Scavenging Activities of α-, β- and γ-Thujaplicins against Active Oxygen Species

Yaeno ARIMA, Akihiko HATANAKA, Sachiko TSUKIHARA, Kaori FUJIMOTO, Keiko FUKUDA, and Hiromu SAKURAI*

Department of Analytical and Bioinorganic Chemistry, Kyoto Pharmaceutical University, Nakauchi-cho 5, Misasagi, Yamashina-ku, Kyoto 607, Japan. Received June 13, 1997; accepted September 22, 1997

α-, β- and γ-Thujaplicins are three tropolone-related compounds purified from the woods of *Chamaecyparis obtusa* SIEB. *et* ZUCC. and *Thuja plicata* D. DON. Based on our recent finding that β-thujaplicin inhibits the formation of sunburn cells (SBC), which appear in the epidermis after UVB irradiation, we have speculated that β-thujaplicin is a potent scavenger against active oxygen species. ESR spin-trapping, chemiluminescence and fluorescence methods, and cyclic voltammetry were used to evaluate the abilities of β-thujaplicin and its isomers, α- and γ-thujaplicins, as scavengers of five kinds of active oxygen species such as superoxide anion radicals, hydroxyl radicals, singlet oxygens, *tert*-butyl peroxyl radicals and hydrogen peroxides. These compounds were found to have comparable inhibitory effects of hydroxyl radicals generated by the Fenton reaction and high scavenging activities against *tert*-butyl peroxyl radicals as compared with those of *l*-ascorbic acid and *dl*-α-tocopherol, and α-thujaplicin was found to have the highest activity against hydrogen peroxides among the thujaplicins.

However, the effects of these compounds on superoxide anion radicals and singlet oxygens were less than those of *l*-ascorbic acid and *dl*-α-tocopherol. These facts suggest that thujaplicins have effective antioxidant ability. This ability is thought to be due in part to the chelating ability and redox potential of the compounds, which in turn are related to the prevention of UV-induced photo-damage *in vivo*.

Key words β-thujaplicin; hinokitiol; scavenging active; active oxygen species; ESR spin-trapping; chemiluminescence

α-, β- and γ-Thujaplicins (Fig. 1) are three tropolone-related compounds purified from the woods of *Chamaecyparis obtusa* SIEB. *et* ZUCC.[1,2] and *Thujaplicata* D. DON.[3] Although the biological activities of these compounds are not yet fully understood, they have been revealed to have potent anti-microbacterial activity.[4,5] Among them, β-thujaplicin (hinokitiol)[1,2] is used as an anti-microbial agent in foods and cosmetics due to its broad antifungal activity[6,7] and low toxicity in animals.[8] It has also been shown to have anti-tumor activity[9,10] and a strong inhibitory effect on DNA synthesis in the very early stage of culture.[11]

Recently, it has been found that β-thujaplicin is able to inhibit the formation of UVB-induced apoptotic cells, *i.e.*, formation of sunburn cells (SBC),[12,13] that appear in the epidermis after UVB irradiation.[14] Since UVB-induced active oxygen species are thought to be involved in SBC formation,[15,16] UVB-induced active oxygen species in the skin might have the characteristics of a chain reaction in the presence of molecular dioxygen, H_2O_2, lipids and Fe^{2+}.[17,18] It has also been reported that the formation of SBC is prevented by antioxidative enzymes such as superoxide dismutase (SOD) and catalase *in vivo*,[15,16] and that the depletion of glutathione in hairless mice increases SBC formation.[19] These findings suggest that β-thujaplicin is related to scavenge active oxygen species that are generated in cells.

In this paper, we report the results on chemical reactivities of β-thujaplicin and its isomers (α- and γ-thujaplicins) to active oxygen species such as superoxide anion radicals ($\cdot O_2^-$), hydroxyl radicals ($\cdot$OH), singlet oxygens ($^1O_2$), *tert*-butyl peroxyl radicals (ROO$\cdot$) and hydrogen peroxides (H_2O_2). The results were compared with the reactivities of tropolone, the parent compound of the thujaplicins; EDTA, diethylenetriamine pentaacetic acid (DTPA) and deferoxamine, which are chelating agents; and *l*-ascorbic acid and *dl*-α-tocopherol, which are well-known antioxidants.

Experimental

Materials α-Thujaplicin (natural product, purity 90%), β-thujaplicin (synthetic product, purity 100%) and γ-thujaplicin (synthetic product, purity 100%) were obtained from Takasago International Corporation (Tokyo, Japan), and tropolone and 2,2,6,6-tetramathyl-4-piperidone

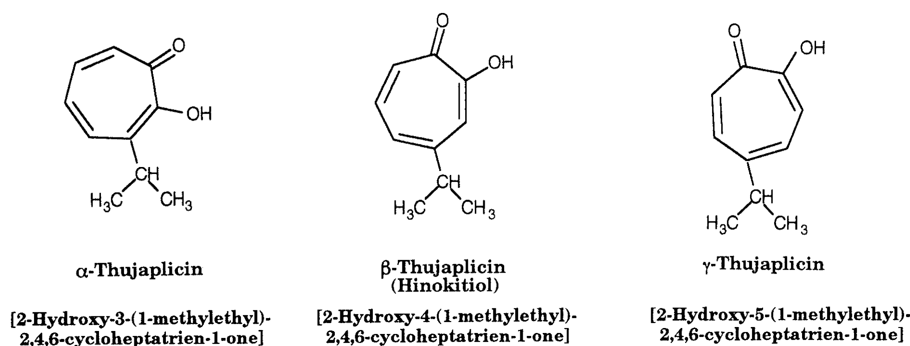

Fig. 1. Chemical Structures of α-, β- and γ-Thujaplicins

* To whom correspondence should be addressed.

