

# Nonoxidizable ubiquinol derivatives that are suitable for the study of the ubiquinol oxidation site in the cytochrome *bc*<sub>1</sub> complex

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## Abstract

Recent X-ray crystallographic analyses of the mitochondrial cytochrome *bc*<sub>1</sub> complex show ubiquinone binding at the Q<sub>i</sub> site, but attempts to show binding of ubiquinol or ubiquinone at the Q<sub>o</sub> site have been unsuccessful, even though the binding of noncompetitive Q<sub>o</sub> site inhibitors near the putative ubiquinol binding pocket is well established. We speculate that ubiquinol binds transiently to the Q<sub>o</sub> site only when both heme b<sub>L</sub> and the iron sulfur cluster are in the oxidized form, an experimental condition difficult to obtain since ubiquinol will be oxidized once bound to the site. Stable binding at the Q<sub>o</sub> site might be achieved by a nonoxidizable ubiquinol-like compound. For this purpose, the isomers 2,3,4-trimethoxy-5-decyl-6-methyl-phenol (TMDMP) and 2,3,4-trimethoxy-5-methyl-6-decyl-phenol (TMMDP) were synthesized from 2,3-dimethoxy-5-methyl-6-decyl-1, 4-benzoquinol (Q<sub>o</sub>C<sub>10</sub>) by controlled methylation and separated by TLC and HPLC. The structures of TMDMP and TMMDP were established by <sup>1</sup>H-<sup>13</sup>C-two-dimensional NMR. Both are competitive inhibitors of the cytochrome *bc*<sub>1</sub> complex, with TMDMP being the stronger one. Preliminary results suggest that TMDMP binds tightly enough to make X-ray crystallography of inhibitor-*bc*<sub>1</sub> complex co-crystals feasible. The binding site of TMDMP does not overlap with the binding sites of stigmatellin, MOA-stilbene (MOAS), undecylhydroxydioxobenzothiazole (UHDBT) and myxothiazol.

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**Keywords:** Nonoxidizable; Ubiquinol; Cytochrome *bc*<sub>1</sub> complex

## 1. Introduction

The mitochondrial respiratory chain, which provides more than 90% of the energy needed for aerobic cells, contains four electron transfer complexes and an ATP synthase complex [1–3]. The cytochrome *bc*<sub>1</sub> complex is the central segment of the respiratory chain in mitochondria. It oxidizes ubiquinol and reduces cytochrome *c* with concomitant generation of a proton gradient and membrane potential for ATP synthesis by ATP synthase [4,5].

The first crystal structure of cytochrome *bc*<sub>1</sub> complex from bovine heart mitochondria was solved at 2.9-Å resolution in 1997 [6]. One year later, more complete structures from different crystalline forms were reported [7–10]. These X-ray crystallographic analyses revealed binding of ubiquinone at the Q<sub>i</sub> site of the complex. Surprisingly, none of the reported structures for cytochrome *bc*<sub>1</sub> complex shows ubiquinone or ubiquinol binding at the Q<sub>o</sub> site, although they show binding for Q<sub>o</sub> site inhibitors [11]. Since most of the Q<sub>o</sub> site inhibitors are reported to be noncompetitive [12,13] inhibitors or having too high binding affinity that they cannot be released by substrate or other inhibitors, their binding sites cannot be assumed to be the binding site for ubiquinol at the Q<sub>o</sub> site without severe reservation. Establishing the nature of ubiquinol binding at the Q<sub>o</sub> site is very important in the mechanistic study of this complex. The key feature of the Q-cycle mechanism [14–16] is the bifurcation of ubiquinol oxidation at the Q<sub>o</sub> site. This step cannot be established without detailed information of ubiquinol binding at Q<sub>o</sub> site.

A possible explanation for the failure to detect ubiquinol or ubiquinone binding at the Q<sub>o</sub> site is that ubiquinol

*Abbreviations:* DM, *n*-dodecyl-β-D-maltoside; 2Fe2S, iron-sulfur cluster; ISP, iron-sulfur protein; K<sub>i</sub>, the inhibition constant; MOAS, MOA-stilbene; Q<sub>o</sub>C<sub>10</sub>, 2,3-dimethoxy-5-methyl-6-decyl-1, 4-benzoquinone; Q<sub>o</sub>C<sub>5</sub>, 2,3-dimethoxy-5-methyl-6-pentyl-1, 4-benzoquinone; Q<sub>2</sub>, 2,3-dimethoxy-5-methyl-6-geranyl-1, 4-benzoquinone; TMDMP, 2,3,4-trimethoxy-5-decyl-6-methyl-phenol; TMMDP, 2,3,4-trimethoxy-5-methyl-6-decyl-phenol; UHDBT, undecylhydroxydioxobenzothiazole

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binding at the  $Q_o$  site is transient, occurring only when the iron–sulfur cluster (2Fe2S) of the Reiske iron–sulfur protein (ISP) and heme  $b_L$  are both in the oxidized state. This experimental condition is difficult to obtain because once ubiquinol binds at the  $Q_o$  site, it immediately gives electrons to 2Fe2S and to heme  $b_L$  to become ubiquinone, which may be only loosely bound to the  $Q_o$  site and thus is not detectable in the crystal structure. If this speculation is correct, one would expect to see Q-binding at the  $Q_o$  pocket in cytochrome  $bc_1$  complex crystals loaded with a non-oxidizable reduced Q derivatives, since the conditions for ubiquinol binding, 2Fe2S and heme  $b_L$  in oxidized state and Q derivative in reduced state, are preserved.

