Bis-(2-amino-5-selenazoyl) Ketone as a Superoxide Anion-Scavenger

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Received February 14, 2006; accepted April 13, 2006; published online April 18, 2006

We investigated the superoxide anion scavenging effects of 2-amino-1,3-selenazoles and bis-(2-amino-5-selenazoyl) ketones using a highly sensitive quantitative chemiluminescence method. At 166 μM , the 2-amino-1,3-selenazoles and bis-(2-amino-5-selenazoyl) ketones scavenged in the range of 10.0 to 80.0% of O_2^- . Bis[2-dimethylamino-5-(1,3-selenazoyl)] ketone exhibited the strongest superoxide anion-scavenging activity among the six kinds of 2-amino-1,3-selenazoles and three kinds of bis-(2-amino-5-selenazoyl) ketones. The 50% inhibitory concentration (IC_{50}) of bis[2-dimethylamino-5-(1,3-selenazoyl)] ketone was determined to be 37.1 μM . Thus, bis[2-dimethylamino-5-(1,3-selenazoyl)] ketone acted *in vitro* as effective and potentially useful O_2^- scavenger.

Key words 2-amino-1,3-selenazole; heterocycle; superoxide radical; scavenging effect; superoxide anion-scavenging activities (SOSAs)

Accumulating evidence suggests that even under normal physiological conditions, reactive oxygen species (ROSs) are generated in aerobic cells, and therefore, exposure to ROSs is inevitable.¹⁾ ROSs also react with biomacromolecules, including DNA and proteins, leading to the degeneration of these molecules.^{2,3)} In addition, phagocytes (granulocytes, monocytes/macrophages, eosinophils, and basophils) generate large amounts of ROSs, leading to cell/tissue injuries due to excessive inflammation.^{4–6)} In extreme instances such as endotoxin shock, neutrophils kill the infected host.⁷⁾

Antioxidative enzymes and compounds such as superoxide dismutases (SODs), catalase, glutathione peroxidase (GPX), and some vitamins act as antioxidants.^{4,6)} GPX is an important antioxidant enzyme and effectively eliminates H_2O_2 *in vitro* and *in vivo*.^{6,8)} GPX essentially contains selenium atoms in active domain.⁸⁾ Various studies have reported that selenoproteins including GPX reduce oxidative stress in cells.^{9,10)} Thus, various organic selenium compounds may be a candidate of ROSs scavenger.

The ebselen, a five-membered ring selenium-containing heterocyclic compound showing glutathione peroxidase-like activity,¹¹⁾ is a synthetic antioxidant due to a ROSs scavenger.¹²⁾ The antioxidative effect of the ebselen is selective blockade of leukocyte infiltration and activation, resulting in reduction of the H_2O_2 level.¹²⁾ This compound is a multifunctional antioxidant and a potential chemopreventive agent in inflammation-associated carcinogenesis.¹³⁾ Thus, various types of organic selenium compounds may be applicable to reduce oxidative stress.^{14–16)}

We have synthesized various organic selenium compounds including selenoamides,¹⁴⁾ selenoureas, thioureas,¹⁵⁾ and selenocarbamates.¹⁶⁾ Moreover, most of these compounds have also effectively scavenged O_2^- *in vitro*.^{15,16)} In this study, superoxide anion-scavenging activities (SOSAs) of a newly synthesized series of five-membered ring selenium-containing heterocyclic compounds, 2-amino-1,3-selenazoles and bis-(2-amino-5-selenazoyl) ketones were tested *in vitro*.

MATERIALS AND METHODS

Materials 2-Amino-1,3-selenazoles and bis-(2-amino-5-selenazoyl) ketones were prepared according to procedures previously reported.¹⁷⁾ A *Cypridina* luciferin analogue, 2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo-[1,2-*a*]-pyrazin-3-one hydrochloride (MCLA) was obtained from Tokyo Kasei (Tokyo, Japan) for a use as a chemiluminescent probe for superoxide radicals. MCLA was dissolved in doubly distilled water and stored at -80°C prior to usage. The concentration of MCLA solution was determined by absorbance at 430 nm using an absorbance coefficient value of $\epsilon=9600\text{ M}^{-1}\text{ cm}^{-1}$, as previously described.¹⁸⁾ SOD (Lyophilized powder, 3400 units/mg protein) and xanthine oxidase (XOD grade III) were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). Hypoxanthine was purchased from Wako Pure Chemical (Osaka, Japan) and used without further purification. All other chemicals and solvents were analytical grade and used without further purification.

