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3,5-Di-*tert*-butyl-4-hydroxybenzylidenemalononitrile; a new powerful uncoupler of respiratory-chain phosphorylation

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SUMMARY

3,5-Di-*tert*-butyl-4-hydroxybenzylidenemalononitrile (SF 6847) was found to be one of the most powerful uncouplers of respiratory-chain phosphorylation ever reported. Structure–activity studies of the derivatives indicated the role of three functional groups in SF 6847: (1) the malononitrile group as an electron withdrawing group, which may cause uncoupling by interaction with a primary energy conservation site, (ii) a free hydroxyl group as an electron donator, and (iii) bulky tertiary butyl groups of hydrophobic character located at a certain spacial distance from the electron withdrawing center.

Various hypotheses for the molecular mechanism of uncoupling have been developed[1–4], depending on the model of primary energy conservation for the case of classical weak acid uncouplers, such as dinitrophenol. However, it is uncertain whether these hypotheses cover the action of new types of powerful uncouplers[5–7], which have been discovered mainly from biocidal drugs. Recently, Horiuchi *et al.*[8] reported that 3,5-dialkyl-4-hydroxybenzylidenemalononitrile derivatives had fungicidal and acaricidal activities. This paper reports that these compounds showed a potent uncoupling activity in rat-liver mitochondria. Among them, 3,5-di-*tert*-butyl-4-hydroxybenzylidenemalononitrile (SF 6847) was found to have the most powerful uncoupling activity.

3,5-Dialkyl-4-hydroxybenzylidenemalononitrile derivatives were kindly supplied by Dr Y. Nishizawa, Sumitomo Chemical Industry, Osaka and were used as solutions in ethanol. Mitochondria from rat liver were prepared according to a previously published method[9]. Oxygen consumption was measured with Galvani electrodes as described by Utsumi *et al.*[10]. The rate of respiration in State 4 mitochondria with succinate as substrate was titrated with successive additions of uncoupler at 1-min intervals in the absence of rotenone. The degree of reduction of cytochrome *c* was measured with a Hitachi, Model 356

Two-wavelength Spectrophotometer using a wavelength pair of 550 *minus* 540 nm. These experiments were performed in medium of the following composition: 25 mM Tris–HCl buffer (pH 7.4), 50 mM sucrose, 5 mM $MgCl_2$, 2 mM EDTA and 15 mM KCl in a final volume of 3 ml. ATPase activity was determined by incubation of mitochondria for 10 min at 25° C in a medium containing 200 mM sucrose, 5 mM Tris–HCl buffer (pH 7.4), 20 mM KCl, 5 mM ATP and 3 mM $MgCl_2$ in a total volume of 1.6 ml.

Table I shows the effects on ATPase and on State 4 respiration of a series of di-*tert*-butylphenols, in which various groups were introduced at the *para* position. The uncoupling activities of the derivatives were taken to be the same as the concentrations giving maximal effects on ATPase and respiration. It was noted that analogues possessing electron withdrawing groups, such as $-CN$, $-COOCH_3$, $-NO_2$ and $-CONH_2$ displayed high activity, while the compounds with non-electron withdrawing groups, such as $-CH_2OH$ and $-CH_3$ demonstrated very little activity in either system. Among them, SF 6847, a malononitrile derivative, showed the most potent uncoupling activity, but considerable difference in its potency was noticed according to the experimental methods and conditions used (*i.e.* $8.6 \cdot 10^{-8}$ M for ATPase and $2.9 \cdot 10^{-8}$ M in the respiration method). This may partly be due to the stability of SF 6847 in aqueous solution. When a stock solution of SF 6847 was prepared in 10 % ethanol, its potency decreased to approximately 1/10 of the initial value within 2 h. However, the activity of stock solution in 100 % ethanol scarcely changed on standing for several hours. On measurement of the absorption spectrum of SF 6847, 50 % decrease in absorbance at the maximum (458 nm) was observed within 2 min in 10 % ethanol solution at room temperature, but no appreciable changes in the peaks at 363 and 464 nm were observed within several hours with a solution in 100 % ethanol.

