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Inhibition of electron transport of rat-liver mitochondria by synthesized antimycin A analogs

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A series of antimycin A analogs was synthesized by replacement of a dilactone-ring moiety of natural antimycin A by various alkyl, substituted phenyl, substituted diphenyl ether, or amino acid ester groups. The structure-inhibitory activity relationship was studied with rat-liver mitochondria to identify roles of the dilactone-ring moiety in the inhibitor binding to a Q_i reaction center of cytochrome bc_1 complex. All derivatives caused further reduction of cytochrome *b* reduced by succinate and the oxidant-induced reduction, showing that the derivatives inhibited electron transport by interacting with a Q_i reaction center. The inhibition tended to increase as the hydrophobicity of the inhibitor increased. The mode of binding of inhibitor molecules to a Q_i center, which was reflected in, for example, a sigmoidal titration curve for respiratory inhibition and a time-dependent change in inhibitory activity, varied depending on structure. These results suggested that the role of the dilactone-ring moiety of antimycin A may be not only to support hydrophobic interaction with the binding domain by increasing the hydrophobicity of the molecule, as proposed earlier, but also to regulate close fitting of the salicylic acid moiety to the binding domain.

Introduction

Antimycin A inhibits cyt. bc_1 complex activity by interacting with a Q_i center with high specificity [1]. This inhibitor has been useful in the development of mechanistic concepts and evolutionary aspects of this complex [1–3]. Information about the antimycin A binding domain on cyt. *b* has been obtained with use of mitochondrial mutants resistant to this inhibitor [2,4–6], but much remains to be learned. To identify the structure of the antimycin A binding domain of cyt. *b*, investigations on structural aspects of the antimycin A molecule needed for inhibition would be helpful.

Structure-inhibitory activity studies of antimycin A and its analogs showed that a salicylic acid moiety, especially the hydroxy group, is essential for inhibitory activity [7–10]. A dilactone-ring moiety, on the other hand, has been thought to have a supporting role in inhibitor binding to a Q_i center by increasing the hydrophobicity of the molecule [11,12]. However, structural modifications of antimycin A analogs studied so far seem to be insufficient to evaluate details of the structure required for inhibition action by antimycin A. In earlier studies it was not specified whether or not the antimycin A analogs synthesized had inhibitory characteristics similar to those elicited by natural antimycin A (such as giving a sigmoidal titration curve for respiratory inhibition and affecting the redox status of cyt. *b*).

Using an unnatural (−)-antimycin A_3, we found that the configuration of the dilactone-ring moiety is important for inhibition (i.e., for its binding to a Q_i center) [13]. The configuration of the antimycin A molecule, when appropriate, may allow tight fitting of the salicylic acid moiety into the binding domain. That study suggested that the dilactone-ring moiety may do more than simply increase the hydrophobicity of the molecule.

In this study, we synthesized a series of antimycin A analogs that have the natural salicylic acid moiety but not the dilactone-ring moiety, and examined electron transport inhibition with isolated rat-liver mitochondria. The inhibition caused by natural antimycin A and the synthesized analogs was compared.

Materials and Methods

Materials

Compound 2 was the generous gift of the Wellcome Research Laboratories (Kent, U.K.) [10]. Rotenone,

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Abbreviations: cyt, cytochrome; DCC, dicyclohexylcarhodiimide; SF6847, 3,5-di-*tert*-butyl-4-hydroxybenzylidene malononitrile.

myxothiazol and antimycin A_3 were obtained from Sigma. Other reagents were of the purest grade commercially available.

Methods

Mitochondria were isolated as described by Myers and Slater [14] from the livers of adult male Wistar rats in a medium containing 250 mM sucrose and 2 mM Tris-HCl (pH 7.4). The amount of mitochondrial protein was measured by the method of Bradford [15] with bovine serum albumin as the standard. Mitochondrial respiration with 10 mM succinate as the respiration substrate was measured with a Clark oxygen electrode at 25°C, the final mitochondrial protein concentration in the medium being 0.7 mg/ml. The incubation medium consisted of a mixture of 200 mM sucrose, 2 mM $MgCl_2$, 1 mM EDTA, and 2.5 μM rotenone in 2.5 mM potassium phosphate buffer (pH 7.4), and the total volume was 2.5 ml.