Synthetic Methods for the Preparation of 2-Amino-1,3-selenazoles 1 and Bis-(2-amino-5-selenazoyl) Ketones 2 5-Acetyl-2-dimethylamino-1,3-selenazole **1a**: Chloroacetone (24 μl , 0.3 mmol) was added to stirred solution of *N,N*-dimethyl-*N'*-(dimethylselenocarbamoyl)formamidine (0.62 g, 0.3 mmol) in dry methanol (3 ml) under an argon atmosphere. The reaction mixture was refluxed for 2 h. The mixture was extracted with dichloromethane. The extract was evaporated to dryness. The residue was purified by flash chromatography on silica gel with ethyl acetate–hexane (1:2) to give **1a** (64 mg, 98%) as pale red crystals. mp: $100\text{--}102^\circ\text{C}$. IR (KBr): 1622 cm^{-1} . $^1\text{H-NMR}$ (500 MHz, CDCl_3 , rt): $\delta=2.44$ (s, 3H, CH_3), 3.19 (br s, 6H, N- CH_3), 7.78 (s, 1H, CH). $^1\text{H-NMR}$ (500 MHz, CDCl_3 , -40°C): $\delta=2.49$ (s, 3H, CH_3), 3.14 (br s, 3H, N- CH_3), 3.33 (br s, 3H, N- CH_3), 7.83 (s, 1H, CH). $^{13}\text{C-NMR}$ (125 MHz, CDCl_3 , rt): $\delta=25.3$, 41.2, 134.2, 149.6, 178.4, 190.3. $^{13}\text{C-NMR}$ (125 MHz, CDCl_3 , -40°C): $\delta=25.4$, 39.1, 43.7, 133.5, 149.9, 178.2, 190.8. $^{77}\text{Se-NMR}$ (95 MHz, CDCl_3): $\delta=523.1$. MS (FAB): $m/z=219$ [M^++1]. Anal. Calcd for $\text{C}_7\text{H}_{10}\text{N}_2\text{OSe}$:

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C, 38.72; H, 4.64; N, 12.90. Found; C, 38.99; H, 4.95; N, 12.63.

5-Acetyl-2-piperidino-1,3-selenazole **1b**: Yield 93%. White crystals. mp: 74–76 °C. IR (KBr): 1624 cm⁻¹. ¹H-NMR (500 MHz, CDCl₃, rt): δ=1.70 (m, 6H, CH₂), 2.43 (s, 3H, CH₃), 3.57 (m, 4H, CH₂), 7.75 (s, 1H, CH). ¹³C-NMR (125 MHz, CDCl₃, rt): δ=23.9, 25.2, 25.3, 51.0, 133.1, 149.7, 178.1, 190.3. ⁷⁷Se-NMR (95 MHz, CDCl₃): δ=522.4. MS (FAB): *m/z*=259 [M⁺+1]. *Anal.* Calcd for C₁₀H₁₄N₂OSe: C, 46.70; H, 5.49; N, 10.89. Found; C, 46.88; H, 5.51; N, 10.70.

5-Acetyl-2-morpholino-1,3-selenazole **1c**: Yield 74%. Pale yellow crystals. mp: 134–135 °C. IR (KBr): 1636 cm⁻¹. ¹H-NMR (500 MHz, CDCl₃, rt): δ=2.45 (s, 3H, CH₃), 3.59 (t, *J*=5.2 Hz, 4H, CH₂), 3.81 (t, *J*=5.2 Hz, 4H, CH₂), 7.77 (s, 1H, CH). ¹³C-NMR (125 MHz, CDCl₃, rt): δ=25.4, 49.5, 65.9, 134.6, 149.0, 178.6, 190.4. ⁷⁷Se-NMR (95 MHz, CDCl₃): δ=532.4. MS (FAB): *m/z*=261 [M⁺+1].