© 1997 Pharmaceutical Society of Japan

NII-Electronic Library Service

hydrochloride (TMPD) were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI, U.S.A.). *dl*-α-Tocopherol, EDTA-2Na, sodium lauryl sulfate (SDS) and tetra-*n*-butylammonium perchlorate (TBAP) were from Nacalai Tesque Inc. (Kyoto, Japan). *l*-Ascorbic acid, ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), H_2O_2 (30% solution), hypoxanthine (HPX), 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol) and scopoletin were obtained from Wako Pure Chemical Industries (Tokyo, Japan); and deferoxamine mesylate (desferal), hematoporphyrin, xanthine oxidase (XOD), methemoglobin (MetHb), hematin and horseradish peroxidase (HRP) were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). 5,5-Dimethyl-1-pyrroline-1-oxide (DMPO) was obtained from Labotec Company (Tokyo, Japan), DTPA from Dojindo Laboratories (Kumamoto, Japan), *tert*-butyl hydroperoxide (BHP) from Katayama Chemical Industries Co., Ltd. (Osaka, Japan), and 2-methyl-6-phenyl-3,7-dihydroimidazo [1,2-*a*] pyrazin-3-one (CLA) from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). All other chemicals used in this study were commercially available products of analytical reagent grade.

Sample Preparations α-, β- and γ-Thujaplicins or tropolone were dissolved in ethanol or NaOH solution at an equimolar concentration and diluted with water to various concentrations. The ethanol solution was used for the assay of hydroxyl radicals, singlet oxygens and *tert*-butyl peroxyl radicals in the ESR spin-trapping method. Other experiments were carried out in NaOH solution. The final concentrations of ethanol and NaOH were less than 0.1% and 2 mM, respectively. The addition of 0.1% or 2 mM NaOH showed no remarkable effect on these assay systems. *l*-Ascorbic acid and *dl*-α-tocopherol were dissolved in water and 1% SDS solution, respectively, and diluted with water to various concentrations.

Estimation of Active Oxygen ($\cdot O_2^-$, $\cdot OH$, $^1O_2$ and $ROO\cdot$) Scavenging Activity by ESR Spin-Trapping Method ESR spectra were recorded on a JEOL JES-RE1X spectrometer or a JEOL FR-30 freeradical monitor (Tokyo, Japan) at a field modulation frequency of 100 kHz, modulation amplitude of 0.1 mT, and an output power of 5 mW. Mn(II) doped in magnesium oxide (MgO) was used as a standard. All experiments were carried out at 23 °C. Analysis of spin-trapped adducts of $\cdot O_2^-$, $\cdot OH$, $^1O_2$ or $ROO\cdot$ was performed as described below. After recording the ESR spectra, each signal intensity was normalized as a relative signal height against the standard signal due to the Mn(II) maker. Scavenging activity was expressed as the 50% inhibition concentration (IC_{50}) of active oxygen species generated, which was calculated from regression lines, where the abscissa represented the concentration of the test compound and the ordinate represented the relative signal height for independent triplicate tests.

Superoxide-Anion ($\cdot O_2^-$) Superoxide anion scavenging activity of the test compounds was determined by the method of Kitagawa *et al.*[20] XOD (0.12 units/ml) was added to 0.4 mM HPX in 80 mM phosphate buffer (pH 7.4) containing 1 mM DTPA and various concentrations of the test compounds. Almost simultaneously, 90 mM DMPO was added to the solution and then mixed on a vortex mixer. After allowing the solution to react for 30 s, ESR spectra due to DMPO-OOH were recorded under the conditions described above.

Hydroxyl-Radical ($\cdot OH$) Hydroxyl radical scavenging activity of the test compounds was determined using the Fenton system (Fe(II)+H_2O_2).[21] First, 2.25, 3.38, 4.50 or 5.63 μM $FeSO_4$, various concentrations of the test compound, and 1, 10 or 100 mM DMPO were mixed. Then, 2.25, 22.5 or 225 μM H_2O_2 was added and mixed on a vortex mixer. After 30 s, ESR spectra due to DMPO-OH were recorded under the same conditions as those above.

Singlet-Oxygen ($^1O_2$) Singlet oxygen quenching activity of the test compounds was estimated with TMPD, which was used as a singlet oxygen-trapping reagent, and by the method of Masaki *et al.*[22] Various concentrations of the test compounds were added to a solution containing 50 μM hematoporphyrin and 50 mM TMPD in 100 mM Tris–HCl buffer (pH 8.0). After UVA irradiation (0.65 J/cm^2, 1 kW xenon lamp, Ushio Inc.) filtered with a UV-33 filter (Toshiba Glass Co.) for 1 min, ESR spectra due to 2,2,6,6-tetramathylpyperidone-1-oxyl (TEMPONE) were recorded under the conditions as those above.

***tert*-Butyl Peroxyl Radical ($ROO\cdot$)** *tert*-Butyl peroxyl radical scavenging activity of the test compounds was determined by the method of Akaike *et al.*[23] BHP (25 mM) was added to MetHb (250 μg/ml) in 100 mM phosphate buffer (pH 7.4) containing 25 μM DTPA and various concentrations of the test compounds. Almost simultaneously, 10 mM DMPO was added to the solution. After mixing the solution on a vortex mixer for 30 s, ESR spectra due to DMPO-OOR were recorded under the conditions described above.

