

Inhibition of Succinoxidase and Reduced Nicotinamide Adenine Dinucleotide (NADH) Oxidase by 2,3-Ethylenedioxy-1,4-benzoquinones Having Alkylthio and Arylthio Side Chains¹⁾

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A series of 2,3-ethylenedioxy-1,4-benzoquinones having an alkylthio or arylthio side chain at the 5-position or two alkylthio or arylthio side chains at the 5,6-positions was synthesized. These compounds were tested for inhibition of the succinoxidase and reduced nicotinamide adenine dinucleotide (NADH) oxidase systems in the mitochondrial respiratory chain. 5-Arylthio- and 5,6-diarylthio-2,3-ethylenedioxy-1,4-benzoquinones were found to show potent inhibitory activities toward both enzyme systems. However, 5-alkylthio- and 5,6-dialkylthio-2,3-ethylenedioxy-1,4-benzoquinones showed weak inhibitory activities. The substitution of a 2,3-ethylenedioxy group in place of the 2,3-dimethoxy group of coenzyme Q (CoQ) was found to be more favorable than the previously reported 2,3-dimethyl compound or 1,4-naphthoquinones for the inhibition of both enzyme systems. The effects of 2,3-ethylenedioxy-1,4-benzoquinones on the reduced minus oxidized difference spectra of submitochondrial particles with succinate or NADH as a substrate were investigated to identify their inhibitory sites in the respiratory chain. The spectral changes suggested that 5-arylthio- and 5,6-diarylthio-2,3-ethylenedioxy-1,4-benzoquinones inhibit some sites between succinate and CoQ, as well as after cytochrome $a + a_3$. On the other hand, 5,6-dialkylthio-2,3-ethylenedioxy-1,4-benzoquinones were found to inhibit some sites between NADH and CoQ, as well as after cytochrome $a + a_3$.

Keywords coenzyme Q analog; succinoxidase; NADH oxidase; beef heart mitochondria; difference spectra; respiratory chain; 5-alkylthio-2,3-ethylenedioxy-1,4-benzoquinone; 5-arylthio-2,3-ethylenedioxy-1,4-benzoquinone; 5,6-dialkylthio-2,3-ethylenedioxy-1,4-benzoquinone; 5,6-diarylthio-2,3-ethylenedioxy-1,4-benzoquinone

Coenzyme Q (CoQ) has been reported to play an important role in the mitochondrial respiratory chain. As described in the previous paper,¹⁾ many kinds of CoQ analogs have been synthesized and several analogs were found to have a variety of biological activities such as antitumor activity,²⁾ inhibition of lipid peroxidation,³⁾ improvement of brain metabolism,⁴⁾ antimetabolite activity,⁵⁾ *etc.* We have previously reported the inhibitory activities toward the succinoxidase and reduced nicotinamide adenine dinucleotide (NADH) oxidase systems of 2,3-dimethyl-1,4-benzoquinones having alkylthio and arylthio side chains. Among these compounds, 5-arylthio-2,3-dimethyl-1,4-benzoquinones were found to show potent inhibitory activities toward both enzyme systems. In this paper, we report the inhibition of the succinoxidase and NADH oxidase systems by 2,3-ethylenedioxy-1,4-benzoquinones having an alkylthio or arylthio side chains at the 5-position (Ia–e) and two alkylthio or arylthio side chains at the 5,6-positions (IIa–d). A series of 5-hydroxy-6-alkyl-2,3-ethylenedioxy-1,4-benzoquinones has been previously synthesized and tested by Bowman *et al.*⁶⁾ as inhibitors of the succinoxidase and reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase systems. They concluded that the 2,3-ethylenedioxy group on 5-hydroxy-6-alkyl-1,4-benzoquinone is less favorable than the 2,3-dimethoxy group for inhibition of both enzyme systems. Roberts *et al.*⁷⁾ suggested that the introduction of a sulfur atom between the quinone ring and the side chain has little effect on the inhibitory activities. However, Porter *et al.*^{8,9)} indicated that the several quinone analogs with an alkylthio or arylthio group in the quinone ring were potent inhibitors. Therefore, it is of interest to examine the correlation between the inhibition of the enzyme systems and the introduction of ethylenedioxy, alkylthio and arylthio groups. In addition, the effects of these quinones on the reduced minus oxidized difference spectra of submitochondrial particles with succinate or NADH as a substrate were

investigated to identify their inhibitory sites in the respiratory chain.

