Pergamon

SCIENCE DIRECT®

Bioorganic & Medicinal Chemistry 11 (2003) 2329–2337

BIOORGANIC & MEDICINAL CHEMISTRY

Manganese-Based Complexes of Radical Scavengers as Neuroprotective Agents

Opa Vajragupta,[a,*] Preecha Boonchoong,[a] Yaowared Sumanont,[a] Hiroshi Watanabe,[b] Yuvadee Wongkrajang[a] and Naparat Kammasud[a]

[a]*Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Mahidol University, 447 Sri-Ayudhya Road, Bangkok 10400, Thailand*
[b]*Department of Pharmacology, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-0194, Japan*

Received 19 November 2002; accepted 28 January 2003

Abstract—Manganese was incorporated in the structure of the selected antioxidants to mimic the superoxide dismutase (SOD) and to increase radical scavenging ability. Five manganese complexes (**1**–**5**) showed potent SOD activity in vitro with IC_{50} of 1.18–1.84 μM and action against lipid peroxidation in vitro with IC_{50} of 1.97–8.00 μM greater than their ligands and trolox. The manganese complexes were initially tested in vivo at 50 mg/kg for antagonistic activity on methamphetamine (MAP)-induced hypermotility resulting from dopamine release in the mice brain. Only manganese complexes of kojic acid (**1**) and 7-hydroxyflavone (**3**) exhibited the significant suppressions on MAP-induced hypermotility and did not significantly decrease the locomotor activity in normal condition. Manganese complex **3** also showed protective effects against learning and memory impairment in transient cerebral ischemic mice. These results supported the brain delivery and the role of manganese in SOD activity as well as in the modulation of brain neurotransmitters in the aberrant condition. Manganese complex **3** from 7-hydroxyflavone was the promising candidate for radical implicated neurodegenerative diseases.

Introduction

Free radical represents one of the main causes of cellular damage and numerous degenerative diseases.[1–3] A predominant cellular free radical is oxygen-derived species, superoxide anion ($O_2^{\bullet -}$) that is formed by leakage of high-energy electrons along mitochondrial electron transport chain and by a variety of cytosolic and membrane-bound enzymes, including xanthine oxidase and the cytochrome P_{450} complex.[4] $O_2^{\bullet -}$ mediates direct cell damage and reacts with hydrogen peroxide (H_2O_2) and nitric oxide radical to generate the extremely reactive species; hydroxyl radical ($HO^{\bullet}$) and peroxynitrite ($ONOO^-$),[4,5] respectively. The first line of cellular defense is the superoxide dismutase (SOD), the metalloenzymes, which catalyze the dismutation of superoxide anion into hydrogen peroxide and dioxygen. Increasing intracellular levels of SOD or administering exogenous SOD have been investigated for protection against oxidative injury but they were disappointed because, as a protein, SOD has a number of delivery and stability shortcomings. To circumvent such limitations, there has been considerable interest in developing synthetic SOD mimics that have low molecular weight, biological stability, membrane permeability, nontoxic and cost-effectiveness.[6,7]

Depending on the metal at the active center, there are three general types of SOD enzymes; Cu/Zn-SOD, Mn-SOD and Fe-SOD. The search for SOD mimics yielded a variety of chelates of transition metals such as copper,[8,9] iron[10,11] or manganese[12–29] which behaved like the metal center in native SOD. Certain copper chelates were found to react rapidly with $O_2^{\bullet -}$ but proved to be biologically ineffective because they are prone to dissociation in the presence of cellular proteins, such as serum albumin, which are related to not only causing loss of SOD mimic activity but also mobilization of potentially toxic redox-active metals. Copper and iron have the potential of participating in fenton chemistry with production of highly toxic hydroxyl radical, as it seemed safer to base such mimic upon manganese,

*Corresponding author. Tel.: +662-644-8677; fax: +662-247-4696; e-mail: pyovj@mahidol.ac.th

0968-0896/03/$ - see front matter © 2003 Elsevier Science Ltd. All rights reserved.
doi:10.1016/S0968-0896(03)00070-1

which does not do so. There are many manganese based SOD mimics that have been developed, such as manganese porphyrins,[12–17] manganese macrocyclics,[18–22] manganese desferroxamines[23,24] and salen manganese complexes.[25–29]

In an effort to develop novel SOD mimics as potent radical scavengers, structure modification of antioxidants to possess SOD activity was carried out in this study. Manganese was designed to incorporate in the structure of selected antioxidants to mimic SOD. The selected antioxidants or ligands were hydroxyl containing compounds with various nucleus systems: pyran, pyridine, benzopyran and quinoline i.e., kojic acid (**L1**), 6-hydroxynicotinic acid (**L2**), 7-hydroxyflavone (**L3**), 8-hydroxyquinoline (**L4**) and 8-hydroxyquinoline ethylenediamine (**L5**), respectively, as shown in Figure 1. Radical scavenging activity of the newly synthesized compounds was evaluated for both in vitro SOD activity and for in vitro antilipid peroxidation. The neuropharmacological experiment on antagonistic activity on methamphetamine (MAP)-induced hypermotility resulting from dopamine release in the CNS was also conducted to determine the brain delivery and the role of SOD mimics in the modulation of brain neurotransmitters in the aberrant condition. The most potent complex was further investigated for the neuroprotective effect against learning and memory impairment in transient cerebral ischemic mice.

Materials and Methods

Chemical

Melting points of the compounds were determined on a Buchi capillary melting point apparatus and uncorrected. Infrared (IR) spectra were run on FTIR Nicolet 500 as a potassium bromide pellet. Proton nuclear magnetic resonance (^{1}H NMR) spectra were obtained with a Perkin–Elmer (300 MHz). Chemical shifts were reported in ppm related to the internal standard, tetramethylsilane. Elemental analysis of all compounds were obtained from an elemental analyser (Perkin–Elmer PE 400) for C, H, N and with atomic absorption spectroscopy (Perkin–Elmer 1100B) for manganese, The results were within ±0.4% of the theoretical values.

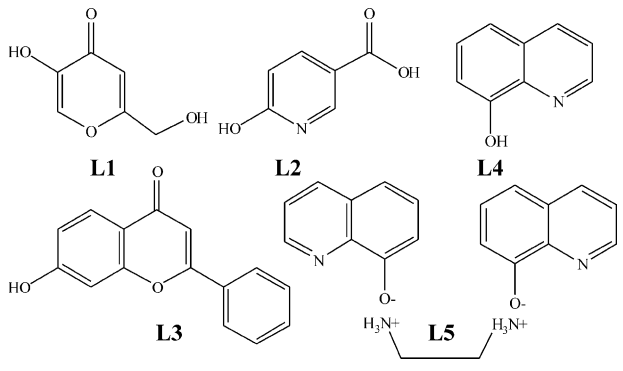

Figure 1. Structures of the selected antioxidants and ligands.