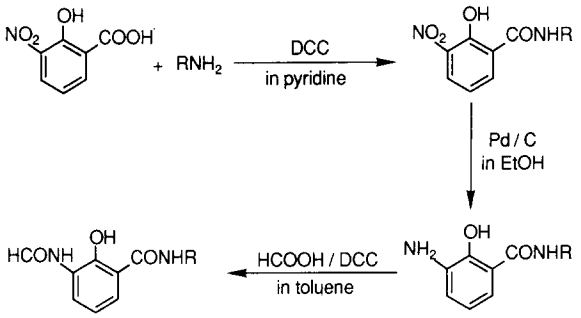
The redox status of cytochrome *b* of the intact mitochondria was measured before and after each treatment with the wavelength pair of 563 and 577 nm [16]. Absorbance spectra were measured with a Shimadzu UV-3000 spectrophotometer with a 1-nm bandwidth. The reaction medium was the same as that used for the respiration experiment except that 1 mM KCN was included. The final mitochondrial protein was 1.4 mg/ml.

The retention time in HPLC was measured at 40°C with a TSK-ODS (80Tm) column (4.6 × 150 mm, Tosoh Corp., Japan) and $CH_3CN:H_2O$ (70:30, v/v) as the eluent, with a flow rate of 1 ml/min.

Synthesis

Compounds were prepared as shown in Scheme I. 2-Hydroxy-3-nitrobenzanilides were obtained by the condensation of 2-hydroxy-3-nitrobenzoic acid, which was prepared by the method of Meldrum and Hirre [17], with appropriate amines in the presence of dicyclohexylcarbodiimide (DCC) in dry pyridine [18]. The resultant amides were hydrogenated over 10% palladium on carbon in ethanol at atmospheric pressure [10]. Formylation of 2-hydroxy-3-aminobenzamides was done by the addition of formic acid and DCC in toluene at room temperature [10].

The amines (the precursors of compounds 1, 3, and 4) were commercially available. The *p*-(substituted phenoxy)anilines (the precursors of compounds 5–11) were prepared by the reduction of *p*-(substituted phenoxy)nitrobenzenes, which were obtained by the condensation of 1-bromo-4-nitrobenzene with the corresponding substituted phenols (which were commercially available) in the presence of K_2CO_3 in dimethyl sulfoxide [19]. The same method was used for the preparation of aniline (the precursor of compound 12) except that 1-bromo-2-chloro-4-nitrobenzene was used.



Scheme 1.

The amino acid esters for the preparation of compounds 17–23 were obtained by the condensation of glutamic acid and the corresponding alcohol in the presence of *p*-toluensulfonic acid in benzene. The same conditions were used for the preparation of aspartates (the precursors of compounds 13–16) with aspartic acid. L-Amino acids were used for the preparation of all amino acid ester derivatives except compound 14.

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 ±0.3%.

Results

Effects of antimycin A analogs on redox status of cytochrome b

The binding site of natural antimycin A is a Q_i center of the cyt. bc_1 complex [3]. We set out to identify the binding site of synthesized antimycin A analogs from their effects on the redox status of cyt. *b* (Fig. 1). The control experiments on the effects peculiar to natural antimycin A_3 (i.e., the extra reduction of cyt. *b* reduced by succinate and an oxidant-induced reduction of cyt. *b* [3]) are shown in Fig. 1A. The oxidant-induced reduction was prevented by the pres-

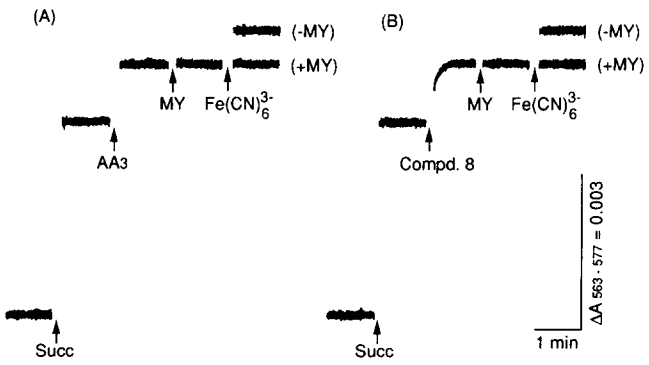


Fig. 1. Effects of compound 8 on the reduction of cytochrome *b* by succinate. Mitochondria were incubated in the reaction medium for 10 min before the addition of succinate. Where indicated, 10 mM succinate (Succ), 0.1 μM antimycin A_3 (AA_3), 0.1 μM myxothiazol (MY), 1 μM compound 8, and an excess of potassium ferricyanide ($Fe(CN)_6^{3-}$) were added.

