Electronic Supplementary Material (ESI) for Molecular BioSystems

Supporting Information

From Cerius^2 based stereoview to mouse and enzyme: The model systems for discovery of novel urokinase inhibitors

Shenghui Mei, Jiawang Liu,† Ming Zhao,* Wenjing Wang, Yuji Wang, Guofeng Wu, Meiqing Zheng and Shiqi Peng*

College of Pharmaceutical Sciences, Capital Medical University, Beijing, PR China

†Co-first author

SUPPORTING INFORMATION ENCLOSED

Experimental Section

General

All chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO, U.S.) and were purified when necessary. Column chromatography was performed with silica gel. (200-300 mesh, Qingdao Haiyang Chemical Co., Qingdao, P.R. China). The purities of the intermediates and the products were measured by TLC analysis (Merck silica gel plates of type 60 F_{254}, 0.25 mm layer thickness) and HPLC analysis (*hp* HEWLETT PACKARD SERIES 1050, Waters C_{18} column 4.6×150 mm). Melting points were determined in capillary tubes on an electrothermal SM/XMP apparatus without correction. ESI-MS was determined by Micromass Quattro micro TM API (Waters Co.). Infrared spectra were run on a Shimadzu 8010 M Spectrophtometer, using the KBr disk method. ^{1}H NMR (500 Hz) and ^{13}C NMR (125 Hz) spectra were recorded on a Bruker Advance 500 spectrometer in $CDCl_3$ with TMS as internal standard, and chemical shifts are expressed in ppm. Optical rotations were determined with a Jasco P-1020 Polarimeter. Biological data was analyzed using ANOVA test and $p < 0.05$ was considered statistical significance.

(S)-methyl 1-(2,2-dimethoxyethyl)-2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole-3-carboxylate (1)

A suspension of 5.0 g (24.5 mmol) of L-tryptophan methylester, 50 mL of MeOH, and 6.0 mL (23.6 mmol) of 1,1,3,3-tetramethoxypropane was adjusted to pH 1-2 with HCl (5 N), and stirred at 45 ºC for 48 h. After evaporation of solvent under

vacuum the residue was diluted with water, and the aqueous solution was extracted with EtOAc (30 mL × 3). The organic phase was separated, washed successively with 10% sodium carbonate and saturated NaCl, dried over with anhydrous sodium sulfate, and evaporated under vacuum. The residue was purified by column chromatography using chloroform/methanol (30:1) to provide 4.8 g (89%) of diastereomeric mixture of the title compound as pale yellow oil. ESI-MS (*m*/*e*) 319 $[M + H]^+$.

12-(2,2-Dimethoxyethyl)-(5aS,12S,14aS)-1,2,3,5a,6,11,12,14a-octahydro-5H,14H-Pyrrolo[1'',2'':4',5']pyrazino[1',2':1,6]pyrido[3,4-b]indole-5,14-dione (2-12S)

To 10.0 g (29.7 mmol) of Fmoc-Pro-OH, 50 mL of $SOCl_2$ was added dropwise. The reaction mixture was refluxed for 5 h and then evaporated under vacuum to remove excess $SOCl_2$. The residue was treated with ether to provide Fmoc protected prolinyl chloride as colourless powder, which reacted with 6.3 g (20 mmol) of (S)-methyl 1-(2,2-dimethoxyethyl)-2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole-3-carboxylate (**1**) in 50 mL of CH_2Cl_2 at 0 ºC. After 0.5 h, the reaction mixture was adjusted to pH 9 with diisopropylamine, and stirred at room temperature for 24 h. The reaction mixture was evaporated under vacuum and the residue was separated on column chromatography using chloroform/methanol (40:1). The powders from the corresponding fractions were crystallized in acetone to provide 1.30 g (20%) of **2-12S**. Mp 180 - 183 ºC. IR (KBr): 3346, 2933, 2832, 1683, 1645, 1462, 1335, 744 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 10.14 (s, 1H), 8.67 (s, 1H), 7.50 (t, *J* = 7.5 Hz, 1H), 7.34 (t, *J* = 7.5 Hz, 1H), 7.09 (d, *J* = 7.5 Hz, 1H), 7.22 (d, *J* = 7.5 Hz, 1H), 5.82 (t, *J* =

