Article

C$_{60}$-based Ebselen Derivative: Synthesis by Bingel Cyclopropanation and Enhanced Antioxidative and Neuroprotective Activity

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Um derivado de ebselen baseado em C$_{60}$, **3**, foi sintetizado pela reação de ciclopropanação de Bingel envolvendo C$_{60}$ e manolato de ebselen, **2**. O produto foi sintetizado em três etapas, partindo de cloreto de 2-cloro-selenobenzoila e 2-(2-aminoetoxietanol), com rendimento de 42% (baseado no consumo de C$_{60}$). O composto **3** foi caracterizado estruturalmente por análises espectroscópicas de RMN de ^{1}H e de ^{13}C, IV, UV e FAB-MS. Com o objetivo de verificar um possível aumento na atividade antioxidante e neuroprotetora do derivado **3**, um derivado de C$_{60}$ (**4**), um derivado de ebselen (**2**) e sua mistura (**4** + **2** em razão equimolar) foram selecionados para o tratamento de células neurais pelo mesmo procedimento adotado para **3** e na mesma concentração final (30 µmol L^{-1}). As viabilidades celulares dos quatro grupos de tratamento foram estimadas por ensaios de LDH (desidrogenase láctica) e MTT (brometo de 3-[4,5-dimetil-tiazol-2-il]-2,5-difenil-tetrazólio). Os resultados mostraram que a atividade antioxidante e neuroprotetora de **3** frente à lesão neural mediada por H$_2$O$_2$ (MTT(OD) 0,364 ± 0,028; LDH liberado (UL^{-1}) 4,66 ± 0,28) foi significativamente mais alta que a do derivado de C$_{60}$ **4** (MTT(OD) 0,324 ± 0,025; LDH liberado (UL^{-1}) 5,39 ± 0,17), do derivado de ebselen **2** (MTT(OD) 0,294 ± 0,021; LDH liberado (UL^{-1}) 5,71 ± 0,27), e da sua mistura (**4** + **2**) (MTT(OD) 0,310 ± 0,018; LDH liberado (UL^{-1}) 5,54 ± 0,39). Esses resultados mostram que a combinação de dois componentes moleculares com atividades biológicas similares (C$_{60}$ e ebselen) pode ser uma forma desejável de preparação de novos compostos biologicamente efetivos derivados de C$_{60}$.

C$_{60}$-based ebselen derivative **3** was synthesized through Bingel cyclopropanation of C$_{60}$ with the ebselen malonate **2**. Compound **3** was obtained in 42% yield (based on consumed C$_{60}$) in a three-step synthesis starting from 2-(chloroseleno)benzoyl chloride and 2-(2-aminoethoxy)ethanol. Its structure was confirmed by ^{1}H NMR, ^{13}C NMR, IR, UV and FAB-MS spectroscopy analyses. In order to verify the enhanced antioxidative and neuroprotective activity of **3**, a C$_{60}$ derivative (**4**), an ebselen derivative (**2**), and their mixture (**4** plus **2** in equimolar ratio) were employed to treat cortical neuronal cells, following the same procedure used with **3** and at the same final concentration (30 µmol L^{-1}). Cell viabilities of the four treated groups were estimated by LDH (lactic dehydrogenase) leakage and MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assays. Results showed that the antioxidative and protective activities of C$_{60}$-based ebselen derivative **3** against H$_2$O$_2$-mediated neuronal injury (MTT(OD) 0.364 ± 0.028; LDH release (UL^{-1}) 4.66 ± 0.28) were significantly higher than those of C$_{60}$ derivative **4** (MTT(OD) 0.324 ± 0.025; LDH release (UL^{-1}) 5.39 ± 0.17), ebselen derivative **2** (MTT(OD) 0.294 ± 0.021; LDH release (UL^{-1}) 5.71 ± 0.27), and the mixture of **4** and **2** (MTT(OD) 0.310 ± 0.018; LDH release (UL^{-1}) 5.54 ± 0.39). These findings demonstrated that the combination of two molecular units with similar biological activities (C$_{60}$ and ebselen) may be a desirable way of obtaining new and more biologically effective C$_{60}$-based compounds.

Keywords: fullerene, ebselen derivative, Bingel cyclopropanation, neuroprotective activity, cell viability

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Introduction

The investigation of modern medical sciences has suggested that free radicals are associated with a number of neurodegenerative diseases,[1] such as Parkinson and Huntington's diseases and Alzheimer's dementia. Some reactive oxygen species (ROS), including superoxide ($O_2^{-\cdot}$) and hydroxyl ($\cdot OH$) radicals, and the H_2O_2 molecule, are believed to be major contributors to oxidative damage in neurodegenerative diseases. Reactive oxygen species, produced during oxidative stress, may cause different kinds of biological damage, such as lipid peroxidation, protein oxidation and protein cross-linking, and lead to neuronal cell death.[2] In the search for a link between active oxygen species and neurodegenerative conditions, the development of chemopreventive agents with antioxidative activities could help preventing neurological injury caused by the ROS.

C_{60}-fullerenes, owing to their electronic structure as electron-poor polyolefines, are highly reactive towards various organic radicals[3] and have drawn considerable attention in the area of antioxidant research. An electron paramagnetic resonance analysis has shown that C_{60} derivatives, such as carboxyfullerene, fullerenol and hexa(sulfobutyl)fullerene, have excellent capabilities to scavenge deleterious reactive oxygen species.[4] In addition, neuroprotective effects of some water-soluble C_{60} derivatives on oxidative injuries have also been extensively studied in several cell cultures and animal models of CNS (central nervous system) damage. For example, carboxyfullerenes prevent apoptotic injury of cultured cortical neurons evoked by *N*-methyl-*D*-aspartate and β-amyloid peptide,[5] and inhibit iron-induced oxidative stress in rat brain.[6] The same carboxyfullerenes were also reportedly able to inhibit cerebellar granule cell apoptosis, possibly by reducing the generation of ROS.[7] Other fullerene derivatives, such as fullerenol, hexasulfobutylated C_{60} (FC4S) and C_{60}(glucosamine)$_6$, also showed neuroprotective activity related to their antioxidative property. Fullerenol demonstrated to be a powerful scavenger for the free radicals induced by I/R (ischemia/reperfusion) injury of the small intestine.[8] Hexasulfobutylated C_{60} (FC4S) can be employed as a free radical remover in the biological system.[9] Recently, C_{60}(glucosamine)$_6$ has been found to prevent renal I/R-induced apoptosis formation and superoxide generation.[10] All these results reveal that fullerene and its derivatives possess antioxidative activity and may be useful as neuroprotective agents in several acute or chronic neurodegenerative diseases.

