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Comparison of the inhibitory action of synthetic capsaicin analogues with various NADH-ubiquinone oxidoreductases

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Abstract

Capsaicin is a new naturally occurring inhibitor of proton-pumping NADH-ubiquinone oxidoreductase (NDH-1), that competitively acts against ubiquinone. A series of capsaicin analogues was synthesized to examine the structural factors required for the inhibitory action and to probe the structural property of the ubiquinone catalytic site of various NADH-ubiquinone reductases, including non-proton-pumping enzyme (NDH-2), from bovine heart mitochondria, potato tuber (*Solanum tuberosum*, L) mitochondria and *Escherichia coli* (GR 19N) plasma membranes. Some synthetic capsaicins were fairly potent inhibitors of each of the three NDH-1 compared with the potent rotenone and piericidin A. Synthetic capsaicin analogues inhibited all three NDH-1 activities in a competitive manner against an exogenous quinone. The modification both of the substitution pattern and of the number of methoxy groups on the benzene ring, which may be superimposable on the quinone ring of ubiquinone, did not drastically affect the inhibitory potency. In addition, alteration of the position of dipolar amide bond unit in the molecule and chemical modifications of this unit did not change the inhibitory potency, particularly with bovine heart and potato tuber NDH-1. These results might be explained assuming that the ubiquinone catalytic site of NDH-1 is spacious enough to accommodate a variety of structurally different capsaicin analogues in a dissimilar manner. Regarding the moiety corresponding to the alkyl side chain, a rigid diphenyl ether structure was more inhibitory than a flexible alkyl chain. Structure–activity studies and molecular orbital calculations suggested that a bent form is the active conformation of capsaicin analogues. On the other hand, poor correlations between the inhibitory potencies determined with the three NDH-1 suggested that the structural similarity of the ubiquinone catalytic sites of these enzymes is rather poor. The sensitivity to the inhibition by synthetic capsaicins remarkably differed between NDH-1 and NDH-2, supporting the notion that the sensitivity against capsaicin inhibition correlates well with the presence of an energy coupling site in the enzyme (Yagi, T. (1990) Arch. Biochem. Biophys. 281, 305–311). It is noteworthy that several synthetic capsaicins discriminated between NDH-1 and NDH-2 much better than natural capsaicin.

Keywords: NADH-ubiquinone reductase; Capsaicin; Structure–activity relationship

1. Introduction

Rotenone and piericidin A are well-known, naturally occurring potent inhibitors of NADH-ubiquinone oxidoreductase, acting at, or close to, the ubiquinone catalytic site [1,2]. These inhibitors have been widely used to probe the mechanistic concepts and structural aspects of this protein complex [3–5]. The new naturally occurring inhibitor, capsaicin (Fig. 1), the pungent principle of red pepper species, has been shown to act as a competitive inhibitor for ubiquinone with NADH-ubiquinone oxidoreductase isolated from bovine heart mitochondria [6]. Using submitochondrial particles prepared from bovine heart mitochondria and several bacterial membranes, Yagi [7] has demonstrated that the inhibition of NADH-ubiquinone oxidoreductase activities of several organisms by capsaicin correlated well with the presence of an energy coupling site in this segment of the respiratory chain. That is, the sensitivity to capsaicin inhibition of NADH-ubiquinone oxidoreductase that bears an energy coupling site (NDH-1) is much higher than that of the enzyme that lacks an energy coupling site (NDH-2). Yagi consequently concluded that capsaicin inhibition correlates better with the presence of a coupling site than that by rotenone,

Abbreviations: NDH, NADH-ubiquinone oxidoreductase; NDH-1, NDH that bears an energy coupling site; NDH-2, NDH that lacks an energy coupling site; DB, 2,3-dimethoxy-5-methyl-6-*n*-decyl-1,4-benzoquinone; deaminoNADH, reduced nicotinamide hypoxanthine dinucleotide.

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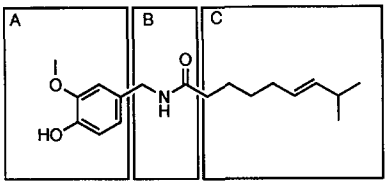


Fig. 1. Structure of natural capsaicin. Capsaicin is split into three sections (A, B and C) for simplicity.

piericidin A or DCCD (*N,N'*-dicyclohexylcarbodiimide) [7], which had been thought to discriminate fairly well between NDH-1 and NDH-2 [8]. Thus capsaicin appeared to be a useful reagent for investigating the mechanism of an energy coupling site associated with NADH-ubiquinone oxidoreductase.

To examine the mode of action of capsaicin and to probe the structural property of its binding site which might overlap the ubiquinone binding site, identification of the structural factors of this inhibitor required for the inhibition is essential. The structural requirements of capsaicin analogues for other pharmacological effects, such as analgesic activity have been examined closely (for example, Refs. [9,10]), whereas structure–activity studies of capsaicin analogues regarding electron-transfer inhibition have been reported only twice [6,11], to our knowledge. Chudapongse and Janthasoot [11] found that methyl capsaicin is several times more potent than natural capsaicin, indicating that the phenolic OH group is not essential for the activity. On the other hand, Shimomura et al. [6] have shown that capsaicin analogues with an acyl group of 10 to 12 carbons are much more potent than capsaicin, indicating that the hydrophobicity of the acyl moiety favors the activity. However, the structural variations of capsaicin analogues in these studies seemed insufficient to evaluate details of the structural factors required for the inhibitory action or to probe the structure of their binding sites. For instance, the roles of the substitution pattern of methoxy groups on the benzene ring, which may be superimposable on the ubiquinone ring, and of the amide bond unit in the inhibitory action have not been defined. Furthermore, considering that natural capsaicin is not necessarily a potent inhibitor of NDH-1 compared to the potent one such as rotenone and piericidin A [7], the development of the potent synthetic capsaicin analogues that possess a strictly selective activity for either NDH-1 or NDH-2 would be highly useful.