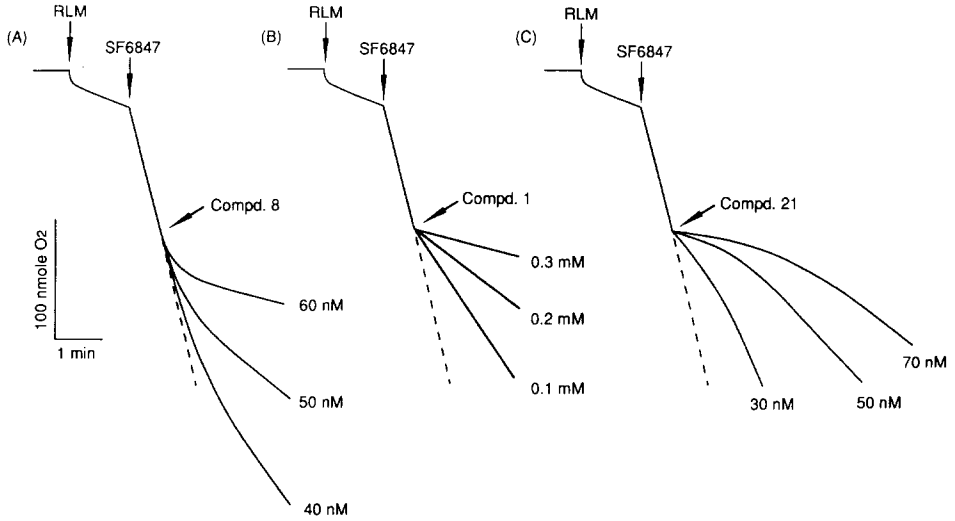


Fig. 2. Effects of compounds 8 (A), 1 (B) and 21 (C) on respiration fully stimulated by an uncoupler, SF6847 (40 nM). The dotted lines show the trace in the absence of an inhibitor. RLM, rat-liver mitochondria.

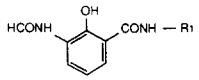
ence of myxothiazol, a Q_o center inhibitor. Compound 8 at 1 μM, which was enough to inhibit uncoupler-stimulated respiration completely, as described later, amplified the reduction of succinate-reduced cyt. *b* to the same level as that observed with natural antimycin A_3 (Fig. 1B). The reduction rate was, however, significantly slower than that observed with natural antimycin A_3. The oxidant-induced reduction of cyt. *b* was observed in the absence of myxothiazol, but not in its presence. Effects with compound 8, for the reduction of cyt. *b*, were similar for all derivatives synthesized (data not shown). The reduction rates with these compounds were different depending on their structure.

Time-dependent changes in inhibition by antimycin A analogs

Natural antimycin A_3 inhibited respiration fully stimulated by SF6847 after a lag, and the inhibition

TABLE I

Chemical structures and inhibition



Compound No.	R_1	pI_{50}	Titration curve [a]	Change in activity [b]	R.T. [c]
1	$(CH_2)_4CH_3$	3.88		±	2.9
2	$(CH_2)_{13}CH_3$	6.80		+	33.4
3	C_6H_5	3.67		±	2.4
4	4-(*n*-C_4H_9)C_6H_4	6.57	S	+	4.8
5	4-(C_6H_5O)C_6H_4	5.76		±	3.6
6	4-(4-*t*-$C_4H_9C_6H_4O$)C_6H_4	6.95	S	+	7.7
7	4-(3-$CF_3C_6H_4O$)C_6H_4	6.80		±	4.8
8	4-(2,6-di-*s*-$C_4H_9C_6H_3O$)C_6H_4	7.32	S	+	17.9
9	4-(2,4-di-*t*-$C_4H_9C_6H_3O$)C_6H_4	7.20	S	+	19.6
10	4-(3,5-di-*t*-$C_4H_9C_6H_3O$)C_6H_4	7.01	S	+	16.5
11	4-(2,4,6-tri-*s*-$C_4H_9C_6H_2O$)C_6H_4	7.00	S	+	49.8
12	3-Cl-4-(4-ClC_6H_4O)C_6H_3	7.08		+	5.7
Natural (+)-antimycin A_3 [d]		7.42	S	+	8.5
Unnatural (−)-antimycin A_3 [d]		5.38		±	8.5

[a] The derivatives that gave a sigmoidal curve for respiratory inhibition are marked S.