On the other hand, ebselen and its analogues seem to possess glutathione (GSH) peroxidase-like activity, and may act as antioxidants and free radical scavengers.[11] Recently, ebselen and its analogues were reported to have neuroprotective effect against brain ischemic insults and the glutamate-induced neurotoxicity.[12] More recently, ebselen and its derivatives also confirmed to have neuroprotective effects on experimental rat spinal cord injury.[13] All these biological effects shown by ebselen and its analogues are obviously related to their antioxidative properties and free radical scavenge activities.

In view of the above mentioned antioxidative and neuroprotective properties of both C_{60} and ebselen derivatives, it is expected that the covalent attachment of the biologically active ebselen moiety to C_{60}-fullerene may lead to the formation of a new C_{60}-based ebselen derivative, which could present better antioxidative and neuroprotective properties than the parent C_{60} or ebselen molecules. In this article we report the synthesis of the new C_{60}-based ebselen derivative **3**, which contains a solubilizing ethyleneglycol chain, by Bingel cyclopropanation involving C_{60} and ebselen malonate. We also report the neuroprotective activity of **3** against hydrogen peroxide-induced neuronal insults in cortical cell cultures.

Experimental

Reagents and instruments

C_{60} (99% purity) was prepared by the Institute of Fullerenes of Wuhan University. 2-(Chloroseleno)benzoyl chloride was prepared according to the literature.[14] All solvents and reagents were dried prior to use by standard procedures and other commercially available compounds were used as received. Melting points were determined with a Digital Melting Point Apparatus (WRS-IB). Column chromatography was performed on silica Gel (200-300 mesh). IR spectra (KBr pellets) were obtained on a Spectrum One Perkin Elmer spectrometer. UV spectra were recorded on a Perkin-Elmer λ-17 instrument. NMR data were recorded in $CDCl_3$ at room temperature on a Varian INOVA 600 NMR instrument operating at 14.09 T, observing ^1H and ^{13}C at 599.94 and 150.86 MHz, respectively. ^1H and ^{13}C NMR chemical shifts are given in ppm relative to TMS as internal standard at 0.00 ppm. Mass spectra were recorded on a JMS AXS05H instrument using 3-nitrobenzyl alcohol (m-NBA) as a matrix.

Wister rats of 18-day pregnancy were provided by the Experimental Animal Center of Tongji Medical College, Huazhong University of Science & Technology (China). D-Hanks medium, trypsinase and DMEM (Dulbecco's Modification of Eagle's Medium) were purchased from Gibco Co. (USA). Fetal calf serum (FBS) and cytarabine

hydrochloride were from Tianjin Biochemical Factory (China). Poly-*L*-lysine, DMSO, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were obtained from Sigma Chemical Co. (USA). C$_{60}$ derivative **4** (Figure 1) was prepared according to the literature.[15] LDH (lactic dehydrogenase) assay kit was obtained from Beijing Chemical Kit Company (China). The optical density of each well was measured at 550 nm on a BIO-RAD550 (USA) enzyme-linked analyzer.

Synthesis of 2-(2-(2-hydroxyethoxy)ethyl)benzo[d][1,2] selenazol-3(2H)-one (1)

Details of the procedure are described in reference 16. Reaction of diethylene glycolamine (1.456 g, 13.86 mmol) with 2-(chloroseleno)benzoyl chloride gave ebselen derivative **1** (2.5 g, 63%). White needles, mp 116.7~117.5 °C; IR ν$_{max}$/cm^{-1}: 3630 (O-H), 3048 (C-H$_{Ar}$), 1610, 1573 (amide C=O), 1283 (amide C-N), 1170 (C-O-C); ^{1}H NMR (CDCl$_3$, 600 MHz) δ 8.00 (dd, *J* 7.6, 1.5 Hz, 1 H, ArH), 7.86 (dd, *J* 7.1, 1.4 Hz, 1 H, ArH), 7.63 (ddd, *J* 7.3, 7.3, 1.7 Hz, 1 H, ArH), 7.43 (ddd, *J* 7.3, 7.3, 1.7 Hz, 1 H, ArH), 4.92 (br, s, 1 H, OH), 4.10 (t, *J* 6.2 Hz, 2 H, NCH$_2$), 3.76 (t, *J* 6.0 Hz, 2 H, ethylene glycol aliphatic hydrogens), 3.65 (t, *J* 6.0 Hz, 2 H, ethylene glycol aliphatic hydrogens), 3.54 (t, *J* 6.2 Hz, 2 H, ethylene glycol aliphatic hydrogens); ^{13}C NMR (CDCl$_3$, 150 MHz) δ 166.55 (amide C=O), 134.33, 132.54, 132.08, 129.20, 127.85, 127.00 (aromatic C), 72.26 (OCH$_2$CH$_2$OH), 69.23 (NCH$_2$CH$_2$O), 61.52 (OCH$_2$CH$_2$OH), 39.20 (NCH$_2$CH$_2$O).

