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ORIGINAL ARTICLE

Cyclodextrin-derived chalcogenides as glutathione peroxidase mimics and their protection of mitochondria against oxidative damage

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Abstract A series of novel glutathione peroxidase (GPx) mimics based on organochalcogen cyclodextrin (CD) dimer were synthesized. Their GPx-like antioxidant activities were studied using hydrogen peroxide H_2O_2, tert-butylhydroperoxide (BHP), and cumene hydroperoxide (CHP) as substrates and glutathione as thiol co-substrate. The results showed that 6A,6B-ditelluronic acid-A,6B′-tellurium bridged γ-cyclodextrin (6-diTe-γ-CD) had the highest peroxidase activity, which was ~670-fold higher than ebselen, a well-known GPx mimic. Reduction of lipophilic CHP often proceeded much faster than reduction of the more hydrophilic H_2O_2 or BHP, which cannot bind into the hydrophobic interior of the CD. The biological activities were also evaluated for their capacity to protect mitochondria against ferrous sulfate/ascorbate-induced oxidative damage. 6-diTe-γ-CD was the best inhibitor which significantly suppressed ferrous sulfate/ascorbate-induced cytotoxicity as determined by swelling of mitochondria, lipid peroxidation and cytochrome *c* oxidase activity. Our data suggests that 6-diTe-γ-CD has potential pharmaceutical application in the treatment of ROS-mediated diseases.

Keywords Organochalcogen · Gamma-cyclodextrin · Glutathione peroxidase · Artificial enzyme · Mitochondria

Introduction

Glutathione peroxidase (GPx, EC 1.11.1.9) is an important mammalian selenoenzyme that function in cellular redox reactions and plays an essential role in the detoxification of ROOHs in vivo, thereby scavenging active oxygen and protecting biomembranes from oxidative stress [1–3]. It is related to many diseases and regarded as one of the most important antioxidant enzymes in living organisms. However, native GPx has some shortcomings, such as instability, antigenicity and poor availability, which have limited its therapeutic use [4, 5]. Additionally, it is extremely difficult to synthesize selenium-containing proteins by traditional recombinant DNA methods; therefore considerable effort has been spent to find other routes to compounds capable of imitating the properties of GPx [6, 7]. In designing GPx models, the synthetic mimics reported in the literature [8–13] can be divided into three major categories: (i) simple organoselenium compounds, such as ebselen, ebselen homologues, selenenamides, diselenides and α-phenylselenoketones; (ii) a number of organotellurium compounds, including diaryl ditellurides and diorganyl tellurides; (iii) semisynthetic selenoenzymes and antibodies.

CD dimers, forming inclusion complexes with binding constants comparable to that of very strong antibodies [14, 15], affect the rate of various chemical reactions with high substrate specificity, so that they have extensively been exploited as enzyme models [16–18]. Recently, a series of organoselenium- and organotellurium-bridged CD

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dimers [19–25] have been developed as GPx models with high GPx activity and substrate specificity. However, β-CD, rather than α-CD or γ-CD, was used because of general availability, but again it was not clear how important this choice was. Goodness shape/size-fitting in complexes between substrates and GPx mimics must be extremely important. The larger cavity of γ-CD—and the smaller cavity of α-CD—might change not only the catalytic rate constants within the complex but also the affinity of the catalyst to the substrate. In order to extend the model system, we thought it would be necessary to design and synthesize GPx mimics based on γ-CD or α-CD for investigating the relationships on substrate specificity and catalytic activity.

In present paper, we selected γ-CD as the scaffold of enzyme model and introduced catalytic sites Se or Te into the primary or secondary side of CD by using commonly known designs and chemical synthesis methods [19–22, 26–28]. Finally, a series of organoselenium- and organotellurium-bridged CD dimers were made and tested functionally. Based on their activities in reduction of hydroperoxide (ROOH), suppression of ferrous sulfate/ascorbate induced toxicity, the two major enzymatic activities known for glutathione peroxidase, a compound, 6-diTe-γ-CD, was chosen to be the best-mimic for GPx.

Experimental section

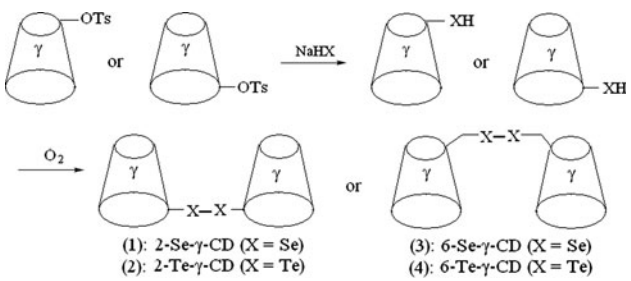
General

γ-CD (C.P.) was obtained from the Tokyo Chemical Industry Co. Ltd., *p*-toluene sulfonylchloride (p-TsCl), 1.3-benzenedisulfonyl chloride, GSH, CHP and BHP, Se-Cys were purchased from Merck. Thiobarbituric acid (TBA) and ferrous sulfate were obtained from Shanghai second reagent plant. Ascorbic acid was purchased from Fluka. β-Nicotinamide adenine dinucleotide phosphate (NADPH), ebselen, and glutathione reductase (type III) were purchased from Sigma. Sephadex G-25 was purchased from Amersham Pharmacia Biotech, Uppsala, Sweden. All other chemicals were from the highest purity available. The concentrations of the ROOH stock solutions were determined by titration with potassium permanganate. Phosphate buffer (PBS) was used in the all experiments.