6.0 Hz, 1H), 4.76 (t, *J* = 6.0 Hz, 1H), 4.41 (t, *J* = 4.5 Hz, 1H), 4.14 (t, *J* = 4.0 Hz, 1H), 3.92 (m, 1H), 3.64 (dd, *J* = 4.5 Hz, *J* = 15.0 Hz, 1H), 3.46 (s, 3H), 3.40 (s, 3H), 2.86 (dd, *J* = 11.1 Hz, *J* = 16.0 Hz, 1H), 2.51 (m, 2H), 1.90 - 2.20 (m, 5H). ESI-MS (*m*/*e*) 384 $[M + H]^+$. $[\alpha]_D^{20}$= 76 (c = 0.33, $CHCl_3$: CH_3OH, 1:1). Anal.Calcd for $C_{21}H_{25}N_3O_4$: C, 65.78; H, 6.57; N, 10.96. Found: C, 66.02; H, 6.74; N, 10.75.

2-((5aS,12S,14aS)-1,2,3,5a,6,11,12,14a-Octahydro-5,14-dioxo-5H,14H-Pyrrolo[1'',2'':4',5']pyrazino[1',2':1,6]pyrido[3,4-b]indol-12-yl)acetaldehyde (3-12S)

To a solution of 200 mg (0.52 mmol) of **2-12S** in 15 mL of acetone, 20 mg of *p*-toluenesulfonic acid was added. The reaction mixture was stirred at 45 ºC for 1 h, and TLC (chloroform/methanol, 20:1) indicated complete disappearance of **2-12S**. The reaction mixture was treated with 0.5 mL of triethylamine, evaporated under vacuum to remove the acetone, and separated on column chromatography using chloroform/methanol (40:1). The powders from the corresponding fractions were crystallized in acetone to provide 158 mg (90%) of **3-12S**. Mp 133 - 135 ºC. IR (KBr): 3340, 2937, 2835, 1726, 1684, 1643, 1460, 1332, 743 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 10.22 (s, 1H), 9.86 (s, 1H), 7.52 (t, *J* = 7.6 Hz, 1H), 7.35 (t, *J* = 7.6 Hz, 1H), 7.25 (d, *J* = 7.6 Hz, 1H), 7.06 (d, *J* = 7.6 Hz, 1H), 5.83 (t, *J* = 6.4 Hz, 1H), 5.12 (t, *J* = 4.1 Hz, 1H), 4.77 (t, *J* = 6.1 Hz, 1H), 4.42 (t, *J* = 4.6 Hz, 1H), 3.90 (m, 1H), 3.65 (dd, *J* = 4.6 Hz, *J* = 14.7 Hz, 1H), 2.88 (dd, *J* = 11.0 Hz, *J* = 15.8 Hz, 1H), 2.52 (m, 2H), 1.92 - 2.21 (m, 4H). EI-MS (*m*/*e*) 338 $[M + H]^+$. $[\alpha]_D^{20}$= 176 (c = 0.71, $CHCl_3$: CH_3OH, 1:1). Anal.Calcd for $C_{19}H_{19}N_3O_3$: C, 67.64; H, 5.68; N, 12.46. Found: C,

67.85; H, 5.53; N, 12.69.

Methyl (S)-3-(1H-indol-3-yl)-2-(2-((5aS,12S,14aS)-1,2,3,5a,6,11,12,14a-octahydro-5,14-dioxo-5H,14H-Pyrrolo[1'',2'':4',5']pyrazino[1',2':1,6]pyrido[3,4-b]indol-12-yl)ethylamino)propionate (4-12S)