Recently we synthesized two isomers of a reduced Q-like compound, 2,3,4-trimethoxy-5-decyl-6-methyl-phenol (TMDMP) and 2,3,4-trimethoxy-5-methyl-6-decyl-phenol (TMMDP), to test this possibility. Unlike other  $Q_o$  site inhibitors of the cytochrome  $bc_1$  complex, both of these Q derivatives inhibit the cytochrome  $bc_1$  complex competitively, suggesting that they bind to the substrate-binding site. Herein we report the synthetic procedure for and structural identification of these two reduced Q-like compounds. The inhibitor potency and the mode of inhibition of the cytochrome  $bc_1$  complex by TMDMP and TMMDP are analyzed with Lineweaver–Burk plots. That TMDMP or TMMDP competes with substrate quinol for binding at the ubiquinol oxidation site of the cytochrome  $bc_1$  complex is further established by measuring the inhibitor potency of TMDMP under various assay conditions. The effect of other  $Q_o$  site inhibitors on the TMDMP inhibition of cytochrome  $bc_1$  complex is also examined. The binding site of TMDMP in the cytochrome  $bc_1$  complex is briefly described.

## 2. Experimental procedure

### 2.1. Materials

Cytochrome  $c$ , Type III, sodium cholate and deoxycholic acid were from Sigma. Dodecyl- $\beta$ -D-maltoside (DM) was purchased from Anatrace. 2,3-Dimethoxy-5-methyl-6-geranyl-1, 4-benzoquinone ( $Q_2$ ), 2,3-dimethoxy-5-methyl-6-decyl-1, 4-benzoquinone ( $Q_{10}$ ), and 2,3-dimethoxy-5-methyl-6-pentyl-1, 4-benzoquinone ( $Q_{5}$ ) were synthesized in our laboratory [17]. Other chemicals were of the highest purity commercially available.

### 2.2. Synthesis of TMDMP and TMMDP

Two isomers, TMDMP and TMMDP, were synthesized from 2,3-dimethoxy-5-methyl-6-decyl-1, 4-benzoquinol ( $Q_{10}C_{10}H_2$ ) by controlled methylation [18].  $Q_{10}C_{10}$ , 77 mg (0.24 mmol), was dissolved in 0.35 ml of methanol and shaken with 45-mg sodium borohydride (1.2 mmol) until the solution became colorless. The mixture was treated with 1.4 ml of water and extracted with diethyl ether. The ether

extract was dried over  $MgSO_4$ , filtered and evaporated to obtain the reduced product,  $Q_{10}C_{10}H_2$ , as colorless oil.

$Q_{10}C_{10}H_2$  was dissolved in 0.5 ml of dry acetone, mixed with 50  $\mu$ l of methyl iodide and 33 mg of  $K_2CO_3$ . This mixture was refluxed for 6–8 h with stirring. All described steps were carried out under argon. The solution was filtered and the filtrate was purified with Silica Gel G plates. The thin layer plate was developed with a hexane/ethyl acetate (9:1 v/v) mixture. TMDMP has a slightly lower mobility ( $R_f=0.53$ ) than TMMDP does ( $R_f=0.55$ ) in this system. Both compounds were eluted with ether; yields were 8.2% and 20.6%, respectively. Since only small amounts of these compounds were needed, no special effort was made to improve the yields. Both TMDMP and TMMDP were further purified by HPLC, using a C-18 column ( $3.9 \times 150$  mm) with 75% acetonitrile as the mobile phase. The retention times were 16.5 and 14.8 min for TMDMP and TMMDP, respectively, at a flow rate of 1.0 ml/min. The structure of TMDMP and TMMDP was determined by  $^1H$ - $^{13}C$ -HMBC (heteronuclear multiple band correlation) NMR using a 600-MHz NMR spectrometer.

### 2.3. Enzyme preparations and assays

Bovine heart mitochondrial cytochrome  $bc_1$  complex was prepared as previously reported [19]. The purified complex was dissolved in 50 mM Tris–Cl buffer, pH 7.8, containing 0.66 M sucrose to a protein concentration of 20 mg/ml and frozen at  $-80$  °C until use. Normally the purified  $bc_1$  complex contains 10 nmol of cytochrome  $b$  and 5.7 nmol of cytochrome  $c_1$  per milligram of protein. It was reported that crystalline cytochrome  $bc_1$  complex has a ratio of cytochrome  $b$  to cytochrome  $c_1$  of 2 and the excess cytochrome  $c_1$  subfraction that appeared in the purified sample was recovered in the mother liquid of crystallization [20]. The concentrations of cytochromes  $b$  and  $c_1$  were determined spectrophotometrically using millimolar extinction coefficients of  $\Delta E_{562-575\text{ nm}} = 28.5\text{ cm}^{-1}\text{ mM}^{-1}$  and  $\Delta E_{552-540\text{ nm}} = 17.5\text{ cm}^{-1}\text{ mM}^{-1}$  for cytochromes  $b$  and  $c_1$ , respectively.

