Supporting Information

Dithiaarsanes inducing oxidative stress-mediated apoptosis in HL-60 cells by selectively targeting the thioredoxin reductase

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Table S1 Characterization of the purity of synthesized compounds.

Sample	Peak time (min)	Purity (%)	Eluent (V/V)
PAO (**2**)	6.333	98.72	MeOH:H_2O=15:85
PAO-EDT (**3**)	7.959	99.09	MeOH:H_2O =7:3
PAO-PDT (**4**)	9.597	98.30	MeOH:H_2O =7:3
APAO-EDT (**5**)	8.949	99.37	MeOH:H_2O =7:3
APAO-PDT (**6**)	11.675	98.99	MeOH:H_2O =7:3
PAO-Nap (**12**)	8.519	95.89	MeOH:H_2O =35:65
PAO-PDT-Nap (**13**)	8.557	93.57	MeOH:H_2O =7:3

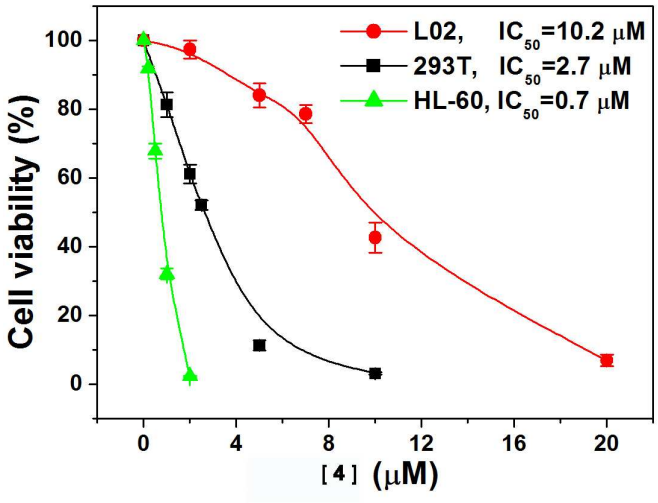

Fig. S1 Cytotoxicity of 4 towards L02, HEK 293T, and HL-60 cells. The cells (1×10^4 cells) were treated with varying concentrations of **4** for 48 h, and the viability was determined by the MTT method. Data are expressed as mean ± S. E. of three experiments.

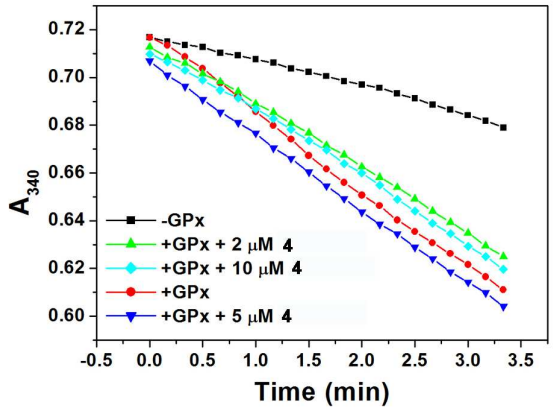

Fig. S2 No inhibition of GPx by 4. The GPx activity was measured indirectly by a coupled reaction with glutathione reductase. The detailed procedure was described in Materials and Methods.

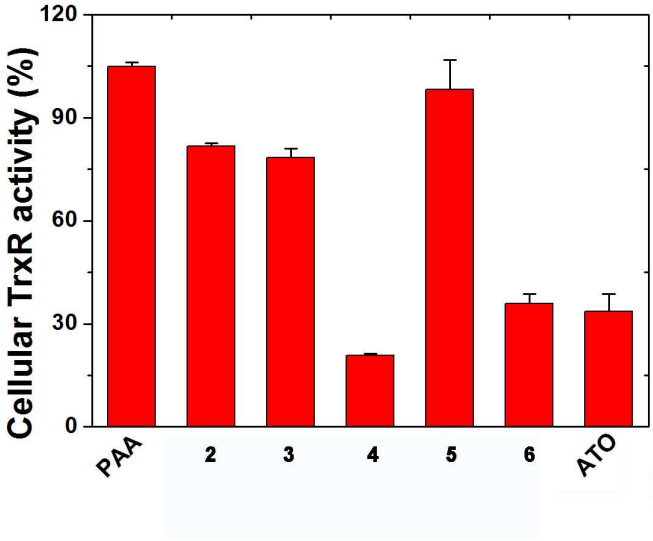

Fig. S3 Inhibition of TrxR activity in HL-60 cells by organoarsenical compounds. After the cells were treated with 2μM of PAO derivatives for 12 h, the enzyme activity of TrxR in the cells was determined by the endpoint insulin reduction assay, and expressed as the percentage of the control. Data are expressed as mean ± S. E. of three experiments.

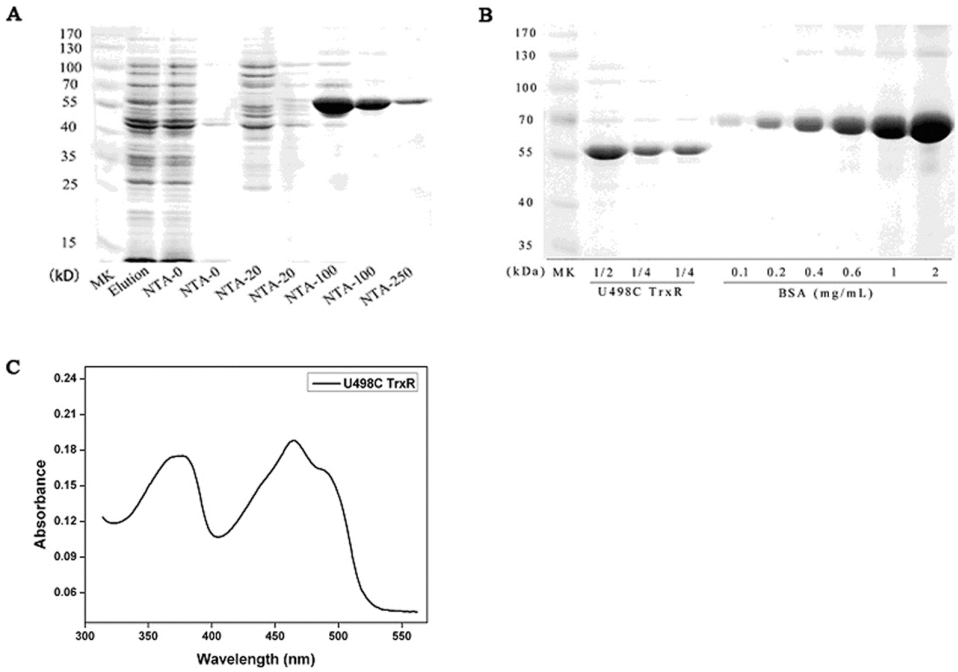

Fig.S4 Preparation and characterization of recombinant proteins. (A) SDS-PAGE analysis of U498C TrxR after elution from Ni-NTA-Sefinose by different elution buffer. For details, refer to the experimental section. **(B) Quantification of the concentration of U498C TrxR by SDS-PAGE using BSA as a standard. (C) Absorbance spectrum of the U498C TrxR.** The spectrum was recorded at 30 °C in 50mM Tris-HCl, 0.15M NaCl, 1mM EDTA, pH 7.5.