Infrared spectra (IR) were obtained as neat films by using a Nicolet NEXUS infrared spectrometer in the 4,000–400 cm^{-1} regions. Elemental analyses were determined on a Perkin-Elmer 240 DS elemental analyzer. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectra were obtained by using a Bruker Avance III 400 Digital NMR spectrometer. Chemical shifts (δ) were reported in ppm and the coupling constants in Hertz (Hz) with respect to $SiMe_4$ as internal (^{1}H and ^{13}C) standard. A PERSEE TU-1900 UV–Vis-near-IR recording spectrophotometer was used to measure the electronic absorption spectra. Data were analyzed by using ultraviolet spectroscopy software. Mass spectral studies were carried out by using a LDI-1700 MALDI-TOF mass spectrometer (MS). The content and valence of selenium and tellurium were determined by means of an ESCALAB MKII X-ray photoelectron spectrometer.

Synthesis of 2,2′-diselenide(2-deoxy-γ-cyclodextrin) (2-Se-γ-CD) (**1**)

The synthesis route of 2-Se-γ-CD was shown in Scheme 1. The regiospecific monotosylation of 2-position hydroxyl of γ-CD was carried out according to the ref [29] to synthesize 2-OTs-2-deoxy-γ-CD (2-OTs-γ-CD). NaHSe was prepared according to the method described by Klayman et al. [30]. 100 mg of 2-OTs-γ-CD was dissolved in 10 mL of 50 mM PBS solution, pH 7.0, and then 100 μL of 1 M NaHSe solution was added under the protection of pure nitrogen. Following a reaction period of 48 h at 60 °C, the reaction mixture was oxidized in air, and then purified by centrifugation and column chromatography on Sephadex G-25 ($\lambda = 254$ nm) with deionized water as the eluent. The product solution was freeze-dried and the lyophilized power provided the product 2-Se-γ-CD with a yield of 73 %. Characterization of 2-Se-γ-CD: Anal. Calcd for $C_{96}H_{158}O_{78}Se_2 \cdot 6H_2O$: C, 40.80; H, 6.06. Found: C, 40.76; H, 6.05; ^{1}H NMR (400 MHz, D_2O): $\delta = 5.39$–4.85 (m, 16H, H-1), 3.67–3.44 (m, 64H, H-3, H-5, H-6), 3.44–3.27 (m, 32H, H-2, H-4); ^{13}C NMR (400 MHz, D_2O): $\delta = 100.5$–98.8(C1), 79.2–77.6 (C4), 71.8–68.1(C2, C3, C5), 60.9(C6), 51.6($C2^{A}$); IR (cm^{-1}, KBr): 3383, 1404 (–OH), 2930 (CH, CH_2), 1636, 1155, 1080, 1030 (–O–); MALDI-MS: calcd. 2718.2 found 2717.6. The content and valence of selenium in 2-Se-γ-CD were measured by X-ray photoelectron spectroscopy. The Se (3d, 5/2) electronic-binding energy of 2-Se-γ-CD was 54.8 eV, which approached the binding energy of SeCys (55.1 eV), indicating that the selenium in 2-Se-γ-CD was present in the form of the diselenium bridge (–Se–Se–). The experiment also gave the C/Se ratio, which was 48.2:1 (calculated 48:1), indicating 2 mol of selenium per mol of mimic.



Scheme 1 Synthetic route of GPx mimics (1–4)

Synthesis of 2,2′-ditellurobis(2-deoxy-γ-cyclodextrin) (2-Te-γ-CD) (**2**)

The synthesis route of 2-Te-γ-CD was shown in Scheme 1. Finely ground elemental tellurium (1.27 g) and sodium borohydride (0.9 g) were heated in ethanol (20 mL) at reflux under nitrogen for 1 h. After cooling to ambient temperature, acetic acid free of oxygen (1.2 mL) was added to the solution. 2-OTs-γ-CD (2 g) dissolved in 50 mM PBS, pH 7.0 (20 mL), was bubbled using pure nitrogen for 30 min and added to the above solution. Under the protection of nitrogen, the mixture was kept for 48 h at 60 °C, then was oxidized in air and finally purified by centrifugation and column chromatography on Sephadex G-25 ($\lambda = 254$ nm) with deionized water as the eluent. The product solution was freeze-dried and the lyophilized power provided the product 2-Te-γ-CD with a yield of 65 %. Characterization of 2-Te-γ-CD: Anal. Calcd for $C_{96}H_{158}O_{78}Te_2{\cdot}6H_2O$: C, 39.44; H, 5.86. Found: C, 39.45; H, 5.84; ^{1}H NMR (400 MHz, D_2O) δ (ppm): 5.44–4.90 (m, 16H, H-1), 3.77–3.42 (m, 64H, H-3, H-5, H-6), 3.39–3.07 (m, 32H, H-2, H-4); ^{13}C NMR (400 MHz, D_2O): δ = 99.8–98.1(C1), 79.5–77.8(C4), 70.7–67.2(C2, C3, C5), 59.5(C6), 50.5($C2^A$); IR (cm^{-1}, KBr): 3370, 1400 (–OH), 2928 (CH, CH_2), 1630, 1155, 1080, 1030 (–O–); MALDI-MS: calcd. 2815.4 found 2815.9. The content and valence of tellurium in 2-Te-γ-CD were measured by X-ray photoelectron spectroscopy. The Te (3d, 5/2) electronic-binding energy of 2-Te-γ-CD was 574.6 eV, which approached the binding energy of diphenyl ditelluride (573.9 eV), indicating that the tellurium in 2-Te-γ-CD was present in the form of the ditellurium bridge (–Te–Te–). The experiment also gave the C/Te ratio, which was 49.3:1 (calculated 48:1), indicating 2 mol of tellurium per mol of mimic.