For activity assay, the cytochrome  $bc_1$  complex was diluted with 50 mM phosphate buffer, pH 7.4, containing 1 mM EDTA and 0.01% dodecylmaltoside to a protein concentration of 0.05 mg/ml. Diluted enzyme solution (10  $\mu$ l) was added to 980  $\mu$ l of an assay mixture containing 50 mM phosphate buffer, pH 7.4, containing 1 mM EDTA and 100 mM cytochrome  $c$  in the presence or absence of inhibitor. The mixture was incubated for 1 min at 25 °C. Activity was determined by measuring the reduction of cytochrome  $c$  after addition of 5  $\mu$ l of  $Q_{10}C_{10}H_2$  or other reduced ubiquinol derivatives. A millimolar extinction coefficient of  $18.5\text{ cm}^{-1}\text{ mM}^{-1}$  was used to calculate the activity. Nonenzymatic reduction of cytochrome  $c$ , determined under the same conditions in the absence of the cytochrome  $bc_1$  complex, was subtracted from the assay.

#### 2.4. Cytochrome *bc*<sub>1</sub> complex–inhibitor co-crystallization and X-ray diffraction data analysis

For co-crystallization of the cytochrome *bc*<sub>1</sub> complex with TMDMP, a fourfold molar excess of inhibitor was added to the protein solution. This solution was set up for crystallization as described [6,9]. Crystals grew in 2–4 weeks; they had a rectangular shape and ranged from 0.2 to 0.6 mm. They could be frozen at high glycerol concentration, and they had the same symmetry and similar unit cell dimensions as the native crystals [6,9]. X-ray diffraction data were collected at the beamline 17-ID in the facilities of the IMCA-CAT at the Advanced Photon Source (APS), Argonne National Laboratory. The data were analyzed by HKL2000, CNS and Xtal View [21–23].

### 3. Results and discussion

#### 3.1. Synthesis, chemical structures and absorption properties of TMDMP and TMMDP

The structures of these two isomers are confirmed by <sup>1</sup>H-NMR, and 2-D NMR <sup>1</sup>H-<sup>13</sup>C-HMBC. <sup>1</sup>H NMR for TMDMP (CDCl<sub>3</sub>) shows: δ 5.52 (s, 1, OH), 3.86 (s, 3H, OMe), 3.84 (s, 3H, OMe), 3.72 (s, 3H, OMe), 2.50 (t, 2H, -CH<sub>2</sub>-decyl), 1.38–1.21 (m, 16H, (CH<sub>2</sub>)<sub>8</sub> of decyl), 0.81 (t, 3H, Me of decyl) and 2.09 (s, 3H, Me); whereas <sup>1</sup>H-NMR for TMMDP (CDCl<sub>3</sub>) shows: δ 5.47 (s, 1, OH), 3.86 (s, 3H, OMe), 3.84 (s, 3H, OMe), 3.69 (s, 3H, OMe), 2.52 (t, 2H, -CH<sub>2</sub>- of decyl), 1.38–1.21 (m, 16H, (CH<sub>2</sub>)<sub>8</sub> of decyl), 0.81 (t, 3H, Me of decyl) and 2.10 (s, 3H, Me). As expected when D<sub>2</sub>O was added to the system, the peaks of δ 5.52 and 5.47 disappeared. Because TMDMP and TMMDP are isomers, 1-D <sup>1</sup>H-NMR spectrum is not adequate for complete structure elucidation. Hence, 2-D <sup>1</sup>H-<sup>13</sup>C HMBC NMR spectra were used to identify the structure of and confirm the position of the substituents in these two isomers. Table 1 shows NMR data in low field (aromatic ring region) of TMDMP and TMMDP. Fig. 1A and B shows 2-D NMR <sup>1</sup>H-<sup>13</sup>C-HMBC spectrograms of TMDMP and TMMDP, respectively.

In TMDMP (Fig. 1A), the hydroxyl H (δ 5.52) shows heteronuclear multiple bond couplings with C-1, C-2 and

C-6, indicating direct bonding to C-1, which bonds to both C-2 and C-6. Furthermore, the methylene H (δ 2.50) in the decyl group shows correlation with C-5, C-4 and C-6, showing bonding to C-5, which bonds to both C-2 and C-6. The remaining three 3H singlets at δ 3.86, 3.84 and 3.72 are assigned to the hydrogens of methoxy groups at C-2, C-3 and C-4 based on <sup>1</sup>H-<sup>13</sup>C HMBC correlations. In TMMDP (Fig. 1B), the phenol ring is confirmed by the presence of hydroxyl resonance at δ 5.47, correlations with ring carbons C-1, C-2 and C-6. Moreover, the 3H of the methyl group interact with aromatic carbons C-4, C-5 and C-6, while -CH<sub>2</sub> (δ 2.52) of the decyl group in TMMDP interacts with aromatic carbons C-6, C-1 and C-5. These NMR data confirm the chemical formula of TMDMP and TMMDP given in Fig. 1.