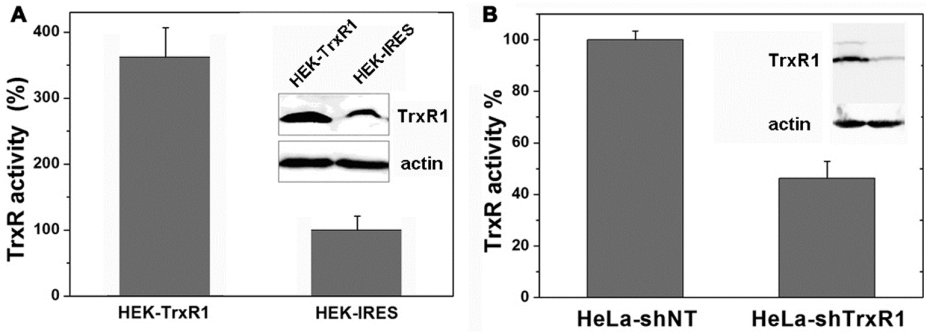

Fig S5 Overexpressing and knocking down TrxR1 in cells. (A) Quantification of TrxR activity in HEK-IRES and HEK-TrxR1 cells. The TrxR activity in different cells was determined by the endpoint insulin reduction assay, and the relative activity was expressed compared to TrxR activity in HEK-IRES cells. Data are expressed as mean ± S. E. of three experiments. Inset: Cell extracts were prepared and analyzed by Western blotting with an antibody against TrxR1 and actin. **(B) TrxR1 gene expression in HeLa-shNT and HeLa-shTrxR1 cells.** The mRNA levels of TrxR1 were quantified by RT-PCR and normalized using GAPDH as an internal standard. Inset: Cell extracts were prepared and analyzed by Western blotting with an antibody against TrxR1 and actin.

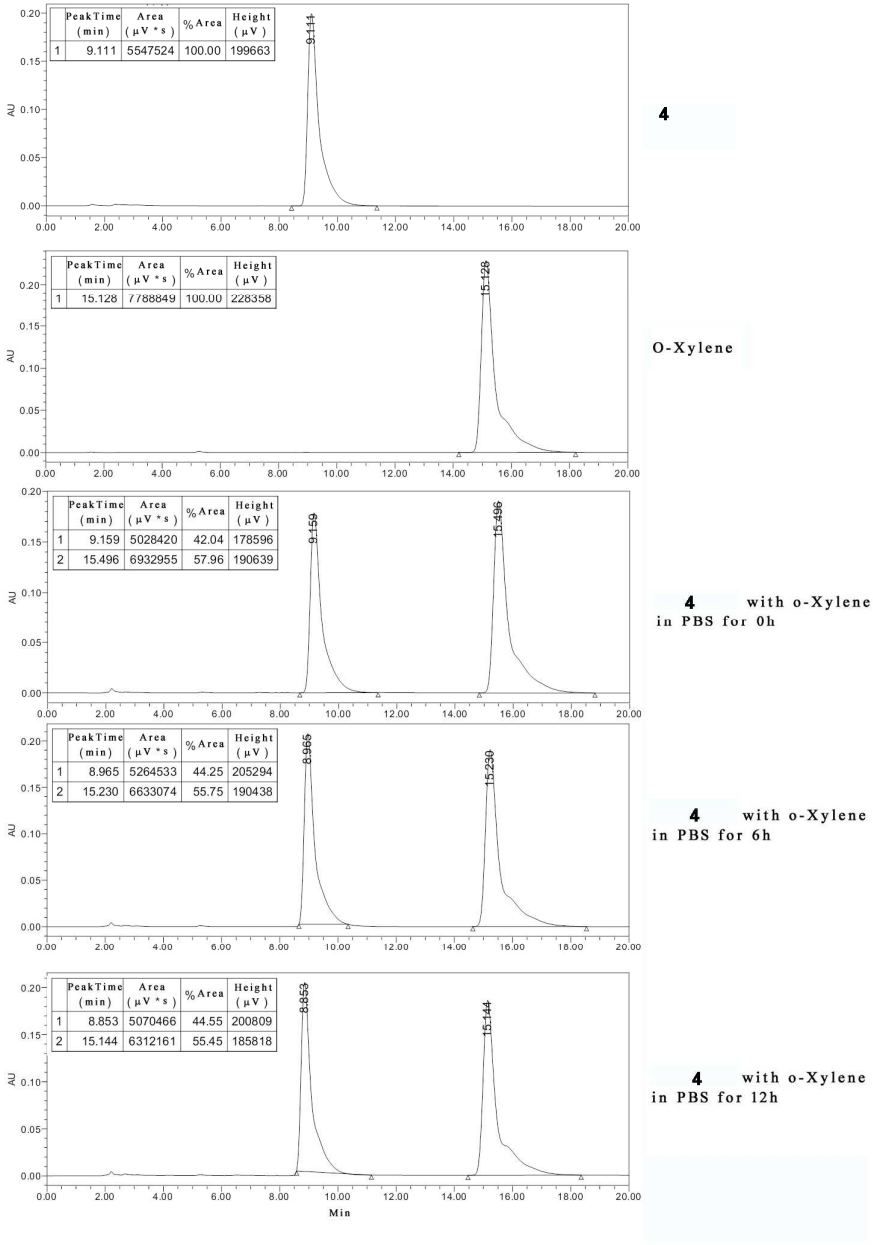

Fig. S6 Stability of 4 in PBS. Compound **4** was dissolved in PBS buffer and incubated at 37 °C. At indicated time, 100μL of the **4** solution was mixed with 100μL of o-xylene in CH_3OH (an internal standard), and analyzed by HPLC.

