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Structural factors of rotenone required for inhibition of various NADH-ubiquinone oxidoreductases

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Abstract

We performed a structure-activity study of a series of synthetic rotenone analogues to elucidate the structural factors of rotenone required for inhibition and to probe the structural properties of the rotenone binding site of various NADH-ubiquinone oxidoreductases (NDH), including both proton-pumping (NDH-1) and non-proton-pumping (NDH-2) enzymes, from bovine heart mitochondria, potato tuber (*Solanum tuberosum* L.) mitochondria and *Escherichia coli* (GR 19N) plasma membranes. Using a benzyloxy group as a substitute for the E-ring moiety of natural rotenone, systematically selected structural modifications of the A-ring became feasible. The inhibitory potency of bovine NDH markedly varied depending upon structural modifications of the A-ring. The native chemical structure (2,3-dimethoxy substitution) appeared to be the most favorable for the activity. The spatial location of the hydrogen-bond acceptable methoxy oxygens may be important for tight fitting into the binding site. However, replacing one of the two methoxy groups by an ethoxy group almost completely retained the activity, indicating that the binding environment of the A-ring moiety is spacious enough to accommodate a substituent larger than the methoxy group. The manner of action of the derivative lacking the 12-C = O group in the C-ring differed from that of natural rotenone, indicating that this functional group is important for supporting the inhibitory action of natural rotenone itself. Regarding potato tuber and *E. coli* NDH-1, the sensitivity of the two enzymes to the inhibition by rotenone analogues was much lower than that of the bovine enzyme. The 2,3-dimethoxy substitution was the most favorable for the activity with potato NDH-1, whereas this substitution pattern was not necessarily the best with *E. coli* NDH-1. A rule governing inhibitory potency depending upon structural modifications was ambiguous for the two enzymes because of a small variation in the inhibitory potencies. These findings indicated that the local binding environment of the A-ring moiety of rotenone in bovine NDH is specific and differs considerably from that in potato and *E. coli* NDH-1.

Keywords: NADH-ubiquinone oxidoreductase; Rotenone; Mitochondrial respiratory chain; Structure–activity relationship

1. Introduction

Mitochondrial NADH-ubiquinone oxidoreductase (NDH) is a large enzyme that catalyzes the oxidation of NADH by ubiquinone coupled to proton translocation across the inner membrane. Bovine heart NDH consists of over 40 different subunits, noncovalently bound flavin mononucleotide (FMN) and at least four distinct electron paramagnetic resonance (EPR)-detectable iron-sulfur clusters [1–4]. This enormous complexity has hampered progress in studies into both the pathway of electron flow and the mechanism of proton pumping. To characterize the structural and mechanistic features of the ubiquinone catalytic site, specific inhibitors acting at the quinone catalytic site should be effective probes. There are a number of potent inhibitors that act at (or close to) this site [5,6]. Among them, commercially available rotenone is the most widely applied. However, despite the important role of rotenone as a useful inhibitor of this enzyme, the mode of inhibitory action of this inhibitor remained to be determined. For instance, studies on the site and the stoichiometry of rotenone binding have yielded puzzling results [7–9].

Studies of the structural requirements for rotenone in the inhibitory action are important for elucidating the structural properties of the rotenone binding site and the inhibitory mechanism. For this purpose, a structure-activity

Abbreviations: NDH, NADH-ubiquinone oxidoreductase; NDH-1, NDH that bears an energy coupling site; NDH-2, NDH that lacks an energy coupling site; PB, 2,3-dimethoxy-5-methyl-6-*n*-pentyl-1,4-benzoquinone; SMP, submitochondrial particles.

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study of a structurally systematic set of rotenone analogues is useful, as shown for other types of electron-transfer inhibitors [10–13]. Burgos and Redfearn [14] have shown that the bent form of rotenone at the face of contact between the B and C rings (i.e., [6aβ, 12aβ] configuration, Fig. 1) is very important for inhibiting the NDH activity of mammalian mitochondria. Using highly purified rotenone stereoisomers (5′α- and 5′β-epirotenones), we confirmed this notion and further showed that the significance of the stereochemical properties varies with NDH from different biological sources [15].

Besides the stereochemical properties of rotenone, the structural factors required for the inhibitory action remain ambiguous since the structural variations of rotenone analogues in the structure-activity studies performed so far [14,16,17] are limited. In particular, substituent effects in the A-ring on the inhibition are entirely unknown, since derivatives that possess different substitution patterns in the A-ring have not been investigated. This is primarily due to the fact that almost all rotenone derivatives used in the earlier studies were natural rotenoids and their transformation products. Therefore, a study using a systematic set of synthetic rotenones is required. However, so long as the portion of the rotenone corresponding to the E-ring is fixed as the native chemical structure, wide structural modifications of the A-ring moiety might be not easy to achieve, because of difficulties involved in the stereospecific synthesis of this moiety (*R* configuration at the 5′-position) [18]. To overcome this obstacle, the E-ring and the attached isopropenyl group should be replaced by the proper mimetic structure which can be synthesized by a simpler route.

