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Structural factors of antimycin A molecule required for inhibitory action

Nobuya Tokutake, Hideto Miyoshi *, Takashi Satoh, Taku Hatano, Hajime Iwamura

Department of Agricultural Chemistry, Kyoto University, Kyoto 606, Japan

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Abstract

A series of antimycin A analogues was synthesized by modifying the salicylic acid moiety, whereas the portion of the molecule corresponding to the natural dilactone-ring moiety was fixed as di-n-octyl L-glutamate. To probe the structure of the antimycin A binding site, the structural factors of the salicylic acid moiety required for inhibitory action were examined by means of structure-activity studies with intact rat-liver mitochondria and the cytochrome bc_1 complex isolated from bovine heart mitochondria. As suggested earlier (Rieske, J.S. (1976) Biochim. Biophys. Acta 456, 195–247), the phenolic OH was very important for inhibition. For the derivatives which do not possess a formylamino group in the 3-position (*ortho* to the phenolic OH), the inhibitory activity tended to increase as the electron-withdrawing property of the substituent increased, i.e., as the acidity of the phenolic OH group increased. This indicates that the acidity of the phenolic OH is an important factor governing inhibition. While the electron-withdrawing property of the formylamino group itself is rather poor, 3-formylamino derivatives elicited potent activity. The conformation of the 3-formylamino group was also found to be a very important factor in establishing inhibitory activity. In addition, the bulkier the moiety corresponding to the 3-formylamino group, the lower the activity. These results demonstrate that the presence of the 3-formylamino group, and its proper conformation, are needed for a close fitting of antimycin A to its binding domain. Although the inhibitors that lack a 3-formylamino group retained fairly potent activity, their effects on the reduction of cytochromes *b* and c_1 were somewhat different from those of natural antimycin A, indicating that the 3-formylamino group is essential for inhibitor binding to the cytochrome bc_1 complex in the same manner as natural antimycin A. It is concluded that both the 3-formylamino group and the phenolic OH of antimycin A make important contributions to specific interactions with the amino acid residues of the cytochrome *b*.

Key words: Antimycin A; Cytochrome bc_1 complex; Mitochondrion; Structure–activity relationship

1. Introduction

The ubiquinol-cytochrome *c* oxidoreductase (cytochrome bc_1 complex) is a membrane-bound, protonmotive multisubunit complex involved in cellular energy transduction. The properties of several specific inhibitors have been helpful in the development of models for structure and mechanism of the bc_1 complex. Among these, the affinity of antimycin A in submitochondrial particles ($K_d = 3.2 \times 10^{-11}$ M [1]) is the highest measured so far of all the inhibitors of the cytochrome bc_1 complex [2]. Antimycin A binds to the Qi center of the cytochrome bc_1 complex and blocks electron transfer from the heme b_h center to ubiquinone [3]. Numerous yeast mutants resistant to antimycin A have been isolated [4–7]. By genetic mapping and sequence analyses of these resistance mutations, information about the antimycin A binding domain of cytochrome *b* has been obtained [6–8], but much still remains to be learned. For instance, the portion of the antimycin A molecule that interacts with the mutated position in cytochrome *b* remains unknown. To probe the structure of the antimycin A binding domain of cytochrome *b*, investigations into the structural features of antimycin A molecule required for inhibitory action will be helpful.

Antimycin A consists of a 3-formylaminosalicylic acid moiety linked via an amide bond to an alkyl- and acyl-substituted dilactone-ring (Fig. 1). Based upon

* Corresponding author. Fax: +81 75 7536128.

Abbreviations: cytochrome bc_1 complex, ubiquinone-cytochrome *c* reductase; DBH, 2,3-dimethoxy-5-methyl-6-n-decyl-1,4-benzoquinol; Q_o, ubiquinol-oxidizing site; Q_i, ubiquinone-reducing site; SF6847, 2,6-di-*tert*-butyl-4-(2,2-dicyanovinyl)phenol.

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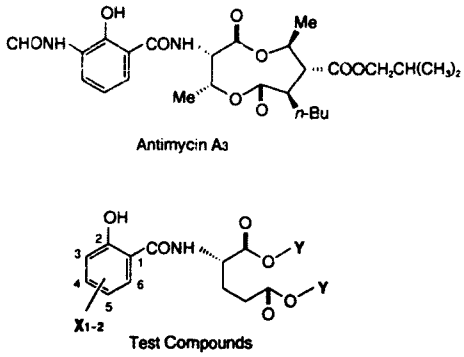


Fig. 1. Chemical structure of antimycin A_3 and synthetic antimycin A analogs. In compounds **1–23**, Y = n-octyl. Compound **24**: X = 5-NO_2, Y = n-propyl, compound **25**: X = 3-NO_2, Y = n-propyl, compound **26**: X = 3-NHCHO, Y = n-propyl.