Preparation of ebselen malonate 2

A solution of malonyl dichloride (0.37 mL, 3.87 mmol) in dry CHCl$_3$ (50 mL) was added dropwise to a stirred solution of ebselen derivative **1** (2.44 g, 8.51 mmol) and pyridine (0.84 mL, 10.2 mmol) at 0 °C in dry CHCl$_3$ (150 mL) and the resulting mixture was allowed to warm slowly to room temperature. After stirring overnight, the mixture was washed with aqueous NaHCO$_3$ (5%), dried over MgSO$_4$ and evaporated in *vacuum*. The residue was chromatographed on silica gel, and elution with ethyl acetate/petroleum ether (3:1) gave ebselen malonate **2** (183.5 mg, 74%) as a pale yellow oil. TLC R$_f$ = 0.48 (ethyl acetate/petroleum ether 4:1); IR ν$_{max}$/cm^{-1}: 3045 (C-H$_{Ar}$), 1750, 1735 (ester C=O), 1613, 1568 (amide C=O), 1330, 1276 (amide C-N), 1160, 1170 (C-O-C); ^{1}H NMR (CDCl$_3$, 600 MHz) δ 8.04 (dd, *J* 7.8, 1.5 Hz, 2 H, ArH), 7.86 (dd, *J* 7.2, 1.5 Hz, 2 H, ArH), 7.66 (ddd, *J* 7.3, 7.3, 1.8 Hz, 2 H, ArH), 7.45 (ddd, *J* 7.5, 7.5, 1.8 Hz, 2 H, ArH), 4.22 (t, *J* 7.0 Hz, 4 H, COOCH$_2$), 4.08 (t, *J* 6.0 Hz, 4 H, NCH$_2$),

3.74 (t, *J* 6.0 Hz, 4 H, ethylene glycol aliphatic hydrogens), 3.70 (t, *J* 7.2 Hz, 4 H, ethylene glycol aliphatic hydrogens), 3.30 (s, malonic CH$_2$); ^{13}C NMR (CDCl$_3$, 150 MHz) δ 168.32 (ester C=O), 167.85 (amide C=O), 134.85, 132.43, 132.12, 129.16, 127.70, 127.02 (aromatic C), 70.73 (OCH$_2$CH$_2$O), 69.46 (NCH$_2$CH$_2$O), 65.32 (OCH$_2$CH$_2$O), 41.27 (malonic CH$_2$), 39.53 (NCH$_2$CH$_2$O).

Bingel cyclopropanation of C$_{60}$ with ebselen malonate 2 to give 3

To a solution of C$_{60}$ (150 mg, 0.208 mmol), CBr$_4$ (103.5 mg, 0.312 mmol), and ebselen malonate **2** (159.5 mg, 0.249 mmol) in 200 mL of toluene, 63.33 mg of DBU (0.416 mmol) was added. The reaction mixture was stirred under N$_2$ at room temperature for 10 h. The solvent was distilled, and the residue was chromatographed on silica gel, and eluting first with toluene (to remove unreacted C$_{60}$), then with toluene/CHCl$_3$ (8:1) provided the fullerene-ebselen mono-adduct **3** (95.23 mg, 42%, based on consumed C$_{60}$) as a dark brown solid. TLC R$_f$ = 0.51 toluene/chloroform 8:1); UV-Vis λ$_{max}$/nm (CHCl$_3$) 433, 332, 228.5; IR ν$_{max}$/cm^{-1}: 3052 (C-H$_{Ar}$), 1756, 1740 (ester C=O), 1610, 1570 (amide C=O), 1433 (C$_{60}$), 1334, 1272 (amide C-N), 1185 (C$_{60}$), 1158, 1174 (C-O-C), 528 (C$_{60}$); ^{1}H NMR (CDCl$_3$, 600 MHz) δ 8.06 (dd, *J* 7.8, 1.5 Hz, 2 H, ArH), 7.88 (dd, *J* 7.1, 1.5 Hz, 2 H, ArH), 7.65 (ddd, *J* 7.3, 7.3, 1.8 Hz, 2 H, ArH), 7.46 (ddd, *J* 7.5, 7.5, 1.8 Hz, 2 H, ArH), 4.35 (t, *J* 6.6 Hz, 4 H, COOCH$_2$), 4.17 (t, *J* 6.3 Hz, 4 H, NCH$_2$), 3.80 (t, *J* 7.5 Hz, 4 H, ethylene glycol aliphatic hydrogens), 3.76 (t, *J* 7.8 Hz, 4 H, ethylene glycol aliphatic hydrogens); ^{13}C NMR (CDCl$_3$, 150 MHz) δ 168.80 (ester C=O), 167.88 (amide C=O), 145.70, 145.66, 145.53, 145.46, 145.16, 144.92, 144.86, 144.14, 143.34, 143.26, 143.24, 142.46, 142.17, 141.20, 139.24 (15 C$_{60}$-sp^2-carbons), 134.87 (aromatic C), 132.44, 132.14, 129.13, 127.73, 127.11 (aromatic C), 72.37 (C$_{60}$-sp^3-carbons), 70.80 (OCH$_2$CH$_2$O), 69.80 (NCH$_2$CH$_2$O), 65.71 (OCH$_2$CH$_2$O), 52.51 (malono bridge), 39.61 (NCH$_2$CH$_2$O); FAB-MS [(+), m-NBA] *m/z* 1361 [(M+H)$^+$, 13%], 720 [(M+H-ebselen malonate)$^+$, 100].

Primary cortical neuronal cultures

Primary cultures of rat cortical neurons were established using a modification of previous methods[17] which is briefly outlined below. Cortical tissue from 18-day fetuses of Wistar rats was dissociated and digested for 20 min at 37 °C in D-Hanks medium with 0.125% trypsinase. Trypsinase was then removed and the dissociated neurons were washed with DMEM / 10% fetal calf serum (FCS) and filtrated

through a 200-order stainless steel sieve. After centrifugation at 4000 g for 10 min, the cells were gently resuspended in DMEM / 10% FCS and vaccinated to poly-*L*-lysine (0.01%) coated 96-well plates at the density of 1×10^5 cells per well. All cells were incubated in DMEM culture medium containing 10% fetal calf serum (FCS), penicillin (100 U mL^{-1}) and streptomycin (100 μg mL^{-1}) at 37 °C in a 95% air / 5% CO_2 humidified culture box. On the third day of vaccination, cytarabine (final concentration 10 μmol L^{-1}) was added to the culture medium to prevent proliferation of non-neuronal cells.

