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Quantitative analysis of electron transport inhibition of rat-liver mitochondrial cytochrome bc_1 complex by nitrophenols

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A series of nitrophenolic electron transport inhibitors (2-*sec*-butyl-4-nitro-6-substituted phenols and 2-*sec*-butyl-4-substituted-6-nitrophenols) of rat-liver mitochondrial cytochrome bc_1 complex (cyt. bc_1 complex) was synthesized. To obtain information on the three-dimensional structure of the ubiquinone redox site of cyt. bc_1 complex, the structure-inhibitory activity relationship was examined by regression analysis using physicochemical substituent parameters. The inhibitory activity increased as the hydrophobicity and the electron-withdrawing ability of the 4- and 6-substituents increased. These results indicated that hydrophobic interaction between the inhibitor molecule and the binding domain should be important and that an anionic form of nitrophenols may be the active form at the binding domain. Hydrogen-bond-acceptable 4-substituents such as methoxy and nitro groups, but not cyano group, were favorable to the inhibitory activity. This result, along with the fact that phenolic OH group was essential for the activity, suggested that nitrophenols occupy the ubiquinone redox site by forming two hydrogen-bond bridges as proposed for natural ubiquinone binding. Although a cyano group is hydrogen-bond-acceptable, hydrogen-bond formation between the 4-cyano group and the binding domain was not suggested. This result and molecular orbital calculation studies on electrostatic potential of the inhibitors suggested that hydrogen-bond donating residue may be not located in the region where the rod-like cyano (C≡N) bond directs.

Introduction

The cytochrome bc_1 complex (cyt. bc_1 complex) is a redox protein complex common to both respiratory and photosynthetic electron-transport systems. To obtain functional and structural information of quinone redox sites in this complex, substantial amount of mutagenesis [1–3] and biophysical studies [4,5] have been performed. The inhibitors of cyt. bc_1 complex such as antimycin A and myxothiazol have been important tools for the development of mechanistic and structural concepts to this ubiquitous protein complex [6]. Studies of the variation in inhibitory activities of systematically selected inhibitors could help to elucidate the three-dimensional structure of quinone redox domain.

We previously showed that typical Photosystem II inhibitor nitrophenols (2-alkyl-4,6-dinitrophenols) inhibit mitochondrial cyt. bc_1 complex activity at Q_o reaction center by working as an antagonist of ubiquinol [7]. The conformation of alkyl substituents at the 2-position, which correspond to the isoprenoid side-chain of ubiquinol, was significant factor for the inhibitor binding. That is, the conformation in which the 2-alkyl substituent is almost perpendicular to the benzene-ring plane was favorable for the activity. Molecular orbital calculation studies demonstrated that this conformation of the 2-alkyl substituent well corresponds to that of the isoprenoid side-chain of ubiquinol. Looking at this in another way, the ubiquinone redox site of cyt. bc_1 complex may strictly recognize the configuration of the side-chain. Yu et al. [8] also demonstrated the important role of the conformation of alkyl side-chain near the quinone ring of synthetic ubiquinol derivatives in the electron-transport activity.

Structural variations, however, were limited to the substituent at 2-position of 4,6-dinitrophenol in the previous study [7]. To obtain further information on the structural characteristics of the ubiquinone redox site of cyt. bc_1 complex, further studies of derivatives with systematically modified structure are needed. In the present study, we synthesized various nitrophenols fixing the 2-position as a *sec*-butyl group which was shown to be one of the most favorable substituents for

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Abbreviations: cytochrome bc_1 complex, ubiquinol-cytochrome *c* oxidoreductase; Q_o, ubiquinol-oxidizing site; SF6847, 3,5-di-*tert*-butyl-4-hydroxybenzylidene malononitrile.

the inhibition in the previous study [7]. Variations in the inhibitory activity of these compounds with rat-liver mitochondria were analyzed quantitatively by regression analysis using physicochemical substituent parameters.

Materials and Methods

Materials

Rotenone was obtained from Sigma. SF6847 was the same sample as that used in a previous study [9]. Other reagents were of the purest grade commercially available.

