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A Long-lived *o*-Semiquinone Radical Anion is Formed from *N*-β-alanyl-5-S-glutathionyl-3,4-dihydroxyphenylalanine (5-S-GAD), an Insect-derived Antibacterial Substance

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***N*-β-Alanyl-5-S-glutathionyl-3,4-dihydroxyphenylalanine (5-S-GAD), an insect-derived antibacterial peptide, generates hydrogen peroxide (H_2O_2) that exerts antitumour activity. We have investigated the precise mechanism of H_2O_2 production from 5-S-GAD by autoxidation aiming to understand its action toward tumour cells. Using the electron spin resonance (ESR) technique, we detected a strong signal due to radical formation from 5-S-GAD. Surprisingly, the ESR signal of the radical derived from 5-S-GAD appeared after incubation for 30 min at 37°C in the buffer at pH 7.4; the signal was persistently detected for 10 h in the absence of catalytic metal ions. The computer simulation of the observed ESR spectrum together with the theoretical calculation of the spin density of the radical species indicates that an *o*-semiquinone radical anion was formed from 5-S-GAD. We demonstrated that H_2O_2 is produced via the formation of superoxide anion ($O_2^{\bullet-}$) by the electron-transfer reduction of molecular oxygen by the 5-S-GAD anion, which is in equilibrium with 5-S-GAD in the aqueous solution. The radical formation and the subsequent H_2O_2 production were inhibited by superoxide dismutase (SOD), when the antitumour activity of 5-S-GAD was inhibited by SOD. Thus, the formation of the *o*-semiquinone radical anion would be necessary for the antitumour activity of 5-S-GAD as an intermediate in the production of cytotoxic H_2O_2.**

Key words: electron spin resonance, hydrogen peroxide, *o*-semiquinone radical anion, superoxide dismutase, 5-S-GAD.

Abbreviations: 5-S-GAD, N-β-alanyl-5-S-glutathionyl-3,4-dihydroxyphenylalanine; H_2O_2, hydrogen peroxide; $O_2^{\bullet-}$, superoxide anion; SOD, superoxide dismutase; GSH, reduced glutathione.

N-β-Alanyl-5-S-glutathionyl-3,4-dihydroxyphenylalanine (5-S-GAD) was originally isolated as an inducible antibacterial peptide from infected *Sarcophaga peregrina* adults (*1*). The major components of 5-S-GAD are glutathione and β-alanyl-L-dopa, which are covalently conjugated by catechol oxidase during bacterial infection. 5-S-GAD was found to exert antitumour activity against some human melanomas and breast carcinomas *in vitro* and *in vivo* (*2*). We recently found that 5-S-GAD produces hydrogen peroxide (H_2O_2) (*3*) and exhibits selective cytotoxicity toward certain tumour cells with low levels of antioxidant molecules, namely, catalase (EC1.11.1.6) (*3*, *4*). The inhibitory effect of 5-S-GAD on the growth of MDA-MB-435S, a breast cancer cell line with low levels of catalase, was effectively blocked by the addition of catalase or reduced glutathione (GSH) (*3*). These results were reasonable since catalase and GSH detoxified H_2O_2 that was produced from 5-S-GAD. Furthermore, we found that exogenously added superoxide dismutase (CuZnSOD from bovine erythrocytes, EC1.15.1.1) also cancelled the effect of 5-S-GAD (*3*). Generally, the SOD enzyme is known to dismutate the superoxide anion ($O_2^{\bullet-}$) to H_2O_2; therefore, we assumed that $O_2^{\bullet-}$ might be associated with cytotoxicity of 5-S-GAD. However, the precise mechanisms by which SOD cancels the effect of 5-S-GAD are yet to be clarified.

The cytotoxic activity of compounds L-dopa and L-dopamine that possess a catechol moiety such as 5-S-GAD has been reported (*5–9*). The toxic mechanisms of L-dopa and L-dopamine were speculated to involve the generation of some reactive oxygen species (ROS) such as superoxide radicals, H_2O_2, and hydroxyl radicals; these

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ROS may be produced via autoxidation of the *o*-dihydoxyl groups and formation of *o*-semiquinone radicals. These ROS may adversely affect cellular functions, thereby causing cell death. It was suggested that the cytotoxic effects of L-dopamine were associated with the generation of *o*-semiquinone radicals by autoxidation (*8*, *9*), and the generation of these radicals was prevented by adding SOD (*8*).

In this study, we attempted to clarify the autoxidation process of 5-S-GAD that is closely associated with its antitumour activity. We found that 5-S-GAD formed a unique *o*-semiquinone radical anion that slowly and persistently appeared under physiological conditions without any redox-active metal ions. We investigated the precise mechanism of the radical formation and subsequent H_2O_2 production from 5-S-GAD. The results obtained in this study suggest that the radical formation accompanied by H_2O_2 production from 5-S-GAD would be an indispensable step for its antitumour activity.

MATERIALS AND METHODS

Chemicals—5-S-GAD was synthesized as reported previously (*1*). L-Dopamine was purchased from Nacalai Tesque (Kyoto, Japan), and bovine erythrocyte catalase was obtained from Wako Pure Chem. Ind. Ltd (Osaka, Japan). CuZnSOD from bovine erythrocytes, manganese- or iron-containing isozymes from *Escherichia coli*, L-dopa, GSH, and PeroxiDetect^{TM} kit were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The RPMI 1640 culture medium was obtained from Invitrogen Corp., (Carlsbad, CA, USA). All buffers and solutions except the RPMI 1640 medium were made using certified distilled water (Otsuka Pharmaceutical Co., Tokyo, Japan) to avoid the effect of metal ions.

Electron Spin Resonance Measurements—The electron spin resonance (ESR) signal intensity of radicals derived from 5 mM 5-S-GAD, L-dopamine or L-dopa was determined at 25°C in 0.1 M Tris–HCl (pH 7.4, 8.0 or 8.5) or in 0.1 M $NaHCO_3/Na_2CO_3$ buffer (pH 9.0 or 9.5) by ESR spectroscopy (JEOL JES-RE1X, X-band, 100 kHz modulation frequency) in the ESR flat cell (Radical Research Inc., Tokyo, Japan) (*9*, *10*). 5-S-GAD, L-dopamine, or L-dopa was dissolved in distilled water at a concentration of 10 mM and mixed with 0.2 M buffered solution in a volume ratio of 1:1. Prior to the measurement, the solutions (pH 7.4) were incubated at 37°C for the specified time indicated in the figures. To detect superoxide radicals using spin trapping tequnique, we added 15 μl of 8% DMPO solution to the 5 mM 5-S-GAD solution (pH 7.4) just before the measurement. For examining the effects of antioxidants on radical formation, the 5-S-GAD solutions (pH 7.4) were incubated at 37°C in the presence of antioxidants for 30 min. CuZnSOD was inactivated by overnight incubation in 1 M H_2O_2 (pH 10). The ESR measurement settings were as follows: centre field, 335 ± 5 mT; microwave power, 8 mW; modulation amplitude, 0.1 mT; gain, $1.25–6.3 \times 100$; time constant, 0.03 s; and scanning time, 2 min. The signal intensity was defined as the ratio of peak heights of these radicals to that of an Mn^{2+} marker. The radical's *g*-value was calculated by interpolation between the Mn^{2+} signals.

