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EFFECTIVE INHIBITION OF HYDROXYL RADICALS BY HYDROXYLATED BIPHENYL COMPOUNDS

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In aqueous media, approximate rate constants for the reactions between hydroxyl radicals (·OH) and biphenyl compounds such as dehydrodieugenol, magnolol, honokiol, dehydrodidihydroeugenol, dehydrodivanillyl alcohol, and dehydrodicreosol were estimated by competition reactions for ·OH between these biphenyls and 5,5-dimethyl-1-pyrroline-N-oxide (DMPO). By measuring the decrease in the height of the EPR signals of the ·OH spin adduct, rate constants in the order of 10^9 to 10^{10} M were measured.

KEY WORDS: DMPO, Biphenyl compounds, Radical scavenging, ·OH, Hydroxyl radical, EPR.

INTRODUCTION

It has been reported that spontaneous free radical reactions occurring in living bodies such as lipid peroxidation are directly related to a variety of diseases[1-4]. Lipid peroxidation leads to the fatal damage of cellular organelles. Active oxygen species such as hydroxyl radicals (·OH), superoxide anion radicals, and singlet oxygen are reported to take part in the induction of lipid peroxidation[5-8]. Skin is a target organ of environmental oxidative damages. One of the active oxygen species yielding severe skin damage could be ·OH which may be induced by UV light[9-10]. If effective scavengers for ·OH can be used as raw materials for cosmetics, such skin damage could be prevented.

We have reported previously that phenyl propanoids in essential oils are effective ·OH scavengers[11]. Generally, however, these phenolic compounds are hardly used as cosmetic materials because they cause sensitization of skin. It has been reported that dehydrodieugenol, which has been found in our previous paper to be a good ·OH scavenger[11], leads to low sensitization[11]. This result may suggest that biphenyl compounds could be used as cosmetic raw materials. In the present study, therefore, our aim was placed on examining the ·OH scavenging ability of biphenyl compounds.

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MATERIALS AND METHODS

Materials

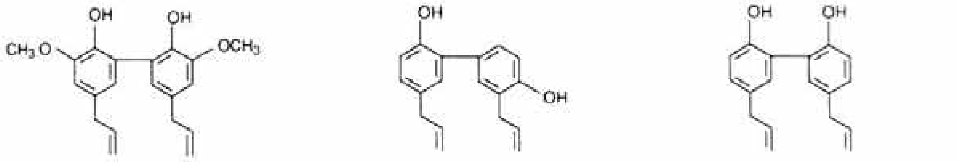
Six biphenyl compounds were examined. Magnolol and honokiol were purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). Dehydrodieugenol, dehydrodidihydroeugenol, dehydrodivanillyl alcohol, and dehydrodicreosol were prepared in our laboratories using a modification of a previous procedure as follows[13].

Dehydrodieugenol: To eugenol (20 mL) contained pyridine (20 g) was added ferrous sulfate (0.5 g). With vigorous stirring, hydrogen peroxide (100 mL, 30%) was added dropwise over a period of 8 hr at 60–70°C. The reaction product was extracted with ether. To the ether extract was added 5% sulfuric acid. The solvent was evaporated and recrystallization from ethanol gave white crystals, m.p. 108.5–109°C (lit., 104–105°C[12]).

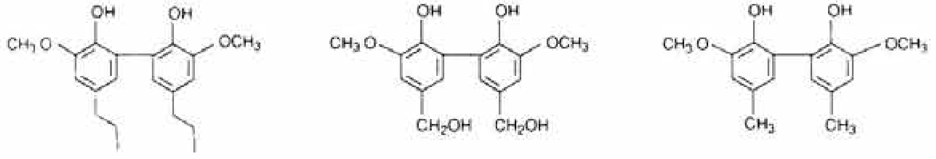
Dehydrodidihydroeugenol: To ethanol (60 mL) containing of 4-propyl guiacol (3.0 g), was added peroxidase (60 mL, 0.025%). With vigorous stirring, hydrogen peroxide (10.5 mL, 3%) was added dropwise over a period of 30 min and the precipitate was filtered. Recrystallization from ethanol gave white crystals, m.p. 156.5–158°C (lit., 150–152°C[12]).