To a solution of 176 mg (0.52 mmol) of **3-12S** in 10 mL of chloroform, 106 mg (0.52 mmol) of tryptophan methyl ester, 0.5 mL of triethylamine and 3 g of anhydrous sodium sulfate were added. The reaction mixture was stirred at room temperature for 3 h, mixed with a solution of 100 mg (1.85 mmol) of potassum borohydride in 5 mL of methanol, and stirred for another 1 h. TLC (chloroform/ methanol, 20/1) indicated complete disappearance of **3-12S**. The reaction mixture was evaporated under vacuum to remove the solvent, and the residue was mixed with 10 mL of chloroform and 10 mL of deionised water. The two-phase solution was treated with 1 mL of hydrochloric acid (5N) to exhaust the excess potassium borohydride, and adjusted to pH 9 with aqueous ammonia. The chloroform phase was separated, and the aqueous phase was extracted with chloroform (5 mL × 3). The combined chloroform phase was washed with 20 mL of saturated aqueous sodium chloride and dried over with anhydrous sodium sulfate. After filtration the filtrate was evaporated under vacuum and the crude product was purified on column chromatography using chloroform/methanol (30:1) to provide 261 mg (91%) of **4-12S**. Mp. 141 - 142 ºC. IR (KBr): 3341, 2932, 2830, 1735, 1681, 1646, 1462, 1333, 1299, 745 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 10.91 (s, 1 H), 7.62 (d, *J* = 7.8 Hz, 1 H), 7.51 (d, *J* = 8.0 Hz, 1 H), 7.32 (m, 2 H), 7.26 (s, 1 H),

7.06 (m, 2 H), 6.94 (m, 2 H), 5.68 (d, *J* = 8.1 Hz, 1 H), 4.29 (m, 2 H), 3.65 (s, 3 H), 3.59 (m, 2 H), 3.43 (m, 1 H), 3.25 (dd, *J* = 5.1 Hz, *J* = 15.0 Hz, 1 H), 3.11 (m, 2 H), 2.95 (m, 2 H), 2.66 (m, 1 H), 2.29 (m, 2 H), 2.03 (m, 1 H), 1.90 (m, 5 H). ESI-MS (*m*/*e*) 540 $[M + H]^+$. $[\alpha]_D^{20}$= 33 (c = 0.59, $CHCl_3$: CH_3OH 1:1). Anal.Calcd for $C_{31}H_{33}N_5O_4$: C, 69.00; H, 6.16; N, 12.98. Found: C, 69.22; H, 6.01; N, 12.76.

(S)-3-(1H-indol-3-yl)-2-(2-((5aS,12S,14aS)-1,2,3,5a,6,11,12,14a-octahydro-5,14-dioxo-5H,14H-Pyrrolo[1'',2'':4',5']pyrazino[1',2':1,6]pyrido[3,4-b]indol-12-yl)ethylamino)propanoic acid (CIPPC)

At 0 ºC to a solution of 300 mg (7.5 mmol) of sodium hydroxide in 5 mL of methanol 270 mg (0.5 mmol) of **4-12S** was added. The reaction mixture was stirred at room temperature for 2 h and TLC (chloroform/methanol, 20:1) indicated complete disappearance of **4-12S**. The reaction mixture was adjusted to pH 3 with hydrochloric acid (2N) and evaporated under vacuum. The residue was purified on column chromatography using chloroform/methanol (30:1) to provide 250 mg (95%) of CIPPC. Mp. 230-235 ºC. IR (KBr): 3346, 3133, 3001, 2932, 2835, 2712, 1682, 1641, 1462, 1331, 741 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 11.55 (s, 1 H), 10.89 (s, 1 H), 7.60 (d, *J* = 7.9 Hz, 1 H), 7.49 (d, J = 8.1 Hz, 1 H), 7.31 (m, 2 H), 7.24 (s, 1 H), 7.04 (m, 2 H), 6.97 (m, 2 H), 5.66 (d, *J* = 8.3 Hz, 1 H), 4.27 (m, 2 H), 3.57 (m, 2 H), 3.41 (m, 1 H), 3.23 (dd, *J* = 5.3 Hz, 15.1 Hz, 1 H), 3.08 (m, 2 H), 2.93 (m, 2 H), 2.69 (m, 1 H), 2.27 (m, 2 H), 2.01 (m, 1 H), 1.88 (m, 5 H). ^{13}C-NMR ($CDCl_3$, 125 Hz): δ 185.6, 167.2, 164.4, 136.7, 136.4, 133.6, 127.8, 126.4, 124.4, 121.8, 121.4, 119.3,