The prototype capsaicin has been split into three sections for simplicity as follows: the substituted benzene ring (A-section), the dipolar amide bond region (B-section) and the hydrophobic side chain (C-section), as shown in Fig. 1. In this study, we synthesized 49 capsaicin analogues by modifying each region in isolation. It was of interest to compare the effects of various synthetic capsaicin analogues on NDH-1 and NDH-2 activities, the latter being supposedly insensitive to inhibition by natural capsaicin. We investigated their ability to inhibit NDH-1 as well as NDH-2 from bovine heart mitochondria, potato tuber mitochondria and *E. coli* plasma membranes, and compared it with that of potent inhibitors such as rotenone and piericidin A.

2. Experimental procedures

2.1. Materials

NADH, deaminoNADH (reduced nicotinamide hypoxanthine dinucleotide), antimycin A, capsaicin and HQNO (heptylhydroxyquinoline *N*-oxide) were purchased from Sigma. Rotenone was obtained from Tokyo Chemical Industry Co. DB (2,3-dimethoxy-5-methyl-6-*n*-decyl-1,4-benzoquinone) was the same sample as described [12]. Piericidin A was a generous gift from Dr. Shigeo Yoshida (The Institute of Physical and Chemical Research (RIKEN), Japan). Fenpyroximate (*tert*-butyl-α-(1,3-dimethyl-5-phenoxypyrazol-4-ylmethyleneaminooxy)-*p*-toluate) was synthesized as reported [13].

2.2. Synthesis

The structures of capsaicin analogues synthesized here are shown in Fig. 2. All compounds were prepared by condensation of the corresponding amines and acid chlorides in the presence of triethylamine in dichloromethane or sodium carbonate in water/dichloromethane (0.8:1.0, v/v) at 4°C (Scheme 1, reactions 1 and 2). The substituted benzylamines (the precursors of series I, IV, V and VI compounds) were synthesized by reduction with $LiAlH_4$ in dry ether [14] from corresponding substituted benzonitriles (Scheme 1, reaction 3), which were obtained from corresponding commercially available substituted benzaldehydes in the presence of NH_2OH-HCl and sodium acetate in acetic acid at 130°C [15]. 3,4-Dimethoxyaniline (precursor of compound 15) and 3,4-dimethoxyphenethylamine (the precursor of compound 16) were commercially available. 2-(3,4-Dimethoxybenzyl)ethylamines (the precursors of compound 17) was prepared by reducing with $LiAlH_4$ of 3,4-dimethoxypenethylnitrile, which was synthesized by reacting of 3,4-dimethoxypenethylbromide and sodium cyanide in dimethylsulfoxide at 100°C. 3,4-Dimethoxybenzoic acid (the precursor of compound 18), 3,4-dimethoxyphenylacetic acid (the precursor of 19) and 3,4-dimethoxylphenetyl acid (the precursor of compound 20) were also commercially available. 4-(Substituted phenoxy)benzoylchlorides (the precursors of series V compounds) were prepared by reacting in dimethylformamide of thionylchloride and corresponding methyl 4-(substituted phenoxy)benzoates, which were synthesized from methyl 4-bromobenzoate and corresponding commercially available substituted phenols in the presence of potassium carbonate and copper [16]. The substituted benzoylchlo-