Manganese complex of kojic acid (1). A solution of manganese acetate (0.490 g, 2 mmol) in ethanol (3 mL) was added to a solution of kojic acid (0.568 g, 4 mmol) in ethanol 10 mL. The mixture was stirred for 1 h at room temperature, the manganese complexes were precipitated. The suspended solid was collected by filtration and washed by a small quantity of cold ethanol. The obtained solid was dried in vacuo over silica gel to give 0.670 g (96.78% yield) of product as a yellow solid, mp >300 °C. IR (KBr) (cm^{-1}): 3340 (O–H), 2929–2850 (aliphatic C–H), 1621–1587 (C=O ketone), 1527 (aromatic C=C), 1297–1250 (C–O), 917–800 (aromatic C–H). Elemental analysis for Mn-complex **1**, $C_{12}H_{10}O_8Mn{\cdot}1/2H_2O$; Calc: C, 41.64; H, 3.20; Mn, 15.87. Found: C, 41.48; H, 3.39; Mn = 16.06.

Manganese complex of 6-hydroxynicotinic acid (2). 6-Hydroxynicotinic acid (0.278 g, 2 mmol) was dissolved in mixed solvent (ethanol 8 mL and water 2 mL), heat was applied at 60 °C until the solution was clear. The solution was adjusted to pH 11 with weak base, ethylene diamine. Manganese chloride (0.198 g, 1 mmol) was dissolved in ethanol (1 mL) and added dropwise to the prepared 6-hydroxynicotinic solution. The reaction mixture was stirred for 1 h at 60 °C. The precipitated solid was collected by filtration, washed with cold ethanol and dried. The solid was recrystallized with water to give 0.267 g. (60.68% yield) of product as white crystal, mp >300 °C, IR (cm^{-1}): 3460 (O–H), 1650 (C=O carboxylic), 1565 (aromatic C=C, cyclic C=N), 1387 (aromatic C–N), 1242–1118 (C–O), 795-656 (aromatic C–H). ^{1}H NMR (DMSO-d_6): broad spectrum. Elemental analysis for Mn-cpx **2**, $C_{12}H_{10}N_2O_6MnCl_2{\cdot}2H_2O$; Calcd: C, 32.75; H, 3.21; N, 6.37; Mn, 12.50. Found: C, 33.10; H, 3.55; N, 6.62; Mn, 12.47.

Manganese complex of 7-hydroxyflavone (3). 7-Hydroxyflavone (0.476 g, 2 mmol) was dissolved in ethanol (10 mL) and heated at 60 °C to clear the solution. The solution of manganese chloride (0.198 g., 1 mmol) in ethanol (1 mL) was added dropwise to the solution of 7-hydroxyflavone. The reaction mixture was stirred at 60 °C for 1 h. The precipitated solid was collected by filtration, washed with cold ethanol and dried. The solid was recrystallized with ethanol to give 0.349 g (58.04% yield) of product as pale-yellow needle crystal, mp >300 °C, IR (cm^{-1}): 3300 (O–H), 3091–3050 (aromatic C–H), 1620 (C=O ketone), 1446–1390 (aromatic C=C), 1157 (C–O), 837–682 (aromatic C–H). ^{1}H NMR (DMSO-d_6): broad spectrum. Elemental analysis for Mn-cpx **3**, $C_{30}H_{20}O_6MnCl_2$; Calcd: C, 59.82; H, 3.35; Mn, 9.13. Found: C, 59.88; H, 3.29; Mn, 9.48.

Manganese complex of 8-hydroxyquinoline (4). Complex **4** was prepared in the same way as **3** but using 8-hydroxyquinoline as ligand. Mn-cpx **4** was obtained as yellow powder (58.29% yield). IR (KBr) (cm^{-1}): 3420–3300 (O–H), 3040 (aromatic C–H), 1580–1470 (aromatic C=C, C=N), 1380 (aromatic C–N), 1120 (C–O), 850–720 (aromatic C–H). ^{1}H NMR (DMSO-d_6): broad spectrum. Elemental analysis for Mn-cpx **4**, $C_{18}H_{12}N_2O_2MnCl_2{\cdot}1.5H_2O$; Calcd: C, 53.29; H, 3.72; N, 6.91; Mn, 13.54. Found: C, 53.44; H, 3.72; N, 6.94; Mn, 13.47.

8-hydroxyquinoline.ethylene diamine (L5). 8-Hydroxyquinoline (0.580 g, 4 mmol) was dissolved in ethanol (7 mL) and heated to clear the solution at 60 °C. Ethylene diamine 0.13 mL (0.120 g, 2 mmol) was added to the prepared 8-hydroxyquinoline solution and stirred for 1 h at 60 °C. The precipitated solid were filtered and recrystallized with ethanol to give the yellow product (0.593 g, 84.57% yield), mp(d) 100 °C. IR (KBr) (cm^{-1}): 3017 (aromatic C–H), 2908 (aliphatic C–H), 3031–2440 (NH_3^+ stretching), 1642 (NH_3^+ bending), 1500–1460 (aromatic C=C, N–H), 1395–1351 (aromatic C–N), 1104–1088 (C–O), 832–700 (aromatic C–H). ^1H NMR (DMSO-d_6): δ 3.68 (broad, 4H, $^+NH_3$–C$\underline{H}_2$–C$\underline{H}_2$–$^+NH_3$), 7.11 (dd, $J=7.25$ and 0.95 Hz, 2H, quinoline-H7), 7.43 (dd, $J=7.86$ and 0.78 Hz, 2H, quinoline-H5), 7.47 (t, $J=7.40$ and 7.96 Hz, 2H, quinoline-H6), 7.56 (dd, $J=8.34$ and 4.20 Hz, 2H, quinoline-H3), 8.02 (broad s, 6H, $^+N\underline{H}_3$–CH_2–CH_2–$^+N\underline{H}_3$ exchangeable with D_2O) 8.31 (d, $J=8.32$ Hz, 2H, quinoline-H4), 8.84 (d, $J=3.97$ Hz, 2H, quinoline-H2). Elemental analysis for **L5**, $(C_9H_6NO)_2.H_2N(CH_2)_2NH_2$; Calcd: C, 68.56; H, 6.32; N, 15.99. Found: C, 68.13; H, 6.23; N, 16.14.