Estimation of Active Oxygen ($\cdot O_2^-$, $\cdot OH$, $^1O_2$ and H_2O_2) Scavenging Activity by Chemiluminescence (CL) Method Luminescence was measured with an ALOKA Luminescence Reader RLR-20 (Tokyo, Japan). The reactivities of the test compounds towards $\cdot O_2^-$, $\cdot OH$, $^1O_2$ or H_2O_2 were determined by the following method. Each cuvette (12 mm in diameter) containing the resulting solutions was placed in a photomultiplier tube at 30 °C in a dark cuvette chamber, and the intensities of luminescence were recorded. Scavenging activity was expressed as the 50% inhibition concentration (IC_{50}) of active oxygen species generated, which was calculated from regression lines, where the abscissa represented the concentration of the test compound and the ordinate represented the intensities of luminescence for independent triplicate tests.

Superoxide-Anion ($\cdot O_2^-$) Superoxide anion scavenging activity of the test compounds was determined by a modified procedure of Suzuki *et al.*[24] Experiments were performed on solutions consisting of 100 μl of CLA (final conc., 0.44 μM), 100 μl of DTPA (1.1 mM), 200 μl of HPX (0.2 mM) in 25 mM phosphate buffer (pH 7.1), and 100 μl of various concentrations of the test compounds. The CL reaction was initiated by injection of 1 μl XOD (0.02 units/ml) in the buffer solution. Superoxide anion scavenging effects were evaluated by the intensities due to CLA-dependent chemiluminescence.

Hydroxyl-Radical ($\cdot OH$) Hydroxyl radical scavenging activity of the test compounds was determined by using the Fenton system.[25] After allowing a reaction containing 100 μl of luminol (final conc., 41.0 μM) to stand in the dark for 1 h, 50 μl of DTPA (0.2 mM) in 50 mM phosphate buffer (pH 7.8) and 50 μl of $FeSO_4 \cdot 7H_2O$ (8.2 μM) in water, 300 μl of the buffer solution, and 100 μl of test compounds at various concentrations were added to the luminol solution. After the CL reaction was initiated by injection of 10 μl H_2O_2 (0.5 mM) in water, the intensities due to luminescence (A) were then recorded. In a similar manner to that of A, the intensities of luminescence (B) were recorded after addition of 300 μl of ethanol instead of the buffer solution. Hydroxyl radical scavenging effects were evaluated by subtracting B from A.

Singlet-Oxygen ($^1O_2$) Singlet oxygen quenching activity of the test compounds was determined by a modified method of Nakano *et al.*[26] Experiments were performed on solutions containing 100 μl of CLA (final conc., 0.43 μM), 100 μl of DTPA (1.1 mM), 200 μl of H_2O_2 (78.4 mM) in 100 mM acetate buffer (pH 4.5), and 100 μl of the test compounds at various concentrations. The CL reaction was initiated by injection of 10 μl sodium hypochlorite (58.8 mM) in the buffer solution. Singlet oxygen scavenging effects were evaluated by the intensities due to CLA-dependent chemiluminescence.

Hydrogen Peroxide (H_2O_2) Hydrogen peroxide scavenging activity of the test compounds was measured by the luminol–H_2O_2 system.[27] After allowing a solution containing 200 μl of luminol (final conc., 196 μM) and 200 μl of hematin (58.8 μM) in 100 mM borate buffer (pH 11.0) to stand in the dark for 1 h, 100 μl of the test compounds at various concentrations was added to the cuvette. The CL reaction was initiated by injection of 10 μl of H_2O_2 (3.9 μM) in water. Hydrogen peroxide scavenging effects were evaluated by the intensities due to luminol-dependent chemiluminescence.

Estimation of H_2O_2 Scavenging Activity of the Compounds by Fluorescence Method Fluorescence was measured with a CORONA microplate reader MTP-100F (Tokyo, Japan). Hydrogen peroxide scavenging ability of the compounds was measured by the decrease in H_2O_2 according to the scopoletin method.[28] Scopoletin (1 mM) was prepared in 100 mM phosphate buffer (pH 7.4) at 37 °C and stored at 4 °C in the dark. After mixing the solution containing test compounds at various concentrations with or without 5 μM H_2O_2 mixed for 5 min at room temperature (23 °C), a solution containing 5 μM scopoletin and 0.5 units/ml HRP in 100 mM phosphate buffer (pH 7.4) was added and incubated for 5 min at 37 °C. Determination of H_2O_2 was carried out by measuring fluorescence intensities (Ex: 360 nm, Em: 450). After correcting these values by excluding the effects without H_2O_2, the concentrations of H_2O_2 were calculated from the calibration curve. Scavenging activity was expressed as the 50% scavenging concentration (IC_{50}) of H_2O_2 added, which was calculated from regression lines, where the abscissa represented the concentration of the test compound and the ordinate represented the concentration of H_2O_2 for independent triplicate tests.