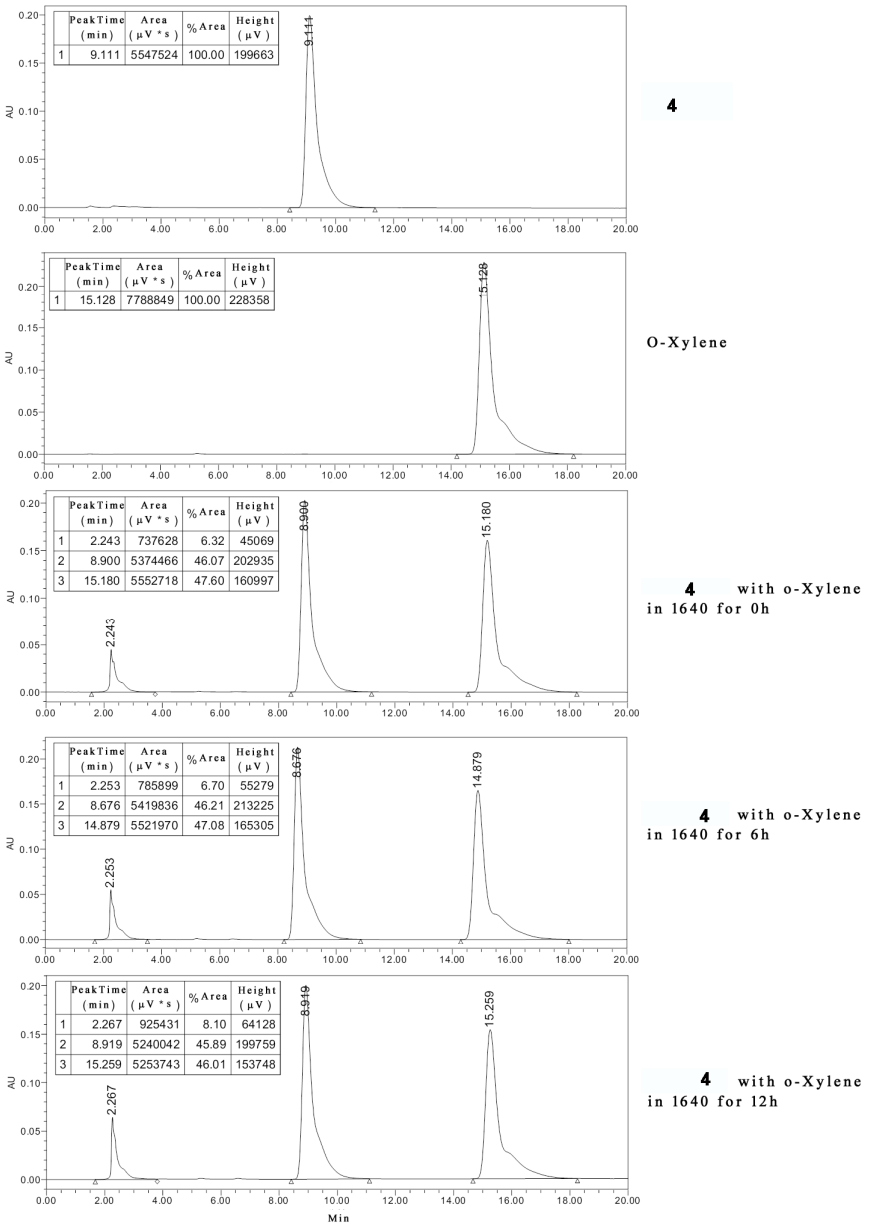

Fig. S7 Stability of 4 in cell culture medium (RPMI 1640). Compound **4** was dissolved in RPMI 1640 and incubated at 37 °C. At indicated time, 100μL of the **4** solution was mixed with 100μL of o-xylene in CH_3OH (an internal standard), and analyzed by HPLC.

Materials and methods

Chemicals and enzymes

The recombinant rat TrxR1 was essentially prepared as described,[1] and is a gift from Prof. Arne Holmgren at Karolinska Institute, Sweden. The recombinant U498C TrxR mutant (Sec→Cys) and truncated TrxR (T-TrxR) were produced as described.[2] Proteins were pure as judged by Coomassie-stained SDS-polyacrylamide gel electrophoresis (PAGE), and the recombinant TrxR1 had a specific activity of 50% of the wild TrxR1 with 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB) assay. *E. coli* Trx was purchased from IMCO (Stockholm, Sweden, www.imcocorp.se). RPMI 1640 Medium, Dulbecco's modified Eagle's medium (DMEM), G418, *N*-acetyl-L-cysteine (NAC), bovine insulin, L-buthionine-(*S*,*R*)-sulfoximine (BSO), N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Ac-DEVD-pNA), reduced and oxidized glutathione (GSH and GSSG), dimethyl sulfoxide (DMSO), yeast glutathione reductase (GR), glutathione peroxidase (GPx), superoxide dismutase (SOD) puromycin, 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and 2', 7'-dichlorfluorescein diacetate (DCFH-DA) were obtained from Sigma-Aldrich (St. Louis, USA). Cytochrome c and Ni-NTA-Sefinose were obtained from Sangon Biotech (Shanghai, China). NADPH was obtained from Roche (Mannheim, Germany). Sepharose 4B and Sephadex G-25 were purchased from GE Healthcare Life Sciences. Fetal bovine serum (FBS) was obtained from Sijiqing (Hangzhou, China). Aniti-TrxR1 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), penicillin and streptomycin were obtained from Amresco (Solon, USA). Bovine serum albumin (BSA), phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate (Na_3VO_4) and anti-actin antibody were obtained from Beyotime (Nantong, China). 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB) and ethylene diamine tetraacetic acid (EDTA) were obtained from J&K Scientific (Beijing, China). All other reagents were of analytical grade.