Synthesis of 6,6′-diselenide(6-deoxy-γ-cyclodextrin) (6-Se-γ-CD) (**3**)

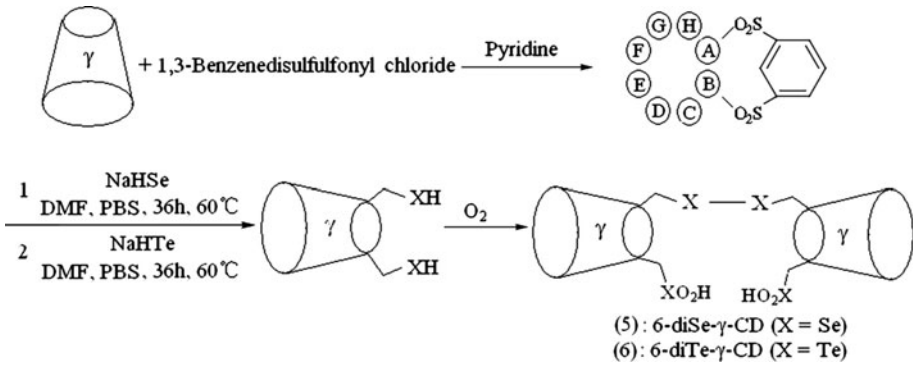
The synthesis route of 6-Se-γ-CD was shown in Scheme 1. The regiospecific monotosylation of 6-position hydroxyl of γ-CD was carried out according to the ref [31] to synthesize 6-OTs-6-deoxy-γ-CD (6-OTs-γ-CD). The selenolation of 6-OTs-γ-CD was carried out as follows. 100 mg of 6-OTs-γ-CD was dissolved in 100 mL of 50 mM, pH 7.0, PBS solution, then 100 mL of 1 M NaHSe was added under the protection of pure nitrogen and the mixture was kept for 48 h at 60 °C. The reaction mixture was oxidized in air, and then purified by centrifugation and column chromatography on Sephadex G-25 ($\lambda = 254$ nm) with deionized water as the eluent. The product solution was freeze-dried and the lyophilized power provided the product 6-Se-γ-CD with a yield of 82 %. Characterization of 6-Se-γ-CD: Anal. Calcd for $C_{96}H_{158}O_{78}Se_2{\cdot}6H_2O$: C, 40.80; H, 6.06; Found: C, 40.78, H, 6.04. ^{1}H NMR (400 MHz, D_2O): $\delta = 5.14$ (m, 16H, H-1), 3.75–3.53 (m, 64H, H-3, H-5, H-6), 3.50–3.07 (m, 32H, H-2, H-4); ^{13}C NMR (400 MHz, D_2O): δ101.1(C1), 81.7–78.4(C4), 73.5–69.7(C2,C3,C5), 61.1 ($C6^{B-H}$), 39.6($C6^{A-H}$); IR (cm^{-1}, KBr): 3394, 1404 (–OH), 2930 (CH, CH_2), 1634, 1155, 1080, 1030 (–O–); MALDI-MS: calcd. 2718.2 found 2717.7. The content and valence of selenium in 6-Se-γ-CD were measured by X-ray photoelectron spectroscopy. The Se (3d, 5/2) electronic-binding energy of 6-Se-γ-CD was 54.9 eV, which approaches the binding energy of SeCys (55.1 eV), indicating that the selenium in 6-Se-γ-CD was present in the form of the diselenium bridge (–Se–Se–). The experiment also gave the C/Se ratio, which was 48.3:1 (calculated 48:1), indicating 2 mol of selenium per mol of mimic.

Synthesis of 6,6′-ditellurobis(6-deoxy-γ-cyclodextrin) (6-Te-γ-CD) (**4**)

The synthesis route of 6-Te-γ-CD was shown in Scheme 1. 6-OTs-γ-CD was dissolved in PBS (50 mM, pH 7.0) and DMF (cosolvent) and then excess NaTeH was added to the above solution. The mixture was kept under nitrogen for 72 h at 60 °C, then oxidized in air and finally purified by centrifugation and Sephadex G-25 column chromatography with distilled water as the eluent. The resultant solution was freeze-dried and the lyophilized powder provided the yellow product in 57 % yield. Characterization of 6-Te-γ-CD: Anal. Calcd for $C_{96}H_{158}O_{78}Te_2{\cdot}6H_2O$: C, 39.44; H, 5.86. Found: C, 39.42; H, 5.85; ^{1}H NMR (400 MHz, D_2O): $\delta = 5.12$ (m, 16H, H-1), 3.95–3.43 (m, 64H, H-3, H-5, H-6), 3.38–3.05 (m, 32H, H-2, H-4); ^{13}C NMR (400 MHz, D_2O): δ102.2(C1), 80.2–77.6(C4), 73.5–69.7(C2,C3,C5), 60.5($C6^{B-H}$), 38.4($C6^{A-H}$); IR (cm^{-1}, KBr): 3344, 1400 (–OH), 2928 (CH, CH_2), 1627, 1150, 1080, 1030 (–O–); MALDI-MS: calcd. 2815.4 found 2815.7. The content and valence of tellurium in 6-Te-γ-CD were measured by X-ray photoelectron spectroscopy. The Te (3d, 5/2) electronic-binding energy of 6-Te-γ-CD was 574.9 eV, which approached the binding energy of diphenyl ditelluride (573.9 eV), indicating that the tellurium in 6-Te-γ-CD was present in the form of the ditellurium bridge (–Te–Te–). The experiment also gave the C/Te ratio, which was 48.6:1 (calculated 48:1), indicating 2 mol of tellurium per mol of mimic.