Synthesis

Compounds **1**, **11**, **13**, **21–23** and **26** were synthesized by nitration of corresponding commercially available substituted phenols. Compounds **2–4**, **7**, **14–16** and **19** were synthesized by nitration of corresponding 2-*sec*-butyl-4-substituted phenols, which were prepared by reduction of 2-(1-methylallyl)-4-substituted phenols by Pd/C under hydrogen gas. 2-(1-Methylallyl)-4-substituted phenols were obtained from corresponding commercially available 4-substituted phenols and crotyl chloride [10]. Compounds **17**, **18**, **24** and **25** were synthesized by nitration of corresponding 2-alkyl-6-chlorophenols, which were prepared from corresponding commercially available 2-alkylphenols and SO_2Cl_2 [11]. Compounds **5**, **6** and **27** were synthesized by halogenation of corresponding 2-alkyl-6-nitrophenols [12], which were obtained by nitration of corresponding commercially available 2-alkylphenols. Compound **20** was prepared by nitration of 2-*sec*-butyl-3-hydroxybenzaldehyde, which was obtained from 2-*sec*-butylphenol and acetic acid in the presence of hexamethylenediamine. Compounds **9** and **28** were prepared from corresponding 2-alkyl-6-nitrophenols and trifluoroacetic acid in the presence of hexamethylenediamine [13]. Compounds **10** and **29** were derived from **9** and **28**, respectively, by the method reported [14]. Compounds **12** and **30** were obtained from **9** and **28** by the condensation with malononitrile [13], respectively. Compound **8** was synthesized by nitration of 2-*sec*-butyl-4-acetylphenol, which was derived from 2-*sec*-butyl-4-acetylanisole [15]. 2-*sec*-Butyl-4-acetylanisole was prepared from 2-*sec*-butylanisole and acetyl chloride in the presence of $AlCl_3$.

All synthesized compounds were characterized by ^{1}H-NMR spectra (JEOL PMX-60) and elemental analyses for C, H and N within an error of $\pm 0.3\%$.

Methods

Mitochondria were isolated from the liver of adult male Wistar rats in a medium containing 250 mM sucrose and 2 mM Tris-HCl (pH 7.4), as described by Myers and Slater [16]. The amount of mitochondrial protein was measured by the method of Bradford [17] with bovine serum albumin as the standard. Mitochondrial respiration with 10 mM succinate as the respiration substrate was measured with a Clark-type oxygen electrode at 25°C, the final mitochondrial protein concentration in the medium being 0.7 mg/ml. The incubation medium consisted of a mixture of 200 mM sucrose, 2 mM $MgCl_2$, 1 mM EDTA and 2.5 μM rotenone in 2.5 mM potassium phosphate buffer (pH 7.4), and the total volume was 2.5 ml.

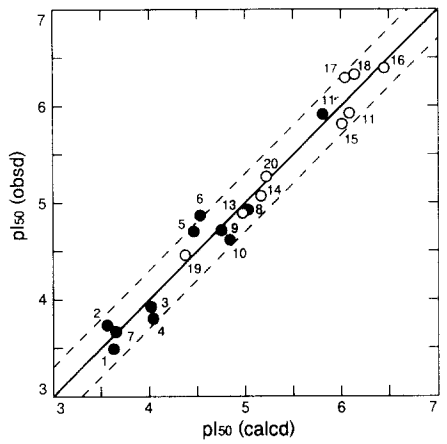


Fig. 1. Plot of pI_{50} values observed versus calculated. •; by Eqn. 1, ○; by Eqn. 2. The numbering is in accordance with Tables I and II. The standard deviation ($s = 0.30$) is indicated by dashed lines.

Nitrophenols synthesized in this study have both respiratory acceleration and inhibition effects with rat-liver mitochondria at the same time. To evaluate only the inhibitory activity by neglecting the uncoupling effect, we examined the effects of nitrophenols on the respiration fully stimulated by SF6847 (35 nM). This uncoupler-stimulated respiration is susceptibly reduced by respiration inhibitors [7,9]. The molar concentration (I_{50}) in the incubation medium needed to half the fully stimulated respiration rate was measured. pI_{50}, the log of the reciprocal of I_{50}, was used as the index of the inhibitory potency of cytochrome bc_1 complex activity.

