Antioxidative Properties of Probucol Estimated by the Reactivity with Superoxide and by Electrochemical Oxidation

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The reaction of probucol with superoxide ($O_2^{\cdot-}$) was investigated in acetonitrile using both electron spin resonance (ESR) and electrochemical techniques. The formation of phenoxyl radical was observed during the reaction of probucol with $O_2^{\cdot-}$ by ESR spectroscopy. The reaction of probucol with $O_2^{\cdot-}$ in acetonitrile was followed by cyclic voltammetry. With the addition of probucol, the oxidation peak current of $O_2^{\cdot-}$ decreased concentration dependently. This suggests that probucol reacts with $O_2^{\cdot-}$, that is, probucol scavenges $O_2^{\cdot-}$ in acetonitrile. 2,6-Di-*tert*-butyl-*p*-benzoquinone was identified as the major product of the reaction of probucol with $O_2^{\cdot-}$ in acetonitrile. Electrochemical oxidation of probucol was also performed. Probucol gives an irreversible oxidation peak at *ca.* +1.4 V *vs.* the saturated calomel electrode in the cyclic voltammogram. Controlled-potential electrolysis was carried out at +1.2 V in a divided cell. 2,6-Di-*tert*-butyl-*p*-benzoquinone, 4,4′-dithiobis(2,6-di-*tert*-butylphenol), and 4,4′-trithiobis(2,6-di-*tert*-butylphenol) were identified as the products of anodic oxidation. These redox properties of probucol may correlate with the physiological activities.

Key words probucol; antioxidant; cyclic voltammetry; superoxide; ESR; anodic oxidation

Probucol (4,4′-isopropylidenedithio-bis(2,6-di-*tert*-butylphenol),[1] (Chart 1) is a hypolipidemic agent that has been widely used in the treatment of heart and blood vessel disease.[1] Oxidation of low-density lipoprotein (LDL) in blood is implicated in the development of human atherosclerosis.[2] Probucol is an effective antioxidant transported in lipoproteins that blocks the oxidative modification of LDL *in vivo*.[3] A commonly discussed mechanism of the pharmacological effects of hypolipidemic agents, especially phenolic antioxidants, is their antioxidative activities such as suppressing the formation of active species like reactive oxygens and free radicals.[4] Reactive oxygen species, such as superoxide anion radical ($O_2^{\cdot-}$), hydroxyl radical, and nitric oxide, and other reactive oxygen species, such as hydrogen peroxide, are formed *in vivo*. An imbalance between the production of these reactive oxygen species and antioxidant defense can result in oxidative stress. Among the reactive oxygen species, $O_2^{\cdot-}$, which is easily produced by the one-electron reduction of molecular oxygen, is implicated in several harmful biological processes such as lipid peroxidation and protein denaturation. The reactivity of $O_2^{\cdot-}$ is different in an aqueous media than in an aprotic solvent.[5] $O_2^{\cdot-}$ spontaneously and disproportionately forms hydrogen peroxide and molecular oxygen in aqueous systems. On the other hand, $O_2^{\cdot-}$ shows many reactivities, *e.g.*, as an electrogenerated base, nucleophile, reductant, and oxidant in aprotic media.[6]

The direct scavenging action of probucol on hydroxyl radicals, $O_2^{\cdot-}$, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals was examined by electron spin resonance (ESR) spectrometry.[7] Probucol scavenged DPPH radicals dose dependently but showed no effect on hydroxyl radicals or on superoxide generated by Fenton reaction and by the hypoxanthine–xanthine oxidase system. From a pharmacological aspect, probucol is a lipophilic antioxidant; thus one of the antioxidative properties of probucol plays a role in an aprotic system similar to the lipophilic domain of the liposomal membrane. To clarify the reactivity of probucol with $O_2^{\cdot-}$ in the lipophilic phase, we studied the mechanism of redox reactions of probucol with $O_2^{\cdot-}$ in acetonitrile. Electrochemical techniques are useful for investigating electron-transfer reactions. The electrochemical behavior appears to be a good model for biological oxidation of pharmacologically active substances. In this study, anodic oxidation of probucol was undertaken to determine the possible relationships between the electrochemical and physiological properties.