structure-activity studies performed in several laboratories [9–12], Rieske reviewed the structural factors of antimycin A required for the inhibitory activity [13,14]. He concluded that (1) the natural dilactone-ring is not essential to inhibition and has a supporting role in binding to the Qi center by increasing the hydrophobicity of antimycin A, and that (2) the phenolic OH group with proper pK_a value modified by an electron-withdrawing substituent such as a nitro group in the *ortho* or *para* position is absolutely essential for the activity. Both of these views, however, seem to be somewhat incomplete as the descriptions of the structural features of antimycin A that determine inhibition. Regarding the first conclusion, using a synthetic stereoisomer of natural antimycin A_3 ((−)-antimycin A_3), Miyoshi et al. showed that the configuration of antimycin A (i.e., the spatial position of the benzene-ring plane relative to that of the dilactone-ring) is very important for its inhibitory activity [15]. That study indicated that the natural dilactone-ring moiety may do more than simply increase the hydrophobicity of the molecule. According to the latter conclusion of Rieske's study, the formylamino group (*ortho* to the phenolic OH) of the natural salicylic acid moiety is not essential for inhibition. Furthermore, the formylamino group has to be an electron-withdrawing substituent to increase the acidity of the phenolic OH group. However, the Hammett-type σ value of the formylamino in *ortho* position (σ_{ortho}), which is the index of the electron-withdrawing property and taken to be equal to σ_{para} [16], is zero [17], indicating that the formylamino group is not necessarily an electron-withdrawing substituent. It is likely, therefore, that Rieske's view of the structural aspects of antimycin A is insufficient and requires modification.

We recently showed that the natural dilactone-ring is not essential for the activity of antimycin A [18], suggesting that its binding to a Qi center is primarily governed by specific interaction(s) between the salicylic acid moiety and some amino acid residue(s) in the binding site. As substitutes for the natural dilactone-ring, flexible and hydrophobic structures such as long alkyl chains and substituted diphenyl ethers are appropriate [18]. Although the importance of the salicylic acid moiety in activity has been suggested by several structure-activity studies of antimycin A and its analogues [9–12], structural modifications of antimycin A analogues in those studies seem to be insufficient to evaluate details of the structural factors required for the inhibitory action. So long as the portion of the molecule corresponding to the dilactone-ring moiety is fixed as the natural chemical structure, wide structural modifications of the salicylic acid moiety might be not easy to achieve because total synthesis of the natural dilactone-ring is still difficult dispite recent progress [19,20].

In this study, we synthesized a series of antimycin A analogues by modifying the salicylic acid moiety, while fixing the portion corresponding to the dilactone-ring as a di-n-octyl L-glutamate (Fig. 1). This substitute is a mimetic structure of the natural dilactone-ring moiety [18]; that is, the synthetic antimycin A analog carrying this substitute and natural salicylic acid moiety binds to the Qi center of the cytochrome bc_1 complex and has similar inhibitory potency to that of natural antimycin A_3. To identify the structural factors of antimycin A molecule required for its inhibitory action, the structure-inhibitory activity relationship was examined with intact rat-liver mitochondria and the cytochrome bc_1 complex isolated from beef heart mitochondria.

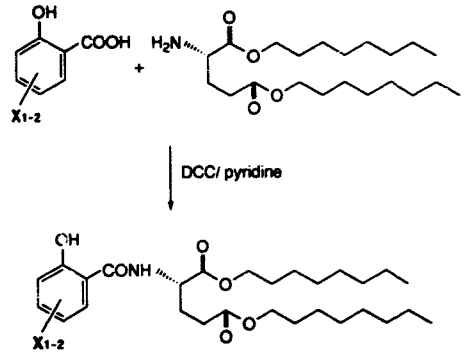
2. Experimental procedures

Materials

Rotenone, antimycin A_3, and cytochrome *c* (horse heart) were purchased from Sigma. Myxothiazol was obtained from Boehringer. SF6847 and 2,3-dimethoxy-5-methyl-6-n-decyl-1,4-benzoquinol (DBH) were the same samples as those used previously [21]. Other reagents were of the purest grade commercially available.

Synthesis

Compounds **1–23** were prepared by condensing the substituted salicylic acids with di-n-octyl L-glutamate in the presence of dicyclohexylcarbodiimide (DCC) in dry pyridine [18] (Scheme 1). Di-n-octyl L-glutamate was synthesized as previously described [18]. The substituted salicylic acids (the precursors of compounds **1–7** and **12**) were commercially available. To drive the nitrosalicylic acids (the precursors of compounds **8–11** and **13–15**), the corresponding commercially available



Scheme 1.

salicylic acids were first converted to methyl salicylates in the presence of 4-toluenesulfonic acid in methanol to facilitate the separation of *ortho* (3-position) and *para* (5-position) nitro derivatives by silica gel column chromatography. These methylsalicylates were nitrated by the method of Meldrum and Hirre [22]. Following the separation of nitro salicylates, the compounds were hydrolyzed to the acid form. The 3-formylamino derivatives (compounds **16**, **17**, **18** and **19**) were derived from the corresponding nitro derivatives (compounds **8**, **10**, **9** and **11**, respectively) by hydrogenation of the nitro group over 10% palladium on carbon in ethanol and then formylation of the resulting amine derivatives [12,18]. Compounds **20**, **21** and **22** were synthesized by acylation of the 3-amino derivative, which was prepared from compound **8** by reduction over 10% palladium carbon in ethanol [12], with acetic, propionic and cyclohexyl acetic acids, respectively, in the presence of DCC in toluene. 2-Methoxy-3-nitrosalicylic acid (the precursor of compound **23**) was obtained by hydrolyzing methyl 2-methoxy-3-nitrosalicylate, which had been prepared by nitration of methyl 2-methoxysalicylate.