The molecular orbital calculations was done by use of AMPAC (QCPE no. 523) with AM1 parameterization [18,19]. The starting coordinations were obtained from ANCHOR (Kuraha Chemical Industory, Co., Ltd.; Fujitsu Ltd., Tokyo), a program system for molecular modeling. The electrostatic potentials were calculated for the optimum geometries from the point charges on the atomic centers and plotted as shown in Fig. 2.

Results

We have already showed that the action site of nitrophenols is ubiquinone redox site of cyt. bc_1 complex, probably in Q_o center, by examining effects of the inhibitors on the redox status of cyt. *b* [7]. The inhibition of cyt. bc_1 complex activity by the inhibitors was sensitively detected by measuring the effects on the fully stimulated respiration by uncoupler [7]. In this

study, we also recognized the inhibition of this respiration by the inhibitors, in terms of pI_{50}, as the index of inhibition of cyt. bc_1 complex activity. The pI_{50} was measured at least twice and averaged. The standard deviation was within +0.08.

Quantitative structure-inhibitory activity analyses

The effects of substituents on the electron-transport inhibition of series I compounds (Table I) were examined by regression analyses with physicochemical substituent parameters shown in Table I. For 11 compounds, Eqn. 1 was derived as the equation of the best quality.

$$pI_{50} = \underset{(0.35)}{0.41}\pi + \underset{(0.71)}{2.14}\sigma + \underset{(0.50)}{0.52}HB + \underset{(0.38)}{3.67} \quad (1)$$

$(n = 11, s = 0.26, r = 0.95)$

In this and following equations, n is the number of compounds included in the regression analysis, s is the standard deviation and r is the correlation coefficient. The figures in parentheses are the 95% confidence intervals. The π and σ is the index of hydrophobicity [20] and electron-withdrawing ability [21] of substituent, respectively. *HB* is the indicator variable that takes one for hydrogen-bond-acceptable substituent and zero for others. If the *HB* term is excluded, the correlation was slightly but significantly poorer; for instance, $s = 0.33$, $r = 0.91$ for the counterpart of Eqn. 1. To derive Eqn. 1, compound **12** was not included, as discussed later. In Fig. 1, the pI_{50} value observed was plotted against that calculated by Eqn. 1 (closed circles). Eqn. 1 indicates that inhibitory activity increases as the hydrophobicity and the electron-withdrawing ability of the 4-substituent (X_1) increase. The positive sign of *HB* term indicates that the hydrogen-bond-acceptable substituents were favorable to the activity, and the increase in activity is estimated to be 3.3 ($10^{0.52}$)-fold. In Eqn. 1, *HB* of cyano derivative (**10**) takes zero although this functional group is hydrogen-bond-acceptable [22]. When we reanalyzed the data used to derive Eqn. 1 taking one as *HB* for compound **10** instead of zero, the quality of the correlation was poorer; for instance, $s = 0.34$, $r = 0.92$ for the counter part of Eqn. 1. This result suggested that cyano group could not form hydrogen-bond to the binding site. Use of σ^-, which takes into account the through-resonance effect between the dissociable group and the *para* substituent [21], instead of σ did not improve the correlation, indicating that the through-resonance system between the phenolic O^- and the 4-substituent is not formed.

