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Hybrid ubiquinone: novel inhibitor of mitochondrial complex I

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Abstract

We synthesized novel ubiquinone analogs by hybridizing the natural ubiquinone ring (2,3-dimethoxy-5-methyl-1,4-benzoquinone) and hydrophobic phenoxybenzamide unit, and named them hybrid ubiquinones (HUs). The HUs worked as electron transfer substrates with bovine heart mitochondrial succinate–ubiquinone oxidoreductase (complex II) and ubiquinol–cytochrome *c* oxidoreductase (complex III), but not with NADH–ubiquinone oxidoreductase (complex I). With complex I, they acted as inhibitors in a noncompetitive manner against exogenous short-chain ubiquinones irrespective of the presence of the natural ubiquinone ring. Elongation of the distance between the ubiquinone ring and the phenoxybenzamide unit did not recover the electron accepting activity. The structure/activity study showed that high structural specificity of the phenoxybenzamide moiety is required to act as a potent inhibitor of complex I. These findings indicate that binding of the HUs to complex I is mainly decided by some specific interaction of the phenoxybenzamide moiety with the enzyme. It is of interest that an analogous bulky and hydrophobic substructure can be commonly found in recently registered synthetic pesticides the action site of which is mitochondrial complex I.

Keywords: Mitochondrial complex I; Ubiquinone; Structure–activity relationship

1. Introduction

A large number of inhibitors of mitochondrial NADH–ubiquinone oxidoreductase (complex I) are known [1–3]. With the exception of rhein [4] and diphenyleneiodonium [5], which inhibit electron input into complex I, all inhibitors act at the terminal electron transfer step of this enzyme. Radioligand binding [6] and photoaffinity labeling [7] studies indicated that a wide variety of structurally different inhibitors act at a common binding domain in complex I. These findings suggest that the domain is a large cavity-like structure that enables occupation by a variety of inhibitors in a dissimilar manner depending on their structural specificity, in analogy with different types of Q_o center inhibitors of the cytochrome bc_1 complex (ubiquinol–cytochrome *c* oxidoreductase—complex III) [8]. Recent mutagenesis studies using the yeast *Yarrowia lipolytica* and *Rhodobacter capsulatus* suggested that both PSST and 49-kDa subunits contribute to the inhibitor binding domain and ligate iron–sulfur cluster N2 [9–11]. It should, however, be realized that there is still no hard experimental evidence to verify whether the inhibitors of the terminal electron transfer step indeed occupy the ubiquinone reduction site.

Some complex I inhibitors, such as piericidin A and capsaicin, are certainly analogous in chemical structure to the ubiquinone molecule. However, taking into consideration the fact that these inhibitors do not block all the ubiquinone redox sites of mitochondrial respiratory enzymes, one should not necessarily conclude that an inhibitor which is chemically analogous to ubiquinone occupies the ubiquinone reduction site of complex I. In this context, an inhibitor that possesses the natural ubiquinone ring itself (i.e., 2,3-dimethoxy-5-methyl-1,4-benzoquinone) could be useful to probe the structural and functional characteristics of the ubiquinone reduction site of the enzyme, although no such chemical has been described to date. In the present study, we synthesized novel ubiquinone analogs by hybridizing the natural ubiquinone ring and hydrophobic phenoxybenzamide unit (*N*-

Abbreviations: Complex I, NADH–ubiquinone oxidoreductase; Complex II, succinate–ubiquinone oxidoreductase; Complex III, ubiquinol–cytochrome *c* oxidoreductase; DB, 6-*n*-decyl-2,3-dimethoxy-5-methyl-1,4-benzoquinone; DBH_2, a reduced form of DB; HU, hybrid ubiquinone; HUH_2, a reduced form of HU; SMP, submitochondrial particles

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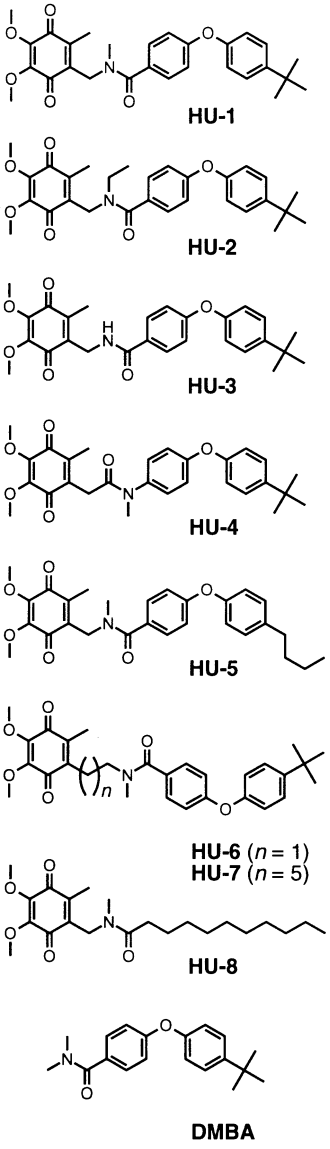

Fig. 1. Structures of HUs synthesized in this study.

methyl-4-(4-*tert*-butylphenoxy)benzamide), and named them hybrid ubiquinones (HUs, see Fig. 1). The phenoxybenzamide unit was chosen as a partner of the ubiquinone ring as this structural unit was expected to enhance the affinity of the ligand to complex I, as discussed later. The electron transfer and/or inhibitory efficiencies of HUs with bovine heart mitochondrial complexes I, II and III were investigated.