To determine the exact amount of SF 6847 available for uncoupling, the redox states of cytochrome *c* were determined under the conditions previously reported by Muraoka and Slater[11], where the uncoupler added to State 4 mitochondria induced reduction of cytochrome *c* in the presence of azide. Fig. 1 shows that the first addition of

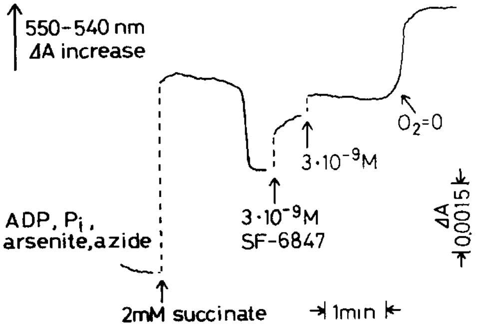
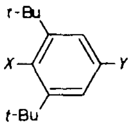


Fig. 1. Effect of SF 6847 on the redox state of cytochrome *c* added to rat-liver mitochondria in State 4 oxidizing succinate in the presence of rotenone. Succinate was added to State 2 mitochondria induced by 10 mM inorganic phosphate and 150 μM ADP in the presence of 500 μM azide and 1 mM of arsenite. Uncoupler was added to State 4 mitochondria, induced by exhaustion of added ADP, resulting in reduction of cytochrome *c*. Rat-liver mitochondria, 3.9 mg protein in a total volume of 3 ml.

TABLE I

UNCOUPLING ACTIVITIES OF 3,5-DI-*tert*-BUTYL-4-HYDROXYBENZYLIDENEMALONONITRILE AND ITS DERIVATIVES



No.	*X*	*Y*	*ATPase method*		*Respiration method*	
			Concn (M) [a]	*R.A.* [b]	*Concn (M)* [c]	*R.A.* [d]
IP-1008	–OH	–H	$3.2 \cdot 10^{-4}$	0.31	$7.0 \cdot 10^{-5}$	0.69
SF-6811	–OH	–COOH	$1.3 \cdot 10^{-3}$	0.08	$1.8 \cdot 10^{-3}$	0.03
SF-6795	–OH	$-CH_2OH$	$9.8 \cdot 10^{-4}$	0.10	$9.0 \cdot 10^{-4}$	0.05
SF-7475	–OH	–CH=C(CN)COOH	$1.2 \cdot 10^{-4}$	0.83	$1.1 \cdot 10^{-4}$	0.44
SF-6793	–OH	–CH=NOH	$2.0 \cdot 10^{-4}$	0.50	$8.5 \cdot 10^{-5}$	0.56
SF-6819	–OH	$-CH{=}NN(CH_3)_2$	$2.7 \cdot 10^{-4}$	0.37	$7.5 \cdot 10^{-5}$	0.64
SF-6949	–OH	–CH=CHCN	$1.2 \cdot 10^{-4}$	0.83	$3.6 \cdot 10^{-5}$	1.33
SF-7477	–OH	$-CH{=}C(COCH_3)COOC_2H_5$	$1.2 \cdot 10^{-4}$	0.83	$2.4 \cdot 10^{-5}$	2.00
SF-7479	–OH	$-CH{=}C(CN)CONH_2$	$2.3 \cdot 10^{-5}$	4.30	$7.0 \cdot 10^{-6}$	6.86
SF-6822	–OH	$-CH{=}C(CN)COOCH_3$	$2.3 \cdot 10^{-6}$	43.0	$5.0 \cdot 10^{-7}$	96.0
IP-1010	–OH	$-NO_2$	$2.1 \cdot 10^{-6}$	47.1	$7.3 \cdot 10^{-7}$	65.8
SF-6847	–OH	$-CH{=}C(CN)_2$	$8.6 \cdot 10^{-8}$	1151	$2.9 \cdot 10^{-8}$	1655
SF-6884	$-CO_2CH_3$	$-CH{=}C(CN)_2$	$1.4 \cdot 10^{-6}$	70.7	$7.2 \cdot 10^{-7}$	66.7
	2,4-Dinitrophenol		$9.9 \cdot 10^{-5}$	1.0	$4.8 \cdot 10^{-5}$	1.0

[a] Concentration at the maximal ATPase activity. Mean value of three experiments using rat-liver mitochondria of 2.1–3.5 mg protein per ml.

[b] Relative activity taking concentration of 2,4-dinitrophenol as reference.