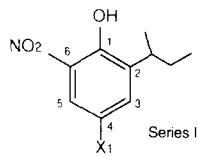
Next, we examined the substituent effects on the inhibitory activity of series II compounds (Table II), deriving Eqn. 2 as the best equation. In Fig. 1, the pI_{50} value observed was plotted against that calculated by Eqn. 2 (open circles).

$$pI_{50} = \underset{(0.27)}{0.85}\pi + \underset{(0.68)}{1.75}\sigma + \underset{(0.26)}{4.95} \quad (2)$$

$(n = 9, s = 0.23, r = 0.96)$

TABLE I

Inhibitory activity and physicochemical substituent parameters

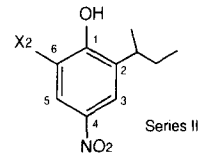


Compd. No.	X_1	pI_{50} obsd.	pI_{50} calcd. [c]	π [a]	σ [b]	*HB*
1	H	3.46	3.67	0.00	0.00	0
2	CH_3	3.75	3.53	0.54	−0.17	0
3	$CH(CH_3)_2$	3.85	4.02	1.49	−0.13	0
4	$C(CH_3)_3$	3.78	4.06	1.98	−0.20	0
5	Cl	4.70	4.46	0.71	0.23	0
6	Br	4.89	4.52	0.86	0.23	0
7	OCH_3	3.73	3.60	−0.02	−0.27	1
8	$COCH_3$	4.89	5.03	−0.55	−0.50	1
9	CHO	4.72	4.82	−0.65	0.42	1
10	CN	4.67	4.85	−0.57	0.66	0
11	NO_2	5.84	5.74	−0.28	0.78	1
12	$CH=C(CN)_2$	5.05	6.01 [d]	0.05 [e]	0.84 [e]	1

[a] Unless otherwise noted, from Ref. 22.
[b] Unless otherwise noted, from Ref. 21.
[c] By Eqn. 1.
[d] Not included in the analysis of Eqn. 1.
[e] From Hansch, C., Rockwell, S.D., Jow, P.Y., Leo, A. and Steller, E.E. (1977) J. Med. Chem. 20, 304–306.

TABLE II

Inhibitory activity and physicochemical substituent parameters



Compd. No.	X_2	pI_{50} obsd.	pI_{50} calcd. [c]	π [a]	σ [b]
11	NO_2	5.84	6.08	−0.28	0.78
13	H	4.88	4.95	0	0
14	CH_3	4.97	5.11	0.54	−0.17
15	$CH(CH_3)_2$	5.77	5.98	1.49	−0.13
16	$CH(CH_3)CH_2CH_3$	6.34	6.39	2.03	−0.16
17	Cl	6.30	5.95	0.71	0.23
18	Br	6.32	6.08	0.86	0.23
19	OCH_3	4.55	4.46	−0.20	−0.02
20	CHO	5.18	5.14	−0.65	0.42

[a] From Ref. 22.
[b] From Ref. 21.
[c] By Eqn. 2.

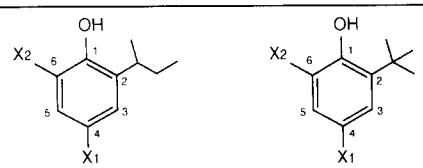
Eqn. 2 indicates that the greater the hydrophobicity and the electron-withdrawing ability, the greater was the inhibitory activity. This result suggests that the physicochemical factors deciding the inhibitory activity are almost similar for the 4- and 6-substituents except for the hydrogen-bond interaction of the 4-substituent. The nitro group at 6-position was not essential to the activity, but could be replaced by halogen. This is compatible with the study on the inhibition of Photosystem II (Q_B site) activity by nitrophenols [23]. The addition of any steric parameter terms representing steric dimensions of substituents was insignificant.

Comparison of inhibitory activity between 2-sec-butyl and 2-tert-butyl derivatives