[b] The derivatives with inhibition that increased or decreased with time are marked + or −, respectively. The derivatives with inhibition that did not change are marked ±.

[c] Retention time (min) by HPLC with a TSK-ODS column.

[d] From Ref. 13.

increased with time, as in our previous study [13]. Time dependency of inhibition was similar for some derivatives in the present study. As an example, inhibition by compound 8 is illustrated in Fig. 2A. Potent inhibitors such as compounds 9, 12, and 23 caused this kind of time-dependent inhibition. However, the extent of inhibition by poor inhibitors such as compounds 1, 3 and 17 did not change with time, as shown in Fig. 2B, with compound 1 given as an example. The derivatives that caused the time-dependent inhibition like natural antimycin A_3 are marked + in Tables I and II. The derivatives with inhibition not changing with time are marked ±.

It is of interest that the inhibition by some amino acid ester derivatives such as compounds 20 and 21 decreased with time, as shown in Fig. 2C, with compound 21 given as an example. The inhibition was not abolished; rather the final respiration rate was lower than that before the addition of the inhibitor. This kind of decrease in inhibition by compound 21 was also observed for the state 4 respiration (data not shown). The derivatives with activity decreasing with time are marked in − Tables I and II.

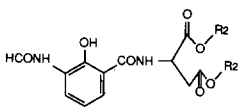
The time-dependent change in inhibition by compound 13 (L-form) was compared with that of the D-form derivative (compound 14) (Fig. 3). Inhibition by the L-form in terms of pI_{50} was more potent than that of the D-form, as described later. In Fig. 3, the concentrations of compounds 13 and 14 were chosen to cause the same extent of initial inhibition (immediately after addition to the mitochondrial suspension). Inhibition by the L-form decreased faster than that by the D-form; that is, the respiration rate, once reduced by the L-form, recovered faster. The final respiration rate caused by the L-form was faster than that caused by the D-form.

Inhibitory titration curve for antimycin A analogs

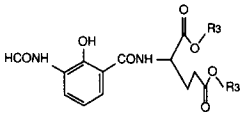
Natural antimycin A_3 gives a sigmoidal curve in respiration inhibitory titration when succinate or NADH is used as the respiration substrate [11–13]. Van Ark and Berden proposed that the sigmoidicity might be due to conformational change of cyt. *b* by

TABLE II

Chemical structures and inhibition [a]



Compound No.		pI_{50}	Titration curve [b]	Change in activity [c]	R.T. [d]
	R_2				
13	$(CH_2)_4CH_3$	7.04 (6.42) [e]		−	5.2
14	$(CH_2)_4CH_3$ [f]	6.63 (6.33)		−	5.2
15	$CH_2C(CH_3)_3$	6.14		+	4.6
16	$(CH_2)_8CH_3$	6.91	S	+	53.0



	R_3				
17	$(CH_2)_2CH_3$	5.32		±	2.6
18	$(CH_2)_3CH_3$	6.62 (6.31)		−	3.8
19	$(CH_2)_4CH_3$	6.97 (6.63)		−	5.6
20	$(CH_2)_5CH_3$	7.31 (7.06)		−	10.2
21	$(CH_2)_6CH_3$	7.50 (7.29)		−	16.8
22	$(CH_2)_7CH_3$	7.28	S	+	38.2
23	$(CH_2)_8CH_3$	6.99	S	+	62.0

[a] Unless otherwise noted, L-form amino acids were used.
[b] The derivatives that gave a sigmoidal curve for respiratory inhibition are marked S.
[c] The derivatives with inhibition that increased or decreased with time are marked + or −, respectively. The derivatives with inhibition that did not change are marked ±.
[d] Retention time (min) by HPLC with a TSK-ODS column.
[e] The value in parenthese is the pI_{50} value estimated from the final respiration rates.
[f] D-form aspartic acid was used.

