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A model of antimycin A binding based on structure–activity studies of synthetic antimycin A analogues

Hideto Miyoshi [a,*], Nobuya Tokutake [a], Yasuhiro Imaeda [a], Toshio Akagi [b], Hajime Iwamura [a]

[a] *Department of Agricultural Chemistry, Kyoto University, Kyoto 606, Japan*
[b] *Central Research Laboratory, Ishihara Sangyo Kaisha, Ltd., Kusatsu 525, Japan*

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Abstract

The structural factors of antimycin A molecule required for inhibitory action were studied using newly synthesized antimycin A derivatives with bovine heart submitochondrial particles, in order to probe the interaction between antimycin A and its binding site. In particular, we focused upon the roles of the amide bond bridge, which connects the salicylic acid and dilactone ring moieties, and the 3-formylamino group in the salicylic acid moiety. The lack of formation of an intramolecular hydrogen-bond between phenolic OH and amide carbonyl groups resulted in a remarkable loss of the activity (by four orders of magnitude), indicating that this hydrogen-bond is essential for the inhibition. This result suggested that both the phenolic OH and the carbonyl groups form a hydrogen-bond with some residues at a fixed conformation. In addition, the inhibitory potency was remarkably decreased by *N*-methylation of the amide bond moiety, indicating that the NH group might function in hydrogen-bond interaction with the binding site. The *N*-methylation of 3-formylamino group also resulted in a decrease in the activity, probably due to a loss of the rotational freedom of this functional group. Molecular orbital calculation studies with respect to the conformation of the 3-formylamino group indicated that this group takes an active conformation when the formyl carbonyl projects to the opposite side of the phenolic OH group. Based upon a series of structure–activity studies of synthetic antimycin A analogues, we propose a tentative model for antimycin A binding in its binding cavity.

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

1. Introduction

The antibiotic antimycin A is a natural product of various species of *Streptomyces*. This compound inhibits cytochrome bc_1 complex activity by blocking electron transfer from the heme b_H center to ubiquinone [1]. For the development of mechanistic concepts and structural aspects of cytochrome bc_1 complex, antimycin A has been widely used [1–6]. Information about the antimycin A binding domain of cytochrome *b* has been obtained using mitochondrial mutants resistant to this inhibitor [4,7–9], whereas the portion of antimycin A molecule that interacts with the mutated positions remains unknown. To gain insight into the interaction between antimycin A and its binding domain, the structural factors of antimycin A essential for the inhibition should be identified.

The structure of natural antimycin A is shown in Fig. 1, taking antimycin A_3 as an example. The benzene and dilactone-ring planes locate on and above the page plane in Fig. 1, respectively. Using a synthetic stereoisomer of natural antimycin A_3 ((−)-antimycin A_3, in which the spatial position of the benzene ring plane relative to that of the dilactone ring is opposite to the position for natural antimycin A_3), we showed that the configuration of antimycin A is important for its inhibitory action [10]. This strongly suggested that the shape of whole antimycin A molecule is strictly recognized by the binding site. However, the natural dilactone ring moiety itself is not essential for the activity; that is, flexible and hydrophobic structures such as dialkyl L-glutamate and substituted diphenyl ether are favorable substituents for the dilactone ring [11]. It is therefore likely that antimycin A binding to a Q_i center is primarily governed by a specific interaction between the salicylic acid moiety and the binding site. In fact, it has been shown that antimycin A-resistant yeast mitochondrial

* Corresponding author. Fax: +81 75 7536128.