Fig. 2 shows the absorption spectra of TMDMP in neutral and alkaline aqueous solution. As expected the absorption spectra of TMMDP are very similar (data not shown). Both compounds have an absorption peak at 283 nm. The millimolar extinction coefficient for TMDMP and TMMDP was determined to be 2 cm<sup>-1</sup> mM<sup>-1</sup> at 283 nm in ethanol. Upon alkalization with sodium hydroxide, this 283-nm peak shifts to a high wavelength with a significant increase in absorption intensity, indicating the dissociation of a proton from the hydroxyl group. This result confirms that these two isomers are indeed phenolic compounds. The pK<sub>a</sub> of both compounds is estimated to be around 12.5.

#### 3.2. Mode of inhibitions of cytochrome *bc*<sub>1</sub> complex by TMDMP and TMMDP

Fig. 3 shows the concentration-dependent inhibition of the cytochrome *bc*<sub>1</sub> complex by TMDMP and TMMDP. When a given amount of complex was added to an assay mixture containing 50 mM Na/K phosphate buffer, pH 7.0, 100 μM cytochrome *c* and varying amounts of TMDMP or TMMDP, the rate of cytochrome *c* reduction, upon addition of 50 μM of substrate ubiquinol-2, decreased as the concentration of TMDMP or TMMDP in the assay mixture increased. These compounds are not very strong inhibitors. Inhibitions of 60% and 40% were observed when 60 μM TMDMP or TMMDP was present in the assay mixture (see Fig. 3). The need for a high concentration of TMDMP or

Table 1  
NMR data in lower field (aromatic ring region) of TMDMP and TMMDP

TMDMP				TMMDP			
Groups	<sup>1</sup> H δ <sub>H</sub>	<sup>13</sup> C δ <sub>C</sub>	<sup>1</sup> H- <sup>13</sup> CHMBC	Groups	<sup>1</sup> H δ <sub>H</sub>	<sup>13</sup> C δ <sub>C</sub>	<sup>1</sup> H- <sup>13</sup> CHMBC
C-1(-OH)	5.52	143.2	C-1,C-2,C-6	C-1(-OH)	5.47	143.1	C-1,C-2,C-6
C-2(-OCH <sub>3</sub> )	3.86	137.5	C-2,C-3,C-1	C-2(-OCH <sub>3</sub> )	3.86	137.5	C-2,C-1,C-3
C-3(-OCH <sub>3</sub> )	3.84	143.4	C-3,C-2,C-4	C-3(-OCH <sub>3</sub> )	3.84	143.5	C-3,C-2
C-4(-OCH <sub>3</sub> )	3.72	144.4	C-4	C-4(-OCH <sub>3</sub> )	3.69	144.6	C-4
C-5(-CH <sub>2</sub> -)	2.50	130.7	C-4,C-5,C-6	C-5(-CH <sub>3</sub> )	2.10	125.3	C-5,C-4,C-6
C-6(-CH <sub>3</sub> )	2.09	117.1	C-1,C-5,C-6	C-6(-CH <sub>2</sub> -)	2.52	122.7	C-1,C-5,C-6

