

# ANTIBIOTICS FOR NON-HUMAN USES

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## ABSTRACT

Antibiotics are often associated with medicinal uses only; still, 30 to 40 per cent of the total production of antibiotics are intended for non-human applications. The first and most important non-medical use is in animal production for disease prevention and growth promotion. Another important application is in plant protection to control various bacterial and fungal diseases. Antibiotics are also used to a limited extent in food preservation. These applications are giving rise to some concern because of their possible implications on public health. Antibiotics are valuable adjuncts in microbiological technique, especially in tissue culture. They contribute significantly to the advance in our knowledge of molecular biology.

A new, less known, application of antibiotics is that of antimycin A in fish management. The teleocidal spectrum of antimycin A makes it a selective toxicant for eradication of undesirable species and replacement with game fish. High efficiency and rapid action even at low temperature, rapid and complete degradation are inherent characteristics of antimycin A. Treated waters are not hazardous to man, livestock, and wildlife other than fish. Recent work on the chemistry of antimycin A components, biosynthesis, mode of action and fermentation is reported.

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Antibiotics are commonly and incorrectly associated with medicinal uses only. These substances, produced by microorganisms, are highly active and greatly selective to kill or to prevent the development of other microorganisms that threaten man's and animal health. The spectacular successes scored with antibiotics for curing of acute infections have made the term 'miracle drugs' a familiar expression for these substances.

Antibiotics are also valuable tools in laboratory research and contribute incessantly and not less spectacularly to the advance in our knowledge of molecular biology. The benefits that have accrued from their application in elucidating various mechanisms of protein synthesis, nucleic acid synthesis, respiration and so forth have been praised by several authors at this symposium.

Antibiotics are further used in animal management and production, in plant protection, and as preservatives for food and other biological materials. These applications are much debated nowadays. In this connection, I will make a brief exposé; then, I will discuss a new application of antibiotics, that of antimycin A in fish management.

ANTIBIOTICS IN ANIMAL PRODUCTION

The first broad and still most important non-medical use of antibiotics is in animal production. It emerged some twenty-five years ago from the observation of Moore *et al.*<sup>1</sup> that addition of certain antibiotics to feed stimulates the growth rate of a variety of livestock. Some facts on this now routine practice were collected from the recent reviews of Goldberg<sup>2</sup> and Elliott<sup>3</sup> and are summarized in *Table 1*. In the United States, more than a dozen

*Table 1.* Antibiotics used in animal production†

Antibiotics	Disease prevention (g/ton)	Growth promotion (10-50 g/ton)				
		Poultry	Swine	Dairy calves	Beef cattle	Sheep
Bacitracin	500	yes	yes			
Chlortetracycline	400	yes	yes	yes	yes	yes
Oxytetracycline	300	yes	yes	yes	yes	
Penicillin	200	yes	yes			
Tylosin	100	yes	yes			
Erythromycin	—	yes				
Nystatin	50-100	yes				
Oleandomycin	—	yes	yes			
Streptomycin	—	yes				
Novobiocin	—					
Spiramycin	—					
Spectinomycin	—					
Lincomycin	—	2-4 g/ton				
Chlortetracycline						
Sulphamethazine	—		yes			
Penicillin						
Increased growth rate and feed utilization (per cent)		2-15	2-13	10-30	3-4	—

† Total value in USA for 1969 was \$90 600 000.

antibiotics are registered by FDA for animal production. As for spectinomycin and lincomycin, they have been introduced only recently. Lincomycin is used for growth promotion of poultry at 2-4 g/ton. In 1969, 11 000 tons of antibiotics, representing a value of \$90 600 000, were added to animal feed. This is roughly 15 per cent of the total US market. The value of \$58 000 000 for antibiotics administered under veterinary control is partly included in this figure.

All the antibiotics listed in the table may be used against specific infections in flocks and herds at feed concentration of 50-400 g/ton. However, only the first five have been used widely as growth promoters at 10-50 g/ton. The economic benefits ascribed to this practice vary from 2 per cent to 30 per cent, as tabulated in *Table 1*. Certain combinations would show a marked improvement in performance over single antibacterials. The magnitude of the antibiotic effect on growth rate and feed utilization depends not only on

the species and age of the animal, but also on hygienic conditions, stresses, nutrition and general health. The response to antibiotics is clear in the early growing period; furthermore, it increases under the modern practices toward increasingly larger units with attendant stresses, and under sub-optimum hygienic conditions and incomplete diets. Nevertheless, reduced morbidity and mortality, heavier animals at market, higher meat quality and feed efficiency result in an appreciable economic benefit in this highly competitive field.

Antibiotics in feed continue to be effective, even after 20 years, but the mechanism of growth promotion is not completely understood. Indeed, the elimination of pathogenic organisms by antibiotics allows animals to express more completely their genetic potential for growth<sup>3</sup>. The mode of action is often associated with an alteration in the enteric flora, since germ-free animals are insensitive to antibiotics in feed<sup>2</sup>. Toxic amines from amino acid decarboxylase activity of *Escherichia coli* and *Clostridium perfringens* in the gut would reduce the availability of nutrients, and Hill<sup>4</sup> reported that prolonged administration of chlortetracycline much reduced the population of these microbes and promoted animal growth. Holtzman and Visek<sup>5</sup> have implicated ammonia from the microbial hydrolysis of urea as a growth-depressing toxin in the gastro-intestinal tract; growth promoters would act by depressing these microbes. Alteration of the intestinal flora is not a complete explanation, however, and there are several reports<sup>2,6</sup> that antibiotics in feed act directly on the animal by thinning the intestinal wall and increasing its permeability; these effects would result in better absorption of nutrients.