Computer simulation of the ESR spectra was carried out using a program (Winsim) developed in National Institutes of Health, MD, USA (*11*). To obtain the experimental signals derived from 5-S-GAD for comparing with the computer simulation signals, the measurement setting was modified as follows: modulation amplitude, 0.0032 mT; gain, 400; scanning time, 4 min.

To detect radical intermediates involved in the autoxidation of 5-S-GAD, the direct detection of superoxide radicals was carried out in the frozen solution at −196°C (*12*). 5-S-GAD (5 mM) solution in a deaerated $NaHCO_3/Na_2CO_3$ buffer (pH 9.5) was incubated at 0°C. Then the reaction of 5-S-GAD radical anion was started with O_2 bubbling, the ESR spectrum was recorded at −196°C. The measurement setting of modulation amplitude was 0.5 mT. The radicals' *g*-values are directly determined via the field strength and frequency, 9.255 GHz.

Theoretical Calculation—Density-functional theory (DFT) calculations were performed on a Compaq DS20E computer. Geometry optimizations were carried out using the B3LYP functional and 6-31G* basis set with the unrestricted Hartree-Fock (UHF) formalism as implemented in the Gaussian 03 program.

Assay for H_2O_2 Production—5-S-GAD was dissolved in sodium carbonate buffer (pH 9.5) or phosphate buffered saline (PBS, pH 7.4) at a final concentration of 80 μM, and the solutions were incubated at room temperature (pH 9.5) or at 37°C (pH 7.4) for the specified time indicated in the figures. For examining the effect of antioxidants on H_2O_2 production from 5-S-GAD, the RPMI 1640 culture medium containing 30 μM of 5-S-GAD was incubated with or without antioxidant molecules for 24 h. The H_2O_2 concentration in the medium was determined by the PeroxiDetect^{TM} kit (*3*). In brief, 10 μl of the culture supernatant was mixed with 100 μl of the reagent in the kit and incubated for 30 min at room temperature. The absorbance at 570 nm was measured, and the H_2O_2 concentration in the culture medium was determined by a standard curve.

Spectral Measurements—UV-visible absorption spectra of 5-S-GAD in the buffer solution (pH 9.5–7.4) were measured using a DU® 640 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA, USA).

Electrochemical Measurements—The second-harmonic alternating current voltammetry (SHACV) measurements (*13*) of 5-S-GAD were performed on an ALS 630A electrochemical analyzer in deaerated 0.1 M buffer solutions (pH 9.5 or 7.4) at 25°C. The gold working electrode was polished with a BAS polishing alumina suspension and rinsed with acetone before use. A platinum wire was used as the counter electrode. The measured potentials were recorded with respect to an Ag/AgCl reference electrode.

RESULTS

Identification of the Radical Species Derived from 5-S-GAD—To investigate the autoxidation mechanism of 5-S-GAD, we first attempted to detect radical species generated from 5-S-GAD by ESR spectroscopy.

As reported previously (*8–10*), we detected a signal of *o*-semiquinone radicals that were formed from L-dopamine (Fig. 1A, left) or L-dopa (data not shown) in the buffer at pH 9.5; the signal was characterized by four lines. Under the same experimental conditions, the ESR signals originating from 5-S-GAD were more complicated and stronger (Fig. 1A, right) than those from L-dopamine or L-dopa. Interestingly, the signals from L-dopamine or L-dopa rapidly decayed during the measurements, whereas the signals from 5-S-GAD were stronger and more persistent. The maximum signal intensity of 5-S-GAD was observed at 2 min after the 5-S-GAD solution was mixed with the buffered solution at pH 9.5. This signal intensity derived from 5-S-GAD was approximately ten times larger than that from L-dopamine under the same experimental conditions.

The *g*-value for the signal from 5-S-GAD was 2.0051 (Fig. 1B, top), which seemed a little higher than L-dopamine or L-dopa (2.0037–2.0046) due to the influence of the sulphur ring substituent (*14*, *15*). As shown in Fig. 1B, the observed hyperfine structure of the ESR spectrum was well reproduced by the computer simulation estimated containing two species, 43% with four hyperfine coupling constants (*hfc*): $a_H = 0.382$, 0.305, 0.215 and 0.029 mT, 57% with four *hfc*: $a_H = 0.308$, 0.253 and two 0.145. In order to assign the *hfc* values, the DFT calculation was performed for the radical species derived from 5-S-GAD (Fig. 1C). The observed ESR spectrum may be due to an *o*-semiquinone radical anion of 5-S-GAD, which is known to be much more stable than the neutral radical. By comparing the *hfc* values with the spin densities calculated by the DFT

