ANTIMYCIN A COMPONENTS. I

ISOLATION AND BIOLOGICAL ACTIVITY

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Antimycin A is a complex of four major components designated as A_1, A_2, A_3 and A_4, which were isolated, A_4 for the first time, in pure crystalline form. All components exhibited the same fungicidal activity, using *Saccharomyces cerevisiae* Y-30 as the test organism; the higher activity of A_3 reported before was found to be only apparent, and could be attributed to its higher diffusion coefficient in agar. The teleocidal activity was also the same for all components. Mutations to increased production of antimycin A do not necessarily lead to a change of composition; but, in the various mutants examined, when a change in composition was observed, it was in favor of A_1. Three new minor components, designated as A_0, A_5 and A_6, have been detected which represent not more than 1 % of the complex.

Antimycin A was isolated by DUNSHEE *et al.*[4] in 1949 as an apparently homogenous antibiotic, but subsquent studies by LOCKWOOD *et al.*[8] revealed its complexity: in bioautographs (paper chromatography and yeast), four different components were detected and designated as antimycin A_1, A_2, A_3 and A_4 in the order of their increasing R_f values. HARADA *et al.*[5] first succeeded in isolating fractions A_1 and A_3 in pure form, and one year later LIU and STRONG[7] further obtained pure fraction A_2 which appeared to consist of two isomers, A_{2A} and A_{2B}. Subsequent studies by STRONG and his collaborators led to the complete structure elucidation (VAN TAMELEN *et al.*[14]; DICKIE *et al.*[3]). When pure fractions were tested against *Saccharomyces cerevisiae* Y-30 in the cylinder plate assay, A_3 seemed to be much more active than either A_1 or A_2[7]; TAPPEL[13], however, reported that all three fractions have identical activity as electron-transport inhibitors. For a review on the mechanism of action of antimycin A, see RIESKE[9].

DERSE and STRONG[2] in 1963 discovered that antimycin A exhibits potent teleocidal activity, and VÉZINA[15] has reviewed its applications in fish management. Therefore, it was deemed desirable to determine precisely the relative activity of individual fractions against fish as a criterion for selecting higher producing strains of *Streptomyces* sp. AY-B-265 and fermentation conditions best suited to the production of this potent fish toxicant. Preliminary studies on enriched fractions in our laboratory (SEHGAL, MEYERS and BOULERICE, unpublished data) indicated in yeast and in goldfish the equivalent activity of components A_1, A_2 and A_3.

The present paper deals with the isolation and purification of individual compo-

nents of the antimycin A complex and their biological characterization; chemical properties have also been determined and will be reported in the next paper[10].

Materials and Methods

Fermentation. Antimycin A producing streptomycetes and fermentation conditions were those published by VÉZINA[15]. Strain AY-B-265 is an isolate which led in our laboratory to a family of higher producing strains selected after ultraviolet irradiation, and successively designated as AY-B-303, AY-B-312 and AY-B-314; strain AY-B-346 is a selectant from AY-B-314.

Fermentation was carried out in New Brunswick Ferma-Cell 250-L fermenters in medium 5 (VÉZINA[15], Table 3): aeration 1 vol air/vol medium/min; agitation, 250 rpm; antifoaming agent, 3 % Alkaterge C in mineral oil (automatic addition). At the end of fermentation, the beer was adjusted to pH 2.5, filtered, and the filtrate discarded; the mycelium was extracted twice with methylene dichloride, the combined extracts evaporated *in vacuo* to an oily residue, the antimycin A complex precipitated with hexane from the residue, and the crude precipitate was washed with ether and dried (SEHGAL, unpublished data).

Separation of Components. The precipitates of antimycin A complex were charged at 1 g per run into a Quickfit Steady State apparatus (120 10-ml tubes). Analytical grade (A. R.) solvents were used throughout; solvent systems were prepared by vigorous stirring of a suitable mixture for 10 minutes and settling for 30 minutes. The two equilibrated phases were separated carefully and each phase was dispensed into each tube at the rate of 10 ml per tube. The apparatus was then programmed for the desired agitation, settling time, and number of transfers. After varying numbers of transfers, every fifth tube (upper and lower phases) was sampled for the spectrophotofluorometric assay of antimycin A[11,12]; distribution curves were drawn and enabled not only to position the various fractions but also calculate the centesimal composition of antimycin A complex.

Isolation and Purification of Components. For the isolation of individual components the central zones under each peak were estimated, after careful exclusion of those tubes which contained more than about 0.1 % of any one of the neighbouring components, the corresponding tubes pooled and the phases separated. To the upper (aqueous) phase half a volume of water was added; the mixture was extracted thrice with half a volume of methylene chloride. The methylene chloride extracts were combined with the lower (organic) phase, dried over sodium sulfate and evaporated to dryness under vacuum. The dry residue was finally dissolved in diethyl ether from which the antimycin A component was crystallized. All fractions were recrystallized from ether.

Bioautography. The method was essentially that of LOCKWOOD *et al.*[8] Samples were spotted on Whatman No. 1 paper strips which were developed for 40 hours in a solvent system containing water, ethanol and acetone (7 : 2 : 1). Developed strips were deposited on large agar plates seeded with *S. cerevisiae* Y-30. After overnight incubation at 30°C, individual components could be detected.