Thus far, antibiotics used as feed supplements have not led to untoward effect in man<sup>2</sup>. Residues are not encountered in the tissues of animals that have received recommended levels of antibiotics in feed throughout their life span, and were slaughtered much after the antibiotics used for growth promotion were discontinued. However, since antibiotics for non-human uses were in general developed for human use, it is normal that their impact on man's health is continually re-examined. The possible hazards are toxicity, hypersensitivity, super-infection, by *Candida* for example, and emergence of microbial resistance. Mutational resistance and multiple resistance, which results from the transmission of the R factor by cell contact, may be encouraged by sub-therapeutic levels of antibiotics (see review by Smith<sup>7</sup> in this symposium). Great importance was given to the R factor by the Swann Committee in elaborating recommendations<sup>8</sup> which, if implemented, may lead to the ban of antibiotics, especially those in use for humans, in animal feed. On the other hand, the Pharmaceuticals Committee of the US Animal Health Association<sup>9</sup> has pointed out that there is no evidence that antibiotic residues have been detrimental to human health, and has warned against recommendations that would overlook the benefits derived from the use of antibiotics and over-emphasize the theoretical or speculative hazards from the use of these substances.

The suggestion that antibiotics be developed specifically for growth promotion has not been found economical until now, mainly because of the cost of their development; nevertheless, virginiamycin and flavomycin have been developed for animal growth promotion and will be introduced in England.

ANTIBIOTICS IN PLANT PROTECTION

The second most important non-medical use of antibiotics is in plant disease control. It is not too surprising that antibiotics, such as streptomycin and griseofulvin which are used in humans, are also active against several bacteria and fungi that cause disease in plants. The main advantage of antibiotics over previous control methods has been the fact that antibiotics are absorbed by the plant and are effective within the plant<sup>2</sup>. They are systemic in their action, and in fact have much contributed to the understanding of translocation in plants<sup>10,11</sup>. Prior to the advent of antibiotics, chemical protectants were applied externally and were protective in their action. Antibiotics are both protective and eradicator<sup>12</sup>.

The value of the most used antibiotics for plant protection in Japan is shown in *Table 2*. Streptomycin, polyoxin, blastidicin S and kasugamycin

*Table 2. Main antibiotics used for plant protection in Japan in 1969†*

Antibiotics	Volume (tons)	Value (US dollars)
Streptomycin	209	420000
Polyoxin	7190	2150000
Blasticidin S	22773	3000000
Kasugamycin	60793	8430000

† Ministry of Agriculture and Forestry, Japan.

represented in 1969 a total value of \$14000000, which is about 5 per cent of the total value of antibiotics in Japan. The figures are those compiled by the Department of Agriculture and Forestry of Japan. In a recent review, Goodman<sup>13</sup> listed 25 bacterial and 50 fungal diseases of plants that are amenable to antibiotics for treatment and prevention. However, only four antibiotics are used broadly, and their applications are summarized in *Table 3*. Streptomycin, at 100 to 1000 p.p.m., is preferred to the tetracyclines which degrade more rapidly under ultraviolet irradiation.

Streptomycin is mainly active against the genera *Erwinia*, *Pseudomonas* and *Xanthomonas*, and against the fungus *Peronospora tabacina* which like other oomycetes has a cellulose cell-wall; eumycetes have a chitinous cell-wall and are resistant to streptomycin<sup>10</sup>. Streptomycin is used mostly in the United States to control bacterial diseases of apple, pear, tomato, bean and tobacco. In India, streptomycin finds limited use in controlling bacterial infections of cotton, rice and citrus (Dr G. Rangaswami, personal communication). Erythromycin is used in India to control *Pseudomonas solanacearum* which causes very severe diseases of vegetables. Cycloheximide is used in the United States at 1 to 2 p.p.m. against fungal diseases of cherry, rose and whitepine. Blasticidin S and kasugamycin have been developed in Japan specifically for plant protection; they are mostly used against *Piricularia oryzae* which causes the rice blast disease, and have replaced the mercurial compounds that were accumulating dangerously in rice fields.

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Table 3. Some plant diseases controlled by antibiotics<sup>2,10</sup>

Antibiotics	Causative agents	Diseases
Streptomycin (100-1000 p.p.m.)	<i>Erwinia amylovora</i>	Fire blight of apple and pear
	<i>Xanthomonas juglandis</i>	Bacterial spot of walnut
	<i>X. vesicatoria</i>	Bacterial spot of tomato and pepper
	<i>Pseudomonas phaseolicola</i>	Seed-borne pathogen of bean
	<i>Peronospora tabacina</i>	Tobacco plant disease
	<i>Ps. tabacini</i>	Tobacco plant disease
	<i>X. malvacearum</i> †	Seed-borne infection of cotton
Cycloheximide (1-2 p.p.m.)	<i>X. oryzae</i> †	Rice blight
	<i>X. citri</i> †	Citrus canker
	<i>Coccomyces hiemalis</i>	Sour cherry disease
	<i>Sphaerotheca pannosa</i> var. <i>rosae</i>	Rose mildew
Blasticidin S (20 p.p.m.)	<i>Cromartium ribicola</i>	White pine blister rust
	<i>Piricularia oryzae</i>	Rice blast disease
Kasugamycin		

† G. Rangaswami, personal communication.