Electrochemical Measurements Cyclic voltammetry was carried out using a BAS 100B/W (Lafayette, IN, U.S.A.) with a three-electrode

NII-Electronic Library Service

Table 1. $\cdot O_2^-$, $\cdot OH$, $^1O_2$, ROO· and H_2O_2 Scavenging Effects of α-, β- and γ-Thujaplicins and Related Compounds as Evaluated by the ESR Spin-Trapping, Chemiluminescence and Fluorescence Methods

Compound	IC50 (μM)							
	$\cdot O_2^-$		·OH		$^1O_2$	ROO·	H_2O_2	
	ESR	Chemiluminescence	ESR[a]	Chemiluminescence	Chemiluminescence	ESR	Chemiluminescence	Fluorescence
α-Thujaplicin	>1000	N.D.	8.1± 0.3	12.7±0.6	718.3±43.9	88.4± 2.1	7.8±0.2	35.4±2.9
β-Thujaplicin	>1000	>1000	5.1± 0.1	7.1±1.8	>1000	46.9± 1.2	61.6±6.3	73.8±3.3
γ-Thujaplicin	>1000	N.D.	5.1± 0.2	10.8±0.5	>1000	64.2± 2.2	159.1±9.9	61.4±0.9
Tropolone	>1000	N.D.	5.9± 0.2	7.1±0.5	603.0±75.5	41.7± 1.8	163.2±2.3	151.3±8.1
l-Ascorbic acid	17.4±1.0	3.0±0.5	2.3± 0.1	2.5±0.6	9.5± 1.7	105.8± 5.5	N.D.	N.D.
dl-α-Tocopherol	>500	>500	170.0±33.0	134.3±8.3	215.7±30.1	141.0±10.0	N.D.	N.D.

Data are expressed as the means±S.D.s of 3 experiments. N.D.: IC_{50} was not determined under the conditions. *a*) Hydroxy radicals by the Fenton system were generated and detected as follows, 2.25 μM $FeSO_4$, 22.5 μM H_2O_2 and 100 mM DMPO.

system. A Pt rod as a working electrode, Pt-wire as an auxiliary electrode, and Ag/AgCl or Ag/Ag^+ electrode as a reference electrode were used. The reference electrode was separated from the bulk of the solution by a glass frit and was immersed in an aqueous solution containing 2% ethanol and 0.1 M KCl or in an acetonitrile solution containing 0.1 M TBAP. All experiments were carried out at 22 °C in each solution purged with N_2 gas. Potentials of *dl*-α-tocopherol and other test compounds were recorded *vs.* Ag/Ag^+ and *vs.* Ag/AgCl, respectively, in which the sweep rate was 25 mV/s for cyclic voltammetry. The half-wave potential ($E_{1/2}$) was calculated as the midpoint of anode potential (E_{pa}) and cathode potential (E_{pc}): $E_{1/2}=(E_{pa}+E_{pc})/2$. The oxidation-reduction potential as the standard hydrogen electrode (NHE) was evaluated from the following equation: $E^0(\mathrm{NHE})=E_{1/2}(\mathrm{Ag/AgCl})-210\,(\mathrm{mV})$ or $E^0(\mathrm{NHE})=E_{1/2}(\mathrm{Ag/Ag^+})-490\,(\mathrm{mV})$.[29]

Results

Scavenging Activities of α-, β- and γ-Thujaplicins against Active Oxygen Species Scavenging activities of α-, β- and γ-thujaplicins and related compounds against superoxide anion radicals ($\cdot O_2^-$), hydroxyl radicals (·OH), singlet oxygens ($^1O_2$), *tert*-butyl peroxyl radicals (ROO·) and hydrogen peroxides (H_2O_2) were examined by the ESR spin-trapping, chemiluminescence and fluorescence methods, as summarized in Table 1.

The IC_{50} values for the superoxide anion radical scavenging activities of α-, β- and γ-thujaplicins and tropolone as evaluated by the ESR spin-trapping and chemiluminescence methods were found to be higher than 1000 μM, indicating that thujaplicins were not as effective as *l*-ascorbic acid, which is a well-known antioxidant.

The hydroxyl radical inhibitory effects of α-, β- and γ-thujaplicins and tropolone were also evaluated by both ESR spin-trapping and chemiluminescence methods. The IC_{50} values of α-, β- and γ-thujaplicins and tropolone were found to be 8.1±0.3, 5.1±0.1, 5.1±0.2 and 5.9±0.2 μM, respectively, by the ESR spin-trapping method using 100 mM DMPO; while by the chemiluminescence method, the IC_{50} values were 12.7±0.6, 7.1±1.8, 10.8±0.5 and 7.1±0.5 μM, respectively. There were no notable differences in the IC_{50} values evaluated by the above two methods except for that of γ-thujaplicin, which was evaluated by the chemiluminescence method to be approximately twice that evaluated by the ESR method. In terms of the IC_{50} values, the inhibitory effects of the test compounds were found to be lower than that of *l*-ascorbic acid but higher than that of *dl*-α-tocopherol. No changes in the IC_{50} value of β-thujaplicin were observed by changing the concentration of DMPO (Table 2). To know the mechanism of inhibitory effects of β-thujaplicin on hydroxyl radicals, we compared the results with other chelating agents such as EDTA, DTPA and deferoxamine (Fig. 2). β-Thujaplicin inhibited the formation of hydroxyl radicals generated by the Fenton reaction in a dose-dependent manner, being more effective than EDTA, DTPA and deferoxamine.