In this study, we found that a benzyloxy group can functionally substitute for the E-ring moiety of natural rotenone. By replacing the E-ring moiety with this substructure, various modifications of the substitution pattern in the A-ring became feasible. To examine the structural factors required for the inhibition and to probe the structural properties of the rotenone binding site, we compared the structure-activity profile of the rotenone analogues among various NDH from bovine heart mitochondria, potato tuber mitochondria and *E. coli* plasma membranes.

2. Experimental procedures

2.1. Materials

Antimycin A and deamino-NADH (reduced nicotinamide hypoxanthine dinucleotide) were purchased from Sigma. 2,3-Dimethoxy-5-methyl-6-*n*-pentyl-1,4-benzoquinone (PB) was prepared as described [15]. Rotenol, dihydrorotenone and dihydrodeguelin were kindly provided by Dr. H. Fukami (Kyoto University). These compounds were purified before use by silica-gel column chromatography and recrystalization from ethanol. Other reagents were of the purest grade commercially available.

2.2. Synthesis

Rotenone analogues (compounds 1–12, Fig. 1) were synthesized based on the procedures for the synthesis of (±)-9-demethylmundeserone reported by Omokawa et al. [19]. All synthetic compounds were characterized by ^{1}H NMR (Bruker ARX-300), mass spectrometry (JEOL JMS-DX300) and elemental analyses for C and H, within an error of ±0.3%.

Compounds 1, 2, 3 and 4 were prepared starting from commercially available 3,4-dimethoxybenzaldehyde, as shown in Scheme 1. In step *c*, 4-methoxy-2-hydroxybenzaldehyde, 4-ethoxy-2-hydroxybenzaldehyde, 4-benzyloxy-2-hydroxybenzaldehyde and 4-(4′-*tert*-butyl)benzyloxy-2-hydroxybenzaldehyde were used to prepare compounds 1, 2, 3 and 4, respectively. These substituted benzaldehydes were obtained by reacting 2,4-dihydroxybenzaldehyde and corresponding commercially available alkylchlorides in the presence of K_2CO_3 and KI in acetone under reflux for 7 h. Compound 1, ^{1}H NMR ($CDCl_3$) δ 3.76 (s, 3H, OCH_3), 3.80 (s, 3H, OCH_3), 3.80 (s, 3H, OCH_3), 3.81 (d, $J = 4.0$ Hz, 1H, 12a-H), 4.19 (d, $J = 12.0$ Hz, 1H, 6-H), 4.62 (dd, $J = 12.0$, 3.1 Hz, 1H, 6-H), 4.94 (m, 1H, 6a-H). Compound 2, ^{1}H NMR ($CDCl_3$) δ 1.40 (t, $J = 7.0$ Hz, 3H, $OCH_2\mathbf{CH_3}$), 3.76 (s, 3H, OCH_3), 3.80 (s, 3H, OCH_3), 3.84 (d, $J = 4.1$ Hz, 1H, 12a-H), 4.02 (q, $J = 7.0$, 3H, $\mathbf{OCH_2}CH_3$), 4.18 (d, $J = 12.0$ Hz, 1H, 6-H), 4.62 (dd, $J = 12.0$, 3.2 Hz, 1H, 6-H), 4.94 (t, $J = 3.1$ Hz, 1H, 6a-H). Compound 3, ^{1}H NMR ($CDCl_3$) δ 3.74 (s, 3H, OCH_3), 3.81 (s, 3H, OCH_3), 3.87 (d, $J = 6.2$ Hz, 1H, 12a-H), 4.18 (d, $J = 11.8$ Hz, 1H, 6-H), 4.62 (dd, $J = 11.0$, 3.0 Hz, 1H, 6-H), 4.95 (t, $J = 2.6$ Hz, 1H, 6a-H) 5.06 (s, 2H, CH_2Ph). EI-MS, m/e 418 (M^+). Compound 4, ^{1}H NMR ($CDCl_3$) δ 1.31 (s, 9H, $C(CH_3)_3$), 3.75 (s, 3H, OCH_3), 3.79 (s, 3H, OCH_3), 3.83 (d, $J = 3.6$ Hz, 1H, 12a-H), 4.16 (d, $J = 12.0$ Hz, 1H, 6-H), 4.60 (dd, $J = 12.0$, 2.7 Hz, 1H, 6-H), 4.91 (m, 1H, 6a-H), 4.98 (s, 2H, CH_2Ph).