Thus far, the use of antibiotics in plant protection has been limited by economic factors and by public health aspects, since many plants so treated ultimately are used for food. Antibiotics may find a place of choice in the field, when new, active biodegradable substances are needed to replace less desirable compounds.

### SOME MINOR USES OF ANTIBIOTICS

At one time antibiotics have been advocated in food preservation<sup>2</sup>. Streptomycin, oxytetracycline and chlortetracycline were shown to be effective in fruit and vegetable preservation. In order for these agents to be successful, they must be present in the product throughout its shelf life. Thus, an antibiotic residue of 2 to 40 p.p.m. is necessarily to be encountered<sup>2</sup>. This level may endanger man's health, and this practice was discontinued in the United States. Nisin and pimarin, however, may have some future. The latter is receiving great attention because of its effectiveness against mould growth on cheese and sausage. It is not only active against *Penicillium*, *Aspergillus* and *Rhizopus* which spoil cheese, but also against *Aspergillus flavus* which grows on grain and other foods of plant origin and produces aflavoxin B<sup>10</sup>.

Antibiotics are also used as adjuncts in several microbiological techniques and procedures<sup>2</sup>. They are useful for the selective isolation of specific microorganisms. Chick embryo cultures and tissue cultures require penicillin and streptomycin or chloromycetin for bacteria-free conditions and amphotericin B or nystatin for yeast- and fungi-free conditions<sup>14</sup>. Antibiotics also play a role via antibiograms in the classification of microorganisms.

From the foregoing it can be concluded that antibiotics play a significant role in non-medical fields. In recent years, these applications have accounted for 25 to 40 per cent of the total antibiotic production in the United States.

In 1969, the world production (Russia and China not included) had a value of \$1 800 000 000. Assuming a conservative proportion of 20 per cent for non-human uses, one can visualize the economic importance of these applications.

### ANTIMYCIN A

It is well recognized in North America that efforts devoted to the preservation and improvement of game fish resources are a realistic investment in conservation, future recreational facilities and continued economic growth<sup>15</sup>. Among the most important reclamation measures recommended are partial or total removal of raw fish, and subsequent restocking with other, more desirable species in accordance with local fishing preferences and environmental conditions. The most promising approach to management problems has been elimination of unbalanced fish populations through application of toxicants<sup>16</sup>. Carp is often the most important target in our waters, and lamprey in our Great Lakes. Until now, rotenone and toxaphene have been most widely used in Canada and the United States to control carp and other undesirable fish (Dykstra and Lennon, personal communication). It is now established from more than 50 field experiments in lakes and streams in 19 States, and in Canada and Guatemala that antimycin A most nearly meets the criteria of an ideal fish toxicant<sup>17</sup>. These criteria most frequently specified by fishery biologists, are listed in *Table 4*. Criteria

*Table 4*. Criteria of the ideal fish toxicant<sup>15</sup>

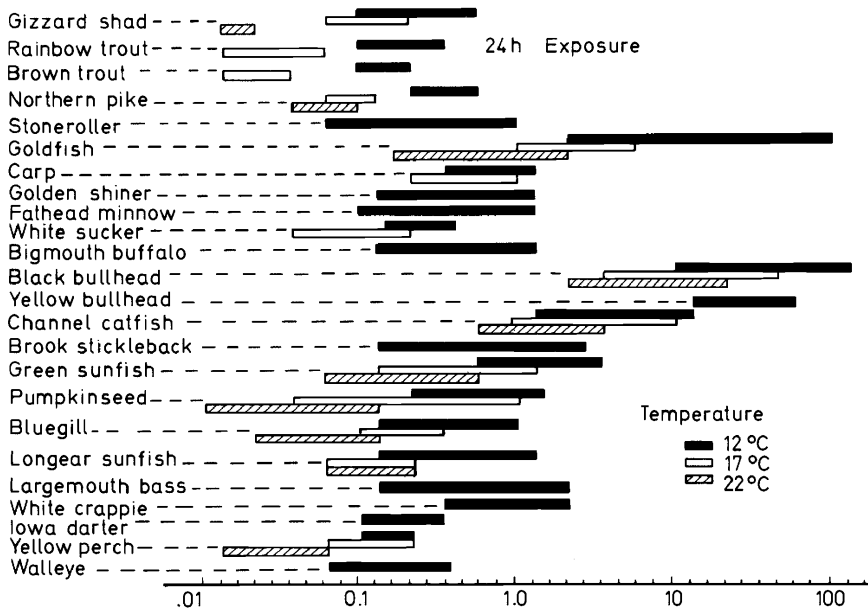
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1. Efficiency after a short exposure against all common fish
  2. Minimum hazard to personnel applying the formulation
  3. Treated waters non-hazardous to man, livestock, wildlife
  4. Rapid degradation to permit early re-stocking
  5. Killed fish non-toxic to man and wildlife
  6. Efficiency in cold, warm, soft, hard, acid, alkaline, clear, turbid waters
  7. Survival of plankton, algae, insects, bottom fauna, aquatic plants
  8. Sterilization of fish eggs
  9. Rapid detoxication by chemicals
  10. Non-repellency to fish
  11. Irreversible action
  12. No odour or taste to water of affected fish
  13. Easy application with available equipment
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1, 4, 6, 8, 9, 10, 11, 12 and 13 relate to efficacy; criteria 2, 3, 5 and 9 relate to safety; criterion 5 is a necessity in chemical fishing; and criteria 3 and 7 should satisfy the antipollution measures that are likely to be enforced by governments in the near future.