Experimental

General ^{1}H- and ^{13}C-NMR spectra were recorded on a JEOL JNM-GSX-500 spectrometer, ESR spectra on a JEOL JES-FE-2XG spectrometer, and MS spectra on a JEOL JMS-HX-110 spectrometer. Cyclic voltammetry and anodic oxidation were carried out by using a dual potentio-galvanostat (DPGS-1, Nikko Keisoku, Kanagawa, Japan), a potential sweeper (NPS-2, Nikko Keisoku), and a digital coulomb meter (NDCM-3, Nikko Keisoku). The preparative high-performance liquid chromatography (HPLC) consisted of a Hitachi L-7100 pump and a Hitachi L-4000 UV detector (Hitachi, Tokyo, Japan). The single-crystal X-ray structure determinations were carried out using a Rigaku AFC-7R diffractometer (Rigaku, Tokyo, Japan) with monochromated Cu$K\alpha$ radiation.

Materials Probucol was synthesized at Aventis Pharma AG (Frankfurt, Germany). Potassium superoxide (KO_2) and 18-crown-6 were purchased from Sigma Co., Ltd., (St. Louis, MO, U.S.A.). Tetraethylammonium perchlorate (TEAP) and DL-alpha-tocopherol were purchased from Aldrich Chemical Co., Ltd., (Milwaukee, WI, U.S.A.). 2,6-Di-*tert*-butylhydroxytoluene (BHT) was purchased from Kanto Kagaku Co., Ltd. (Tokyo, Japan). These materials were used without further purification.

ESR Measurement Thirty milliliters of a dry acetonitrile solution containing probucol 113.56 mg (0.22 mmol) and 18-crown-6 56.5 mg (0.21 mmol) was prepared, and then potassium superoxide powder approximately 100 mg was gradually added to the solution. The resultant solution was transferred to capillary tubes [Drummond Microcaps, 50 μl, (Drummond Scientific Co. PA, U.S.A.)], sealed with Terumoseal (Terumo, Tokyo, Japan), and measured by ESR. ESR spectral conditions were as follows: magnetic field, 327±5 mT; response, 0.3 s; modulation, 100 kHz; modulation amplitude, 0.063 mT; temperature, ambient; microwave power, 8.0 mW; sweep time, 2 min.

Cyclic Voltammetry The measurements were performed on a dual po-

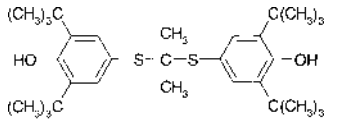

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Chart 1

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tentio-galvanostat and potential sweeper in dry acetonitrile containing TEAP 0.1 M (supporting electrolyte). A glassy carbon electrode was used for the working electrode, a platinum electrode for the counter electrode, and a saturated calomel electrode (SCE) for the reference electrode. Probucol (50 mM) was dissolved in dry acetonitrile containing 0.1 M TEAP. The scan speed was 5 s/V. The potential scan range was from −1.6 V to 2.2 V *vs*. SCE.

$O_2^{\cdot-}$ scavenging activity was estimated by cyclic voltammetry. The conditions were as follows: The scan speed was 5 s/V. The potential scan range was from −1.0 V to 0.0 V *vs*. SCE. The solution containing various concentrations of probucol (28.3, 56.4, 113, 226, 451, 902, 1804 μM) was previously saturated by air for 15 min to maintain a constant concentration of oxygen. $O_2^{\cdot-}$ scavenging activity was determined by relative anodic current, which was defined as the cathodic peak current of oxygen normalized against the anodic peak current of $O_2^{\cdot-}$.