Synthesis of trivalent arsenic compounds (Scheme 1)

The synthesis was essentially carried out as described in the literature.[3, 4]

Synthesis of 4-aminophenyl arsenoxide (PAO, 2)

p-Arsanilic acid (PAA) (10.9 g) was added to a solution containing 30 mL of methanol, 24 mL of HCl, and 100 mg of potassium iodide. Sulfur dioxide was bubbled through the stirred solution for 30 min during which time the color changed from orange to pale yellow and precipitation of 4-aminophenyldichloroarsine·HCl (**1**) ensued. The solution was cooled in ice and the precipitate was collected and washed with diethyl ether. Then **1** was dissolved in 200 mL of 10% ammonium hydroxide. After stirring for 15 min, **2** began to precipitate and the solution was cooled in ice. It was collected by filtration, washed with diethyl ether which gave **2** as white powder (6 g, 56% yield). ^{1}HNMR (400 MHz , DMSO-d6) δ 7.882 - 7.862(d , J = 8 Hz , 2H), δ 7.455 – 7.435 (d , J = 8 Hz , 2H). MS-ESI: 184.0 ($M+H^{+}$)

Synthesis of 4-aminophenyl dithiaarsanes (3 & 4)

To a solution of **2** (6.42 mmol) in absolute ethanol (10 mL), 1, 2-ethanedithiol or 1,3-propanedithiol (6.6 mmol) was added dropwise, then the solution was stirred for 10 min under reflux. The solution was chilled in ice and filtrated. A white crystal was recrystallized from ethanol (10 mL) and afforded **3** and **4** .

Synthesis of 4- acetaminophenyl dithiaarsanes (5 & 6)

To a solution of CH_3COOH (60 mg, 1 mmol , in 10 ml DMF), a mixture of 1-hydroxybenzotriazole (HOBT) (135 mg, 1 mmol) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI) (192 mg, 1 mmol) were added. After stirring for 20 min, dithiaarsanes (0.732 mmol) and N, N-diisopropylethylamine (DIPEA) (129mg, 1 mmol) were added. The solution was stirred for 2h. Then the solution was extracted with EtOAc and water. The organic layer was taken to dryness under reduced pressure. Chromatography on silica gel (petroleum ether : EtOAc = 4:1), afforded the target compound as white solid.

2- p-Aminophenyl-1, 3, 2-Dithiarsenolane (PAO-EDT, 3)

Yield: 68%. mp 86.8-86.9 ^{o}C. ^{1}HNMR (400 MHz, $CDCl_3$), δ 7.441 – 7.408 (d, J = 8.4 Hz, 2H) , δ 6.679 – 6.646 (d , J = 8.4 Hz, 2H) , δ 3.792 (s, NH_2, 2H) , δ 3.380 - 3.310 (m, 2H) , δ 3.252 – 3.182 (m, 2H) . ^{13}C NMR ($CDCl_3$), δ 147.54, 132.06, 131.21, 114.84, 41.55. MS-ESI: 260.2 ($M+H^{+}$).

2- p-Aminophenyl-1, 3, 2-Dithiarsinane (PAO-PDT, 4)

Yield: 70%. mp 127.8-128.2 ^{o}C. ^{1}HNMR(400 MHz, $CDCl_3$) , δ 7.659 – 7.638 (d , J = 8.4 Hz , 2H) , δ 6.788 – 6.768 (d , J = 8.2 Hz , 2H) , δ 3.863 (s , NH_2 , 2H) , δ 2.938 –

2.871 (dt , J = 12.8, 2 Hz , 2H) , δ 2.753- 2.695 (ddd, J = 14, 7.2, 2.4 Hz, 2H) , 2.192 – 2.103 (m , 1H) , 1.968 – 1.895 (m , 1H) . ^{13}C NMR ($CDCl_3$), δ 147.53 , 133.73, 125.68, 115.58, 28.68, 26.43. MS-ESI: 274.4 ($M+H^+$).

2- p-Acetaminophenyl-1, 3, 2-Dithiarsenolane (APAO-EDT, 5)

Yield: 75%. mp 153.0-153.2 ^{o}C. ^{1}HNMR(400 MH,CDCl3) , δ 7.597 - 7.576 (d , J = 8.4 Hz , 2H) , δ 7.510 -7.489 (d ,J = 8.4 Hz , 2H) , δ 7.465 (s , NH , 1H), δ 3.367 - 3.339 (m , 2H) , δ 3.180 - 3.152 (m , 2H) , δ 2.172 (s , CH_3 , 3H) . ^{13}C NMR ($CDCl_3$) , δ 168.42, 138.76, 131.52, 119.60, 41.79, 24.58. MS-ESI: 302.3 ($M+H^+$).

2- p-Acetaminophenyl-1, 3, 2-Dithiarsinane (APAO-PDT, 6)

Yield: 65%. mp 134.1-134.5 ^{o}C. ^{1}HNMR(400 MH,CDCl3) , δ7.850 - 7.829 (d, J = 8.4 Hz , 2H) , δ 7.651-7.630 (d , J = 8.4 Hz , 2H) , δ 7.529 (s ， NH ， 1H) ， δ 2.860 - 2.797 （m , 2H ）, δ 2.715 - 2.692 (m , 2H) , δ 2.216 (s , 3H), δ 2.197 - 2.127 (m , 1H) , δ 1.966 - 1.913 (m , 1H) . ^{13}C NMR ($CDCl_3$), δ 168.49 , 138.73, 133.55, 133.28, 120.32, 28.31, 25.98, 24.64. MS-ESI: 316.1 ($M+H^+$).

Preparation of fluorophore-labeled PAO (Scheme 2)[5]

Synthesis of compound 7

6-Aminocaproic acid (1.12 g, 8.54 mmol) was dissolved in 2 M aqueous solution of NaOH solution (7 mL), followed by 1, 4-dioxane (14 mL). The reaction mixture was cooled to 0 °C and Boc_2O (2.05 g, 9.40 mmol) was added. The reaction mixture was stirred at room temperature (rt) for 3 hours. The solution was concentrated under reduced pressure. The basic aqueous residue was washed once with acetic ether (EtOAc, 25 mL). The aqueous layer was acidified by 1 M aqueous HCl until pH 1 and then extracted with dichloromethane (DCM , 25 mL×2). The combined organic layers were dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to afford the compound **7** as a colourless oil which slowly crystallized (3.76 g, 90% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.161 (br , 1H), 4.516 (br s, 1H), 3.080 (br, 2H), 2.320 (t, J = 7.2 Hz, 2H), 1.672-1.593 (m, 2 H), 1.511-1.460 (m, 2H), 1.424 (s, 9H), 1.384-1.313 (m, 2H). MS-ESI: 232.1 ($M+H^+$).