The application of antimycin in fish management is based on the original observation of Derse and Strong<sup>18</sup> that the antibiotic kills goldfish, now considered as a relatively resistant species, at a very low concentration (1 p.p.b.), and degrades rapidly, thus enabling prompt restocking. Antimycin A was reviewed by Strong<sup>19</sup> and Vézina<sup>20</sup>.

**Teleocidal spectrum**

To study the teleocidal spectrum of antimycin A, Walker *et al.*<sup>21</sup> of the Fish Control Laboratories, Bureau of Sport Fisheries and Wildlife, at La Crosse, Wisconsin, used 24 species from 9 families of cold water and warm water fish. Antimycin A was dissolved in acetone and the solution added to slightly alkaline, medium hard, reconstituted water. Responses of the fish to the toxicant were observed at 24, 48, 72 and 96 hours, at 12, 17 and 22C. Three groups of susceptibility were recognized among fish (*Figure 1*). The first group



*Figure 1.* Responses (24 h) of 24 fishes in laboratory to antimycin A (p.p.b.). Solid, plain and cross-hatched bars span ranges between  $EC_{50}$  and  $EC_{100}$  at 12, 17 and 22C<sup>21</sup>. Courtesy of Bureau of Sport Fisheries and Wildlife, Fish and Wildlife Service, US Department of the Interior, Washington, D.C.

represented extreme sensitivity. All fish in that category perished at 0.8 p.p.b. of antimycin A at 12C. Families and species in the category are: trout (rainbow and brown trout), perch (Iowa darter, yellow perch, and walleye), herring (gizzard shad), and suckers (white sucker). The second group represented intermediate sensitivity. All fish in that category perished when exposed to 0.8 to 1.6 p.p.b. of antimycin B at 12C. Families and species in that category are: pike (northern), sunfish (green, pumpkinseed, bluegill, longear, largemouth bass and white crappie), suckers (white and bigmouth buffalo), stickleback (brook), minnows and carp (stoneroller, goldfish, fathead minnow, carp and golden shiner). The third group represented low sensitivity. Fish in that category resisted 20 to 120 p.p.b. of antimycin A at 12C. They belong to

the group of fresh-water catfishes (channel catfish, black bullhead and yellow bullhead). For a more detailed teleocidal spectrum the reader is referred to Walker *et al.*<sup>21</sup> and Berger *et al.*<sup>22</sup>.

In these experiments<sup>21, 22</sup> it was noted that age of fish had little, if any, effect on susceptibility. Eggs, fry, fingerlings, juveniles and adults were equally sensitive. Effect on fish is obviously dependent on time of exposure and concentration of antimycin A. Subsequent experiments<sup>22</sup> revealed the influence of pH, light, hardness and temperature. Increasing the value of these factors speeds up degradation and may require higher concentrations. Degradation products are completely inactive on fish and other forms of life. It is significant that all specimens which displayed symptoms of distress eventually died, even if they were transferred to antimycin-free water. This suggests that the action of the toxicant on fish is irreversible.

In these experiments great attention was given to possible effects of antimycin A on the other fauna and on the flora. Waterfleas, crayfish and damselfly nymphs were not harmed: turtles, tiger salamanders and bullfrog tadpoles were not affected. No change could be detected on filamentous algae, aquatic plants and phytoplankton.

### Formulation and application

Early in this work, antimycin A was recognized as a precision tool in fish management. Its effect on fish is species-dependent, and its concentration in water must be precisely controlled, if advantage of its selective properties is to be taken. For instance, antimycin A is lethal to carp at a concentration which does not affect largemouth bass. Also, it could be applied to shallow waters only where a carp population is spawning, or to a whole body of water to kill all fish. Therefore, it was found necessary to prepare the toxicant in a formulation that would assure immediate and uniform concentration to a pre-determined layer of water with maximal convenience for and minimal hazard to the operator.

Milosovich and Beall, at Ayerst Laboratories, found that inert particles, such as sand grains, coated with a uniform layer of polyethylene glycols containing suitable concentrations of antimycin A, serve the purpose admirably. The preparation of several formulations has been described by Vézina<sup>20</sup>. One formulation, designated as Fintrol-5<sup>®</sup>, assures a uniform concentration of 5 p.p.b. when applied to 5 feet deep ponds. Fintrol-10, Fintrol-15, Fintrol-30 give the same concentration when applied to 10, 15, 30 feet deep bodies of water. These formulations are conveniently applied by helicopters or by means of electric grass-seed spreaders powered by the battery of the boat.

### Field trials

Field trials with acetone solutions and with sand formulations (Fintrol-5, Fintrol-15, Fintrol-30) were conducted by Gilderhus *et al.*<sup>23</sup> and Lennon *et al.*<sup>17</sup> under a great variety of conditions at lakes and streams located in the United States, and in Canada and Guatemala. Results in the field essentially corroborated those obtained in the laboratory. Antimycin A showed the same efficiency and irreversible action. It could be used in fresh and marine waters, in acid and alkaline, cold and warm waters, and in flowing and static

waters. The formulations contributed no colour or odour to water and did not repel fish. It degraded rapidly, usually within a week.

