Pergamon

Bioorganic & Medicinal Chemistry 8 (2000) 2561–2569

BIOORGANIC & MEDICINAL CHEMISTRY

Reaction of β-Alkannin (Shikonin) with Reactive Oxygen Species: Detection of β-Alkannin Free Radicals

Dayuan Gao, Miyako Kakuma, Shigenori Oka, Keiji Sugino and Hiromu Sakurai*

Department of Analytical and Bioinorganic Chemistry, Kyoto Pharmaceutical University, 5 Nakauchi-cho, Misasagi, Yamashina-ku, Kyoto 607-8414, Japan

Received 20 April 2000; accepted 28 June 2000

Abstract—β-Alkannin (shikonin), a compound isolated from the root of *Lithospermum erythrorhizon* Siebold Zucc., has been used as a purple dye in ancient Japan and is known to exert an anti-inflammatory activity. This study aimed to understand the biological activity in terms of physico-chemical characteristics of β-alkannin. Several physico-chemical properties including proton dissociation constants, half-wave potentials and molecular orbital energy of β-alkannin were elucidated. This compound shows highly efficient antioxidative activities against several types of reactive oxygen species (ROS), such as singlet oxygen ($^1O_2$), superoxide anion radical ($\cdot O_2^-$), hydroxyl radical (•OH) and *tert*-butyl peroxyl radical (BuOO•) as well as iron-dependent microsomal lipid peroxidation. During the reactions of β-alkannin with $^1O_2$, $\cdot O_2^-$ and BuOO•, intermediate organic radicals due to β-alkannin were detectable by ESR spectrometry. Compared with the radicals due to naphthazarin, the structural skeleton of β-alkannin, the β-alkannin radical observed as an intermediate in the reactions with $^1O_2$ and $\cdot O_2^-$ was concluded to be a semiquinone radical. On the other hand, during the reactions of β-alkannin and naphthazarin with BuOO•, ESR spectra different from the semiquinone radical were observed, and proposed to result from the abstraction of hydrogen atoms from phenolic hydroxyl groups of β-alkannin by BuOO•. Based on the ROS-scavenging abilities of β-alkannin, the compound was concluded to react directly with ROS and exhibits antioxidative activity, which in turn exerts anti-inflammatory activity.

Introduction

Recently, many phenol and hydroquinone compounds have been found to exhibit antioxidative activities.[1–4] For example, vitamin K-hydroquinone has been proposed to inhibit microsomal lipid peroxidation.[5] HTHQ (1-*O*-hexyl-2,3,5-trimethylhydroquinone), which is a synthetic analogue of α-tocopherol, has been reported to react directly with peroxyl radicals to form stable HTHQ radical that manifests strong anti-lipid peroxidative effects.[6] Such antioxidative abilities have also been found in quinones. For example, idebenone (6-(10-hydroxydecyl)-2,3-dimethoxy-5-methyl-1,4-benzoquinone), an analogue of coenzyme Q, has been found to participate partially in antioxidative activity.[7,8] In contrast, it has also been reported that quinones generate reactive oxygen species (ROS), including superoxide anion ($\cdot O_2^-$), hydroxyl radical (•OH) and hydrogen peroxide (H_2O_2), via reactions between dioxygen and semiquinone radicals.[9]

On the basis of these findings, it is of interest to investigate the reaction between ROS and compounds having both hydroquinone and quinone moieties in their molecules. Many studies have been undertaken on the generation and identification of radicals derived from naphthazarin (5,8-dihydroxy-1,4-naphthoquinone) derivatives[10–18] as well as their chemical and biological properties,[19] providing useful information on the characterization of interactions between such compounds with ROS.

β-Alkannin is a purple dye extracted from *Lithospermum erythrorhizon* Siebold Zucc. (Japanese name: Shikon), which has been used in ancient Japan as both a dye and an ointment for healing of cut and burns. In 1918, Kuroda purified a purple pigment from the plant, identified its structure, and named it shikonin.[20] Later, shikonin was found to be a kind of alkannin with a naphthazarin structure (Fig. 1), and thus was named β-alkannin (2-(1-hydroxy-4-methyl-3-pentenyl)-5,8-dihydroxy-1,4-naphthoquinone).[21] Pharmacological studies revealed that this compound has abilities such as those of accelerating tissue granulation proliferation,[22,23] and exerting antibacterial,[24] anti-inflammatory[25] and anti-tumor effects.[26] β-Alkannin ointment also has the ability to accelerate wound healing[27] via an anti-inflammatory

*Corresponding author. Tel.: +81-75-595-4629; fax: +81-75-595-4753; e-mail: sakurai@mb.kyoto-phu.ac.jp

0968-0896/00/$ - see front matter
PII: S0968-0896(00)00191-7

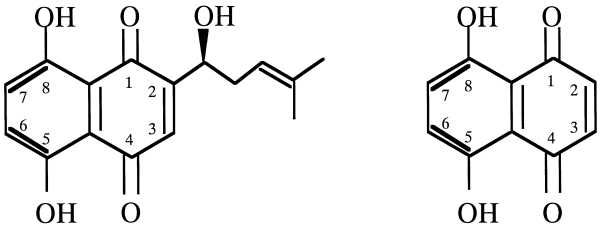

Figure 1. Structures of β-alkannin (shikonin) and naphthazarin.

effect involving ROS.[28–30] These observations and its structure suggest that β-alkannin exerts strong antioxidative activities against various types of ROS. For this reason, the reaction between β-alkannin and $\cdot O_2^-$ [31] and the chelate formation of β-alkannin and iron were examined.[32] However, the basic chemical properties of β-alkannin and its reactions with ROS are almost unknown. Thus we examined the physico-chemical properties of β-alkannin and its interaction with ROS, and found that β-alkannin has high antioxidative abilities against $\cdot O_2^-$, $^1O_2$, BuOO$\cdot$ and lipid peroxidation, resulting in the formation of its free radical species. This paper reports the basic physico-chemical features and antioxidative activities of β-alkannin.

Results

Because the physico-chemical properties of β-alkannin are not yet known, we examined first the chemical characteristics of both β-alkannin and naphthazarin, which is the structural skeleton of β-alkannin, in terms of the acid dissociation constants (pK_a) and the half wave potentials ($E_{1/2}$). Table 1 shows the acid dissociation constants of β-alkannin and naphthazarin in different concentrations of acetonitrile solution. The proton dissociations from the two phenolic hydroxyl groups were found to increase with increasing acetonitrile concentration.