119.0, 118.8, 118.3, 111.8, 110.0, 106.1, 62.9, 58.4, 55.3, 47.7, 45.4, 44.4, 31.3, 29.9, 27.3, 25.8, 22.8, 22.0. ESI-MS (*m*/*e*) 526 $[M + H]^+$. $[\alpha]_D^{20}$= 62 (c=0.39, $CHCl_3$:CH_3OH 1:1).

Animals

Male ICR mice weighing 28 - 31 g and male Wister rats weighing 250 - 300 g, purchased from Peking University Health Science Center, were maintained at 21°C with a natural day/night cycle in a conventional animal colony. The assessments described here were performed based on a protocol reviewed and approved by the ethics committee of Capital Medical University. The committee assures the welfare of the animals was maintained in accordance to the requirements of the animal welfare act and according to the guide for care and use of laboratory animals.

***In vivo* tail bleeding time assay of orally administration of CIPPC**

Male imprinting control region (ICR) mice (purchased from Experimental Animal Center of Peking University) were housed in a 12/12 light/dark cycle at a room temperature of 21 ± 2°C for one day before operation. Each of them was orally administered 0.6 mL of normal saline (NS) containing four doses (1000, 100, 10 and 1 nmol/kg) of CIPPC or 0.6 mL of NS containing aspirin (165 μmol/kg) or 0.6 mL of NS alone. Thirty minutes after administration, the mouse was placed in a tube holder with its tail protruding, and a 2 mm cut was made on the tail. Flowing blood was gently wiped away with a tissue every 30 sec until bleeding ceased, and the observed

bleeding time was recorded.

In vivo thrombogenesis assay of rats orally receiving CIPPC

Male Wistar rats (purchased from Experimental Animal Center of Peking University) were fed with four doses (1000, 100, 10 and 1 nmol/kg) of CIPPC in normal saline (NS) or aspirin (165 μmol/kg) in NS or NS (0.6 ml) alone, and then the rats were anesthetized with pentobarbital sodium (80.0 mg/kg, ip). Thirty minutes later the right carotid artery and left jugular vein of the rat were separated. A weighed 6-cm thread was inserted into the middle of a polyethylene tube. The polyethylene tube was filled with heparin sodium (50 IU/mL in NS) and one end was inserted into the left jugular vein while another end was inserted into the right carotid artery. Blood flowed from the right carotid artery to the left jugular vein through the polyethylene tube for 15 min. The thread was taken out and the weight of the wet thrombus was recorded.

In vitro thrombus clot lysis assay of UK with and without CIPPC

The cylindrical thrombus prepared from rat blood was carefully taken out of the cylindrical thrombus forming tube and hanged into a incubation bottle filled with 8 mL of distilled water for 1 h. Then the thrombus was taken out of the bottle, weighed precisely to record its initial weight and hung in another incubation bottle filled with 8 mL of normal saline (NS) or the solution of urokinase (UK, 100 IU/mL, Sigma, UK) in 8 mL of NS or UK (100 IU/mL) plus CIPPC (1000, 100, 10 or 1 nM) in 8 mL of

NS. The bottle was incubated at 37 °C and 70 rpm on a rocking-bed for 1 h and the thrombus was precisely weighed to record its final weight. Subtracting the final weight from the initial weight, the reduced weight of the thrombus was obtained and used to represent the *in vitro* thrombus lysis potency.