On the 10th day of culture, the neural cells were divided at random into a control group, an H_2O_2-injured group, and a compound-treated group. The compound-treated group was divided into 4 subgroups according to the different chemicals employed (C_{60} derivative **4**, ebselen derivative **2**, mixture of **4** and **2** (equimolar ratio) and C_{60}-based ebselen derivative **3**) (Figure 1).

Culture treatments

Stock solutions (10 mmol L^{-1}) of compounds **4**, **2**, mixture (**4** + **2**) and **3** prepared in DMSO and diluted with sterile water were kept at 4 °C. At the time of medium change, stock solutions were further diluted and applied to the cultures.

Oxidative injury was induced by adding H_2O_2 (final concentration 150 μmol L^{-1}) to the serum-free DMEM culture medium containing 0.2% DMSO and incubating at 37 °C for 2 h.[18] After that treatment, the culture medium was replaced with fresh serum-free DMEM and the cells were incubated for 24 h in culture box. For the four compound-treated groups, cortical neuronal cultures were pretreated with compound **4**, **2**, mixture (**4** + **2**), or **3** (final concentration: 30 μmol L^{-1}) for 2 h and then exposed to 150 μmol L^{-1} H_2O_2 for other 2 h. Afterwards, the cell cultures received the same treatment as the H_2O_2-injured group. Control cells were not exposed to H_2O_2 nor to the various compounds, but cultured in serum-free DMEM medium supplemented with 0.2% DMSO for 24 h.

Measurement of cell viability[19]

Cell viability was determined by measuring the mitochondrial dehydrogenase activity (MDHA) through the reduction of MTT. After the above described procedure, an MTT solution (final concentration 0.5 mg mL^{-1}) was added to each well, and the cells were incubated at 37 °C for 4 h. After removal of the medium, formazan crystals, produced by MDHA in viable cells, were dissolved in dimethyl sulfoxide (200 μL). The optical density of each well was measured at 550 nm using an enzyme-linked analyzer.

Determination of LDH release[20]

Cell damage was evaluated by measuring the release of lactate dehydrogenase (LDH) into the culture medium. LDH contents in the extracellular medium were determined using the LDH assay kit according to the kit instructions.

Results and Discussion

Synthesis and spectroscopic properties of C_{60}-based Ebselen derivative 3

The synthesis of **3** is achieved after a three-step process as outlined in Scheme 1. Reaction of 2-(chloroseleno)benzoyl chloride with slight excess 2-(2-aminoethoxy)ethanol (1.1 equiv.) in dry CH_3CN afforded ebselen derivative **1**, which is *N*-substituted with an ethylene glycol chain, in 63% yield. The ebselen derivative **1** was esterified with malonyl dichloride in 2.2:1 molar ratio in dry $CHCl_3$ in the presence of pyridine (1.2 equiv. to **1**). After purification by flash chromatography (silica gel, eluent: ethyl acetate/petroleum ether 3:1), ebselen malonate **2** was obtained in 74% yield. Finally, Bingel cyclopropanation[21] of C_{60} (toluene, room temperature, 10 h) with the ebselen malonate **2** (1.2 equiv. to C_{60}) in the presence of 1,8-diaza-bicyclo[5.4.0]undec-7-ene (DBU, 2.0 equiv. to C_{60}) and CBr_4 (1.5 equiv. to C_{60}) gave rise to the fullerene-ebselen mono-adduct **3** in a 42% yield (based on consumed C_{60}). Mono-adduct **3** was isolated by column chromatography on silica-gel, eluted with pure toluene, followed by toluene/$CHCl_3$ (8:1, v/v).

The structure of **3** was confirmed by standard spectroscopic methods. Its ^{13}C NMR spectrum (150MHz, $CDCl_3$) displays 15 peaks between δ 139.24 and δ 145.70 owing to the sp^2-fullerene C-atoms, indicating that compound **3** has C_{2v} symmetry with a [6,6]-closed structure.[22] The signal at 72.37 ppm was assigned to the sp^3-fullerene C-atoms. The six carbons of the phenyl ring in the ebselen moiety give rise to 6 peaks between δ 127.11 and δ 134.87. The carbon of the bridge is observed at 52.51 ppm. The remaining 4 signals in the δ 39.61 ~ δ 70.80 region are assignable to the aliphatic carbons of the ethyleneglycol chain. The carbons of the carboxylate and amide groups are detected at 168.80 and 167.88 ppm, respectively. The ^1H NMR spectrum (600 MHz, $CDCl_3$) of compound **3** exhibits the aromatic hydrogens as two triplets (δ 7.65, *J* 7.3 Hz; δ 7.46, *J* 7.5 Hz) and two doublets (δ 8.06, *J* 7.8 Hz; δ 7.88, *J* 7.1 Hz). The hydrogens of the

ethyleneglycol chain give rise to two triplets (δ 4.35, *J* 6.6 Hz; δ 4.17, *J* 6.3 Hz) and two doublets between δ 3.76 and δ 3.80. The FTIR spectrum of compound **3** shows strong carbonyl absorptions at 1756, 1740 cm^{-1} (ester) and 1610, 1570 cm^{-1} (imide), and three characteristic absorptions of the C$_{60}$ skeleton at 1433, 1185, and 528 cm^{-1}. The FAB-MS analysis of compound **3** gives the molecular ion peak at *m/z* 1361 (M+H)$^+$ and a relevant fragment at *m/z* 720 (M+H-ebselen malonate)$^+$. The UV-Vis spectrum of compound **3** contains a typical medium intensity absorption band at around 430 nm, which is characteristic of most dihydrofullerenes[23] and further proves that compound **3** is a [6,6]-closed methano-fullerene.