Table 1. Acid dissociation constants of β-alkannin and naphthazarin at various ratios of acetonitrile and water at 25 °C[a]

$CH_3CN:H_2O$	β-Alkannin		Naphthazarin	
(volume/volume)	pK_{a1}	pK_{a2}	pK_{a1}	pK_{a2}
4:6	9.27	11.95	9.34	11.98
5:5	9.52	12.08	9.59	12.21
6:4	9.80	12.28		
7:3	10.07	12.53	10.18	12.56
8:2	10.56	12.88		

[a]Potentiometric titrations were carried out at I = 0.1 (KC1) and 2.5 mM of the compounds. Data are expressed as the mean values of duplicate measurements.

β-Alkannin and naphthazarin both exhibited reversible cyclic voltammograms in acetonitrile (Figure 2, left); cyclic voltammograms were then measured for both compounds at various mixing ratios of acetonitrile and water. Cyclic voltammograms of the compounds at $CH_3CN:H_2O$=4:6 are shown in Figure 2 (right). The occurrence of a two-step redox reaction can be clearly observed, probably due to the quinone groups, indicating that β-alkannin participates in redox reactions. From the voltammograms, the apparent $E_{1/2}$ of β-alkannin and naphthazarin were calculated and summarized in Table 2. Increasing of acetonitrile concentrations from 40 to 80% in the solvent affected little the $E_{1/2}$ values, and existence of water affected the second $E_{1/2}$ value rather than the first $E_{1/2}$.

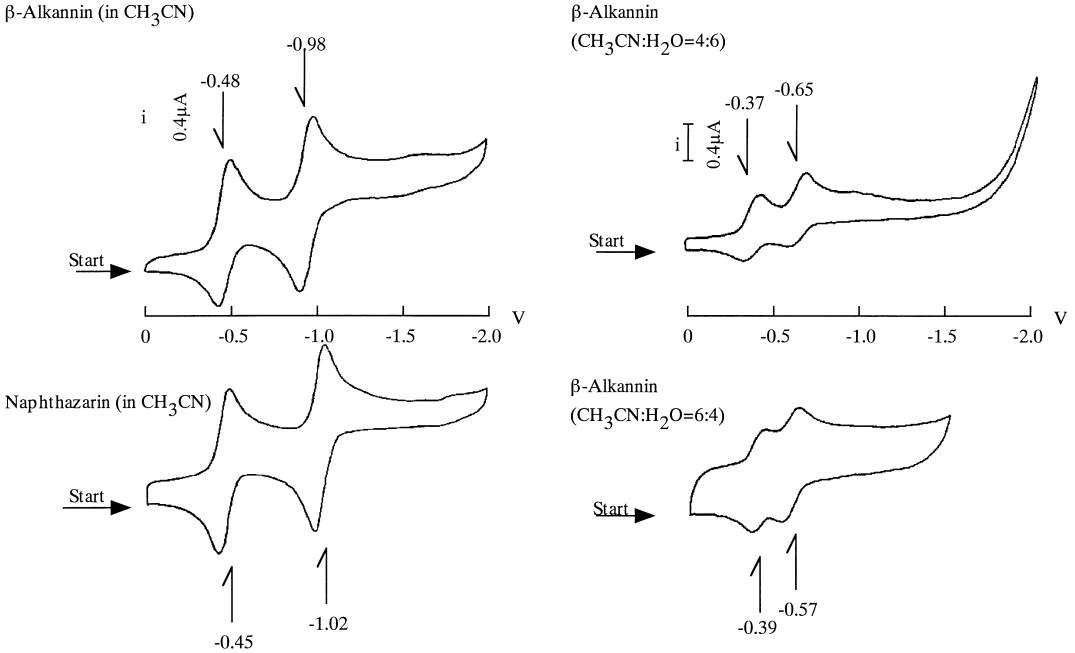

Figure 2. CV voltammograms of β-alkannin and naphthazarin. CV scans were carried out in acetonitrile (left) for β-alkannin and naphthazarin or in different ratios of acetonitrile/water for β-alkannin (right). Vertical arrows with number indicate the respective $E_{1/2}$ values. Experimental conditions were the same as described in the Experimental.

Table 2. $E_{1/2}$ (V) of β-alkannin and naphthazarin in mixing solvent of acetonitrile and water[a]

$CH_3CN:H_2O$	β-Alkannin		Naphthazarin	
(v/v)	$E(1)_{1/2}$	$E(2)_{1/2}$	$E(1)_{1/2}$	$E(2)_{1/2}$
4:6	−0.37	−0.65	−0.35	−0.63
5:5	−0.38	−0.55	−0.35	−0.54
6:4	−0.39	−0.57	−0.36	−0.56
7:3	−0.40	−0.58	−0.36	−0.57
8:2	−0.40	−0.62	−0.38	−0.61
10:0	−0.48	−0.98	−0.45	−1.02

[a]CV measurements were carried out with GCE-Pt-SCE electrodes at 1 mM of the compounds in different ratios of acetonitrile–water solution containing 0.1 M TBAP as described in the Experimental. Data are expressed as the mean values of duplicate measurments.

The antioxidative activity of β-alkannin was then examined in both chemical and biochemical systems by the ESR spin-trapping and lipid peroxidation methods. The ROS scavenging and anti-lipid peroxidation abilities of β-alkannin were evaluated by comparing the IC_{50} values of β-alkannin with those of such typical antioxidants as ascorbic acid and α-tocopherol under the same conditions. The results are summarized in Table 3. β-Alkannin exhibited stronger scavenging effects against $\cdot O_2^-$ and $^1O_2$ than both ascorbic acid and α-tocopherol, being more effective against BuOO• than α-tocopherol. The inhibition of iron-dependent microsomal lipid peroxidation by β-alkannin was more enhanced than that by α-tocopherol.

During the ESR spin-trapping of $^1O_2$ by TMPD, the signal intensity due to a spin adduct of $^1O_2$ was decreased by addition of β-alkannin in a concentration-dependent manner (see IC_{50} value in Table 3). By addition of higher concentrations of this compound, new signals were observed. A well resolved spectrum of the radical was recorded in 40% acetonitrile buffer solution (Fig. 3a). The signals, whose intensities depended on both β-alkannin concentration and UVB irradiation time, were not observed without β-alkannin (data not shown) or by addition of sodium azide, a quencher of $^1O_2$ (Fig. 3b). β-Alkannin alone without irradiation gave no ESR signal. These results suggest that β-alkannin reacts with $^1O_2$. In order to analyze the radical, H_2O was replaced with D_2O to exclude any possible contribution of hydrogen atoms of the 5,8-dihydroxyl groups in β-alkannin. The addition of D_2O simplified the spectrum to an eight-line signal (Fig. 3c). Hyperfine coupling constant (hfc) values of the radical were found as follows by computer simulation (Fig. 3d and e): a quadruplet of 0.223 mT (a_1) and a doublet of 0.132 mT (a_2) and a triplet of 0.056 mT (a_3).