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mutants are specifically resistant to the inhibition by synthetic antimycin A analogues that possess a natural salicylic acid moiety but not a natural dilactone ring [12]. Furthermore, we demonstrated that both the 3-formylamino group in its proper conformation and the phenolic OH group are essential for the inhibitory action [13]. This study indicated that these two substituents are strictly recognized by the binding cavity, although the 3-formylamino group is thought to be not essential for the activity [14,15],

Antimycin A consists of 3-formylaminosalicylic acid linked via an amide bond to a dilactone ring moiety. The roles of both ring moieties have been elucidated [10,11,13], whereas the roles of the amide bond moiety remain unknown. In this study, we synthesized three new antimycin A derivatives (compounds B, D and E, Fig. 1) by modifying the distance between the benzene ring and the amide bond or by *N*-methylation. The substituent pattern on the benzene ring was set as that of natural antimycin A (3-formylamino salicyl). The portion corresponding to the dilactone ring was replaced by suitable mimetic structures [11,13], since total synthesis of the natural dilactone ring is still difficult despite recent progress [16,17]. Their inhibitory potencies of ubiquinol-cytochrome *c* oxidoreductase activity were compared with those of the corresponding control compounds (A and C, Fig. 1) using bovine heart submitochondrial particles. We also performed molecular orbital calculation studies on the conformation of the 3-formylamino group, which is important for the inhibitory action [13], to investigate its active conformation. Based upon a series of structure–activity studies of synthetic antimycin A analogues, we proposed a tentative model of antimycin A binding in the receptor cavity.

2. Experimental procedures

2.1. Materials

Antimycin A and cytochrome *c* (horse heart) were purchased from Sigma and 2,3-dimethoxy-5-methyl-6-*n*-decyl-1,4-benzoquinol (DBH) was prepared as described [18]. Compounds A and C are the same as those used in the previous studies [11,13]. Other reagents were of the purest grade commercially available.

2.2. Synthesis

Compound B. The precursor of compound B was synthesized by condensation of di-*n*-octyl L-glutamate with 2-hydroxy-3-nitrophenylacetic acid, which was prepared from commercially available 2-hydroxyphenylacetic acid by nitration, in the presence of dicyclohexylcarbodiimide in dry pyridine [11]. This precursor was reduced by 5% palladium on carbon in ethylacetate under hydrogen gas, and formylated according to the previous method [11], to obtain compound B. The final product was purified by silica-gel column chromatography using ethyl acetate/hexane (1:4) as the eluting solvent. The structure was characterized by ^{1}H-NMR spectra (JEOL GSX-400) and elemental analyses for C, H and N, within an error of $\pm 0.3\%$. ^{1}H-NMR ($CDCl_3$) δ 3.61 (s, 2H, $ArCH_2CO$), 6.78–6.83 (m, 2H, ArH), 7.07 (d, $J = 7.6$ Hz, 1H, CONHCH), 8.03 (dd, $J = 2.0$, 7.3 Hz, 1H, ArH), 8.19 (br s, 1H, NHCHO), 8.37 (d, $J = 2.1$ Hz, 1H, NHCHO), 10.44 (s, 1H, OH). Anal. Calcd for $C_{29}H_{46}O_7N_2$: C, 65.67; H, 8.82; N, 5.11. Found: C, 65.47; H, 8.82; N, 4.89.