Effects of C$_{60}$ derivative 4, ebselen derivative 2, mixture (4 + 2), and C$_{60}$-based ebselen derivative 3 on cell viability and LDH release

In order to verify the enhanced neuroprotective activity of C$_{60}$-based ebselen derivative **3**, related compounds (the C$_{60}$ derivative **4**, the ebselen derivative **2**, and a mixture of **4** and **2**) were also used to treat cortical neuronal cells according to the same procedures employed for **3** (Figure 1).

Cell viability and cell damage were estimated by MTT and LDH leakage assays, respectively. Results of LDH release and MTT(OD) measurements are shown in Figure 2. Compared with the control group, treatment of cortical culture with 150 µmol L^{-1} H$_2$O$_2$ for 2 h caused a decrease of MTT(OD) from 0.458 ± 0.024 to 0.215 ± 0.024 (P < 0.01) and an increase of LDH content in the extra-cellular media from 3.69 ± 0.17 to 8.02 ± 0.27 (P < 0.01), indicating H$_2$O$_2$-induced neuronal injury. Cytotoxicity tests demonstrated that compounds **4**, **2** and **3**, as well as the mixture **4** + **2**, at a concentration up to 30 µmol L^{-1}, have no toxic effect on cortical neurons. Therefore, 30 µmol L^{-1} was selected to detect their protective effects on H$_2$O$_2$-mediated neuronal injury. As expected, they were effectively able to attenuate the H$_2$O$_2$-mediated increase of LDH release and decrease of MTT(OD) value (Figure 2). MTT(OD) was elevated from 0.215 ± 0.024 (H$_2$O$_2$ alone) to 0.324 ± 0.025 (**4**), 0.294 ± 0.021 (**2**), 0.310 ± 0.018 (**4** + **2**), 0.364 ± 0.028 (**3**) (P < 0.01) (Figure 2a), and LDH release was reduced from 8.02 ± 0.27 (H$_2$O$_2$ alone) to 5.39 ± 0.17 (**4**), 5.71 ± 0.27 (**2**), 5.54 ± 0.39 (**4** + **2**) and 4.66 ± 0.28 (**3**) (P < 0.01) (Figure 2b).

Cell viability in compound **3**-treated group (MTT(OD) 0.364 ± 0.028) was higher than those obtained for compound **4** (0.324 ± 0.025), compound **2** (0.294 ± 0.021), or mixture (**4** + **2**) (0.310 ± 0.018). LDH release (**3**: 4.66 ± 0.28 UL^{-1}), in its turn, was consistently lower: compound **4** (5.39 ± 0.17), compound **2** (5.71± 0.27), or mixture (**4**
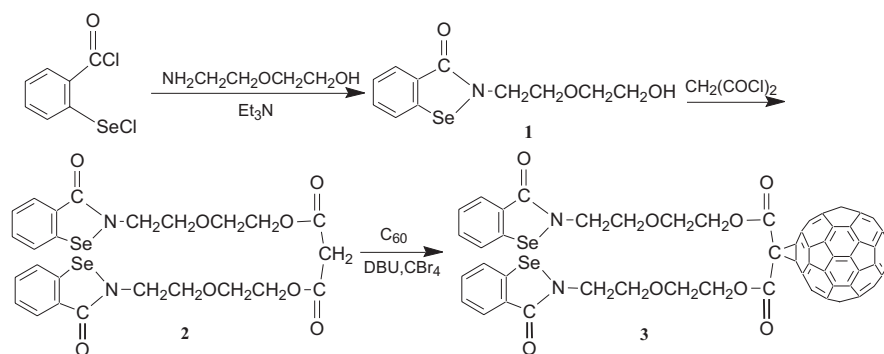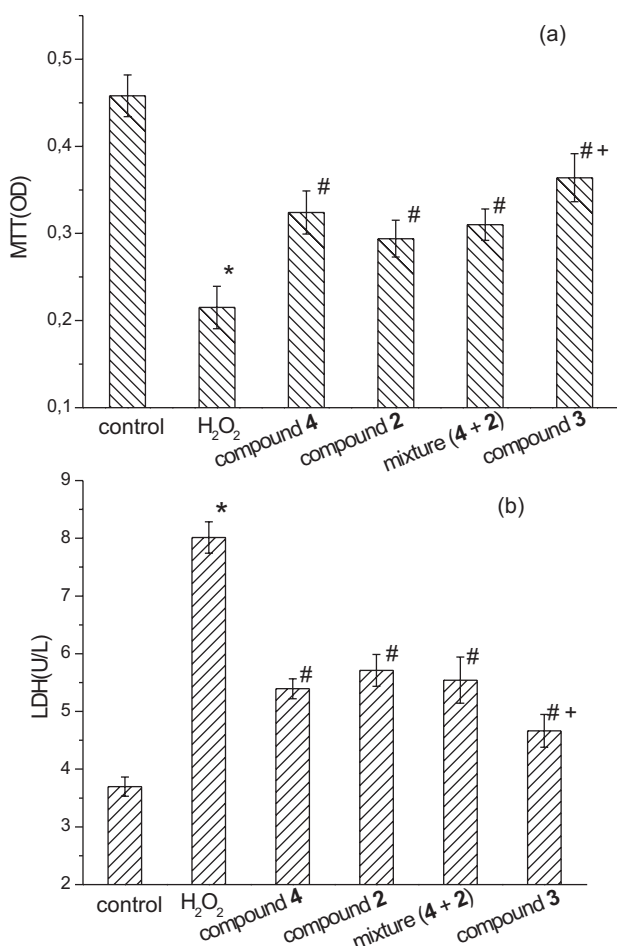