In addition, ESR spectrum of the β-alkannin radical formed in $^1O_2$ generating system was compared with that of naphthazarin. A naphthazarin radical was also obtained as a fifteen-line signal under the same conditions (Fig. 4a), and that in D_2O gave a five-line signal (Fig. 4b). The hfc values for the naphthazarin radical were 0.236 mT (a_1) and 0.056 mT (a_2), being consistent with the reported semiquinone radical obtained by electrochemical reduction,[12,14–16] and similar to those of β-alkannin radical (Fig. 3a and c). From these results, we concluded that the β-alkannin radical observed in $^1O_2$ generating system was a semiquinone radical.

On the other hand, formation of β-alkannin radicals was studied in two $\cdot O_2^-$ generating systems, a DMSO-alkaline solution and an HPX–XOD system containing 40% acetonitrile (Fig. 5). The signal intensity due to the radical was increased in a concentration-dependent manner with β-alkannin as well as the DMSO-alkaline solution (data not shown). In addition, SOD but not catalase inhibited this radical formation in mixtures containing DMSO-alkaline solution and phosphate buffer (data not shown). These results showed that β-alkannin radical was formed by the reaction with $\cdot O_2^-$. Although the ESR spectrum due to β-alkannin radical (Fig. 5a) was not well resolved in the solvent system containing DMSO and H_2O in a ratio of 99:1, addition of D_2O gave a relatively simple eight-line signal (Fig. 5(b)). The results together with the observed naphthazarin radicals (Fig. 5c and d) were consistent with those in the $^1O_2$ generating system (Figs. 3 and 4). In the second $\cdot O_2^-$ generating system containing HPX and XOD, we observed a complicated ESR signal due to β-alkannin radical (Fig. 5e) as previously reported.[31] These results indicated that β-alkannin is reduced by $\cdot O_2^-$ and forms a semiquinone radical.

In the BuOO• generating system, a four-line β-alkannin radical (hfc value: 0.048 mT) was observed (Fig. 6a). The appearance of the signal depended on the concentration of β-alkannin, and the signal was not observed if either BuOOH or MetHb was omitted (data not shown). The naphthazarin radical showed a five-line signal (Fig. 6c) with hfc value of 0.044 mT, being distinct from the semiquinone radical, suggesting that the former radical results from the reaction of naphthazarin with BuOO•. Addition of D_2O did not alter either the β-alkannin or the naphthazarin radicals (Fig. 6b and d),

Table 3. ROS scavenging and lipid peroxidation inhibitory activities of β-alkannin

Compound	IC_{50} (μM)				
	·OH	$\cdot O_2^-$	$^1O_2$	*t*-BuOO·	Lipid peroxidation
β-Alkannin	108±3[a]	17±1[a]	27±1[a]	113±5[b]	9±1
Ascorbic acid	59±3	33±2	171±4	106±6	ND
α-Tocopherol	ND	>500	ND	141±10	11% inhibition at 50 μM

Experimental conditions were as described in the Experimental. Each value is expressed as the mean±SD of 3–4 experiments. ND: not determined.
[a]$P < 0.0l$ versus ascorbic acid.
[b]$P < 0.0l$ versus α-tocopherol.

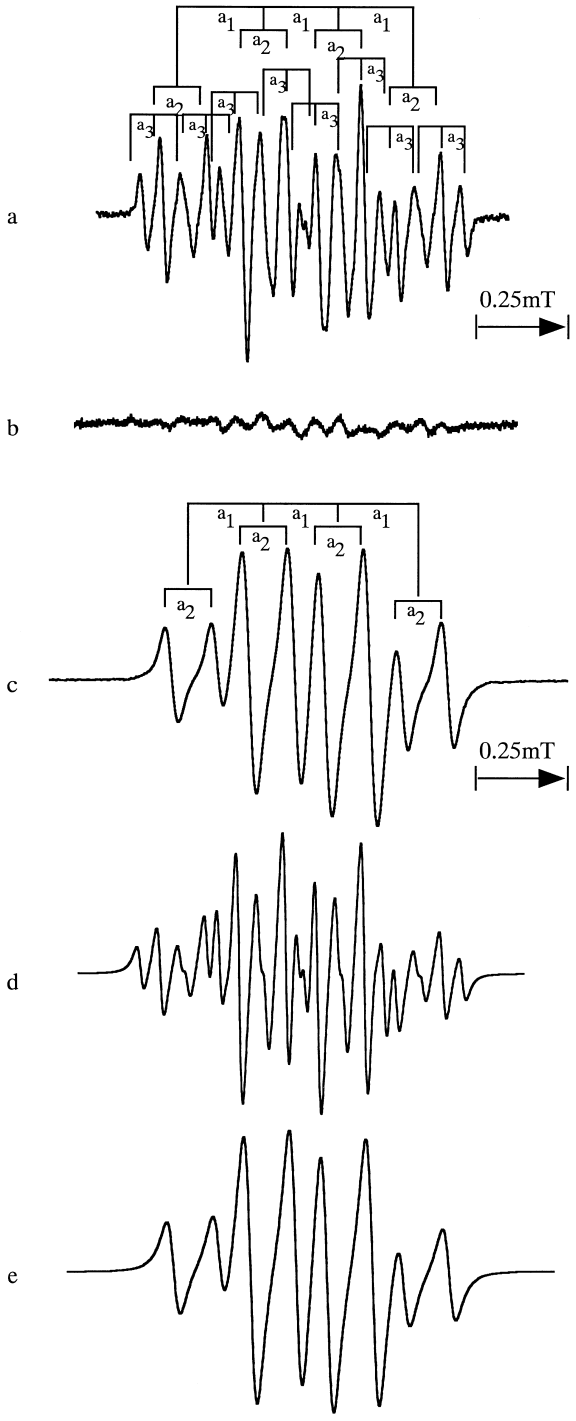

Figure 3. β-Alkannin radical formation in $^1O_2$ generating system. Reaction mixtures consisting of β-alkannin (2 mM), HP (62.5 μM) and phosphate buffer (20 mM, pH 7.4) containing 40% acetonitrile were exposed to UVB for 3 min. (a) β-Alkannin; (b) a but in the presence of NaN_3 (7 mM); (c) a but H_2O was replaced with D_2O; (d) and (e) computer simulations of a and c, respectively.

indicating that phenolic hydroxyl groups did not contribute to the radical formation.

The reaction of β-alkannin with •OH was also examined. In the Fenton reaction, the ESR signal due to the DMPO-OH adduct, evaluated by spin trapping, was decreased by addition of β-alkannin (Table 3). However, no β-alkannin radical signal was observed under the present conditions.