Manganese complex of L5 (5). The intermediate product (**L5** 0.350 g, 1 mmol) was dissolved in ethanol (10 mL) and heated at 60 °C until clear. The solution of manganese chloride (0.198 g, 1 mmol) in ethanol 1 mL was added dropwise into the reaction mixture and stirred for 1 h at room temperature. The precipitated solid were removed by vacuum filtration and washed with cold ethanol. The solid was dried to give 0.374 g. (76.34% yield) of product as a yellow solid, mp > 300 °C. IR (KBr) (cm^{-1}): 3447 (O–H), 3045 (aromatic C–H), 2927 (aliphatic C–H), 1578–1466 (aromatic C=C, N–H), 1387–1321 (aromatic C–N), 1104 (C–O), 834–729 (aromatic C–H). ^1H NMR (DMSO-d_6): δ 3.80 (broad s, 4H,–NH–C$\underline{H}_2$–C$\underline{H}_2$–NH–), 7.11 (d, $J=7.24$ Hz, 2H, quinoline-H7), 7.43 (dd, $J=7.70$ and 0.57 Hz, 2H, quinoline-H5), 7.53 (t, $J=7.47$ and 7.94 Hz, 2H, quinoline-H6), 7.56 (dd, $J=8.29$ and 4.15 Hz, 2H, quinoline-H3), 8.32 (dd, $J=8.02$ and 1.22 Hz, 2H, exchangeable with D_2O, quinoline-H4), 8.78 (dd, $J=3.93$ and 1.16 Hz, 2H, quinoline-H2). Elemental analysis for Mn-cpx **5**, $C_{20}H_{20}N_4O_2MnCl_2.H_2O$; Calcd: C, 48.80; H, 4.50 ; N, 11.38; Mn, 11.26. Found: C, 48.99; H, 4.12; N, 10.96; Mn, 11.58.

Evaluation of radical scavenging ability

Superoxide dismutase activity assay.[30] All test compounds were determined for SOD activity by indirect assay using nitroblue tetrazolium (NBT) reduction method. The assay components of 1100 μL of phosphate buffer pH 7.8, 500 μL of 1% gelatin, 100 μL of catalase (6 unit/mL) and 300 μL of NBT solution (4 mg/mL) were mixed in 1 cm light path cuvette. Then, 250 μL of xanthine oxidase (0.2 unit/mL) and 250 μL of solution of test compound were added. After 30 s, the reaction was started by adding 500 μL of 1 mM of xanthine solution. Xanthine and xanthine oxidase system in an assay mixture produced superoxide anion which reduced NBT yielding blue formazan. The forming blue formazan is a spectrophotometrically detectable product that was monitored at 540 nm, the absorbance of the reduced NBT or blue formazan at 540 nm was recorded at 1 min interval for 7 min. The SOD activities of blank (vehicle), positive control (with SOD) and test compounds (four concentrations) were assayed in triplicate. The IC_{50} values (50% inhibition of NBT reduction) were obtained from linear regression plots between percent SOD activity versus their concentrations.

In vitro antilipid peroxidation.[32] Mice brains were obtained from freshly slaughtered mice. The brains were homogenized in ice-cold phosphate buffer 40 mM pH 7.4 (1:19 w/v) and kept in ice bath. Lipid peroxidation was assessed by the formation of thiobarbituric acid-reactive substances as described previously. The fluorescence of TBARS was measured at 553 nm while excitation wavelength settled at 515 nm. Test compounds were prepared in DMSO and were diluted serially. The antilipid peroxidation was expressed in term of IC_{50}, which obtained from linear regression plot between concentrations of test compound and percent inhibitions.

Evaluation of Neuropharmacological Effects

Determination of locomotor activity in mice.[33] The 25–35 g of male Swiss albino mice were used and divided into 4 groups of 6–10 animals. In the first group, manganese complexes **1**–**5** were administered subcutaneously at the dose of 50 mg/kg. One hour after treatment with test compounds, MAP dissolved in normal saline solution (NSS) at the dose of 2 mg/kg was administered subcutaneously. In the second group, mice were treated as group 1 except that NSS was injected instead of MAP. The other two groups are negative control and positive control. The drug vehicle and then the MAP vehicle (NSS) were administered in negative control group. The drug vehicle and then MAP (2 mg/kg) were administered as positive control group. Three min after administration of MAP or NSS in drug treated group and positive control or MAP's vehicle (NSS) in negative control group, locomotor activity of mice in the $12'' \times 12''$ chamber was observed for 5 min. The area in the chamber was divided into 16 squares, the area of each square was about the size of the mice. The locomotor activity was recorded in term of the number of squares per min that the mice occupied in 5 min. Significant increase in locomotor activity induced by MAP of the positive control group was observed. The locomotor activity of mice in drug treated groups were determined to evaluate the effect of test compounds on the locomotor activity in normal saline (group 2) as well as on the MAP induced hypermotility (group 1). Two tail student's *t*-test was used for statistical analysis.

Transient Cerebral Ischemia and Cognitive Deficit in Mice[34,35]

Animals. Eight-week-old 30–40 g male ICR mice (SLC, Japan) were subjected to a 12:12 h light–dark cycle (light on 7.30–19.30). All experimental procedures were performed in accordance with the standards established by the Guide for the Care and Use of Laboratory Animals of Toyama Medical and Pharmaceutical University.

Training and selection before 2VO-reperfusion. A circular blue pool, 70 cm in diameter, was filled to a depth of 13 cm with water maintained at 25±1 °C. Two days before the cerebral ischemic operation, mice were given a pretraining session in which they were allowed to swim freely in pool for 60 s without an escape platform. One day later, a dark platform (5 cm diameter) was placed in the middle of the pool, 1 cm above the surface of the water. A mouse was allowed to swim from 4 different starting points and the latency for escaping onto the platform was recorded with a maximum of 60 s. Mice with bad memory were excluded from the experiment. In each experiment, the mice were divided into 4 groups of 8–10 animals in each. One group served as a sham-control receiving vehicle only, one was the ischemia-control group receiving the vehicle and other two groups were treated with different doses of test compounds before 2VO surgery.

Transient cerebral ischemia. Mice were subjected to transient cerebral ischemia by occlusion of the bilateral common carotid arteries (2-vessel-occlusion, 2VO) plus hypotension at 1 h after administration (i.p.) of drugs or vehicle . The mice were anesthetized with urethane. The bilateral common carotid arteries were then exposed, and carefully separated from adjacent veins and sympathetic nerves before being followed by occlusion with artery clips. During clamping of the arteries, 0.3 mL of blood was withdrawn to induce hypotension by cutting off the tip of the tail. The artery clips were then removed and cerebral blood flow was restored after 20 min of clamping. The skin incision was closed and the mice were kept in a temperature-controlled room. Sham-operated mice were subjected to the same procedure with the exception of carotid clamping and bleeding. After 24 h, the water maze task experiment was repeated.

Water maze learning performance. A dark platform was situated 1 cm below the surface of the water. The pool was divided into four quadrants with the platform in a fixed position in the middle of one quadrant. Daily training consisted of four trials in which the mouse was placed in the water from four different starting points and the latency for escaping onto the platform with a maximum of 60 s was recorded. This was conducted for 5 consecutive days. On the sixth day, the platform was removed, mice were allowed to swim for 1 min, and the time for crossing the quadrant where the platform was formerly located was recorded. On day 7, the platform was placed 1 cm above the surface of the water in different position. The mouse was allowed to swim from four different starting points and the latency for swimming on to the platform was recorded.