***In vivo* thrombolytic assay of UK on the rats pretreated with oral CIPPC**

Male Wistar rats were fed with four doses (1000, 100, 10 and 1 nmol/kg) of CIPPC in normal saline (NS) or NS (0.6 mL) alone. Then the rats were anesthetized with pentobarbital sodium (80.0 mg/kg, ip). Thirty minutes later the right carotid artery and left jugular vein of the animals were separated. The cylindrical thrombus supporting helix (15 circles, pitch 1.2 mm, diameter 1.0 mm) was put into the cylindrical thrombus forming tube, and this tube was then filled with artery blood (0.2 mL) from the right carotid artery of the animal immediately. After 15 min the cylindrical thrombus was carefully taken out of the thrombus forming tube, weighed precisely to record its initial weight, and then put into the middle of a polyethylene tube. The polyethylene tube was filled with heparin sodium (50 IU/mL, in NS) and one of the ends was inserted into the left jugular vein. Heparin sodium was injected via the other end of the polyethylene tube as the anticoagulant, following which a 3 mL solution of UK (20000 IU/kg) in NS was injected. The blood was circulated through the polyethylene tube for 90 min, after which the cylindrical thrombus was carefully taken out of the polyethylene tube, and weighed precisely to record its final weight. Subtracting the final weight from the initial weight, the reduced weight of the

thrombus was obtained and used to represent the *in vivo* thrombolytic potency.

Effect of CIPPC on electrophoresis of UK and plasminogen

Protein electrophoresis is a general technology for detecting the action of urokinase on plasminogen,[1] and was carried out in a vertical slab gel unit DYY-6C. The separation gel (sodium dodecyl sulphate, GE healthcare, USA) was 10 cm high, and had a total concentration of 12%, with a crosslinking of 4% and contained 10% of glycerol (GE healthcare, USA). A stacking gel of about 1.2 cm with 4% T and 3% C were used. The gel thickness was always 1.5 mm. A solution of plasminogen (PLG, 5 μL, final concentration 0.1 U/mL, Sigma, USA) and normal saline (NS) (10 μL) or a solution of plasminogen (PLG, 5 μL, final concentration 0.1 U/mL, Sigma, USA) and UK (5 μL, final concentration 100 U/mL) plus 5 μL of NS or a solution of plasminogen (PLG, 5 μL, final concentration 0.1 U/mL, Sigma, USA) and UK (5 μL, final concentration 100 U/mL) plus 5 μL of CIPPC (in a series of final concentration ranging from 0.025 to 2500 ng/μL) or PLG (5 μL, final concentration 0.1 U/mL) plus UK (5 μL, final concentration 100 U/mL) and EACA (5 μL, final concentration 2500 ng/μL) or PLG (5 μL, final concentration 0.1 U/mL) plus UK (5 μL, final concentration 100 U/mL) and EACA (5 μL, final concentration 1250 ng/μL) was incubated at 37 ºC for 30 min. The separation of these solution was performed at a constant current of about 100 mA, starting at 90 V, rising up to about 120 V and lasting for 2 h until the bromophenol blue (as a tracer, Sigma, USA) band had reached the bottom of the gel. Gels were stained in methanol/acetic acid/water (45/10/45) with

coomassie brilliant blue R250 (0.1%, Amresco, USA) for 4 h. The gels were kept in methanol/acetic acid/water (10/10/80) for about 14 h for destaining.

***In vitro* ·OH and ·ON scavenging assay**

·OH and ·ON scavenging assays were carried out on an electron spin resonance spectrometer, JES-300 (JEOL, Tokyo) and a JEOL quartz tuber were used. The conditions were: field (336 mT ± 5 mT width), power (4 mW), field modulation (0.200 mT), time constant (0.1) and amplitude (300). A manganese signal was used for the external standard.

For ·OH scavenging assay, the reaction mixture contained 25 µM of Fe^{2+}, 200 µM of H_2O_2, 200 mM of DMPO and 25 µM of CIPPC (final concentration 50 µM) was introduced into a quartz tuber and ESR spectrum was recorded and shown in Figure S1.

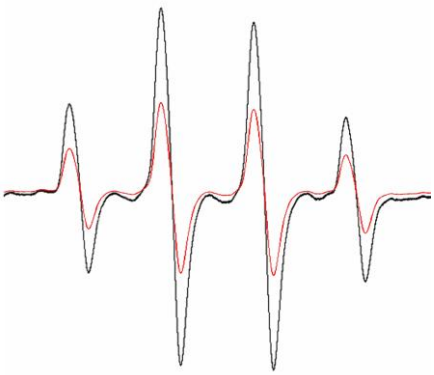


Figure S1 ESR spectrum without CIPPC (black) and ESR spectrum with CIPPC (red), 53% of ·OH was scavenged.