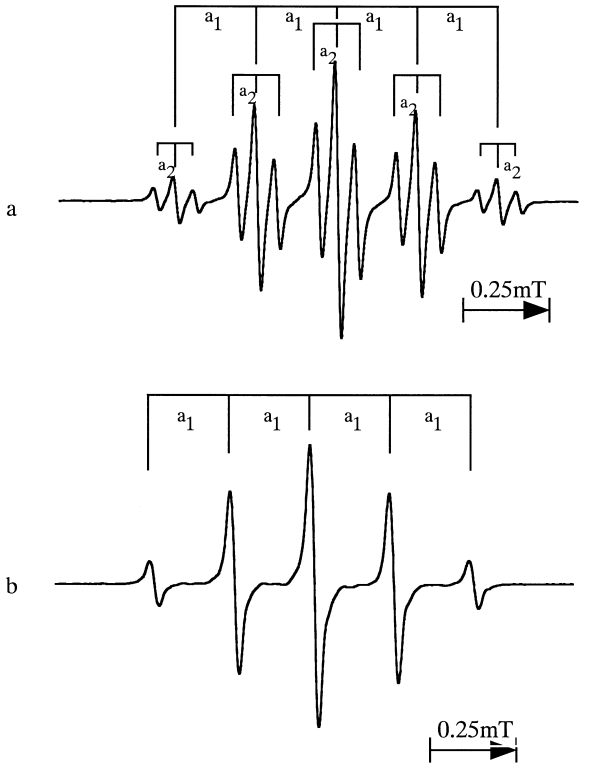

Figure 4. Naphthazarin radicals in $^1O_2$ generating system. All conditions were the same as those in Fig. 3. (a) Naphthazarin (1.2 mM); (b) a but H_2O was replaced with D_2O.

Finally, to presume the possible reaction site of β-alkannin and ROS, molecular orbital (MO) calculations were carried out. In β-alkannin molecule, carbon atoms at positions 5 and 8 showed the biggest highest occupied MO (HOMO) and singly occupied MO (SOMO) values, while those at positions 1 and 3 showed the biggest lowest unoccupied MO (LUMO) densities (Table 4). The contribution of the side chain to these MO energies was estimated to be quite small (data not shown), suggesting that the initial reaction sites in the molecule with ROS are at the ring structure but not at side chain of β-alkannin. Naphthazarin showed a trend similar to that of β-alkannin, except that its highest LUMO was formed at positions 2 and 3 (Table 4), because of the lack of side chain.

Discussion

β-Alkannin scavenged well both $\cdot O_2^-$ and $^1O_2$ and inhibited lipid peroxidation (Table 3). Generally, quinones (Q) have been assumed to undergo the following reaction:[9]

$$Q + e^- \rightarrow \cdot Q^- \quad (1)$$

A previous pulse radiolysis study demonstrated the occurrence of a one electron reduction of naphthazarin by $\cdot O_2^-$.[33] Our CV study suggested a two-electron

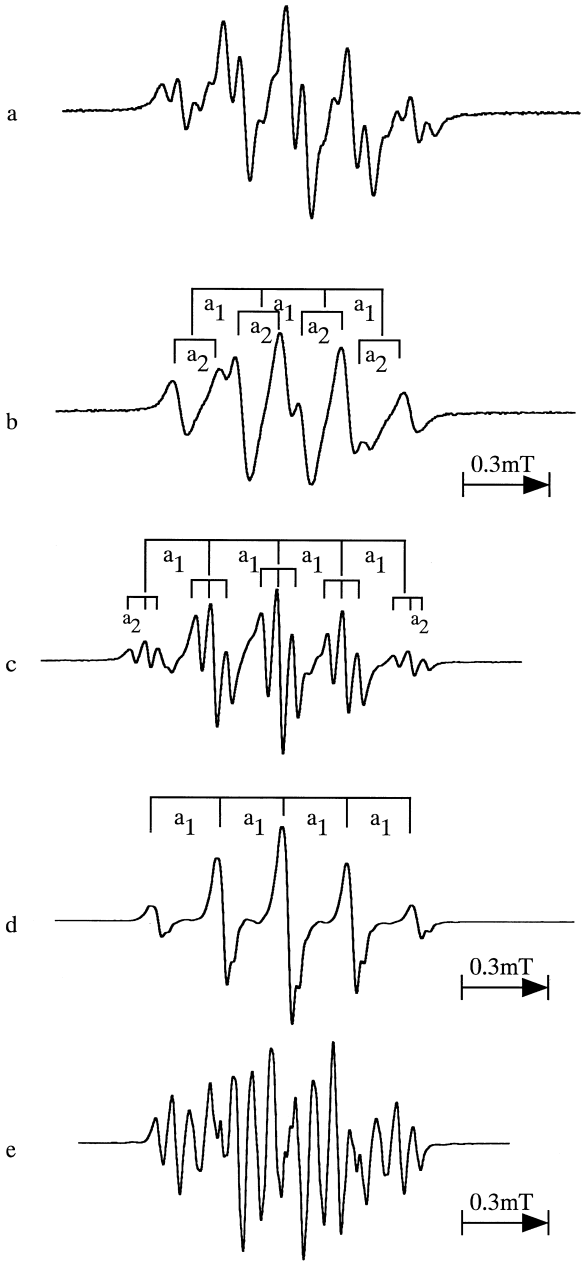

Figure 5. β-Alkannin and naphthazarin radicals in $\cdot O_2^-$ generating systems. (a) β-Alkannin (2 mM) in DMSO-alkaline solution (final volume was 200 μL) prepared as described in the Experimental; (b) a but 10 μL D_2O was added; (c) naphthazarin (2 mM) in DMSO-alkaline solution; (d) c but D_2O was added; (e) β-alkannin semiquinone radical in HPX–XOD system.