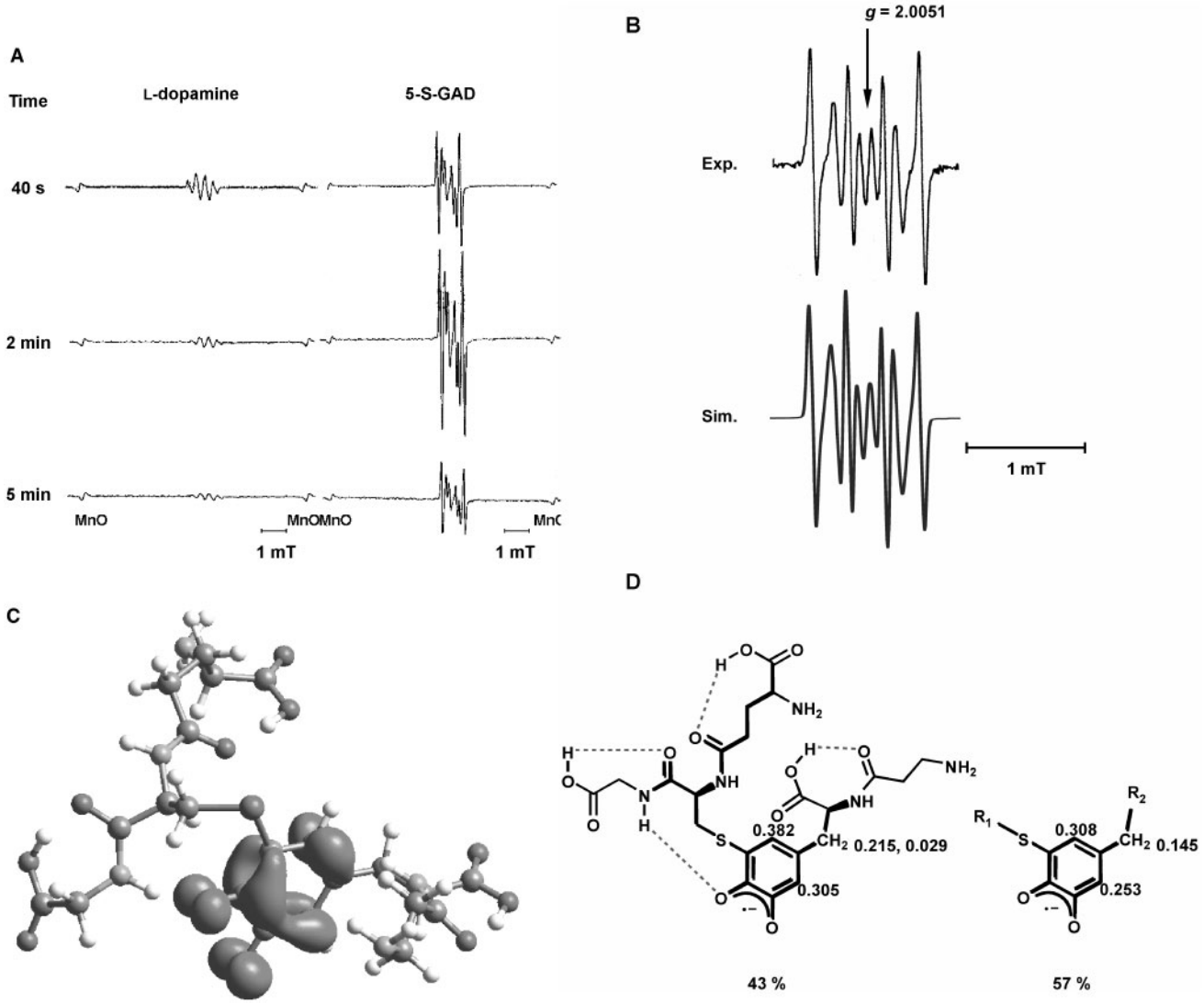


Fig. 1. **Identification of the radical species derived from 5-S-GAD** (A) Time course of the ESR spectra obtained from 5 mM 5-S-GAD or L-dopamine in 0.1 M sodium carbonate buffer with pH adjusted to 9.5. (B) Top, the ESR spectrum obtained from 5-S-GAD in sodium carbonate buffer at pH 9.5. The *g*-value was calculated by interpolation between the Mn^{2+} signals. Bottom, the computer-simulated spectrum. (C) Spin distribution of 5-S-GAD radical anion calculated by the DFT method (B3LYP/6-31G* basis set). (D) The hyperfine coupling constants for the two estimated *o*-semiquinone radical anions derived from 5-S-GAD (in mT), and the estimated intramolecular hydrogen bonding of the 5-S-GAD radical anion (dotted line).

for the *o*-semiquinone radical anion of 5-S-GAD, we have assigned the *hfc* values as shown in Fig. 1D. The *hfc* values of benzyl protons can be intrinsically magnetically non-equivalent, because they are adjacent to a chiral centre (Fig. 1D, left). However, the simulation spectrum by assuming 57% of the radical anion with the equivalent *hfc* values of the benzyl protons seemed closely approximate to the experimental spectrum. Therefore, we speculate the existence of a different conformer (rotamer) of the radical anion. The β-alanyl moiety of the radical anion may be rotated even though there are four intramolecular hydrogen bondings, resulting in conformational changes and the modulation of the $-CH_2-$ splittings (Fig. 1D, right).

We expected that this radical formation occurred by the autoxidation of 5-S-GAD, and other unstable radicals could be formed from molecular oxygen (*16*, *17*). We first tried to detect superoxide anion by the spin-trapping technique using DMPO. Superoxide radical anion is readily trapped by DMPO and quickly protonated to DMPO-OOH in the buffered solution. However, we had not observed a distinctive spectral pattern of DMPO–OOH (data not shown). This is probably because DMPO can react with other organic radicals, not only with superoxide radical anion. Next, we attempted to measure ESR at −196°C to identify $O_2^{\bullet-}$ (*12*). We expected that the superoxide anion could be directly detected using this method, and the signals could be more discriminative than using the spin trapping ones in case of quinone compounds. As shown in Fig. 2, a characteristic ESR spectrum of $O_2^{\bullet-}$ having a $g_{||}$-value of 2.0945 was observed for the frozen buffer solution (pH 9.5) containing 5-S-GAD (5 mM) at −196°C. This result was well reproducible. In addition, strong signals were observed with *g*-values of 2.0700, 2.0511 and 2.0049. We deduced that these signals were derived from 5-S-GAD semiquinone radical anion (*14*), possibly, and/or its decompositions.

When oxygen was removed from the reaction buffer by bubbling N_2, the *o*-semiquinone radical anion derived from 5-S-GAD was hardly detected (data not shown). In the buffer at pH 9.5 the amount of O_2 was limited by sealing the ESR cell, the weak signal of 5-S-GAD-radical anion was stable for 1 h at 25°C (data not shown). These results indicate that the production and the decay of the radical anion are attributed to the reaction with oxygen.

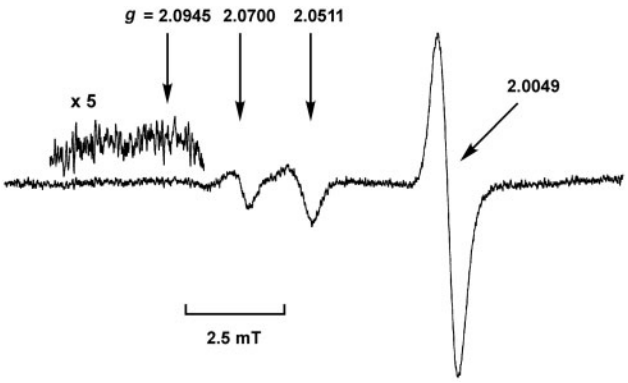


Fig. 2. **ESR spectrum of $O_2^{\bullet-}$ generated from a buffer solution (pH 9.5) containing 5-S-GAD (5 mM) at −196°C.** The signal assigned to $O_2^{\bullet-}$ ($g_{||} = 2.0945$) was five-times magnified. The major signals with $g_1 = 2.0700$, $g_2 = 2.0511$, and $g_3 = 2.0049$ will be assigned to 5-S-GAD radical anion and/or its decompositions. The radicals' *g*-values are directly determined via the field strength and frequency, 9.255 GHz.