5-Benzoyl-2-dimethylamino-1,3-selenazole **1d**: Yield quantitatively. Yellow crystals. mp: 69–70 °C. IR (KBr): 1603 cm⁻¹. ¹H-NMR (500 MHz, CDCl₃, rt): δ=3.22 (br s, 6H, N-CH₃), 7.43–7.46 (m, 2H, Ar), 7.50–7.53 (m, 1H, Ar), 7.68 (s, 1H, CH), 7.73–7.75 (m, 2H, Ar). ¹³C-NMR (125 MHz, CDCl₃, rt): δ=128.5, 128.7, 131.6, 134.0, 138.6, 152.3, 178.4, 188.3. ¹³C-NMR (125 MHz, CDCl₃, -40 °C): δ=39.0, 43.6, 128.2, 128.4, 131.4, 133.0, 137.8, 152.5, 177.9, 188.2. ⁷⁷Se-NMR (95 MHz, CDCl₃): δ=533.8. MS (CI): *m/z*=281 [M⁺+1]. *Anal.* Calcd for C₁₂H₁₂N₂OSe: C, 51.62; H, 4.33; N, 10.03. Found; C, 51.57; H, 4.65; N, 9.93.

5-Benzoyl-2-piperidino-1,3-selenazole **1e**: Yield quantitatively. Light yellow crystals. mp: 123–125 °C. IR (KBr): 1611 cm⁻¹. ¹H-NMR (500 MHz, CDCl₃, rt): δ=1.71 (m, 6H, CH₂), 3.60 (m, 4H, CH₂), 7.44–7.47 (m, 2H, Ar), 7.51–7.54 (m, 1H, Ar), 7.65 (s, 1H, CH), 7.74–7.76 (m, 2H, Ar). ¹³C-NMR (125 MHz, CDCl₃, rt): δ=24.0, 25.2, 51.1, 128.3, 128.5, 131.4, 132.9, 138.6, 152.3, 178.0, 188.2. ⁷⁷Se-NMR (95 MHz, CDCl₃): δ=532.8. MS (CI): *m/z*=321 [M⁺+1].

5-Benzoyl-2-morpholino-1,3-selenazole **1f**: Yield quantitatively. White crystals. mp: 130–132 °C. IR (KBr): 1612 cm⁻¹. ¹H-NMR (500 MHz, CDCl₃, rt): δ=3.63 (t, *J*=5.2 Hz, 4H, CH₂), 3.82 (t, *J*=5.2 Hz, 4H, CH₂), 7.45–7.48 (m, 2H, Ar), 7.53–7.56 (m, 1H, Ar), 7.68 (s, 1H, CH), 7.75–7.77 (m, 2H, Ar). ¹³C-NMR (125 MHz, CDCl₃, rt): δ=49.6, 66.0, 128.4, 128.6, 131.6, 134.3, 138.3, 151.5, 178.5, 188.3. ⁷⁷Se-NMR (95 MHz, CDCl₃): δ=543.5. MS (CI): *m/z*=323 [M⁺+1].

Bis[2-dimethylamino-5-(1,3-selenazolyl)] Ketone **2a**: 1,3-Dichloroacetone (13 mg, 0.1 mmol) was added to stirred solution of *N,N*-dimethyl-*N'*-(dimethylselenocarbamoyl)formamidine (42 mg, 0.2 mmol) in dry acetonitrile (3 ml) under an argon atmosphere. The reaction mixture was stirred at 50 °C for 1 h. The mixture was cooled off until 25 °C. Then triethylamine (0.10 ml, 0.7 mmol) was added into the mixture. The reaction mixture was stirred for 30 min. at 25 °C. The mixture extracted with dichloromethane. The extract was concentrated to dryness. The residue was purified by flash chromatography on silica gel with ethyl acetate–hexane (1 : 1) to give **2a** (33 mg, 87%) as orange crystals. mp: 225–227 °C. IR (KBr): 1572 cm⁻¹. UV/VIS (CHCl₃) λ_{max} (log ε): 392 (2.74). ¹H-NMR (500 MHz, CDCl₃, rt): δ=3.20 (br s, 12H, N-CH₃), 7.89 (s, 2H, CH). ¹³C-NMR (125 MHz, CDCl₃, rt):