Synthesis of 6A,6B-diseleninic acid-6A′,6B′-selenium bridged γ-cyclodextrin (6-diSe-γ-CD) (**5**)

The synthesis route of 6-diSe-γ-CD was shown in Scheme 2. The regiospecific ditosylation of 6A, 6B-hydroxyl positions of γ-CD was carried out according to the method described



Scheme 2 Synthetic route of GPx mimics (5) and (6)

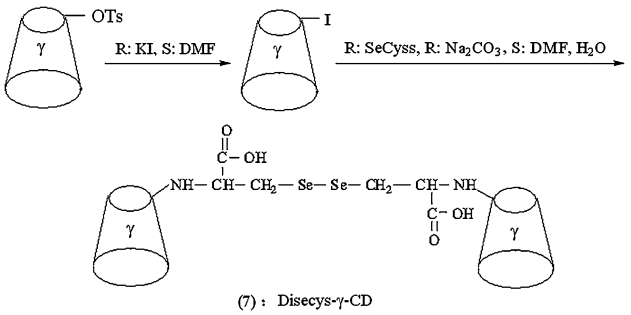
by Fujita et al. [32] to obtain 6A,6B-capped-γ-CD using 1, 3-benzenedisulfonyl chloride as a specific reagent. The selenolation of 6A,6B-capped-γ-CD was carried out as follows: 100 mg of 6A,6B-capped-γ-CD was dissolved in 2 mL of 50 mM PBS, pH 7.0. The reaction mixture was bubbled using pure nitrogen for 20 min and then 100 μL of 1 M NaHSe solution was added. Under the protection of pure nitrogen the mixture was kept for 36 h at 60 °C. The reaction mixture was oxidized in air and then purified by centrifugation and column chromatography on Sephadex G-25 ($\lambda = 254$ nm) with deionized water as the eluent. The product solution was freeze-dried and the lyophilized power provided the product 6-diSe-γ-CD with a yield of 13 %. Characterization of 6-diSe-γ-CD: Anal. Calcd for $C_{96}H_{158}O_{80}Se_4{\cdot}6H_2O$: C, 38.23; H, 5.68; Found: C, 38.36; H, 5.76. ^{1}H NMR (400 MHz, D_2O): $\delta = 4.96$–4.84 (1-H), 3.90–3.53 (3-, 5-, 6-H), 3.48-3.17 (2-, 4-, 6-H). ^{13}C NMR (400 MHz, D_2O): δ103.70–101.75 (C1); 80.56–79.2 (C4); 73.88–71.90 (C2, C3, C5); 60.33 (C6); 41.05 ($C6^A$). IR (cm^{-1}, KBr): 3385, 1393 (–OH), 2930 (CH, CH_2), 1634, 1155, 1080, 1025 (–O–); MALDI-MS: calcd. 2908.1 found 2907.0. The content and valence of selenium in 6-diSe-γ-CD were measured by X-ray photoelectron spectroscopy. The Se (3d, 5/2) electronic-binding energy of 6-diSe-γ-CD was 54.9 and 55.3 eV. The 54.9 eV approached the binding energy of SeCys (55.1 eV), indicating that the selenium in 6-diSe-γ-CD was partly presented in the form of the diselenium bridge (–Se–Se–). The 55.3 eV was assigned to be the binding energy of seleninic acid occurred in native GPx [33]. The experiment also gave the C/Se ratio, which was 20.8:1 (calculated 21:1), indicating 4 mol of selenium per mol of mimic.

Synthesis of 6A,6B-ditelluronic acid-6A′,6B′-tellurium bridged γ-cyclodextrin (6-diTe-γ-CD) (**6**)

The synthesis route of 6-diTe-γ-CD was shown in Scheme 2. 100 mg of 6A,6B-capped-γ-CD was dissolved in 2 mL of 50 mM PBS, pH 7.0. The reaction mixture was bubbled using pure nitrogen for 20 min and then 100 mL of 1 M NaHTe solution was added. Under the protection of pure nitrogen the mixture was kept for 72 h at 60 °C. Then oxidized in air and finally purified by centrifugation and Sephadex G-25 column chromatography with distilled water as the eluent. The resultant solution was freeze-dried and the lyophilized powder provided the yellow product in 9 % yield. Characterization of 6-diTe-γ-CD: Anal. Calcd for $C_{96}H_{158}O_{80}Te_4{\cdot}6H_2O$: C, 35.91; H, 5.34; Found: C, 36.02; H, 5.42. ^{1}H NMR (400 MHz, D_2O): $\delta = 5.04$–4.87 (1-H), 3.95–3.56 (3-, 5-, 6-H), 3.53–3.21 (2-, 4-, 6-H). ^{13}C NMR (400 MHz, D_2O): δ104.5 (C1), 85.5, 83.2 (C4), 75.7, 75.3, 74.3, 72.7(C2,3,5), 62.8 ($C6^{B-H}$), 42.9 ($C6^A$). IR (cm^{-1}, KBr): 3363, 1395 (–OH), 2930 (CH, CH_2), 1634, 1155, 1080, 1028 (–O–); MALDI-MS: calcd. 3102.6 found 3101.5. The content and valence of tellurium in 6-diTe-γ-CD were measured by X-ray photoelectron spec troscopy. The Te (3d, 5/2) electronic-binding energy of 6-diTe-γ-CD was 574.9 and 575.5 eV. The 574.9 eV approached the binding energy of diphenyl ditelluride (573.9 eV), indicating that the tellurium in 6-Te-γ-CD was present in the form of the ditellurium bridge (–Te–Te–). The 575.5 eV was assigned to be the binding energy of telluronic acid. The experiment also gave the C/Te ratio, which was 21.7:1 (calculated 21:1), indicating 4 mol of tellurium per mol of mimic.