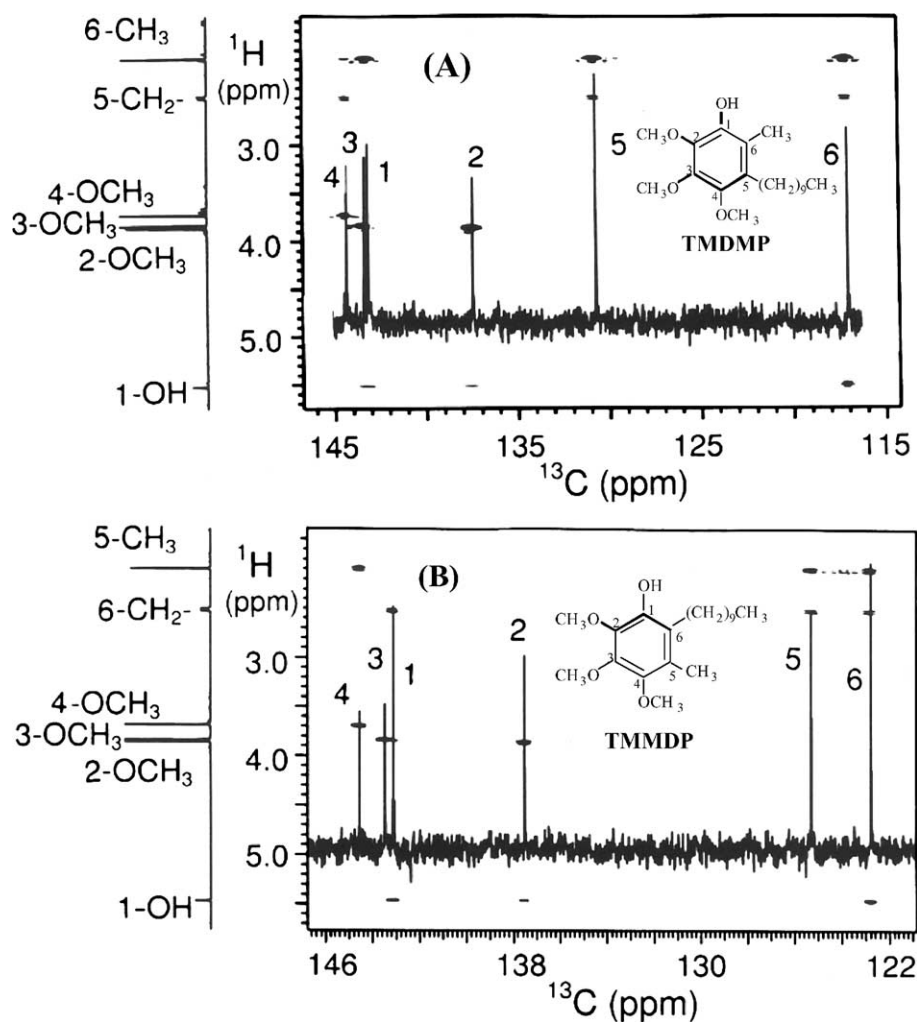


Fig. 1.  $^1\text{H}$ - $^{13}\text{C}$  HMBC spectrum of the aromatic ring region of TMDMP (A) and TMMDP (B).

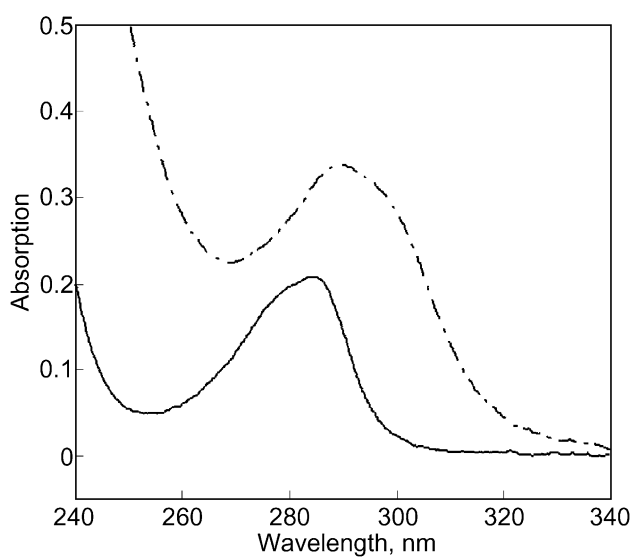


Fig. 2. Absorption spectra of TMDMP. The solid line shows the spectrum measured in 20 mM phosphate buffer, pH 7.0, containing 0.05% DM. The dashed line shows the spectrum in 0.1 N NaOH.

TMMDP to show partial inhibition is expected since these two compounds are competitive inhibitors and they must compete with the excess substrate ubiquinol-2 present in the assay mixture.

Lineweaver–Burk analyses show that both TMDMP and TMMDP are competitive inhibitors (Fig. 4) with a calculated  $K_i$  of 6.8  $\mu\text{M}$  for TMDMP and 71.6  $\mu\text{M}$  for TMMDP, indicating that TMDMP is the stronger inhibitor than TMMDP. The  $K_m$  for  $\text{Q}_0\text{C}_{10}\text{H}_2$  is increased from 20 to 51.3  $\mu\text{M}$  upon treatment with TMDMP. A slightly larger increase in  $K_m$  for  $\text{Q}_0\text{C}_{10}\text{H}_2$  (to 66.7  $\mu\text{M}$ ) was observed with the TMMDP-treated enzyme.

The difference in the inhibitory potency of TMDMP and TMMDP suggests that the two hydroxyl groups of ubiquinol do not play an equal role in substrate binding. The hydroxyl in the position *meta* to the long alkyl side chain is apparently more important for binding than is hydroxyl group in the position *ortho* to the side chain.