Results and Discussion

Synthesis of 2,3-Ethylenedioxy-1,4-benzoquinones Having Alkylthio and Arylthio Side Chains New 5-alkylthio- (Ia–c), 5-arylthio- (Id, e), 5,6-dialkylthio- (IIa–c) and 5,6-diarylthio-2,3-ethylenedioxy-1,4-benzoquinones (II d) were prepared by treating 2 eq of 2,3-ethylenedioxy-1,4-benzoquinone (III) in ethanol with a hexane solution of alkyl- or arylthiol followed by oxidation with Fremy's salt (Chart 1). The reactions gave 10–45% yields of Ia–e and 7–19% yields of IIa–d. In the case of the reaction of III with β -naphthalenethiol, the expected product, 5,6-di- β -naphthylthio-2,3-ethylenedioxy-1,4-benzoquinone was not obtained.

Inhibition of the Succinoxidase and NADH Oxidase Systems The synthesized quinones were evaluated for the inhibition of the mitochondrial succinoxidase and NADH oxidase systems. Figure 1 shows the enzyme activity as a function of the concentration of 5- β -naphthylthio-2,3-ethylenedioxy-1,4-benzoquinone (Ie). Compound Ie exhibited 50% inhibition of succinoxidase and NADH oxidase activities at the concentrations of 8 and 6 nmol per flask,

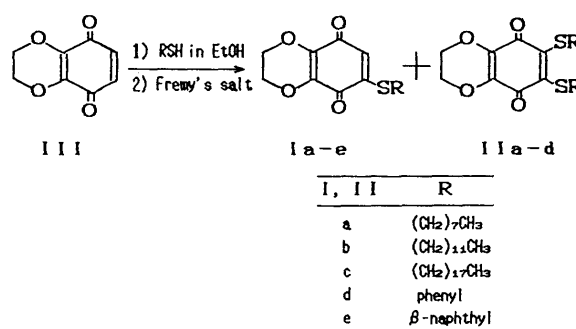


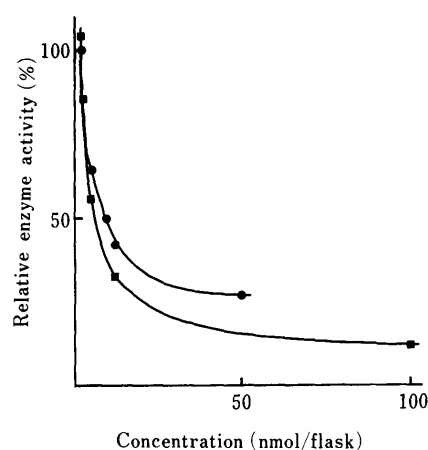
Chart 1

TABLE I. Effects of Quinones on Succinoxidase and NADH Oxidase Activities in Beef Heart Mitochondria