δ=41.2, 132.8, 147.1, 177.1, 178.5. ⁷⁷Se-NMR (95 MHz, CDCl₃): δ=545.4. MS (FAB): *m/z*=379 [M⁺+1].

Bis[2-piperidino-5-(1,3-selenazolyl)] Ketone **2b**: Yield 91%. Orange crystals. mp: 260–262 °C. IR (KBr): 1541 cm⁻¹. UV/VIS (CHCl₃) λ_{max} (log ε): 399 (3.22). ¹H-NMR (500 MHz, CDCl₃, rt): δ=1.71 (m, 12H, CH₂), 3.57 (m, 8H, CH₂), 7.84 (s, 2H, CH). ¹³C-NMR (125 MHz, CDCl₃, rt): δ=24.1, 25.3, 51.0, 132.1, 147.2, 177.1, 178.5. ⁷⁷Se-NMR (95 MHz, CDCl₃): δ=545.5. MS (FAB): *m/z*=459 [M⁺+1].

Bis[2-morpholino-5-(1,3-selenazolyl)] Ketone **2c**: Yield 82%. Orange crystals. mp: 262–263 °C. IR (KBr): 1554 cm⁻¹. UV/VIS (CHCl₃) λ_{max} (log ε): 391 (3.20). ¹H-NMR (500 MHz, CDCl₃, rt): δ=3.59 (t, *J*=5.2 Hz, 8H, CH₂), 3.82 (t, *J*=5.2 Hz, 8H, CH₂), 7.87 (s, 2H, CH). ¹³C-NMR (125 MHz, CDCl₃, rt): δ=49.5, 66.1, 133.2, 146.9, 177.7, 178.7. ⁷⁷Se-NMR (95 MHz, CDCl₃): δ=555.0. MS (FAB): *m/z*=463 [M⁺+1]. *Anal.* Calcd for C₁₅H₁₈N₄O₃Se₂: C, 39.14; H, 3.94; N, 12.17. Found; C, 38.86; H, 4.28; N, 11.97.

Assay of Superoxide Anion-Scavenging Activities (SOSAs) The SOSAs of 2-amino-1,3-selenazoles **1** and bis-(2-amino-5-selenazolyl) ketones **2** were measured by a previously reported method.¹⁸ In brief, the reaction mixture contained 5.8×10⁻⁷ M MCLA, 5×10⁻⁵ M hypoxanthine, xanthine oxidase (6.5 U), and 50 mM Tris–HCl buffer containing 0.1 mM EDTA at pH 7.8, in the presence or absence of various concentrations of compounds **1** and **2**. To evaluate certain SOSAs, we added SOD standard solutions (0.6 to 30 ng/ml) to the reaction mixture instead of compounds **1** and **2**. Total volume was 3.0 ml. Compounds **1** and **2** (25 mM) were dissolved in DMSO and stored at -80 °C prior to use. Chemiluminescence was measured using a luminometer (Aloka, BLR201) at 25 °C. Chemiluminescence measurement was initiated by the addition of 2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one hydrochloride (MCLA) to the standard incubation mixture excluding XOD, continued for 2 min without XOD and for an additional 2 min after addition of XOD. A representative example of a measurement of the effect of **2a** on MCLA-dependent luminescence is shown in Fig. 1. When the compounds had strong SOSAs at 166 μM, we also measured at 4.17, 16.7, 41.7 and 83.3 μM. Percentage of inhibition of MCLA dependent chemiluminescence was calculated as a previously described.¹⁸ Inhibition concentration of 50% (IC₅₀) was calculated by six concentrations of **2a** (4.17, 16.7, 27.7, 41.7, 83.3 and 166 μM). In this study, it was measured twice about the same sample and average value was used.