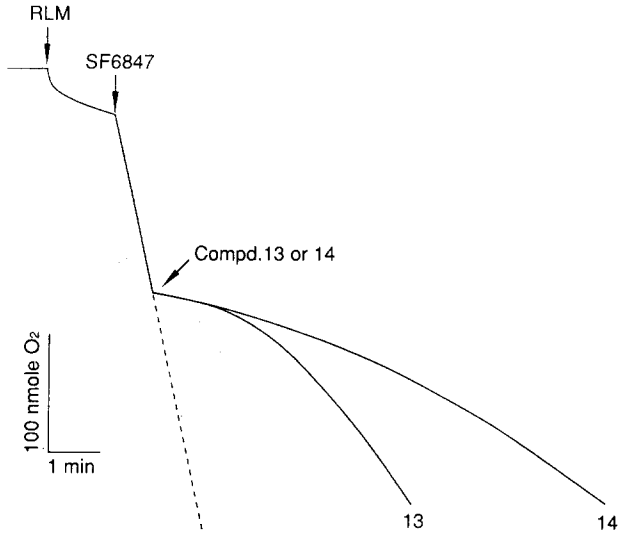


Fig. 3. Effects of compounds 13 (L-form) and 14 (D-form) on respiration fully stimulated by an uncoupler. The concentrations of SF6847, compound 13 and compound 14 were 40 nM, 0.25 μM and 0.42 μM, respectively. RLM, rat-liver mitochondria.

antimycin A binding [20]. Some synthesized derivatives also had a typical sigmoidal relationship for the inhibition of respiration induced by SF6847 (40 nM), as shown in Fig. 4 (open circles), with compound 8 given as an example. The respiration rate changed with time after the addition of compound 8, so the rate was read when it had become stable. In general, strong inhibitors gave this kind of sigmoidal titration curve. The derivatives that did are marked S in Tables I and II. The relationship was not sigmoidal for poor inhibitors such as compounds 1, 3 and 17, as shown in Fig. 4

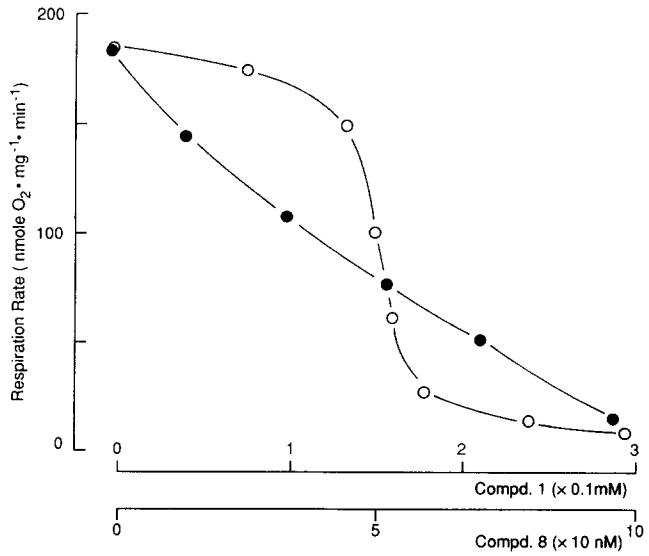


Fig. 4. Titration curve for inhibition of the uncoupler-stimulated respiration by compound 8 (open circles) or compound 1 (closed circles). SF6847 (40 nM) was used as the uncoupler. The respiration rate after the addition of the inhibitor was read when it had become stable.

(closed circles) with compound 1 given as an example. The derivatives with inhibition decreasing with time such as compounds 20 and 21 did not give a sigmoidal titration curve. For these derivatives, the initial respiration rates were read.

Structure-inhibitory activity relationship

To evaluate the ability of synthesized antimycin A analogs to inhibit respiration, we examined their effects on respiration fully stimulated by SF6847 (40 nM). This uncoupler-stimulated respiration is susceptibly inhibited by electron transport inhibitors [21]. The molar concentration (I_{50}) in the incubation medium needed to reduce the respiration rate at full stimulation to half was measured. To obtain the I_{50} value, the final and initial respiration rates were read for derivatives with inhibition that increased and decreased, respectively, with time. The log of the reciprocal of the I_{50}, pI_{50}, was used as the index of inhibition of cyt. bc_1 complex activity. The pI_{50} was measured at least twice and the averaged value is listed in Tables I and II; the standard deviation was within ± 0.07.