[c] Concentration at the maximal release of respiration. Mean value of three experiments using rat-liver mitochondria of approximately 1.7 mg protein per ml.

[d] Relative activity taking concentration of 2,4-dinitrophenol as reference.

$3 \cdot 10^{-9}$ M SF 6847 caused approximately 75 % uncoupling activity (taking the effect of $3 \cdot 10^{-5}$ M dinitrophenol as 100 % activity) and that the second addition of $3 \cdot 10^{-9}$ M SF 6847 was equivalent to the maximal response. By this method it was indicated that the amount of SF 6847 giving half-maximal uncoupling (S_{50}) is less than 1.2 nmoles/g protein and the potency of this compound is more than 8300 times that of dinitrophenol (S_{50}: 10 μmoles/g protein).

As shown in Table I, a strict relationship was found between the chemical structures and the uncoupling activities of the derivatives, since slight modifications of the malononitrile group or hydroxyl group caused remarkable decreases in the uncoupling activity. It seems probable that the two tertiary butyl groups are necessary for the uncoupling activity, since Horiuchi *et al.*[8] reported that substitution of two bulky *tert*-butyl groups for iodine in 3,5-diiodo-4-hydroxybenzonitrile increased biocidal activity. This is consistent with results obtained with some uncouplers, such as alkyl dinitrophenols[12] and *N*-phenylanthranilic acid derivatives[9,13], in which the essential role of hydrophobic groups was emphasized. It was noted that the introduction of electron withdrawing groups at the *para* position caused remarkable increases in the activities of these compounds. A similar fact has already been reported by Williamson and Metcalf[7] for powerful uncouplers, such as 5-chloro-3-*tert*-butyl-2′-chloro-4′-nitrosalicylanilide(S-13), 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole(TTFB) and carbonyl cyanide *m*-chlorophenylhydrazone(CCCP) in which the strong electron-withdrawing centers are located within a certain spacial distance from a halogenated aryl ring. The strong electron withdrawing property of the malononitrile group in SF 6847 was confirmed by solvent effect experiments; a large shift of the absorption peak was observed when the absorption spectrum of SF 6847 was measured in various non-polar solvents with different refractive indices, however, the shift was small in the derivatives with less activity and was negligible in some known uncouplers, such as dinitrophenol and flufenamic acid. A wide range of spectral changes of malononitrile compounds in mixtures of alcohols and water was also described by Lauerer *et al.*[14], who proposed the formation of charge transfer complexes between the compounds and the solvent molecules. A free hydroxyl group in the aromatic ring is probably not necessary for uncoupling activity, since the 4-acetoxy congener of the malononitrile derivative (SF 6884), which has no free hydroxyl group, still exhibits considerable activity (relative activity, 71 times that of dinitrophenol in the ATPase method), and since an abrupt decrease of activity is found even in the presence of a free hydroxyl group when the electron withdrawing force at the *para* position is weak or absent.

It is possible that the electron withdrawing group interacts with the energy conservation site in a direct manner such as nucleophilic attack or formation of a charge–transfer complex. This may easily be explained by a chemical[1,2] or conformational hypothesis, but scarcely in a chemiosmotic hypothesis[3]. The hydroxyl group acts as an electron donor to the electron withdrawing group as illustrated in Fig. 2. The figure also shows that bulky hydrophobic groups are necessary for binding with the hydrophobic region located in the vicinity of the energy conservation site. The distance between these groups may be about 5 Å. It is likely that the scheme reflects a topological aspect of the

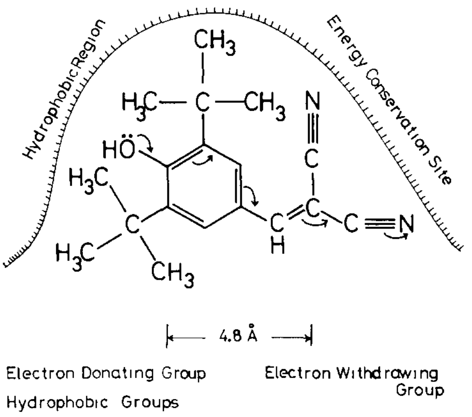


Fig. 2. Possible mechanism of uncoupling by SF 6847.

coupling site in the mitochondrial membrane.

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