Synthesis of compound 8

A suspension of **7** (315.7 mg, 1.36 mmol) in acetonitrile (50 mL) at -5 °C was treated

with triethylamine (0.19 mL, 1.36 mmol) and ethyl chloroformate (0.13 mL, 1.32 mmol). The suspension was stirred at that temperature for 5 h. To a suspension of p-aminophenyldichloroarsine·HCl (**2**) (375.5 mg, 1.37 mmol) in acetonitrile/MeOH 10:1 (11 mL) was added triethylamine (0.38 mL), and this suspension was dropped slowly into the suspension. The reaction mixture was stirred at -5 °C, then warmed to room temperature and stirred for 5h. The solvent was removed under vacuum and 2 N NH_4OH (6 mL) was added. The suspension was stirred for 10 min, and cold water (15 mL) was added. The suspension was cooled in ice, and the solid was collected by filtration, washed with cold water and dried. Then the solid was stirred with a solution (10 mL) of trifluoroacetic acid (TFA)/ DCM (30:70, v/v) for 45 min. The solution was concentrated under reduced pressure and the residue was purified by silica gel column chromatography (DCM: methanol=4:1, v/v) to get **2** as gray solid (200 mg, 47% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.016 (s, 1H), 7.749 (s, 2H), 7.641-7.620 (d, J = 8.4 Hz, 2H), 7.546-7.526 (t, J = 8.0 Hz, 2H), 2.786-2.504 (m, 2H), 2.496-2.300 (m, 2H), 1.608-1.570 (m, 4H), 1.550-1.326 (m, 2H). MS-ESI: 315.2 ($M+H^+$).

Synthesis of compound 9

To the mixture of acenaphthene (7.7g, 50 mmol) and acetic acid (60 mL) was added nitric acid (5.5 mL) with stirring at room temperature. After 4 h, the reaction mixture was poured into ice water. The precipitate was filtered, washed with water. The crude product was recrystallized from acetic acid, and then ethanol to afford compound **9** (6.27g, 63％ yield). ^{1}H NMR (400 MHz, CDCl3) δ: 8.560 (d, J = 8.6 Hz, 1H), 8.502 (dd, J = 7.7, 2.2 Hz, 1H), 7.731 (t, J = 7.8 Hz, 1H), 7.455 (d, J = 7.0 Hz, 1H), 7.330 (d, J = 7.7 Hz, 1H), 3.481 (dd, J = 16.1, 6.7 Hz, 4H). ^{13}C NMR (CDCl3) δ: 155.74, 146.61, 140.14, 131.98, 127.74, 124.39, 121.22, 120.13, 117.93, 30.61, 30.57. EI-MS m/z (％): 199 (M^+, 86), 152 (100).

Synthesis of compound 10

To a stirred solution of sodium dichromate (2.24 g, 7.5 mmol) and acetic acid (5 mL) was added the solution of **9** (0.60 g, 3 mmol in 5 mL acetic acid). After heating at reflux for 5 h, the reaction mixture was mixed with cold water (50 mL) at 0 ^{o}C, and then filtered and washed with water until the filtrate was neutral. The residue was dried in vacuo to afford the product (0.63 g, 87 % yield). ^{1}H NMR (400 MHz, DMSO) δ 8.761 (dd, *J* = 8.7, 0.9 Hz, 1H),

8.700 – 8.613 (m, 2H), 8.562 (d, *J* = 8.0 Hz, 1H), 8.123 (dd, *J* = 8.7, 7.3 Hz, 1H). ^{13}C NMR (DMSO-d6) δ 160.03, 159.43, 149.55, 133.17, 131.04, 130.58, 130.26, 129.76, 124.34, 124.09, 122.77, 120.06. EI-MS *m*/*z* (%): 243 (M^+, 100), 199 (66), 153 (82), 125 (61).

Synthesis of compound 11

To a stirred cloudy solution of **10** (120 mg, 0.5 mmol) in ethanol (5 mL) was added dropwisely the solution of $SnCl_2 \cdot 2H_2O$ (677 mg, 3 mmol) in concentrated hydrochloric acid (1 mL) at room temperature. The reaction was heated to reflux for 8 h. After cool down to room temperature, aqueous solution of Na_2CO_3 (10%) was added to quench the reaction. The precipitate was collected by filtration, washed with water (3 × 10 mL), and dried in vacuo to afford the crude product **11**, which was directly used for the preparation of compound **12** without further purification.

Synthesis of compound PAO-Nap (12)

Compound **8** (157 mg, 0.5 mmol) was added to a cloudy solution of **11** (108 mg, 0.5 mmol) in DMF (10 mL). After heating at 100 °C for 6 h, the reaction was allowed to cool to room temperature. The solution was added to ice water, filtered and dried in vacuo. The crude product was purified by silica gel column chromatography (chloroform/ methanol=4:1, v/v) to give 100 mg of compound **12** (40 %) as a brown yellow solid. mp 199.0-200.5 °C. ^{1}H NMR (400 MHz, DMSO) δ 8.597- 8.576 (d, J = 8.4 Hz , 1H), 8.403 - 8.386 (d, J = 6.8 Hz , 1H), 8.180- 8.159 (d, J = 8.4 Hz, 1H), 7.633-7.526 (m, 4H), 6.910-6.871 (d, J = 8.0 Hz, 1H), 4.011-3.976 (m, 2H), 2.334-2.300 (m, 2H), 1.628-1.615 (m, 4H), 1.366-1.349 (m, 2H). MS-ESI: 510.2 ($M+H^+$).

Synthesis of compound PAO-PDT-Nap (13)

Compound **12** (51mg, 0.1 mmol) was added to 1, 3-propanedithiol (10 μL) in EtOH (10 mL). After heating at reflux for 6 h, the reaction was allowed to cool to room temperature. The solution was concentrated under reduced pressure. The crude product was purified by silica gel column chromatography (chloroform/ methanol=6:1, v/v) to give 40 mg of compound **13** (69 %) as a brown yellow solid. mp 139.3-140.8 °C. ^{1}H NMR (400 MHz, DMSO) δ 10.075 (s, 1H), 8.602- 8.582 (d , J = 8.0 Hz , 1H), 8.407 - 8.390 (d , J = 6.8 Hz , 1H), 8.182- 8.161 (d , J = 8.2 Hz , 1H), 7.741-7.606 (m , 4H), 7.433 (s, 2H), 6.838-6.818 (d , J = 8.0 Hz, 1H), 4.011-3.976 (m, 2H), 2.736-2.720 (m, 2H), 2.334-2.300 (m, 2H), 2.075-1.833

(m, 2H), 1.628-1.615 (m, 4H), 1.366-1.349 (m, 2H), 1.160-1.143 (m, 2H). ^{13}C NMR (DMSO-d6) δ 172.00, 164.22, 163.36, 153.14, 140.82, 134.39, 133.19, 131.43, 130.12, 129.73, 124.40, 122.22, 120.13, 119.81, 108.61, 108.01, 36.75, 28.36, 28.01, 26.69, 26.10, 25.32. MS-ESI: 604.2 ($M+Na^+$).