2. Materials and methods

2.1. Materials

MOA-stilbene was provided by Aburahi Laboratories, Shionogi (Shiga, Japan). Piericidin A was provided by Dr. Shigeo Yoshida (RIKEN, Japan).

2.2. Synthesis

2.2.1. Intermediates 1a, 1b and 1c

N-Methyl-4-bromobenzamide was prepared by reacting 4-bromobenzoyl chloride and methylamine hydrochloride in the presence of K_2CO_3 in a water/Et_2O mixture (1:1) at 0 °C (Fig. 2). Intermediate 1a was synthesized by reacting *N*-methyl-4-bromobenzamide and 4-*tert*-butylphenol in the presence of a catalytic amount of Cu at 160 °C for 3 h. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.33 (s, 9H), 3.00 (d, J = 4.8 Hz, 3H), 6.08 (br s, 1H), 6.96 (m, 4H), 7.37 (m, 2H), 7.71 (m, 2H) (Fig. 2).

Intermediates 1b (R = Et) and 1c (R = H) were prepared by the same procedures, except that ethylamine hydrochloride and ammonium chloride were used in place of methylamine hydrochloride, respectively. 1b: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.25 (t, J = 7.2 Hz, 3H), 1.33 (s, 9H), 3.49 (dq, J = 6.0, 7.2 Hz, 2H), 6.05 (br s, 1H), 6.96 (m, 4H), 7.37 (m, 2H), 7.72 (m, 2H). 1c: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.34 (s, 9H), 5.85 (br s, 1H), 6.98 (m, 4H), 7.38 (m, 2H), 7.77 (m, 2H).

2.2.2. Intermediates 2, 3 and 4

Intermediate 2 was synthesized as described previously [13]. Intermediate 3 was obtained by reacting intermediate 2 and NaCN in the presence of triethylbenzylammonium chloride (0.05 molar equiv.) in an acetone/water mixture (8:3) at 90 °C overnight. ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.26 (s, 3H), 3.67 (s, 2H), 3.79 (s, 3H), 3.90 (s, 3H), 3.92 (s, 3H), 3.95 (s, 3H). Intermediate 4 was prepared by the reduction of intermediate 3 with $LiAlH_4$ in dry THF at room temperature (r.t.). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.47 (br s, 2H), 2.18 (s, 3H), 2.80 (m, 4H), 3.78 (s, 3H), 3.83 (s, 3H), 3.89 (s, 3H), 3.92 (s, 3H).

2.2.3. HU-1, HU-2, HU-3, HU-5 and HU-8

To a solution of intermediate 1 (0.2 g, 0.71 mmol) in DMSO (7 ml) was added NaH (20 mg, 0.83 mmol), and the mixture was stirred at r.t. for 1 h. To the reaction mixture was added a solution of intermediate 2 (0.26 g, 0.85 mmol) in DMSO (1 ml) dropwise and stirred at r.t. for 1 h. The reaction mixture was extracted with ethyl acetate and washed with brine. The crude product was purified by silica gel column chromatography (ethyl acetate/hexane = 7:3) to give the precursor of HU-1 in an 84% yield. The precursor was oxidized by ceric ammonium nitrate (CAN) to give HU-1 as described previously [14]. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.33 (s, 9H), 2.19 (br s, 3H), 3.03 (br s, 3H), 4.00 (s, 3H), 4.03 (s, 3H), 4.55 (br s, 2H), 6.97 (m, 4H), 7.36 (m, 4H). ESIMS m/z 478 $[M+H]^+$.

HU-2 and HU-3 were synthesized by the same procedures, except that intermediates 1b and 1c were used, respectively, in place of intermediate 1a. HU-2: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.12 (t, J = 7.0 Hz, 3H), 1.33 (s, 9H), 2.18 (br s, 3H), 3.42 (q, J = 7.0 Hz, 2H), 4.00 (s, 3H), 4.02 (s, 3H), 4.53 (br s, 2H), 6.96 (m, 4H), 7.32 (m, 4H). ESIMS m/z 492 $[M+H]^+$. HU-3: ^{1}H NMR (300 MHz, $CDCl_3$) δ

