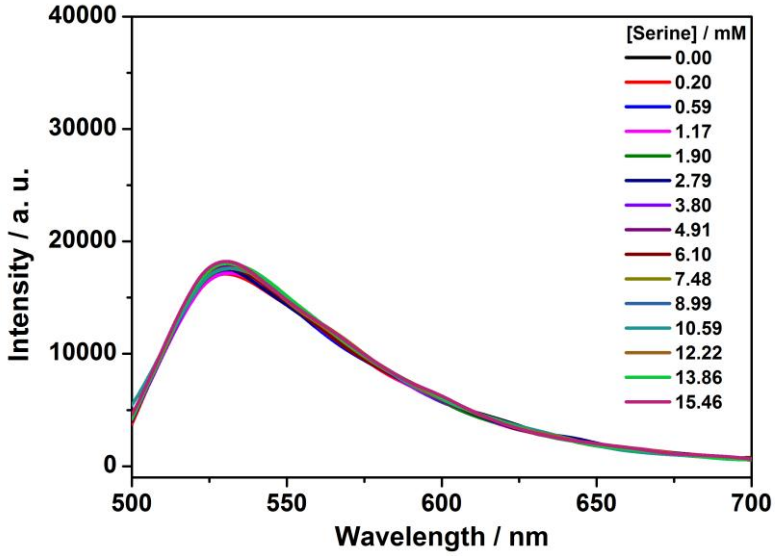


Fig. S21. Competitive fluorescence titration of serine in the presence of AO (1.0 μM)/CP6A (2.5 μM) in 10 mM PBS buffer at pH 7.4, λ_{ex} = 493 nm. The recovery of fluorescence is too small relative to range of quenching, so no reasonable association constant can be obtained.

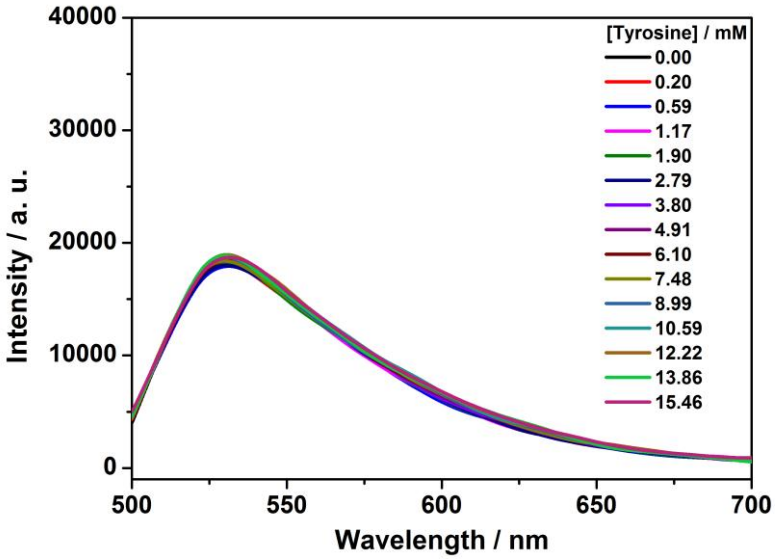


Fig. S22. Competitive fluorescence titration of tyrosine in the presence of AO (1.0 μM)/CP6A (2.5 μM) in 10 mM PBS buffer at pH 7.4, λ_{ex} = 493 nm. The recovery of fluorescence is too small relative to range of quenching, so no reasonable association constant can be obtained.