Compd. No.	Succinoxidase			NADH oxidase		
	Concen- tration ^{a)}	Relative enzyme activity ^{b)}	Antime- tabolite CoQ index ^{c)}	Concen- tration ^{a)}	Relative enzyme activity ^{b)}	Antime- tabolite CoQ index ^{c)}
None	—	100	—	—	100	—
5-RS-2,3-Ethylenedioxy- 1,4-benzoquinones						
R						
Ia <i>n</i> -C ₈ H ₁₇	500	76	> 873	10	97	37
	1000	73		20	72	
	1500	72		50	43	
				100	35	
				200	33	
			500	13		
Ib <i>n</i> -C ₁₂ H ₂₅	840	94	> 489	840	87	> 696
Ic <i>n</i> -C ₁₈ H ₃₇	330	96	> 192	330	130	> 399
Id C ₆ H ₅	1	88	5	0.1	107	3
	5	68		1	88	
	10	39		3	59	
	50	16		5	37	
				10	18	
			100	14		
Ie β -C ₁₀ H ₇	1	100	5	1	103	5
	5	66		3	84	
	8	50		5	56	
	10	42		10	34	
	50	28		100	13	
5,6-Di-RS-2,3-ethylenedioxy- 1,4-benzoquinones						
R						
IIa <i>n</i> -C ₈ H ₁₇	200	71	174	50	73	58
	300	50		70	51	
	400	37		100	43	
	1000	15		200	33	
IIb <i>n</i> -C ₁₂ H ₂₅	500	59	> 582	500	7	356
	1000	54		200	76	
				500	41	
				800	38	
			1000	28		
IIc <i>n</i> -C ₁₈ H ₃₇	330	100	> 192	330	138	> 399
IId C ₆ H ₅	1	95	4	1	101	4
	5	60		2	84	
	10	31		5	50	
	50	14		10	30	
				100	19	

a) nmol in a flask. b) Percentage of specific activity in the presence of inhibitor to that of the control. c) The mark (>) means that antimetabolite CoQ index is greater than the number shown.

respectively. However, the inhibitory activities on the mitochondrial succinoxidase and NADH oxidase systems depend not only on the concentration of inhibitors, but also on the concentration of mitochondrial CoQ. Therefore the inhibitory activities were expressed as an antimetabolite coenzyme Q₁₀ (CoQ₁₀) index (A.I.) defined by Bowman *et al.*,¹⁰⁾ i.e., nmol of inhibitor per nmol of mitochondrial CoQ which causes 50% inhibition of enzyme activity. The results are summarized in Table I. The following characteristics were revealed. (1) 5-Arylthio- (Id, e) and 5,6-diarylthio-2,3-ethylenedioxy-1,4-benzoquinones (IId) exhibited potent inhibitory activities toward both enzyme systems. On the other hand, quinones with 5-alkylthio (Ib, c) and 5,6-dialkylthio groups (IIb, c) showed weak inhibitory activities toward both enzyme systems. (2) A dependence of the

Fig. 1. Inhibitory Effects of 5- β -Naphthylthio-2,3-ethylenedioxy-1,4-benzoquinone (Ie) on Succinoxidase and NADH Oxidase

●, succinoxidase; ■, NADH oxidase.

TABLE II. Antimetabolite CoQ Indices (A.I.) of Quinones

Compd.	Succinoxidase	NADH oxidase
	A.I. ^{a)}	A.I. ^{a)}
5-RS-2,3-Dimethyl-1,4-benzoquinones ^{b)}		
R		
<i>n</i> -C ₈ H ₁₇	552	105
<i>n</i> -C ₁₂ H ₂₅	> 575	151
<i>n</i> -C ₁₈ H ₃₇	> 287	> 291
C ₆ H ₅	49	20
β -C ₁₀ H ₇	132	10
5-R ₁ S-6-R ₂ -2,3-Dimethoxy-1,4-benzoquinones ^{c)}		
R ₁	R ₂	
<i>n</i> -C ₈ H ₁₇	H	> 425
<i>n</i> -C ₁₂ H ₂₅	H	> 425
<i>n</i> -C ₁₈ H ₃₇	H	> 425
<i>n</i> -C ₈ H ₁₇	Cl	9
<i>n</i> -C ₁₂ H ₂₅	Cl	21
β -C ₁₀ H ₇	H	11
β -C ₁₀ H ₇	CH ₃	20
β -C ₁₀ H ₇	Cl	7
2-R ₁ S-3-R ₂ -1,4-Naphthoquinones ^{d)}		
R ₁	R ₂	
<i>n</i> -C ₁₈ H ₃₇	H	> 37
<i>n</i> -C ₁₂ H ₂₅	NH ₂	365
<i>n</i> -C ₁₈ H ₃₇	NH ₂	> 183
<i>n</i> -C ₁₈ H ₃₇	OH	6
β -C ₁₀ H ₇	H	> 73
β -C ₁₀ H ₇	NH ₂	> 147
β -C ₁₀ H ₇	OH	> 147

a) The mark (>) means that A.I. is greater than the number shown. b) Ref. 1. c) Ref. 9. d) Ref. 11.