Compounds **24–26** were synthesized by the same method as described above, except that di-n-propyl L-glutamate was used in place of di-n-octyl L-glutamates.

All synthesized compounds were characterized by ^{1}H-NMR spectra (JEOL PMX-60 and JEOL GSX-400) and elemental analyses for C, H and N, within an error of ±0.3%.

Methods

Mitochondria were isolated from the livers of adult male Wistar rats as described by Myers and Slater [23]. Rat-liver mitochondrial respiration with 10 mM sodium succinate as the substrate was measured with a Clark-type oxygen electrode at 25°C, the final mitochondrial protein concentration in the medium being 0.7 mg/ml. The assay buffer consisted of 200 mM sucrose, 2 mM $MgCl_2$, 1 mM EDTA, and 2 μM rotenone in 2.5 mM potassium phosphate (pH 7.4), and the total volume was 2.5 ml. Protein concentration was determined by the biuret method [24] with bovine serum albumin as the standard. The molar concentration of the inhibitor (I_{50}) needed to halve the uncoupled respiration rate by SF6847 (40 nM) was measured. The pI_{50}, the log of the reciprocal of I_{50}, is used here as the index of the inhibitory potency.

The cytochrome bc_1 complex was prepared according to Rieske [25] from bovine heart mitochondria and stored at −75°C in medium consisting of 20% glycerol and 25 mM Tris-HCl buffer (pH 8.0). The concentration of heme *b* was determined from the reduced minus oxidized spectrum using 562–575 nm wavelength pair ($\epsilon = 28.5\ mM^{-1}\ cm^{-1}$) [26].

The relative extent of reduction of the heme centers of the isolated cytochrome bc_1 complex (2.5 μM) was determined under varying conditions using a stirred cuvette in a Shimadzu UV3000 spectrophotometer in the dual-wavelength mode; the wavelength pairs 562–575 nm for cytochrome *b* and 553–540 nm for cytochrome c_1 were used [26]. The incubation medium consisted of a mixture of 2 mM NaN_3, 0.5 mM EDTA, 20 mM $MgCl_2$ and 0.01% Tween-20 in 55 mM Tris-HCl (pH 7.4). After adding 1.25 μM $K_3[Fe(CN)_6]$, and preincubation with an excess of inhibitor, 9 μM DBH was added.

Cytochrome bc_1 complex activity was measured at 25°C as the rate of cytochrome *c* (horse heart) reduction with the wavelength pair 550–540 nm [26] using a stirred cuvette in a final volume of 2 ml. After adding a cytochrome *c* (20 μM), cytochrome bc_1 complex was added to a final concentration of 2.6 nM cytochrome *b*. The protein was incubated for 2 min with inhibitor, then the reaction was started by adding DBH (20 μM). The assay medium consisted of a mixture of 2 mM NaN_3, 0.5 mM EDTA, 20 mM $MgCl_2$ and 0.01% Tween-20 in 55 mM Tris-HCl (pH 7.4). The molar concentration of inhibitor (I_{50}) needed to reduce cytochrome *c* reductase activity by half was measured.

Inhibition by antimycin A_3 and compound **16** was also measured with submitochondrial particles prepared from bovine heart mitochondria by the method of Matsuno-Yagi and Hatefi [27]. The particles were treated with sodium deoxycholate (0.3 mg/ mg protein) before dilution with reaction medium [28]. Cytochrome bc_1 complex activity was measured at 25°C as the rate of cytochrome *c* reduction with DBH as substrate. The reaction medium consisted of a mixture of 0.25 M sucrose, 1 mM $MgCl_2$, 2 mM KCN, 20 μM DBH, 20 μM cytochrome *c* and 50 mM phosphate buffer (pH 7.4), the final mitochondrial protein concentration being 30 μg/ ml.

The acid dissociation constant (K_a) was measured in 10% ethanol/water (v/v) spectrophotometrically (25°C) with a Shimadzu UV3000 spectrophotometer.

The pH of the buffers was measured with a Horiba pH meter M-12. The pH readings were not corrected for the effect of ethanol on the apparent acid dissociation constant, since comparisons between dissociation constants of the related compounds in a mixed solvent are valid if the amount of the organic solvent present is constant [29].