Dehydrodivanillyl alcohol: To a solution prepared by dissolving vanillyl alcohol (3.0 g) in hot water (30 mL), peroxidase (30 mL, 0.05%) was added. With vigorous stirring, hydrogen peroxide (22 mL, 1%) was added dropwise over a period of 30 min. The product was extracted by ethyl acetate (50 mL). The ethyl acetate extract was dried over anhydrous magnesium sulfate and the solvent was evaporated. Then, obtained brown oil was dissolved in acetone/hexane and subjected to recrystallization. Subsequent recrystallization from ethyl acetate/hexane gave white crystals, m.p. 187–189°C (lit., 192–194°C[12]).

Dehydrodicreosol: To a solution consisting of 2-methoxy-4-methyl phenol (50 g) and ethanol (25 mL), was added peroxidase (25 mL, 0.05%). With vigorous stirring, hydrogen peroxide (15 mL, 3%) was added dropwise over a period of 30 min. The residue was twice extracted with 100 mL of chloroform and dried over anhydrous magnesium sulfate. After evaporation, the obtained brown oily residue was loaded on silica gel (100 g). The eluent used was benzene/acetone (8/1 in v/v). Fractions were collected by monitoring the spot with the Rf value of 0.58 on thin layer chromatography coupled with benzene/acetone (6/1 in v/v). The collected fractions were evaporated to dryness. Recrystallization from benzene/hexane gave white crystals, m.p. 137–138.5°C. ^{1}H-NMR spectroscopy was carried out in $CDCl_3$ on a JEOL GX-270 FT (JEOL, Tokyo). The chemical shifts in ppm obtained are as follows: 3.4 (d, 4H, 6.6 Hz), 3.9 (s, 6H), 5.0–5.1 (m, 4H), 5.9–6.0 (m, 4H), and 6.7 (m, 2H) for dehydrodieugenol, 1.0 (t, 6H, 7.2 Hz), 1.6–1.7 (m, 4H), 2.6 (t, 4H, 8.0 Hz), 3.9 (s, 6H), 6.0 (s, 2H), 6.7 (d, 2H, 1.9 Hz), and 6.8 (d, 2H, 1.9 Hz) for dehydrodidihydroeugenol, 4.0 (s, 6H), 4.5 (s, 2H), 4.6 (s, 2H), 7.4 (d, 2H, 1.9 Hz), and 7.5 (d, 2H, 1.9 Hz) for dehydrodivanillyl alcohol, 2.4 (s, 6H), 3.9 (s, 6H), 6.0 (s, 2H), and 6.7 (d, 4H, 2.5 Hz) for dehydrodicreosol. Mass spectra were measured on a JOEL JMS-DX303 mass spectrometer (JEOL). The mass numbers obtained by electron ionizing mass spectroscopy are 326, 330, 306, and for 274 dehydrodieugenol, dehydrodidihydroeugenol, dehydrodivanillyl alcohol, and dehydrodicreosol, respectively. The structures of the biphenyl compounds tested here are illustrated in Figure 1.



Dehydrodieugenol Honokiol Magnolol



Dehydrodidihydroeugenol Dehydrodivanillyl alcohol Dehydrodicreosol

FIGURE 1 Structures of biphenyl compounds tested in the present study.

5,5-Dimethyl-1-pyrroline-N-oxide (DMPO) with high purity was obtained from Mitsui Toatsu Co. (Tokyo). Peroxidase (from horse radish) with high purity and other chemicals of reagent grade were obtained from Wako Pure Chemicals, Ltd. Water was purified sequentially by Milli R/Q and Milli QII (Millipore, MA, USA).

EPR Measurements

EPR measurements were carried out on a JOEL PE-3X spectrometer (X-band and 100 kHz field modulation, JOEL). EPR settings are as follows: microwave power, 5 mW; modulation amplitude, 0.05 mT; time constant, 0.1 sec; gain, 160; sweep time, 2 min. The EPR intensities were calculated by double integration. The marker used for the measurements of hyperfine splitting constants and *g*-values was Mn^{2+} in MgO.