Synthesis of Selenium-bridged-6,6′-amino-selenocystine-6,6′-deoxy-γ-CD (Disecys-γ-CD) (**7**)

The synthesis route of disecys-γ-CD was shown in Scheme 3. 6-Iodo-6-deoxy-γ-CD was synthesized by using the reaction of 6-OTs-6-deoxy-γ-CD and potassium iodide in dried DMF at 80 °C [34]. The synthesis of disecysCD was accomplished as follows. SeCyss (10 mg, 0.03 mmol) in water solution (4 mL) containing Na_2CO_3 (13 mg, 0.12 mmol) was added to 6-Iodo-6-deoxy-γ-CD (400 mg, 0.32 mmol) in DMF (4 mL). The mixture was bubbled using pure nitrogen for 30 min. Under the protection of



Scheme 3 Synthetic route of disecys-γ-CD

pure nitrogen the mixture was kept for 36 h at 60 °C. Then oxidized in air and finally purified by centrifugation and Sephadex G-25 column chromatography with distilled water as the eluent. The resultant solution was freeze-dried and the lyophilized powder provided the yellow product in 23 % yield. Characterization of disecys-γ-CD: Anal. Calcd for $C_{102}H_{168}O_{82}N_2Se_2{\cdot}6H_2O$: C, 41.83; H, 6.05; N, 0.93;Found: C, 41.91; H, 5.93; N, 0.94. IR (cm^{-1}, KBr): 3365, 1396 (–OH), 2928 (CH, CH_2), 1675(C=O), 1618, 1155, 1082, 1028 (–O–). ^{1}H NMR (400 MHz, D_2O) d: 5.12–4.90 (m, 16H, 1-H), 3.92–3.63 (m, 60H, 3-, 5-, 6-H, and CH of Se-Cys), 3.57–3.32 (m, 32H, 2-, 4-H), 3.28–2.59 (m, 10H, 6-H and CH_2 of Se-Cys); ^{13}C NMR (400 MHz, D_2O): δ164.1 (amide C=O), 104.56(C1), 85.4–83.1 (C4), 76.3–73.0 (C2, C3, C5), 63.1 ($C6^{B-H}$), 43.3 ($C6^{A}$). MALDI-MS: calcd. 2892.3 found 2891.2. The content and valence of selenium in disecys-γ-CD were measured by X-ray photoelectron spectroscopy. The Se (3d, 5/2) electronic-binding energy of disecys-γ-CD was 54.8 eV, which approached the binding energy of SeCys (55.1 eV), indicating that the selenium in disecys-γ-CD was present in the form of the diselenium bridge (–Se–Se–). The experiment also gave the ratio of C/Se and C/N: C/Se, 45.5:1 (calculated 45:1), and C/N, 45.8:1 (calculated 45:1), indicating 2 mol of selenium and nitrogen per mol of mimic.

Procedures of estimation of GPx-like activity

The GPx-like activity of the mimics was assessed by using coupled reductase assay with minor modification [35]. The sample and control cuvettes both contained PBS (50 mM, pH 7.0), sodium azide (1 mM), EDTA (1 mM), GSH (0.1 mM), ROOH (0.25 mM), NADPH (0.25 mM), GSH reductase (1 U), and a moderate amount of test compound at 37 °C. This reaction was started by the subsequent addition of ROOH and the absorbance at 340 nm was recorded for a few minutes to determine the rate of NADPH consumption. One unit of enzyme activity was defined as the amount of enzyme mimic that used 1 μmol NADPH per min.

Procedures of evaluation of antioxidative properties and biological effects

Preparation of mitochondria

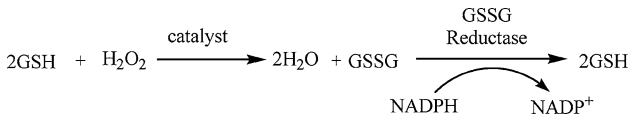
Mitochondria were isolated from fresh bovine heart according to ref [36], suspended in sucrose (0.25 M), EDTA (10 mM), and HEPES/NaOH buffer (25 mM, pH 7.4), and maintained at 0 °C. The concentration of mitochondrial protein was determined by Coomassie brilliant blue, using bovine serum albumin as the standard [37].

Ferrous sulfate/ascorbate-induced mitochondria damage

Mitochondria (0.5 mg protein mL^{-1}) were suspended in PBS (10 mM, pH 7.4) containing $MgCl_2$ (1 mM), KCl (0.125 M), GSH (1 μM), glutamate (5 mM), and the appropriate amount of enzyme mimic at 25 °C. Swelling of the mitochondria and malondialdehyde (MDA) content were determined at intervals after the addition of ascorbate (0.5 mM) plus ferrous sulfate (12.5 μM). Damage experiments were performed without the enzyme mimic, while control experiments were carried out in the absence of enzyme mimic, ferrous sulfate, and ascorbate.