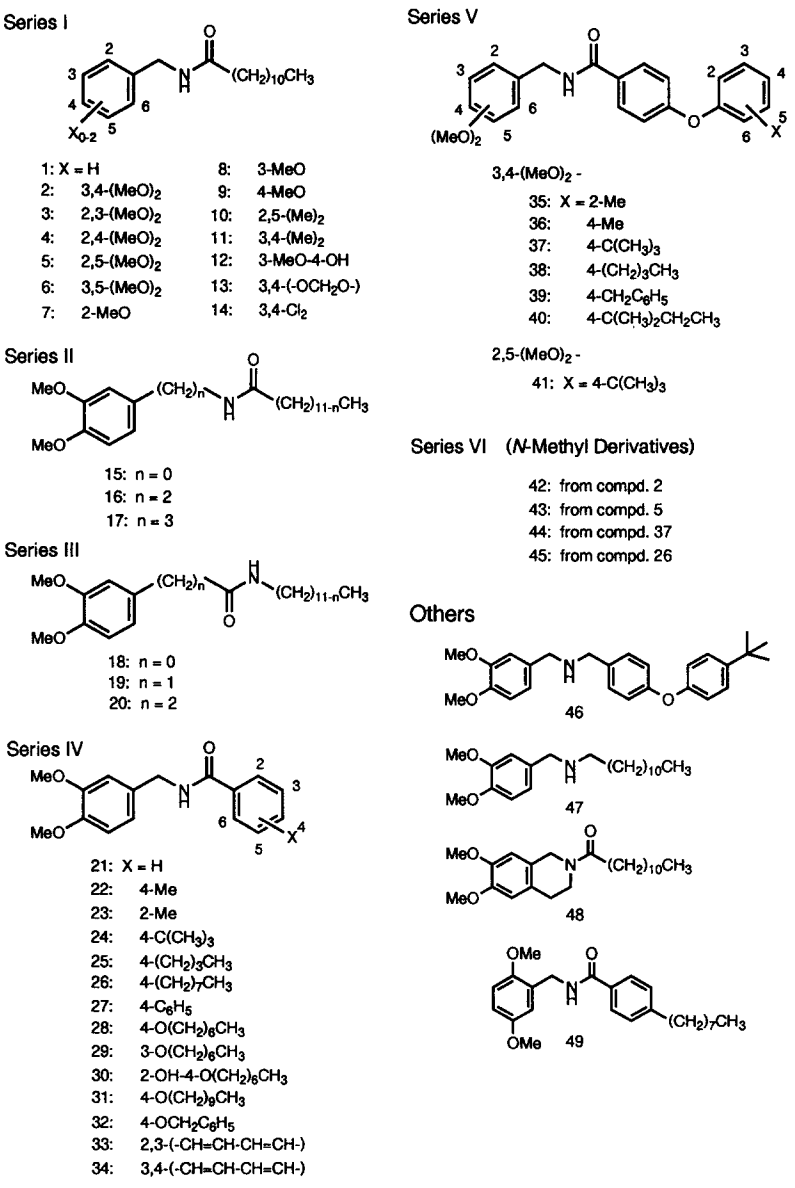
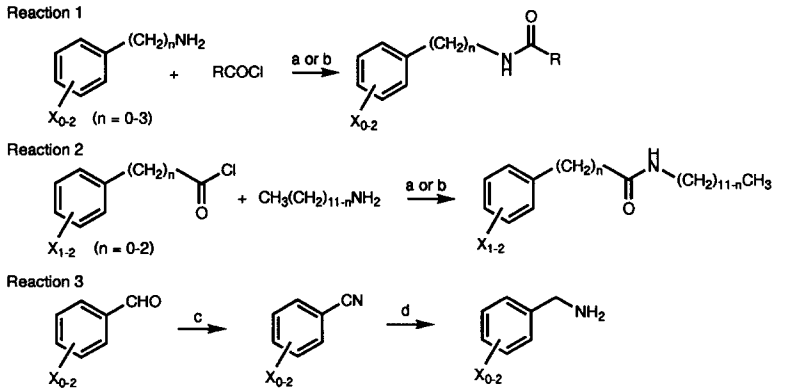


Fig. 2. Structures of all capsaicin analogues synthesized in the present study.



Scheme 1. Reaction conditions. (a) Et_3N/CH_2Cl_2; (b) $K_2CO_3/H_2O/CH_2Cl_2$; (c) NH_2OH-HCl/CH_3COONa/ CH_3COOH/Δ; (d) $LiAlH_4/Et_2O$.

rides (the precursors of series IV compounds) were obtained by reacting of thionylchloride and corresponding commercially available substituted benzoic acids in the presence of dimethylformamide in dichloromethane at 40°C. Compounds 42, 43, 44 and 45 were obtained by *N*-methylation of compounds 2, 5, 37 and 26, respectively, with methyl iodide in the presence of triethylamine and sodium hydride in dimethylsulfoxide. Compounds 46 and 47 were synthesized by reducing 37 and 2, respectively, with $LiAlH_4$ in tetrahydrofuran. 6,7-Dimethoxy-1,2,3,4-tetrahydroisoquinoline (the precursor of compound 48) was commercially available.

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 $\pm 0.3\%$.

2.3. Methods

Bovine heart submitochondrial particles were prepared by the method of Matsuno-Yagi and Hatefi [17]. NADH-DB reductase 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 DB, the final mitochondrial protein concentration being 15 μg/ml. The enzyme reaction was started by the addition of 50 μM NADH.

Membranes of *E. coli* (GR19N) were prepared as described [18] and were a gift from Dr. K. Matsushita (Yamaguchi University, Japan). The deaminoNADH-DB reductase activity of NDH-1 was measured as described [12] in reaction medium containing 50 mM phosphate buffer (pH 7.4), 2.5 mM KCN, 5 mM $MgSO_4$ and 10 μM DB, 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 method as that for the NDH-1 assay, except 50 μM NADH (plus 5 μM compound 39) was added in place of deaminoNADH. We used compound 39 to selectively inhibit NDH-1 activity, because it is a far more potent inhibitor of NDH-1 than rotenone (see Results).

Potato tuber (*Solanum tuberosum*, L) submitochondrial particles were prepared according to Møller et al. [19]. The deaminoNADH-DB reductase activity of NDH-1 was measured as described [12] in a reaction medium consisting of 50 mM phosphate buffer (pH 7.4), 0.25 M sucrose, 2 mM KCN, 1 mM $MgCl_2$, 2 μM antimycin A and 40 μM DB, the final protein concentration being 15 μg/ml. The enzyme reaction was started by the addition of 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. In all assays, the inhibitor was incubated for 4 min in advance of the reaction.