3. Results

Structure–activity relationship of synthetic antimycin A analogues

Table 1 summarizes the compound structures and inhibitory activities in terms of the pI_{50} with intact rat-liver mitochondria and the isolated cytochrome bc_1 complex. The pI_{50} was measured at least twice and the values then averaged. In all cases, the standard deviation were no more than ± 0.07 and ± 0.05 with mitochondria and the cytochrome bc_1 complex, respectively. The sum of Hammett-type σ constants of the substituents is also listed in Table 1. Compound **16** was the most potent inhibitor of all synthetic antimycin A analogues. The activity of this compound was about 1/2 and 1/20th that of antimycin A_3 with intact mitochondria and the isolated cytochrome bc_1 complex, respectively. With submitochondrial particles prepared from beef heart mitochondria, the activity of **16** was about 1/2 that of antimycin A_3 (I_{50} value of **16** and antimycin A_3 was 0.42 and 0.22 nmol/mg protein, respectively). The poorer activity with the isolated cytochrome bc_1 complex may be due to nonspecific binding of a part of the compound added to some hydrophobic region of the protein complex owing to hydrophobic interaction, since this compound is much more hydrophobic than antimycin A_3 [18]. With intact mitochondria and submitochondrial particles, the hydrophobic compound distributes into lipid bilayer phase, enabling it to access the action site of the cytochrome bc_1 complex. It has been suggested that phospholipids are involved in inhibitor binding to cytochrome bc_1 complex [30,31]. Taking this into account, we cannot exclude the possibility that a decrease in binding affinity of the inhibitor to the isolated cytochrome bc_1 complex, due to some sort of structural change of the complex, occurs when enzyme complex is extracted by detergents.

Table 1
Structures, inhibitory activities and physical properties of test compounds [a]

No.	Substituents (X_{1-2})	pI_{50}		m.p. (°C)	$\Sigma\sigma_{o,m,p}$ [b]
		cyt. bc_1 [c]	RLM [d]		
1	H	3.80	3.92	oil	0
2	4-CH_3	3.90	3.68	oil	−0.07
3	5-CH_3	3.85	3.50	35–36	−0.17
4	3-Cl	5.74	4.94	oil	0.23
5	4-Cl	3.99	3.97	oil	0.37
6	5-Cl	4.03	4.28	38–40	0.23
7	5-Br	3.94	3.83	49–50	0.23
8	3-NO_2	7.06	6.70	35–36	0.78
9	3-NO_2-5-Cl	6.93	6.63	oil	1.01
10	3-NO_2-4-CH_3	7.19	6.68	oil	0.71
11	3-NO_2-5-CH_3	6.99	6.56	35–36	0.61
12	5-NO_2	7.15	6.87	33–34	0.78
13	5-NO_2-4-CH_3	6.45	6.59	oil	0.71
14	5-NO_2-3-CH_3	7.16	6.94	oil	0.61
15	3,5-$(NO_2)_2$	6.75	6.96	71	1.56
16	3-NHCHO	7.81	7.20	35	0
17	3-NHCHO-4-CH_3	5.27	5.72	48–50	−0.07
18	3-NHCHO-5-Cl	7.66	7.14	85	0.23
19	3-NHCHO-5-CH_3	7.59	7.19	33–34	−0.17
20	3-$NHCOCH_3$	6.64	6.48	oil	0
21	3-$NHCOCH_2CH_3$	5.39	4.46	74–75	0 [e]
22	3-$NHCOCH_2$-$\widehat{CH_2(CH_2)_4CH_2}$	5.37	4.14	28	0 [e]
23	3-NHCHO-2-OCH_3 [f]	5.30	3.90	41–42	0
Natural antimycin A_3		9.15 [g]	7.38		

[a] In Fig. 1, Y = n-octyl.
[b] The sum of Hammett-type σ constants. Unless otherwise noted, the σ constants were cited from Ref. [17]. The σ_{ortho} was taken to be equal to σ_{para} (Ref. [16]).
[c] Assays with isolated cytochrome bc_1 complex.
[d] Assays with intact rat-liver mitochondria.
[e] Taken as the same as that of $NHCOCH_3$.
[f] The phenolic OH group was replaced by a methoxy group.
[g] This concentration is equal to 0.55 mol antimycin A_3/ mol cytochrome c_1.

The methylation of the phenolic OH on the salicylate moiety resulted in a decrease in inhibition (**16** *vs.* **23**), demonstrating that the free OH group is very important for inhibition as suggested earlier [13,14]. To examine whether the methoxy derivative can occupy the binding site without inhibiting the catalytic reaction, we measured the inhibitory activity of **16** using cytochrome bc_1 complex treated with an excess of the methoxy derivative (**23**, 720 nM). Compound **23** at this concentration elicited a 2–5% inhibition of the control activity in preliminary studies. As seen in Fig. 2, the inhibitory activity of **16** was not reduced in the presence of **23**, indicating that excess **23** could not replace **16** from its binding site. It is concluded, therefore, that the phenolic OH group is important for the inhibitor binding to the binding site.