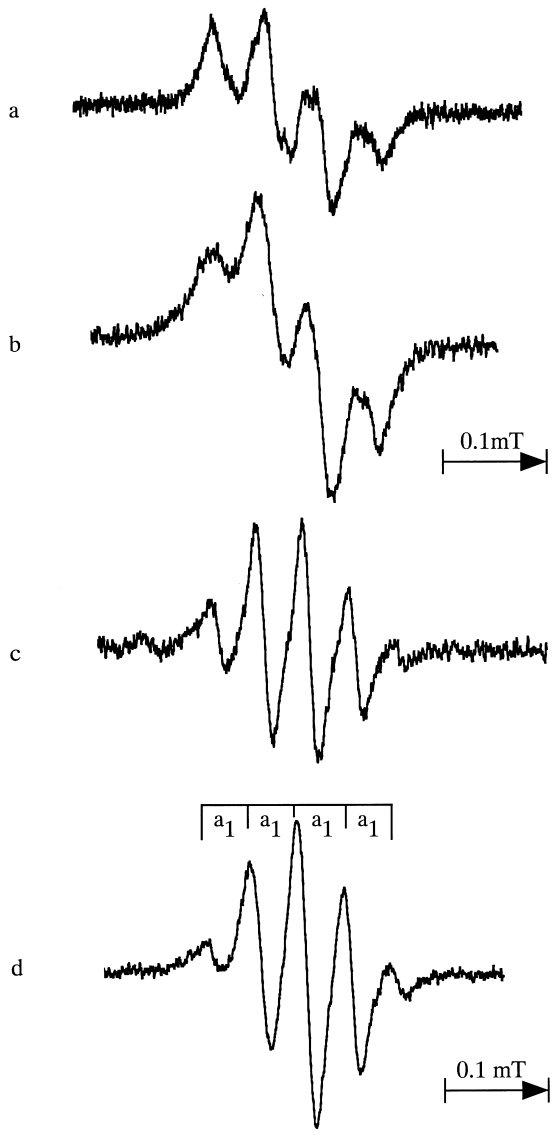

Figure 6. β-Alkannin and naphthazarin radicals in BuOO· generating system. BuOO· was generated by reaction of MetHb (1 mg/mL) and BuOOH (25 mM) in phosphate buffer (125 mM, pH 7.4) containing 40% acetonitrile. (a) β-Alkannin (2 mM); (b) as a but in D_2O; (c) naphthazarin (1.2 mM); (d) c but in D_2O.

reduction of β-alkannin (Fig. 2), where the first $E_{1/2}$ value for the one-electron reduction of the compound in 100% CH_3CN was −0.48 V, while that of naphthazarin was −0.45 V (Table 2). When we compared the value with that of $E_{1/2} = -0.78$V for $O_2/\cdot O_2^-$ under similar conditions,[34] both β-alkannin and naphthazarin were presumed to receive one electron from $\cdot O_2^-$.

When β-alkannin was treated with an XOD-HPX aqueous system, a complicated ESR signal was observed (Fig. 5e). This signal was assigned to a semiquinone radical as previously reported.[31] On the other hand, β-alkannin and naphthazarin radicals were observed as broad signals in DMSO-alkaline solution (Fig. 5a and c), with the signal due to the latter radical being identical to those of the semiquinone radicals prepared by electrochemical reduction.[11,13–15] We therefore concluded that the β-alkannin radical is a semiquinone radical formed during the reaction with $\cdot O_2^-$ as follows:

$$\beta\text{-Alkannin} + \cdot O_2^- \rightarrow \cdot\beta\text{-Alkannin (semiquinone radical)} + O_2 \quad (2)$$

$^1O_2$ is known to oxidize a number of compounds (A):[35]

$$^1O_2 + A \rightarrow \cdot O_2^- + A^{\cdot +} \quad (3)$$

β-Alkannin suppressed $^1O_2$ production in a concentration-dependent manner as evaluated by ESR spin-trapping

Table 4. MO energy of β-alkannin and naphthazarin molecules

Position	Element	β-Alkannin			Naphthazarin		
		HOMO	LUMO (eV)	SOMO	HOMO	LUMO (eV)	SOMO
1	C	0.006	0.248	0.127	0.005	0.229	0.117
	O	0.043	0.220	0.131	0.043	0.223	0.133
2	C	0.001	0.231	0.116	0.001	0.240	0.120
3	C	0.001	0.243	0.122	0.001	0.240	0.120
4	C	0.005	0.221	0.113	0.005	0.230	0.118
	O	0.046	0.219	0.132	0.044	0.224	0.134
5	C	0.347	0.058	0.203	0.348	0.061	0.204
	O	0.238	0.012	0.125	0.236	0.013	0.124
6	C	0.096	0.130	0.113	0.098	0.128	0.113
7	C	0.099	0.124	0.112	0.100	0.129	0.115
8	C	0.344	0.066	0.205	0.348	0.060	0.204
	O	0.233	0.013	0.123	0.237	0.012	0.125
Side chain	C	0	0–0.001	0–0.002			
	O	0	0.007	0.003			

method (Table 3), suggesting the reaction of β-alkannin with $^1O_2$. UV irradiation in the presence of certain photosensitizers is known to include the following two types of reactions:[36,37] type I process that includes redox reactions of excited photosensitizers with certain substrates or with ground state sensitizers, and type II process that involves energy transfer reactions of excited sensitizers to dioxygen to produce $^1O_2$. When β-alkannin was irradiated with UV light in the absence of HP, β-alkannin radical was detected. The obtained radical intensity was weak, but greatly enhanced by addition of HP. In both cases, the addition of sodium azide, a $^1O_2$ quencher, dispelled the signal (data not shown), suggesting a major contribution of $^1O_2$. However, the β-alkannin radical observed under the present condition was semiquinone radical, and cation radical shown in reaction (3) was not detected, suggesting the β-alkannin semiquinone radical is not a direct reaction intermediate with $^1O_2$.

The ESR spectrum of β-alkannin radical produced during the reaction with $^1O_2$ was very similar to that formed in the HPX–XOD system (Figs 3a and 5a). Thus, the radical was assigned to the semiquinone radical. In addition, the naphthazarin radical formed in the $^1O_2$ generating system (Fig. 4a) was identical to the previously reported semiquinone radical.[11,13–15] Therefore, the β-alkannin radical found in the $^1O_2$ generating system was also indicated to be a semiquinone radical. SOD inhibited the formation of the β-alkannin semiquinone radical in part under the same conditions (data not shown). Based on these results, we propose that the observed semiquinone radical is derived from the reaction with $\cdot O_2^-$, which is photochemically generated by transfer of an electron from excited-state to photosensitizer to molecular oxygen.[38] $\cdot O_2^-$ may be alternatively generated via reaction of β-alkannin with $^1O_2$ (as reaction (3)). It was also reported that the side chain of β-alkannin was photo-oxidized.[39] The MO calculation showed that the side chain of β-alkannin contributed far less in electron densities, suggesting the initial reaction site with $^1O_2$ was at the ring structure.

Peroxyl radicals are known to abstract hydrogen atom from scavengers such as α-tocopherol, resulting in the generation of both hydroperoxide and scavenger radicals.[40] In the reaction with BuOO•, β-alkannin radical was observed (Fig. 6a and c), different from its semiquinone radical (Fig. 3a). The fact that D_2O does not exhibit an isotopic effect (Fig. 6b and d) excluded the participation of the hydrogen atoms of the phenolic hydroxyl groups. The structural skeleton, naphthazarin, gave ESR signal (Fig. 6c), suggesting the contribution of phenolic hydroxyl groups to form the naphthazarin radical, as supported by the MO calculation (Table 4). From these results, we assume that β-alkannin radical may be formed by abstraction of hydrogen atoms in the phenolic hydroxyl groups.

The failure to detect β-alkannin radical in the Fenton reaction was assigned to its iron chelating effect at a ratio of 2:1.[32] It is known that some phenolic compounds possess an iron chelating effect.[41–44] Our results showed that the IC_{50} of β-alkannin was twice the value of the ferrous concentration in the Fenton reaction (Table 3). Considering the high stability constant of DTPA with ferrous ion ($\log K = 17$), as well as the high redox potential for •OH ($E^{\bullet}OH/H_2O = 2.73$ V),[45] it is reasonable to speculate that β-alkannin reacts with this highly reactive radical species.