The stable conformation of synthetic capsaicins was studied by molecular orbital methods. Computations were achieved using a semi-empirical molecular orbital program package (MOPAC ver. 6.01) with AM1 parameterization [20]. Initial conformations were constructed using standard bond lengths and angles.

3. Results

3.1. Assays with bovine heart mitochondrial NDH

The inhibitory potencies of all synthetic capsaicin analogues for NADH-DB 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 halve the control enzyme activity, was used as the index of inhibitory potency. The pI_{50} measurement was repeated at least twice and the result was averaged. The standard inhibitors were natural capsaicin, rotenone, piericidin A, HQNO and fenpyroximate, which is a new synthetic pesticide acting upon mitochondrial NADH-ubiquinone oxidoreductase [13,21]. Almost all synthetic capsaicins were more potent than natural capsaicin.

The 3- and 4-positions of the benzene ring of natural capsaicin are substituted by methoxy and hydroxy groups, respectively. Chudapongse and Janthasoot [11] showed that the 4-hydroxy group is not essential for the activity, and can be replaced with a methoxy group. Shimomura et al. [6] have demonstrated that capsaicin analogues with acyl groups of 10 to 12 carbons are more potent than natural capsaicin. Considering these findings, we synthesized series I compounds to examine whether a 3,4-dimethoxy substitution pattern is important for the activity; in other words, whether these methoxy groups are strictly recognized by the binding site. Looking at the variation of inhibitory potencies of series I compounds from this perspective (Table 1), it is clear that 3,4-dimethoxy substitution pattern is not necessarily essential for the activity, since inhibitors possessing other substitutions including monomethoxy substitution retained the activity. Replacing the methoxy group by methyl or chloride resulted in a decrease in the activity (2 vs. 11 or 14, and 5 vs. 10), indicating that existence of the methoxy group itself is important for the activity, whereas its substitution pattern is not limited.

The dipolar amide bond (-NHCO-), which can act as a hydrogen-bond donor and acceptor, is linked to benzene ring through one methylene unit for natural capsaicin. It was of interest to examine whether the relative position of the amide bond unit and the benzene ring is strictly recognized by the binding site. We therefore synthesized the series II and III compounds. The hydrophobicity of the derivatives was standardized by fixing the number of total methylene units to a total of 13 carbons. Except for the derivatives of $n = 0$ (compounds 15 and 18), the relative

Table 1
Inhibition of various NDH activities by synthetic capsaicin analogues

Compd.	Bovine SMP	Potato SMP		*E. coli*	
	NDH-1 pI_{50}	NDH-1 pI_{50}	NDH-2 [a] inhibition(%) [b]	NDH-1 pI_{50}	NDH-2 inhibition(%) [b]
1	4.66	4.92	– [c]	< 4 [d]	–
2	5.00	4.43	n.t.	4.30	–
3	5.11	4.00	–	< 4	–
4	4.59	5.05	–	5.40	–
5	5.30	5.00	100	< 4	–
6	< 4	4.92	–	5.30	–
7	4.00	4.52	–	4.10	–
8	4.90	4.00	60	4.08	–
9	4.42	4.80	100	4.59	80
10	< 4	4.52	–	4.00	60
11	< 4	4.66	–	4.00	60
12	5.20	–	–	< 4	70
13	4.58	4.90	–	4.20	60
14	3.78	4.74	–	4.15	70
15	< 4	4.90	–	< 4	–
16	5.26	4.46	–	< 4	–
17	4.60	–	–	< 4	40
18	< 4	5.00	–	< 4	–
19	5.75	4.80	–	4.52	–
20	5.20	4.52	–	4.77	40
21	3.44	–	–	5.10	–
22	3.85	3.52	–	5.30	–
23	3.52	–	–	< 4	–
24	5.06	–	–	5.71	–
25	5.22	–	–	6.74	–
26	5.54	5.30	–	6.82	50
27	< 4	5.40	–	6.15	–
28	5.49	4.86	–	6.30	60
29	5.43	4.89	–	4.70	60
30	5.22	5.28	30	5.30	90
31	4.87	–	–	5.82	70
32	5.44	–	–	< 4	–
33	4.10	4.15	–	< 4	–
34	4.99	4.43	–	5.72	–
35	5.77	–	n.t.	5.30	90
36	5.96	–	–	6.35	–
37	7.03	5.50	100	6.72	60
38	5.63	5.40	–	6.60	60
39	5.78	5.15	–	7.00	50
40	6.78	5.62	n.t.	7.00	60
41	6.38	5.56	n.t.	4.46	60
42	5.49	4.52	n.t.	4.00	–
43	5.45	3.92	n.t.	4.30	30
44	7.12	5.27	–	5.70	40
45	5.59	5.00	n.t.	4.70	60
46	6.92	5.01	–	5.58	60
47	5.40	4.00	n.t.	4.96	–
48	5.74	4.74	n.t.	4.03	–
49	5.20	4.60	n.t.	5.26	60
Capsaicin	4.28	3.87	–	4.77	20
Rotenone	7.59	5.30	–	4.90	70
Piericidin	8.22	7.82	n.t.	6.63	50
Fenpyrox.	7.74	6.26	n.t.	5.00	60
HQNO	< 4	< 4	n.t.	5.15	80