Results and Discussion

Synthesis

The novel manganese-based complexes were synthesized by the reaction of manganese acetate or mangenese chloride with the selected antioxidants or ligand, for example, kojic acid (**L1**), 6-hydroxynicotinic acid (**L2**), 7-hydroxyflavone (**L3**), 8-hydroxyquinoline (**L4**) and 8-hydroxyquinoline–ethylene diamine (**L5**). All manganese complexes were mononuclear structure. The elemental analyses data confirmed the stoichiometry 1:2 of Mn:**L1**–**L4**, except that the stoichiometry of Mn:**L5** was 1:1. The formation of manganese complexes occurred within 10 min. The addition of $MnCl_2$ solution to the ligand solution occasionally resulted in a few amount of insoluble brown precipitates (presumably MnO_2), which could be eliminated from reaction mixture by filtration at the beginning as the manganese complexes were later precipitated. Manganese acetate was used as the manganese source in the preparation of Mn-cpx **1** because of kojic acid (**L1**) did not react with $MnCl_2$ to form manganese complex (Fig. 2). Manganese complexes **2**–**5** used manganese chloride as the manganese source. These complexes were chloro complexes as confirmed by the positive result from the chloride ion test using silver nitrate ($AgNO_3$). Manganese complex **2** was prepared from the reaction between the 6-hydroxynicotinic acid (**L2**) and $MnCl_2$ in ethanolic aqueous solution. After addition of ethylene diamine that acted as counter ion, the product was precipitated. The complex structure was solvated with two molecules of water. The manganese complex of 7-hydroxyflavone (**L3**) or $[Mn(L3)_2Cl_2]$ was formed in anhydrous ethanol at temperature of 60 °C (Fig. 2). Mn-cpx **3** was not formed in aqueous solution. Manganese complex of 8-hydroxyquinoline (**L4**) and 8-hydroxyquinoline–ethylenediamine (**L5**) were prepared from the reaction of $MnCl_2$ with **L4** and **L5**, respectively. **L5** was synthesized in order to add N-atoms of ethylene diamine into **L4**. The reaction was run in ethanolic solution at the temperature of 60 °C. **L5** was precipitated as quaternary salt form (NH_3^+). **L5** was purified by recrystallization before complexation with $MnCl_2$. The broad absorption at

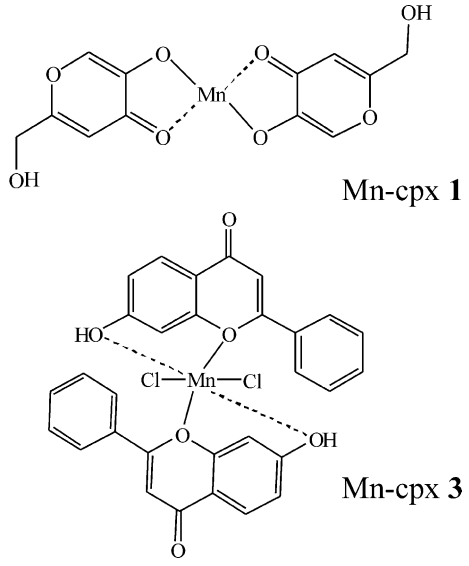

Figure 2. Structures of Mn-Cpx **1** and Mn-Cpx **3**. The configuration of Mn-Cpx **1** should be tetrahedral structure because of the monodeprotonated form of kojic acid is bidentate ligand, while Mn-Cpx **3** should be formed from two cyclic oxygens of two molecules of 7-hydroxyflavone and dichloro atoms coordinated with manganese in addition to the charge transfer complexes between manganese atom and benzene rings and/or hydroxyl group.

3460–3447 cm^{-1} in the spectra of Mn-cpx **2** and Mn-cpx **5** was attributed to the presence of solvated water. The infared absorption spectra of **L5** showed the characteristic peak of quaternary salt (NH_3^+). Salts of primary amines were found to be broad, strong NH_3^+ stretching band in the 3031–2440 cm^{-1} region arose from asymmetrical and symmetrical stretching of the NH_3^+ group. In addition, combination of multiple bands of medium intensity occurred in the 2700–2100 cm^{-1} region, the most prominent being the band at 2220 cm^{-1}. A weak asymmetrical NH_3^+ bending band at 1642 cm^{-1} and the fairly strong symmetrical bending band at 1460 and 1494 cm^{-1} are also the characteristic peak of NH^{3+}. These data indicated that ethylene diamine was added into the **L4** ligand. The IR spectrum of Mn-cpx **5** showed aliphatic C–H stretching at 2927 cm^{-1} and N-H bending vibration of primary amine at 1473–1579 cm^{-1}. The characteristic proton peak of **L5** and Mn-cpx **5** are shown in ^1H NMR spectra whereas the NMR spectra of Mn-cpx **1**–**5** are found to be broad. The broad spectra of **1**–**4** may be due to two coordinate bonds between metal ion and the ligand that brought the nucleus of the ligand close to the manganese ion. These attributed to the broadening of the NMR peaks of Mn-cpx **1**–**5**. The ^{13}C spectrum suggested that ethylenediamine reacted with 8-hydroxyqunoline to form **L5** because of the carbon signal of two methylene group appeared at 44.89 ppm.

Evaluation of radical scavenging ability

New manganese complexes **1**–**5** and the related compounds, for example, selected antioxidants or ligands, manganese chloride, manganese acetate, trolox and SOD were investigated for their radical scavenging ability. The nitro blue tetrazolium method and thiobarbituric acid method were used for the determination of SOD activity and lipid peroxidation inhibitory action, respectively.

SOD activity

SOD activity was assayed by nitro blue tetrazolium (NBT) method which was an indirect assay. The mixture of the enzyme xanthine oxidase and its substrate xanthine was used to generate superoxide radical ($O_2^{\bullet-}$). $O_2^{\bullet-}$ was allowed to reduce NBT to the blue formazan that was a spectrophotometrically detectable product at 540 nm. SOD enzyme or compound having SOD activity catalyzed the dismutation of $O_2^{\bullet-}$ and consequently inhibited the $O_2^{\bullet-}$ mediated NBT reduction by competitive process. According to reaction of SOD and $O_2^{\bullet-}$, hydrogen peroxide were generated and disturbed the SOD activity monitoring. Hydrogen peroxide inhibited and inactivated SOD activity. From the kinetic point of view, H_2O_2 is an unfavorable by-product. The assay should be run with the product (H_2O_2) at a concentration as low as possible so that the equilibrium was not shift in favor of $O_2^{\bullet-}$ production.[31] Therefore, the adding of catalase in assay mixture was necessary to prevent these problems. Test compounds were initially screened for the appropriate cycle time that gave the linear regression plot between absorbance and time. The appropriate cycle time was found to be within 7 min. Xanthine and xanthine oxidase system was used to generate superoxide radical which reduced NBT yielding blue formazan. The blue formazan was a spectrophotometrically detectable product that was monitored at 540 nm. Plots between percent SOD activity values versus the concentrations of all test compounds were linear with r^2 greater than 0.97. The relationship of percent SOD activity and concentration of test compounds is dose dependent as same as the SOD enzyme. The IC_{50} values relating to SOD activity were determined from the regression plots.