Isolation of the Reaction Product of Probucol with Superoxide Thirty milliliters of a dry acetonitrile solution containing probucol 113.56 mg (0.22 mmol) and 18-crown-6 56.5 mg (0.21 mmol) was prepared, and then potassium superoxide powder approximately 100 mg was gradually added to the solution. The reaction solution was allowed to stand at room temperature for 1 d in the dark. Then the volume of the reaction solution was concentrated to approximately 10 ml under reduced pressure, and the residue was subjected to preparative HPLC. The preparative HPLC was conducted on a reversed-phase column with TSKgel ODS-80T_M (21.5 mm i.d.×300 mm). Ninety-three percent (v/v) acetonitrile was used as the eluent at a flow rate of 8.0 ml/min. Product detection was monitored at 242 nm. The product yield was determined by HPLC.

Controlled Potential Electrolysis of Probucol A platinum mesh was used for the working electrode, a platinum wire for the counter electrode, and SCE for the reference electrode. Probucol 106.9 mg (2.1 mmol) was dissolved into acetonitrile 100 ml containing TEAP 0.1 M in an H-shaped glass cell divided by a methyl cellulose plug and a sintered glass disk. The solution was subjected to electrolysis at +1.2 V *vs*. SCE until 350 °C, which corresponded to 1.7 F per mol of probucol, had been consumed. The electrolysis was carried out under nitrogen at atmospheric pressure.

Isolation of Anodic Oxidation Products The electrolyzed solution was concentrated to approximately 10 ml under reduced pressure, and the residue was subjected to preparative HPLC under the same HPLC conditions as described above. The collected fractions were evaporated to dryness under reduced pressure at 40 °C. Three major products were isolated: product **2** 10.5 mg (12% yield), product **3** 29.0 mg (30%), and product **4** 24.4 mg (23%).

X-Ray Structure Determination of Anodic Oxidation Product 4 A yellowish, columnar crystal of $C_{28}H_{42}O_2S_3$ with the approximate dimensions of 0.40×0.15×0.10 mm was grown from acetonitrile. The compound crystallized in monoclinic space group $P2_1/n$ with cell parameters a=10.152 Å, b=27.771 Å, and c=12.462 Å. The structure was solved by the direct method with the program TEXSAN. The final R value was 3.8%.

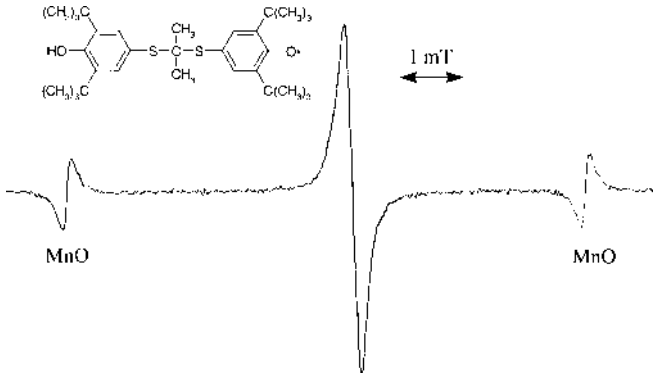

Fig. 1. ESR Spectrum of the Reaction Mixture of Probucol with KO_2 in the Presence of 18-Crown-6 in Acetonitrile at Room Temperature

Magnetic field, 327±5 mT; response, 0.3 s; modulation, 100 kHz; modulation amplitude, 0.063 mT; temperature, ambient; microwace power, 8.0 mW; sweep time, 2 min.

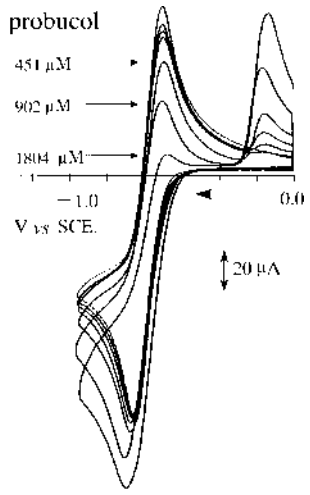

Fig. 2. Cyclic Voltammograms of $O_2/O_2^{\cdot-}$ in Oxygen-Saturated Acetonitrile Containing TEAP 0.1 M in the Presence of Several Concentrations of Probucol (28.3, 56.4, 113, 226, 451, 902, 1804 μM) at a Glassy Carbon at Room Temperature

Voltage sweep rate, 5 s/V.