Compounds 5–12 were prepared by the same reaction conditions as shown in Scheme 1, but starting from the corresponding commercially available substituted benzaldehydes or substituted phenols. In step *c*, 4-benzyloxy-2-hydroxybenzaldehyde was used. The precursors of compounds 5, 6, 9 and 10 were 4-methoxyphenol, 3-methoxyphenol, 3,4-dimethylphenol and 4-methylphenol, respectively. The precursors of compounds 7, 8, 11 and 12, respectively, were 3,5-dimethoxy, 2,3-dimethoxy, 4-ethoxy-3-methoxy and 3-ethoxy-4-methoxybenzaldehydes. Compound 5, ^{1}H NMR ($CDCl_3$) δ 3.71 (s, 3H, OCH_3), 3.89 (d, $J = 2.1$ Hz, 1H, 12a-H), 4.17 (d, $J = 13.1$ Hz, 1H, 6-H), 4.62 (dd, $J = 13.1$, 2.7 Hz, 1H, 6-H), 4.94 (t, $J = 2.7$ Hz, 1H, 6a-H), 5.03 (s, 2H, CH_2Ph). EI-MS, m/e 388 (M^+). Compound 6, ^{1}H NMR ($CDCl_3$) δ 3.72 (s, 3H,

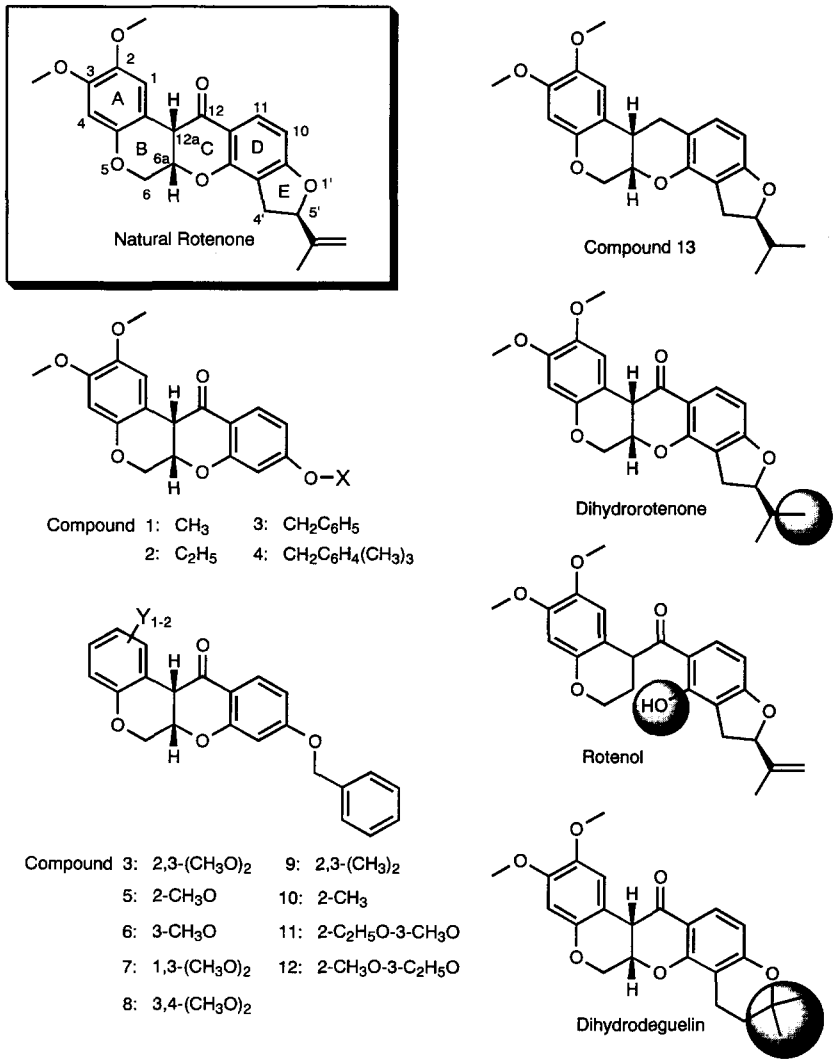
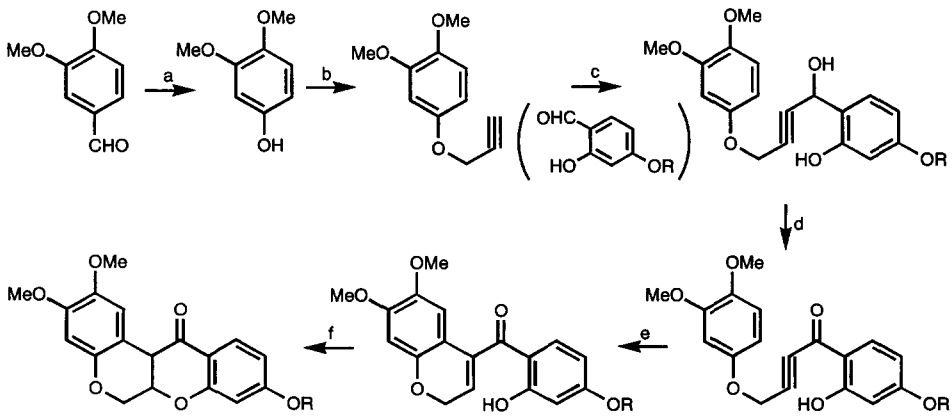


Fig. 1. Structure of natural rotenone and the analogues studied here. The shadowed circles in dihydrorotenone, rotenol and dihydrodeguelin represent the moieties that differ from natural rotenone.