Although TMDMP and TMMDP are rather weak competitive inhibitors, the structural similarity between them and the substrate ubiquinol make them valuable for studying



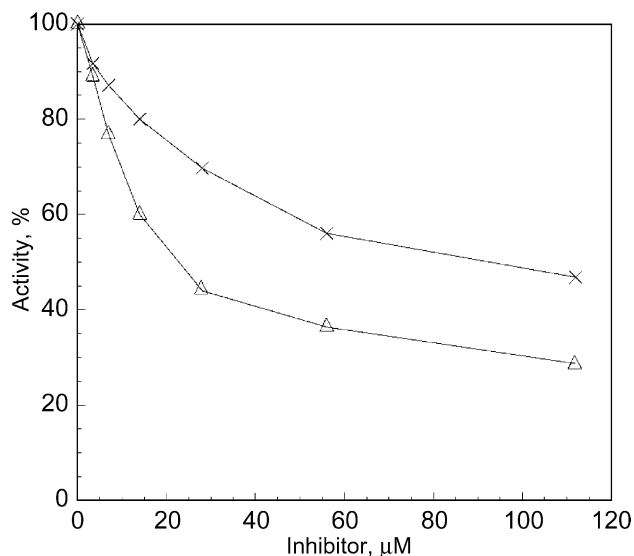


Fig. 3. TMDMP and TMMDP concentration-dependent inhibition of ubiquinol-cytochrome *c* reductase. Twenty microliters of cytochrome *bc*<sub>1</sub> complex, 0.05 mg/ml, in 50 mM phosphate buffer, pH 7.4, containing 1 mM EDTA and 0.01% dodecylmaltoside, was added to 975 μl of an assay mixture with indicated amounts of TMDMP (Δ) or TMMDP (×). The reaction was started by the addition of 5 μl of 10 mM Q<sub>0</sub>C<sub>10</sub>H<sub>2</sub>. One hundred percent activity represents 200 μmol cytochrome *c* reduced per minute per milligram of protein.

the ubiquinol oxidation site in the cytochrome *bc*<sub>1</sub> complex. Preliminary results indicate that TMDMP binds tightly enough to make feasible X-ray crystallography on inhibitor-protein complex co-crystals.

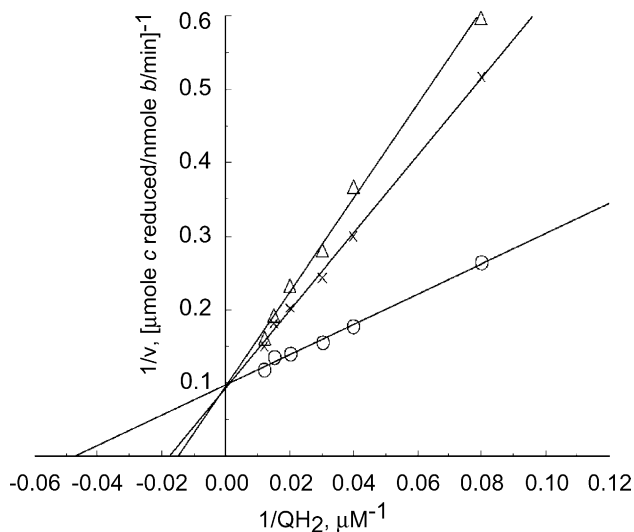


Fig. 4. Lineweaver-Burk analysis of TMDMP and TMMDP inhibition on cytochrome *bc*<sub>1</sub> complex. Ten microliters of the *bc*<sub>1</sub> complex, 0.05 mg/ml, in 50 mM phosphate buffer, pH 7.4, containing 1 mM EDTA and 0.01% dodecylmaltoside, was added to 975 μl of an assay mixture with no inhibitor (○), 11.8 μM TMDMP (Δ) or 130 μM TMMDP (×). The reaction was started by the addition of various amounts of Q<sub>0</sub>C<sub>10</sub>H<sub>2</sub>.

### 3.3. The effect of the alkyl side-chain of ubiquinol on the inhibition of the cytochrome *bc*<sub>1</sub> complex by TMDMP

It has been reported [24] that the cytochrome *bc*<sub>1</sub> complex catalyzes the oxidation of quinol derivatives with an efficiency directly proportional to the number of carbons in the quinol side chain, up to 10 carbons; e.g. Q<sub>0</sub>C<sub>10</sub>H<sub>2</sub> or Q<sub>2</sub>H<sub>2</sub>. Apparently lower homologs are less effective substrates because of a lesser affinity for the oxidation site. If TMDMP indeed competes with the substrate for the binding at the Q<sub>0</sub> site of the cytochrome *bc*<sub>1</sub> complex, we would expect it to be a more potent inhibitor when a less effective substrate (lower homologs of ubiquinol) is used. To test this idea we measured the inhibitory potency of TMDMP against ubiquinol substrates with varying lengths of the alkyl side chain (see Table 2). As predicted, when the alkyl side chain length of substrate ubiquinol was decreased, activity decreased and the percent activity inhibited by TMDMP increased. These results further support binding of TMDMP at the quinol oxidation site of the *bc*<sub>1</sub> complex.