Results and Discussion

ESR Spectrum of the Reaction of Probucol with Superoxide Figure 1 shows the ESR spectrum of the reaction mixture of probucol with potassium superoxide in acetonitrile at room temperature. A broad singlet was observed in the ESR spectrum. $O_2^{\cdot-}$ is not detectable by ESR spectrometry at room temperature. The result shows that probucol reacts with $O_2^{\cdot-}$ in acetonitrile and forms stable free radicals during the reaction. Several reports have discussed the radicals derived from probucol. A one-line spectrum formed during the Ag_2O-catalyzed oxidation of probucol and was assigned to the probucol phenoxyl radical.[8] Probucol also reacted with DPPH radicals and gave a new signal in the ESR spectra.[7] Therefore the obtained signal was thought to be a phenoxyl radical. The primary oxidation step of probucol by $O_2^{\cdot-}$ is identified as the formation of free radicals.

Superoxide Scavenging Activity of Probucol Estimated Using Cyclic Voltammetry We have already reported that the $O_2^{\cdot-}$ scavenging activity of antioxidants in acetonitrile could be simply estimated by cyclic voltammetry.[9] A reversible one-electron wave corresponding to the $O_2/O_2^{\cdot-}$ redox couple is seen in cyclic voltammograms in dried acetonitrile. The reduction potential of the $O_2/O_2^{\cdot-}$ is −0.80 V *vs.* SCE, and the oxidation potential of $O_2^{\cdot-}/O_2$ is −0.65 V *vs.* SCE. The reproducibility on cyclic voltammograms for $O_2/O_2^{\cdot-}$ is quite high, because $O_2^{\cdot-}$ is stable in an aprotic solvent.[5] Figure 2 shows cyclic voltammograms of various concentrations of probucol in acetonitrile containing TEAP 0.1 M. With the addition of probucol, the oxidation peak current of $O_2^{\cdot-}$ decreased, while the reduction current (cathodic current [$O_2^{\cdot-}$ formation]) slightly increased. This suggests that $O_2^{\cdot-}$ is scavenged within the time scale of cyclic voltammetry in acetonitrile in a concentration-dependent manner.

The scavenging activity of probucol was compared with those of the other antioxidants BHT and DL-α-tocopherol (Fig. 3). The scavenging concentration of the antioxidant from the relative anodic current (SC_{50}) was estimated. The SC_{50} for these compounds was in the following order: probucol (593 μM)>BHT (1052 μM)$\cong$DL-α-tocopherol (1089 μM). Probucol scavenges $O_2^{\cdot-}$ the most efficiently, because probucol has two phenolic groups. These results suggest that the antioxidant property of probucol in acetonitrile is partly due to its free radical scavenging effect.

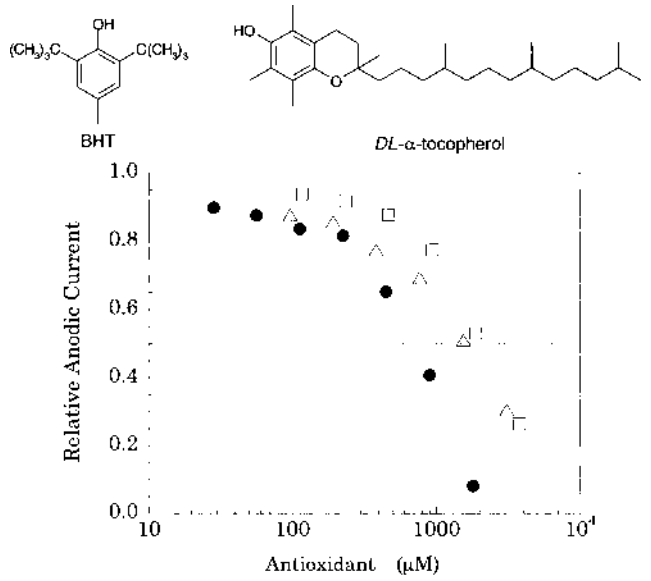

Fig. 3. $O_2^{\cdot-}$ Scavenging Abilities of Antioxidants Determined by the Cyclic Voltammetry