The inhibition of NADH (or deaminoNADH)-DB reductase activity was determined, as described in Experimental procedures. (a) NDH-2 located inside the inner mitochondrial membrane was assayed. (b) The relative inhibition (%) in the presence of 100 μM inhibitor is shown. (c) Compounds that did not elicit inhibition at 100 μM are marked by "–". (d) Compounds that did not attain 50% inhibition at 100 μM are marked by "< 4". The effects of higher concentrations were not studied due to limited solubility.
n.t., not tested; Fenpyrox, Fenpyroximate.

position of the amide bond unit and benzene ring as well as the order of NH and CO (-NHCO- or -CONH-) appeared not to significantly affect the activity. Moreover, *N*-methylation of amide bond retained or rather potentiated the activity (2 vs. 41, 5 vs. 43, 37 vs. 44, and 26 vs. 45). These observations indicated that the existence of a dipolar amide bond itself and its spatial distance from the benzene ring contribute relatively little to the inhibitor binding.

To understand the roles of the alkyl side chain of capsaicin, we synthesized series IV and V compounds. The hydrophobic and rigid diphenyl ether structure tended to favor the activity. Elongation of the side chain beyond that of compound 37 (*tert*-butyl group) decreased the activity (37 vs. 38, 39 or 40). The structural modifications of potent inhibitor compound 37 confirmed the above conclusions about the roles of the A- and B-sections in the inhibition. That is, the fact that compounds 41, 44 and 46 retained the inhibitory potency compared to compound 37 indicated that an amide bond unit and a 3,4-dimethoxy substitution are not necessarily important for the activity.

Next, we examined the kinetics of the inhibition of NADH-DB reductase by some compounds. Fig. 3 shows double-reciprocal plots demonstrating the effect of a fixed concentration of compound 37 on the rate of NADH-DB reductase activity. The double-reciprocal plots were curvilinear in the presence of a fixed concentration of compound 37. In the lower concentration range of DB, compound 37 appeared to be a noncompetitive inhibitor. At higher concentrations of DB, compound 37 acted as a competitive inhibitor regarding DB. Compounds 44 and 46 had similar inhibition profiles (data not shown).

Yagi [7] has shown that the I_{50} values of natural capsaicin for the inhibition of NADH oxidase activity of bovine submitochondrial particles and *Paracocus* membranes decrease as the pH increases. Based upon the pH profile of the I_{50} values, Yagi suggested that the ionic

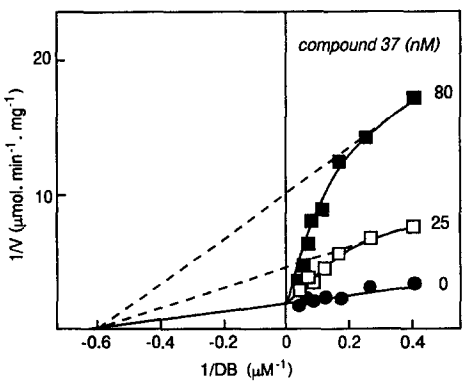


Fig. 3. Double-reciprocal plots of kinetic data of NADH-DB reductase in bovine heart submitochondrial particles in the presence of compound 37. The reaction medium, in a final volume 2.5 ml, contained 50 mM potassium phosphate (pH 7.4), 0.25 M sucrose, 1 mM $MgCl_2$, 2 mM KCN, 0.4 μM antimycin A and 50 μM NADH. The final mitochondrial protein concentration was 15 μg/ml.

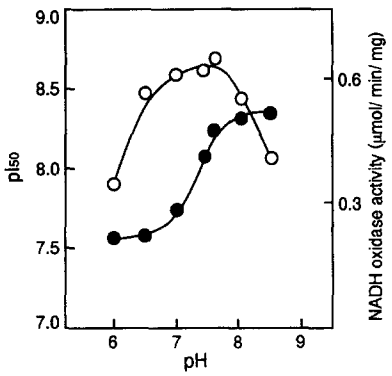


Fig. 4. The effect of pH on the NADH oxidase activity of bovine submitochondrial particles (○) and its inhibition by compound 37 (●). The pH values of the buffer containing 50 mM potassium phosphate, 0.25 M sucrose and 1 mM $MgCl_2$ were adjusted by adding HCl. The NADH concentration was 50 μM.

residue(s) with a pK_a about 7.5 is involved in the inhibition by capsaicin. To confirm this for synthetic derivatives, we examined the pH dependence of the inhibition of NADH oxidase activity of bovine submitochondrial particles by potent synthetic capsaicin (compound 37). As shown in Fig. 4, the optimum pH for NADH oxidase activity was around 7.5. The pH profile of pI_{50} was similar to that reported by Yagi, suggesting that the ionic residue(s) involved in natural capsaicin inhibition is also involved in the inhibition by synthetic capsaicins. About 5 to 10-fold lower I_{50} values were obtained in NADH oxidase assay compared with that measured in NADH-DB reductase assay, as reported [1,21].

Natural capsaicin does not inhibit succinate oxidase activity of rat liver mitochondria [11]. To confirm this for synthetic derivatives, we investigated the effect of synthetic capsaicins on succinate oxidase activity of bovine heart submitochondrial particles. Although some compounds partially inhibited succinate oxidase activity at high concentrations close to solubility limit, they did not elicit inhibition at the concentration range wherein they inhibit NADH-DB reductase activity.