The Radical Formation is Dependent on pH—Next, we examined the dependency of signal intensity on pH (Fig. 3A). The ESR signals appeared more slowly with a decrease in pH, and the maximum signal intensity decreased. Under physiological conditions at pH 7.4, 5-S-GAD produced signals after incubation at 37°C for 30 min (Fig. 3B), while no signal was detected after incubation for 6 h at 25°C (data not shown). Therefore, the 5-S-GAD radical formation was apparently accelerated by incubation at 37°C. No signal was observed from L-dopamine or L-dopa under the same experimental conditions at pH 7.4 (data not shown). The ESR signal derived from 5-S-GAD at 37°C was persistently detected for 10 h, and the relative signal intensity to that of an Mn^{2+} was about one during this period. Therefore, both of the radical formation and decay seemed in equilibrium under these conditions. After incubation for 24 h, the signal from 5-S-GAD was completely disappeared.

It was well known that alkaline conditions and trace metals strongly catalyse the oxidation of the catechol compounds (*5*, *18*). To avoid the catalytic effect of redox-active metal ions, we used commercial distilled water, which was certified containing no metal ions, to make all buffers and solutions (except the below-mentioned RPMI1640 medium). We observed that added copper metal ions enhanced the radical formation, and facilitated H_2O_2 production from 5-S-GAD (data not shown).

To confirm whether the mechanism of ROS formation from 5-S-GAD is dependent on pH, we first attempted to investigate the p*K*a value of 5-S-GAD by UV-visible spectroscopy (Fig. 4A). In the buffer at pH 7.4, 5-S-GAD showed an absorption band at 293 nm, whereas in the buffer at pH 9.5, the absorption band shifted to 308 nm. Such a red shift in the absorption band indicates that 5-S-GAD undergoes deprotonation at one of the two phenolic OH groups and exists mainly as the corresponding mono-anion form (phenolate anion) at pH 9.5. The isosbestic point was at 293 nm. Based on the absorption band shift, the observed p*K*a value of 5-S-GAD was approximately 8.6. This value appears to be reasonable because the p*K*a values of each functional group of L-dopa, as a reference compound, were reported to be 9.2–9.7 (3-OH) and 11.5–13.4 (4-OH) (*19–21*). The glutathionyl residue at position five possibly acts as an electron withdrawing group (*22*); therefore, the actual p*K*a values of 5-S-GAD may be lower than those of L-dopa.

Next, we examined the effect of pH on the one-electron oxidation potential (E^0_{ox}) of 5-S-GAD by SHACV (*13*) (see MATERIALS AND METHODS) because the first process of formation of a mono-anion and reaction with oxygen might be pH dependent. The E^0_{ox} value of 5-S-GAD in the buffer at pH 7.4 was determined to be 0.87 V and that at pH 9.5 was 0.24 V versus Ag/AgCl (Fig. 4B). These results indicate that the 5-S-GAD anion at pH 9.5 is a considerably stronger electron donor

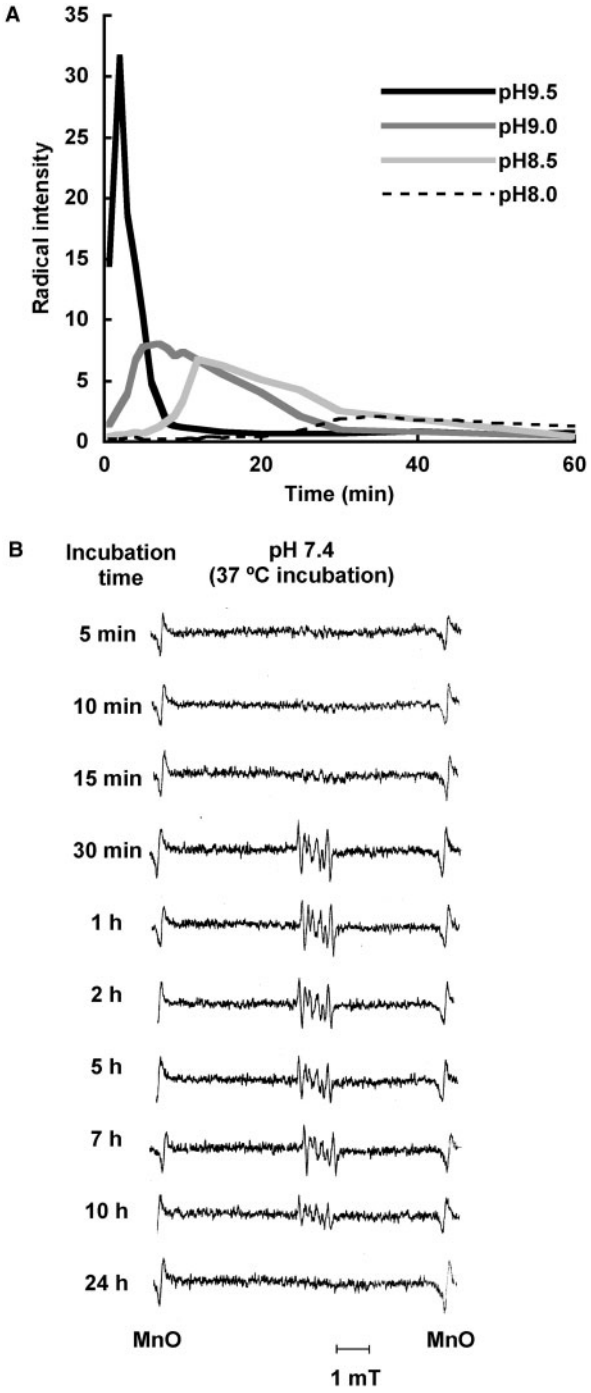


Fig. 3. **Time- and pH- dependent ESR signal intensity of 5-S-GAD in the buffered solution.** (A) Samples containing 5mM 5-S-GAD in the buffer at pH 8.0–9.5 were incubated at 25°C. Signal intensity was defined as the ratio of the second peak height of 5-S-GAD to the peak height of an Mn^{2+} marker. (B) Time course of the ESR spectra obtained from 5 mM 5-S-GAD in 0.1 M Tris-HCl buffer at pH 7.4 after incubation at 37°C.