Scheme 1. Reaction conditions. (a) *m*-$ClPhCO_3H$/EtOAc, (b) $HC{\equiv}CCH_2Br$, NaH/dry THF, reflux 6 h, (c) EtMgBr, 4-substituted-2-hydroxybenzaldehyde/dry THF, (d) H_2MnO_3/benzene, (e) HCl/1,2,4-trichlorobenzene, 220°C 1 h under N_2, (f) CH_3COONa/EtOH, reflux 3 h.

OCH_3), 3.82 (d, $J = 7.6$ Hz, 1H, 12a-H), 4.20 (d, $J = 12.0$ Hz, 1H, 6-H), 4.62 (dd, $J = 12.0$, 3.0 Hz, 1H, 6-H), 4.91 (m, 1H, 6a-H), 5.02 (s, 2H, CH_2Ph). EI-MS, m/e 388 (M^+). Compound 7, ^1H NMR ($CDCl_3$) δ 3.76 (s, 3H, OCH_3), 3.79 (s, 3H, OCH_3), 4.05 (m, 1H, 12a-H), 4.18 (dd, $J = 20.7$, 9.9 Hz, 1H, 6-H), 4.36 (dd, $J = 7.1$, 1.0 Hz, 1H, 6a-H), 5.03 (dd, $J = 18.8$, 7.1 Hz, 1H, 6-H), 5.08 (s, 2H, CH_2Ph). EI-MS, m/e 418 (M^+). Compound 8, ^1H NMR ($CDCl_3$) δ 3.81 (s, 3H, OCH_3), 3.86 (s, shoulder, 1H, 6a-H), 3.86 (s, shoulder, 1H, 12a-H), 3.87 (s, 3H, OCH_3), 4.23 (d, $J = 12.1$ Hz, 1H, 6-H), 4.75 (d, $J = 12.1$ Hz, 1H, 6-H), 5.63 (s, 1H, 6a-H), 5.63 (s, 2H, CH_2Ph). EI-MS, m/e 418 (M^+). Compound 9, ^1H NMR ($CDCl_3$) δ 2.09 (s, 3H, CH_3), 2.19 (s, 3H, CH_3), 3.83 (d, $J = 3.5$ Hz, 1H, 12a-H), 4.18 (d, $J = 12.2$ Hz, 1H, 6-H), 4.62 (dd, $J = 12.2$, 3.5 Hz, 1H, 6-H), 4.91 (t, $J = 3.5$ Hz, 1H, 6a-H), 5.03 (s, 2H, CH_2Ph). EI-MS, m/e 386 (M^+). Compound 10, ^1H NMR ($CDCl_3$) δ 2.20 (s, 3H, CH_3), 3.88 (d, $J = 3.5$ Hz, 1H, 12a-H), 4.21 (d, $J = 11.3$ Hz, 1H, 6-H), 4.63 (dd, $J = 11.3$, 3.5 Hz, 1H, 6-H), 4.93 (t, $J = 3.5$ Hz, 1H, 6a-H), 5.03 (s, 2H, CH_2Ph). EI-MS, m/e 372 (M^+). Compound 11, ^1H NMR ($CDCl_3$) δ 1.37 (t, $J = 6.7$, 3H, $OCH_2C\mathbf{H}_3$), 3.79 (s, 3H, OCH_3), 3.83 (d, $J = 4.0$ Hz, 1H, 12a-H), 3.96 (m, 3H, $OC\mathbf{H}_2CH_3$), 4.17 (d, $J = 12.7$ Hz, 1H, 6-H), 4.62 (dd, $J = 12.7$, 3.3 Hz, 1H, 6-H), 4.92 (t, $J = 3.3$ Hz, 1H, 6a-H), 5.04 (s, 2H, CH_2Ph). EI-MS, m/e 432 (M^+). Compound 12, ^1H NMR ($CDCl_3$) δ 1.41 (t, $J = 7.0$ Hz, 3H, $OCH_2C\mathbf{H}_3$), 3.75 (s, 3H, OCH_3), 3.88 (d, $J = 3.9$ Hz, 1H, 12a-H), 4.01 (m, 3H, $OC\mathbf{H}_2CH_3$), 4.15 (d, $J = 12.3$ Hz, 1H, 6-H), 4.90 (t, $J = 3.0$ Hz, 1H, 6a-H), 5.03 (dd, $J = 12.3$, 3.0 Hz, 1H, 6-H), 5.03 (s, 2H, CH_2Ph). EI-MS. m/e 432 (M^+).

Compound 13 was prepared by reducing natural rotenone with 10% palladium on carbon in methanol with a catalytic amount of HCl under hydrogen gas at room temperature. Compound 13, ^1H NMR ($CDCl_3$) δ 3.30 (m, 1H, 12a-H), 3.81 (s, 3H, OCH_3), 3.81 (s, 3H, OCH_3), 4.25 (shoulder, 1H, 6a-H), 4.48 (dd, $J = 16.9$, 8.7 Hz, 1H, 6-H), 4.67 (dd, $J = 8.7$, 5.7 Hz, 1H, 6-H). EI-MS, m/e 382 (M^+).