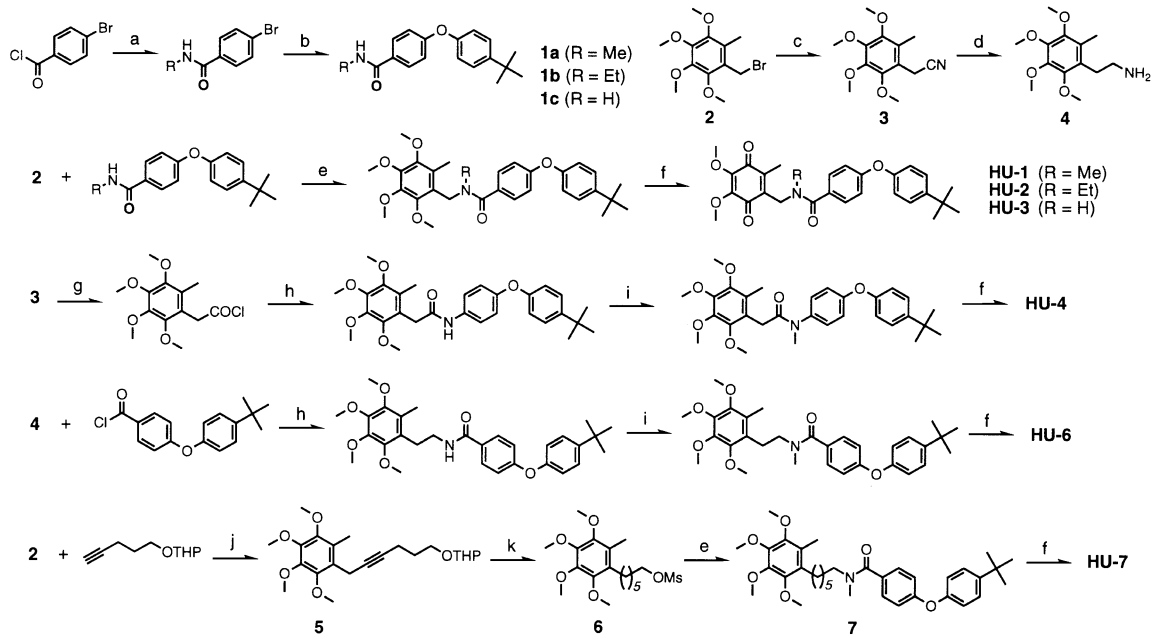

Fig. 2. Reaction conditions; (a) $CH_3NH_3^+Cl^-$, $C_2H_5NH_3^+Cl^-$, or $NH_4^+Cl^-$, K_2CO_3, H_2O-Et_2O; (b) *tert*-butylphenol, K_2CO_3, Cu; (c) NaCN, triethylbenzylammonium chloride, acetone–H_2O; (d) $LiAlH_4$, THF; (e) NaH, DMSO; (f) ceric ammonium nitrate, CH_3CN-H_2O; (g) (i) KOH, *n*-butanol–H_2O, (ii) $SOCl_2$, DMF, toluene; (h) Et_3N, CH_2Cl_2; (i) CH_3I, NaH, Et_3N, DMSO; (j) *n*-BuLi, CuI, HMPA, THF; (k) (i) *p*-toluenesulfonic acid, MeOH, (ii) H_2, Pd/C, (iii) MsCl, Et_3N, THF.

1.33 (s, 9H), 2.30 (s, 3H), 3.98 (s, 3H), 4.01 (s, 3H), 4.46 (d, $J=6.3$ Hz, 2H), 6.70 (br t, $J=6.3$ Hz, 1H), 6.96 (m, 4H), 7.37 (d, $J=8.7$ Hz, 2H), 7.69 (d, $J=8.7$ Hz, 2H). ESIMS *m/z* 486 $[M+Na]^+$.

HU-5 was synthesized by the same procedures, except that *N*-methyl-4-(4-*n*-butylphenoxy)benzamide was used in place of *N*-methyl-4-(4-*tert*-butylphenoxy)benzamide. ^1H NMR (300 MHz, $CDCl_3$) δ 0.94 (t, $J=7.3$ Hz, 3H), 1.36 (sext, $J=7.2$ Hz, 2H), 1.60 (m, 2H), 2.19 (br s, 1H), 2.60 (t, $J=15$ Hz, 2H), 3.03 (br s, 3H), 4.00 (s, 3H), 4.03 (s, 3H), 4.55 (br s, 2H), 6.95 (m, 4H), 7.15 (m, 2H), 7.37 (m, 2H). ESIMS *m/z* 478 $[M+H]^+$.

HU-8 was synthesized by the same procedures, except that *N*-methylundecanamide was used in place of intermediate 1a. ^1H NMR (300 MHz, $CDCl_3$) δ 0.88 (t, $J=6.5$ Hz, 3H), 1.22–1.38 (m, 14H), 1.58 (m, 2H), 2.15 (s, 3H), 2.27 (t, $J=7.5$ Hz, 2H), 3.02 (s, 3H), 3.98 (s, 3H), 4.01 (s, 3H), 4.40 (s, 2H). ESIMS *m/z* 416 $[M+Na]^+$.