### 3.4. The effect of pH on the inhibition of the cytochrome *bc*<sub>1</sub> complex by TMDMP and TMMDP

Titration of the activity of *bc*<sub>1</sub> complex against pH shows a bell-shaped curve with the pH ranging from 6.5 to 9.0. The maximal activity is observed at pH 8.0, suggesting the optimal condition for the substrate binding and the product releasing at this pH, even though other factors may also contribute to the maximal activity observed. If TMDMP competes with substrate quinol for the Q<sub>0</sub> site of the *bc*<sub>1</sub> complex, one would expect inhibition to be minimal at pH 8.0. To test this hypothesis, the inhibitor potencies of TMDMP and TMMDP were determined at pH ranging from 6.5 to 9.0 (see Fig. 5). In the presence of TMDMP the enzyme complex exhibits bell-shaped pH curve, like the free enzyme, but with lower activity. In the presence of 16.3 μM of TMDMP and a cytochrome *bc*<sub>1</sub> complex concentration of 2.0 nM, inhibition is 63.0% at pH 7.0, but only 33.0% at pH 8.0. An inverted bell-shaped curve is seen when percent inhibition is plotted against pH. That is, TMDMP inhibits least effectively when the complex is fully active at pH 8.0. Similar pH dependence is observed with TMMDP. These results further confirm that TMDMP or TMMDP compete with substrate for its binding site in the

Table 2

Effect of the length of the alkyl side chain of ubiquinol on the inhibition of cytochrome *bc*<sub>1</sub> complex by TMDMP

Substrate	Activity (%)	Activity with TMDMP	Percent inhibition
Q <sub>0</sub> C <sub>10</sub>	100	78.4	21.6
Q <sub>0</sub> C <sub>7</sub>	66	43.0	34.8
Q <sub>0</sub> C <sub>5</sub>	28	15.0	46.4

The concentrations of substrate and TMDMP used were 50 and 20 μM, respectively.

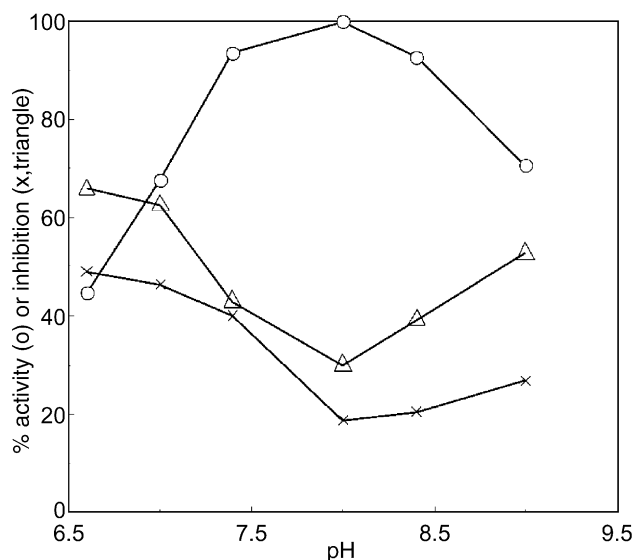


Fig. 5. The effect of pH on the activity of the cytochrome  $bc_1$  complex in the absence or presence of TMDMP or TMMDP. Ten microliters of the  $bc_1$  complex, 0.05 mg/ml, in 50 mM phosphate buffer, pH 7.4, containing 1 mM EDTA and 0.01% dodecylmaltoside, was added to 900  $\mu$ l of an assay mixture with the indicated pH's containing no inhibitor (○), 16.3  $\mu$ M TMDMP (Δ) or 163.0  $\mu$ M TMMDP (×). The reaction was started by addition of 48.5  $\mu$ M  $Q_0C_{10}H_2$ . The inhibition (Δ, ×) was calculated as percent of the control activity in the absence of the inhibitor, at the indicated pH values.

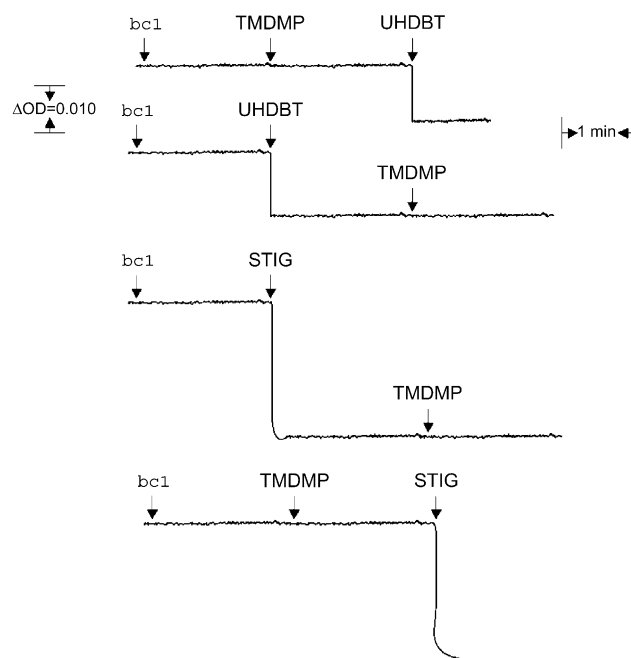


Fig. 6. Binding competition between TMDMP and stigmatellin or UHDBT. One milliliter of partially reduced cytochrome  $bc_1$  complex in 20 mM Tris–Cl, pH 7.8, containing 100mM KCl, 0.05% dodecylmaltoside, was treated sequentially with different inhibitors (fivefold molar excess). The redox state of cytochrome  $c_1$  was monitored (553 minus 545 nm). Cytochrome  $c_1$  was about 70% reduced before addition of inhibitor. The final concentration of cytochrome  $c_1$  is 5  $\mu$ M.

cytochrome  $bc_1$  complex since they compete less effectively when the complex is at optimal pH condition, a situation where substrate is most effectively bound.