Biological analysis of GPx mimics against mitochondria damage

Swelling of mitochondria was assessed as described by Hunter et al. [38]. Changes in light scattering were correlated with mitochondrial swelling. The increase in the mitochondria swelling induced the decrease in turbidity of the reaction mixture at 520 nm, indicating a decrease in the mitochondria integrity. Lipid peroxidation was analyzed by TBA assay [39]. The level of lipid peroxidation was determined as the formation of MDA, the final product of lipid peroxidation. In this assay, TBA reacts with MDA to give a 2:1 molar ratio of colored conjugates. The reaction was terminated by addition of trichloroacetic acid (0.1 mL, 0.5 % (w/w)). In plots of lipid peroxide formation, the absorbance reading was plotted directly rather than by means of a fixed conversion into MDA and/or other carbonyl byproduct equivalents. Assay of cytochrome *c* oxidase (CCO) activity was determined according to ref [40]. An aliquot of incubation mixture was taken at different time intervals and centrifuged (10,000g, 4 °C, 2 min). The pellet was washed with PBS (10 mM, pH 7.4) containing KCl (125 mM), MgCl (1 mM), and glutamate (5 mM), then suspended in a small amount of PBS (100 mM, pH 7.0) and an aliquot taken to assay the cytochrome *c* oxidase



Scheme 4 The presence of GSSG reductase and NADPH allows the progress of the catalyzed reduction reaction to be spectrophotometrically followed by observation of NADPH absorption at 340 nm

Table 1 The activity for the ROOH by GSH in the presence of various catalysts

Compound	Activity (U/μmol)		
	Hydroperoxide		
	H_2O_2	BHP	CHP
Ebselen	0.99 ± 0.01	0.34 ± 0.01	1.24 ± 0.01
2-Se-γ-CD	12.21 ± 0.11	14.97 ± 0.17	18.32 ± 0.51
2-Te-γ-CD	76.71 ± 0.36	104.5 ± 0.73	143.4 ± 0.82
6-Se-γ-CD	6.08 ± 0.16	7.39 ± 0.08	8.63 ± 0.09
6-Te-γ-CD	7.83 ± 0.25	9.82 ± 0.26	12.33 ± 0.33
6-diSe-γ-CD	22.57 ± 0.40	12.88 ± 0.21	38.51 ± 0.35
6-diTe-γ-CD	145.8 ± 0.71	645.3 ± 1.15	832.2 ± 2.56
Disecys-γ-CD	7.23 ± 0.16	3.76 ± 0.08	10.28 ± 0.10

One unit of enzyme activity is defined as the amount of mimic that utilizes 1 μmol of NADPH per minute. All values are means of at least five times with standard deviation

activity [40]. The CCO activity was measured in 2 mL of reaction system, in which the cytochrome *c* concentration was 15 μM. The absorbance decreased with oxidation of cytochrome *c* in the sample cell. $K_3Fe(CN)_6$ (5 μL of a 10 mM solution) was added to oxidize the cytochrome *c* thoroughly when the reaction was complete. The absorbance intensity at this time was recorded as A_8. A plot of $\ln(A_t–A_8)$ versus time was made (A_t = absorbance at time t). The absolute value of the line slope, K_{app}, was the apparent rate constant of cytochrome *c* oxidation and was used to express the COO activity.

Results and discussion

Glutathione peroxidase-like properties

The GPx-like activities of different mimics were determined according to the Wilson's procedure with minor modification, in which the oxidation of NADPH was spectrophotometrically monitored at 340 nm by coupling to the reduction of the oxidized glutathione (GSSG) catalyzed by glutathione reductase (Scheme 4).

The GPx activities measured in this method were shown in Table 1. For the peroxidase activity, the enzymatic catalytic activities were corrected for the background (nonenzymic) reaction between ROOH and GSH. Analyzing the data from Table 1, we could conclude four important findings. The first one is that CD-derived GPx mimics with hydrophobic cavity were much better catalysts than ebselen carrying no binding group for substrates. 6-diTe-γ-CD showed the most efficient peroxidase activity, which reduction of CHP that was almost 670-fold than ebselen. Obviously, substrate binding played a vital role in GPx catalysis. Secondly, CD-derived tellurium model compounds showed much higher catalytic efficiency than their respective selenium analogues. For example, BHP decomposing capacity of 6-diTe-γ-CD was determined to be 645.3 U/μmol, and the catalytic efficiency was remarkably higher than 6-diSe-γ-CD (12.88 U/μmol). Thirdly, it was significant to report that 2-position of CD-derived GPx models displayed much more efficiently than the 6-position analogues. For example, H_2O_2 decomposing capacity of 2-Te-γ-CD was nearly 10-fold than that of 6-Te-γ-CD, meanwhile, 2-Se-γ-CD also showed much higher catalytic efficiency than that 6-Se-γ-CD. The difference of catalytic capacity may be made by geometric preference. The last and the most notable result from the coupled reductase assay was the specificity for the reduction of ROOH substrates. With all of the mimics based on CDs, CHP was reduced much faster than H_2O_2 and BHP. A similarly modest selectivity for CHP was also observed for the CD-derived diselenide and ditelluride GPx mimics [24, 27, 41, 42]. The relatively high GPx activity in organic peroxide (CHP) assays as compared to the H_2O_2 assay could be ascribed to the CD part of the enzyme mimics acting as a binding site for the lipophilic ROOH. It is expected that the bulky aromatic group of CHP binded fairly strongly into 6-diTe-γ-CD, so as to bring the HOO group into close proximity to tellurium at the secondary face of the CD.