2.2.4. HU-4

Intermediate 3 was driven to acid chloride by hydrolysis with KOH in 1-butanol/water (1:1) followed by $SOCl_2$ treatment in toluene at 70 °C. To a solution of 4-(4-*tert*-butylphenoxy)aniline (0.92 g, 3.8 mmol) and triethylamine (0.93 g, 9.2 mmol) in dry CH_2Cl_2 was added a solution of crude 2,3,4,5-tetramethoxy-6-methylphenylacetyl chloride in CH_2Cl_2, and the reaction mixture was stirred at r.t. for 10 h. After working up the reaction mixture, the crude product was purified by silica gel column chromatography (ethyl acetate/hexane = 3:7) to give *N*-4-(4-*tert*-butylphenoxy)phenyl-2-(2,3,4,5-tetramethoxy-6-methyl)phenylethanamide in an 87% yield. *N*-Methylation of this product using CH_3I and NaH, and sequential oxidation by CAN afforded HU-4 in a 65% yield. ^1H NMR (300 MHz, $CDCl_3$) δ 1.33 (s, 9H), 1.98 (br s, 3H), 3.25 (br s, 3H), 3.27 (br s, 2H), 3.97 (s, 3H), 3.99 (s, 3H), 6.99 (m, 4H), 7.28 (m, 2H), 7.38 (m, 2H). ESIMS *m/z* 500 $[M+Na]^+$.

2.2.5. HU-6

To a solution of intermediate 4 (0.14 g, 0.55 mmol) and triethylamine (0.11 g, 1.1 mmol) in 20 ml of CH_2Cl_2 was added a solution of 4-(4-*tert*-butylphenoxy)benzoylchloride (0.16 g, 0.55 mmol) in CH_2Cl_2 (2 ml) dropwise at 0 °C and the mixture was stirred at r.t. overnight. The reaction mixture was extracted with ethyl acetate and washed with 1 N HCl, saturated sodium bicarbonate solution and brine. The crude product was purified by silica gel column chromatography (ethyl acetate/hexane = 1:1) to give the amide intermediate in a 60% yield. *N*-Methylation and sequential oxidation of this amide by the procedures described above afforded HU-6 in a 45% yield for two steps. ^1H NMR (300 MHz, $CDCl_3$) δ 1.33 (s, 9H), 1.76 (br s, 1H), 2.17 (br s, 2H), 2.69 (br s, 0.6 H), 2.86 (br s, 1.4 H), 3.08 (br s, 3H), 3.43 (br s, 0.6 H), 3.58 (br s, 1.4 H), 3.99 (s, 6H), 6.96 (m, 4H), 7.36 (m, 4H). ESIMS *m/z* 514 $[M+Na]^+$.

2.2.6. HU-7

To a mixture of tetrahydro-2-(4-pentynyloxy)-2*H*-pyrane (0.94 g, 5.6 mmol) and CuI (1.06 g, 5.6 mmol) in dry THF (7 ml) was added 4.5 ml of *n*-BuLi (1.50 M, 6.7 mmol) dropwise at −78 °C and stirred for 20 min. To the mixture was added 1.2 ml of hexamethylphosphoric triamide and

stirred for 30 min at −78 °C. A solution of intermediate 2 (1.14 g, 3.73 mmol) in 10 ml of dry THF was added to the mixture dropwise, and the mixture was allowed to warm to r.t. over a period of 1 h. The reaction mixture was quenched with saturated ammonium chloride solution, extracted with Et_2O and washed with brine. The crude product was purified by silica gel column chromatography (ethyl acetate/hexane = 15:85) to give intermediate 5 in a 58% yield. Deprotection of the THP group was achieved by *p*-toluenesulfonic acid in MeOH. The alcohol was hydrogenated with 10% palladium on carbon in EtOH under hydrogen gas. The resulting saturated alcohol was mesylated in the presence of triethylamine in dry THF to afford intermediate 6. Amide formation, *N*-methylation, and sequential oxidation were carried out by the procedures described for HU-1 to afford HU-7. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.33 (s, 9H), 1.25–1.70 (m, 8H), 2.00 (br s, 3H), 2.44 (br s, 2H), 3.02 (br s, 3H), 3.28 (br s, 1H), 3.50 (br s, 1H), 3.99 (s, 6H), 6.97 (m, 4H), 7.36 (m, 4H). ESIMS *m*/*z* 548 $[M+H]^+$.

2.3. Electron transfer activity in submitochondrial particles

Bovine heart submitochondrial particles (SMP) were prepared by the method of Matsuno-Yagi and Hatefi [12] using a sonication medium containing 0.25 M sucrose, 1 mM succinate, 1.5 mM ATP, 10 mM $MgCl_2$, 10 mM $MnCl_2$ and 10 mM Tris–HCl (pH 7.4), and stored in a buffer containing 0.25 M sucrose and 10 mM Tris–HCl (pH 7.4) at −82 °C.