3.2. Assays with potato tuber mitochondrial NDH

We next examined variations of the inhibitory potency depending upon structural modifications with potato tuber mitochondrial NDH-1 (Table 1). The variation in the activity was somewhat smaller than that of bovine heart mitochondrial NDH. The most potent inhibitor was compound 40, the activity of which was comparable with that of rotenone. Like bovine heart mitochondrial NDH, both the 3,4-dimethoxy substitution pattern and the relative position of the amide bond unit were not necessarily important for the activity. Contrary to bovine enzyme, the activity was almost totally retained when the methoxy group was replaced with a methyl or chloride group (2 vs. 11 or 14, and

5 vs. 10). The difference in the inhibitory potency between flexible alkyl derivatives and rigid diphenyl ether derivatives was somewhat reduced compared to that of bovine heart mitochondrial NDH. The pattern of inhibition by compounds 37 and 40 was investigated using DB as an electron acceptor. These compounds inhibited the enzyme activity in a competitive manner against exogenous quinone (DB) regardless of its concentrations (data not shown).

Two species of NADH-ubiquinone reductases are located on the inner surface of the inner membrane. One of them lacks an energy coupling site (NDH-2) and is insensitive to the inhibition by rotenone or piericidin A [22]. The inhibition of NDH-2 by synthetic capsaicins was also investigated. The relative inhibition (%) in the presence of 100 μM of each compound was shown in Table 1. This concentration was nearly at the solubility limit of almost all tested compounds. Although compounds 5, 9 and 37 were fairly potent inhibitors of NDH-2, the structural factors required for the inhibition could not be identified, since few compounds inhibited this enzyme. It was nevertheless apparent that the sensitivity to the inhibition by capsaicin analogues is remarkably different between NDH-1 and NDH-2.

3.3. Assays with Escherichia coli NDH

The inhibition of *E. coli* NDH-1 by synthetic capsaicins was also examined (Table 1). Like the two proton-pumping NDH described above, a clear rule governing the inhibitory potency depending upon structural modifications of the A- and B-sections was not defined. However, structural modifications of the potent inhibitors (compounds 26 and 37) indicated that the roles of 3,4-dimethoxy group and amide bond unit in the activity are more important than those in the inhibition of bovine heart and potato tuber mitochondrial NDH-1 (26 vs. 45 or 49, and 37 vs. 41, 44 or 46).

Compounds 39 and 40 potently inhibited NDH-1, even compared with piericidin A, which is one of the most potent inhibitors of this enzyme [21]. The inhibitory potencies of these compounds differed remarkably (by two to three orders of magnitude) between NDH-1 and NDH-2. Natural capsaicin also selectively inhibited NDH-1 activity, being consistent with an earlier report [7], whereas the inhibitory potency was much less than that of the above potent synthetic derivatives. Considering both the inhibitory potency for NDH-1 and the negligibly small effect on NDH-2 activity, we concluded that compounds 25 and 36 are more selective inhibitors of NDH-1 than those known to date. Although some compounds inhibited NDH-2 at high concentrations, NDH-1 and NDH-2 have remarkably different sensitivities to inhibition by capsaicin analogues, as observed with potato tuber mitochondrial enzymes. The inhibition by compounds 39 and 40 of NDH-1 was competitive against DB regardless of its concentration (data not shown).

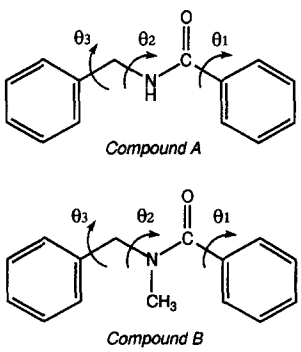


Fig. 5. Structure of compounds A and B. The compounds were rotated along θ_1 and θ_3 in steps of 120° and along θ_2 in steps of 60°, and the amide bond was fixed in the *trans* conformation.

3.4. Conformational study of capsaicin analogues

The structure–activity study suggested that strict electrostatic interactions between the functional groups on capsaicins (i.e., vicinal methoxy groups and amide bond unit) and their binding site make relatively small contributions to the inhibitor binding, in particular with bovine heart and potato tuber mitochondrial NDH-1. Therefore, it seems likely that the whole molecular shape plays an important part in the inhibitor binding. To obtain information on the active conformation of capsaicin analogues, we performed a conformational energy study upon compounds A and B (Fig. 5), which are simplified models of series IV-V compounds and their *N*-methyl derivatives, respectively. Compound A was rotated along θ_1 and θ_3 in steps of 120° and along θ_2 in steps of 60° and NH-CO bond was fixed in the *trans* conformation. The total energy of the molecule was calculated for each of 54 conformations. Six stable conformations existed within an energy difference in about 1 kcal mol^{-1} arising from a difference in θ_2 and θ_3. In each conformation, the carbonyl plane was coplanar to that of the attached benzene ring and the two benzene ring planes were almost perpendicular to each other.

Although the total energy was increased for each of the six stable conformations of compound B due to steric congestion by *N*-methylation, the same conclusion about the conformation was derived; namely, the molecule is stable when the two benzene ring planes are almost perpendicular to each other. This conclusion seems to be supported from NMR spectroscopic and X-ray crystallographic experiments on the conformational behavior of synthetic capsaicin analogues in which the relative disposition of the B-section with respect to that of the A-section was varied [10]. Based on one of the minimal conformations, the structure of compound 37 was optimized, as shown in Fig. 6 ($\theta_1 = -32.84$, $\theta_2 = 100.13$ and $\theta_3 = -84.18$ degree). Regarding the conformation of the diphenyl ether structure, one of the stable conformations in which two phenyl rings are perpendicular to each other was adopted from the literature [23].