All manganese complexes were SOD mimics as expected and showed much greater SOD activity than their corresponding antioxidants or ligands (**L1**–**L4**) and related compounds. IC_{50} values of the prepared Mn-cpx **1**–**5** were in the range of 1.18–2.84 μM, as presented in Table 1. SOD activity of all prepared manganese complexes was in the same order of magnitude. Mn-cpx **3** was the most potent SOD mimic with IC_{50} of 1.18 μM was in agreement of the reported studies[26,27] that the presence of aromatic rings in the vincity of metal center led to the enhanced SOD activity. The SOD activity of its ligand, 7-hydroxyflavone (**L3**), was reported as% inhibition of NBT reduction instead of IC_{50} due to their low SOD activity and poor solubility in the assay media. The results indicated that the SOD activity of Mn-cpx **1** of kojic acid was 10650 times more potent than kojic acid (**L1**) and Mn-cpx **4** and Mn-cpx **5** were 18522 and 16220 times more potent than the corresponding ligand (8-hydroxyquinoline, **L4**). Trolox, a reference antioxidant compound, showed lower SOD activity with IC_{50} of 2821 μM. SOD enzyme from bovine liver was also assayed and found that SOD at 5.19 units/mL inhibited 50 NBT reduction. These indicated that IC_{50} value or inhibitory concentration of 50% NBT reduction was equivalent to 5 units/mL of SOD enzyme. Manganese chloride and manganese acetate also possessed high SOD activity with IC_{50} of 12.21 and 11.91 μM, respectively, but 10 times lower than the prepared

Table 1. SOD activity and antilipid peroxidation

Compd[a]	IC_{50} (μM) or inhibition ($n=3$)	
	SOD activity	Antilipid peroxidation
Mn-cpx **1**	2.84	8.00
L1	30780.00	350.00
Mn-cpx **2**	1.42	7.93
L2	ND[b]	ND[b]
Mn-cpx **3**	1.18	6.15
L3	34.91%[c]	418.00
Mn-cpx **4**	1.62	3.92
Mn-cpx **5**	1.85	1.97
L4	30006.00	10.80
$MnCl_2$	12.21	1.85
$Mn(OAc)_2$	11.91	4.23
Trolox	2821.24	59.23
SOD	5.19 units/mL	—

[a]Ligand of the complex: **L1** = kojic acid, **L2** = 6-hydroxynicotinic acid, **L3** = 7-hydroxyflavone, **L4** = 8-hydroxyquinoline and **L5** (ligand of Mn-cpx **5**) = **L4**–ethylene diamine.
[b]ND = not determined due to poor solubility in test media.
[c]% Inhibition of NBT reduction at the maximum concentration, 10 mg/mL of 7-hydroxyflavone.

manganese complexes. These results supported that manganese atom is the essential part for SOD activity and the incorporation of manganese into the structure of antioxidant tremendously increased the SOD activity. The mechanism of action may involve in the changing of oxidation state of manganese atom. A number of manganese based SOD mimics have been previously reported, for example, salen manganese complexes[25–29] and manganese porphyrins.[12–17] As manganese complexes **1**–**5** were found to be potent SOD mimics, the SOD activity of the prepared manganese complexes were compared to the reported activity of salen manganese complexes and manganese porphyrins in term of specific activity, for example, unit of the SOD activity per mg of compound. When comparing with the low molecular weight, Mn-cpx **1**–**5** exhibited higher specific activity values from 5281–8287 units/mg whereas salen manganese complexes exhibited specific activity values in the range of 618–5383 units/mg.[26–29] Manganese complexes of high molecular weight, $Mn^{II}OBTMPyP^{4+}$ showed highest SOD activity of 18460 units/mg. However, the SOD activities of all prepared manganese complexes (Table 2) were comparable with MnTM-2-PyP^{5+} (*ortho*). Mn-cpx **2** (8287 units/mg) was as active as MnTM-2-PyP^{5+} (8500 units/mg),[16,17] thus the prepared manganese complexes of low molecular weight and simple structures were the potent SOD mimics as proposed.

In vitro antilipid peroxidation

The inhibition of lipid peroxidation activity represented in term of concentration inhibiting 50% of lipid peroxidation, IC_{50}, which was obtained by measuring the pink color of the thiobarbituric acid reactive substances (TBARS). The linear regression plots between per cent inhibition values and the concentration of all test compounds showed r^2 values ranging from 0.97–0.99 and the IC_{50} values were presented in Table 1. All manganese complexes at low concentrations in micromolar level exhibited the great capacity to protect brain lipid against in vitro peroxidation. The manganese complexes were found to be more active than their ligands and trolox, the reference antioxidant according to the lower IC_{50} values. Among five manganese complexes, Mn-cpx **5** from 8-hydroxyquinoline ethylenediamine was the most potent compound against lipid peroxidation with IC_{50} of 1.97 μM, however IC_{50} values of other manganese complexes were comparable to Mn-cpx **5**. They are in the same order of magnitude with Mn-cpx **5**. When IC_{50} of manganese complexes were compared with its corresponding ligands, Mn-cpx **4** and Mn-cpx **5** were only 2.7 and 10 times more potent than 8-hydroxyquinoline (**L4**). IC_{50} of Mn-cpx **1** and Mn-cpx **3** was 44 times and 70 times lower than kojic acid (**L1**) and 7-hydroxyflavone (**L3**), respectively. So, the incorporation of manganese into **L3** most increased the antilipid peroxidation action when comparing the complex with its ligand. IC_{50} of Mn-cpx **2** from 6-hydroxynicotinic acid was 7.93 μM while IC_{50} of 6-hydroxynicotinic acid could not determined because of its poor solubility in test media. Mn-cpx **4** and **5** had 8-hydroxyquinoline (**L4**) as its ligand but ethylene diamine was added to Mn-cpx **5** in addition to **L4**. Mn-cpx **5** that contained ethylene diamine showed greater inhibitory effect than Mn-cpx **4**. It was indicated that adding N-atom to **L4** yielded better results than direct complexation of manganese with **L4**. The increase in the inhibitory action against lipid peroxidation of all manganese complexes was due to the incorporation of manganese atom in the structure of antioxidants; because manganese chloride and manganese acetate showed high activity against lipid peroxidation with IC_{50} of 1.85 and 4.23 μM, respectively.

Table 2. Specific activity of manganese complexes

Mn Cpx[a]	Specific activity[b] (units/mg)
1	5281
2	8287
3	7280
4	7703
5	5717

[a]Ligand of the complex: **1** = kojic acid, **2** = 6-hydroxynicotinic acid, **3** = 7-hydroxyflavone, **4** = 8-hydroxyquinoline (L4) and **5** = L4.ethylene diamine.
[b]Unit of activity was calibrated with purified SOD using NBT reduction method.