The NADH–HU oxidoreductase activity in SMP was determined following NADH oxidation at 30 °C with a Shimadzu UV-3000 (340 nm, $\varepsilon = 6.2\ mM^{-1}\ cm^{-1}$). The reaction medium (2.5 ml) contained 0.25 M sucrose, 1 mM $MgCl_2$, 0.2 μM antimycin A, 0.2 μM MOA-stilbene and 2 mM KCN and 50 mM phosphate buffer (pH 7.4). The final mitochondrial protein concentration was 30 μg of protein/ml. After equilibration of SMP with the HU, the reaction was started by the addition of 50 μM NADH.

The inhibition of NADH–decyl ubiquinone (DB) oxidoreductase activity by HUs was determined under the same experimental conditions, except that the 50 μM DB was used as a substrate. The reaction was started by adding 50 μM NADH after equilibration of SMP with HU for 5 min.

The succinate–HU oxidoreductase activity was coupled to the reduction of DCIP ($\varepsilon = 21\ mM^{-1}\ cm^{-1}$), and the rate was followed spectrophotometrically at 600 nm and 30 °C. The reaction medium contained 0.25 M sucrose, 20 mM succinate, 2.0 mM KCN, 1.0 mM $MgCl_2$, 1 μM rotenone, 0.1 μM antimycin A, 50 μM DCIP, and 50 mM phosphate buffer (pH 7.4). The final mitochondrial protein concentration was 30 μg of protein/ml. The effects of HUs on succinate–Q_2 oxidoreductase activity was examined under the same experimental conditions, except that 50 μM Q_2 was used as a substrate.

The HUH_2–cytochrome *c* oxidoreductase activity was measured at 30 °C as the rate of cytochrome *c* reduction with the wavelength pair 550–540 nm ($\varepsilon = 19\ mM^{-1}\ cm^{-1}$). SMP were treated with sodium deoxycholate (0.2 mg/mg of protein) before dilution with reaction medium. The reaction medium contained 0.25 M sucrose, 2.0 mM KCN, 1.0 mM $MgCl_2$, 1 μM rotenone, 50 μM cytochrome *c*, and 50 mM phosphate buffer (pH 7.4). The reaction was started by adding HUH_2. The final mitochondrial protein concentration was 30 μg of protein/ml. The effects of HUH_2s on Q_2H_2–cytochrome *c* oxidoreductase activity was examined under the same experimental conditions, except that 50 μM Q_2H_2 was used as a substrate.

3. Results

3.1. Design concept of HUs

The design strategy for the HU is illustrated in Fig. 3. The *N*-methyl-4-(4-*tert*-butylphenoxy)benzamide unit was chosen as a partner for the ubiquinone ring since this structural unit markedly enhances the affinity of synthetic capsaicin derivatives to bovine heart mitochondrial complex I [6,15], as shown such a potent capsaicin analog as an example. If the phenoxybenzamide unit, in general, supports

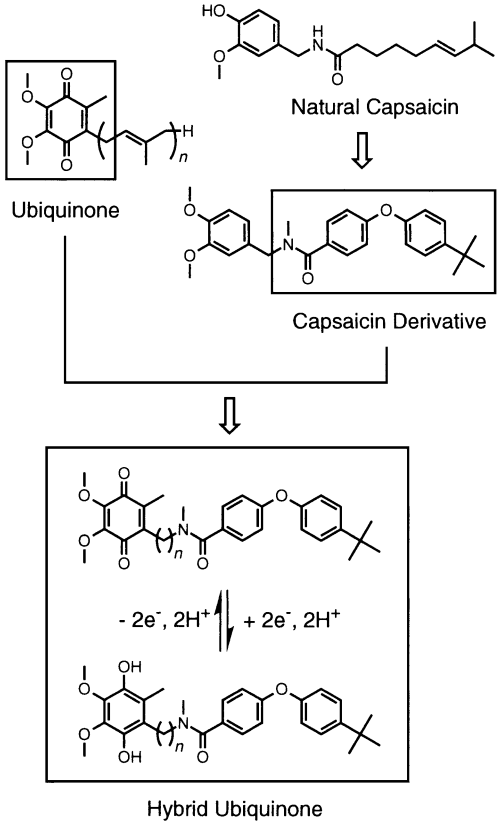

Fig. 3. Design concept of HUs. The synthetic capsaicin possessing the *N*-methyl-4-(4-*tert*-butylphenoxy)benzamide unit has a much higher affinity to complex I than natural capsaicin. The isoprenyl tail of ubiquinone was replaced by the *N*-methyl-4-(4-*tert*-butylphenoxy)benzamide unit which is expected to enhance the affinity of the ligand to complex I.

tight binding of the ligand to complex I regardless of the remaining structure, HU is expected to bind to the enzyme with high affinity.

We synthesized eight HUs shown in Fig. 1. HU-1 was prepared as a lead compound, wherein the ubiquinone ring and *N*-methyl-4-(4-*tert*-butylphenoxy)benzamide unit were directly linked. To examine the function of the *N*-Me amide group, HU-2, HU-3 and HU-4 were synthesized. HU-5 possessing an *n*-butyl group in place of the *tert*-butyl group was prepared to examine the effect of bulkiness of the substituent attached to the benzene ring. To vary the distance between the ubiquinone ring and *N*-methyl-4-(4-*tert*-butylphenoxy)benzamide moiety, HU-6 and HU-7 were synthesized. In addition, to know the effect of hydrophobic phenoxyphenyl moiety, HU-8 possessing a long alkyl chain was prepared.