●, probucol; △, DL-α-tocopherol; □, BHT. $O_2^{\cdot-}$ scavenging activity was determined by relative anodic current, which was defined as the cathodic peak current of oxygen normalized against the anodic peak current of $O_2^{\cdot-}$.

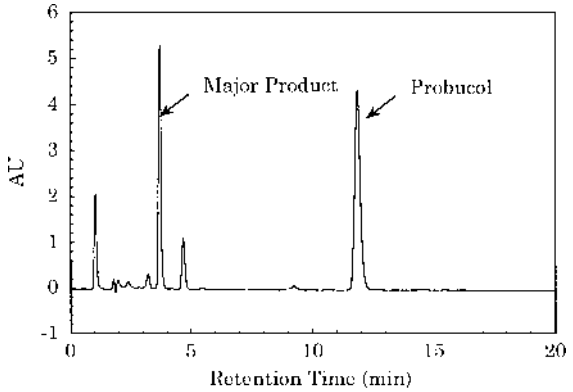

Fig. 4. High-Performance Liquid Chromatogram of the Reaction Solution of Probucol with $O_2^{\cdot-}$

Reaction of Probucol with Superoxide First, we attempted to generate $O_2^{\cdot-}$ by electrolytic reduction of O_2 in acetonitrile, but the reaction of probucol with $O_2^{\cdot-}$ did not proceed by this method because the concentration of $O_2^{\cdot-}$ was too low. Therefore we elected to use the commercially available potassium superoxide (KO_2) in the presence of 18-crown-6 in acetonitrile. The high-performance liquid chromatogram obtained from the reaction of probucol with $O_2^{\cdot-}$ is shown in Fig. 4. The reaction product was observed at $t_R = ca.$ 3.8 min. This product was isolated and identified as 2,6-di-*tert*-butyl-*p*-benzoquinone (Chart 2) by NMR, MS: ^{1}H-NMR ($CDCl_3$) δ: 1.26 (18H, s), 6.51 (2H, s). ^{13}C-NMR ($CDCl_3$) δ: 29.4, 35.6, 130.2, 157.9. MS (EI) *m/z*: 220 (M^+), (CI) 221 ($M+H^+$). The structure was confirmed by X-ray crystal analysis. After the reaction mixture was stored for 1 d at room temperature, 53% of the probucol was recovered and 34% of 2,6-di-*tert*-butyl-*p*-benzoquinone was produced.

Phenoxyl radical was generated in the reaction of probucol with $O_2^{\cdot-}$, and the stable reaction product of probucol with $O_2^{\cdot-}$ was elucidated to be a 2,6-di-*tert*-butyl-*p*-benzoquinone. The oxidation of hydroxynaphthalenes with $O_2^{\cdot-}$ leads to the formation of naphthoquinones.[10] Therefore the mechanism of probucol with $O_2^{\cdot-}$ was considered. The reaction is initiated by abstracting a hydrogen atom from phenol to give a phenoxyl radical. The phenoxyl radical may be resonated with the carbon-centered radical at the 4-position. The carbon-centered radical reacts with O_2, and subsequently the carbon–sulfur bond is cleaved. Finally 2,6-di-*tert*-butyl-*p*-benzoquinone is formed. Chart 3 shows the proposed mechanism for the reaction of probucol with $O_2^{\cdot-}$. One of the antioxidative properties of probucol is explained by the stable phenoxyl radical that was formed by the attack of free radicals in the aprotic system. The ability of probucol to participate in redox reactions probably plays a role in its pharmaco-