Scheme 1. Synthesis of C$_{60}$-based ebselen derivative **3**.
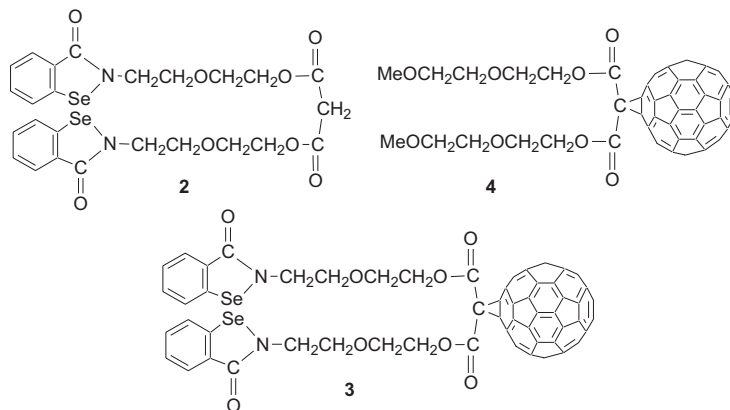
Figure 1. C$_{60}$ derivative **4**, ebselen derivative **2** and C$_{60}$-based ebselen derivative **3** used in this study.

+ **2**) (5.54 ± 0.39). These results indicated that the antioxidative and protective activities of C_{60}-based ebselen derivative **3** against H_2O_2-mediated neuronal injury are significantly higher than those shown by C_{60} derivative **4**, ebselen derivative **2**, and their mixture (**4** + **2**). Hence, the covalent incorporation of fullerene and ebselen units into a new molecule is of significance for enhancing antioxidative activity.

C_{60}-fullerene is a redox-active compound, since it has low LUMO and high HOMO levels. Okuda *et al.*[24] have observed that a water-soluble fullerene derivative (C_{60}-dimalonic acid, $C_{62}(COOH)_4$) has cytochrome *c* peroxidase-like activity. They confirmed that $C_{62}(COOH)_4$ was essential for the oxidation of reduced cytochrome *c* in the presence of H_2O_2. Hence, C_{60}-fullerene should also display glutathione peroxidase-like (Gpx) activity. Besides, C_{60}-fullerene possesses an excellent antioxidative property derived from its polyolefine bonds, which are highly reactive towards deleterious reactive oxygen species. On the other hand, ebselen has excellent Gpx activites and limited radical scavenging ability. Therefore, we think that the enhanced neuroprotective activity of C_{60}-based ebselen derivative **3** can reasonably be attributed to the improvement of both its free radical scavenging and Gpx-like activities.

Conclusions

The present work describes the synthesis of a C_{60}-based ebselen derivative (**3**), which contains an ethyleneglycol chain between the fullerene and the biologically active ebselen moiety. The C_{60}-based ebselen derivative was characterized by NMR, MS, IR and UV spectroscopy analyses. In addition, it showed stronger antioxidative and neuroprotective activities than parent C_{60} derivative **4**, ebselen derivative **2**, and a mixture of **4** and **2**. From this encouraging result, it can be concluded that the selection and incorporation of appropriate antioxidant components into C_{60}-fullerene molecules can give rise to new C_{60}-based derivatives with better antioxidative ability, and this is also an effective strategy for designing and synthesizing C_{60}-based antioxidants.

Acknowledgment

Supplementary Information

Relevant spectral data, including NMR and mass spectra, are available free of charge at http://jbcs.sbq.org.br, as a PDF file.

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Figure 2. (a) MTT(OD) values and (b) LDH release, both caused by H_2O_2 in cortical neurons, after treatment with compounds **4**, **2**, mixture (**4** + **2**) and **3**. Values are mean ± SD of 8 experiments. Statistical comparisons were made using one way ANOVA followed by Newman-keuls test as a post-hoc analysis. Differences were accepted as significant at p < 0.05 and p < 0.01. *p < 0.01 *vs.* control, #p < 0.01 *vs.* H_2O_2 group, +p < 0.05 *vs.* compound **4**, **2** or mixture (**4** + **2**) group.

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C₆₀-based Ebselen Derivative: Synthesis by Bingel Cyclopropanation and Enhanced Antioxidative and Neuroprotective Activity