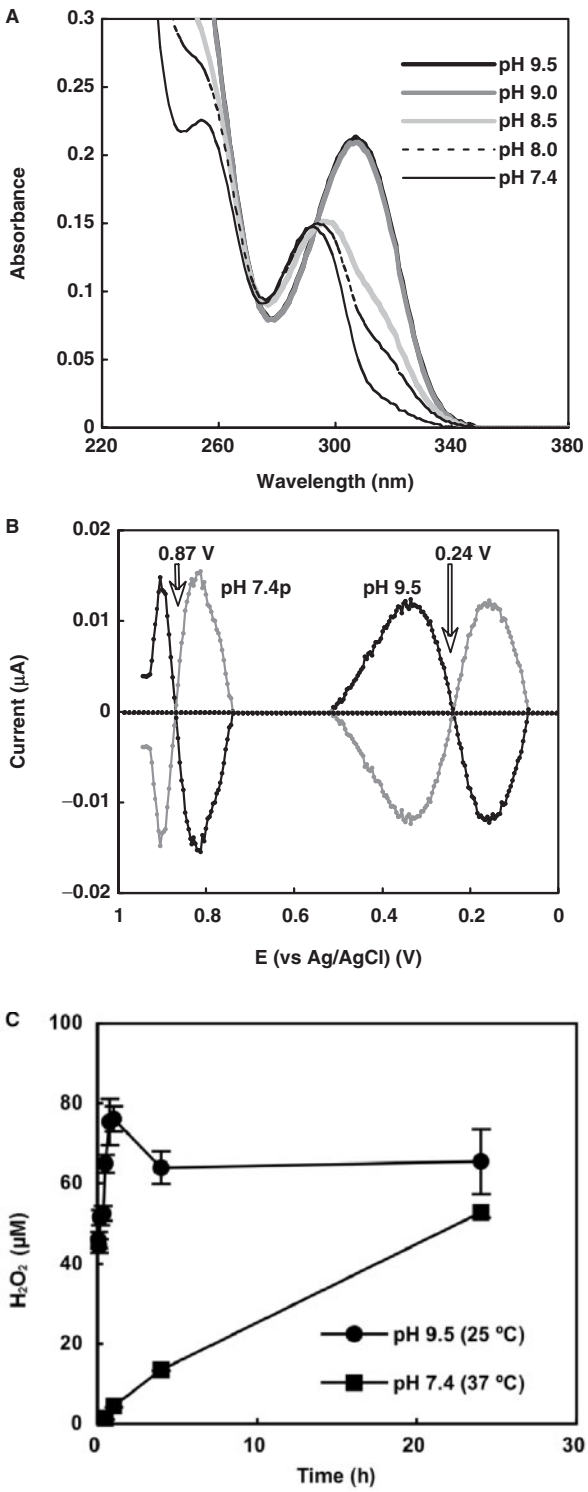


Fig. 4. **The chemical properties of 5-S-GAD dependent on pH.** (A) UV-visible spectra of 5-S-GAD in the buffer at various pH. (B) SHACVs of 5-S-GAD in deaerated 0.1 M buffer solutions (pH 9.5 or 7.4) at 25°C. Scan rate, 4 mV/s; working electrode, Au; phase shift, 160 (grey)/340 (black) degree (pH 9.5) and 170 (grey)/350 (black) degree (pH 7.4). (C) The time course of H_2O_2 production from 80 μM 5-S-GAD was dependent on pH. The amount of H_2O_2 was estimated by a standard using the PeroxiDetect™ kit. Data shown are the average of triplicates with error bars (±SD).

than the parent 5-S-GAD at pH 7.4, when the formation of the *o*-semiquinone radical anion from 5-S-GAD at pH 9.5 was considerably faster than that at pH 7.4. On the other hand, the deprotonation of 5-S-GAD has hardly occurred in the buffer solution at pH 7.4.

Thus, the deprotonation of 5-S-GAD becomes the rate-determining step for the generation of the *o*-semiquinone radical anion from 5-S-GAD.

Furthermore, we verified that the rate of producing H_2O_2 from 5-S-GAD was dependent on pH (Fig. 4C). At pH 9.5, H_2O_2 production from 5-S-GAD was rapid and reached a maximum level after incubation for 1 h at room temperature. On the other hand, H_2O_2 production from 5-S-GAD at pH 7.4 gradually increased during the incubation period at 37°C for 24 h. Thus, the rate of pH-dependent H_2O_2 production from 5-S-GAD was in parallel with that of radical formation. The total amount of H_2O_2 produced from 5-S-GAD was almost equivalent to that of the starting material (80 μM 5-S-GAD). We confirmed that this reaction was completely inhibited by adding 10 μg/ml catalase (data not shown) (*3*).

Effects of Antioxidant Molecules on Radical Formation and H_2O_2 Production from 5-S-GAD—We examined the effects of antioxidant molecules on radical formation from 5-S-GAD, because the radical formation from L-dopamine was prevented by SOD (*5*, *8*, *16*). As shown in Fig. 5A, the addition of 10 μg/ml CuZnSOD to 5 mM 5-S-GAD apparently inhibited radical formation in the buffer at physiological pH after incubation for 30 min. CuZnSOD actually delayed rather than inhibited the reaction, because a radical signal generated from 5-S-GAD appeared after incubation for 40 min (data not shown) (*16*). The same results were obtained by the addition of Iron- or Manganese-containing SOD enzymes under the same conditions (data not shown). Radical formation was completely suppressed by adding 2 mM GSH due to inhibition of deprotonation from 5-S-GAD (Fig.5A). However, neither 10–100 μg/ml catalase nor 10 μg/ml inactivated CuZnSOD inhibited radical formation from 5-S-GAD.