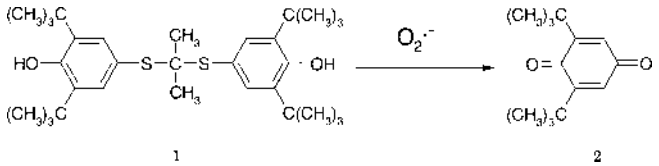

Chart 2

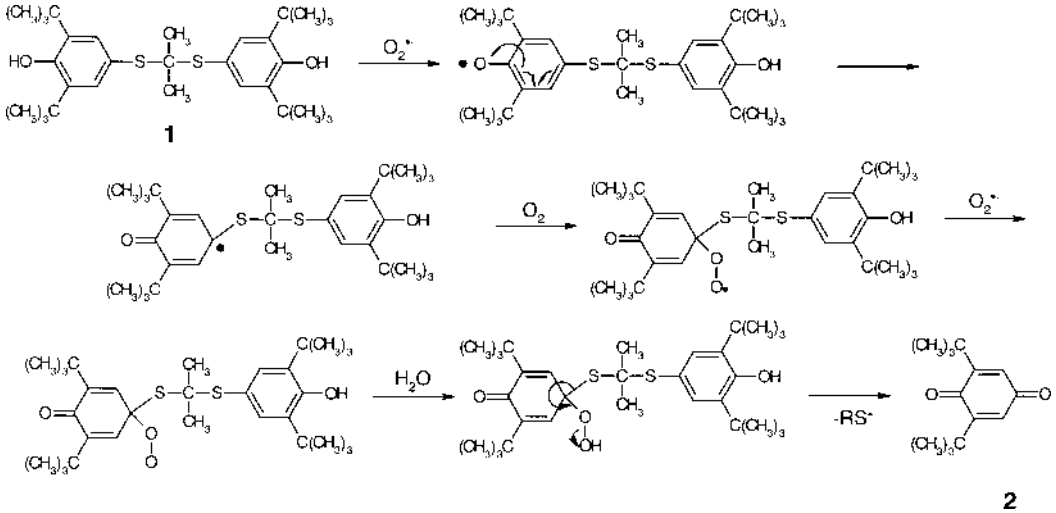

Chart 3

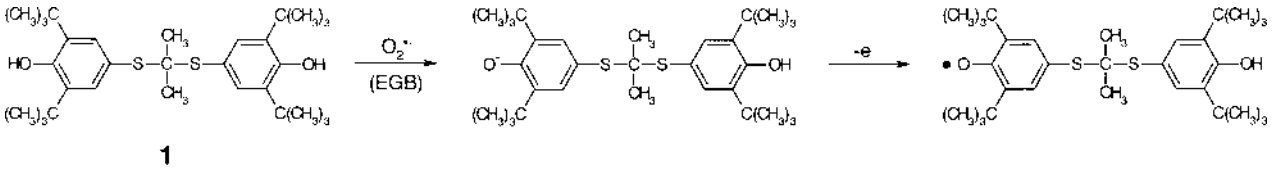

Chart 4

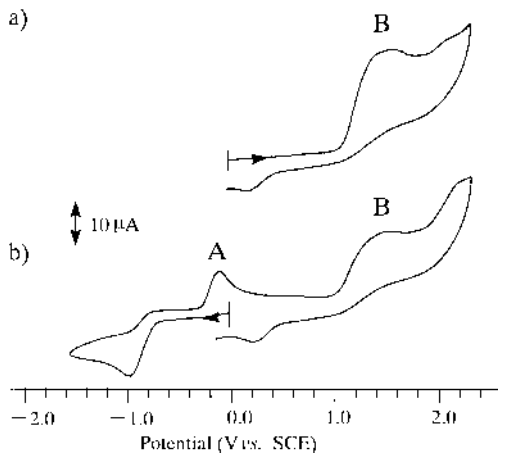

Fig. 5. Cyclic Voltammograms of Probucol 50 mM in Oxygen-Saturated Acetonitrile Containing TEAP 0.1 M at a Glassy Carbon Electrode at Room Temperature

(a) Voltage sweep started from anodic direction. (b) Voltage sweep started from cathodic direcion. Volatge sweep rate, 5 s/V. The voltammetric peak A in (b) is due to the oxidation as shown in Chart 4. Controlled potential electrolysis was done at the voltammetric peak B.