3.5. Comparison of inhibitory potency between different NDH-1

The results described above confirmed that capsaicin analogues act at the ubiquinone catalytic site of NDH-1. If the ubiquinone catalytic sites from different sources share similar structural properties, variations among the inhibitory potencies of systematically selected series of inhibitors would correlate well [24,25]. Then, we compared the inhibitory potencies in terms of pI_{50}, between bovine heart and potato tuber mitochondrial NDH-1 (Fig. 7A) and between bovine mitochondrial and *E. coli* NDH-1 (Fig. 7B). Compounds of which the I_{50} value could not be evaluated due to limited solubility, were not included in the comparison. Correlations among the three enzymes were poor, suggesting that the similarity of the structure of the ubiquinone catalytic sites in these enzymes is not necessarily high.

In all DB reductase activity measurements described, the enzyme reaction was started by the addition of either NADH or deaminoNADH. Thus the test compounds made contact with the oxidized enzymes, after which their inhibitory actions were determined. Van Belzen et al. [26] showed that NADH oxidase activity of bovine heart submitochondrial particles was inhibited by piericidin A more efficiently in the reduced state than in the oxidized state, although a difference was slight. To see whether this is also the case for capsaicins, we compared the sensitivity to the inhibition by compounds 35 and 39 between reduced and oxidized NDH by reversing order of the addition of DB and NADH (or deaminoNADH). For the three NDH-1, the sensitivity to the inhibition was almost same irrespective of the redox state of the enzymes (data not shown). Furthermore, since the inhibitory potencies were treated logarithmically, in terms of pI_{50}, in this study, a small difference, if any, in the sensitivity to other inhibitors depending upon the redox state of the enzymes might not affect our conclusion.

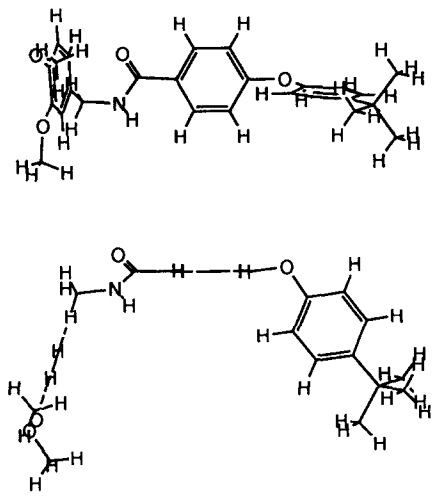


Fig. 6. The optimized conformation of compound 37. The θ_1, θ_2 and θ_3 in Fig. 5 are −32.84, 100.13 and −84.18 degree, respectively. The moiety of the molecule corresponding to diphenylether and the 3,4-dimethoxy groups on the benzene ring were optimized in isolation. Regarding the conformation of the diphenylether structure, a stable conformation in which two phenyl rings are perpendicular to each other was adopted from the literature [23]. Two methoxy groups were optimized from 36 conformers which were obtained by rotation along the MeO-Ph bond in steps of 60°.

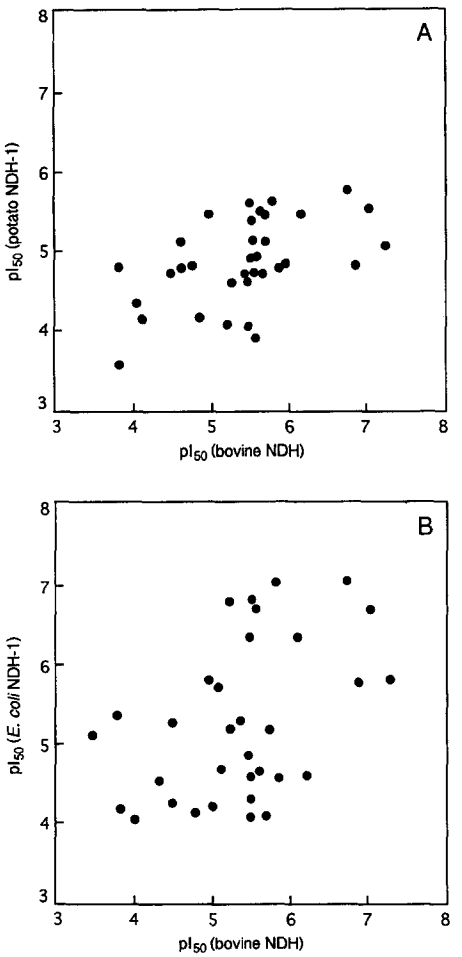


Fig. 7. The relationship between the inhibitory potencies in terms of pI_{50} determined with different enzymes. (A) The pI_{50} determined with bovine heart NDH vs. that with potato tuber NDH-1; (B) the pI_{50} determined with bovine heart NDH vs. that with *E. coli* NDH-1.