3.2. Effects of HUs on complex I activity

The electron accepting efficiency of HUs was examined by NADH–HU oxidoreductase assay with complex I in SMP (Table 1). No HUs, except HU-8, elicited any electron accepting activity at all, indicating that the hydrophobic phenoxyphenyl group is quite unfavorable for electron accepting activity. HU-8, which carries a long alkyl chain in place of the phenoxyphenyl group, worked as a substrate, although the electron accepting activity in terms of the V_{max} value was about one-third that of DB. Considering that HU-8 possesses a long alkyl tail as does DB, the presence of a polar amide group in the tail is not preferable for electron accepting activity. This result is consistent with our previous finding that the role of the alkyl tail of short chain ubiquinones is not only to enhance the hydrophobicity of the ubiquinone molecule, but to contribute to some specific interaction with complex I [13]. The electron accepting activity of HU-8 was almost completely (>95%) inhibited by 0.1 μM piericidin A.

Table 1
Summary of electron transfer and inhibitory activities of the hybrid ubiquinones

	IC_{50} (μM) for complex I[a]		Electron transfer activity			
	Oxidized form	Reduced form	Complex II		Complex III	
			K_m (μM)	V_{max} (μmol/min/mg of protein)	K_m (μM)	V_{max} (μmol/min/mg of protein)
HU-1	0.077	0.088	0.36	0.028	–	26%[b]
HU-2	0.37	0.63	0.90	0.023	4.0	0.50
HU-3	0.34	0.53	–	<5%[c]	2.1	0.91
HU-4	1.8	2.0	1.2	0.010	9.4	0.64
HU-5	0.97	0.84	0.76	0.017	3.8	1.0
HU-6	0.47	0.76	1.3	0.049	–	<5%[b]
HU-7	1.1	0.61	2.0	0.011	5.5	1.1
HU-8[d]	11.8	6.2	33	0.028	–	19%[b]
DMBA	0.65					
Piericidin A	0.003					
Q_2 (or Q_2H_2)			0.36	0.11	1.6	5.2

[a] The inhibitory potency in terms of the IC_{50}, which is the molar concentration needed to halve the control enzyme activity, was obtained from two independent experiments.

[b] The kinetic parameters could not be accurately estimated due to substrate inhibition at high concentration range. The maximum reaction rate normalized by the V_{max} value of Q_2H_2 is listed.

[c] The kinetic parameters could not be accurately estimated due to very poor activity. The maximum reaction rate normalized by the V_{max} value of Q_2 is listed.

[d] The electron accepting activity of HU-8 for complex I in terms of the V_{max} value is about one-third that of DB.

Although HU-8 served as an electron acceptor, both the oxidized and the reduced forms of this compound showed, at the same time, apparent inhibition of NADH–DB oxidoreductase activity at high concentrations (Table 1). This apparent inhibition by the oxidized and the reduced forms is likely due to a low turnover number and product inhibition, respectively, as is the case for the inhibition of NADH–Q_1 oxidoreductase activity by Q_2 and Q_2H_2 [13,14,17].

To elucidate whether HU-1 to HU-7 act as inhibitors of complex I, their effect on NADH–DB oxidoreductase activity was examined. Inhibitory potencies in terms of the IC_{50} value, which is the molar concentration needed to halve the control enzyme activity, are listed in Table 1. The inhibition of complex I activity by HUs at sufficiently high concentrations took place immediately as is the case of ordinary complex I inhibitors. Among 4-(4-*tert*-butylphenoxy)benzamide derivatives (HU-1 to HU-3), the N–Me derivative (HU-1) was the most potent inhibitor. The order of N–Me and C═O (–NMeCO– or –CONMe–) appeared to significantly affect the activity (HU-1 vs. HU-4). Replacement of the *tert*-butyl group by the *n*-butyl group resulted in a marked decrease in inhibitory potency, regardless of the same number of carbon atoms (HU-1 vs. HU-5). Furthermore, elongation of the distance between the ubiquinone ring and the phenoxybenzamide moiety also significantly diminished the inhibitory activity (HU-1 vs. HU-6 and HU-7). Thus, it is obvious that when HU-1 binds to complex I, the *N*-methyl-4-(4-*tert*-butylphenoxy)benzamide unit and its position relative to the ubiquinone ring are strictly recognized by the enzyme.

Next, we examined the inhibitory effect of the reduced form of HUs on NADH–DB oxidoreductase activity (Table 1). The reduced form was prepared by the ordinary method of Rieske [16]. The structure/activity profiles for the reduced form were comparable to those for the oxidized form, indicating that the redox form of the ubiquinone ring has no or, at least, only a slight effect on the inhibitory action of HUs. This result also indicates that the inhibitory action of HUs is mainly decided by the phenoxybenzamide moiety rather than the ubiquinone ring. In support of this idea, solely *N*,*N*-dimethyl-4-(4-*tert*-butylphenoxy)benzamide (DMBA in Fig. 1) elicited inhibition, though its potency was less than that of HU-1.