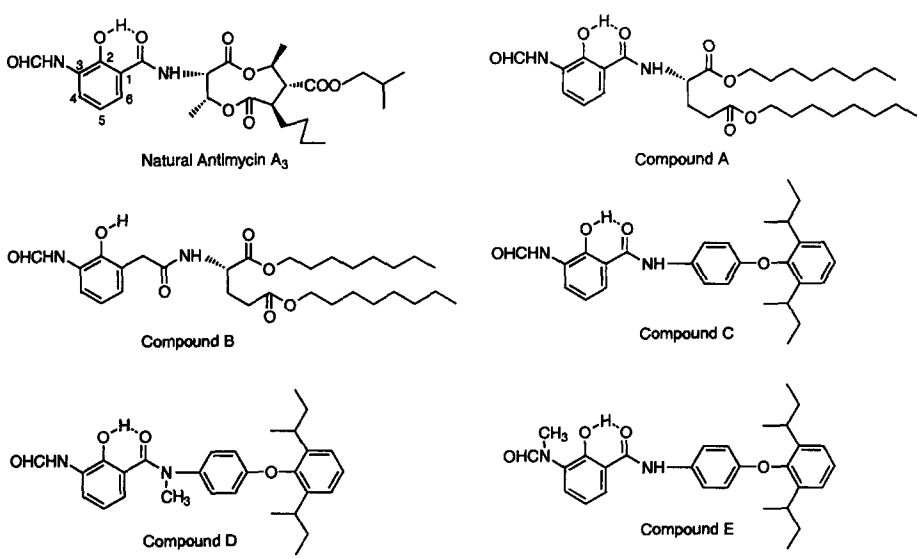

Fig. 1. Structure of the natural antimycin A_3 and synthetic antimycin A analogues studied. The intramolecular hydrogen-bond between the phenolic OH and the carbonyl oxygen is represented by a dotted line.

Compound D. The precursor of compound D was obtained by condensing 2-hydroxy-3-nitrobenzoic acid with 4-(2′,6′-di-*sec*-butylphenoxy)-*N*-methylaniline in the presence of dicyclohexylcarbodiimide in dry pyridine [11]. The compound 4-(2′,6′-di-*sec*-butylphenoxy)-*N*-methylaniline was synthesized by alkaline (K_2CO_3) hydrolysis of 4-(2′,6′-di-*sec*-butylphenoxy)-*N*-methyl-*N*-trifluoroacetylaniline, which was prepared from 4-(2′,6′-di-*sec*-butylphenoxy)-*N*-trifluoroacetylaniline by iodomethane methylation in the presence of K_2CO_3 in dimethylsulfoxide [19]. 4-(2′,6′-Di-*sec*-butylphenoxy)-*N*-trifluoroacetylaniline was prepared by the reaction of 4-(2′,6′-di-*sec*-butylphenoxy)aniline [11] and trifluoroacetic anhydride in dichloromethane. To obtain compound D, the above precursor was reduced, and formylated as described above. ^{1}H-NMR ($CDCl_3$) δ 3.47 (s, 3H, $CONCH_3$), 6.37–6.39 (m, 1H, ArH), 6.43–6.45 (m, 1H, ArH), 6.72 (d, $J = 8.0$ Hz, 1H, ArH), 6.81–6.85 (m, 1H, ArH), 6.98 (s, 1H, ArH), 7.00–7.03 (2H, ArH), 7.13 (d, $J = 7.6$ Hz, 1H, ArH), 7.20 (d, $J = 6.4$ Hz, 1H, ArH), 7.90 (br s, 1H, NHCHO), 8.24–8.27 (m, 1H, ArH), 8.46 (d, $J = 2.0$, 1H, NHCHO), 11.90 (s, 1H, OH). Anal. Calcd for $C_{29}H_{34}O_4N_2$: C, 73.39; H, 7.22; N, 5.90. Found: C, 73.20; H, 7.22; N, 5.95. The structure was also characterized by mass spectra (JEOL JMS-DX300). FAB-MS (*m/e*); 475 ($M^+ + 1$, 70%), 474 (M^+, 27), 310 (18) and 164 (100).

Compound E. Compound E was prepared by the formylation [11] of the corresponding 3-*N*-methylamino derivative which was obtained by methylation of the corresponding 3-amino derivative with paraformaldehyde in the presence of sodium borohydride in sodium methoxide [20]. The synthetic procedures for the 3-amino derivative were the same as those for compound C [11]. ^{1}H-NMR ($CDCl_3$) δ 3.26, 3.27 (3H, NCH_3), 6.84 (d, $J = 8.2$ Hz, 2H, ArH), 6.96–6.98 (3H, ArH), 7.15 (d, $J = 7.2$ Hz, 1H, ArH), 7.28–7.33 (m, 1H, ArH), 7.43–7.51 (m, 2H, ArH), 7.52 (t, $J = 9.5$ Hz, 1H, ArH), 7.92, 7.95 (1H, CHO), 8.24 (s, 1H, CONH), 12.55 (s, 1H, OH). Anal. Calcd for $C_{29}H_{34}O_4N_2$: C, 73.39; H, 7.22; N, 5.90. Found: C, 73.15; H, 7.26; N, 6.15.