The rotenone analogues synthesized in this study except compound 13, were a mixture of two stereoisomers (i.e., [6aα, 12aα] and [6aβ, 12aβ] derivatives) which have the opposite configuration at the B/C ring junction. The ratio of the stereoisomers checked by using a metal chiral HPLC column, Ceramospher RU-1 (4.6 mm × 120 mm, Shiseido, Japan), was almost 1 to 1. Since the [6aα, 12aα] derivative is at least 100 times less inhibitory than the [6aβ, 12aβ] derivative [15], the inhibition by the former was negligible within the applied concentration range.

To examine the electronic effect of the substituent in the A-ring on the inhibition, we tried to prepare derivatives which possess electron-withdrawing substituents such as chloride and nitro groups. However, the desired products were not obtained because of the very low yield in reaction step *e* (Scheme 1), probably due to a decrease in the electron density of the A-ring caused by the electron-withdrawing substituents. Therefore, all substituents on the A-ring of compounds 5–12 were limited to electron-donating groups.

2.3. Assays

Bovine heart SMP were prepared by the method of Matsuno-Yagi and Hatefi [20]. The NADH-PB oxidoreductase activity was measured using a stirred cuvette in a Shimadzu UV3000 spectrophotometer at 30°C, as the rate of NADH oxidation (340 nm, $\epsilon = 6.2\ mM^{-1}cm^{-1}$). The reaction medium, in a final volume of 2.5 ml, contained 50 mM potassium phosphate (pH 7.4), 0.25 M sucrose, 1 mM $MgCl_2$, 2 mM KCN, 0.4 μM antimycin A and 50 μM PB, with a final mitochondrial protein concentration of 30 μg/ml. When the effects of the inhibitors were determined, SMP were incubated with the inhibitors for 4 min at 30°C in the reaction medium, then the enzyme reaction was started by adding 50 μM NADH.

Membranes of *E. coli* (GR19N) were prepared as described [21]. The deaminoNADH-PB reductase activity of NDH-1 was measured as described [15] in a reaction medium containing 50 mM phosphate buffer (pH 7.4), 2.5 mM KCN, 5 mM $MgSO_4$ and 10 μM PB, the final protein concentration being 15 μg/ml. The enzyme reaction was started by adding 50 μM deaminoNADH. The activity of NDH-2 was determined by the same means as that for the NDH-1 assay, except 50 μM NADH (plus 100 μM rotenone) was added in place of deaminoNADH.

Potato tuber (*Solanum tuberosum*, L) submitochondrial particles were prepared according to Møller et al. [22]. The deaminoNADH-PB reductase activity of NDH-1 was measured as described [15] in a reaction medium consisting of 50 mM phosphate buffer (pH 7.4), 0.25 M sucrose, 2 mM KCN, 1 mM $MgCl_2$, 1 μM antimycin A and 40 μM PB. The final protein concentration was 15 μg/ml. The enzyme reaction was started by adding 80 μM deaminoNADH. The activity of NDH-2 located inside the inner membrane was measured using 80 μM NADH (plus 100 μM rotenone and 0.2 mM EGTA) instead of deaminoNADH.

The stable conformation of synthetic rotenones was studied by molecular orbital methods. Computations were achieved using a semi-empirical molecular orbital program package (MOPAC ver. 6.01) with AM1 parameterization [23]. Initial conformations were constructed using standard bond lengths and angles.

3. Results

3.1. Structure-activity relationship of rotenones with bovine heart mitochondrial NDH

As described in the Introduction, a suitable substitute for the portion corresponding to the E-ring and the at-

Table 1
Inhibitory potency (pI_{50}) of rotenone and synthetic analogues for NADH-PB reductase activity

Inhibitor	pI_{50}		
	Bovine NDH	potato NDH-1	*E. coli* NDH-1
compound 1	6.06 (29)	4.53 (2.0)	4.72 (1.3)
compound 2	6.62 (8.0)	5.34 (0.30)	5.17 (0.45)
compound 3	7.92 (0.40)	5.51 (0.21)	4.82 (1.0)
compound 4	6.59 (8.6)	5.68 (0.14)	4.41 (2.6)
compound 5	6.82 (5.0)	4.60 (1.7)	5.44 (0.24)
compound 6	6.01 (33)	4.97 (0.71)	5.30 (0.33)
compound 7	6.90 (4.2)	4.91 (0.82)	5.08 (0.55)
compound 8	5.64 (76)	4.93 (0.78)	4.52 (2.0)
compound 9	6.45 (12)	4.70 (1.3)	5.12 (0.51)
compound 10	5.82 (50)	5.05 (0.59)	4.84 (0.96)
compound 11	7.64 (0.76)	4.42 (2.5)	5.00 (0.67)
compound 12	7.56 (0.92)	5.19 (0.43)	4.52 (2.0)
compound 13	7.98 (0.35)	5.78 (0.11)	5.11 (0.52)
natural rotenone	7.96 (0.37)	4.96 (0.73)	4.68 (1.4)
dihydrorotenone	7.85 (0.47)	4.74 (1.2)	4.59 (1.7)
rotenol	5.52 (101)	< 4.00 (< 6.7)	< 4.00 (< 6.7)
dihydrodeguelin	7.61 (0.82)	5.01 (0.65)	4.65 (1.5)