3.3. Effects of HUs on complexes II and III activities

The electron accepting efficiencies of HUs were examined with succinate–ubiquinone oxidoreductase (complex II) in SMP (Table 1). All HUs worked as electron acceptors, though the efficiency varied widely depending upon structure. The kinetic parameters of HU-3 could not be accurately estimated due to very poor activity. The K_m value of HU-8 was markedly larger than that of the phenoxybenzamide derivatives. Some HUs showed slight inhibition of succinate–Q_2 oxidoreductase activity at high concentration range due to competition for the Q_2 binding site, as reported for other ubiquinone analogs being poor electron acceptors [13,14,18].

The electron transfer activities of HUs were also examined with complex III in SMP using the reduced form of HUs (Table 1). In this assay, electron donating activity is mainly evaluated since the ubiquinol oxidation at the Q_o site is a rate-limiting step in the overall reaction of complex III [19]. All HUs elicited electron donating activity, though substrate inhibition was observed at high concentration range for HU-1, HU-6 and HU-8. The reduced form of some HUs showed slight inhibition of Q_2H_2–cytochrome *c* oxidoreductase activity at high concentration range due to competition for the Q_2H_2 binding site, as is the case for complex II assay.

4. Discussion

The HUs, except HU-8, worked as specific inhibitors solely with complex I. HU-1 is the most potent inhibitor among the HUs synthesized in this study. Structural modification of the *N*-methyl-4-(4-*tert*-butylphenoxy)benzamide unit of HU-1 markedly decreased the inhibitory potency, regardless of the redox form of the ubiquinone ring. Expectedly, this structural unit appears to enhance the binding affinity of the ligand to complex I. Elongation of the distance between the *N*-methyl-4-(4-*tert*-butylphenoxy)benzamide unit and the ubiquinone ring up to six methylene units (HU-7) did not recover the electron accepting activity. It is therefore obvious that irrespective of the presence of the natural ubiquinone ring in the molecule, the binding of HU-1 to complex I is mainly decided by some specific interaction of the *N*-methyl-4-(4-*tert*-butylphenoxy)benzamide unit with the enzyme. However, this does not necessarily exclude a contribution by the ubiquinone ring to the inhibitor binding to the enzyme. The ubiquinone ring, particularly the two carbonyl groups, would behave like the polar substructure which is commonly found in many complex I inhibitors such as the heterocyclic ring of piericidin A and synthetic pesticides shown in Fig. 4.

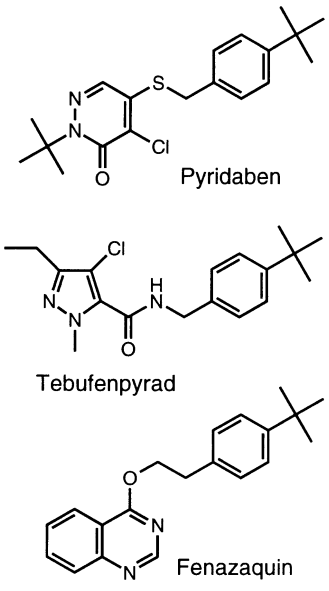

Fig. 4. Structure of Pyridaben (Nissan Chemical Industries), Tebfenpyrad (Mitsubishi Chemical), and Fenazaquin (Dow Elanco).

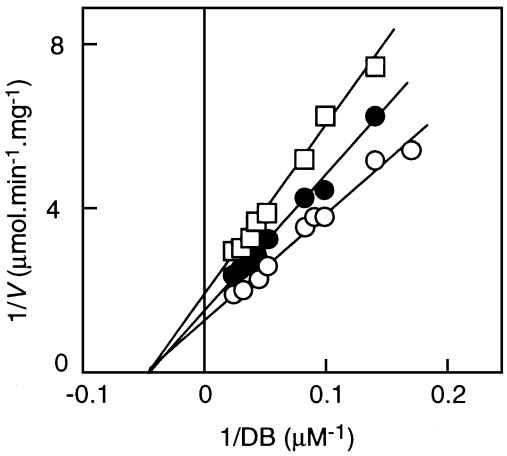

Fig. 5. Lineweaver–Burk plot of NADH–DB oxidoreductase activity in the presence of 0 M (○), 9.6 nM (●), or 19 nM (□) of HU-1.