The present research shows that β-alkannin has potent ROS scavenging abilities. However, the reported biological effects of quinones remain somewhat confusing. For example, menadione (2-methyl-1,4-naphthalenedione), which shows an antioxidative effect in microsomal lipid peroxidation,[46] is a well-known ROS generator and thus toxic to cells.[47,48] Both the metabolic fate of β-alkannin and the toxicity of β-alkannin remain to be elucidated. Further studies will therefore be needed to determine whether β-alkannin is a potent agent in ROS-involving pathology.

Conclusion

We conclude that β-alkannin can react directly with $^1O_2$, $\cdot O_2^-$ and BuOO•, and has higher reactivity towards $^1O_2$ and $\cdot O_2^-$ than either ascorbic acid or α-tocopherol. In addition, the scavenging ability of β-alkannin against

BuOO• is higher than that of α-tocopherol. In microsomal lipid peroxidation, the IC_{50} of β-alkannin was estimated to be 9 μM (Table 2), far less than the value for α-tocopherol, indicating that β-alkannin has a high anti-lipid peroxidative ability.

Experimental

Materials. β-Alkannin (shikonin), hematoporphyrin hydrochloride (HP), hypoxanthine (HPX), xanthine oxidase (XOD), diethylenetriamine-*N*, *N,N′,N″,N″*-pentaacetic acid (DTPA), ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$) and hydrogen peroxide (H_2O_2) were purchased from Wako Pure Chemical Industry (Tokyo, Japan). Naphthazarin (5,8-dihydroxy-1,4-naphthoquinone) was obtained from Tokyo Kasei Industry (Tokyo, Japan). Methemoglobin (MetHb), superoxide dismutase (SOD) and catalase were products of the Sigma Chemical Company (St. Louis, MO, USA) and 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) was from the Labotec Company (Tokyo, Japan). Deuterium oxide (D_2O, 99.9%) and 2,2,6,6-tetramethyl-4-piperidone hydrochloride (TMPD) were purchased from the Aldrich Chemical Company, Inc. (Milwaukee, WI, USA). TMPD was recrystallized from ethanol. *tert*-Butyl hydroperoxide (BuOOH) was obtained from Katayama Chemical Industry (Osaka, Japan). Tetra-*N*-butylamine perchlorate (TBAP) was from Nacalai Tesque, Inc. (Kyoto, Japan). All other chemicals used were of commercially available analytical reagent grade.

Potentiometric titration and determination of proton dissociation constants. Potentiometric titrations were carried out with a Radiometer TTT60 Titrator equipped with an REC61/REA160 Titrigraph and TTA60 Titrator Assembly and associated Autoburette ABU12 using a G2040C glass electrode and a K4040 calomel electrode. The ionic strength was adjusted to 0.1 with KCl. The solution was stirred with a motorized stirring stick, and nitrogen gas was passed slowly into the solution during the titration. For the determination of proton dissociation constants, the solution containing 2.5 mM of β-alkannin or naphthazarin dissolved in mixed solvents consisting of various ratios (v/v) of acetonitrile and water was titrated with carbonate-free 0.05 M KOH. The titrations were done at 25±0.1 °C (all). Proton dissociation constants of the compounds were calculated as reported previously.[49,50]

Cyclic voltammetry (CV). CV measurements were performed at room temperature with an electrochemical cell consisting of a GCE (glassy carbon electrode) working electrode, an SCE (saturated calomel electrode) reference electrode and a platinum wire auxiliary electrode. A computer controlled potentiostat (BAS 100B/W, Lafayette, IN, USA) and accompanying software package were used. Acetonitrile was supplemented with 0.1 M TBAP as a supporting electrolyte. The concentrations of β-alkannin and naphthazarin were fixed at 1 mM in mixed solvents consisting of various ratios of acetonitrile and water. CV scans were carried out at 20 mV/s after purging the reaction mixture with nitrogen gas for 10 min. The half-wave potential ($E_{1/2}$) was calculated as the midpoint of the anode and cathode potentials.[44]

Molecular orbital (MO) calculation. MO calculations of β-alkannin and naphthazarin were performed with a MOPAC semi-empirical molecular orbital package program (version 7.0). MOPAC internal files were made with MOLDA, a molecular-model building program. Optimized geometrical structures obtained using the PM3 Hamiltonian were used to determine the MO energy of HOMO (highest occupied MO), LUMO (lowest unoccupied MO) and SOMO (singly occupied MO).[51]

ESR spin-trapping. Four kinds of ROS were prepared and trapped as described below. Scavenging activities of β-alkannin and other compounds were expressed as IC_{50} values, which were the 50% inhibition concentrations of the compounds against generated ROS.

Singlet oxygen ($^1O_2$) was generated by ultraviolet light B (UVB) irradiation in the presence of HP.[44] UVB was irradiated through a UVB filter at the dose of 0.65 J/cm^2 using a Supercure-203S (San-Ei Electric MFG Co. Ltd., Osaka, Japan), which was connected to the ESR cavity. Reaction mixtures contained HP (62.5 μM), TMPD (50 mM), various concentrations of β-alkannin (in acetonitrile) and phosphate buffer (20 mM, pH 7.4). The solvent system consisted of acetonitrile and H_2O in a ratio of 2.5:97.5 (v/v), respectively. ESR spectra were measured from 30 s after the start of irradiation of the reaction mixtures.

HPX (0.42 mM) and XOD (0.1 U/mL) were used to generate $\cdot O_2^-$ as described previously.[35] β-Alkannin dissolved in acetonitrile was added so that the acetonitrile:H_2O ratio of the solvent system was 2.5:97.5 (v/v). Alternatively, $\cdot O_2^-$ was also prepared by the dimethyl sulfoxide (DMSO)-alkaline method as described previously.[52] Briefly, DMSO containing water (1%, v/v) and NaOH (5 mM) was allowed to stand at 25 °C for 30 min to give $\cdot O_2^-$ solution. This solution was mixed with phosphate buffer (100 mM, pH 7.4), DTPA (0.1 mM) and DMPO (112.5 mM) in the presence or absence of β-alkannin (in DMSO) at final volume of 240 μL. ESR spectra were recorded at 30 s after addition of the $\cdot O_2^-$ solution (100 μL). The solvent system consisted of DMSO and H_2O in a ratio of 41.7:58.3 (v/v), respectively.

tert-Butyl peroxyl radical (BuOO•) was prepared by the method of Akaike et al.[53] Briefly, BuOOH was added to a reaction mixture (200 μL final volume) consisting of BuOOH (25 mM), MetHb (0.25 mg/mL), phosphate buffer (100 mM, pH 7.4), DTPA (25 μM), DMPO (11.25 mM) and various concentrations of β-alkannin (in DMSO). The solvent system consisted of acetonitrile and H_2O in a ratio of 2.5:97.5 (v/v), respectively. ESR spectra were measured at 30 s after addition of BuOOH.