As expected, inhibition of the cytochrome  $bc_1$  complex by the currently available  $Q_0$  site inhibitors, such as stigmatellin and myxothiazol, showed no such pH dependent inhibition curve because they are noncompetitive inhibitors. Although inhibition of cytochrome  $bc_1$  by undecylhydroxydioxobenzothiazole (UHDBT) is pH-dependent [25–27], the inhibition curve differs from that observed with TMDMP.

### 3.5. The lack of binding competition between TMDMP or TMMDP and $Q_0$ site inhibitors

It was reported [26,28] that stigmatellin or UHDBT binding induces cytochrome  $c_1$  oxidation in a partially reduced cytochrome  $bc_1$  complex due to the elevation of the  $E_m$  of the ISP. An inhibitor, which competes with stigmatellin but does not (or only slightly) elevate the  $E_m$  of ISP upon binding, should decrease or abolish the oxidation of cytochrome  $c_1$  caused by stigmatellin. Addition of TMDMP to a partially reduced cytochrome  $bc_1$  complex

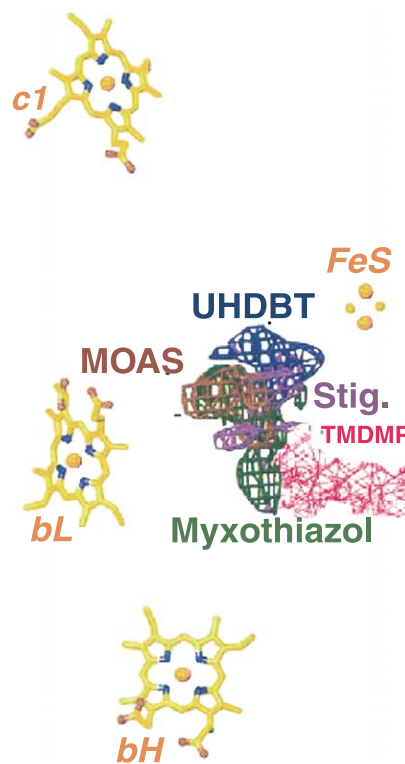


Fig. 7. The relative binding location of TMDMP to those of other  $Q_0$  site inhibitors and redox centers in the cytochrome  $bc_1$  complex. The densities of inhibitors at the  $Q_0$  site are color-coded with TMDMP in red, myxothiazol in green, stigmatellin in purple, UHDBT in blue and MOAS in brown. The density for TMDMP comes from a Fourier synthesis electron density map of TMDMP-treated crystals minus native  $bc_1$  crystals showing a  $6\sigma$  peak for TMDMP. Residues (F325, W326, V329, L333 and T336) of helix G and residues (M96, Y95, and I92) of helix D surround the cavity opening for the TMDMP binding site.

does not induce cytochrome  $c_1$  oxidation, suggesting that binding of this inhibitor does not elevate the  $E_m$  of ISP. The lack of binding competition between TMDMP and stigmatellin or UHDBT is shown in Fig. 6. Note that the degree of cytochrome  $c_1$  oxidation induced by treatment with stigmatellin or UHDBT is not altered by the presence of TMDMP, regardless of the addition sequence. This result further indicates nonidentical binding sites for these inhibitors. Although the lack of effect of TMDMP or TMMDP on UHDBT or stigmatellin binding can also be explained by the large difference in binding affinity among these inhibitors, preliminary results from X-ray crystallographic analysis of TMDMP loaded crystals of cytochrome  $bc_1$  complex support the nonidentical binding site hypothesis.

### 3.6. Preliminary results on the TMDMP binding site

Fig. 7 shows the binding location of TMDMP in relation to other  $Q_o$  site inhibitors and the redox centers in the complex. The binding site of TMDMP does not overlap with the binding sites of stigmatellin, MOA-stilbene (MOAS), UHDBT and myxothiazol. The binding site of TMDMP coincides with one of the two peptides (P-47, residues 142–155, and P-49, residues 326–336) of cytochrome  $b$  identified by the photoactivated affinity labeling technique using a radioactive azido- $Q$  derivative [29]. P-47 is located in cd1 helix, which is close to the PEWY sequence in the ef-loop of the 3-D structure of cytochrome  $b$ . Stigmatellin and several other  $Q_o$  site inhibitors are located between cd1 and ef-loop. P-49 is located in the transmembrane helix G, which is located at the opening of the TMDMP binding site. These results seem to be in line with the idea that more than one quinol binding site [30,31] are involved in the oxidation of quinol catalyzed by the cytochrome  $bc_1$  complex; one at or near the stigmatellin binding site and another at the TMDMP binding site. Detailed X-ray crystallographic study of TMDMP (ubiquinol) binding site is currently in progress and will be reported later.

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