The inhibitory activities of methyl (**2**, **3**) and halogen (**4–7**) derivatives are, in general, much weaker than those of nitro derivatives (**8–15**). This might be due to the electron-donating and weak electron-withdrawing properties of the methyl and halogen groups, respectively, compared with the nitro group. Irrespective of the same hydrophobic and electronic properties of the substituents, compound **13** was slightly, but significantly, poorer than compound **14**. Considering that the electron-withdrawing property of the 5-nitro group is decreased by the presence of the vicinal methyl (4-methyl), but not by 3-methyl, since the plane of the

5-nitro group is twisted against the benzene-ring plane by steric congestion arising from vicinal methyl [32], this result also indicates that the electron-withdrawing property of nitro group at the 5-position favors the activity. The activity of the 3,5-dinitro derivative (**15**) was poorer than that of mononitro derivatives (**8** and **12**), suggesting that there is an optimal acidity of the phenolic OH for inhibitory activity.

Despite the weak electron-withdrawing property of formylamino group (i.e., σ_{para} $(=\sigma_{ortho})=0$ and $\sigma_{meta}=0.19$ [17]), the activities of 3-formylamino derivatives, except compound **17**, were greater than those of the nitro derivatives with both assay systems. This suggests that inhibition is not governed solely by the electron-withdrawing property of that substituent. To identify the role of the formylamino group at the 3-position (*ortho* to phenolic OH) in the inhibitory action, we investigated the poorer activity of compound **17** relative to that of **19**. A methyl group was introduced to the 4- (vicinal to 3-formylamino) and 5-positions in compounds **17** and **19**, respectively. Despite the difference in the position of substitution, the hydrophobic and electronic properties of the two compounds can be considered to be similar. The vicinal methyl group might affect the conformation of the 3-formylamino group by steric congestion, resulting in interference of the specific interaction between the 3-formylamino group and the binding domain. To obtain information about the conformation of the 3-formylamino group, 400 MHz ^{1}H-NMR spectra of compound **17** and related compounds were recorded in chloroform-d_1 at 25°C. The signal for the phenolic OH proton of compound **16** appeared at 12.93 ppm. The ^{1}H-NMR spectra of compound **17** showed signals at 12.60 and 12.75 ppm, assigned to the phenolic OH, and at 2.29 and 2.34 ppm, assigned to 4-methyl protons, in a ratio of 4:5, respectively (Fig. 3A). Upon increasing the temperature to 50°C, the signals of the OH proton broadened (12.54 and 12.69 ppm) and the two signals of methyl protons coalesced, giving a broad single resonance at 2.30 ppm (Fig. 3B). In contrast, the phenolic OH proton and 5-methyl protons of compound **19** showed a single resonance at 12.64 and 2.30 ppm (25°C), respectively. These results indicated that the 3-formylamino group of compound **17**, but not **19**, takes two stable conformations in the presence of a vicinal (4-position) methyl group under the experimental conditions, probably by steric congestion. Since the NH proton of the formylamino group showed a single resonance at 7.58 ppm, these two conformers may not simply reflect the two isomers arising from a rotation around the N–C bond axis of the formylamino group. Thus, it is confirmed that a conformational change of the 3-formylamino group occurs by introducing a methyl group to the 4-position. Although the two conformations of the 3-formylamino group are still not characterized, the drastic decrease in the activity of compound **17** should be due to the conformational changes of 3-formylamino group. In other words, the 3-formylamino group is not able to take an active conformation due to a decrease in rotational freedom. On the other hand, the downfield-shifted signal of the phenolic OH proton was observed irrespective of the sub-

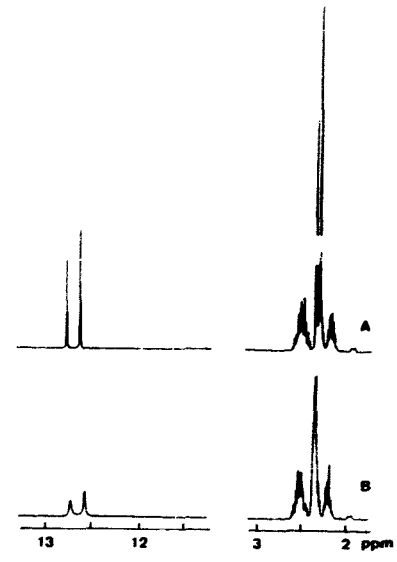


Fig. 3. 400 MHz ^{1}H-NMR spectrum of compound **17** in chlorform-d_1. Variable-temperature studies were performed using the standard temperature-regulation apparatus of the spectrometer. Temperature: 25°C (A) and 50°C (B).