Preparation of immobilized PAO (Scheme 3)[6]

1 g of suction-dried Sepharose 4B was washed on a glass filter-funnel with water and then mixed with 1 mL of 1,4-butanediol diglycidyl ether and 1 mL of 0.6 M sodium hydroxide solution containing 2 mg of sodium borohydride per millilitre. The suspension was mixed by rotation for 8 h at 25 ^{o}C and the reaction stopped by washing the Sepharose 4B on a glass filter-funnel with large volumes of water (500 mL). The obtained Sepharose 4B was named A-Seph. And the coupled product of A-Seph and 4-aminophenylarsenoxide which was called Seph-PAO was accomplished by suspending 1 g of suction-dried A-Seph in 2 mL of sodium carbonate buffer (pH 10.6) containing 5 mg of 4-aminophenylarsenoxide. The suspension was mixed by rotation for 10 h at 25 ^{o}C. Then block remaining excess groups with 1 M ethanolamine in sodium carbonate buffer (pH 10.6) for at least 4 h and wash away excess 4-aminophenylarsenoxide with sodium carbonate buffer (pH 10.6) followed by distilled water, 0.1 M acetate, pH 4.0. Repeat four or five times. And Seph-PAO-PDT was obtained by adding 6 μL 1,3-propanedithiol (PDT) in 20% ethanol to 1mL suction-dried Seph-PAO. The suspension was mixed by rotation for 30min at 25 ^{o}C and washed with 20% ethanol followed by distilled water, sodium carbonate buffer (pH 10.6). Repeat four or five times. The three kinds of sepharose were saved in 20% ethanol at 4 ^{o}C.

Preparation of the recombinant U498C TrxR and T-TrxR proteins [2]

The plasmids encoding U498C TrxR ($pET28^+$-TrxR1) and T-TrxR ($pET28^+$-T-TrxR) genes were synthesized from Sangon. The $pET28^+$-TrxR1 plasmid was transformed into competent E. coli BL21(DE3) cells using the heat shock method. After a short incubation in ice, a mixture of E. coli BL21(DE3) (40 μL) and the plasmid (1 μL, 50 ng/μL) was placed at 40°C for 45 seconds (heat shock) and then placed back in ice for 2 minutes. 500 μL pre-warmed LB liquid medium was added and the transformed E. coli strain was incubated at

37 ℃ for 1 h with agitation (210 rpm). Using a fresh sterile pipette tip, drag through streak and spread the E. coli strain over a plate with LB solid medium containing 50 μg/mL kanamycin. After incubation at 37 ℃ overnight, single colonies should be visible. A single colony should look like a white dot growing on the solid medium. Then transferring one colony to 6 mL LB liquid medium containing 50 μg/ml kanamycin incubated at 37 ℃ overnight. To express the recombinant U498C TrxR, 3 mL of the transformed E. coli cells were grown in 600 mL LB liquid medium containing 50 μg/mL kanamycin at 37 ℃ for 12 h with agitation (210 rpm) and induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 30 ℃ for another 4 h with agitation (210 rpm) before harvesting cells. The cells were harvested by centrifugation and stored at -80 ℃ until used.

Purification of the Recombinant U498C TrxR and T-TrxR Proteins

E. coli cells were resuspended in 1/20 growth volume of 50 mM Tris-HCl pH 8.0, 0.15 M NaCl, 1 mg/mL lysozyme and 1 mM PMSF. After placed in ice for 30 minutes, the cells were sonicated four times for 1.5 min with 2-minute intervals. Then 0.1% Triton X-100 was added and placed in ice for 15 minutes. Following centrifugation for 15 min at 13,000 rpm at 4 ℃, the supernatant fraction was loaded on a column of Ni-NTA-Sefinose. U498C TrxR was eluted with 10 ml of NTA-0 (50 mM Tris-HCl pH 8.0, 0.5 M NaCl and 10% glycerin), 10 ml of NTA-20 (50 mM Tris-HCl pH 8.0, 0.5 M NaCl，10% glycerin and 20 mM imidazole), 10 ml of NTA-100 (50 mM Tris-HCl pH 8.0, 0.5 M NaCl，10% glycerin and 100 mM imidazole), and 10 ml of NTA-250 (50 mM Tris-HCl pH 8.0, 0.5 M NaCl，10% glycerin and 250 mM imidazole). Fractions were analyzed by SDS-PAGE (Fig S4). According to the result, NTA-20 and NTA-250 were combined and dialyzed at 0 ℃ for 4 h against 500 mL solution (50 mM Tris-HCl pH 7.5, 0.15M NaCl, 1mM EDTA，10% glycerin，0.1 mM DTT), then dialyzed again at 0 ℃ for 4 h with 500 mL solution (50mM Tris-HCl pH 7.5, 0.15M NaCl, 1mM EDTA，10% glycerin). The U498C TrxR was stored at -80 ℃. The preparation of T-TrxR was exactly followed the procedure described for U498C TrxR.

Protein Analyses

U498C TrxR concentration was determined either by measuring the absorbance of flavin at 460 nm (ε=11.3 $mM^{-1}cm^{-1}$)[2] or using SDS-PAGE method (Fig. S4).

Cell cultures

SMMC-7721, HeLa, HepG2, HL-60, L02, and HEK 293T cells were obtained from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. HL-60 cells were cultured in RPMI 1640 supplemented with 10% FBS, 2 mM glutamine, and 100 units mL^{-1} penicillin/streptomycin and maintained in an atmosphere of 5% CO_2 at 37 °C. 7721, HeLa, HepG2, HL-60, L02, and HEK 293T cells were cultured in DMEM with 10% FBS under the same conditions. HEK-TrxR1 and HEK-IRES cells,[7-10] kindly provided by Prof. Constantinos Koumenis from University of Pennsylvania School of Medicine, were cultured in DMEM with 10% FBS, 2 mM glutamine, 100 units mL^{-1} penicillin/streptomycin, 0.1 μM sodium selenite, and 0.4 mg/mL G418 and maintained in an atmosphere of 5% CO_2 at 37 °C. The concentrations of DMSO in all cell experiments are 0.1% (V/V).