2.3. Methods

Submitochondrial particles (SMP) were prepared from bovine heart mitochondria by the method of Matsuno-Yagi and Hatefi [21]. The particles were treated with sodium deoxycholate (0.3 mg/ mg of protein) before dilution with the reaction medium [22]. Cytochrome bc_1 complex activity was measured at 30° C as the rate of cytochrome *c* reduction with DBH as an electron donor. 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 15 μg/ ml. The molar concentration (I_{50}) in the incubation medium required to halve the control enzyme activity was measured. The I_{50} value was the average of the values of three series measurements.

Table 1
Inhibition of cytochrome bc_1 complex activity by antimycin A and synthetic analogues

Compounds	I_{50} (nM)	OH signal, δ (ppm) [a]
A	4.5	12.93
B	47800	10.44
C	2.8	12.89
D	2700	11.90
E	460	12.60
Antimycin A [b]	2.4	12.57 [c]

[a] ^{1}H-NMR (400 MHz) chemical shift (ppm) of the phenolic OH proton.
[b] Antimycin A_3 was used.
[c] From Ref. 24.

The stable conformation of 3-formylamino group was studied by molecular orbital methods. Computations were achieved by use of AMPAC (QCPE No. 523) with AM1 parameterization [23]. Initial conformations were constructed using standard bond lengths and angles. The torsion angle of the amide bond was set at 180° in the initial conformation.

3. Results

3.1. The role of the amide bond bridge in the inhibitory action

The 400 MHz ^{1}H-NMR spectra showed a remarkable difference in the phenolic OH proton signals between compounds A and B. Signals of the OH proton of the former and latter compounds appeared at 12.93 and 10.44 ppm, respectively, in chloroform-d_1 at 25° C (Table 1). Judging from the downfield-shifted signal of the phenolic OH proton of compound A, this OH group should form an intramolecular hydrogen-bond. Since such a remarkable downfield-shift signal was not observed with compound B, it is reasonable to consider that a six-membered hydrogen-bonded ring is formed between the phenolic OH and the carbonyl groups in the 1-position. On the other hand, since the signal for the phenolic OH proton of natural antimycin A appears at 12.57 ppm [17,24]; this kind of intramolecular hydrogen-bond is also formed with natural antimycin A.

Thus, to examine whether the formation of the intramolecular hydrogen-bond is important for the inhibitory action of antimycin A, we compared the inhibitory activities between compounds A and B. The inhibition of cytochrome bc_1 complex activity by compounds A and B was examined with bovine heart submitochondrial particles. As shown in Table 1, the inhibitory potency of compound B was about 10000-times less than that of compound A. This indicated that the formation of an intramolecular hydrogen-bond is essential for the in-

hibitory action. This idea is supported by the previous observation that replacing the phenolic OH by methoxy group resulted in a large loss of inhibitory potency [13].

It is unclear whether the formation of the intramolecular hydrogen-bond is important for occupying the antimycin binding site or for inhibiting the catalytic reaction at the Q_i center. To see whether compound B can occupy the binding site without inhibiting the catalytic reaction, we measured the inhibition by antimycin A or compound A using submitochondrial particles treated with at least about 1000-fold excess of compound B (7.6 μM). Compound B alone at this concentration did not inhibit control enzyme activity. The inhibitory potencies of antimycin A and compound A were not affected in the presence of excess compound B (data not shown), indicating that compound B can not replace these inhibitors from their binding site. It is therefore concluded that the formation of an intramolecular hydrogen-bond is important for inhibitor fitting to the binding site.