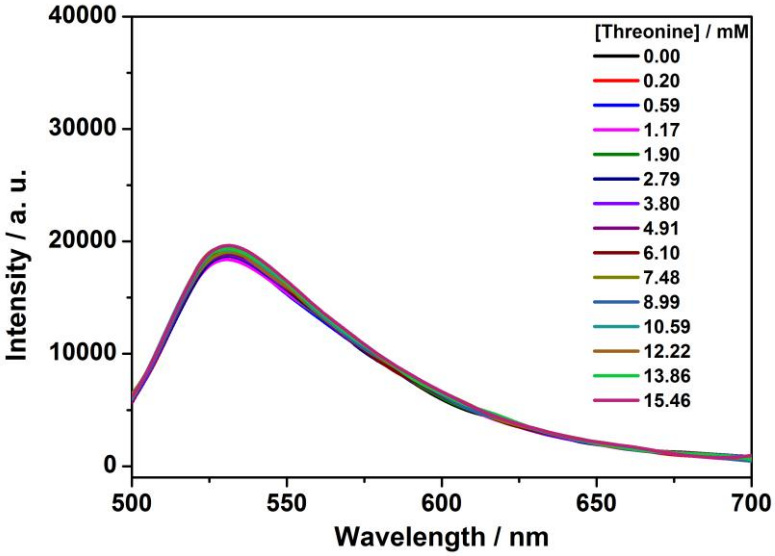


Fig. S23. Competitive fluorescence titration of threonine in the presence of AO (1.0 μM)/CP6A (2.5 μM) in 10 mM PBS buffer at pH 7.4, λ_{ex} = 493 nm. The recovery of fluorescence is too small relative to range of quenching, so no reasonable association constant can be obtained.

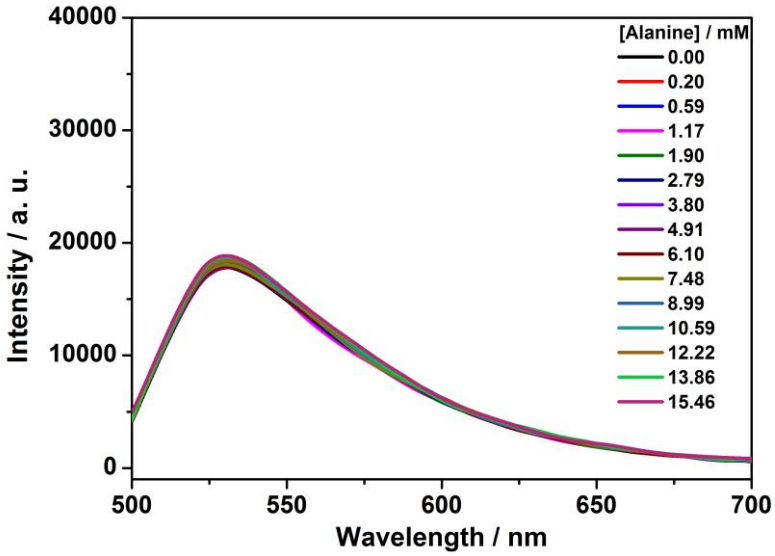


Fig. S24. Competitive fluorescence titration of alanine in the presence of AO (1.0 μM)/CP6A (2.5 μM) in 10 mM PBS buffer at pH 7.4, λ_{ex} = 493 nm. The recovery of fluorescence is too small relative to range of quenching, so no reasonable association constant can be obtained.

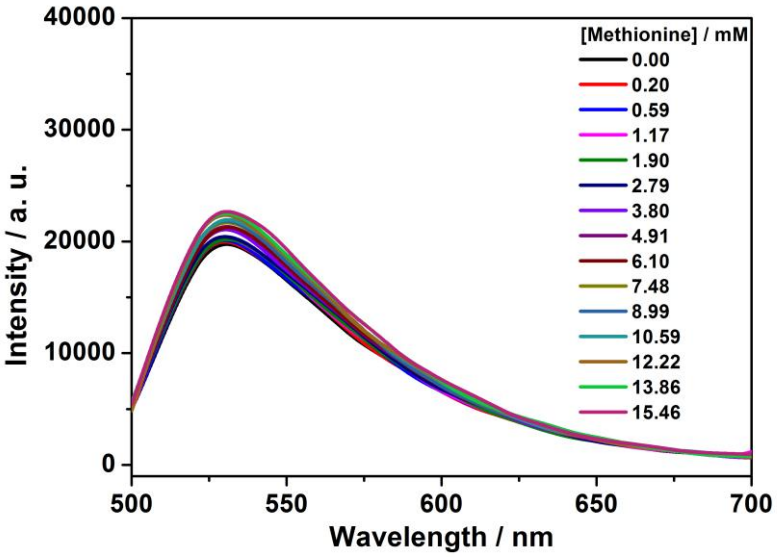


Fig. S25. Competitive fluorescence titration of methionine in the presence of AO (1.0 μM)/CP6A (2.5 μM) in 10 mM PBS buffer at pH 7.4, λ_{ex} = 493 nm. The recovery of fluorescence is too small relative to range of quenching, so no reasonable association constant can be obtained.