Cytotoxicity assay

MTT assay[8]

1×10^4 cells were incubated with **2** and its derivatives in triplicate in a 96-well plate for the indicated time at 37 °C in a final volume of 100 μL. Cells treated with DMSO alone were used as controls. At the end of the treatment, 10 μL MTT (5 mg/mL) was added to each well and incubated for an additional 4 h at 37 °C. An extraction buffer (100 μL, 10% SDS, 5% isobutanol, 0.1% HCl) was added, and the cells were incubated overnight at 37°C. The absorbance was measured at 570 nm on Multiskan GO (Thermo Scientific).

Trypan blue exclusion assay[8]

HL-60 cells were seeded at 2×10^4 cells per well in 24-well plates and treated with different concentrations of NAC and **4** (0.5, 1 or 2 μM) for 48 h. Cells treated with DMSO alone were used as controls, and cell viability was determined by the trypan blue exclusion assay. After treatment, the cells were stained with trypan blue (0.4%, w/v), and the number of viable (non-stained) and dead (stained) cells were counted under microscope.

In vitro TrxR activity by DTNB assay[7, 8]

The TrxR activity was determined at room temperature using a microplate reader. The NADPH-reduced TrxR (80 nM) or U498C TrxR (1.0 μM) was incubated with different concentrations of **4** for the indicated times at room temperature (the final volume of the

mixture was 50 μL) in a 96-well plate. A master mixture in TE buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 50 μL) containing DTNB and NADPH was added (final concentration: 2 mM and 200 μM, respectively), and the linear increase in absorbance at 412 nm during the initial 3 min was recorded. The same amounts of DMSO (0.1%, v/v) were added to the control experiments and the activity was expressed as the percentage of the control.

GR assay[7, 8]

The NADPH-reduced GR (0.25 U/mL) in TE buffer was incubated with different concentrations of **4** for 0.5 h in a 96-well plate at room temperature in a total volume of 100 μL. Reactions were initiated by the addition of GSSG and NADPH (50 μL, final concentration: 1 mM and 400 μM, respectively). The GR activity was determined by measuring the decrease in absorbance at 340 nm during the initial 3 min. The same amounts of DMSO were added to the control experiments and the activity was expressed as the percentage of the control.

GPx assay[11, 12]

The GPx activity was measured indirectly by a coupled reaction with GR. Oxidized glutathione (GSSG), produced upon reduction of hydroperoxides by GPx, is recycled to its reduced state by GR and NADPH. The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm. The rate of decrease in the A_{340} is directly proportional to the GPx activity. To the wells of a 96-well microliter plate were added 130 μL of TE buffer (50 mM Tris-HCl with 1 mM EDTA, pH 7.5,), 10 μL of a freshly prepared NADPH solution (4.0 mM in TE buffer), 10 μL of bovine erythrocyte GPx solution (1.0 $IU \cdot mL^{-1}$ in TE buffer), and 10 μL of indicated concentrations of **4**. The solution was incubated at 37 °C for 30 min. Then 10 μL of a baker's yeast GR solution (4.0 $IU \cdot mL^{-1}$ in TE buffer) and 10 μL of freshly prepared GSH solution (5.0 mM in TE buffer) were added. After adding 20 μL of H_2O_2 solution (5.0 mM in water), the final volume in each well was 200 μL. The background of GPx-independent NAPDH oxidation was run by replacing the GPx solution with TE buffer. The decreases in absorption of NADPH at λ=340 nm were measured for 4 min at intervals of 10 sec at room temperature.

Determination of TrxR activity in cell lysates[7, 8]

After HL-60 cells were treated with different concentrations of **2** or **4** for 12 h, the cells were harvested, and washed twice with PBS. Total cellular proteins were extracted by RIPA buffer (50 mM Tris-HCl pH 7.5, 2 mM EDTA, 0.5% deoxycholate, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM Na_3VO_4 and 1 mM PMSF) for 30 min on ice. The total protein content was quantified using the Bradford procedure. TrxR activity in cell lysates was measured by the endpoint insulin reduction assay. Briefly, the cell extract containing 20 μg of total proteins was incubated in a final reaction volume of 50 μl containing 100 mM Tris-HCl (pH 7.6), 0.3 mM insulin, 660 μM NADPH, 3 mM EDTA, and 15 μM *E. coli* Trx for 30 min at 37 °C. The reaction was terminated by adding 200 μL of 1 mM DTNB in 6 M guanidine hydrochloride, pH 8.0. A blank sample, containing everything except Trx, was treated in the same manner. The absorbance at 412 nm was measured, and the blank value was subtracted from the corresponding absorbance value of the sample. The same amounts of DMSO were added to the control experiments and the activity was expressed as the percentage of the control.

Imaging TrxR activity in HL-60 cells by TRFS-green[13]

HL-60 cells were treated with indicated concentrations of **4** for 8 h followed by further treated with TRFS-green (10μM) for 4h. Phase contrast (top panel) and fluorescence (bottom panel) images were acquired by fluorescence microscopy (Leica DMI4000). Ten cells were randomly selected, and the fluorescence intensity in individual cells was quantified by the software of Leica Qwin accompanied with the Leica microscope.

Assessment of the intracellular ROS[7, 8]

HL-60 cells were plated in 12-well plates and were treated with 1 μM **4** for 1 h or 2 h. After removal of the medium, the ROS indicator DCFH-DA (10 μM) in fresh FBS-free medium was added, and continued incubation for 30 min at 37 °C in dark. The cells were visualized and photographed under a Leica inverted fluorescent microscopy.