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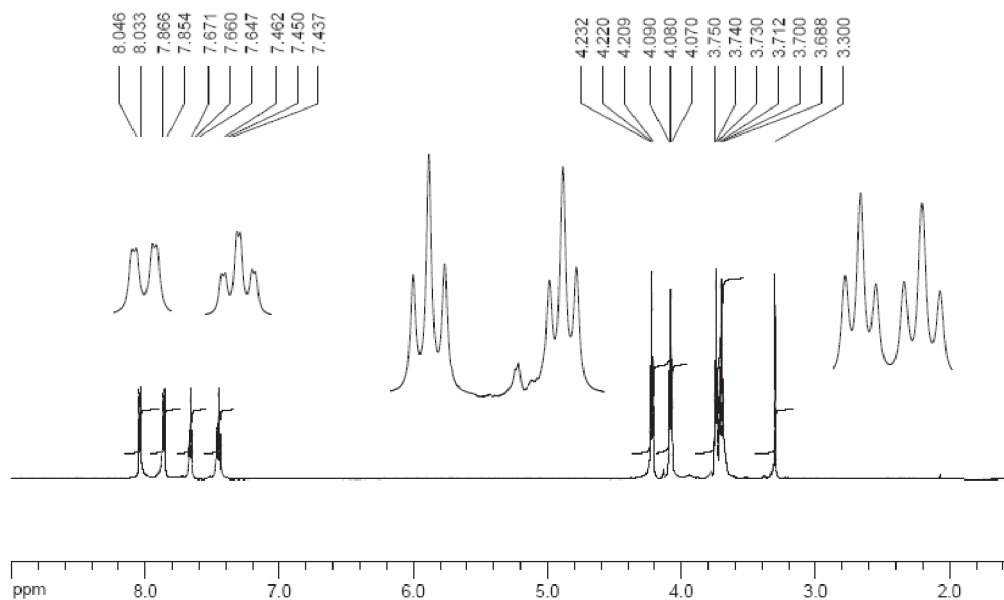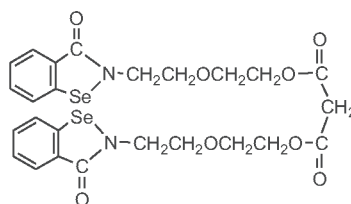
Figure S1. ¹H NMR spectrum of compound **2** at room temperature (600 MHz, CDCl₃).
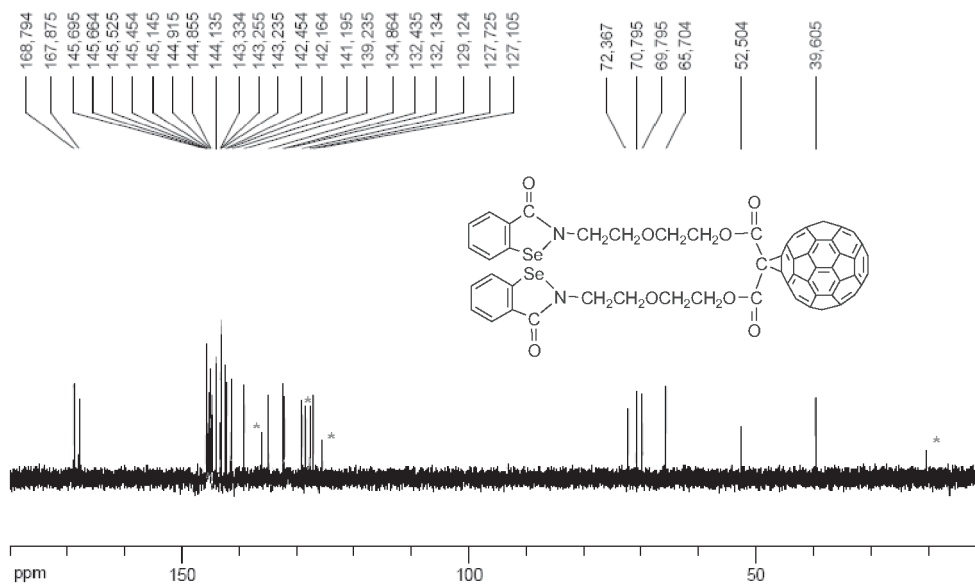
*e-mail: wcguan04@yahoo.com.cn
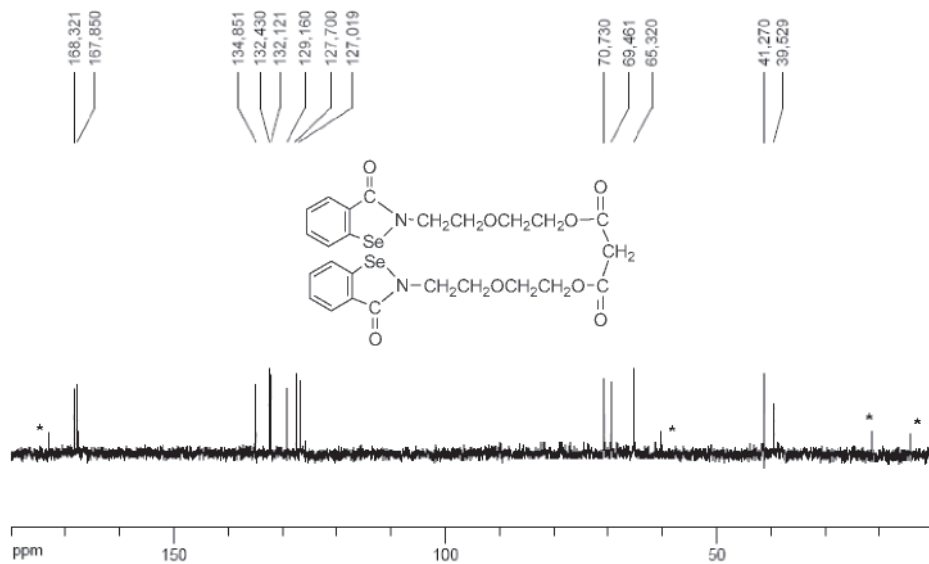
Figure S2. ^{13}C{^{1}H} NMR spectrum of compound **2** at room temperature (150 MHz, CDCl$_3$). The peak noted with * is due to solvent (ethyl acetate).
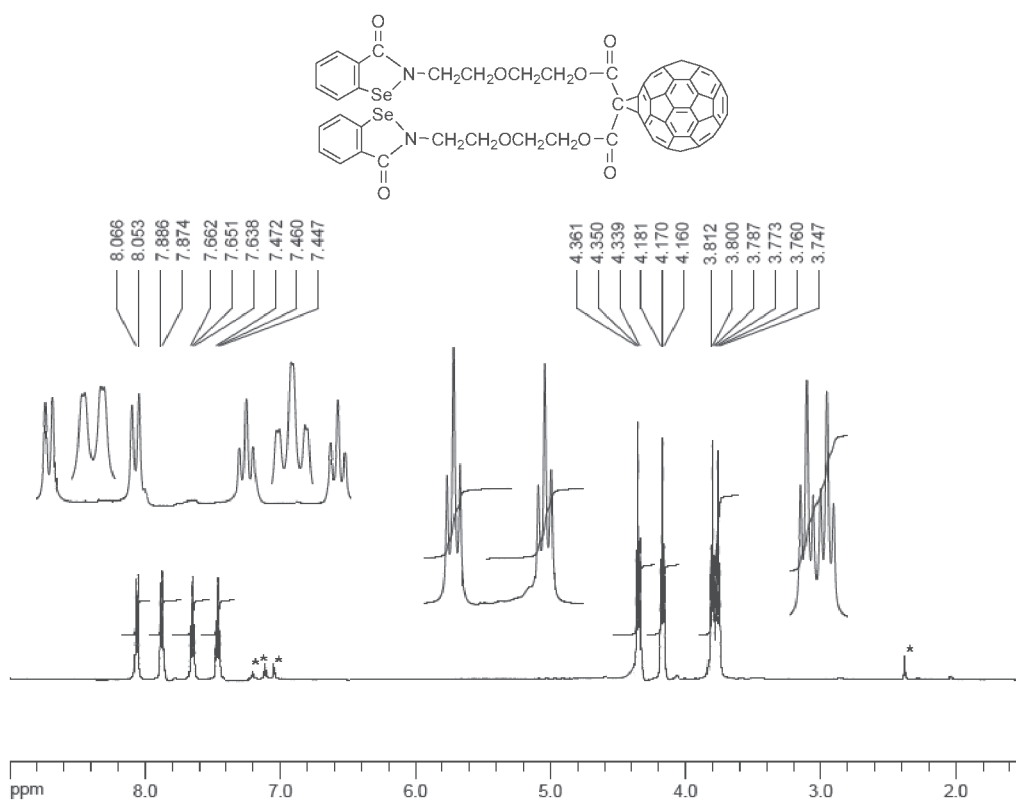
Figure S3. ^{1}H NMR spectrum of compound **3** at room temperature (600 MHz, CDCl$_3$). The peak noted with * is due to solvent (toluene).