The pI_{50} value of natural antimycin A_3 was 7.42. It can be considered that some of the synthesized antimycin A analogs such as compounds 8, 21 and 22 have almost the same inhibitory potency as antimycin A_3 taking an experimental error into consideration. Despite structural variations, inhibition by all of the series of derivatives tended to increase as the hydrophobicity of the inhibitor molecules increased. The hydrophobicity of antimycin A analogs is proportional to their retention times in HPLC with a C_{18} column. The pI_{50} values of amino acid ester derivatives 13–23 are plotted against the retention times in Fig. 5. For the glutamate derivatives, the activity changed parabolically against the hydrophobicity of the compounds, that is, inhibition was maximum with an *n*-heptyl derivative (21). Alkyl chains longer than this were slightly unfavorable for the inhibition. Although differences in the retention times between different series of derivatives did not necessarily correspond to differences in their hydrophobicity because interactions (like hydrogen bonding) between the compound and the reagent packed in the HPLC column differ depending on structure [22], pI_{50} values of compounds 1–12 were also plotted in Fig. 5. Regardless of wide variations in structures, the relationships were almost similar between compounds 13–23 and 1–12, indicating that the activity is primarily dependent on hydrophobicity of the compounds.

Large differences in inhibition were not observed between aspartic and glutamic acid ester derivatives with the same alcohol moiety (13 and 19; 16 and 23). Regardless of hydrophobicity being the same, the inhibitory potency of an L-form of pentyl aspartate derivative (13) was more potent than that of a D-form

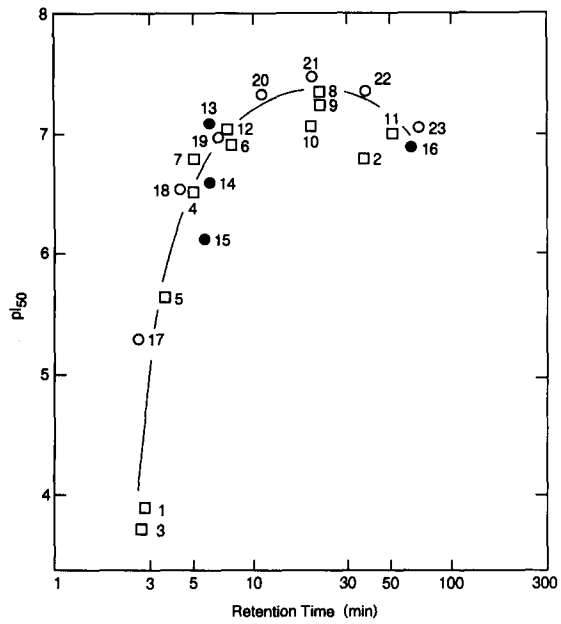


Fig. 5. Relationship between the pI_{50} values and the retention times in HPLC. (○) glutamate derivatives (17–23); (●) aspartate derivatives (13–16); (□) compounds 1–12.

(14). This result together with that shown in Fig. 3 indicates that the hydrophobicity of the inhibitor molecule was not the only factor governing inhibitor binding to the binding domain.

Compounds 13 and 15 had similar hydrophobicity, but 15, with its bulky alkyl groups (R_2 = *neo*-pentyl) caused less inhibition than 13 (R_2 = *n*-pentyl). This result suggested that the bulkiness of the portion corresponding to the dilactone-ring moiety of natural antimycin A may be somewhat unfavorable for close fitting of the inhibitor molecule to the binding domain.

Besides hydrophobicity of the compounds, some steric factors were suggested to be important for the activity from structure-inhibitory activity studies. To separate and quantitatively represent the difference in steric factors of the portion corresponding to the dilactone-ring moiety, we tried to evaluate steric dimension in terms of the liner free-energy related steric parameters such as STERIMOL and E_S [23]. Unfortunately, it was actually impossible to estimate steric parameter by a unique standard because of large flexibility of the structure (especially of alkyl groups).