The effect of antioxidant molecules on H_2O_2 production from 5-S-GAD was also investigated (Fig. 5B) in order to clarify the relationship between radical formation and the production of H_2O_2 from 5-S-GAD. This experiment was also designed assuming the cell culture conditions when 5-S-GAD revealed cytotoxicity, which was inhibited by adding CuZnSOD (*3*). We observed that the rate of H_2O_2 production from 5-S-GAD in the RPMI1640 culture medium was about 1.5 times faster than that in the PBS(–) (data not shown) due to some catalytic components in the medium (*5*, *18*). 5-S-GAD apparently produced H_2O_2 in the culture medium after incubation for 24 h, as previously reported (*3*). When either 1 μg/ml catalase or 2 mM GSH was added to the solution, H_2O_2 was completely eliminated due to enzymatic decomposition or non-enzymatic reduction. Although CuZnSOD did not scavenge H_2O_2, it decreased H_2O_2 production from 5-S-GAD in a dose-dependent manner. These results indicate that radical formation from 5-S-GAD via the formation of $O_2^{\bullet -}$ may be essential for H_2O_2 production, which is intimately associated with its antitumour activity.

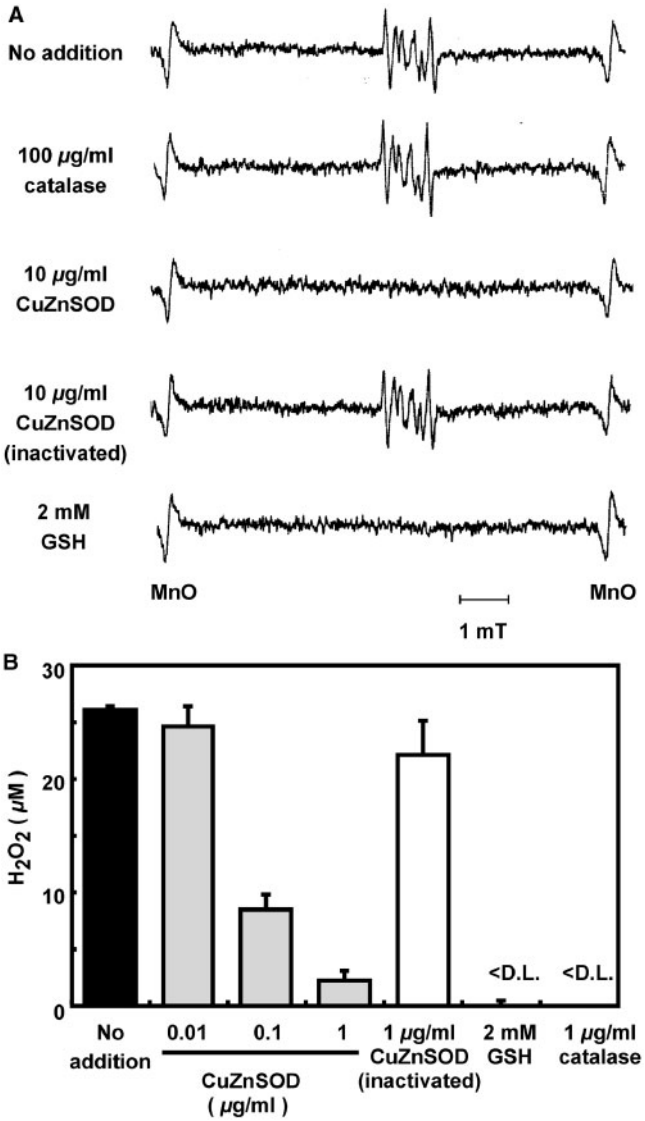
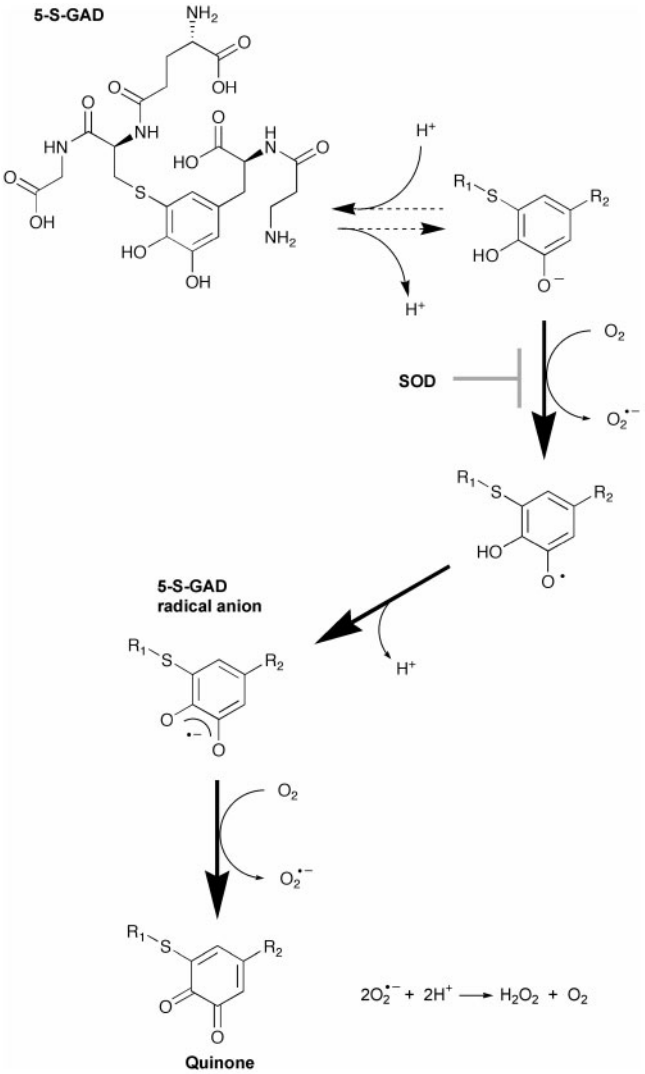


Fig. 5. **Effects of antioxidants on the autoxidation of 5-S-GAD.** (A) Effects of antioxidants on radical formation from 5-S-GAD at physiological pH. 5-S-GAD (5 mM) at pH 7.4 was incubated at 37°C for 30 min with catalase, CuZnSOD, inactivated CuZnSOD or GSH; the spin adduct of the 5-S-GAD-semiquinone radical anion was then recorded. (B) Effects of antioxidants on the production of hydrogen peroxide from 5-S-GAD in the RPMI 1640 culture medium. 5-S-GAD (30 μM) was incubated at 37°C for 24 h in the presence or absence of antioxidant molecules. The figure shows error bars + SD (in triplicates). <D.L. represents values below the detection limit.

DISCUSSION

In this report, we investigated the autoxidation mechanism of 5-S-GAD via radical formation prior to H_2O_2 generation aiming to understand the antitumour activity of 5-S-GAD. We found a unique radical signal that was generated from 5-S-GAD and demonstrated that the generation of an *o*-semiquinone anion from 5-S-GAD plays an important role in the production of H_2O_2.