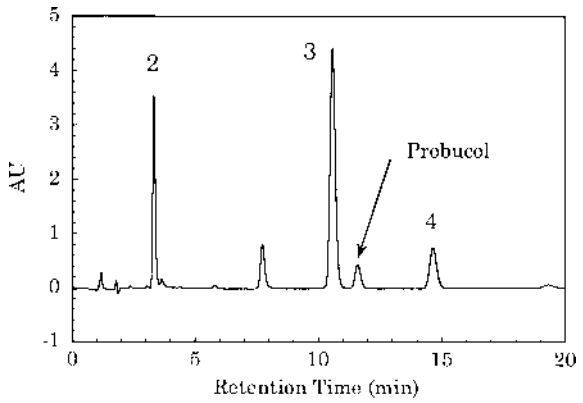

Fig. 6. High-Performance Liquid Chromatogram of an Electrolyzed Solution

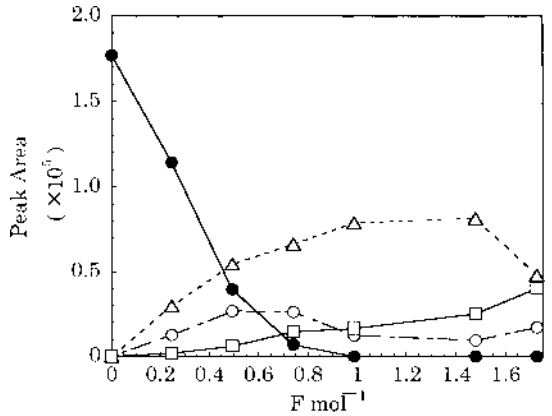

Fig. 7. Consumed Electricity *vs*. Peak Area of Probucol and Reaction Products

●, peak area of probucol; ○, product **2**; △, product **3**; □, product **4**.

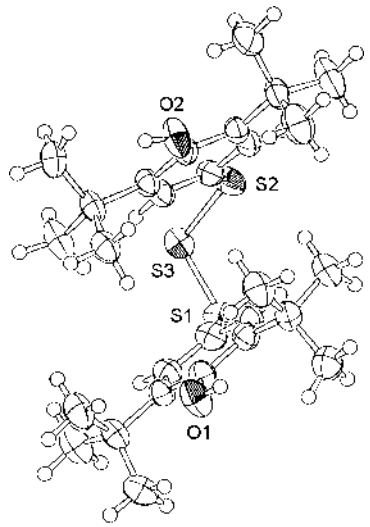

Fig. 8. X-Ray Crystal Structure of Anodic Oxidation Product **4**

logical activity.

Electrochemical Behavior of Probucol Figure 5 shows the cyclic voltammograms of probucol in acetonitrile from both anodic and cathodic scans. A voltammetric peak is seen at *ca*. −0.2 V (Fig. 5b, A). The anodic peak voltage agrees well with that reported by Ohmori *et al*.,[11] and is ascribed to the oxidation of the phenolate ion to the phenoxyl radical. The phenolate ion is formed by $O_2^{\cdot-}$ as an electrogenerated base,[4] that is, a proton is abstracted from phenol by $O_2^{\cdot-}$. Chart 4 shows this reaction.

A large irreversible anodic peak is seen at *ca*. +1.4 V (Fig. 5, B). Controlled potential electrolysis of probucol was carried out in acetonitrile containing TEAP 0.1 M at +1.20 V *vs*. SCE. Figure 6 shows the high-performance liquid chromatogram of probucol in during the electrolysis (0.74 F mol^{-1}). Probucol was oxidized by electrolysis, yielding oxidation products in addition to the three major products **2**, **3**, and **4**. The plot of the consumed electricity *vs*. peak area of probucol and these oxidation products is shown in Fig. 7. Product **2** is obtained in the highest yield by anodic oxidation, followed by product **3**.