We showed that configuration (or steric shape) of the 2-alkyl substituent is significant factor deciding the inhibitory activity, i.e., the close fitting of inhibitor molecule to the ubiquinone redox site [7]. In other words, the ubiquinone redox site of cyt. bc_1 complex could strictly recognize the difference in shape of the 2-substituents. The inhibitory activity of 2-*sec*-butyl-4,6-dinitrophenol (**11** in the present study), for instance, was about ten times greater than that of 2-*tert*-butyl-4,6-dinitrophenol (**21**). In this case, the difference in activity is due to that in the main-chain length of 2-alkyl substituent, that is, the activity increases as the length of main-chain increases [7]. It is of interest whether or not precision of this kind of recognition by the binding site can be maintained against structural modifications of the 4- and 6-substituents. In Table III, the inhibitory activity of a series of *sec*-butyl and *tert*-butyl derivatives are listed. When 4-substituent (X_1) was fixed as a nitro group, *sec*-butyl derivatives were always more potent than the corresponding *tert*-butyl derivatives irrespective of structural variations of the 6-substituent (X_2), ΔpI_{50} being from ca. 0.5 to 1.1. When 4-substituent varied fixing the 6-substituent as a nitro group, the activities of *sec*-butyl derivatives were also greater than those of *tert*-butyl derivatives, ΔpI_{50} being from ca. 0.3 to 1.0, except dicyanovinyl derivatives (**12** *vs* **30**). The activity difference between 2-*sec*-butyl and 2-*tert*-butyl derivatives was abolished ($\Delta pI_{50} = -0.02$) by replacing the 4-substituent as a bulky dicyanovinyl group.

TABLE III

Comparison of inhibitory activity between 2-*sec*-butyl and 2-*tert*-butyl derivatives



X_1	X_2	pI_{50} [a] (*sec*-butyl)	pI_{50} [a] (*tert*-butyl)	ΔpI_{50} [b]
NO_2	NO_2	5.84 (**11**)	4.77 (**21**)	1.07
NO_2	H	4.88 (**13**)	3.96 (**22**)	0.92
NO_2	CH_3	4.97 (**14**)	4.51 (**23**)	0.46
NO_2	Cl	6.30 (**17**)	5.65 (**24**)	0.65
NO_2	Br	6.32 (**18**)	5.82 (**25**)	0.50
H	NO_2	3.46 (**1**)	3.13 (**26**)	0.33
Br	NO_2	4.89 (**6**)	3.85 (**27**)	1.04
CHO	NO_2	4.72 (**9**)	4.28 (**28**)	0.44
CN	NO_2	4.67 (**10**)	4.31 (**29**)	0.36
$CH=C(CN)_2$	NO_2	5.03 (**12**)	5.05 (**30**)	−0.02

[a] The figures in parentheses are compound number.
[b] $\Delta pI_{50} = pI_{50}$ (*sec*-butyl) − pI_{50} (*tert*-butyl).

Study on electrostatic potentials of the inhibitors

From Eqn. 1, the existence of hydrogen-bonding interaction between the hydrogen-bond-acceptable 4-substituent, such as nitro and methoxy groups, and some residue in the binding site was suggested. Cyano group (compound **10**), however, seemed not to form the hydrogen-bond. To explain this difference among hydrogen-bond acceptable 4-substituents, the electrostatic potentials around the 4-substituent were computed from the point charges using 4-methoxy, 4-cyano and 4-nitrophenols as a model compound of **7**, **10** and **11**, respectively. The potential contours are plotted in Fig. 2. Note that the site on the contour at about the van der Waals surface of the molecule that could be assumed to be the frontier of the contact with the hydrogen-bond donating residue in the binding domain. Distribution of the negative potentials in terms of the pattern of the contour map of cyano group (Fig. 2C) slightly differed from that of methoxy (Fig. 2A) and nitro (Fig. 2B) groups. The negative peak of methoxy or nitro group is shifted from the bond connecting the benzene-ring and the substituents, whereas that of cyano group is along the connecting-axis. Moreover, when the methoxy or nitro group twists, the distribution of negative potentials is significantly affected, suggesting that methoxy and nitro groups are able to closely adjust the electrostatic interaction to the hydrogen-bond donating residue by flexibly twisting. Contrarily, the distribution of negative potentials of cyano group is fixed in one direction, i.e., being independent on its twisting.