We propose a mechanism of H_2O_2 production via the formation of a radical from 5-S-GAD (Scheme 1). First, whole and deprotonated 5-S-GAD would be equilibrated in a buffered solution. We assume that a monoanion form of 5-S-GAD reacts with molecular oxygen, thereby generating a $O_2^{\bullet -}$ and an *o*-semiquinone radical (anion). Subsequently, on reaction with oxygen, the



Scheme 1. **Speculated mechanisms of radical formation and H_2O_2 production from 5-S-GAD.**

o-semiquinone anion would form orthoquinone. Finally, $O_2^{\bullet-}$ will undergo rapid conversion to H_2O_2 and O_2.

Our previous reports indicated that H_2O_2 production was necessary for the antitumour activity of 5-S-GAD against a particular tumour cell line because exogenously added catalase or endogenously transfected catalase inhibited the antitumour activity of 5-S-GAD (*3*, *4*). In addition, exogenously added CuZnSOD was reported to cancel the effect of 5-S-GAD on tumour growth (*3*); this indicated that some ROS were involved in the antitumour effects of 5-S-GAD. Here, we have provided conclusive evidence that CuZnSOD clearly suppressed H_2O_2 production as well as radical formation from 5-S-GAD. Therefore, we concluded that radical formation from 5-S-GAD would be an indispensable step for the production of H_2O_2 and for the inhibitory effects of 5-S-GAD on tumour growth. However, we had determined in a previous study that H_2O_2 production from 5-S-GAD was essential for causing cell death since catalase completely inhibited this process. Other radicals, including 5-S-GAD-semiquinone anion and $O_2^{\bullet-}$, may act as intermediates in H_2O_2 production.

However, the precise mechanism by which CuZnSOD apparently inhibits (or delays) both radical formation and H_2O_2 production from 5-S-GAD (Fig. 5) remains unknown. It is very puzzling that CuZnSOD apparently prevents H_2O_2 production (Fig. 5B) because this enzyme is well known for its action in the accumulation of H_2O_2 by the disproportionation of $O_2^{\bullet-}$. However, CuZnSOD has also been reported to prevent the autoxidation of various hydroquinone compounds and functions as a superoxide:semiquinone oxidoreductase (*5*, *8*, *16*, *23–25*). Therefore, we deduce that CuZnSOD may not only reduce semiquinone radicals again but would also scavenge $O_2^{\bullet-}$ (Scheme 1). In fact, the enzymatic action of CuZnSOD was dependent on time, pH, and the ratio of its concentration to that of the substrates (5-S-GAD). It appears that 1 μg/ml CuZnSOD was sufficient to inhibit the H_2O_2 production from 30 μM 5-S-GAD after incubation for 24 h (Fig. 5B). However, catalysation of the 5-S-GAD-semiquinone radical substrates occurred only when the amount of the enzyme was sufficient, and the enzyme has no effect on 50–100 μM 5-S-GAD (data not shown) when the amount of substrate exceeded that of the enzyme. These results indicated that the 5-S-GAD anion was the direct substrate for the enzyme because the rate of the catalytic reaction was determined by the ratio of the concentration of substrates to that of the enzyme.

One novel finding in this report was the unique profile of the 5-S-GAD anion. Surprisingly, the ESR signal intensity of the *o*-semiquinone anion derived from 5-S-GAD was 10 times greater than that from L-dopamine or L-dopa. In addition, the ESR spectrum of the 5-S-GAD anion was observed after incubation at 37°C for 30 min in the buffer (pH 7.4) lacking metal ions, and the ESR signals derived from 5-S-GAD persisted for a period of up to 10 h. However, no signal was observed from L-dopamine or L-dopa under the same experimental conditions. Therefore, the strong signal intensity and the long-lived signal stability of *o*-semiquinone anions from 5-S-GAD should be associated with the structure of the *N*-β-alanyl residue and the 5-S-glutathionyl moiety. On the basis of computer simulations, we proposed that the observed ESR spectrum was attributed to a mixture of the two different conformers of 5-S-GAD anion. We assumed that the rotamers would exist due to the conformational alterations of the β-alanyl group. On the basis of the DFT calculations, we also speculate the existence of a high rotational barrier of the 5-S-glutathionyl group derived from intramolecular hydrogen bonds, particularly due to the bonding between the NH group of the glutathionyl moiety and one of the orthoquinoic oxygens. Both the conformational flexibility of the β-alanyl residue and the hindered rotation of the glutathionyl chain may contribute to the stability of the 5-S-GAD anion. We believe that the stability of the intermediates during the autoxidation of 5-S-GAD would have a very important role in the rate of H_2O_2 production and/or its selective cytotoxicity. Structural studies on the side chains of 5-S-GAD are currently underway to investigate the radical stability, rate of H_2O_2 production and antitumour activity.

5-S-GAD was originally isolated as an inducible antibacterial peptide from infected *S. peregrina* adults (*1*). The characteristic constituents of 5-S-GAD are glutathione and β-alanyl-L-dopa, which are covalently conjugated by catechol oxidase during bacterial infection. We postulate that 5-S-GAD kills bacteria by releasing H_2O_2 at the infection sites, although the location of 5-S-GAD synthesis and the mechanisms by which it releases H_2O_2 remain unknown. However, it is possible that some bacterial oxidases may participate in the synthesis; further, their amine residues or chelating metal ions may promote the deprotonation of 5-S-GAD, and the resulting anion may reduce O_2 to produce $O_2^{\bullet-}$ (H_2O_2) at the region of localization.

Finally, the chemical property of 5-S-GAD is very intriguing with respect to the formation of a stable radical in nature. The formation of a stable anion from 5-S-GAD under physiological conditions may be useful as a radical scavenger for trapping free radicals, which cause oxidative stress in adverse conditions such as aging, cancer and arteriosclerosis. A new pharmacological effect of 5-S-GAD as a radical scavenger may be worthy of further examination (*26*).