Microbiological Assay. The cylinder plate method of LOCKWOOD *et al.*[8], as modified by MURPHY and DERSE (Wisconsin Alumni Research Foundation, personal communication) was followed throughout, using *S. cerevisiae* Y-30 as the test organism. The response is linear for concentrations of 0.5 to 7.5 μg/ml of antimycin A (complex) standard.

Fish Assay. Toxicity of antimycin A components to goldfish was determined by the method of DERSE and STRONG[2]. Two liters of tap water at 20°C (pH 7.4) were added to each of 4-liter beakers. Individual acetone solutions containing 50 μg/ml of antimycin A complex or of the various fractions of antimycin A, were added to separate beakers to give 5 final concentrations (25, 50, 75, 100, 125 ppb) of antimycin A complex and 3 final concentrations (25, 50, 75 ppb) of each fraction. The acetone concentration in each

beaker was kept constant by addition of pure acetone where needed. At zero time, 4 common goldfish approximately 5 cm (2 inches) in length were added to each beaker. The death time for each fish was noted and mean death time at each concentration of antimycin A complex and various fractions calculated. A standard curve was drawn by plotting the logarithm of antimycin A complex concentration against mean death time in minutes. The activity of various fractions was calculated from this curve.

Results

Isolation of Pure Components

Preliminary experiments confirmed the earlier observations of LIU and STRONG[7] that suitable solvent systems for the separation of antimycin A components are limited; systems consisting of methanol, water, carbon tetrachloride and hexane gave the best results. After several trials, we finally adopted a solvent system consisting of the above solvents in the ratio 85 : 15 : 80 : 20; antimycin A complex showed a partition coefficient (K) of 1.05. From the distribution pattern of several preliminary runs on 1 g quantities of antimycin A complex we could calculate the approximate partition coefficient of each major component and programme the apparatus for optimal isolation conditions for each component. In general, 500 upper and lower phase transfers were sufficient to obtain pure components. To isolate larger quantities of individual components, it was found advantageous to run a preliminary separation on a larger quantity of the complex, pool the tubes containing enriched fractions, and a run a second distribution on each pool. With this procedure, four components of increasing polarity were obtained which corresponded to antimycins A_1, A_2, A_3 and A_4 of LOCKWOOD *et al.*[8] Their partition coefficients (K) were 0.757, 0.908, 1.127 and 1.409 respectively. Three further components were detected and designated as A_0, A_5 and A_6 according to their polarity: A_0 has very low polarity, whereas A_5 and A_6 are considerably more polar than A_4. Components A_1, A_2 and A_3, and component A_4 for the first time, were isolated in gram quantities; their purity was ascertained by paper chromatography and by pyrolysis gas-liquid chromatography. In paper partition chromatography the following R_f values were obtained: A_0, 0.03; A_1, 0.156; A_2, 0.247; A_3, 0.468; A_4, 0.701; A_5, 0.866; A_6, 0.940. Structural data obtained from mass spectrometry and GLC analysis are reported in the paper by SCHILLING *et al.*[10] No attempt was made to isolate A_0, A_5 and A_6 which were present in very small quantities.

Fungicidal Activity

Contradictory results have been reported concerning the biological activity of antimycin A components. While LIU and STRONG[7] and HARADA *et al.*[5] found that antimycin A_3 was several times more active than A_1 against yeast, TAPPEL[18] observed that A_1, A_2 and A_3 were identical as electron-transport inhibitors. Therefore, we speculated that in the yeast plate assay some other factor(s) than concentration, especially diffusion in agar, could play a role. To test this hypothesis, individual components at concentrations between 0.1 and 1.0 μg/ml were assayed against the antimycin A standard by the cylinder plate method. Based on diameters of inhibition zones, the results were: A_1, 40 %; A_2, 82 %; complex, 100 %; A_3, 250 %; A_4, 300 %.

According to COOPER *et al.*[1], zone sizes are not only a function of antimicrobial activity; some other factors influencing sizes of inhibition zones must be considered; they expressed this in the following relationship:

$$X^2=4DT_0 \ln (m_0/m_1)$$

where X=zone diameter (mm), D=diffusion coefficient of antibiotic, T_0=critical time for zone formation, m_0=concentration of antibiotic in cylinder, and m_1=critical concentration of antibiotic at zone formation (equivalent to "minimum inhibitory concentration" in the serial dilution method).

From this relationship it appears that all other factors being constant, the size of the inhibition zone depends on m_1 (minimum inhibitory concentration) which is the true biological activity, and D which is the diffusion coefficient of the antibiotic in agar gel. m_1 is simply determined by plotting the values of X^2 for a given antibiotic against the logarithm of its concentration; a straight line ensues which intersects the concentration axis at m_1[1]. When pure antimycin A components were assayed by this method, a family of straight lines were observed which all intersected the concentration axis at the same point, showing the same value of m_1. These results are illustrated in Fig. 1. Therefore, it is concluded that all components have the same fungicidal activity, but different diffusion coefficients which increase with the polarity of components, as one would expect, and are responsible for the larger zone sizes of more polar components.