Determination of locomotor activity

As all newly prepared complexes showed greater SOD activity and action against lipid peroxidation than their corresponding antioxidants, therefore, the neuropharmacological study was continued. MAP administration caused significant increase in striatal dopamine and serotonin levels in mice. Thus, MAP induced the hypermotility as the result from dopamine release in the central nervous system. Dopamine can exacerbate the cell damage produced by cerebral ischemia and trauma, the oxidation of dopamine by molecular oxygen results in the formation of superoxide anion leading to the generation of 6-hydroxydopamine, the neurotoxin. All manganese complexes were tested for antagonizing hypermotility induced by MAP at the initial dose of 50 mg/kg. The test compounds were injected subcutaneously and 1 h after treatment, MAP was injected subcutaneously at a dose of 2 mg/kg.

The result showed that at the dose of 50 mg/kg of test compounds, Mn-cpx **1** and **3** exhibited significant effect against MAP-induced hypermotility while Mn-cpx **2**, **4**, **5** and $MnCl_2$ did not do so. The per cent suppression of MAP-induced hypermotility of Mn-cpx **1** and **3** at 50 mg/kg were 33.91 and 68.33, respectively, while their ligands kojic acid (**L1**) and 7-hydroxyflavone (**L3**) at 100 mg/kg dose did not show significant suppression on MAP-induced hypermotility. The dose of Mn-cpx **1** and **3** were varied and the results in Table 3 indicated that the relationship of per cent suppression and concentration of test compounds was in the dose dependent manner. Mn-cpx **3** showed the highest suppression, 68.33% at a dose of 50 mg/kg. Trolox, the reference antioxidant at 100 mg/kg was not given significant suppression of MAP-induced hypermotility. In addition, Mn-cpx **1**, **3** and trolox were evaluated for the effect on

Table 3. Effect of Mn-cpx on MAP-induced hypermotility[a]

Treatment ($n=6$–10)	Dose (mg/kg)	Inducer	Number of squares/min mean ± SEM	% Suppression of hypermotility[c]
Vehicle[b]	—	Saline	21.80 ± 2.29	—
Vehicle[b]	—	MAP	55.96 ± 2.87*	0.00
Mn-cpx **1**	50	MAP	44.38 ± 2.25$^{\#\#,\phi}$	33.91
	100	MAP	4.08 ± 2.25$^{\#\#,\phi\phi}$	64.07
L1	100	MAP	53.54 ± 1.53*	7.06
Mn-cpx **4**	50	MAP	58.54 ± 3.31*	−7.55
Mn-cpx **5**	50	MAP	56.46 ± 4.73*	−1.46
Vehicle[b]	—	Saline	25.51 ± 1.56	—
Vehicle[b]	—	MAP	54.00 ± 3.31*	0.00
Mn-cpx **2**	50	MAP	55.83 ± 3.18*	−6.38
Vehicle[b]	—	Saline	15.96 ± 1.23	—
Vehicle[b]	—	MAP	35.98 ± 2.24*	0.00
Mn-cpx **3**	25	MAP	29.88 ± 1.80$^{\#}$	30.49
	50	MAP	22.30 ± 0.99$^{\#\#,\phi\phi}$	68.33
L3	100	MAP	34.08 ± 2.39*	9.52
Trolox	100	MAP	33.86 ± 1.58	10.60

[a]The test compound or its vehicle was injected s.c. 1 h before MAP injection (2 mg/kg in saline) and locomotor activity was measured after administration of MAP. *$p<0.001$ MAP vs saline, $p<0.05$, test compound-MAP versus vehicle-MAP treated control, $^{\phi}p<0.01$, $^{\phi\phi}p<0.001$ versus ligand.
[b]The vehicle for Mn-cpx 1 and L1 was 15% Tween 80 and for Mn-cpx 3, L3 and trolox was 80% DMSO.
[c]% Suppression = $\{1-[(N_{test}-N_{saline})/N_{MAP}-N_{saline}]\}\times 100$.

locomotor activity in normal mice. The result (Table 4) showed that these three compounds did not have significant effect on locomotor activity. Although the percent locomotor activity of trolox was reduced to 86.20% of control, but it was not significantly different from the control. The results showed that Mn-cpx **1** from kojic acid (100 mg/kg) and Mn-cpx **3** from 7-hydroxyflavone (50 mg/kg) were able to suppress the MAP-induced hypermotility without reducing the locomotor activity in normal condition.

According to the locomotor result, it also suggested that the investigated radical-scavengers, mimicking SOD were delivered to brain, the target organ and possessed the ability to suppress the aberrant action due to dopamine release. The prepared manganese based SOD mimics were found to give additional benefit as they showed greater radical scavenging ability than their corresponding antioxidants. The result indicated that the incorporation of manganese into the structure of the antioxidants enhanced the radical scavenging ability of those antioxidants. These confirmed that manganese atom is the essential part exerting the SOD activity. Its mechanism possibly involved in redox reaction of manganese atom, the active site. In vitro, all manganese complexes were considered as potent SOD mimics and potent radical scavengers. Mn-cpx **2** from 6-hydroxynicotinic acid, Mn-cpx **4** and **5** from 8-hydroxyquinoline were the potent SOD mimics but they did not show the significant suppression of MAP-induced hypermotility in mice at 50 mg/kg dose. From in vitro antilipid peroxidation and SOD results, Mn-cpx **4** was found to be the most potent compound in the series, however it did not effective in neuropharmacological experiment. It was possible that Mn-cpx **2**, **4** and **5** were poorly delivered to the brain, the target organ. These three complexes were soluble in water, it is suggested that the low lipophilicity made them hardly cross the blood brain barrier. In relation to the lipophilicity of corresponding ligands, log P of 6-hydroxynicotinic acid (**L2**) and 8-hydroxyquinoline (**L4**) were 0.38 and 1.73 (calculated by software Molgen 4.0 using Crippen's method), respectively which indicated low lipophilicity. Kojic acid (**L1**) showed poor lipophilicity with the lowest log P of -2.45 but Mn-cpx I was able to suppress MAP-induced hypermotility. The reason for the in vivo action was thus the stability of the complex in vivo beside the lipophilicity. In case of Mn-cpx **1**, the hydroxyl group at C_3 position of kojic acid was in monodeprotonated form, the deprotonated kojic acid (**L1***) formed stronger bond with manganese center atom than the coordination bond resulting in the stability of Mn-cpx **1** in vivo. The stability of the complexes in the biological system is governed by a number of factors. The metal chelates may dissociate, can catalyze other undesirable redox process in cell, and exhibit high affinity toward proteins

Table 4. Effect of test compounds on locomotor activity[a]

Treatment ($n=6$–10)	Dose (mg/kg)	Number of squares/min mean ± SEM	% Locomotor activity
Vehicle	—	26.80 ± 2.49	100.00
Mn-cpx **1**	100	26.66 ± 2.13	99.47
Vehicle	—	19.46 ± 1.13	100.00
Mn-cpx **3**	50	18.40 ± 1.78	94.55
Trolox	100	16.78 ± 1.55	86.20