RESULTS AND DISCUSSION

Structures of these 2-amino-1,3-selenazoles **1** and bis-[2-dimethylamino-5-(1,3-selenazolyl)] ketones **2** are shown in Table 1, where the SOSAs of the compounds also are summarized (Table 1). As results, bis-[5-(1,3-selenazolyl)] ketones **2** showed stronger activities than 1,3-selenazoles **1**. Among them, bis[2-dimethylamino-5-(1,3-selenazolyl)] ketone **2a** and bis[2-morpholino-5-(1,3-selenazolyl)] ketone **2c** had the highest SOSAs at 166 μM (80.0% and 68.6%, respectively). The effect of **2a** was dose dependent (Fig. 1). Compound **2a** was sufficiently active to suggest further testing, by serial dilutions, 50% inhibitory concentrations (IC₅₀) for the

Table 1. Scavenging Activities of 2-Amino-1,3-selenazoles **1** and Bis-[2-amino-5-(1,3-selenazolyl)] Ketones **2** on the Superoxide Radical Anion

Compound	Inhibition (%)
1a	26.8
1b	22.0
1c	29.7
1d	16.6
1e	20.0
1f	10.0
2a	80.0
2b	45.7
2c	68.6

Fig. 1. Effect of Bis[2-dimethylamino-5-(1,3-selenazolyl)] Ketone **2a** on 2-Methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo-[1,2-a]pyrazin-3-one Hydrochloride (MCLA)-Dependent Luminescence

(A) Chemiluminescence inhibition curves by compound **2a**. Arrows indicate the time at which MCLA or xanthine oxidase (XOD) was added. (B) Fifty percent inhibitory concentrations (IC_{50}) for the compound.

compound **2a** were 37.1 μM (Fig. 1). We have systematically synthesized various organic selenium compounds including selenocarbamates, selenoureas, thioureas, and tertiary se-

Fig. 2. Uric Acid Generation in Hypoxanthine-Xanthine Oxidase System

—●—: Control, —□—: with **2a**. Bis[2-dimethylamino-5-(1,3-selenazolyl)] ketone **2a** of 83.3 μM was added to the standard reaction mixture, and generation rate of uric acid was measured by uric acid measurement kit (L type WAKO UA-M; Wako Pure Chemical Industries, Ltd., Osaka, Japan). The data are representative of three separate experiments and shown by the means ± S.E.

lenoamide compounds, and showed their SOSAs using a same chemiluminescence method. IC_{50} of these compounds were approximately a range of 0.1 to 100 μM.^{14–16} Thus, SOSAs of newly synthesized compounds, bis-[5-(1,3-selenazolyl)] ketones **2a** and **2c** had similar SOSAs to that of previously synthesized other organic selenium compounds such as selenocarbamates and selenoureas. The results suggested that bis-(2-amino-5-selenazolyl) ketones **2** are useful organic selenium compounds as one of scavenger of ROSs. We investigated whether bis[2-dimethylamino-5-(1,3-selenazolyl)] ketone **2a** could eliminate super oxide anion generated by XOD or not. For this purpose, the amounts of uric acid generation which is the by-product of superoxide anion in the standard reaction mixture with or without **2a** were measured. The result was shown in Fig. 2. Generation of uric acid was not significantly affected by the addition of relatively high concentration of a compound **2a** (83.3 μM). Thus, it is suggested that our compounds act as not inhibitor of XOD but scavenger of superoxide anion.

In conclusion, the present work shows the SOSAs of bis[2-dimethylamino-5-(1,3-selenazolyl)] ketone **2a**. Future studies will be directed toward elucidating the mechanism of action of the active compounds and toward synthesizing and studying additional analogues of bis[2-dimethylamino-5-(1,3-selenazolyl)] ketone **2a**.

Acknowledgements This work was supported by a Grant-in-Aid for Science Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (No. 15550030 and 17550099) to which we are grateful.

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