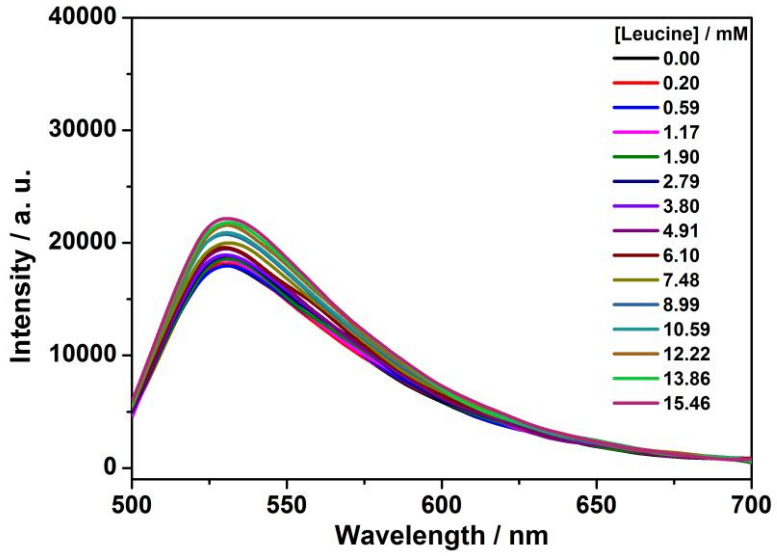


Fig. S26. Competitive fluorescence titration of leucine in the presence of AO (1.0 μM)/CP6A (2.5 μM) in 10 mM PBS buffer at pH 7.4, λ_{ex} = 493 nm. The recovery of fluorescence is too small relative to range of quenching, so no reasonable association constant can be obtained.

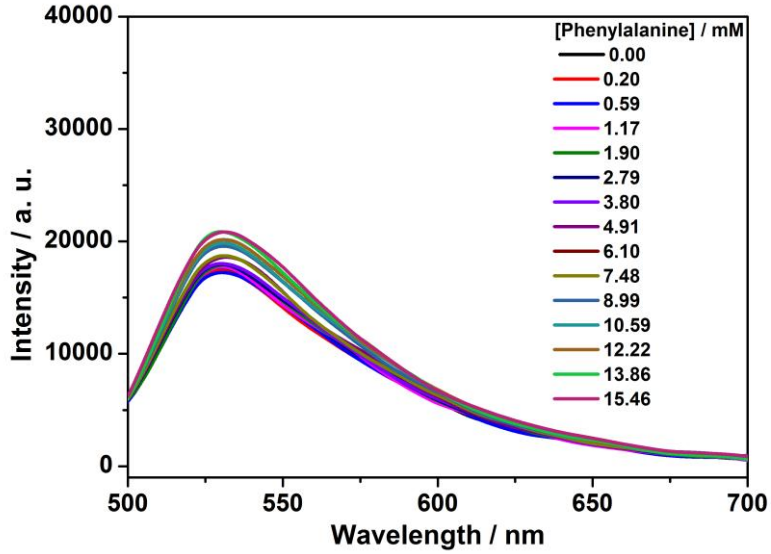


Fig. S27. Competitive fluorescence titration of phenylalanine in the presence of AO (1.0 μM)/CP6A (2.5 μM) in 10 mM PBS buffer at pH 7.4, λ_{ex} = 493 nm. The recovery of fluorescence is too small relative to range of quenching, so no reasonable association constant can be obtained.