inhibitory effects on the length of the alkylthio side chain was observed, and the short chain *n*-octylthio group was more effective than the long chain *n*-dodecylthio and *n*-octadecylthio groups in the NADH oxidase system. These results are in agreement with those for 2,3-dimethyl-1,4-benzoquinone analogs. (3) 5-*n*-Octylthio-2,3-ethylenedioxy-1,4-benzoquinone (Ia) is a specific inhibitor of the NADH oxidase system, because the A.I. value calculated in the NADH oxidase system was 37, but the value could not be determined in the succinoxidase system at the highest concentration tested. (4) There was no apparent difference in the inhibitory activities between 5-mono- and

5,6-disubstituted quinones.

Moreover, our results were compared with previously reported A.I. values of other analogs having similar alkylthio and arylthio side chains (Table II). It can be considered that 5-arylthio- and 5,6-diarylthio-2,3-ethylenedioxy-1,4-benzoquinones are more effective than other benzoquinone and naphthoquinone analogs.

Difference Spectra of Submitochondrial Particles The difference spectra of reduced minus oxidized forms of cytochromes can be applied to investigate the site of action of inhibitors in the respiratory chain. The effects of Ie and 5,6-di-*n*-octylthio-2,3-ethylenedioxy-1,4-benzoquinone (IIa) on the difference spectra of submitochondrial particles with succinate or NADH as a substrate are shown in Fig. 2, together with the absorption peaks of reduced cytochromes a, a₃, b and c. Figure 2(A) shows that when succinate (5 μmol) as a substrate was added to the system, the cytochromes were reduced, that is, the a, a₃, b and c peaks appeared (solid line). These peaks in the difference spectra almost disappeared after addition of Ie (0.1 μmol), which inhibited the succinoxidase activity (broken line), and were restored by subsequent addition of another substrate, NADH (dotted line). Such spectral changes mean that the respiratory chain from succinate to oxygen was inhibited by Ie between succinate and CoQ (Fig. 3), and then restored by replacing the route between succinate and CoQ with a route between NADH and CoQ. Such spectral changes by Ie are similar to those observed after addition of malonic acid,¹²⁾ an inhibitor of succinate dehydrogenase. However, in the NADH oxidase system, the peaks of reduced cytochromes a, a₃, b and c in the difference spectra did not disappear after addition of Ie (1.0 μmol), which decreased NADH oxidase activity to 13% of that of the control (none) as shown in Table I. This suggests that the inhibitory site in the NADH oxidase system is located after cytochrome a + a₃ (Fig. 3). Such spectral changes are similar to those observed after addition of NaN₃,¹³⁾ an inhibitor of cytochrome oxidase. The difference spectra of other quinones were also measured and the inhibitory sites were investigated in the same manner as above. The results are summarized in Fig. 3 together with those for known representative inhibitors, malonic acid, NaN₃ and rotenone¹⁴⁾ (an inhibitor of NADH oxidase). The conclusions to be drawn from the experimental results on the inhibitory sites are as follows. 5-Arylthio- and 5,6-diarylthio-2,3-ethylenedioxy-1,4-benzoquinones inhibit some sites between succinate and CoQ, as well as after cytochrome a + a₃. On the other hand, 5,6-dialkylthio-2,3-ethylenedioxy-1,4-benzoquinones were found to inhibit some sites between NADH and CoQ, as well as after cytochrome a + a₃. Compound Ia, a specific inhibitor of the NADH oxidase system, appears to be inhibit the site only after cytochrome a + a₃.