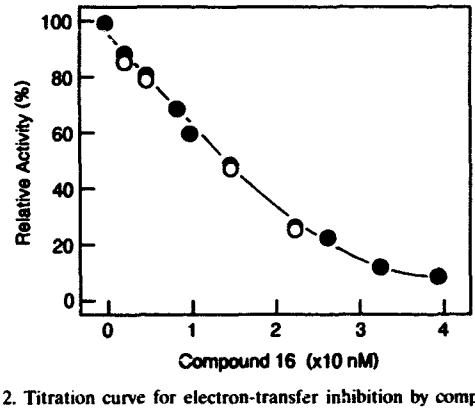


Fig. 2. Titration curve for electron-transfer inhibition by compound **16**. The electron-transfer activity was measured in the presence (○) and absence (●) of 720 nM methoxy derivative (compound **23**). The control DBH-cytochrome *c* reductase activity was 13 μmol of cytochrome *c* reduced per min per nmol cytochrome c_1. For details, see Methods.

stituent in 3-position (data not shown). This suggests that the phenolic OH group forms an intramolecular hydrogen-bond. A six-membered hydrogen-bonded ring may be formed between the phenolic OH and the carbonyl oxygen of amide bond bridge in the 1-position.

The replacement of formyl of 3-formylamino group by a bulkier acetyl group resulted in a decrease in inhibition (**16** vs. **20**). A more clear-cut effect of steric bulkiness of 3-substituent on the activity was observed with compounds **21** and **22**, suggesting that this portion of the analogue fits tightly into the binding cavity.

The effects of synthetic antimycin A analogues upon reduction of heme centers of the cytochrome bc_1 complex

Antimycin A binding to the cytochrome bc_1 complex is characterized by an extra reduction of cytochrome *b* reduced by substrate and by oxidant-induced reduction of cytochrome *b* [3]. We identified the binding site of synthetic antimycin A analogues from their effects upon the reduction of cytochrome *b* and *c* at the concentrations of inhibitors sufficient to exhibit full inhibition. In the absence of inhibitor, about 50 and 70% of cytochromes *b* (Fig. 4A) and c_1 (4G) were reduced by the addition of DBH. The control experiments on the effects peculiar to antimycin A_3 are shown in Fig. 4B and 4H. The extent of rapidly reduced cytochrome *b* was higher than that without inhibitor. The addition of ferricyanide to the system resulted in a transient re-reduction of cytochrome *b* (oxidant-induced reduction). The oxidant-induced reduction was abolished in the presence of myxothiazol (data not shown). The extent of cytochrome c_1 reduction was slightly diminished (Fig. 4H). Compound **16** elicited the same effects as antimycin A_3 on the reduction of cytochrome *b* (Fig. 4C). The inhibition of cytochrome *b* reduction was almost complete in the presence of compound **16** and myxothiazol (Fig. 4D), i.e., under double-kill conditions. These results showed that this compound binds to the Qi center of cytochrome bc_1 complex in the same manner as natural antimycin A. The effects of compound **12**, however, on the reduction of cytochrome *b* and c_1 differed from those of both natural antimycin A and compound **16**. That is, although the extent of reduced cytochrome *b* was slightly higher than without inhibitor, clear oxidant-induced reduction of cytochrome *b* was not observed (Fig. 4E). When compound **12** and myxothiazol act together, the reduction of cytochrome *b* was slowed (Fig. 4F), but faster than with compound **16** and myxothiazol. The fast phase of cytochrome c_1 reduction was slightly slower compared with the control, but not so much as that by myxothiazol (Fig. 4J). The reduction of cytochrome c_1 was almost completely blocked in the presence of compound **12** and myxothiazol (Fig. 4K). Other nitro derivative, compound **8**, exerted almost similar effects to those of **12** (data not shown). These observations indicated that the manner of binding of nitro derivatives to cytochrome bc_1 complex is somewhat different from that of natural antimycin A.