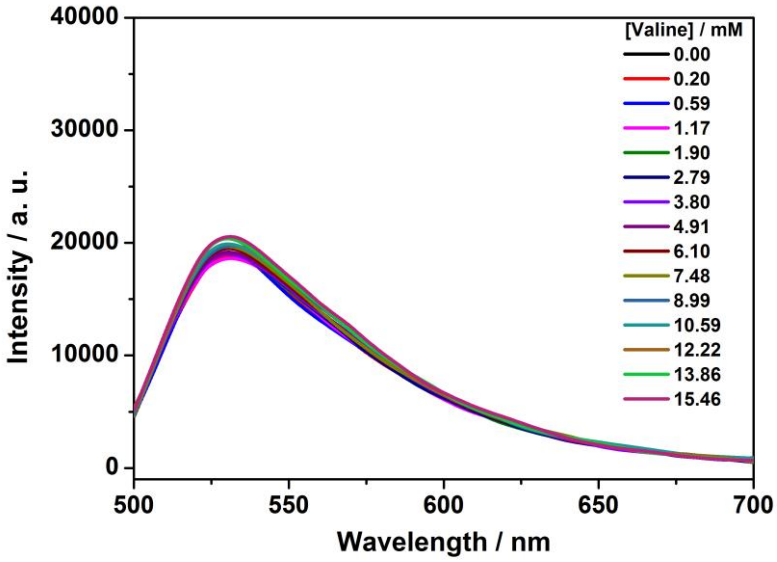


Fig. S28. Competitive fluorescence titration of valine in the presence of AO (1.0 μM)/CP6A (2.5 μM) in 10 mM PBS buffer at pH 7.4, λ_{ex} = 493 nm. The recovery of fluorescence is too small relative to range of quenching, so no reasonable association constant can be obtained.

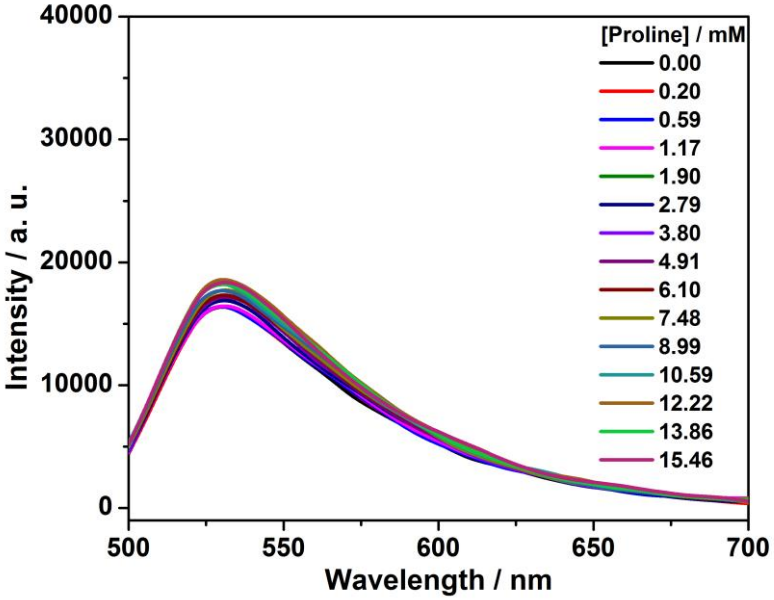


Fig. S29. Competitive fluorescence titration of proline in the presence of AO (1.0 μM)/CP6A (2.5 μM) in 10 mM PBS buffer at pH 7.4, λ_{ex} = 493 nm. The recovery of fluorescence is too small relative to range of quenching, so no reasonable association constant can be obtained.

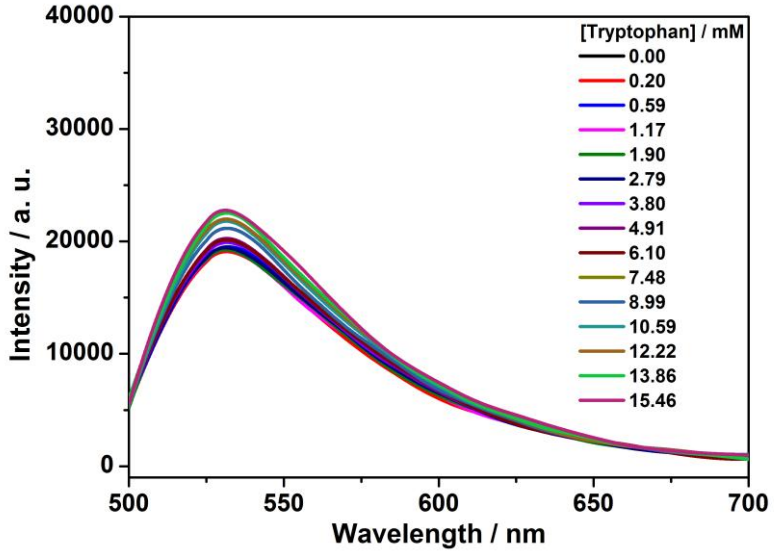


Fig. S30. Competitive fluorescence titration of tryptophan in the presence of AO (1.0 μM)/CP6A (2.5 μM) in 10 mM PBS buffer at pH 7.4, λ_{ex} = 493 nm. The recovery of fluorescence is too small relative to range of quenching, so no reasonable association constant can be obtained.