•OH was generated by the Fenton system consisting of $FeSO_4 \cdot 7H_2O$ (50 μM) and H_2O_2 (0.5 mM). DTPA (100 μM), various amounts of β-alkannin (in acetonitrile),

DMPO (94.5 mM) and phosphate buffer (50 mM, pH 7.4) were added to the reaction systems. The solvent system consisted of acetonitrile and H_2O in a ratio of 2.5:97.5 (v/v), respectively. H_2O_2 was added to start the reaction and ESR spectra were recorded after 30 s.

For ESR spin-trapping, a JES-FR30 free radical monitor (X-band) (JEOL Ltd., Tokyo, Japan) was used and the measurement conditions were as follows: magnetic field, 341±5 mT; field modulation frequency, 100 kHz; modulation amplitude width, 0.1 mT; time constant, 0.1 s; output power, 4 mW; and sweep time, 2 min. Manganese(II) oxide doped in magnesium oxide was used as external standard. Signal intensity due to the free radical spin adducts was normalized as relative signal height against the standard signal of Mn(II).

ESR analysis of free radical species in the reaction between β-alkannin and ROS. β-Alkannin and naphthazarin radicals were formed in the following ROS generating systems without spin trapping reagent.

Singlet oxygen ($^1O_2$) was generated by ultraviolet UVB irradiation in the presence of HP. Reaction mixtures contained HP (62.5 μM), β-alkannin (2 mM, in acetonitrile) and phosphate buffer (20 mM, pH 7.4), in which the solvent system consisted of acetonitrile and H_2O in a ratio of 40:60 (v/v), respectively. ESR spectra were measured from 60 s after the start of UV irradiation.

A DMSO-alkaline solution or HPX–XOD system was used to generate $^{\bullet}O_2^-$. In the former system, β-alkannin dissolved in DMSO was added to DMSO-alkaline solution and the solvent system consisted of DMSO: H_2O in a ratio of 99:1 (v/v), respectively. In the latter system, β-alkannin dissolved in acetonitrile was added to an HPX (0.42 mM)–XOD (0.1 U/mL) system in phosphate buffer (50 mM, pH 7.4) and the solvent system consisted of acetonitrile:H_2O in a ratio of 40:60 (v/v), respectively.

BuOO$^{\bullet}$ was generated by the reaction of BuOOH (25 mM) and MetHb (0.25 mg/mL) in phosphate buffer (50 mM, pH 7.4). β-Alkannin dissolved in acetonitrile was added, and the solvent system consisted of acetonitrile:H_2O in a ratio of 40:60 (v/v), respectively.

$^{\bullet}$OH was generated in a Fenton system consisting of $FeSO_4{\cdot}7H_2O$ (50 μM), H_2O_2 (0.5 mM), DTPA (100 μM) and phosphate buffer (50 mM, pH 7.4). β-Alkannin (in acetonitrile) was added to the reaction systems. The solvent system consisted of acetonitrile and H_2O in a ratio of 40:60 (v/v), respectively.

ESR spectra were recorded on a JES RE-1X (X-band) (JEOL, Ltd., Tokyo, Japan). ESR measurement conditions were as follows: magnetic field, 335±0.75 mT; field modulation frequency, 100 kHz; modulation amplitude width, 0.01 mT; time constant, 0.03 s; output power, 10 mW; and sweep time, 4 min. Computer simulations for the free radical species were made using Isotropic EPR Simulation software (version 2.2A; Labotec Co., Ltd., Japan).

Microsomal lipid peroxidation. Male Wistar rats (200–250 g) were fasted for 24 h and then sacrificed under anesthesia with ether. Livers were perfused with 0.25 M sucrose solution, then rapidly removed and homogenized in a 5-fold volume of 0.25 M sucrose solution. Microsomes were prepared by differential centrifugations followed by washing once with 1.15% KCl solution as described previously.[54] Microsomal protein concentration was measured as reported previously.[55] Ascorbic acid (0.5 mM) was added to solutions containing Fe^{3+}-EDTA (100 μM), microsomes (0.25 mg protein/mL), sodium potassium phosphate buffer (50 mM, pH7.4) and various concentrations of β-alkannin (in DMSO, final volume 0.5% v/v) to start the reactions. The reaction mixtures were incubated at 37 °C for 30 min followed by addition of 0.2% butylated hydroxytoluene, 3% trichloroacetic acid and 0.2% thiobarbituric acid (TBA). Tubes were heated in a boiling water bath for 20 min, then cooled and centrifuged. The supernatants were used to determine the concentrations of TBA reactive substances (TBARS), with absorbance measured at 532 nm.[56] No interference of β-alkannin was observed in the TBA reaction.

Acknowledgements

This investigation was supported in part by grants from the Ministry of Education, Science, Sports and Culture of Japan (H.S.). D.G. was supported in part by postdoctoral fellowships from Kyoto Pharmaceutical University and the Japan Society for the Promotion of Science.

References

1. Rice-Evans, C.; Miller, N. J.; Paganga, G. *Free Radic. Biol. Med.* **1996**, *20*, 933.
2. Neuzil, J.; Witting, P. K.; Stocker, R. *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 7885.
3. Kondo, K.; Kurihara, M.; Miyata, N.; Suzuki, T.; Toyoda, M. *Arch. Biochem. Biophys.* **1999**, *362*, 79.
4. Uchida, M.; Nakajin, S.; Toyoshima, S.; Shinoda, M. *Biol. Pharm. Bull.* **1996**, *19*, 623.
5. Vervoort, L. M.; Ronden, J. E.; Thijssen, H. H. W. *Biochem. Pharmacol.* **1997**, *54*, 871.
6. Hino, T.; Kawanishi, S.; Yasui, H.; Oka, S.; Sakurai, H. *Biochim. Biophys. Acta* **1998**, *1425*, 47.
7. Shivaram, K. N.; Winklhofer-Roob, B. M.; Straka, M. S.; Devereaux, M. W.; Everson, G.; Mierau, G. W.; Sokol, R. J. *Free Radic. Biol. Med.* **1998**, *25*, 480.
8. Mordente, A.; Martorana, G. E.; Minotti, G.; Giardina, B. *Chem. Res. Toxicol.* **1998**, *11*, 54.
9. Weiner, L. M. *Method Enzymol.* **1994**, *233*, 92.
10. Ashworth, P.; Dixon, W. T. *J. Chem. Soc. Perkin Trans. II* **1974**, *1*, 739.
11. Binks, J. H.; Bullock, A. T.; Hopkin, D. J. *J. Chem. Soc. Faraday Trans. II* **1972**, *68*, 814.
12. Flint, N. J.; Tabner, B. J. *J. Chem. Soc. Faraday Trans. I* **1987**, *83*, 167.
13. Piette, L. H.; Okamura, M.; Rabold, G. P.; Ogata, R.; Moore, R. E.; Scheuer, P. J. *J. Phys. Chem.* **1967**, *71*, 29.
14. Gendell, J.; Miller, W. R. Jr.; Fraenkel, G. K. *J. Am. Chem. Soc.* **1969**, *91*, 4369.