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REFERENCES

1. Leem, J.Y., Nishimura, C., Kurata, S., Shimada, I., Kobayashi, A., and Natori, S. (1996) Purification and characterization of N-β-alanyl-5-S-glutathionyl-3, 4-dihydroxyphenylalanine, a novel antibacterial substance of Sarcophaga peregrina (flesh fly). *J. Biol. Chem.* **271**, 13573–13577
2. Akiyama, N., Hijikata, M., Kobayashi, A., Yamori, T., Tsuruo, T., and Natori, S. (2000) Anti-tumor effect of *N*-β-alanyl-5-S-glutathionyl-dihydroxyphenylalanine (5-S-GAD), a novel anti-bacterial substance from an insect. *Anticancer Res.* **20**, 357–362
3. Akiyama, N. and Natori, S. (2003) Involvement of H_2O_2 and $O_2^{\bullet-}$ in the cytotoxicity of *N*-β-alanyl-5-S-glutathionyl-3, 4-dihydroxyphenylalanine (5-S-GAD), a novel insect-derived anti-tumor compound. *Cancer Sci.* **94**, 400–404
4. Nishikawa, T., Nishikawa, S., Akiyama, N., and Natori, S. (2004) Correlation between the catalase level in tumor cells and their sensitivity to *N*-β-alanyl-5-S-glutathionyl-3, 4-dihydroxyphenylalanine (5-S-GAD). *J. Biochem.* **135**, 465–469
5. Bindoli, A., Rigobello, M.P., and Deeble, D.J. (1992) Biochemical and toxicological properties of the oxidation products *o*-catecholamines. *Free Rad. Biol. Med.* **13**, 391–405
6. Basma, A.N., Morris, E.J., Nicklas, W.J., and Geller, H.M. (1995) L-Dopa cytotoxicity to PC12 cells in culture is via its autoxidation. *J. Neurochem.* **64**, 825–832
7. Lai, C.T. and Yu, P.H. (1997) Dopamine- and L-beta-3, 4-dihydroxyphenylalanine hydrochloride (L-Dopa)-induced cytotoxicity towards catecholaminergic neuroblastoma SH-SY5Y cells. Effects of oxidative stress and antioxidative factors. *Biochem. Pharmacol.* **53**, 363–372
8. Haque, M.E., Asanuma, M., Higashi, Y., Miyazaki, I., Tanaka, K., and Ogawa, N. (2003) Apoptosis-inducing neurotoxicity of dopamine and its metabolites via reactive quinone generation in neuroblastoma cells. *Biochim. Biophys. Acta* **1619**, 39–52
9. Kawase, M., Motohashi, N., Kurihara, T., Inagaki, M., Satoh, K., and Sakagami, H. (1998) Relationship between radical intensity and cytotoxic activity of dopamine-related compounds. *Anticancer Res.* **18**, 1069–1074
10. Sakagami, H., Satoh, K., Ida, Y., Hosaka, M., Arakawa, H., and Maeda, M. (1998) Interaction between sodium ascorbate and dopamine. *Free Rad. Biol. Med.* **25**, 1013–1020
11. Duling, D.R. (1994) Simulation of multiple isotropic spin-trap EPR spectra. *J. Magn. Reson. B* **104**, 105–110
12. Bagchi, R.N., Bond, A.M., Scholz, F., and Stösser, R. (1989) Characterization of the ESR spectrum of the superoxide anion in the liquid phase. *J. Am. Chem. Soc.* **111**, 8270–8271
13. Bond, A.M. and Smith, D.E. (1974) Direct measurement of $E^{r}_{1/2}$ with reversible EC electrode processes by second harmonic alternating current polarography and voltammetry. *Anal. Chem.* **46**, 1946–1951
14. Sealy, R.C., Hyde, J.S., Felix, C.C., Menon, I.A., Prota, G., Swartz, H.M., Persad, S., and Haberman, H.F. (1982) Novel free radicals in synthetic and natural pheomelanins: distinction between dopa melanins and cysteinyldopa melanines by ESR spectroscopy. *Proc. Natl Acad. Sci. USA* **79**, 2885–2889
15. Dzierżęga-Lęcznar, A., Pilawa, B., Stępień, K., and Wilczok, T. (1997) EPR studies of synthetic pheo-and mixed type-melanins. *Nukleonika* **42**, 343–352
16. Bors, W., Michel, C., Saran, M., and Lengfelder, E. (1978) The involvement of oxygen radicals during the autoxidation of adrenalin. *Biochim. Biophys. Acta* **540**, 162–172
17. Bolton, J.L., Trush, M.A., Penning, T.M., Dryhurst, G., and Monks, T.J. (2000) Role of quinines in toxicology. *Chem. Res. Toxicol.* **13**, 136–160
18. Gillette, J.R., Watland, D., and Kalnitsky, G. (1954) The catalysis of the oxidation of some dihydroxybenzene derivatives by various metallic ions. *Biochim. Biophys. Acta* **15**, 526–532
19. Karpel, R.L., Kustin, K., Kowalak, A., and Pasternack, R.F. (1971) Relaxation spectra of L-phenylalanine- and L-dopa (3,4-dihydroxyphenylalanine)-copper(II) complex. *J. Am. Chem. Soc.* **93**, 1085–1087
20. Pizer, C. and Babcock, L. (1977) Mechanism of the complexation of boron acids with catechol and substituted catechols. *Inorg. Chem.* **16**, 1677–1681
21. Borgias, B.A., Cooper, S.R., Koh, Y.B., and Raymond, K.N. (1984) Synthetic, structural, and physical studies of titanium complexes of catechol and 3,5-di-*tert*-butylcatechol. *Inorg. Chem.* **23**, 1009–1016
22. McDaniel, D.H. and Brown, H.C. (1958) An extended table of Hammett substituent constants based on the ionization of substituted benzoic acids. *J. Org. Chem.* **23**, 420–427
23. Cohen, G. and Heikkila, R.E. (1974) The generation of hydrogen peroxide, superoxide radical, and hydroxyl radical by 6-hydroxydopamine, dialuric acid, and related cytotoxic agents. *J. Biol. Chem.* **249**, 2447–2452
24. Cadenas, E., Mira, D., Brunmark, A., Lind, C., Segura-Aguilar, J., and Ernster, L. (1988) Effect of superoxide dismutase on the autoxidation of various hydroquinones – a possible role of superoxide dismutase as a superoxide: semiquinone oxidoreductase. *Free Rad. Biol. Med.* **5**, 71–79
25. Bandy, B., Moon, J., and Davison, A.J. (1990) Multiple actions of superoxide dismutase, why can it both inhibit and stimulate reduction of oxygen by hydroquinones? *Free Rad. Biol. Med.* **9**, 143–148
26. Nishikawa, T., Akiyama, N., Kunimasa, K., Oikawa, T., Ishizuka, M., Tsujimoto, M., and Natori, S. (2006) Inhibition of *in vivo* angiogenesis by *N*-β-alanyl-5-S-glutathionyl-3,4-dihydroxyphenylalanine (5-S-GAD). *Eur. J. Pharmacol.* **539**, 151–157