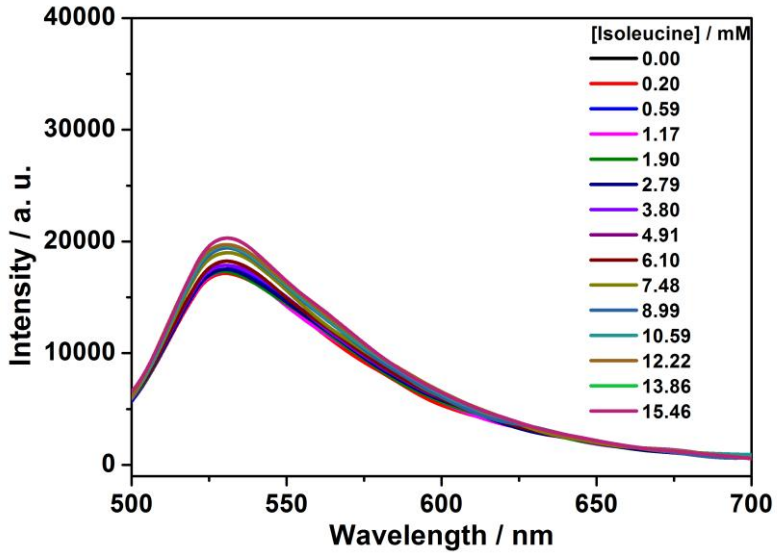


Fig. S31. Competitive fluorescence titration of isoleucine in the presence of AO (1.0 μM)/CP6A (2.5 μM) in 10 mM PBS buffer at pH 7.4, λ_{ex} = 493 nm. The recovery of fluorescence is too small relative to range of quenching, so no reasonable association constant can be obtained.

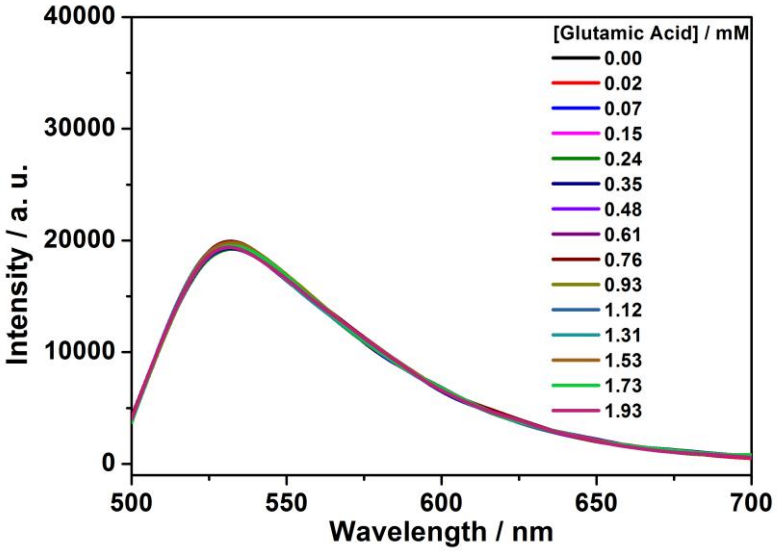


Fig. S32. Competitive fluorescence titration of glutamic acid in the presence of AO (1.0 μM)/CP6A (2.5 μM) in 10 mM PBS buffer at pH 7.4, λ_{ex} = 493 nm. The recovery of fluorescence is too small relative to range of quenching, so no reasonable association constant can be obtained.