The singlet oxygen quenching activities of α-, β- and γ-thujaplicins and tropolone evaluated by the chemiluminescence method were found to be as follows: α-thujaplicin (IC_{50}=718.3±43.9 μM), β- and γ-thujaplicins (IC_{50}> 1000 μM), and tropolone (603.0±75.5 μM). β-Thujaplicin was also examined by the ESR spin-trapping method, and was found to have an IC_{50} value of more than 1000 μM

Table 2. Effect of DMPO Concentration on ·OH Inhibitory Activity of β-Thujaplicin

DMPO Concentration (mM)	IC_{50} (μM)
1	5.0±0.4
10	5.3±0.3
100	5.1±0.1

Data are expressed as the means±S.D.s of 3 experiments. Hydroxy radicals were generated as follows, 2.25 μM $FeSO_4$ and 22.5 μM H_2O_2.

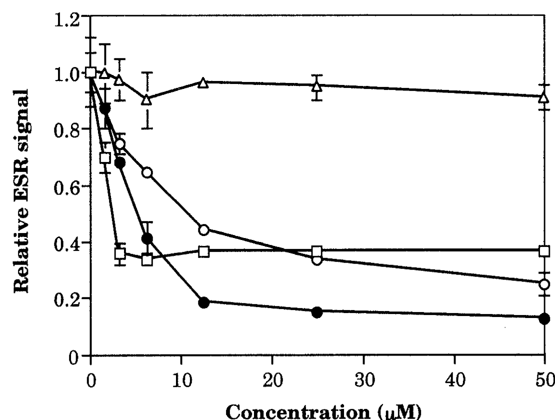

Fig. 2. Inhibitory Effects of β-Thujaplicin and Chelating Agents on Hydroxyl Radicals

Hydroxy radicals by the Fenton system ($FeSO_4$–H_2O_2) were generated and detected as follows, 2.25 μM $FeSO_4$, 22.5 μM H_2O_2 and 100 mM DMPO. After recording ESR spectra, the signal intensity was normalized as the relative signal height against the standard signal height due to Mn(II). Symbols are expressed as the means±S.D.s (n=3). —●—, β-thujaplicin; —△—, DTPA; —○—, deferoxamine; —□—, EDTA.

NII-Electronic Library Service

Table 3. Half-Wave Potentials of α-, β- and γ-Thujaplicins and Related Compounds

Compound	Solvent	Potential (mV)
α-Thujaplicin	KCl	−670[a] (−880)[b]
β-Thujaplicin	KCl	−630[a] (−840)[b]
γ-Thujaplicin	KCl	−640[a] (−850)[b]
Tropolone	KCl	−620[a] (−830)[b]
l-Ascorbic acid	KCl	−440[a] (−650)[b]
dl-α-Tocopherol	Acetonitrile	470[c] (−20)[b]

a) V *vs.* Ag/AgCl, *b)* V *vs.* NHE, *c)* V *vs.* Ag/Ag^+.

(data not shown). The results therefore indicated that α-, β- and γ-thujaplicins were not as effective as *l*-ascorbic acid or *dl*-α-tocopherol for quenching $^1O_2$.

tert-Butyl peroxyl radical scavenging activity of the test compounds was evaluated by the ESR spin-trapping method. Scavenging activity was found to be in the following order: tropolone ($IC_{50}=41.7\pm1.8$ μM) > β-thujaplicin (46.9 ± 1.2 μM) > γ-thujaplicin (64.2 ± 2.2 μM) > α-thujaplicin (88.4 ± 2.1 μM). Thujaplicins and tropolone exhibited higher *tert*-butyl peroxyl radical scavenging activities than those of *l*-ascorbic acid ($IC_{50}=105.8\pm5.5$ μM) and *dl*-α-tocopherol ($IC_{50}=141.0\pm10.0$ μM), both of which are well-known antioxidants. β-Thujaplicin, as estimated by ESR spin-trapping, was found to have the highest activity among the test compounds.

The hydrogen peroxide scavenging activity of the compounds was evaluated by both chemiluminescence and fluorescence methods. Quenching activity in terms of the IC_{50} value estimated by the chemiluminescence method was found to be in the following order: α-thujaplicin ($IC_{50}=7.8\pm0.2$ μM) > β-thujaplicin (61.6 ± 6.3 μM) > γ-thujaplicin (159.1 ± 9.9 μM) > tropolone (163.2 ± 2.3 μM). The results of the fluorescence method, on the other hand, exhibited the following order: α-thujaplicin ($IC_{50}=35.4\pm2.9$ μM) > γ-thujaplicin (61.4 ± 0.9 μM) > β-thujaplicin (73.8 ± 3.3 μM) > tropolone (151.3 ± 8.1 μM). Although the IC_{50} values for α- and γ-thujaplicins were not in agreement, α-thujaplicin, as estimated by both chemiluminescence and fluorescence methods, was found to have the highest activity among the test compounds.

Redox Potentials of α-, β- and γ-Thujaplicins and Related Compounds The reducing ability of α-, β- and γ-thujaplicins and related compounds was studied by cyclic voltammetry. The half-wave potentials of the compounds are given in Table 3. In terms of the half-wave potential as estimated by the standard hydrogen electrode (NHE), reducing ability was found to be in the following order: α-thujaplicin (−880 mV), γ-thujaplicin (−850 mV), β-thujaplicin (−840 mV) and tropolone (−830 mV). This indicates that tropolone derivatives have a higher reducing ability than that of *l*-ascorbic acid (−650 mV) and *dl*-α-tocopherol (−20 mV). Among them, α-thujaplicin is the strongest reducing agent. A linear relationship ($y=0.938x+861$, $r=0.997$ for a total of three compounds in triplicate measurements) (data not shown) between the half-wave potential ($E_{1/2}$) of thujaplicins and their H_2O_2 scavenging activities in terms of the IC_{50} values as evaluated by fluorescence method was found to depend on the position of the substituent. While, a reverse correlation ($y=-0.984x-777$, $r=0.983$ for a total of three compounds in triplicate measurements) (data not shown) between the $E_{1/2}$ of thujaplicins and their *tert*-butyl peroxyl radical scavenging activity evaluated by ESR spin-trapping method was obserbed.