In summary, our experimental results revealed that: (1) synthetic arylthiolated 2,3-ethylenedioxy-1,4-benzoquinone analogs are potent inhibitors of the mitochondrial respiratory chain; (2) their inhibitory sites in the respiratory chain appear to be located between succinate or NADH and CoQ, as well as after cytochrome a + a₃. Further investigations should be done on various 5-arylthio and 5,6-diarylthio homologs in order to elucidate their precise mechanisms of inhibition in the respiratory chain and to

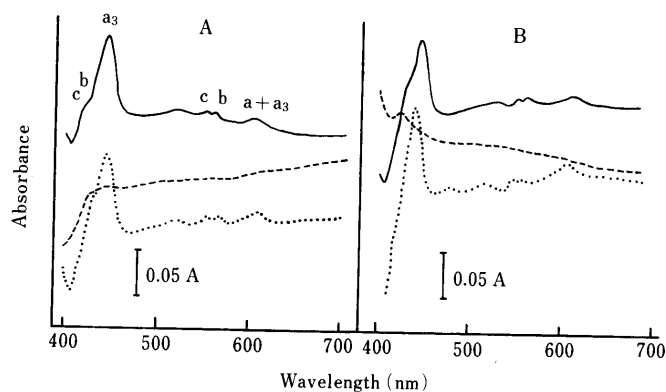


Fig. 2. Effects of 5-β-Naphthylthio- (Ie) and 5,6-Di-*n*-octylthio-2,3-ethylenedioxy-1,4-benzoquinone (IIa) on the Reduced Minus Oxidized Spectra of Submitochondrial Particles with Succinate or NADH as the Substrate

A: Difference spectra after addition of succinate (5 μmol) (—), subsequent addition of Ie (0.1 μmol) (---), and further addition of NADH (1.3 μmol) (.....). B: Difference spectra after addition of NADH (1.3 μmol) (—), subsequent addition of IIa (0.2 μmol) (---), and further addition of succinate (5 μmol) (.....).

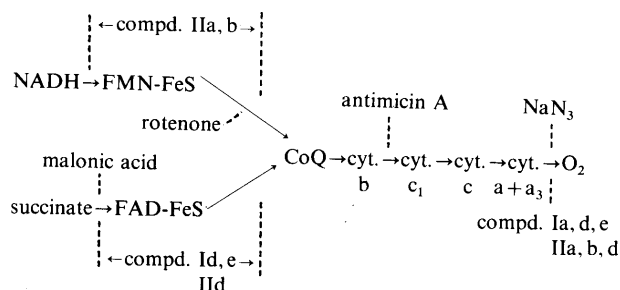


Fig. 3. Mitochondrial Electron Transfer Chain and Its Inhibitors
----, inhibitory site.

design more potent inhibitors.

Experimental

Melting points were determined on a Yanagimoto micro melting point apparatus, and are uncorrected. Infrared (IR) spectra were taken in KBr with a Hitachi 260-30 spectrophotometer. Difference spectra were recorded with a Hitachi 340 double-beam spectrophotometer. Nuclear magnetic resonance (NMR) spectra were measured with a Bruker AM-400 spectrometer in CDCl₃ with tetramethylsilane (TMS) as an internal standard. Chemical shifts are given as δ values (ppm): s, singlet; t, triplet; br, broad; m, multiplet. Mass spectra (MS) were measured with a Hitachi M-60 spectrometer. Beef heart mitochondria were isolated by the usual procedures.¹⁵⁾ The final particles was suspended in 0.25 M sucrose and was used immediately or kept frozen until used. Phospholipid micelles were prepared by sonication of commercial soybean phospholipids (Asolectin)¹⁶⁾ and used instead of mitochondrial phospholipids. Protein was determined by Lowry's method.¹⁷⁾ The amount of CoQ₁₀ in the mitochondrial preparation was determined by the modified Craven's assay¹⁸⁾ after extraction with pentane.¹⁹⁾ The mitochondria contained 2.91 nmol of CoQ₁₀/mg of mitochondrial protein.

General Procedure for the Synthesis of 5-RS- and 5,6-Di-RS-2,3-ethylenedioxy-1,4-benzoquinone (R = Alkyl, Aryl) Nine new 2,3-ethylenedioxy-1,4-benzoquinones were synthesized by the methods which were previously described.¹⁾ The following method for the synthesis of 5-phenylthio-(Id) and 5,6-diphenylthio-2,3-ethylenedioxy-1,4-benzoquinone (IIa) is a typical example of general procedure.