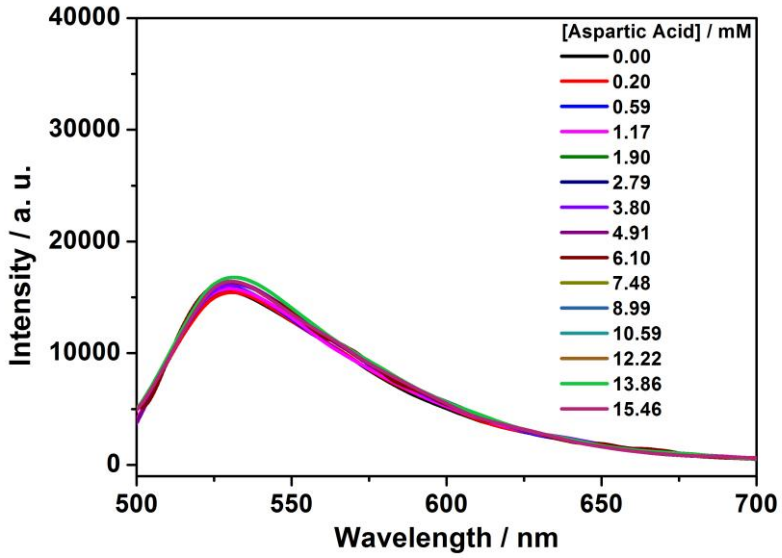


Fig. S33. Competitive fluorescence titration of aspartic acid in the presence of AO (1.0 μM)/CP6A (2.5 μM) in 10 mM PBS buffer at pH 7.4, λ_{ex} = 493 nm. The recovery of fluorescence is too small relative to range of quenching, so no reasonable association constant can be obtained.

3.3 Binding of CP6A with Ang I II and Cpep I II III.

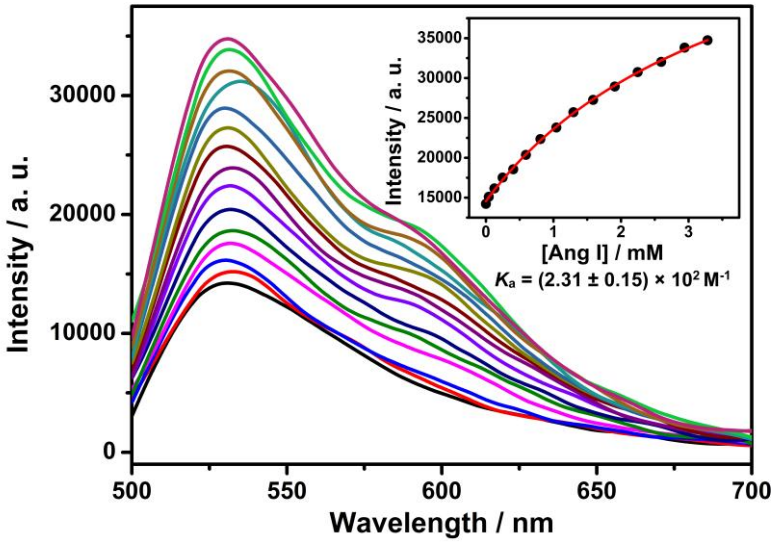


Fig. S34. Competitive fluorescence titration of Ang I in the presence of AO (1.0 μM)/CP6A (2.5 μM) in 10 mM PBS buffer at pH 7.4, λ_{ex} = 493 nm. Insert: the associated competitive titration curve at λ_{em} = 530 nm and fit according a 1:1 competitive binding model.

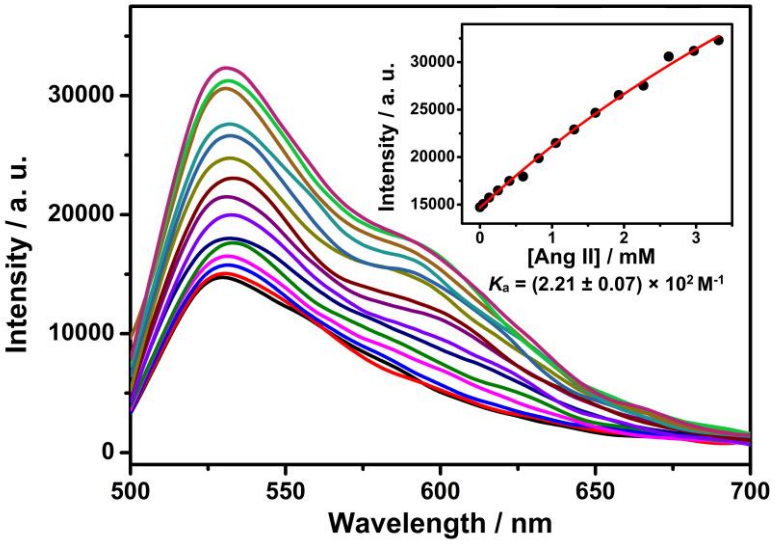


Fig. S35. Competitive fluorescence titration of Ang II in the presence of AO (1.0 μM)/CP6A (2.5 μM) in 10 mM PBS buffer at pH 7.4, λ_{ex} = 493 nm. Insert: the associated competitive titration curve at λ_{em} = 530 nm and fit according a 1:1 competitive binding model.