Discussion

We investigated the chemical reactivities of β-thujaplicin and its isomers α- and γ-thujaplicins (Fig. 1) to five kinds of active oxygen species such as superoxide anion radicals ($\cdot O_2^-$), hydroxyl radicals ($\cdot OH$), singlet oxygen ($^1O_2$), *tert*-butyl peroxyl radicals (ROO·) and hydrogen peroxide (H_2O_2) (Table 1).

Some of the obtained results on active oxgen scavenging activities of the compounds in terms of the IC_{50} value were not in good agreement between the evaluation methods. This method dependent-discrepancy of data will be examined in a future study. This study, however, did show certain tendencies in the active oxygen scavenging activity of the test compounds. The test compounds were found to have comparable inhibitory effects of hydroxyl radicals and high scavenging activities against *tert*-butyl peroxyl radicals as compared with those of *l*-ascorbic acid and *dl*-α-tocopherol and α-thujaplicin was found to have the highest activity against hydrogen peroxides among the thujaplicins. However, the effects of the compounds on superoxide anion radicals and singlet oxygens were less than those of *l*-ascorbic acid and *dl*-α-tocopherol.

Hydroxyl radicals, which are responsible for biological damage, are thought to be formed in cells mainly *via* the reaction of metal ions and hydrogen peroxide (Fenton reaction[21,25]: $Fe^{2+}+H_2O_2\rightarrow Fe^{3+}+\cdot OH+OH^-$).[30,31] Samuni *et al.*[32] proposed a "site-specific" Fenton mechanism in which the binding of transition metal ions with biological targets is a prerequisite for the production of hydroxyl radical-mediated cell damage. In support to this proposal, the Fenton reaction has been reported to be catalyzed by a complex formation between ferrous ion and low-molecular weight compounds.[25,33,34] In addition, the generation of hydroxyl radicals is affected by buffer solutions.[34] Since α-, β-, γ-thujaplicins and tropolone have been known to form stable complexes with ferrous or ferric ion,[35−39] we examined the effect of thujaplicins on the hydroxyl radicals generated in the Fenton system by the ESR spin-trapping method. In the chemiluminescence method, it is presumed that hydroxyl radicals reduce the luminescence through coordination with transition metal ions.[40,41] Thus, the measurement of hydroxyl radical scavenging activity of the compounds by the chemiluminescence method was performed in the presence of a constant concentration of DTPA (50 mM phosphate buffer at pH 7.4).

The hydroxyl radical scavenging activities of thujaplicins in terms of the IC_{50} value were weaker than that of *l*-ascorbic acid but stronger than that of *dl*-α-tocopherol. The stability constants of thujaplicins–Fe complex, especially that of β-thujaplicin–Fe^{3+} complex, are very high, *e.g.*, the value for Fe(β-thujaplicin)$_3$ complex was reported to be log β, 37.7.[37] Thus, it is reasonable to assume that Fe^{2+} chelated with thujaplicins does not cause the Fenton reaction. In the ESR spin-trapping

NII-Electronic Library Service

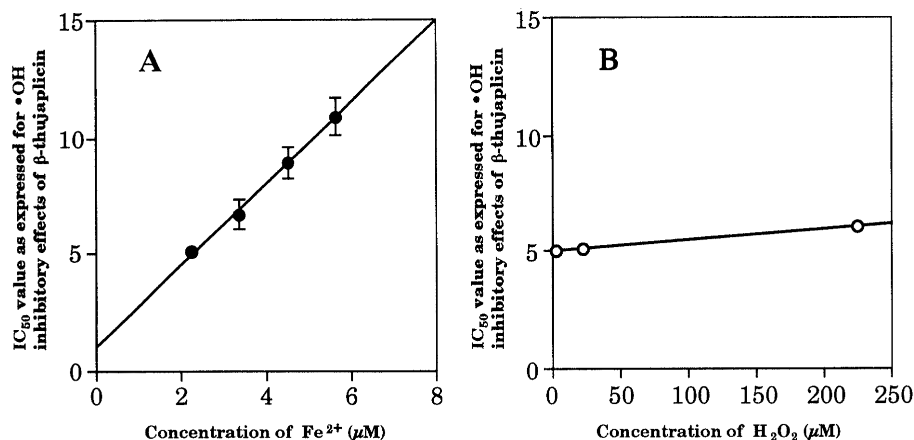

Fig. 3. Effects of Fe^{2+} (A) or H_2O_2 (B) Concentration on the IC_{50} Values of β-Thujaplicin

IC_{50} values of β-thujaplicin for the hydroxy radical inhibitory effect were plotted against concentrations of 2.25, 3.38, 4.50 or 5.63 μM $FeSO_4$ in the presence of 22.5 μM H_2O_2 (constant) (—●—, $y=1.741x+1.051$, $r=0.998$) (A), and 2.25, 22.5 or 225 μM H_2O_2 in the presence of 2.25 μM $FeSO_4$ (constant) (—○—, $y=0.005x+4.990$, $r=1.000$) (B). Hydroxy radicals were generated by the Fenton ($FeSO_4$–H_2O_2) system and trapped with 100 mM DMPO. Symbols are expressed as the means ± S.D.s ($n=3$).