5-Phenylthio- (Id) and 5,6-Diphenylthio-2,3-ethylenedioxy-1,4-benzoquinone (IIa) A solution of benzenethiol (230 mg) in *n*-hexane (15 ml) was added dropwise to a solution of III (300 mg) in EtOH (20 ml) at room temperature under stirring. After being stirred for 1 h, the reaction mixture was evaporated to dryness under reduced pressure. The residue was dissolved in acetone (20 ml) and a solution of Fremy's salt (1.5 g), 1 N sodium acetate (3 ml) and H₂O (90 ml) was added to the above solution

TABLE III. Physicochemical Data for 5-RS- and 5,6-Di-RS-2,3-ethylenedioxy-1,4-benzoquinones

Compd. No.	Yield (%)	mp (°C)	Formula Analysis (%)		MS (M ⁺)	IR (cm ⁻¹ , KBr)	¹ H-NMR (CDCl ₃)
			C	H			
5-RS-2,3-Ethylenedioxy-1,4-benzoquinones							
R							
Ia	<i>n</i> -C ₈ H ₁₇	10.0	145—146	C ₁₆ H ₂₂ O ₄ S 61.91 7.14 (61.66 7.22)	310	2910 (C-H) 1660 (C=O)	0.89 (3H, t, CH ₃), 1.24—1.35 (8H, m, -(CH ₂) ₂ -), 1.41—1.49 (2H, m, -CH ₂ -), 1.68—1.76 (2H, m, S-CH ₂ -CH ₂), 2.75 (2H, t, S-CH ₂), 4.31—4.38 (4H, d, O(CH ₂) ₂ O), 6.13 (1H, s, -CH=)
Ib	<i>n</i> -C ₁₂ H ₂₅	11.34	141—142	C ₂₀ H ₃₀ O ₄ S 65.54 8.25 (65.52 8.28)	366	2900 (C-H) 1665 (C=O)	0.88 (3H, t, CH ₃), 1.22—1.32 (16H, br, -(CH ₂) ₈ -), 1.40—1.48 (2H, m, -CH ₂ -), 1.67—1.76 (2H, m, S-CH ₂ -CH ₂), 2.75 (2H, t, S-CH ₂), 4.30—4.37 (4H, m, O(CH ₂) ₂ O), 6.13 (1H, s, -CH=)
Ic	<i>n</i> -C ₁₈ H ₃₇	13.5	146—147	C ₂₆ H ₄₂ O ₄ S 69.29 9.39 (69.51 9.65)	450	2900 (C-H) 1660 (C=O)	0.88 (3H, t, CH ₃), 1.24—1.34 (28H, br, -(CH ₂) ₁₄ -), 1.40—1.48 (2H, m, -CH ₂ -), 1.67—1.76 (2H, m, SCH ₂ -CH ₂), 2.75 (2H, t, S-CH ₂), 4.31—4.37 (4H, m, O(CH ₂) ₂ O), 6.13 (1H, s, -CH=)
Id	C ₆ H ₅	36.4	220—221	C ₁₄ H ₁₀ O ₄ S 61.31 3.68 (61.03 3.70)	274	1565 (C=C) 1645 (C=O)	4.34 (4H, s, O(CH ₂) ₂ O), 5.65 (1H, s, -CH=), 7.45—7.52 (5H, m, C ₆ H ₅)
Ie	β-C ₁₀ H ₇	45.0	165—166	C ₁₈ H ₁₂ O ₄ S 66.67 3.73 (66.53 3.84)	324	1560 (C=C) 1645 (C=O)	4.34 (4H, s, O(CH ₂) ₂ O), 5.64 (1H, s, -CH=), 7.45—8.06 (7H, m, C ₁₀ H ₇)
5,6-Di-RS-2,3-ethylenedioxy-1,4-benzoquinones							
R							
IIa	<i>n</i> -C ₈ H ₁₇	17.1	61—62	C ₂₄ H ₃₈ O ₄ S ₂ 63.40 8.42 (63.16 8.36)	454	2900 (C-H) 1660 (C=O)	0.88 (6H, t, CH ₃), 1.26—1.33 (16H, m, -(CH ₂) ₄ -), 1.36—1.42 (4H, m, -CH ₂ -), 1.56—1.65 (4H, m, SCH ₂ -CH ₂), 3.17 (4H, t, S-CH ₂), 4.33 (4H, s, O(CH ₂) ₂ O)
IIb	<i>n</i> -C ₁₂ H ₂₅	11.3	76—77	C ₃₂ H ₅₄ O ₄ S ₂ 67.80 9.60 (68.04 9.88)	566	2920 (C-H) 1665 (C=O)	0.88 (6H, t, CH ₃), 1.22—1.32 (32H, m, -(CH ₂) ₈ -), 1.35—1.43 (4H, m, -CH ₂ -), 1.56—1.65 (4H, m, SCH ₂ -CH ₂), 3.17 (4H, t, S-CH ₂), 4.32 (4H, s, O(CH ₂) ₂ O)
IIc	<i>n</i> -C ₁₈ H ₃₇	7.5	88—89	C ₄₄ H ₇₈ O ₄ S ₂ 71.88 10.69 (71.64 10.83)	734	2900 (C-H) 1660 (C=O)	0.88 (6H, t, CH ₃), 1.23—1.32 (56H, m, -(CH ₂) ₁₄ -), 1.36—1.44 (4H, m, -CH ₂ -), 1.56—1.64 (4H, m, SCH ₂ -CH ₂), 3.17 (4H, t, S-CH ₂), 4.33 (4H, s, O(CH ₂) ₂ O)
IId	C ₆ H ₅	7.2	168—169	C ₂₀ H ₁₄ O ₄ S ₂ 62.81 3.69 (62.87 3.69)	382	1565 (C=C) 1645 (C=O)	4.28 (4H, s, O(CH ₂) ₂ O), 7.23—7.37 (10H, m, C ₆ H ₅)