15. Sieiro, C.; Sanchez, A.; Crouigneau, P.; Lamy, C. *J. Chem. Soc. Perkin Trans. II* **1982**, *2*, 1069.
16. Ahmed, I. M.; Hudson, A.; Alberti, A. *J. Orgnometallic Chem.* **1987**, *333*, 9.
17. Ahmed, I. M.; Hudson, A.; Alberti, A. *Chem. Phys. Lett.* **1988**, *151*, 205.
18. Bolton, J. R.; Carrington, A.; Todd, P. F. *Mol. Phys.* **1963**, *6*, 169.
19. Papageorgiou, V. P.; Assimopolou, A. N.; Couladouros, E. A.; Hepworth, D.; Nicolaou, K. C. *Angew. Chem., Int. Ed.* **1999**, *38*, 270.
20. Kuroda, C. *J. Chem. Soc. Japan* **1918**, *39*, 1051.
21. Brockmann, H. *Ann.* **1922**, *521*, 1.
22. Ozaki, Y.; Ohno, A.; Saito, Y.; Satake, M. *Biol. Pharm. Bull.* **1994**, *17*, 1075.
23. Ozaki, Y.; Xing, L.; Satake, M. *Biol. Pharm. Bull.* **1996**, *19*, 233.
24. Tanaka, Y.; Odani, T. *Yakugaku Zasshi* **1972**, *92*, 525.
25. Tanaka, S.; Tajima, M.; Tsukada, M.; Tabata, M. *J. Nat. Prod.* **1986**, *49*, 466.
26. Sankawa, U.; Ebizuka, Y.; Miyazaki, T.; Isomura, Y.; Otuka, H.; Shibata, S.; Inomata, M. *Chem. Pharm. Bull.* **1977**, *25*, 2392.
27. Sekine, T.; Kojima, K.; Ota, S.; Matsumoto, T.; Yamamoto, T.; Maitani, Y.; Nagai, T. *S.T.P. Pharma Sci.* **1998**, *8*, 255.
28. Melikian, V.; Laverson, S.; Zawachi, B. *J. Trauma* **1987**, *27*, 151.
29. White, M. J.; Heckler, F. R. *Clin. Plast. Surg.* **1990**, *17*, 473.
30. Takami, M.; Nishiguchi, K.; Kiyohara, Y.; Komada, F.; Iwakawa, S.; Okumura, K. *Yakuzaigaku* **1993**, *53*, 185.
31. Sekine, T.; Masumizu, T.; Maitani, Y.; Nagai, T. *Int. J. Pharm.* **1998**, *174*, 133.
32. Sekine, T.; Masumizu, T.; Maitani, Y.; Takayama, K.; Kohno, M.; Nagai, T. *Yakugaku Zasshi* **1998**, *118*, 609.
33. Land, E. J.; Mukherjee, T. A.; Swallow, J.; Bruce, J. M. *J. Chem. Soc. Faraday Trans. I* **1983**, *79*, 391.
34. Valentine, J. S.; Miksztal, A. R.; Sawyer, D. T. *Method Enzymol.* **1984**, *105*, 71.
35. Saito, I.; Matsuura, T.; Inoue, K. *J. Am. Chem. Soc.* **1983**, *105*, 3200.
36. Ryter, S. W.; Tyrrell, R. M. *Free Radic. Biol. Med.* **1998**, *24*, 1520.
37. Foote, C. S. *Science* **1968**, *162*, 963.
38. Robert, W.; Wang, S. Y. *J. Am. Chem. Soc.* **1983**, *105*, 4129.
39. Cheng, H.; Chen, H.; Hsu, H.; Chen, C. *Int. J. Pharm.* **1995**, *120*, 137.
40. Burton, G. W.; Ingold, K. U. *J. Am. Chem. Soc.* **1981**, *103*, 6472.
41. Gao, D.; Sakurai, K.; Katoh, M.; Chen, J.; Ogiso, T. *Biochem. Mol. Biol. Int.* **1996**, *39*, 215.
42. Afanas'ev, I. B.; Dorozhko, A. I.; Brodskii, A. V.; Kostyuk, V. A.; Potapovitch, A. I. *Biochem. Pharmacol.* **1989**, *38*, 1763.
43. Morel, I.; Lescoat, G.; Cogrel, P.; Sergent, O.; Pasdeloup, N.; Brissot, P.; Cillard, P.; Cillard, J. *Biochem. Pharmacol.* **1993**, *45*, 13.
44. Arima, Y.; Hatanaka, A.; Tsukihara, S.; Fujimoto, K.; Fukuda, K.; Sakurai, H. *Chem. Pharm. Bull.* **1997**, *45*, 1881.
45. Sonntag, C. V.; Schuchamann, H. P. *Method Enzymol.* **1994**, *233*, 3.
46. Tampo, Y.; Yonaha, M. *Arch. Biochem. Biophys.* **1996**, *334*, 163.
47. Di Monte, D.; Ross, D.; Bellomo, G.; Eklow, L.; Orrenius, S. *Arch. Biochem. Biophys.* **1984**, *235*, 334.
48. Karczewski, .M.; Peters, J.G.; Noordhoek, J. *Biochem. Pharmacol* **1999**, *57*, 27.
49. Flaschka, H. *Talanta* **1960**, *7*, 90.
50. Takeshima, S.; Sakurai, H. *Inorg. Chim. Acta* **1982**, *66*, 119.
51. Stewart, J. J. P. *J. Comp. Chem.* **1989**, *10*, 221.
52. Hyland, K.; Voisin, E.; Banoun, H.; Auclair, C. *Anal. Biochem.* **1983**, *135*, 280.
53. Akaike, T.; Sato, K.; Ijiri, S.; Miyamoto, Y. *Arch. Biochem. Biophys.* **1992**, *294*, 55.
54. Ernster, L.; Siekevitz, P.; Palade, G. *J. Cell Biol.* **1962**, *15*, 541.
55. Markwell, M. A. K.; Haas, S. M.; Tolbert, N. E.; Bieber, L. L. *Method Enzymol.* **1981**, *72*, 296.
56. Esterbauer, H.; Cheeseman, K. H. *Method Enzymol.* **1990**, *186*, 407.