[a]The test compound or its vehicle (15% Tween 80 for Mn-cpx **1** and 80% DMSO for Mn-cpx **3** and trolox) was injected s.c., locomotor activity was measured after 1 h administration.

and amino acids, which lead to the loss of in vivo action. Among five manganese complexes, Mn-cpx **1** and **3** exhibited the better action in both of in vitro radical scavenging testings (SOD action and antilipid peroxidation) and in vivo neuropharmacological experiments. They showed significant suppression of hypermotility induced by MAP when compared with its corresponding antioxidants and trolox, the reference antioxidant without the effect on locomotor activity in normal condition. Mn-cpx **3** from 7-hydroxyflavone was more effective than Mn-cpx **1** from kojic acid on the suppression of MAP-induced hypermotility in spite of the comparable in vitro activity. These were possibly due to the greater lipophilicity of **L3** over **L1** (log P of 2.68 vs −2.45). Moreover, the structure of Mn-cpx **3** was more steric than the structure of Mn-cpx **1** which caused Mn-cpx **3** more stable and more lipophilicity.

Transient cerebral ischemia and water maze task in mice

Cerebral ischemia resulting from a reduction of cerebral blood flow, often produces neural necrosis especially in the CA1 sector of the hippocampus. A number of studies have suggested a strong link among cerebral stroke, superoxide free radical and the occurrence of spatial cognitive deficits.[35,36] In this experiment, the 2VO model to induce transient cerebral ischemia in mice was performed to investigate the protective effect of the manganese complexes against cognitive impairment. Before the 2VO operation, mice were given a pretraining session in water maze task and the bad memory mice which could not escape onto the platform within 45 s were excluded from the next experiment steps. Mice were subjected to 2VO and the spatial learning was tested 1 day later using the water maze task. The 2VO mice required a significantly longer time to locate the hidden platform than the sham-operated control mice during the training trials for 5 consecutive days. On day 6, when the platform was removed and each mouse was subjected to swim in a pool for 60 s, the 2VO mice spent significantly less time crossing into the quadrant where the platform was formerly located compared to the sham-operated controls. This indicated that the transient ischemia impaired learning and memory, as the mice used more time to locate the platform during 5 days (Fig. 3).

In the absence of platform (on day 6), the sham control mice remembered the quadrant that formerly contained the platform, and spend more time in this quadrant (Fig. 4). The compounds that had protective effect against memory impairment were able to improve the performance of 2VO mice to a level close to that of the sham control group. The unhealthy mice (i.e., blind, weakness), which could not escape onto a visible platform (1 cm above water surface) on day 7, were excluded from the experiment groups.

Tacrine, an anticholinesterase agent for dementia treatment and has been reported its protective effect against memory impairment,[35] was used as a reference drug at concentration of 1 and 2.5 mg/kg. Tacrine 2.5 mg/kg,

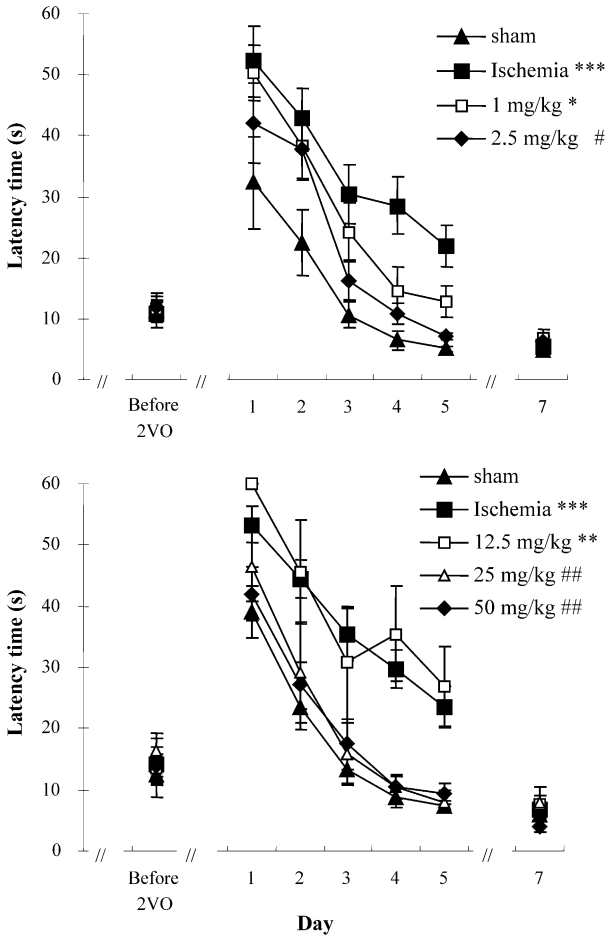

Figure 3. Effect of tacrine and Mn-cpx **3** on water maze learning performance of transient cerebral ischemia mice. The latency for escaping onto the platform was measured for 5 consecutive days. Each data point represents the mean of four trials, $n = 7$–9. *p, **p, ***$p < 0.05$, 0.01, 0.001 versus sham group; ##p, ##p, ###$p < 0.05$, 0.01, 0.001, versus ischemia group, respectively.

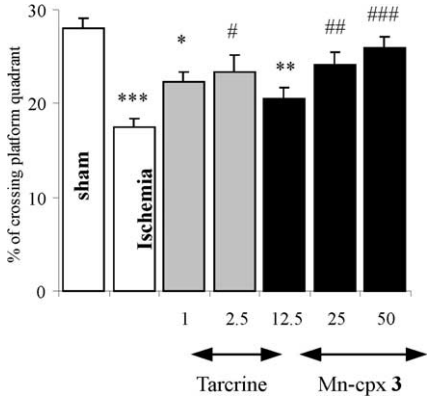

Figure 4. After the platform was removed on day 6, the time spend crossing into the quadrant where the platform was formerly located was recorded, each bar represented the mean ± SEM ($n = 7$–9). *p, **p, ***$p < 0.05$, 0.01, 0.001 versus sham group; ##p, ##p, ###$p < 0.05$, 0.01, 0.001, versus ischemia group, respectively.

ip, shortened the latency of escaping onto the platform during the 5 day training trials and also significantly increased the crossing time to the former platform quadrant on the sixth day (without platform) as shown in Figure 3. Mn-cpx **3** showed a protective effect at 12.5 mg/kg (1.6 mg of manganese/kg). Mn-cpx **1** and manganese acetate had no effect at the dose of 50 mg/kg (8.0 and 11.3 mg of manganese/kg, respectively). These results indicated that antioxidant with SOD activity played an important role for the cognitive improvement after transient ischemia. The learning and memory impairment induced by cerebral ischemia was improved by pretreatment with the Mn-cpx **3**.