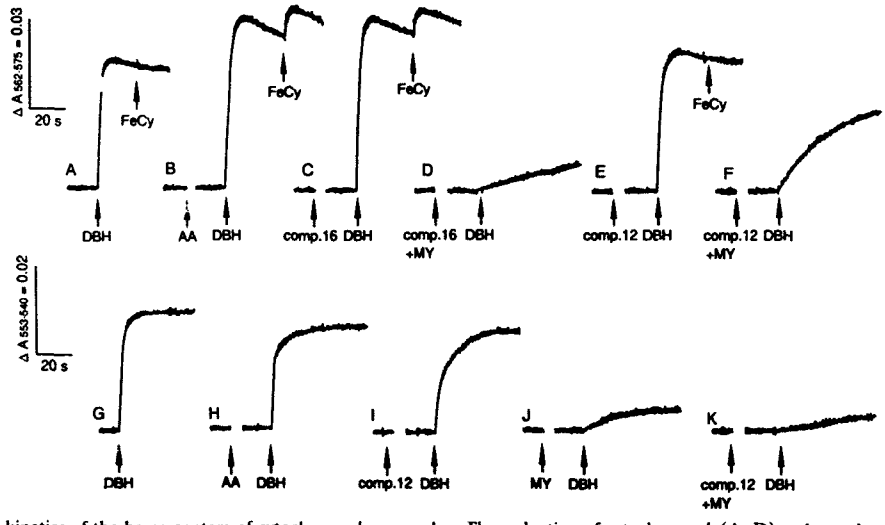


Fig. 4. Reduction kinetics of the heme centers of cytochrome bc_1 complex. The reduction of cytochrome *b* (A–D) and cytochrome c_1 (E–H) was determined at a concentration of 2 μM cytochrome bc_1 complex. Buffer: 2 mM NaN_3, 0.5 mM EDTA, 20 mM $MgCl_2$, 0.01% Tween-20 and 55 mM Tris-HCl (pH 7.4). After adding 2 μM $K_3[Fe(CN)_6]$ and preincubation with inhibitor, 25 μM DBH was added. To test for oxidant-induced reduction, 4 μM $K_3[Fe(CN)_6]$ (FeCy) was added. The maximal amount of reducible cytochrome was determined by adding a few grains of dithionite after the experiment. Where indicated, 25 μM DBH, antimycin A_3 (AA, 5 μM), myxothiazol (MY, 7.5 μM), compound **16** (comp. **16**, 55 μM) and compond **12** (comp. **12**, 0.21 mM) were added.

4. Discussion

Using di-n-octyl L-glutamate as a substitute for the natural dilactone-ring moiety, systematically selected structural modifications of the salicylic acid moiety became feasible in this study. The activity of derivatives which do not possess 3-formylamino group tended to increase as the electron-withdrawing property of the substituents increased, indicating that the acidity of the phenolic OH is important for the activity as suggested earlier [13,14]. The nitro derivatives such as compound **8** retained fairly high activity. However, while electron-withdrawing property *per se* of the formylamino group is much poorer than that of the nitro group (σ_{para}(NHCHO) = 0, $\sigma_{para}(NO_2) = 0.78$ [17]), 3-formylamino derivatives, except compound **17**, induced more potent inhibition than those of the nitro derivatives. It is, therefore, likely that the formylamino group in the 3-position plays some important roles in the inhibitory action which are not explained solely by an ordinary electronic effect.

To examine the role of 3-formylamino group, we compared the pK_a value of the 3-formylamino derivative with that of the nitro derivative. Since the solubility of di-n-octyl L-glutamate derivatives was very low, the pK_a value of compounds **24**, **25** and **26** (Fig. 1, Y = n-propyl) in 10% ethanol-water (v/v) was measured as the model compounds of **12**, **8** and **16**, respectively. It was assumed that the alteration of the ester alcohol moiety (n-octyl to n-propyl) did not affect the pK_a value. The pK_a values of compounds **24**, **25** and **26** were 4.9, 5.0, and 6.2, respectively. The pK_a of **26** was fairly low, which seems not to be accounted for by the electron-withdrawing property (the ordinary electronic effect) of the formylamino group alone. The effects of *ortho* substitution upon an acid dissociation group are complex in contrast to those of *para* and *meta* substituents [16]. The *para* and *meta* substituent effects on an acid dissociation are accounted for by the ordinary electronic effect alone. To elucidate the *ortho* substitution effects, so called proximity effects (or *ortho* effects), which are in general composed of steric and secondary electronic effects, have to be taken into account, in addition the ordinary electronic effect [16]. Thus, the fairly low pK_a value of compound **26** may result from the proximity effects of the formylamino group.

It is not clear, however, whether the role of the 3-formylamino group is solely to modulate the pK_a of the phenolic OH, because a conformational change in the 3-formylamino group and an increase in the steric bulkiness of this portion drastically affected the activity. It seems unlikely that such structural modifications would result exclusively in a drastic increase in pK_a value (i.e., a marked decrease in acidity). Regarding only the acidity of the phenolic OH, nitro derivatives should be more potent inhibitors than 3-formylamino derivatives, but an opposite was observed here. Furthermore, for 3-formylamino derivatives, modifications of the acidity of the phenolic OH by introducing methyl (electron-donating) or halogen groups (electron-withdrawing) in *para* position to the OH group did not significantly affect the activity (**16** vs **18** or **19**). These results indicate that the extent of the acidity of the phenolic OH group does not directly relate to the activity when the inhibitors possess a formylamino group in the 3 position. The 3-formylamino group, therefore, might have a more extensive role in the inhibitory action besides regulating the pK_a value of the phenolic OH. We suggest that there are specific interactions, such as electrostatic and hydrogen-bonding interactions, between the 3-formylamino group and some amino acid residues in the binding domain. In other words, the inhibitor binding domain might have stringent structural requirements.

Irrespective of the lack of a formylamino group in the 3-position, nitro derivatives retained fairly potent activity. This seems to be in conflict with the above conclusion. It is, however, worth noting that 3-formylamino derivatives showed similar effects on the reduction of cytochrome *b* and c_1 to those of natural antimycin A, whereas the effects of nitro derivatives differed from those of antimycin A. These results indicate that the precise manner of action of the inhibitor lacking the 3-formylamino group differs from that of natural antimycin A. This argument seems to be consistent with the recent observations by Xu et al [33], as they reported that 3-nitrosalicylic *N*-decylamide, which has been thought to be a good mimic of natural antimycin A [13,14,34], does not bind to a Qi center. To interact with the cytochrome bc_1 complex in the same manner as natural antimycin A, a 3-formylamino group (along with the phenolic OH group) may be essential. Therefore, it may be inappropriate, in a strict sense, to classify inhibitors lacking a 3-formylamino group as cytochrome *b* inhibitors without designation of the binding site (Qi or Qo) until more detailed studies are completed.