Antimycin A has been used most successfully as a selective toxicant. Burress and Luning<sup>24</sup> have applied 0.4 p.p.b. antimycin A to remove most of the sunfishes in soft-water ponds; largemouth bass and large bluegills were little affected. Antimycin A effectively and economically controlled<sup>25</sup> heavy infestations of green sunfish and golden shiners from catfish ponds at fish farms. Treated ponds yielded 27 per cent more catfish than untreated ponds.

During these field trials, no grossly toxic effects on phytoplankton, bottom fauna or aquatic plants were noted. Snails, mayflies and larvae of damselflies, water beetles, midges and others showed no significant changes as a result of treatment. No gross effects were observed on frogs, bullfrog tadpoles, salamanders, turtles, daphnia or crayfish. Freshwater shrimps were likewise unaffected.

### Toxicity to animals

Acute and chronic toxicity of antimycin A to laboratory animals and to wildlife was thoroughly investigated<sup>26</sup> (also see reference 20). LD<sub>50</sub> (ip) in mouse, rat, and goldfish was respectively 1.7, 1.6, and 0.18 mg/kg. LD<sub>50</sub> (oral) ranged from 1.8 to 160 mg/kg in a variety of animals; it was 0.5 mg/kg in goldfish. The toxicity by immersion ID<sub>50</sub> was 0.00072 in goldfish. Therefore, antimycin A was about 700 times more toxic by immersion than by oral administration.

Chronic toxicity was studied in dogs and rats<sup>26</sup>. Animals were fed fish (50 per cent of the diet) killed with 125 p.p.b. of antimycin A and further enriched with 2500 p.p.b. of the antibiotic. Other animals received water containing 125 p.p.b. of antimycin A. The animals were observed for 3 months, then sacrificed. No sign of toxicity, no change in haematology and no abnormal histopathological finding were noted.

From the results of acute and chronic toxicity studies Herr *et al.*<sup>26</sup> concluded that antimycin A at normal use levels has very little toxicity to mammals, and that consumption of antimycin A-killed fish or antimycin A-containing water at normal use levels would not be harmful to humans. These results corroborate those of Ritter and Strong<sup>27</sup> who measured the tissue residues of antimycin A in fish killed with tritium-labelled material.

### Fungicidal and teleocidal activity of antimycin A components

Antimycin A was discovered in 1948 by Leben and Keitt<sup>28</sup> as an antifungal agent. Its minimum inhibitory concentration (MIC) against a variety of fungi varied from 0.02 to 1 µg/ml. It had almost no action on bacteria. Strong and his collaborators<sup>29</sup> isolated it as an apparently homogeneous antibiotic but soon recognized<sup>30</sup> in bioautographs the presence of four components which were designated as antimycin A<sub>1</sub>, A<sub>2</sub>, A<sub>3</sub> and A<sub>4</sub> in the order of their increasing R<sub>f</sub> values. These studies culminated in the complete structure elucidation<sup>31</sup> of the four fractions (*Figure 2*). It consists of a 3-formamido-salicylic acid residue linked through an amide group to a dilactone ring which bears an acyl side chain and a *n*-butyl or *n*-hexyl side chain. When pure fractions of A<sub>1</sub>, A<sub>2</sub> and A<sub>3</sub> were tested against *Saccharomyces cerevisiae* Y-30 in the cylinder plate assay, A<sub>3</sub> seemed to be much more active than either

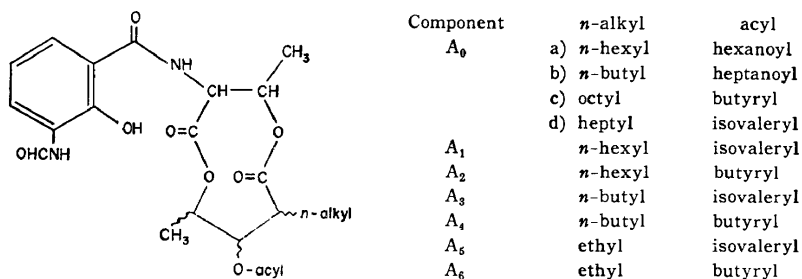


Figure 2. Proposed structures for antimycin A components. Data of Schilling *et al.*<sup>38</sup>. Reproduced with the permission of Japan Antibiotics Research Association.

A<sub>1</sub> or A<sub>2</sub><sup>32,33</sup>. It was deemed desirable to determine more precisely the relative activity of individual fractions against fish as a criterion for selecting higher producing strains of *Streptomyces* sp. and fermentation conditions best suited to the production of this potent fish toxicant.

The antimycin A complex was charged into a Quickfit Steady-State apparatus and the separation of components was achieved after 500 phase transfers in a system consisting of methanol, water, carbon tetrachloride and hexane in the ratio of 85 : 15 : 80 : 20<sup>34</sup>. Antimycin A was assayed spectrophotofluorometrically<sup>35,36</sup>. The central zones under each peak were estimated, the corresponding tubes pooled, and the phases separated. The 4 components were obtained in pure form after crystallization from ether. Three new fractions were detected and designated as A<sub>0</sub>, A<sub>5</sub> and A<sub>6</sub> according to their polarity<sup>34</sup>. These new fractions represented less than one per cent of the complex and were not isolated in pure form.