under stirring. After being stirred for 10 min, the reaction mixture was extracted with CHCl₃. The extract was treated in the usual manner and the residue was purified by column chromatography on silica gel with CHCl₃ as the eluent. The first eluate afforded diphenyldisulfide. The second eluate (yellow in color) was treated as usual, and oxidized again with Fremy's salt to afford IId as black crystals. Yield 50 mg (7.2%). NMR (CDCl₃) δ : 4.28 (4H, s, CH₂), 7.23—7.37 (10H, m, phenyl). The third eluate (brown in color) was treated in the usual way and the residue was purified by column chromatography on silica gel with CH₂Cl₂ as the eluent. The solvent was evaporated off and the residue was recrystallized from EtOH to afford Id as red crystals. Yield 180 mg (36.4%). NMR (CDCl₃) δ : 4.34 (4H, s, CH₂), 5.65 (1H, s, H on the quinone ring), 7.45—7.52 (5H, m, phenyl). The physicochemical data are summarized in Table III.

Inhibition of the Mitochondrial Succinoxidase and NADH Oxidase Systems⁵⁾ Succinoxidase and NADH oxidase activities were determined manometrically in a Gilson respirometer¹⁵⁾ using beef heart mitochondria by a procedure described previously,¹⁾ except that the 2.6 ml reaction mixture in the main compartment of each flask contained 0.590 mg of protein for succinoxidase and 0.415 mg for NADH oxidase. The inhibitory activities of quinones were measured at several concentrations and the concentration required for 50% inhibition of enzyme activity was estimated by interpolation.

Difference Spectra of Submitochondrial Particles The reduced minus oxidized difference spectra of the submitochondrial particles were measured by the method of Chance.²⁰⁾ A pair of reference and sample cuvettes contained 1 mg of submitochondrial particles as protein and 50 μ mol of potassium phosphate buffer (pH 7.4) in a total volume of 0.95 ml, and were maintained at 37°C. Difference spectra at 400—700 nm of the control were recorded 3 min after addition of succinate (5 μ mol) or NADH (1.3 μ mol) as a substrate into the sample cuvette. Then, the inhibitor was added to both cuvettes, and the spectral changes were measured again after a 3 min incubation.

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