method, the concentrations of DMPO were changed, but the IC_{50} values of β-thujaplicin were found to be independent of the DMPO concentration (Table 2). With a two-fold increase in the concentration of Fe^{2+}, the IC_{50} value of β-thujaplicin increased by about two fold (Fig. 3A), but no remarkable change in the IC_{50} value was observed with a ten-fold increase in the amount of H_2O_2 (Fig. 3B). The results indicate that the reaction depends on the concentration of Fe^{2+}. Thujaplicins and tropolone thus suppress the generation of hydroxyl radicals by preventing the progress of the Fenton reaction, suggesting that the chelate formation between thujaplicins and Fe^{2+} inhibits the reaction between Fe^{2+} and H_2O_2. Thus, it is necessary to examine the reaction of thujaplicins with hydroxyl radicals produced in a chemically pure system.

The chemiluminescence and fluorescence methods were used to measure hydrogen peroxide scavenging activity of the compounds, and the IC_{50} values obtained by these two methods were different (Table 1). Although the reactions between thujaplicins and H_2O_2 have yet been investigated, β-thujaplicin and tropolone are known to be oxidized by alkyl peroxides in alkaline solution to produce dicarbonic acids such as *cis,cis*-muconic acid.[2,42] In support of this observation, a good relationship between the redox potential of thujaplicins measured under the same conditions and their hydrogen peroxide scavenging activities in terms of the IC_{50} values as evaluated by fluorescence method was found, indicating that the reducing ability of the compound might be one of factors contributed in the reaction. Relationship between the half-wave potential ($E_{1/2}$) of α-, β- or γ-thujaplicin and their *tert*-butyl peroxyl radical scavenging activity in terms of the IC_{50} value was also observed. Superoxide anion radical scavenging activity of thujaplicins was not found, but low singlet oxygen quenching activities were shown by thujaplicins (Table 1). In support of those observations, it has been reported that β-thujaplicin reacts with singlet oxygen *via* a photosensitized oxygenation reaction, resulting in the formation of tetracyclo $[7.3.2.0^{2,8}.0^{4,14}]$ tetradecane.[43]

Although the biochemical activity of H_2O_2 itself is not so high,[44] H_2O_2 penetrates the cell membrane[45] and provides a source for the formation of hydroxyl radicals in the presence of trace metal ions such as iron and copper.[21,25,30] On the other hand, when H_2O_2 is formed in the $\cdot O_2^-$ generation system catalyzed by SOD, hydroxyl radicals are also formed by Fenton-like reactions (the superoxide-driven Fenton reaction).[33] In addition, hydroxyl radicals are catalyticaly produced by the reaction between H_2O_2 and $\cdot O_2^-$ in the presence of metal ions (the Haber–Weiss reaction).[46] Consequently, metal chelators such as EDTA and deferoxamine are thought to act as a type of quencher to inhibit hydroxyl radical formation.[47] In addition, Bissett *et al.*[48,49] have reported that iron is a factor in skin photodamage, participating in oxygen radical production, in which a high level of protection is provided by metal chelater 2-furildioxime. The results also indicate the important role of metal ions in active oxygen formations. On the basis of these results, it is concluded that β-thujaplicin at a low concentration has a stronger inhibitory effect on the formation of hydroxyl radicals than that of deferoxmine, EDTA or DTPA (Fig. 2).

Since thujaplicins exhibit stronger inhibitory effects of hydroxyl radicals generated by the Fenton reaction and scavenging activities against *tert*-butyl peroxyl radicals than those of *l*-ascorbic acid or *dl*-α-tocopherol and in addition they have scavenging activities against hydrogen peroxides, they are proposed to be effective antioxidants for endogenously produced active oxygen species, which in turn contribute to the prevention of UV-induced photodamage *in vivo*.

Acknowledgment The authors particularly acknowledge Mr. Michiaki Kawasaki and Dr. Susumu Watanabe of Takasago International Corporation for the kind gift of α- and γ-thujaplicins. Y. A. expresses grateful thank to Otsuka Pharmaceutical Co., Ltd. to give a chance to work with the investigation.

References

1) Nozoe T., *Ber Bull. Chem. Soc. Jpn.*, **11**, 295—298 (1936).
2) Nozoe T., Katura S., *Yakugaku Zasshi*, **64**, 181—185 (1944).
3) Erdtman H., Gripenberg J., *Nature* (London), **161**, 719 (1936).
4) Southam C. M., *Proc. Soc. Exp. Biol. Med.*, **61**, 391—396 (1946).
5) Kamp B. J., *Wood and Fiber Science*, **18**, 421—427 (1986).
6) Trust T. J., Coombs R. W., *Can. J. Microbial.*, **19**, 1341—1346 (1973).