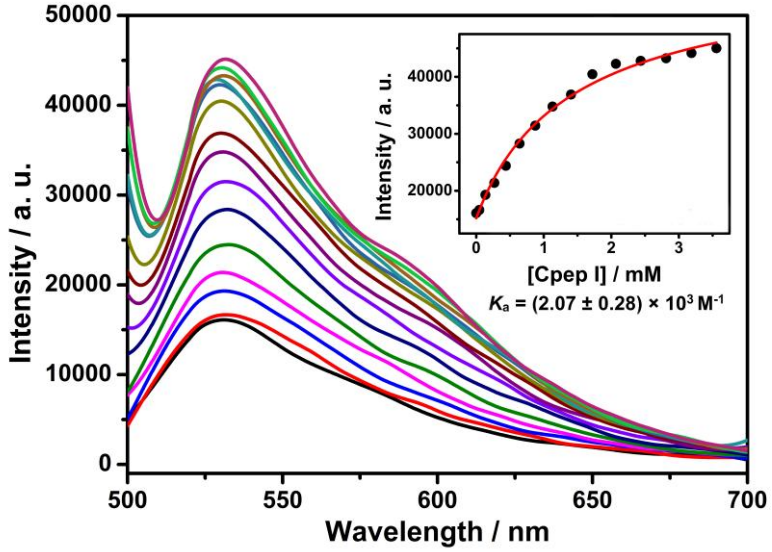


Fig. S36. Competitive fluorescence titration of Cpep I in the presence of AO (1.0 μM)/CP6A (2.5 μM) in 10 mM PBS buffer at pH 7.4, λ_{ex} = 493 nm. Insert: the associated competitive titration curve at λ_{em} = 530 nm and fit according a 1:1 competitive binding model.

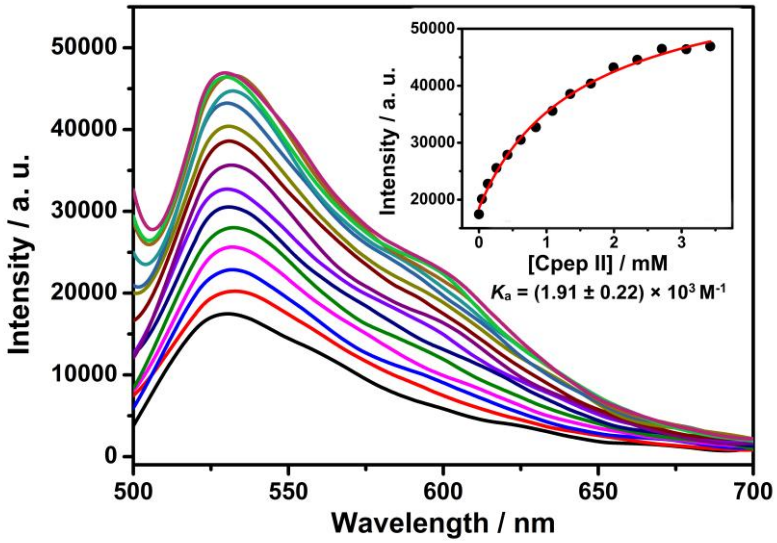


Fig. S37. Competitive fluorescence titration of Cpep II in the presence of AO (1.0 μM)/CP6A (2.5 μM) in 10 mM PBS buffer at pH 7.4, λ_{ex} = 493 nm. Insert: the associated competitive titration curve at λ_{em} = 530 nm and fit according a 1:1 competitive binding model.