The proton-pumping NDH was from bovine heart mitochondrial, potato tuber mitochondrial and *E. coli* plasma membrane. The pI_{50}, the logarithm of the reciprocal of I_{50} which is the molar concentration in the reaction medium required to reduce the control enzyme activity by 50%, was used as an index of inhibitory potency. The pI_{50} was measured at least twice and the results were averaged. The I_{50} value is also expressed in terms of nmol inhibitor/mg protein for bovine NDH and μmol inhibitor/mg protein for potato and *E. coli* NDH-1 in parentheses, respectively.

tached isopropenyl group is desired to enable wide structural modifications of the A-ring. Therefore, we first synthesized compounds 1–4 by fixing the substitution pattern of the A-ring as that of natural rotenone (2,3-dimethoxy substitution). The inhibitory potencies of these inhibitors for NADH-quinone (PB) reductase activity are listed in Table 1. The pI_{50}, the logarithm of the reciprocal of I_{50} which is the molar concentration in the reaction medium required to reduce the control enzyme activity by 50%, was used as the index of inhibitory potency. The I_{50} measurements were repeated at least twice and the results were averaged.

The inhibitory potency in terms of pI_{50} increased with an increase of the hydrophobicity of the portion corresponding to the E-ring moiety (compounds 1–3). The inhibitory potency was entirely retained by replacing the E-ring moiety by a benzyloxy group (rotenone vs compound 3). In addition, the inhibition mechanism of compound 3 was identical with that of natural rotenone, being noncompetitive against exogenous ubiquinone PB [5,9,15]. Elongation of this moiety beyond that of compound 3 (a benzyloxy group) drastically decreased the activity (compound 3 vs compound 4), suggesting the existence of steric restriction around the portion corresponding to the E-ring. These results indicated that a benzyloxy group is a functionally appropriate substitute for the E-ring moiety. Considering that the configuration at the 5′-carbon atom (5′*R* configuration) is important for the activity [15], a high rotational freedom of O-CH_2-C_6H_5 bonds of the benzyloxy group might make this group suitably fit the binding site.

On the basis of the above findings, the substituents of the A-ring were modified by fixing the E-ring moiety as a benzyloxy group (compounds 5–12). A lack of one of 2,3-dimethoxy groups dramatically decreased the inhibitory potency (compound 3 vs 5 or 6), in particular, of a methoxy group in the 3-position. Modifying the substitution position of methoxy groups also decreased the activity (compound 3 vs 7 or 8). Replacing 2,3-methoxy group(s) with a methyl group(s) decreased in the inhibitory potency (compound 3 vs 9 and 5 vs 10). These findings indicated that the native chemical structure (2,3-dimethoxy substitution) is the most favorable for tight fitting to the binding site. In other words, the portion of the molecule corresponding to the A-ring is strictly recognized by the binding environment. On the other hand, it is notable that replacing one of the two methoxy groups with an ethoxy group only slightly reduced the inhibitory potency (compound 3 vs 11 or 12), suggesting that the binding environment of the A-ring moiety is spacious enough to accommodate a substituent larger than the methoxy group.

Burgos and Redfearn [14] have studied the effect of chemical modifications of the polar 12-C = O group in the C-ring on the inhibitory potency. The reduction of this group of natural rotenone (conversion 12-C = O to 12-C-OH) retained the inhibitory potency, whereas the oxime (12-C = N-OH) and methyl (12-C-CH_3) derivatives drastically lost the activity. Moreover, although 5′β-epirotenone (6aα, 12aα-stereoisomer of rotenone) was much less potent than rotenone, the 12-C-OH derivative of 5′β-epirotenone restored the inhibitory potency. Thus, the structure-activity profile for this group was complicated, and it is consequently unclear whether or not the 12-C = O group is essential for the activity of rotenone. These complex results may be attributed to the notion that chemical modifications of 12-C = O group bring about a conformational change of B/C ring junction at the same time owing to steric congestion arising from the A-ring moiety. We therefore synthesized compound 13 to minimize the conformational change. Although the isopropenyl group attached to the E-ring of rotenone was transformed to the isopropyl group in compound 13 during synthetic process, effect of this structural modification on the inhibitory potency is almost negligibly small, as described later.