Assay with Spin Trapping

Since these biphenyl compounds are poorly soluble in water, samples were prepared by the following procedure: To aqueous ethanol solutions (100 mM, 100 mL), biphenyl compounds dissolved in ethanol (20 mM, 1 mL) were added, and 1 mL of these prepared aqueous solutions were mixed with KH_2PO_4-Na_2HPO_4 buffer (pH 7.4, 100 mM, 1 mL) containing $FeSO_4 \cdot 7H_2O$ (1 mM), diethylenetriamine-N,N,N′,N″,N″-pentaacetic acid (DETAPAC) (50 mM), and DMPO (100 mM). Then, the total volume was adjusted to 9 mL by adding water. Immediately prior to the EPR measurements, 1 mL of H_2O_2 (145 mM) was added to the finally prepared sample solutions to initiate Fenton reactions. EPR spectra were recorded 2 min after the addition of H_2O_2 at room temperature. The final sample solution contained $FeSO_4 \cdot 7H_2O$ (0.1 mM), H_2O_2 (14.5 mM), DETAPAC (5 mM), DMPO (10 mM), ethanol (32 mM), and biphenyl compounds (0.02 mM).

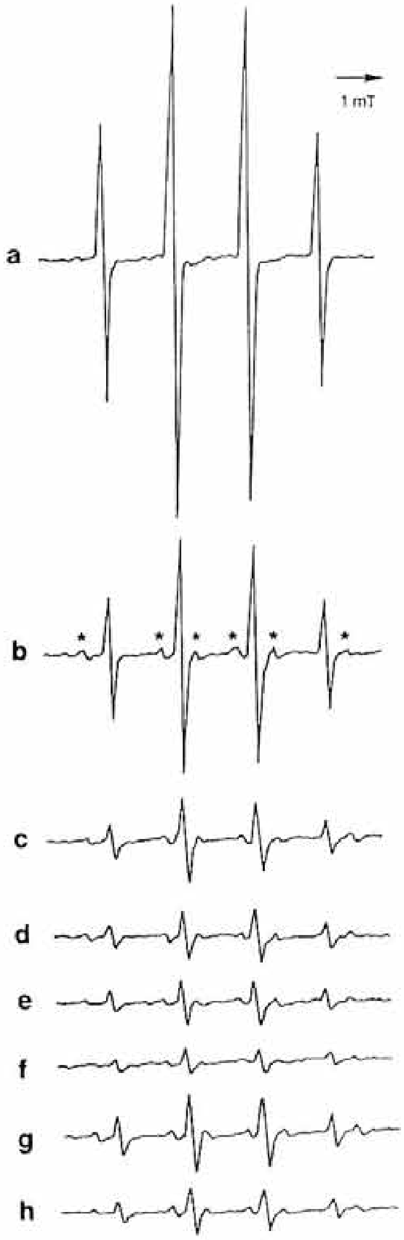


FIGURE 2 EPR spectra obtained from KH_2PO_4-Na_2HPO_4 buffer solutions (10 mM) containing $FeSO_4$ (0.1 mM), H_2O_2 (14.5 mM), DETAPAC (5 mM), DMPO (10 mM), and (a) none, (b) ethanol (100 mM), and (c-h) ethanol (32 mM) and 0.02 mM biphenyl compounds ((c) dehydrodieugenol, (d) magnolol, (e) honokiol, (f) dehydrodidihydroeugenol, (g) dehydrodivanillyl alcohol, and (h) dehydrodicreosol). EPR settings appear in the text.

RESULTS AND DISCUSSION

In order to evaluate how effectively the biphenyl compounds scavenge ·OH, the method of DMPO spin trapping was taken. ·OH was generated by the Fenton systems.

Shown in Figure 2a is an EPR spectrum obtained in the absence of ethanol and biphenyl compounds. In the spectrum, only a 1:2:2:1 qurtet due to a DMPO spin adduct of ·OH (DMPO-OH: $g = 2.0073$, $a(N) = a(\beta H) = 1.5\,mT$) is appearing, indicating the generation of ·OH in this experiment: Although DETAPAC-Fe^{2+} chelate oxidizes DMPO[14-15], no EPR line appeared in the absence of H_2O_2.