When individual components, as well as the complex, were assayed by the yeast cylinder plate method and the diameters of inhibition zones determined, the following results were obtained: A<sub>1</sub> read 40 per cent of the complex; A<sub>2</sub>, 82 per cent; the complex, 100 per cent; A<sub>3</sub>, 250 per cent; A<sub>4</sub>, 300 per cent. This confirmed the apparent superiority of the more polar components. However, inhibition zones are not only a function of minimal inhibitory concentration, but also of the diffusion coefficient of the antibiotic in agar gel. The value  $m_1$ , also called the minimum inhibitory concentration, can be determined by plotting the values of the square of zone diameters against the logarithm of their concentrations. When pure components of antimycin A and the complex were assayed by this method, a family of straight lines were observed which all intersected the concentration axis at the same point  $m_1$  (Figure 3). Therefore, all components have the same fungicidal activity, but different diffusion coefficients which increase with the polarity of components and are responsible for the larger zone sizes of more polar components.

The relative teleocidal activity of individual components was similarly determined<sup>34</sup> using a modified fish assay<sup>18,37</sup>. Two litres of tap water were added to several of 4-litre beakers. Acetone solutions of the complex and components were added to separate beakers to final concentrations of 25 to 125 p.p.b. of the complex and of 25 to 75 p.p.b. of the fractions. At zero time,

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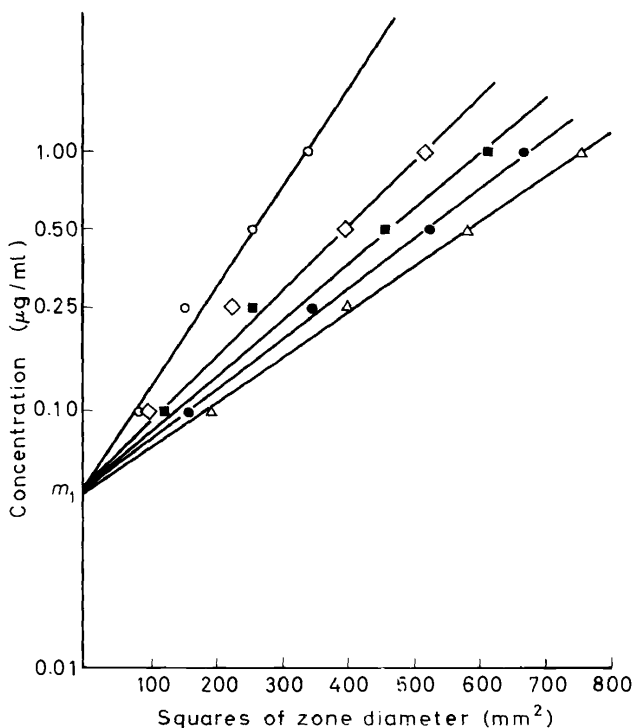


Figure 3. Dose-response relationship of antimycin A complex and components in the cylinder plate assay, using *Saccharomyces cerevisiae* Y-30 as the test organism.  $m_1$  values range from 0.048 to 0.051  $\mu\text{g/ml}$ . Data of Kluepfel *et al.*<sup>34</sup>. Reproduced with the permission of Japan Antibiotics Research Association.

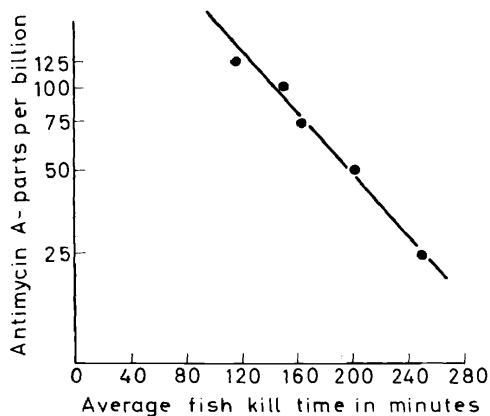


Figure 4. Dose-response relationship of antimycin A complex versus average death time of goldfish (standard curve). Data of Kluepfel *et al.*<sup>34</sup>. Reproduced with the permission of Japan Antibiotics Research Association.

4 goldfish, approximately 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 the complex calculated. A standard curve was drawn (*Figure 4*) and, by interpolation of mean death times, the activity of individual components was determined. The results are shown in *Table 5*. No significant difference in teleocidal activity was found between the complex and the various fractions of antimycin A.

*Table 5.* Teleocidal activity of antimycin A complex and individual components† (average death time in minutes‡).

Antimycin A complex and components	Concentration of complex and components (p.p.b.)				
	25	50	75	100	125
Complex	251 ± 20	201 ± 10	164 ± 12	150 ± 30	116 ± 40
Fraction A <sub>1</sub>	291 ± 40	196 ± 25	188 ± 25	—	—
Fraction A <sub>2</sub>	286 ± 40	175 ± 35	141 ± 20	—	—
Fraction A <sub>3</sub>	282 ± 20	236 ± 50	155 ± 20	—	—
Fraction A <sub>4</sub>	246 ± 10	181 ± 25	166 ± 45	—	—

— Not tested.

† Data of Kluepfel *et al.*<sup>34</sup> (reproduced with the permission of Japan Antibiotics Research Association).