To determine whether or not the conformation of compound 13 is affected by a lack of the 12-C = O group, we performed a conformational study of compound 13. The molecular orbital calculation study indicated that the bent form is the most stable conformation for compound 13 (data not shown), as it is for natural rotenone [24,25]. Therefore, the effect of a conformational change of the B/C ring junction on the inhibitory potency might be

negligibly small, if any. The apparent inhibitory potency of compound 13 for NADH-PB oxidoreductase activity was almost identical to that of natural rotenone. However, the inhibitory action of compound 13 seemed to somewhat differ from that of natural rotenone because of the following. (1) The inhibitory potency of compound 13, in terms of pI_{50} value, for NADH-PB oxidoreductase activity was almost identical to that for NADH oxidase activity, whereas the inhibition by rotenone is less potent for exogenous quinone reductase activity than that for NADH oxidase activity [5,26]. (2) Although rotenone acts in a noncompetitive manner against exogenous quinone [5,9,15], the inhibition pattern of compound 13 was competitive against PB (data not shown). We therefore concluded that the apparent inhibitory potency is indeed retained even in the absence of the 12-C = O group, whereas the manner of binding somewhat differs from that of natural rotenone.

Besides the rotenone analogues synthesized in this study, we also examined the inhibition using natural rotenoids (rotenol, dihydrodeguelin and dihydrorotenone, Fig. 1), as shown in Table 1. The inhibitory potency of rotenol, in which the whole molecular conformation is not fixed due to a lack of C-ring structure, drastically decreased. This result supported the earlier conclusion [14,15] that a rigidly bent form of rotenone at B/C-ring junction is essential for tight fitting into the binding site. The fact that dihydrorotenone retained the activity, indicated that the π-electron system of the isopropenyl group attached to the E-ring does not contribute to the inhibitory action. The inhibitory potency of dihydrodeguelin, which carries geminal methyl groups in the position corresponding to the 5′-isopropenyl group of rotenone, was about one-half of that of rotenone. Since the hydrophobic properties of dihydrodeguelin and rotenone or dihydrorotenone are virtually identical, this finding also suggested that there is steric restriction around the E-ring moiety.

3.2. Structure-activity relationship of synthetic rotenones with potato tuber mitochondrial and E. coli NDH

We examined the structure-activity profiles for NADH-PB oxidoreductase activity of NDH-1 from potato tuber mitochondria and *E. coli* plasma membranes (Table 1). The inhibitory potency of potato NDH-1 increased with an increase of hydrophobicity (or bulkiness) of the E-ring moiety (compounds 1–4). The ethoxy group was most favorable as a substitute for the E-ring moiety for *E. coli* NDH-1. Thus, although a benzyloxy group was not necessarily the best substitute for the two NDH-1, the inhibition by compounds 5–13 was examined in order to compare their structure-activity profile for the two enzymes with that for bovine complex I.

The sensitivity of the two enzymes to the inhibition by rotenone and its analogues was much lower than that of bovine heart mitochondrial NDH. Compound 3 was slightly, but significantly, more potent than natural rotenone for the two enzymes. The 2,3-dimethoxy substitution was the most favorable for the activity with potato NDH-1, whereas this substitution pattern was not necessarily the best with *E. coli* NDH-1. A rule regarding the variation in the activity depending upon structural modifications was ambiguous because of a small variation in the inhibitory potencies. The inhibitory potency of compound 13 was about 3 to 6 times larger than that of natural rotenone for both enzymes. This result also supported the notion that the manner of binding of this inhibitor is not identical with that of natural rotenone.

It is quite likely that the proton pumping machinery is close to the ubiquinone binding site [27,28]. We therefore compared the sensitivity to the inhibition by rotenone analogues between proton-pumping (NDH-1) and non-proton-pumping (NDH-2) enzymes. There are two species of NDH-2 in the inner membrane of potato tuber mitochondria [29]. In this study, the NDH-2 which is located on the inner surface of the inner mitochondrial membrane was assayed in the presence of 100 μM rotenone and 0.2 mM EGTA [29]. The NADH-PB oxidoreductase activity of NDH-2 from potato tuber mitochondria and *E. coli* membranes was not inhibited in the presence of either analogue at a concentration of 100 μM. Since this concentration was nearly at the solubility limit of almost all the test compounds, the effect at higher concentrations was not examined. The sensitivity to inhibition by natural rotenone differs remarkably between NDH-1 and NDH-2 from potato tuber mitochondria [29] and *E. coli* membrane [15,21]. Our results confirmed this notion for a variety of synthetic rotenone analogues.

4. Discussion

The primary structures of all subunits of bovine heart mitochondrial NDH and of many bacterial subunits are now known. Functional and biophysical spectroscopic analyses of the proton-pumping NDH isolated from bovine heart mitochondria [30,31], potato tuber mitochondria [32] and *E. coli* [33] have been reported. However, since details of structural features of ubiquinone reduction site are still vague, the information obtained from structure-activity studies of specific inhibitor rotenone and its analogues is helpful at present to predict the manner of the interaction between rotenone and its binding site. In particular, this kind of information obtained with bovine heart mitochondrial NDH is useful, because rotenone is a very potent and specific inhibitor of this enzyme, and its inhibitory potency is extremely sensitive even to a slight structural change, as shown in this study.