Fig. 1. Dose-response relationship of antimycin A complex and components in the cylinder plate assay, using *Saccharomyces cerevisiae* Y-30 as the test organism. m_1 values range from 0.048 to 0.051 μg/ml.

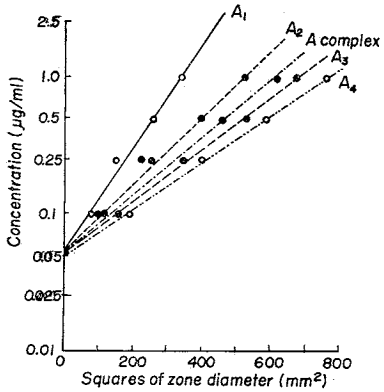
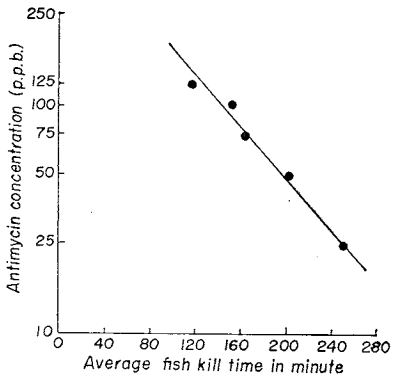


Fig. 2. Dose-response relationship of antimycin A complex *vs.* average death time of goldfish (standard curve).



Teleocidal Activity

The standard curve obtained with antimycin A is shown in Fig. 2. The results obtained with various fractions of antimycin A are given in Table 1. No significant difference in teleocidal activity betwen the complex and the various fractions of antimycin A was found.

Table 1. Teleocidal activity of antimycin A complex and individual components

Antimycin A complex and components	Concentration of complex and components and average death time in minutes*				
	25 ppb	50 ppb	75 ppb	100 ppb	125 ppb
Complex	251±20	201±10	164±12	150±30	116±40
Fraction A_1	291±40	196±25	188±25	—	—
Fraction A_2	286±40	175±35	141±20	—	—
Fraction A_3	282±20	236±50	155±20	—	—
Fraction A_4	246±10	181±25	166±45	—	—

* Average of 4 fish.
— Not tested.

Composition of Antimycin A from Various Strains

Batches of antimycin A produced by various strains, as described in Materials and Methods, were charged into the countercurrent apparatus which was programmed to obtain maximal separation of 4 main components without their migrating out of the apparatus. After 650 transfers samples were assayed by automated SPF as before, distribution curves drawn and the area under each peak determined to calculate the relative quantity of each component in the mixture. Components A_0, A_5 and A_6 were neglected, since their sum amounted to not more than 1 %. Results are tabulated in Table 2. Strain AY-B-312 produced a complex the composition of which is identical to that of antimycin A produced by the wide type AY-B-265. Therefore, the composition was not altered by the mutagenic treatments which significantly increased the antibiotic production. The intermediate mutant AY-B-303 has not been studied, but could be assumed to produce the same complex. AY-B-314 obtained from AY-B-312 through mutation and selection produced an antimycin A complex of a different composition: component A_1 was increased at the expense of A_3 and A_4. Strain AY-B-346, an isolate from AY-B-314, produced, as expected, a complex that showed the same composition as that of the complex produced by its parent.

Table 2. Composition of antimycin complexes produced by four strains of *Streptomyces* sp.

Streptomyces sp. (strain No.)	Antimycin components (%)*			
	A_1	A_2	A_3	A_4
AY-B-265	35.3	28.2	23.5	13.0
AY-B-312	35.5	28.1	23.2	13.2
AY-B-314	50.2	27.8	14.8	7.2
AY-B-346	49.6	26.9	16.8	6.7

* Since components A_0, A_5 and A_6 represented less than 1% of the complex, their values were ignored in this compilation.

Discussion

The complexity of antimycin A has been confirmed and fractions A_1, A_2 and A_3, already described by HARADA *et al.*[5] and LIU and STRONG[7], could be reisolated; furthermore, component A_4 was isolated for the first time in pure crystalline form. Three new components, A_0, A_5 and A_6, were also detected in bioautographs as well as in the corresponding zones of the countercurrent distribution, but were not isolated, since they represented only 1 % of the complex. Their importance is small in practice; the interest in characterizing them, on the other hand, will be stressed in the next communication[10].

All four major components have the same antimicrobial activity, and the apparent difference reported previously could be explained in terms of different diffusion rates of components in the cylinder agar plate method. The teleocidal activity, as determined by DERSE and STRONG's[2] method, is also the same for all four major components and the complex. These results corroborate those of TAPPEL[13] for electron-transport inhibition. Therefore, it appears that the structural differences in the various components only influence the physical characters, but not the site of activity.

Analysis of antimycin A produced by various strains of *Streptomyces* sp. also reveals that mutation which leads to increased production is not necessarily, but sometimes accompanied by a change in centesimal composition. When a change was observed, it was in favor of component A_1. Strains obtained through selection never showed an antibiotic composition different from that of their parent.

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