Product **2** has almost the same retention time as the reaction product of probucol with $O_2^{\cdot-}$. Further, the ^{1}H-, ^{13}C-NMR, and EI-MS spectra of product **2** were similar to those of the reaction product of probucol with $O_2^{\cdot-}$. Thus product **2** was identified as 2,6-di-*tert*-butyl-*p*-benzoquinone.

A single crystal of product **4** was obtained and X-ray analysis was performed. A perspective view of the molecule is shown in Fig. 8. The anodic oxidation product **4** was iden-

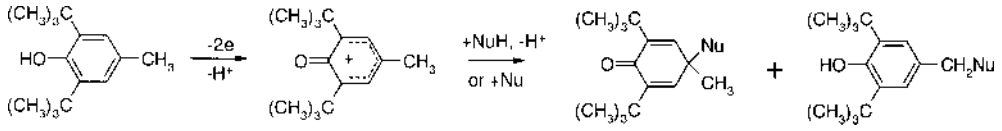

Chart 5

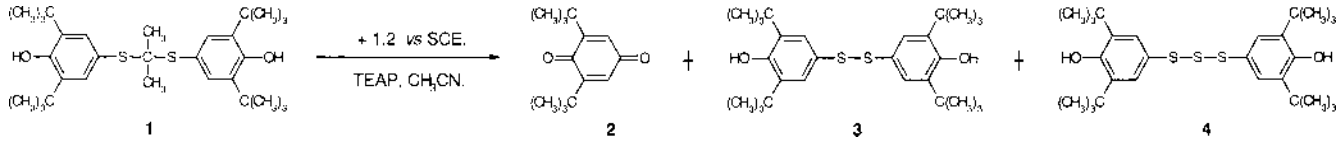

Chart 6

tified as 4,4′-trithiobis(2,6-di-*tert*-butylphenol). Products **3** and **4** had very similar ^{1}H-NMR spectral characteristics; product **3**: ^{1}H-NMR (DMSO-d_6) δ: 1.33 (36H, s), 7.02 (2H, s), 7.22 (4H, s); product **4**: ^{1}H-NMR (DMSO-d_6) δ: 1.31 (36H, s), 7.02 (2H, s), 7.27 (4H, s). A molecular ion of product **4** was observed at *m/z*=506 by EI-, FD- and FAB-MS, and a fragment ion was observed at *m/z*=476 by EI-MS. A peak at *m/z*=476 of product **3** in EI-, FD- and FAB-MS was considered to be a molecular ion of product **3**, with a mass 32 lower than that of product **4**. Therefore product **3** was identified as 4,4′-dithiobis(2,6-di-*tert*-butylphenol). These chemical structures of the anodic oxidation products are summarized in Chart 5.

2,6-Di-tert-butylphenols have often been employed as model compounds for studies on the electrochemical oxidation of phenols. For example, anodic oxidation of 2,6-di-*tert*-butyl-4-methylphenol in acetonitrile predominantly gave the cyclohexadienone, as shown in Chart 6.[12] Product **2** was formed where the phenoxonium ion was suggested to be in the product yield. The formation of product **3** suggests a mechanism involving the organothio radical. The initial one-electron transfer produces the cation radical intermediate and the carbon–sulfur bond is cleaved to give the corresponding carbocation and the organothio radical. The organothio radical dimerizes to give product **3**. Product **3** may undergo further anodic oxidation to give product **4**, but the mechanism remains unclear. There appears to be no report on the formation of the trisulfide form by electrochemical oxidation. One possible mechanism is that probucol reacts with an organothio radical or organothio cation radical and may form product **4** *via* a cyclic intermediate.

We conclude that probucol has favorable antioxidant activity such as $O_2^{\cdot-}$ scavenging activity and redox properties in aprotic media. The ability of probucol to participate in redox reactions probably plays a role in its pharmacological activity.

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