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References

[1] Berden, J.A. and Slater, E.C. (1972) Biochim. Biophys. Acta 256, 199–215.

[2] Von Jagow, G. and Link, T.A. (1986) Methods Enzymol. 126, 253–271.
[3] Trumpower, B.L. (1990) J. Biol. Chem. 265, 11409–11412.
[4] Michaelis, G. (1976) Mol. Gen. Genet. 146, 133–137.
[5] Burger, G., Lang, B., Bandlow, W., Schweyen, R.J., Backhans, B. and Kandewitz, F. (1976) Biochem. Biophys. Res. Commun. 72, 1201–1208.
[6] Di Rago, J.P. and Coison, A.M. (1988) J. Biol. Chem. 263, 12564–12570.
[7] Weber, S. and Wolf, K. (1986) FEBS Lett. 237, 31–34.
[8] Di Rago, J.P., Netter, P. and Slonimski, P.P. (1990) J. Biol. Chem. 265, 15750–15757.
[9] Dickie, J.P., Loomans, M.E., Farley, T.M. and Strong, F.M. (1963) J. Med. Chem. 6, 424–427.
[10] Kluepfel, D., Sehgal, S.N. and Vezina, C. (1970) J. Antibiot. (Tokyo) Ser. A23, 75–80.
[11] Neft, N. and Farley, T.M. (1971) J. Med. Chem. 14, 1169–1170.
[12] Selwood, D.L., Livingstone, D.J., Comley, J.C.W., O'Dowd, A.B., Hudson, A.T., Jackson, P., Jandn, K.S. and Stables, J.N. (1990) J. Med. Chem. 33, 136–142.
[13] Rieske, J.S. (1976) Biochim. Biophys. Acta 456, 195–247.
[14] Rieske, J.S. (1980) Pharmacol. Ther. 11, 415–450
[15] Miyoshi, H., Kondo, H., Oritani, T., Saitoh, I. and Iwamura, H. (1991) FEBS Lett. 292, 61–63.
[16] Fujita, T. and Nishioka, T. (1976) Prog. Phys. Org. Chem. 12, 49–89.
[17] Hansch, C., Leo, A., Unger, S.H., Kim, K.H., Nikaitani, D. and Lien, E.J. (1973) J. Med. Chem. 16, 1207–1216.
[18] Tokutake, N., Miyoshi, H., Nakazato, H. and Iwamura, H. (1993) Biochim. Biophys. Acta 1142, 262–268.
[19] Wasserman, H.H. and Gambale, R.J. (1985) J. Am. Chem. Soc. 107, 1423–1424.
[20] Wasserman, H.H. and Gambale, R.J. (1992) Tetrahedron 48, 7059–7070.
[21] Saitoh, I., Miyoshi, H., Shimizu, R. and Iwamura, H. (1992) Eur. J. Biochem. 209, 73–79.
[22] Meldrum, A. and Hirre, N.W. (1928) J. Indian. Chem. Soc. 5, 95–101.
[23] Myers, D.K. and Slater, E.C. (1957) Biochem. J. 67, 558–572.
[24] Gornall, A.G., Bardawill, C.J. and David, M.M. (1949) J. Biol. Chem. 177, 751–766.
[25] Rieske, J.S. (1967) Methods Enzymol. 10, 239–245.
[26] Brandt, U., Schagger, H. and Von Jagow, G. (1988) Eur. J. Biochem. 173, 499–506.
[27] Matsuno-Yagi, A. and Hatefi, Y. (1985) J. Biol. Chem. 260, 14424–14427.
[28] Esposti, M.D. and Lenaz, G. (1982) Biochim. Biophys. Acta 682, 189–200.
[29] Albert, A. and Serjeant, E.P. (1962) Ionization Constants of Acids and Bases, pp 66. Methuen, London.
[30] Tsai, A.L. and Palmer, G. (1986) Biochim. Biophys. Acta 852, 100–105.
[31] Yang, F.D., Yu, L. and Yu, C.A. (1989) J. Biol. Chem. 264, 891–898.
[32] Guo, Z.J., Miyoshi, H., Nagatani, K., Komyoji, T., Haga, T. and Fujita, T. (1991) J. Org. Chem. 56, 3692–3700.
[33] Xu, J.-X., Xiao, Y., Wang, Y.-H., Li, X. and Gu, L.-Q. (1993) Biochim. Biophys. Acta 1142, 83–87.
[34] Wilson, E., Farley, T.M. and Takemoto, J.Y. (1985) J. Biol. Chem. 260, 10288–10292.