Next we examined the effect of *N*-methylation of the amide bond on the activity. Since the synthesis of a *N*-methyl derivative corresponding to compound A was unsuccessful, that corresponding to compound C was synthesized. Signals for the phenolic OH proton of compounds C and D appeared at 12.86 and 11.90 ppm, respectively. This means that the intramolecular hydrogen-bond of interest is somewhat disturbed for compound D by steric congestion due to *N*-methylation, whereas a weak intramolecular hydrogen bond is still formed. The inhibitory potency of compound D was about 1000-fold less than that of compound C. Although some decrease in the inhibitory potency was predicted from the weakness of the intramolecular hydrogen-bond formation, this large loss of activity might be not accounted for solely from the weakness in the intramolecular hydrogen-bond. We therefore suggest that the amide NH group takes part in hydrogen-bond interaction with the binding site, functioning as a hydrogen-bond donor.

Compounds A and C elicit the same effects as natural antimycin A on the reduction of cytochrome *b*; that is, an extra reduction of cytochrome *b* reduced by substrate, oxidant-induced reduction and a blockage of cytochrome *b* reduction in combination with myxothiazol (under "double kill" conditions) [11,13]. The investigations with compounds B and D were however, experimentally impractical because of the limited solubility of these compounds in the effective concentration range under the experimental conditions (2 μM cytochrome bc_1 complex).

3.2. The role of the 3-formylamino group in the inhibitory action

To examine the role of NH of the 3-formylamino group in the activity, we synthesized compound E. The activity of this compound was significantly decreased compared with that of compound C (Table 1). The ^{1}H-NMR spectra of compound E in chloroform-d_1 at 25° C revealed signals at 7.93 and 7.97 ppm, assigned to the formyl proton, and at 3.26 and 3.27 ppm, assigned to the *N*-methyl protons, in a ratio of 4:5, respectively. This indicated that the 3-formyl

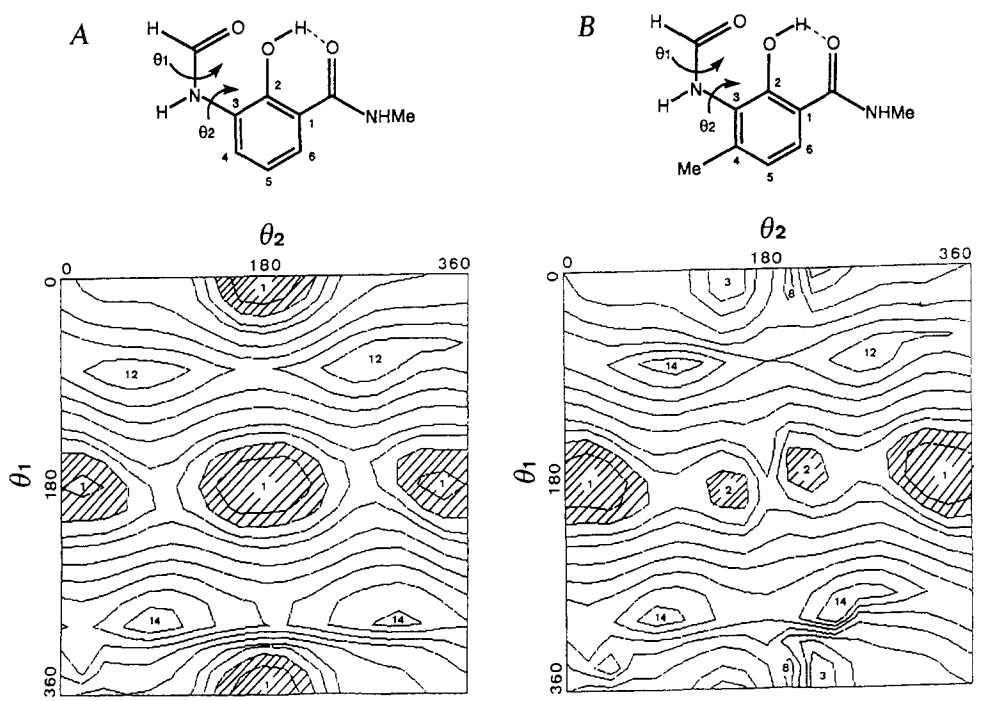

Fig. 2. Conformational energy diagrams of 3-formylamino-2-hydroxy *N*-methylbenzamide (A) and 3-formylamino-2-hydroxy-4-methyl *N*-methylbenzamide (B). The 3-formylamino group was rotated along θ_1 and θ_2 in steps of 20°. The contours represent 1, 2, 3, 4, 6, 8, 10, 12 or 14 kcal mol^{-1} above the global minimum for each molecule. The inside of the shaded region is 2 kcal mol^{-1} or less in energy above the global minimum.