Using an appropriate model compound of natural rotenone and its analogues, we elucidated, for the first time, the substituent effects in the A-ring of rotenone on the inhibition. To elicit potent inhibition with bovine heart mitochondrial NDH, 2,3-dimethoxy substitution in the A-

ring was important, indicating that the portion of the molecule corresponding A-ring is strictly recognized by the binding environment. Specific interactions between the hydrogen-bond accepting methoxy oxygens and some hydrogen-bond donating residues may contribute to rotenone binding. However, 2,3-dimethoxy substitution is not necessarily essential for the activity since analogues possessing different substitutions did not completely lose the activity. In addition, the bulkier ethoxy derivatives retained sufficient activity. These findings suggested that the ubiquinone catalytic site of bovine NDH is spacious enough to accommodate structurally different rotenone analogues in a dissimilar manner. This notion is supported by other studies [5,13,15,34] of various potent inhibitors of bovine NDH (complex I), in which the quinone catalytic site of this enzyme was supposed to be a rather large pocket that can accommodate a wide variety of inhibitors in a dissimilar manner [1]. Furthermore, on the basis of a structure-activity study of a systematic series of synthetic ubiquinone analogues, Sakamoto et al. [35] have suggested that the quinone catalytic site of this enzyme is a large pocket in which bulky quinone analogues can serve as an electron acceptor. In this context, from a study using fluorescent probes that compete for the binding site of ubiquinone, Ahmed and Krishnamoorthy [36] have indicated that the ubiquinone binding site of this enzyme has a high level of local segmental mobility which enables efficient rotational motion of the ligand.

The apparent inhibition by compound 13 is retained even by a lack of the polar and hydrogen-bond acceptable 12-C = O group, whereas the manner of its action is no longer identical with that of natural rotenone. We therefore concluded that the 12-C = O group is important for the inhibitory action of natural rotenone itself. The earlier complicated structure-activity profiles (e.g., Ref. 14) regarding this portion of the molecule would be also explained assuming that the rotenone analogues of interest interact to the binding pocket in a dissimilar manner depending upon the structure of this portion.

Another study [15] as well as this structure-activity relationship study provided information about the structural features of the binding site surrounding the E-ring moiety. The inhibitory potency of rotenone with bovine NDH decreases to about 10% when the 5′*R*-configuration of the isopropenyl group is inverted to a 5′*S*-configuration [15]. Substitute of the E-ring bulkier than a benzyloxy group was unfavorable for the inhibition. Furthermore, the dihydrodeguelin, a naturally occurring rotenone-related compound, was slightly less potent than rotenone. These findings indicated that the E-ring moiety is exposed to steric restriction arising from the binding pocket. This finding provides useful information on the development of rotenone derivatives carrying a probe. Considering the synthetic strategy for introducing some probes into rotenone, the E-ring moiety seems to be suitable portion, like the synthesis of the photoaffinity-labeled rotenone, arylazidoamorphigenin [37]. However, the introduction of a bulky probe into the E-ring must be carefully designed since the possibility that such a structural modification changes the binding manner of the derivative of interest from that of natural rotenone cannot be ruled out. Arylazidoamorphigenin elicits very slow kinetics of inhibition compared with rotenone (Fig. 3 in Ref. 37). This may be due not only to the limited solubility of this compound, as claimed by that authors, but also to a change in the manner of binding.

On the basis of the variation in the inhibitory potencies of rotenone and its stereoisomers (5′α- and 5′β-epirotenones), we concluded that the structure of rotenone binding sites of bovine heart and potato tuber mitochondrial NDH-1 are similar. In other words, both binding sites specifically recognize the bent form of rotenone in common [15]. This study indicated, however, that variation in the inhibitory potency depending upon structural modifications of the A-ring as well as E-ring moiety differs somewhat between the two enzymes, while the 2,3-dimethoxy substitution pattern was the best for the activity of both. Considering the identical conformation at the B/C-ring junction between rotenone and the derivatives synthesized here, it is likely that the overall shape of the rotenone binding cavities is similar between the two enzymes, whereas the local structural nature surrounding the A and E-ring moieties differs to some extent. The specific interactions between the methoxy groups in the A-ring and the binding site might be less important in potato NDH-1 than that in the bovine enzyme. This would explain the significantly low sensitivity of potato NDH-1 to inhibition by rotenone analogues. The structure-activity profiles for rotenone stereoisomers as well as for the derivatives synthesized in this study were totally different between bovine heart mitochondrial and *E. coli* NDH-1, suggesting that the structural similarity of the ubiquinone catalytic sites of the two enzymes is low. A similar conclusion has been obtained from the structure-activity study of a series of synthetic capsaicin analogues [13].

[1] We have to mention our ideas concerning the 'quinone catalytic site' and the 'quinone binding site'. These two terms are used differently in this paper. The quinone catalytic site names generically the reaction site which is believed to catalyze the last electron-transfer step from FeS cluster N-2 to quinone, and naturally comprises the quinone binding site. Since the catalytic site is a rather large pocket as claimed in the text, this site accommodates the inhibitors whose binding site do not necessarily overlap with that of quinone (see Refs. [5] and [15]).

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