Discussion

Despite structural variations in the portion corresponding to the dilactone-ring moiety of natural antimycin A, all antimycin A analogs synthesized in this study inhibited electron transport of cyt. bc_1 complex by interacting with a Q_i reaction center. This fact indicated that the dilactone-ring moiety of antimycin A is not essential for its activity and that antimycin A binding to the binding domain at a Q_i center is primarily governed by the salicylic acid moiety. As a substitute for the natural dilactone-ring, hydrophobic structures were favorable for activity. The relation between the activity and hydrophobicity of the compounds was parabolic, not linear, as shown in Fig. 5. In general, the movement of bioactive compounds through biomembrane to their sites of action has been regarded as important in explaining the action of the compounds. The parabolic change of biological activity against hydrophobicity of the compounds (i.e., parabolic model) is thought to be due to the ease of random movement of the compounds from the part of application to the site of action [24]. Antimycin A derivatives have to penetrate into the matrix side through the inner membrane since the Q_i center locates in the matrix side. Based on the parabolic model [24], the parabolic relation shown in Fig. 5 indicates that the penetration process of antimycin A derivatives through the inner membrane may be one of the important factors deciding the activity. Some portion of added inhibitors which have great hydrophobicity such as compounds 22 and 23 may be trapped in the membrane phase. This is probably the cause of a decrease in their activity. Regardless of the wide variation in structures, the activity of antimycin A derivatives, anyhow, primarily depend on the hydrophobicity of compounds. These results seem to be consistent with the suggestion [11,12] that the dilactone-ring moiety has a supporting role in inhibitor binding, by increasing the hydrophobicity of the molecule.

However, the manner of binding of antimycin A analogs to a Q_i center, seen in the kind of titration curve for respiratory inhibition and in time-dependent changes in the inhibitory activity, varied depending on structure. These findings, along with those of our previous study [13], suggest that role of the dilactone-ring moiety may be not only to contribute to the hydrophobic interaction of the inhibitor to its binding domain as described above, but also to regulate close fitting of the salicylic acid moiety to the binding domain. To support the close fitting, some steric factors such as steric bulkiness and configuration were shown to be important. The existence and positions of the functional groups of the natural dilactone-ring moiety, such as carbonyl groups and ether oxygens, probably make important contributions to close binding of the inhibitor. This argument is supported by the finding that although the hydrophobicity of natural dilactone-ring moiety in terms of retention time was much lower than that of potent synthesized analogs such as compounds 8, 9, 20, 21 and 22, they elicited almost the same extent of activity.

It is of interest that the inhibition by some amino-acid ester derivatives, like compound 21, decreased with time. The decrease in the activity may be due to a

decrease in the binding affinity of the inhibitor molecule to the binding site. The decrease in the concentration of effective inhibitor that arises from metabolic decomposition, such as hydrolysis of an ester bond in the mitochondrial matrix, could not account for the time-dependent decrease in the activity because the inhibition by other amino acid ester derivatives did not necessarily decrease. On the basis of the same idea, the time-dependent increase in the activity observed for natural antimycin A_3 and some synthesized derivatives like compound 8 might be due to an increase in binding affinity. The change in binding affinity may arise from conformational changes in the cyt. bc_1 complex caused by inhibitor binding to the proteinous complex. A conformational change in the cyt. bc_1 complex caused by natural antimycin A binding has been reported [11,20,25,26]. Structural variations in inhibitors might be reflected in differences in manner and extent of the conformational change in the cyt. bc_1 complex.

Van Ark and Berden proposed that the sigmoidal titration curve for respiratory inhibition of Q_i center inhibitors may be related to the binding affinity of the inhibitor to cyt. bc_1 complex [20]. In fact, potent inhibitors tended to elicit the sigmoidal titration curve as shown in Tables I and II. However, it seems to be difficult to explain the sigmoidicity only by the binding affinity of the inhibitor, since potent inhibitors such as compounds 12, 20 and 21 did not necessarily elicit sigmoidicity. It is reasonable to consider that the sigmoidicity may be also related to a conformational change of cyt. bc_1 complex by inhibitor binding.

Many mitochondrial mutants resistant to antimycin A have been isolated. The genetic mapping and sequence analysis of such resistant mutations of cyt. *b* has shown changes in amino acids in the cyt. *b* protein [2,4–6]. It is still not known which portion of the antimycin A molecule interacts with the mutated position in cyt. *b*. Perhaps, close binding of the salicylic acid moiety (probably a phenolic hydroxy group) to the binding domain is affected by the local change in amino acids. However, results of this study remind us not to neglect the possibility that antimycin A resistance might be related to direct or indirect interactions between the dilactone-ring moiety and the mutated site, which regulates tight fitting of the salicylic acid moiety to the binding domain.

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