Protection of mitochondria against oxidative damage

Mitochondria are central to the formation of endogenous reactive oxygen species (ROS), including superoxide anions, hydrogenperoxides, and hydroxyl radicals [43]. Therefore, mitochondria are particularly susceptible to oxidative stress [44, 45]. Oxidative stress decreases the activity of the components of oxidative phosphorylation and promotes the permeability transition of mitochondria [46], a process resulting in the loss of functional integrity. Protection of mitochondria from oxidative damage might be of great importance in the prevention of ROS-related diseases, or therapy for them. In this part, we chose mitochondria as the model for oxidative damage experiments to evaluate the capacity of GPx mimics (1–7) to protect mitochondria against ferrous sulfate/ascorbate-induced oxidative damage. The reactions for ferrous sulfate/ascorbate inducing mitochondrial damage can be proposed as follows:

$$\text{Ascorbic acid} + O_2 \rightarrow \text{dehydroascorbic} + H_2O_2 \quad (1)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\bullet}OH \quad (2)$$

$$\text{Ascorbic acid} + 2Fe^{3+} \rightarrow \text{dehydroascorbic acid} + 2Fe^{2+} + 2H^+ \quad (3)$$

where H_2O_2 was produced by oxidation of ascorbic acid to dehydroascorbic acid (Eq. 1), in addition, a hydroxyl radical was produced via the Fenton reaction (Eqs. 2, 3). The biological molecules in mitochondria are easily attacked by hydroxyl radicals, when changes in morphology, composition, integrity, structure, and function of the mitochondria take place. GPx mimics can scavenge hydroperoxides and block hydroxyl radical production, therefore protect mitochondria against oxidative damage. In the ferrous sulfate/ascorbate-induced mitochondrial damage model system, swelling, MDA content and the activity of cytochrome *c* oxidase were used to determine the injury and extent of protection in mitochondria.

Mitochondria swelling and shrinking is a common and normal physiological phenomena during respiration. However, abnormal swelling may rupture the mitochondrial outer membrane resulting in cell death. Therefore mitochondrial swelling is used to characterize its integrity. Mitochondrial swelling was measured by the decrease in the turbidity of the reaction mixture at 520 nm. The decrease in absorbance indicated an increase in mitochondrial swelling and a decrease in mitochondria integrity (see "Experimental section"). The results in Fig. 1 showed the effect of different mimics on the swelling of mitochondria in the ferrous sulfate/ascorbate-induced mitochondrion damage model system. The absorbance at 520 nm for the damage group decreased considerably over time, indicating that mitochondrial swelling was increased, whereas that for the control group was basically constant. In the protection group, which contained 10 μM different GPx mimics, the swelling was inhibited in a different degree. Under the identical conditions, 6-diTe-γ-CD displayed the highest levels of ability to inhibit the swelling of mitochondria, in agreement with ability to remove ROOH (Table 1).

Lipid peroxidation byproducts are one of the major sources of reactive species (RS), which constantly threaten mitochondrial integrity [47, 48]. Here, we tested the inhibition of lipid peroxidation of mitochondria by our new GPx mimics. The level of MDA, the final product of lipid peroxidation, was measured to show the extent of protection afforded by GPx mimics. In this assay, TBA reacts with malonaldehyde to give 2:1 (mol/mol) colored conjugates, which have an A_{532} value (see "Experimental section"). The results in Fig. 2 showed that our mimics did block the increase in MDA by ferrous sulfate/ascorbate-induced

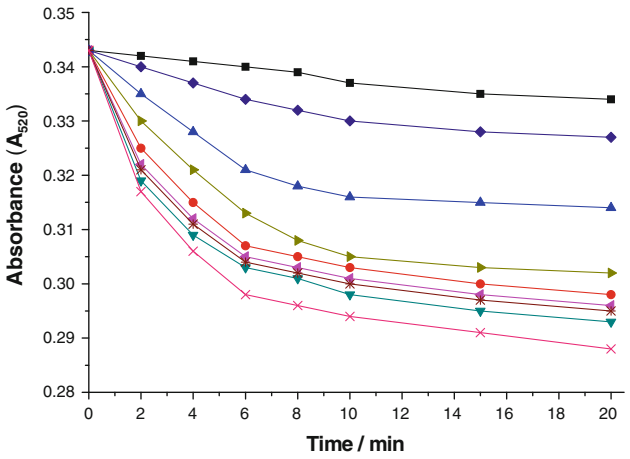


Fig. 1 Effect of different mimics on the swelling of mitochondria. (*filled square*) Control; (*filled diamond*) damage + 10 μM 6-diTe-γ-CD; (*filled triangle*) damage + 10 μM 2-Te-γ-CD; (*black right-pointing triangle*) damage + 10 μM 6-diSe-γ-CD; (*filled circle*) damage + 10 μM 2-Se-γ-CD; (*black left-pointing triangle*) damage + 10 μM 6-Te-γ-CD; (*asterisk*) damage + 10 μM disecys-γ-CD; (*black down-pointing triangle*) damage + 10 μM 6-Se-γ-CD; (×) damage

damage. The amount of MDA in the damage group accumulated considerably over time, whereas that for the protection groups by organochalcogen-bridged CD dimers decreased in different degree with the following order: 6-diTe-γ-CD ⟩2-Te-γ-CD ⟩6-diSe-γ-CD ⟩2-Se-γ-CD⟩6-Te-γ-CD ≈ disecys-γ-CD ⟩6-Se-γ-CD. It was worth noting that 6-diTe-γ-CD decreased the maximal level of MDA accumulated after 50 min to about 30 % of the damage group, indicating that 70 % of MDA production was inhibited.