For ·ON scavenging assay the reaction mixture of 1.0 mM of SNAP, $(MGD)_2$-Fe^{2+} and CIPPC (final concentration 50 µM) was incubated at 37 °C for 30

min and ESR spectrum was recorded and shown in Figure S2.

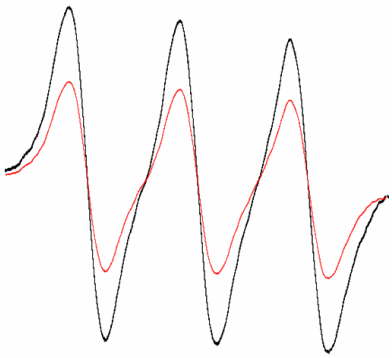
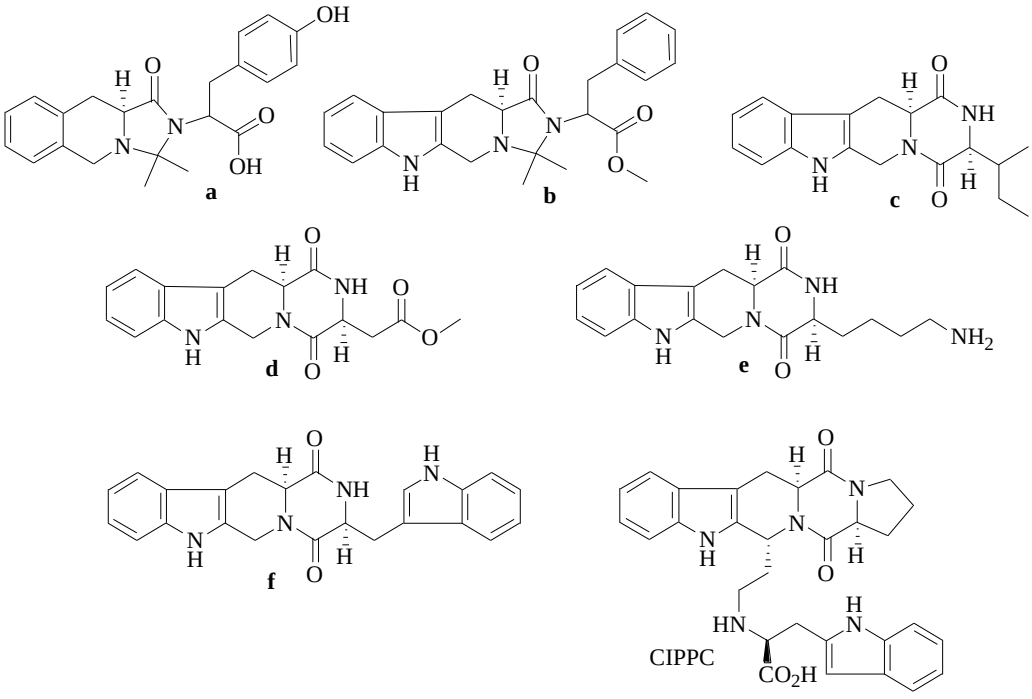


Figure S2 ESR spectrum without CIPPC (black) and ESR spectrum with CIPPC (red), 43% of ·ON was scavenged.

Structures of compounds a-f of Figure 2 in the text



Reference

1.Castanon, M. M.; Gamba, C.; Kordich, L. C., Insight into the profibrinolytic activity of dermatan sulfate: effects on the activation of plasminogen mediated by tissue and urinary plasminogen activators. *Thromb Res* **2007,** 120, (5), 745-52.

2. Guo, Q.; Rimbach, G.; Moini, H.; Weber, S.; Packer, L. ESR and cell culture studies on free radical-scavenging and antioxidant activities of isoflavonoids. Toxicology **2002**, 179, 171-180.