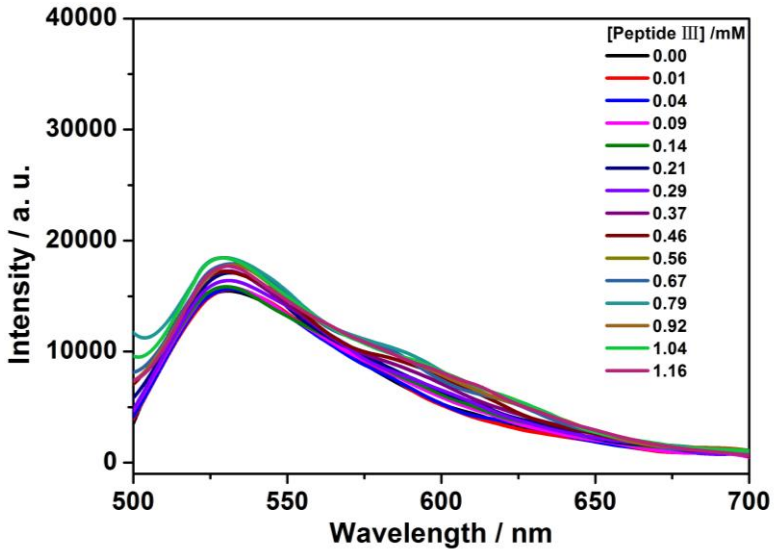


Fig. S38 Competitive fluorescence titration of Cpep III in the presence of AO (1.0 μM)/CP6A (2.5 μM) in 10 mM PBS buffer at pH 7.4, λ_{ex} = 493 nm. The recovery of fluorescence is too small relative to range of quenching, so no reasonable association constant can be obtained.

3.4 Optimized geometries of Arg with CP6A

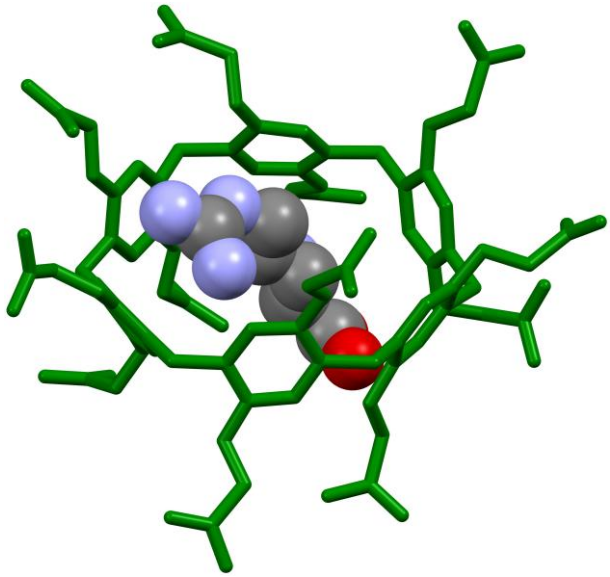


Fig. S39. Optimized structure of MM2 energy-minimized model of Arg/CP6A complex.

4 The standard curves of Ang III as studied by HPLC.

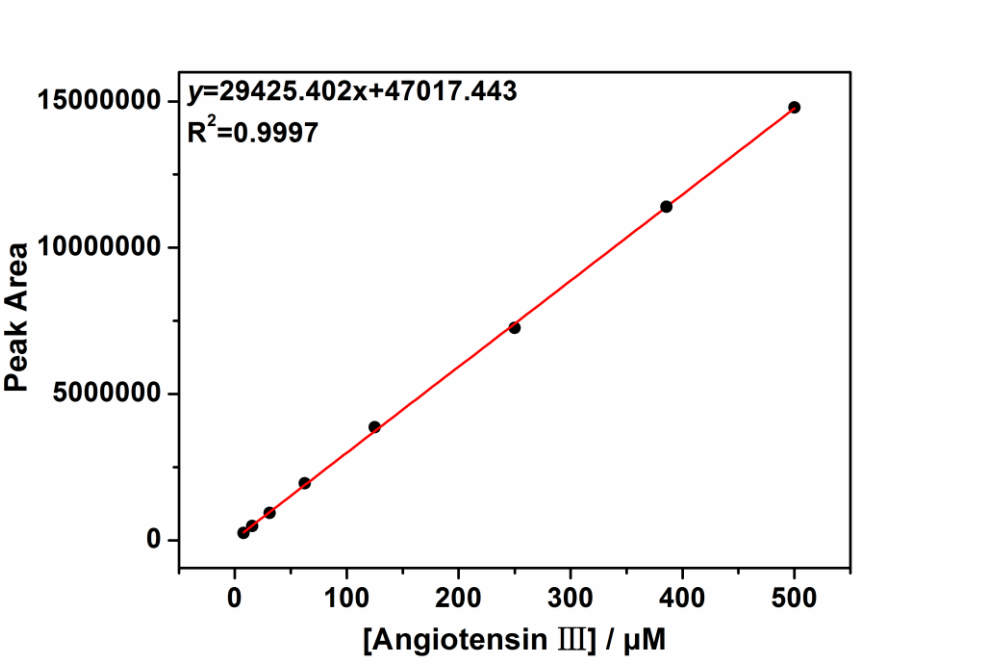


Fig. S40. The calibration curves obtained by HPLC and used for calculating the Ang III concentration in metabolic stability studies, ranged in the concentrations 7.81-500.00 μM.

References

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