‡ Average of 4 fish.

To evaluate the consequence of our strain improvement programme on the composition of the antimycin complex, batches of the antibiotic were produced by various strains and charged into the counter-current apparatus. Distribution curves were drawn and the area under each peak determined to calculate the relative quantity of each component in the mixture (*Table 6*).

*Table 6.* Centesimal composition of antimycin A produced by the original strain and three strains derived from it†.

<i>Streptomyces</i> sp. (strain no.)	Antimycin components (per cent)‡			
	A <sub>1</sub>	A <sub>2</sub>	A <sub>3</sub>	A <sub>4</sub>
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

† Data of Kluepfel *et al.*<sup>34</sup> (reproduced with the permission of Japan Antibiotics Research Association).

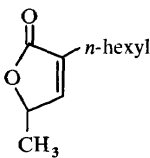
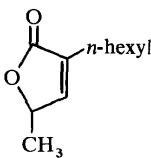
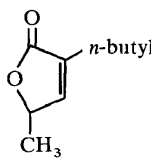
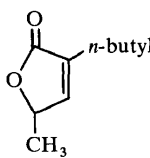
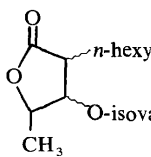
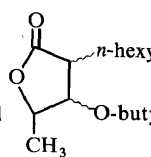
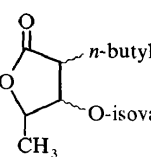
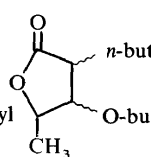
‡ Since components A<sub>0</sub>, A<sub>5</sub> and A<sub>6</sub> represented less than 1 per cent of the complex, their values were ignored in this compilation.

Wild type B-265 and mutant B-312 derived from it produced a complex of identical composition. Mutant B-314 produced a complex of a different composition: A<sub>1</sub> was increased at the expense of A<sub>3</sub> and A<sub>4</sub>. Strain B-346, a natural isolate from B-314 produced a complex identical to that of its parent.

**Pyrolysis-gas liquid chromatography of antimycin A components**

In the isolation of antimycin A components it became apparent that the various methods available<sup>20</sup> were not well suited to the determination of purity of single antimycin A components. The spectrophotofluorometric method itself<sup>35, 36</sup> which was so useful in fermentation work was not rapid and reliable enough when applied to single components. Moreover, standard gas liquid chromatography was not applicable, because of the thermal instability of the molecule. This instability was made use of to develop a method based on pyrolysis and subsequent GLC of the pyrolysate<sup>38</sup>. The pyrolysate of each component showed a characteristic GLC pattern consisting of three major peaks. The complex yielded a pyrolysate which gave a GLC pattern showing the peaks of all components. The thermolytic patterns of fractions A<sub>1</sub>, A<sub>2</sub>, A<sub>3</sub> and A<sub>4</sub> are shown in Table 7. The systematic character of the three peaks, M, N, and O, is reflected in the relative retention times and in the mass numbers of the molecular ions observed in the mass spectrum of each of the collected GLC fractions. Peaks N and O are characteristic of

Table 7. Pyrolysis-gas liquid chromatography†. GLC conditions: 9ft, 2.8 mm ID coiled glass columns. Packing: 15% Se-30 on Chromosorb W 80-100 mesh. Column temperature: 204°. Helium inlet pressure: 50lb/in<sup>2</sup>

	Antimycin A <sub>1</sub>	Antimycin A <sub>2</sub>	Antimycin A <sub>3</sub>	Antimycin A <sub>4</sub>
<i>Peak M</i>	5.65 min (log: 0.752)	5.65 min (log: 0.752)	3.10 min (log: 0.491)	3.10 min (log: 0.491)
<i>Molecular ion observed mass number</i>	182	182	154	154
<i>Proposed structure</i>				
<i>Peak N</i>	20.05 min (log: 1.302)	14.70 min (log: 1.167)	10.70 min (log: 1.029)	7.80 min (log: 0.892)
<i>Molecular ion observed mass number</i>	284	270	256	242
<i>Proposed structure</i>				
<i>Peak O</i>	23.15 min (log: 1.365)	17.05 min (log: 1.232)	12.15 min (log: 1.085)	8.95 min (log: 0.952)
<i>Molecular ion observed mass number</i>	284	270	256	242
<i>Proposed structure</i>	Stereochemical (or positional) isomer of pentanoic acid $\gamma$ -lactone proposed for peak N series.			

† Data of Schilling *et al.*<sup>38</sup> (reproduced with the permission of Japan Antibiotics Research Association).

individual components. Antimycin A<sub>1</sub> and A<sub>2</sub> share the same M peak, whereas A<sub>3</sub> and A<sub>4</sub> share another peak M, different from that of A<sub>1</sub> and A<sub>2</sub>. Note that the mass number for peaks N and O of the various components differs by 14, which corresponds to a methylene group; it differs by 28 for peak M. Pyrolysis-GLC of enriched fractions A<sub>0</sub>, A<sub>5</sub> and A<sub>6</sub> yielded thermolytic patterns which are compatible with the structures shown in *Figure 2*.