NII-Electronic Library Service

7) Osawa K., Matsumoto T., Maruyama T., Takiguchi T., Okuda K., Takazoe I., *Bull. Tokyo Dent. Coll.*, **31**, 17—21 (1990).
8) Ri S., *Niigata Igakkai Zasshi*, **65**, 566—572 (1951).
9) Inamori Y., Tsujibo H., Ohishi H., Ishii F., Mizugaki M., Aso H., Ishida N., *Biol. Pharm. Bull.*, **16**, 521—523 (1993).
10) Yamamoto M., Hashigaki K., Kokubo N., Tsuruo T., Tashiro T., *J. Med. Chem.*, **27**, 1749—1753 (1984).
11) Muto N., Dota A., Tanaka T., Itoh N., Okabe M., Inada A., Nakanishi T., Tanaka K., *Biol. Pharm. Bull.*, **18**, 1576—1579 (1995).
12) Woodcock A., Magnus I. A., *Br. J. Dermatol.*, **95**, 459—468 (1976).
13) Danno K., Horio T., *Photochem. Photobiol.*, **45**, 683—690 (1987).
14) Baba T., Nakano H., Hanada K., Hashimoto I., Arima Y., Proceeding of the 20th Annual Meeting of the Japanese Society for Investigative Dermatology, Okayama, 1995.
15) Miyachi Y., Horio T., Imamura S., *Clin. Exp. Dermatol.*, **8**, 305—310 (1983).
16) Danno K., Horio T., Takigawa M., Imamura S., *J. Invest. Dermatol.*, **83**, 166—168 (1984).
17) Ogura R., Sugiyama M., Nishii J., Haramaki N., *J. Invest. Dermatol.*, **97**, 1044—1047 (1991).
18) Black H. S., *Photochem. Photobiol.*, **46**, 213—221 (1987).
19) Hanada K., Gange R. W., Connor M. J., *J. Invest. Dermatol.*, **96**, 838—840 (1991).
20) Kitagawa S., Fujisawa H., Sakurai H., *Chem. Pharm. Bull.*, **40**, 304—307 (1992).
21) Walling C., *Acct. Chem. Res.*, **8**, 125—131 (1975).
22) Masaki H., Sakaki S., Atsumi T., Sakurai H., *Biol. Pharm. Bull.*, **18**, 162—166 (1995).
23) Akaike T., Sato K., Ijiri S., Miyamoto Y., Kohno M., Ando M., Maeda H., *Arch. Biochem. Biophys.*, **294**, 55—63 (1992).
24) Suzuki N., Suetsuna K., Mashiko S., Yoda B., Nomoto T., Toya Y., Inaba H., Goto T., *Agric. Biol. Chem.*, **55**, 157—160 (1991).
25) Cohen G., Sinet P. M., *FEBS Lett.*, **138**, 258—260 (1982).
26) Nakano M., "Method in Enzymology," Vol. 186, ed. by Packer, A. N. Glazer, 1990, p. 585.
27) Langenbec W., Ruge U., *Ber. Deut. Chem. ges. B*, **70**, 367—369 (1937).
28) Andreae W. A., *Nature* (London), **175**, 859—860 (1955).
29) "The Electrode Handbook for Cyclic Voltammetry," Bioanalitical Systems Inc., Tokyo, 1997, p. 38.
30) Halliwell B., Gutteridge J. M. C., *Biochem. J.*, **219**, 1—14 (1984).
31) Puppo A., Halliwell B., *Biochem. J.*, **249**, 185—190 (1988).
32) Samuni A., *Eur. J. Biochem.*, **137**, 119—124 (1983).
33) Gutteridge J. M. C., *FEBS Lett.*, **185**, 19—23 (1985).
34) Yoshimura Y., Matsuzaki Y., Watanabe T., Utiyama K., Osawa K., Imaeda K., *Magnetic Resonance in Medicine*, **4**, 50—54 (1993).
35) Oka Y., Umehara M., Nozoe T., *J. Chem. Soc. Jpn.*, **83**, 1197—1203 (1962).
36) Oka Y., Yauchi M., Suzuki C., *J. Chem. Soc. Jpn.*, **85**, 873—879 (1964).
37) Menis O., McClellan B. E., Bright D. S., *Anal. Chem.*, **43**, 431—435 (1971).
38) Maclean H., Gardner J. A. F., *Anal. Chem.*, **28**, 509—512 (1956).
39) Bryant B. E., Fernelius W. C., Douglas B. E., *J. Am. Chem. Soc.*, **75**, 3784—3786 (1953).
40) Barb W. G., Baxendale J. H., George P., Hargrave K. R., *Trans. Faraday Soc.*, **47**, 462—500 (1957).
41) Barb W. G., Baxendale J. H., George P., Hargrave K. R., *Trans. Faraday Soc.*, **47**, 591—616 (1957).
42) Cook J. W., Gibb A. R., Raphael R. A., Somerville A. R., *J. Chem. Soc.*, 503—511 (1951).
43) Mihara S., Tateba H., *Biosci. Biotech. Biochem.*, **56**, 1156—1157 (1992).
44) Nakano M., *Mol. Med.*, **30**, 496—502 (1993).
45) Mukherjee S. P., *Biochem. Pharmacol.*, **29**, 1239—1246 (1980).
46) Harber F., Weiss J., *Proc. R. Soc. Lond. A*, **147**, 332—351 (1934).
47) Halliwell B., *Biochem. Pharmacol.*, **34**, 229—233 (1985).
48) Bissett D. L., Chatterjee R., Hannon D. P., *Photochem. Photobiol.*, **54**, 215—223 (1991).
49) Bissett D. L., Oelrich D. M., Hannon D. P., *J. Am. Acad. Dermatol.*, **31**, 572—578 (1994).

NII-Electronic Library Service