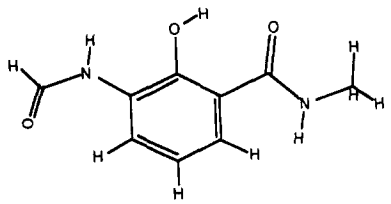

Fig. 3. A possible active conformation of 3-formylamino group. This corresponds to the most stable conformation shown in Fig. 2 ($\theta_1 = 0°$ and $\theta_2 = 180°$).

N-methylamino group of compound E takes two stable conformations, due to a restriction in the rotational motion around OHC-NMe bond. Therefore, a decrease in the activity of compound E might be due to a difficulty in taking an active conformation owing to a loss of flexibility. This result seems to be consistent with previous observations [13] that the 3-formylamino group takes two stable conformations in the presence of a methyl group at the 4-position (but not at 5-position), and that the activity of 3-formylamino-4-methyl derivative is significantly less than that of 3-formylamino derivative.

It was thus suggested that the 3-formylamino group has to be flexible to take a particular active conformation in the binding cavity. To obtain information on the active conformation of the 3-formylamino group, conformational energy studies were performed using 3-formylamino-2-hydroxy *N*-methylbenzamide (Fig. 2A) and 3-formylamino-2-hydroxy-4-methyl *N*-methylbenzamide (Fig. 2B) as model compounds. The latter was chosen to determine the effect of steric congestion around the 3-formylamino group on the conformation.

The 3-formylamino group was rotated along θ_1 and θ_2 (Fig. 2) in steps of 20° and the total energy of the molecule was calculated for each conformation. The conformation of the phenolic OH and amide bond moiety was optimized to form an intramolecular hydrogen-bond between the phenolic OH and the carbonyl oxygen. The lowest energy conformation of 3-formylamino-2-hydroxy *N*-methylbenzamide and 3-formylamino-2-hydroxy-4-methyl *N*-methylbenzamide was found at about $\theta_1 = 0°$, $\theta_2 = 180°$ and $\theta_1 = 180°$, $\theta_2 = 340°$, respectively. The shaded regions represent 2 kcal mol^{-1} or less in energy above the global minimum. We assumed that the conformation of 3-formylamino group, which is stable in the absence, and destabilized by introduction of a 4-methyl group, is a candidate for the active conformation. From Fig. 2, it is clear that the total energy of the conformation ($\theta_1 = 0°$, $\theta_2 = 180°$) is increased by steric repulsion arising from the vicinal (4-position) methyl group. It is therefore likely that the structure, shown in Fig. 3, is a reasonable model for the active conformation of the 3-formylamino group.

4. Discussion

The present study demonstrated that (1) the formation of a six-membered intramolecular hydrogen-bond between the phenolic OH and the amide carbonyl is essential for the inhibitory action; (2) a free NH of the amide bond bridge is important for supporting the formation of the intramolecular hydrogen-bond and also for functioning as a hydrogen-bond donor and (3) the conformation of 3-formylamino group, in which the carbonyl oxygen projects to the opposite side of the phenolic OH group, might be a good model of the active conformation. Based upon the information obtained from a series of structure-activity studies of synthetic antimycin A analogues [10,11,13] and here, we proposed a tentative model for antimycin A binding in its binding cavity (Fig. 4). Since details of three-dimensional structure of the Q_i reaction center are still vague, although models for topological organization of cytochrome *b* have been proposed [4,25–27], the information obtained from the structure-inhibitory activity studies of antimycin A analogues should be helpful at the present time to predict the interaction between antimycin A and its binding site.