The result demonstrated that radical scavengers were the alternative agents against cognitive impairment in transient cerebral ischemia mice beside anticholinesterases. It is interesting to further the additive or synergistic effect between Tacrine and Mn-cpx **3** on the transient cerebral ischemia and water maze task since these two compounds exerted the effect through a different mechanisms.

In conclusion, the incorporation of manganese into the structure of antioxidants made the complexes possessed the SOD activity and increased radical scavenging activity of antioxidants as expected. Manganese atom is therefore the essential part for SOD action. Mn-cpx **3** from 7-hydroxyflavone was promising compounds since it exhibited potent radical scavenging ability and suppressed the MAP-induced hypermotility without reducing the locomotor activity in normal condition, and also improved the impaired learning and memory in transient ischemic mice. It is interesting to further the investigation on their therapeutic efficacy in the diseases that implicated in overproduction of superoxide radical and/or SOD dysfunction.

Acknowledgements

This work was funded by the Royal Golden Jubilee Project, Thailand Research Fund, Mahidol University Research Fund, AIEJ (Monbukagakusho) scholarship and NRCT-JSPS (National Research Council of Thailand Japan Society for Promotion of Sciences project).

References and Notes

1. Roberfroid, M.; Colderon, P. B. *Free radicals and oxidation phenomena in biological system*; University of Catholique de Louvain: Brussels: New York, 1995.
2. Halliwell, B.; Gutteridge, J. M. C. *Free radicals in biology and medicine*; Oxford University: Oxford, 1999.
3. Simonian, N. A.; Coyle, J. T. *Ann. Rev. Pharmacol. Toxicol.* **1996**, *36*, 83.
4. Fridovich, I. *Ann. Rev. Pharmacol. Toxicol.* **1993**, *23*, 239.
5. Beckman, J. S.; Koppenol, W. H. *Am. J. Physiol.* **1996**, *271C1424*.
6. Dowling, E. J.; Chander, C. L.; Claxson, A. W.; Lillie, C.; Blake, D. R. *Free Rad. Res. Com.* **1993**, *18*, 291.
7. Takakura, Y.; Masuda, S.; Tokuda, H.; Nishikawa, M.; Hashida, M. *Biochem. Pharmacol.* **1994**, *47*, 853.
8. Muller, J.; Felix, K.; Maichle, C.; Lengfelder, E.; Strahle, I.; Weser, U. *Inorg. Chim. Acta* **1995**, *233*, 11.
9. Pierre, J. L.; Chautemps, P.; Refaif, S.; Beguin, C.; Marzouki, A. E.; Serratricel, G. *J. Am. Chem. Soc.* **1995**, *117*, 1965.
10. Iuliano, L.; Pederson, J. Z.; Ghiselli, A.; Pratico, D.; Rotlio, G.; Violi, F. *Arch. Biochem. Biophys.* **1992**, *293*, 153.
11. Zhang, D.; Busch, D. H.; Lennon, P. L.; Weiss, R. H.; Neumann, W. L.; Riley, D. P. *Inorg. Chem.* **1998**, *37*, 956.
12. Day, B. J.; Fridovich, I.; Crapo, J. D. *Arch. Biochem. Biophys.* **1997**, *347*, 256.
13. Patel, M. *J. Neurochem.* **1998**, *71*, 1068.
14. Szabo, C.; Day, B. J.; Salzman, A. L. *FEBS Lett.* **1996**, *381*, 82.
15. Gardner, P. R.; Nguyen, D. H.; White, C. W. *Arch. Biochem. Biophys.* **1996**, *325*, 20.
16. Batinic-Haberle, I.; Liochev, S. I.; Spasojevic, I.; Fridovich, I. *Arch. Biochem. Biophys.* **1997**, *343*, 225.
17. Batinic-Haberle, I.; Benov, L.; Spasojevic, I.; Fridovich, I. *J. Biol. Chem.* **1998**, *273*, 24521.
18. Riley, D. P.; Weiss, R. H. *J. Am. Chem. Soc.* **1994**, *116*, 387.
19. Hardy, M. M.; Flickinger, A. G.; Riley, D. P.; Weiss, R. H.; Ryan, U. R. *J. Biol. Chem.* **1994**, *269*, 18535.
20. Weiss, R. H.; Fretland, D. J.; Baron, D. A.; Ryan, U. S.; Riley, D. P. *J. Biol. Chem.* **1996**, *271*, 26149.
21. Riley, D. P.; Henke, S. L.; Lennon, P. J.; Weiss, R. H.; Neumann, W. L.; Rivers, W. *J. Inorg. Chem.* **1996**, *35*, 5213.
22. Riley, D. P.; Lennon, P. J.; Neumann, W. L.; Weiss, R. H. *J. Am. Chem. Soc.* **1997**, *119*, 6522.
23. Beyer, W. F.; Fridovich, I. *Arch. Biochem. Biophys.* **1989**, *271*, 149.
24. Faulkner, K. M.; Steven, R. D.; Fridovich, I. *Arch. Biochem. Biophys.* **1994**, *310*, 341.
25. Zhang, W.; Jacobsen, E. N. *J. Org. Chem.* **1991**, *56*, 2296.
26. Baudry, M.; Etienne, S.; Bruce, A.; Palucki, M.; Jacobsen, E. N. *Biochem. Biophysical Res. Com.* **1993**, *192*, 964.
27. Gonzalez, P. K.; Zhuang, J.; Doctrow, S. R.; Malfroy, B.; Benson, R. F.; Menconi, M. J. *J. Pharmacol. Exp. Ther.* **1995**, *275*, 798.
28. Musleh, W.; Bruce, A.; Malfroy, B.; Baudry, M. *Neuropharmacol* **1994**, *33*, 929.
29. Bruce, A.; Malfroy, A. J.; Baudry, M. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 2312.
30. Younes, M.; Weser, U. *FEBS Lett.* **1976**, *61*, 209.
31. Oberley, L. W.; Spitz, D. R. In *Nitroblue tetrazolium*; Greenwald, R. A., Ed.; CRC Handbook of methods for oxygen radical research, 2nd ed.; CRC Press: New York, 1985; pp 217–220.
32. Vajragupta, O.; Toasaksiri, S.; Boonyarat, B.; Wongkrajang, Y.; Watanabe, H.; Peungvicha, P. *Free Rad. Res.* **2000**, *32*, 145.
33. Vajragupta, O.; Monthakantirat, O.; Wongkrajang, Y.; Watanabe, H.; Peungvicha, P. *Life Sci.* **2000**, *67*, 1725.
34. Jaspers, R. M.; Block, F.; Heim, C.; Sontag, K. H. *Neurosci. Lett.* **1990**, *117*, 149.
35. Xu, J.; Murakami, Y.; Matsumoto, K.; Tohda, M.; Watanabe, H.; Zhang, S.; Yu, Q.; Shen, J. *J. Ethopharmacol.* **2000**, *73*, 405.
36. Nakano, S.; Kato, H.; Kogure, K. *Brain Res.* **1989**, *490*, 178.