The above argument leads to the question of whether the natural ubiquinone ring of HU-1 to HU-7 binds to the physiological ubiquinone reduction site of complex I. If the ubiquinone ring occupies the substrate site, at least one of the following two phenomena would be observed. Firstly, HUs would show electron accepting activity at least to a certain extent, as observed for complexes II and III; and secondly, competitive inhibition pattern of HUs against an exogenous ubiquinone would be observed, in analogy of the case of another series of ubiquinone analogs being poor electron acceptors [13,14]. Regarding the first phenomenon, this is not the case as described in the results. To elucidate the second, we examined the inhibition pattern of HUs by Lineweaver–Burk plots of the kinetic data of NADH–DB (or Q_1) oxidoreductase activity in the presence of HU-1 or HU-6. The inhibition pattern of both HUs was noncompetitive against both exogenous ubiquinones, as

shown in Fig. 5 taking the pair of DB and HU-1 as an example. It should be mentioned that the inhibition pattern of HU-8, a poor electron acceptor, was competitive against both exogenous ubiquinones (data not shown). These results along with the above structure/activity study strongly suggest that the binding site of HUs carrying the phenoxybenzamide moiety does not overlap with the ubiquinone reduction site.

On the other hand, as our assays for complex I activity were performed under steady-state conditions, we cannot entirely rule out the possibility that the ubiquinone part of HUs accepts two electrons from the ubiquinone reduction site in complex I without further electron transfer event. However, we consider this option as unlikely since it seems difficult to suppose that the reduced form of HUs does not leave the ubiquinone reduction site.

Complex I inhibitors are expected to hold important positions in most modern synthetic insecticides and acaricides [20,21]. In fact, a number of chemical companies have recently announced new complex I inhibitors meeting the many standards required for safe agrochemicals [3]. The present and a previous [15] study showed that the *N*-methyl-4-(4-*tert*-butylphenoxy)benzamide unit generally enhances the binding affinity of the ligand to complex I. It is of interest that a bulky and hydrophobic substructure analogous to HUs (i.e., *tert*-butylbenzene) can be commonly found in recently registered synthetic pesticides, as shown in Fig. 4. These chemicals are also potent inhibitors of bovine complex I [3,6,7]. Thus, a bulky and hydrophobic substructure is desirable in an inhibitor acting at the terminal electron transfer step of complex I. This information is helpful for designing novel inhibitors of the enzyme.

References

[1] T. Friedrich, P. Van Heek, H. Leif, T. Ohnishi, E. Forche, B. Kunze, R. Jansen, W. Trowitzsch-Kienast, G. Höfle, H. Reichenbach, H. Weiss, Eur. J. Biochem. 219 (1994) 691–698.
[2] M. Degli Esposti, Biochim. Biophys. Acta 1364 (1998) 222–235.
[3] H. Miyoshi, Biochim. Biophys. Acta 1364 (1998) 236–244.
[4] E.A. Kean, M. Gutman, T.P. Singer, J. Biol. Chem. 246 (1971) 2346–2353.
[5] A. Majander, M. Finel, M. Wikström, J. Biol. Chem. 269 (1994) 21037–21042.
[6] J.G. Okun, P. Lümmen, U. Brandt, J. Biol. Chem. 274 (1999) 2625–2630.
[7] F. Schuler, T. Yano, S.D. Bernardo, T. Yagi, V. Yankovskaya, T.P. Singer, J.E. Casida, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 4149–4153.
[8] H. Kim, D. Xia, C.A. Yu, J.Z. Xia, A.M. Kachurin, L. Zhang, L. Yu, J. Deisenhofer, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 8026–8033.
[9] P.M. Ahlers, K. Zwicker, S. Kerscher, U. Brandt, J. Biol. Chem. 275 (2000) 23577–23582.
[10] N. Kashani-Poor, K. Zwicker, S. Kerscher, U. Brandt, J. Biol. Chem. 276 (2001) 24082–24087.
[11] I. Prieur, J. Lunardi, A. Dupuis, Biochim. Biophys. Acta 1504 (2001) 173–178.
[12] A. Matsuno-Yagi, Y. Hatefi, J. Biol. Chem. 260 (1985) 14424–14427.
[13] K. Sakamoto, H. Miyoshi, M. Ohshima, K. Kuwabara, K. Kano, T. Akagi, T. Mogi, H. Iwamura, Biochemistry 37 (1998) 15106–15113.
[14] M. Ohshima, H. Miyoshi, K. Sakamoto, K. Takegami, J. Iwata, K. Kuwabara, H. Iwamura, T. Yagi, Biochemistry 37 (1998) 6436–6445.
[15] T. Satoh, H. Miyoshi, K. Sakamoto, H. Iwamura, Biochim. Biophys. Acta 1273 (1996) 21–30.
[16] J.S. Rieske, Methods Enzymol. 10 (1967) 239–245.
[17] R. Fato, E. Estornell, S.D. Bernardo, F. Pallotti, G.P. Castelli, G. Lanaz, Biochemistry 35 (1996) 2705–2716.
[18] D.Y. He, L.Q. Gu, L. Yu, C.A. Yu, Biochemistry 33 (1994) 880–884.
[19] A.R. Croft, Z. Wang, Photosynth. Res. 22 (1989) 69–87.
[20] H. Miyoshi, J. Bioenerg. Biomembranes 33 (2001) 223–231.
[21] T.R. Perrior, Chem. Ind. (Lond.) (1993) 883–887.