With all of these individual events resulting biochemical impairment to mitochondrial, cytochrome *c* oxidase (COX) is the only one component of the electron transport chain, which has been consistently found to be defective in the affected tissues [49, 50]. Therefore, COX becomes the marker enzyme of mitochondria. The integrity of the mitochondrion lipid membrane is important for the activity of this enzyme. As shown in Fig. 3, the COX activity decreased drastically in the ferrous sulfate/ascorbate-induced mitochondrion damage model system, about 60 % enzyme activity remained after 60 min. Under identical conditions, however, the COX activity retained in the presence of 10 μM of 6-diTe-γ-CD or 2-Te-γ-CD were 96 and 92 % of the control group activity, respectively.

Conclusions

New organochalcogen-bridged CD dimers were found to catalyze the reduction of hydrogen peroxide, BHP, and CHP in the presence of GSH. Thus, these compounds act as

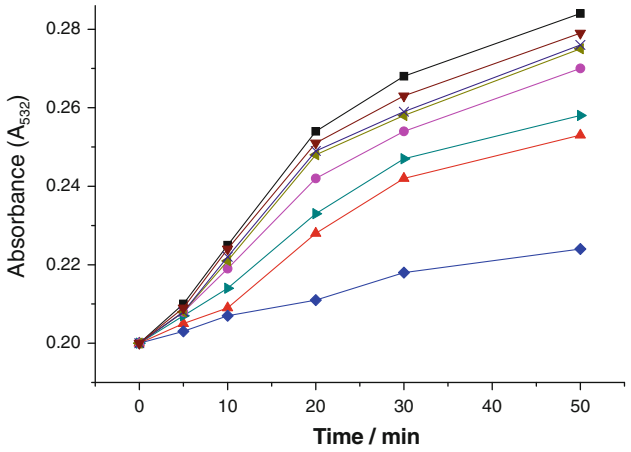


Fig. 2 Dependence of extent of MDA accumulation on different mimics. (*filled square*) damage; (*filled diamond*) damage + 10 μM 6-diTe-γ-CD; (*filled triangle*) damage + 10 μM 2-Te-γ-CD; (*black right-pointing triangle*) damage + 10 μM 6-diSe-γ-CD; (*filled circle*) damage + 10 μM 2-Se-γ-CD; (*black left-pointing triangle*) damage + 10 μM 6-Te-γ-CD; (×) damage + 10 μM disecys-γ-CD; (*black down-pointing triangle*) damage + 10 μM 6-Se-γ-CD

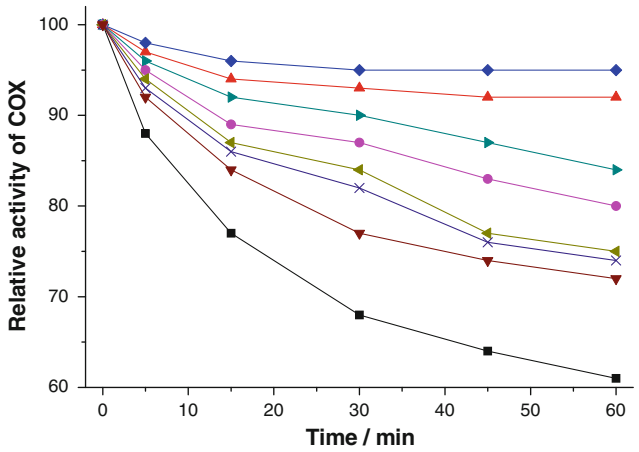


Fig. 3 Effect of different GPx mimics on COX activity in damaged mitochondria. Activity of COX in the control group is defined as 100 %. (*filled diamond*) damage + 10 μM 6-diTe-γ-CD; (*filled triangle*) damage + 10 μM 2-Te-γ-CD; (*black right-pointing triangle*) damage + 10 μM 6-diSe-γ-CD; (*filled circle*) damage + 10 μM 2-Se-γ-CD; (*black left-pointing triangle*) damage + 10 μM 6-Te-γ-CD; (×) damage + 10 μM disecys-γ-CD; (*black down-pointing triangle*) damage + 10 μM 6-Se-γ-CD; (*filled square*) damage

mimics of the glutathione peroxidase antioxidant enzymes. Reduction of the most lipophilic hydroperoxide (CHP) proceeded much faster than reduction of the two more hydrophilic ones which cannot bind into the hydrophobic interior of the CD. It therefore seems that the effect of the substrate binding on catalytic efficiency plays a vital role in enhancing catalytic activity.

We feel that compounds of this type could find use as various types of "antioxidant pharmacotherapy", so we evaluated their biological activities by investigating their capacity to protect mitochondria against ferrous sulfate/ascorbate-induced oxidative damage. And we found that the novel CD compounds, especial for 6-diTe-γ-CD and 2-Te-γ-CD, did interfere with the mitochondrial system and attenuate oxidative stress, consequently protecting against mitochondrial dysfunction. Our data suggests that 6-diTe-γ-CD and 2-Te-γ-CD may have potential pharmaceutical application in the treatment of ROS-mediated diseases.

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