Discussion

Numerous van der Waals interactions seem to contribute to inhibitor binding to the natural quinone redox domain [24]. In the previous study [7], we showed that the configuration of the 2-alkyl substituent, which corresponds to a side-chain of ubiquinone, is the important factor deciding close fitting of nitrophenolic inhibitors to the ubiquinone redox domain (Q_o center)

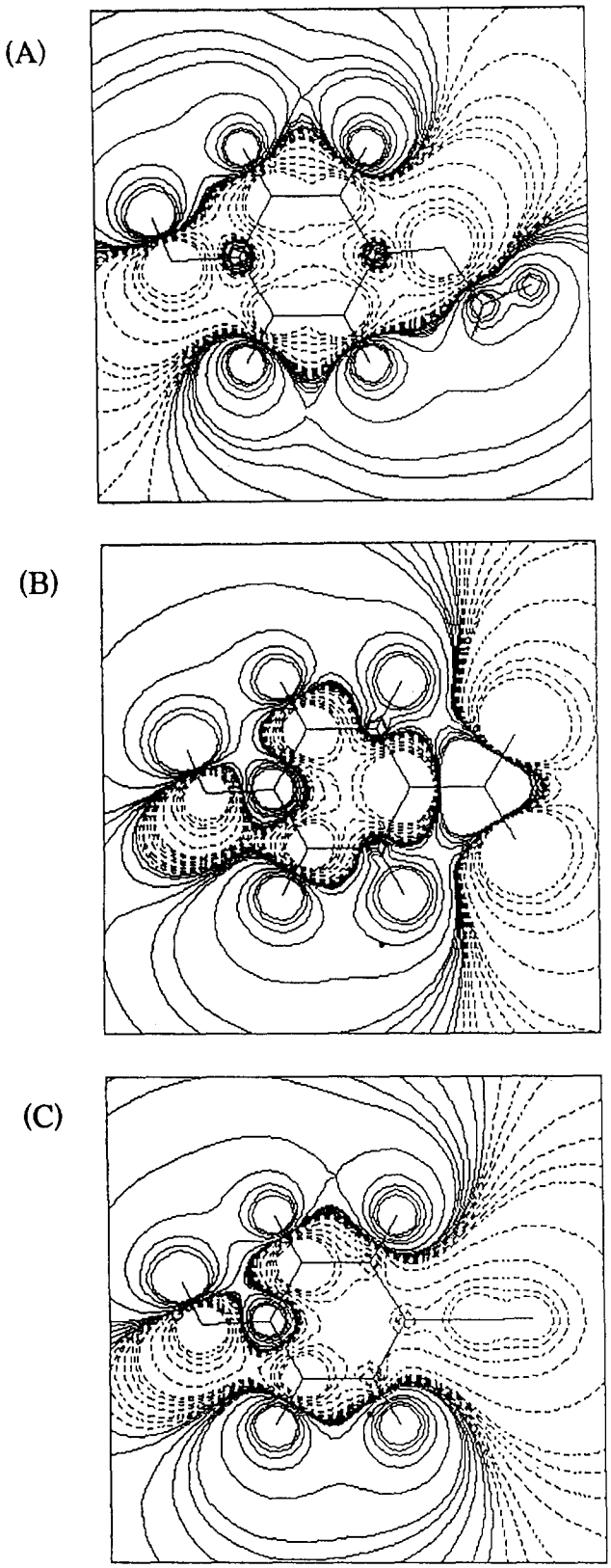


Fig. 2. Electrostatic potentials of 4-methoxy (A), 4-nitro (B), and 4-cyanophenols (C). (———) Positive contours; (— — —) negative contours. Contours are in units of kcal. mol^{-1}.