The configuration of whole antimycin A molecule is very important for inhibitor binding to the cavity [10]. It is notable that the aromatic and dilactone ring planes locate

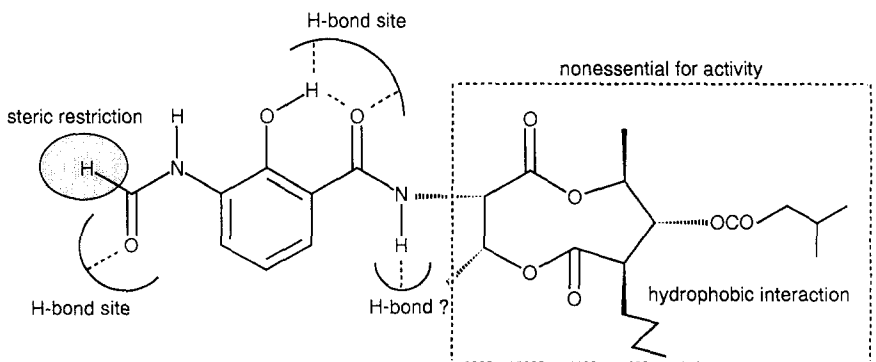

Fig. 4. A tentative model for antimycin A binding in its binding cavity. The aromatic and dilactone-ring planes locate *on* and *above* that of the page, respectively. The dotted lines represent hydrogen-bonded bridges.

on and above the page plane in Fig. 4, respectively. Since the natural dilactone ring is not essential for the activity [11], this moiety may regulate the tight fitting of the salicylic acid moiety into the cavity by fixing the whole molecular configuration and enhancing the hydrophobicity of the molecule. A six-membered hydrogen-bond between the phenolic OH and carbonyl group makes the amide bond plane coplanar to that of aromatic ring. Considering the remarkable loss of the inhibitory potency by the lack of the hydrogen-bond, both the phenolic OH and carbonyl groups may form hydrogen-bond bridges in such a *fixed* conformation to some residues in the binding site, although the residues remains to be determined. One may not rule out the possibility that the fixed conformation itself of antimycin A is required for tight binding to its binding site. On the other hand, a large loss of the activity by *N*-methylation suggests that the amide NH forms a hydrogen-bond to the binding site.

A previous report [13] and this study indicate that when the rotational freedom of 3-formylamino group is reduced by steric congestion, the activity significantly decreases, probably due to the difficulties involved in taking an active conformation. Considering this and the molecular orbital calculations performed here, an active conformation of the 3-formylamino group might be obtained when the formyl carbonyl group projects to the opposite side of the phenolic OH group. With respect to the effect of *N*-methylation of 3-formylamino group, the possibility that the lack of hydrogen-bond formation is responsible for a decrease in the activity would be not excluded. It is, however, reasonable to assume that the contribution of the NH group to the activity as a hydrogen-bond donor is, if there is any at all, not so important, since the degree of a decrease in the activity by *N*-methylation is much less than that which was observed when the hydrogen-bond was lost (compounds A vs. B and compounds C vs. D). On the other hand, it has been shown that the bulkier the portion corresponding to the formyl proton (shadowed in Fig. 4) of the 3-formylamino group, the lower was the activity [13]. Taking these facts into account, the presence of the 3-formylamino group itself in its proper conformation is essential for tight binding. In other words, this functional group is strictly recognized by the binding cavity. There may be a hydrogen-bond between the carbonyl oxygen of 3-formylamino group and the binding site.

Novel antimycin derivatives have the important potential of better defining how the inhibitor is bound to cytochrome *b*, when the mutational analysis of resistance to these compounds is carried out. The inhibition test of a series of synthetic antimycin A derivatives using antimycin-resistant mitochondrial mutants from yeast [4,25] and mouse [9] is currently in progress.

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