Since the sample solutions contain ethanol which is a good scavenger for ·OH ($k_{ethanol + \cdot OH} = 1 \times 10^9\,M^{-1}\,s^{-1}$[16]), as another standard, a solution the same as the sample solution, except the absence of biphenyl compounds, was measured. An EPR spectrum obtained from this standard is depicted in Figure 2b. As is seen in Figures 2a and b, by the addition of ethanol (100 mM), the EPR intensity of the 1:2:2:1 quartet decreased by ca. 44%. This result implies that in the sample solutions containing less amount of ethanol (32 mM), the EPR intensity is sufficient large for the estimation of the scavenging ability of biphenyl compounds. The weak signals indicated by asterisks are due to the spin adduct of $\cdot CH(CH_3)OH$, formed by the H-abstraction from ethanol by ·OH.

When biphenyl compounds (0.02 mM) were present, the EPR intensities of DMPO-OH decreased largely indicating the scavenging of ·OH by the biphenyl compounds. Shown in Figures 2c–h are ESR spectra obtained from the samples containing 0.02 mM of dehydrodieugenol, magnolol, honokiol, dehydrodidihydroeugenol, dehydrodivanillyl alcohol, and dehydrodicreosol, respectively. In the spectra, ca. 95 and 34–56% of the EPR intensities of a 1:2:2:1 quartet due to DMPO-OH was decreased compared to the signal in Figures 2a and b, respectively. This result implies that tested biphenyl compounds are much more efficient scavengers for ·OH than ethanol (the concentrations of biphenyl compounds and ethanol being 0.02 mM and 32 mM, respectively). Using the rate constant of the reaction between ·OH and DMPO ($k_{DMPO + \cdot OH} = 3.4 \times 10^9\,M^{-1}\,s^{-1}$[17]), the rate constants of these biphenyl compounds with ·OH ($k_{s + \cdot OH}$) were calculated by the Equation 1,

$$k_{s + \cdot OH} = k_{DMPO + \cdot OH} \cdot F \cdot [DMPO]/[1 - F] \cdot [S] \qquad (1)$$

where F is the percent decrease in the ESR intensity by the biphenyl compounds, and [S] and [DMPO] are the concentrations of biphenyl compounds and DMPO present, respectively. As summarized in Table 1, the apparent rate constants for the reactions of the biphenyl compounds with ·OH were estimated to be of the order of $10^{12}\,M^{-1}\,s^{-1}$. Since diffusion controls the reaction rate in aqueous solutions, these values are overestimated. The reason may be DMPO-OH was partly reduced by reductive products from the biphenyl compounds. Therefore, the rate constants are roughly equivalent to or higher than the rate constant of the reaction between ·OH and phenol ($k = 10^{10}\,M^{-1}\,s^{-1}$[18]): It should be noted that the concentrations of the biphenyl compounds were very low.

It has been reported that metallothionein has a rate constant of $10^{12}\,M^{-1}\,s^{-1}$ for ·OH: By the diffusion controlled reaction mechanism in aqueous media, this value should be 10^9 to $10^{10}\,M^{-1}\,s^{-1}$. The metallothionein behaves as a sacrificial target for ·OH-mediated cellular damage[19]. In the present study, the biphenyl compounds have also been found to be highly reactive with ·OH. It is suggested, therefore, that the biphenyl compounds may be effective inhibitors for ·OH-mediated cellular

TABLE 1
Rate constants ($M^{-1}s^{-1}$) of hydroxylated biphenyl compounds for ·OH

Compound	Rate constant
Dehydrodieugenol	4×10^{12}
Magnolol	6×10^{12}
Honokiol	5×10^{12}
Dehydrodidihydroeugenol	9×10^{12}
Dehydrodivanillyl alcohol	3×10^{12}
Dehydrodicreosol	6×10^{12}

damage when this radical is the pro-active agent. In conclusion the use of hydroxylated biphenyl compounds, which in formulating cosmetics, may provide the needed protection against skin damage caused by ·OH radicals.

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