of cyt. bc_1 complex [7]. In contrast to this, any contribution of steric factors to the activity was not indicated for the 4- and 6-substituents. The greater the hydrophobicity and the electron-withdrawing ability of the substituents in 4- and 6-positions, the greater was the inhibitory activity. The hydrophobicity of the inhibitors may support hydrophobic interaction between the inhibitor molecule and the binding domain. The electron-withdrawing substituents, in general, increase the acidity of substituted phenols by lowering electron density on a oxygen atom of phenolic OH group. Considering this along with the fact that electron-withdrawing substituents were favorable to the activity, an active form of nitrophenols in the binding domain may be an ionized form. The phenolic O^- atom probably works as a hydrogen-bond acceptor. Based on the analogy between an anionic form of dinoseb (compound **11**) and semiquinone anion of plastoquinone, Gardner [25] suggested that the active form of dinoseb in Photosystem II (Q_B site) may be an anionic form. It is notable that the through-resonance effect of the 4-substituent, which stabilizes an anionic form owing to delocalization of a negative charge into the 4-substituent, was not significant in Eqn. 1. This result does not necessarily exclude the possibility of an anionic form being active form, rather suggesting that quinone form (i.e., extreme canonical form) of phenolate anion may not make an important contribution to the activity. Hydrogen-bonding interaction, anyhow, between the phenolic OH moiety and some residue in the binding domain certainly exists because replacement of the phenolic OH group of compound **11** by methoxy or chloride group resulted in a large loss of inhibitory activity to less than 1/200th the inhibition caused by compound **11** (data not shown). It is, however, not fully understood yet whether sole active form is an anionic form or not.

Eqn. 1 also indicated the existence of hydrogen-bonding interaction between the hydrogen-bond-acceptable 4-substituent and the binding domain. Natural quinones have been thought to occupy the quinone binding domains through two hydrogen-bond bridges with Photosystem II (Q_B site) [26], Q_o center of cyt. bc_1 complex of *Rhodobacter capsulatus* [3] and reaction center of *Rhodobacter sphaeroides* [27]. The present study also indicated that two hydrogen-bond bridges make an important contribution to the inhibitor binding to the ubiquinone redox domain. Since the loss of hydrogen-bonding interaction at phenolic OH moiety resulted in a drastic decrease in the inhibitory activity as observed with methoxy and chloride derivatives of compound **11**, this hydrogen-bond bridge should be appreciably important to anchor the inhibitor molecule in the binding domain. However, since the derivatives lacking hydrogen-bond-acceptable 4-substituent such as compounds **5** and **6** could remain a certain extent of

the activity, the hydrogen-bond bridge between the 4-substituent and the binding domain may be not a detecting factor, but somewhat reinforce inhibitor binding to the domain.

Irrespective of substituent patterns in the 4- and 6-positions, a *sec*-butyl group was more favorable to the activity as the 2-substituent than a *tert*-butyl group. In other words, ubiquinone redox domain recognizes the shape of the 2-substituent in the strict sense irrespective of substituent patterns. This kind of recognition, however, was lost when the 4-position was substituted by a bulky dicyanovinyl group. This suggests that the inhibitor possessing bulky 4-substituent might be crowded out from the ubiquinone binding cavity by steric congestion, resulting in a loss of close steric fitting of the 2-substituent to the binding site. This is supported by the fact that the observed activity of compound **12** was much less than that calculated by Eqn. 1 (see Table I).

It is of interest that although cyano group is hydrogen-bond acceptable, the contribution of hydrogen-bonding interaction to the activity of compound **10** was not indicated in contrast to other hydrogen-bond-acceptable substituents. As shown by electrostatic potentials, the orbitals of lone electron pair on the nitrogen atom of cyano group are fixed in the region where the rod-like cyano (C≡N) bond directs. Thus, electrostatic, complementary interaction with hydrogen-bond donor in the receptor cavity is thought to be best only in this region. On the other hand, the distribution of negative potentials of nitro and methoxy groups is variable toward the position of hydrogen-bond donor by twisting bond angle. Looking at these in another way, the amino acid residue working as a hydrogen-bond donor (probably histidine [3]) may not locate in the region where the rod-like cyano (C≡N) bond directs.

A fair number of information regarding the three-dimensional structure of ubiquinone redox site of cyt. bc_1 complex may be acquired hereafter by various procedures. The binding model of ubiquinone or inhibitor to the ubiquinone redox domain should be consistent with the information obtained from structure-inhibitory activity studies of various electron-transport inhibitors [7,8,28,29]. From the previous [7] and present studies, it is strongly suggested that the ubiquinone redox site of cyt. bc_1 complex may recognize the conformation of the side-chain near the quinol ring in the strict sense and that ubiquinol may be anchored by two hydrogen-bond bridges and hydrophobic interaction.

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