4. Discussion

Capsaicin inhibits NDH-1 activity by acting competitively against ubiquinone. Considering the structural similarity between capsaicins and ubiquinone (vicinal methoxy groups on the aromatic ring and a hydrophobic alkyl side chain), it is reasonable to consider that the spatial position occupied by the benzene ring moiety of capsaicin in the binding site is superimposed upon that occupied by the benzoquinone ring. Therefore, the methoxy groups on the benzene ring and their 3,4-substitution pattern were thought

to play important roles in inhibitor binding, whereas the findings indicated otherwise. It was also indicated that the amide bond unit, which can serve as a hydrogen-bond donor and acceptor in inhibitor binding, contributes very little to the inhibition, particularly for bovine heart and potato tuber mitochondrial NDH-1. Thus, the rule regarding the variation of the inhibitory potency depending upon structural modifications is ambiguous. Considering the structure–activity studies of several potent electron-transfer inhibitors (e.g., piericidin derivatives in Ref. [27], rotenone derivatives in Ref. [28], stigmatellin derivative in Refs. [29,30], methoxyacrylate derivatives in Ref. [31] and antimycin derivatives in Refs. [32,33]), in which structural modifications of functional group(s) cause drastic changes in the activity, the unusual structure–activity profile of the capsaicin analogues studied here would be prominent. This may be explained assuming that the ubiquinone catalytic site of NDH-1 is spacious enough to accommodate a variety of structurally different inhibitors in a dissimilar manner. Other studies [12,21,34] of the inhibitory mechanisms of various potent inhibitors of NDH-1 seem to support this assumption. It is further notable that the fairly high rotational freedom of A- and B-sections of capsaicins make these moieties fit flexibly into the large pocket. This kind of flexibility also enables the inhibitors to bind to the ubiquinone catalytic site in various ways.

On the other hand, taking into account the fact that electrostatic interactions between the functional groups on capsaicins and their binding sites make relatively small contributions to their binding, whole molecular shape itself might be the significant structural factor supporting tight binding. A molecular orbital calculation study suggested that a bent form at the face of contact between A- and B-sections favors inhibitory action. Regarding the structurally rigid inhibitor rotenone, Ueno et al. have shown that the bent form is important for eliciting the inhibitory action [12]. Moreover, a molecular orbital calculation study for fenpyroximate indicated that this compound is also stable when it takes a bent form (data not shown). To confirm whether the bent form is universally required for potent inhibitors of NDH-1, further structure–activity studies of other types of potent inhibitors are needed.

A comparison of variations of inhibitory potency determined with NDH-1 from different sources indicated that structural similarity of the ubiquinone catalytic site of the three NDH-1 is rather poor. Contrary to this, since piericidin A works as a very potent inhibitor with bovine heart and potato tuber mitochondrial NDH-1, and the activity of potent inhibitor compound 37 was almost totally retained after modifying its functional groups with these two enzymes (37 vs. 41, 44 or 46), the two enzymes may share somewhat common structural properties for the ubiquinone catalytic site. This apparent inconsistency may be also explained by assuming a large ubiquinone catalytic site. That is, the ubiquinone catalytic site shares at least a partly similar structure that enables the common inhibitors to fit. It is notable that structural similarity of the ubiquinone catalytic site between bovine heart and potato tuber mitochondrial NDH-1 had been suggested from variations among the inhibitory potencies of rotenone stereoisomers [12]. Herz et al. [35] have reported that potato tuber mitochondrial NDH-1 is similar in complexity to the bovine mitochondrial counterpart and that four of the potato subunits can be aligned by their N-terminal amino acid sequences to corresponding bovine NDH-1 subunits.

The inhibition pattern of some synthetic capsaicins was complicated in bovine heart mitochondrial NDH. It varied depending upon the concentration of exogenous quinone. A similar complexity has been reported for the inhibition by natural capsaicin [7] and synthetic rotenone stereoisomer [12]. This complicated inhibition profile might be interpreted if NDH bears two quinone binding sites with the same affinity for quinone, and the activity of this enzyme is inhibited by the binding of one molecule of inhibitor to one of the two quinone binding sites [7]. In other words, although capsaicins in principle bind to the quinone binding sites, a noncompetitive inhibition pattern might be apparently observed at low levels of quinone (DB) since the quinone can be reduced at one of the two binding sites which is not occupied by the inhibitor when the concentration of quinone is low. Considering the fact that two quinone binding sites on mammal mitochondrial NDH have been suggested by several groups [1,34,36,37], this interpretation seems to be reasonable. The presence of endogenous ubiquinone in submitochondrial particle preparations may not affect the kinetics of DB reduction, since the reduction of short-chain quinone analogues (as CoQ_1 and CoQ_2) does not involve the endogenous ubiquinone [38,39]. In contrast to bovine heart NDH, capsaicins inhibited the NDH-1 activities of *E. coli* and potato tuber mitochondria in a competitive manner against DB, regardless of their concentrations. Therefore, the concept of two quinone binding sites in these enzymes is not supported, at least by our observations.

The sensitivity to inhibition by synthetic capsaicins differed remarkably between NDH-1 and NDH-2 from both potato tuber and *E. coli* enzymes. Yagi [7,8] has indicated that the inhibition by natural capsaicin correlates better with the presence of an energy coupling site of various NDH than the inhibition by rotenone, piericidin A or DCCD. The present study confirmed this notion for a variety of synthetic capsaicin analogues. Considering the fact that a fairly high concentration of natural capsaicin is needed to inhibit NDH-1 activity, it is notable that some synthetic analogues can discriminate between NDH-1 and NDH-2 much better than natural capsaicin.

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