Induction of NADPH oxidase activity and production of superoxide anion by 4-modified enzyme[8, 14, 15]

The NADPH-reduced TrxR (1.3 μM) was incubated with 5 μM **4** at room temperature for 0.5 h in TE buffer. The remaining enzyme activity was less than 10% of the control, as monitored by the DTNB reduction assay. After incubation, the unreacted **4** was removed by a Sephadex G-25 desalting column. To determine the NADPH oxidase activity, 80 μL of modified enzyme were added to 220 μL of TE buffer containing 200 μM NADPH. The production of superoxide anion was determined by addition of 34 μL of 0.82 mM cytochrome *c* to the reaction mixture, and the amount of superoxide anion production was calculated using a molar extinction of 21,000 M^{-1} cm^{-1} at 550 nm due to the reduction of cytochrome *c*.

Assessment of intracellular thiols[8, 14]

HL-60 cells were treated with different concentrations of **4** for 12 or 24 h in 100-mm dishes. The cells were collected, washed twice with PBS, and lysed with RIPA buffer for 30 min on ice. The Protein content was quantified using the Bradford procedure. Total thiol levels were determined by DTNB-titration. Briefly, 10 μL cell lysate was added to cuvettes containing 90 μL of 1 mM DTNB in 6 M guanidine hydrochloride, pH 8.0. After 5 min at room temperature, the absorbance was read at 412 nm. Thiol levels were calculated by a calibration curve using GSH as the standard.

Western Blot Analysis

For Western blot analysis, equal amounts of protein in each lysate sample were separated by SDS-PAGE and electroblotted onto PVDF membrane (Millipore, USA). After blocking with 5% non-fat milk at room temperature for 2 h, membranes were incubated with primary antibodies at 4 ℃ overnight. The membrane was washed with TBST for three times, and then incubated with the peroxidase conjugated secondary antibodies at room temperature for 1 h. The signal was detected using an enhanced chemiluminescence (ECL) kit from GE Healthcare Life Sciences.

Generation of stable TrxR1 knockdown cells[7, 8]

The shRNA plasmid targeting coding regions of the TrxR1 gene (shTrxR1) and the control nontargeting shRNA (shNT) were kindly provided by Prof. Constantinos Koumenis from University of Pennsylvania School of Medicine.[9] HeLa cells were plated in a 6-well plate with 3×10^5 cells/well in DMEM media without antibiotics overnight and were transfected with either shTrxR1 or shNT plasmid using Gene^{TM} □ efficiency Transfection reagent (Biomiga, CA, USA). After 48 h of transfection, the cells were maintained with DMEM medium, 10% FBS, 2 mM glutamine, 100 units/mL penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO_2, and selected by the supplement with 1 μg/mL puromycin.

Annexin V/PI staining[7, 8]

HL-60 cells were treated with 0.5, 2 and 5 μM **4** for 12 or 24 h in 12-well plates. The cells were harvested and washed with PBS. Apoptotic cells were identified by double staining with fluorescein-5-isothiocyanate (FITC)-conjugated Annexin V and PI according to the manufacturer's instructions (Zoman Biotech, Beijing, China). Data were obtained and analyzed using a FACSCanto^{TM} flow cytometer (BD Biosciences, USA) with Cell Quest software (BD Biosciences).

Measurement of caspase-3 activity[7, 8]

HL-60 cells were treated with different concentrations of **4** for 12 h in 100-mm dishes. The cells were collected, washed twice with PBS, and then lysed with RIPA buffer for 30 min on ice. The protein content was quantified using the Bradford procedure. A cell extract containing 50 μg of total proteins was incubated with the assay mixture (50 mM Hepes, 2 mM EDTA, 5% glycerol, 10 mM DTT, 0.1% CHAPS, 0.2 mM Ac-DEVD-pNA, pH 7.5) for 3 h at 37 °C in a final volume of 100 μL. The absorbance at 405 nm was measured using a microplate reader. The same amounts of DMSO were added to the control experiments and the activity was expressed as the percentage of the control.

Hoechst 33342 Staining[7, 8]

HL-60 cells were plated in 12-well plates and were incubated with 1、2 or 5 μM **4** for 24 h followed by addition of 5 μg/mL Hoechst 33342. After incubation for 20 min, the cells were visualized and photographed under a Leica inverted fluorescent microscopy.

12 and 13 labeling assay

Briefly, 100μL of U498C TrxR1 and T-TrxR1 (0.6mg/mL) were reduced by 100μM TCEP for 10min, and then incubated with **12** and **13** (100 μM) for 30min at room temperature. After incubation, 200μL of cold acetone was added to the 100μL of protein solutions. Then the sample was cooled to -20º C for at least 1 hours, and centrifuged at 4℃ for 20 min at 14,000 rpm. After removing the acetonic supernatant and repeating the procedure by washing the pellet with 200μL acetone, the protein samples were prepared by adding 4*loading buffer excluding DTT to the pellet which was dissolved in 10% SDS with vortexing and subjected to 14% SDS-PAGE. Proteins labeled with **12** and **13** were visualized with ImageQuant™ LAS 4000 (GE Healthcare). The gel was further stained with Commassie bright blue G250 (C. B. staining) to confirm the same amount of proteins were loaded.

Seph-PAO-PDT and A-Seph pull-down assay

HL-60 lysate (200 μL, 5 mg total protein/mL) was incubated with 100 μL of Seph-PAO-PDT or A-Seph on a rotator mixer for 30min at room temperature. After centrifugation, the solution was removed and the sepharose was washed three times with TE buffer. The bound protein was eluted with 20 mM DMPS (100 μL), and the eluent was analyzed by Western blot using a specific antibody against TrxR1. The signal was detected using an enhanced chemiluminescence (ECL) kit (GE Healthcare Life Sciences).

Titration of Trx Free thiols with DTNB[16]

The remaining free thiols in the reduced Trx after treatment with **2** or **4** were determined by titration with DTNB assay. Briefly, 25 μM *E. coli* $Trx(SH)_2$ was incubated with 100 μM of **2** or **4** for 0.5 h at 37 °C. After incubation, 1 mM DTNB in 6 M guanidine hydrochloride, pH 8.0, was added, and the absorbance at 412 nm was read against the reference containing the same amount of DTNB, the remaining free thiols were expressed as the percentage of the

control.

HPLC analyses

HPLC analyses were performed on Waters 1525 2998 series HPLC system(C-18 column, Sun Fire, 5 μm, 4.6 mm × 150 mm) under the following conditions: mobile phase: MeOH/H_2O (see Table S1); flow rate: 1.0 mL/min; UV wavelength: maximal absorbance at 210-298 nm; temperature: ambient; injection volume: 10 μL. The purity of the compounds were summarized in Table S1.

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