Figure S4. $^{13}C\{^1H\}$ NMR spectrum of compound **3** at room temperature [150 MHz, CDCl$_3$]. The peak noted with * is due to solvent (ethyl acetate).
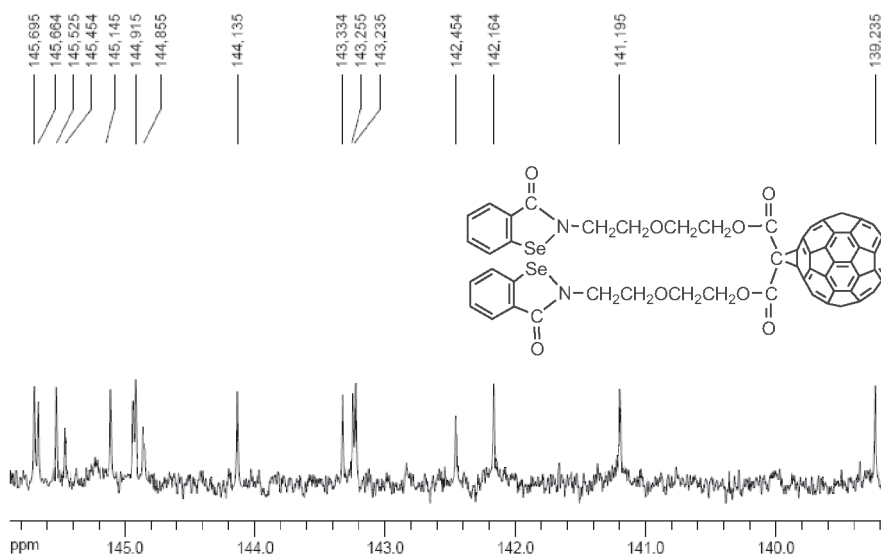
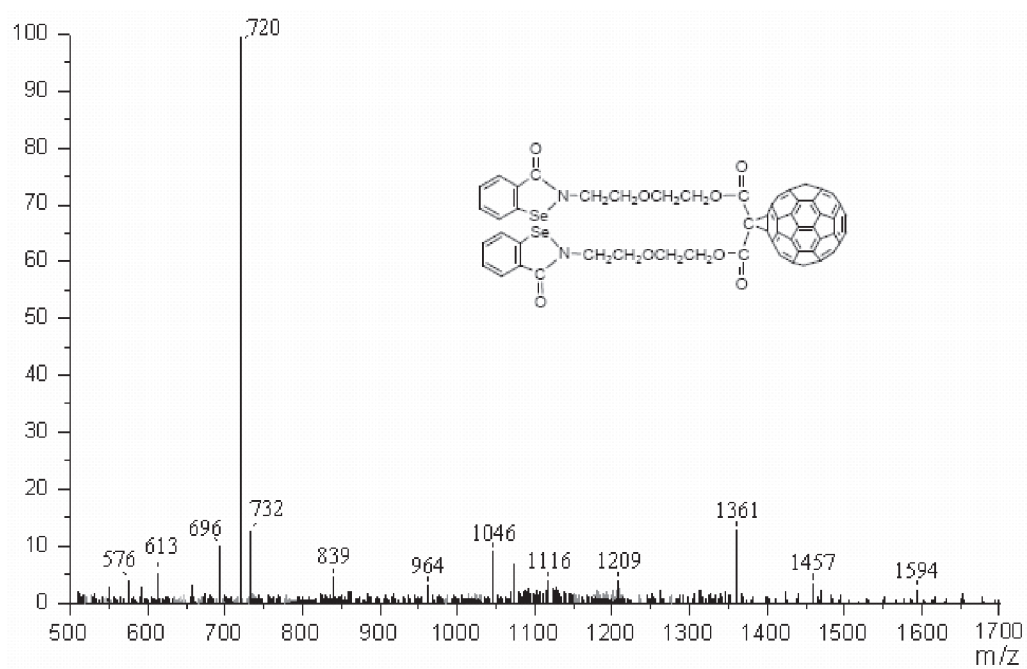**Figure S5.** The expanded $^{13}C\{^1H\}$ NMR spectrum of compound **3** between 139.2 and 145.8ppm (150 MHz, CDCl$_3$).

Figure S6. FAB(+) mass spectrum of compound **3**.