### Fermentation of antimycin A

The genealogy of several antimycin A producing strains is illustrated in *Table 8*. Their productivity in five media is also reported in the table. These

*Table 8.* Family of antimycin A-producing strains†

Ayerst strain no.	Medium no.‡				
	1	2	3	4	5
B-259	65	120		204	316
	↓UV				
B-265	248	503		646	1938
		↓UV			
B-303		690		856	3164
		↓UV			
B-312		870	1260	1960	4492
			↓UV		
B-314			1672	2070	4952
				↓UV	
B-346				2375	6500

† Data were obtained by A. Kudelski (in preparation). Results are expressed as µg/ml.

‡ Medium 1: soybean oil meal, 40 g; cerelese, 20 g; calcium carbonate, 1.5 g; tap water, 1 litre; temperature, 28°C; pH, 6.5; 100 ml per 500-ml Erlenmeyer flask. Medium 2: same as medium 1, except for temperature which was 25°C and pH which was 7.0. Medium 3: same as medium 2, except for aeration-agitation, 50 ml per 500-ml Erlenmeyer flask. Medium 4: same as medium 3, except for addition of 0.3% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. Medium 5: same as medium 4, except for soybean oil meal concentration which was 6 per cent.

results of Mrs A. Kudelski (unpublished data) in our laboratory were obtained in shake flask experiments. When the scale-up in 50-gallon agitated fermenters was started, yields were rather erratic. When the fermentation was successful, pattern "A" in *Figure 5* was always observed. A good fermentation was always associated with a sharp drop in pH during the first two days. After two days, glucose level was barely detectable and amino-nitrogen was also very low. Then the pH climbed regularly up to 8.2, ammonia-nitrogen followed the same trend, and antimycin A reached a maximum at 6 days. Longer incubation does not lead to higher yields, since at pH values above 8 antimycin A is destroyed as fast as it is produced. The sudden, erratic increase in pH in pattern "A" seemed to be associated with the complete exhaustion of the carbon source. Therefore, in subsequent runs, glucose or soybean oil were added continuously at a rate of 1 per cent per day, starting on the second day, when the original glucose had decreased below 0.1 per cent. A typical result is illustrated in pattern "B" of *Figure 5*. As long as a carbon source was added, the pH remained at 4.5; antimycin A was produced between the second and third day, but it remained constant thereafter, as

## ANTIBIOTICS FOR NON-HUMAN USES

### Antimycin A fermentation

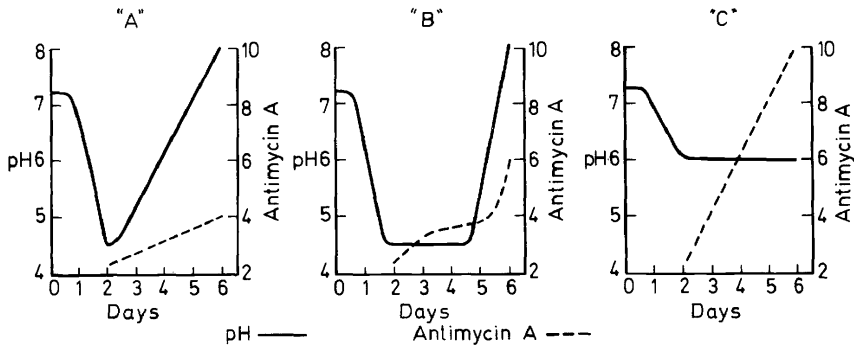


Figure 5. Time course of antimycin A fermentation. Data of Vézina, Saucier and Sehgal (in preparation).

long as the carbon source was added. In a separate experiment, glucose was fed for three weeks; pH remained at 4.5, and antimycin A did not increase. When glucose addition was discontinued (after 4 days in pattern "B") pH started immediately to rise and reached 8.2; ammonia-nitrogen followed a similar trend, and antimycin A was again produced. From pattern "B", it seemed that during the pH drop acidic compounds accumulated from which antimycin A was eventually synthesized. However, at the low pH recorded, the organism could not grow, and the condensing enzymes were probably not present in a large enough quantity to assemble the antibiotic, or were inactive. When the pH rose, the organism started to grow again and antimycin A was assembled until autolysis took place, enzymes were no longer active, or building blocks were exhausted. To solve these difficulties pH was controlled, as illustrated in pattern "C" (Figure 5). The fermentation was started as usual. When the pH had reached 6 (after 2 days), pH was automatically controlled at 6, using ammonium hydroxide as a neutralizing agent. At the same time, glucose or oil addition was started. This resulted in uniform synthesis of antimycin A. Pattern "C" observed in 50-gallon fermenters was reproduced with very slight modification in 10000-gallon fermenters.

### Biosynthesis of antimycin A

In the course of fermentation work it was noted that high producing strains could grow and produce a small quantity of antimycin A in a synthetic medium consisting of glucose, ammonium sulphate, sodium citrate, calcium carbonate and inorganic salts. Single and multiple additions of amino acids, purines, pyrimidines and vitamins had no effect on growth or production. Addition of shikimic acid, however, tripled the yields. Among the fatty acids, myristic acid was the most efficient, and doubled the yields. When added together, shikimic and myristic acids increased yields by a factor of 5 or 6. Birch and his collaborators<sup>39-41</sup> have previously shown that 1-<sup>14</sup>C- and 2-<sup>14</sup>C-acetic acids were highly incorporated into the acyl side chain. They suggested that the acyl side chain was derived from acetate and pyruvate via

valine, leucine and isoleucine or the corresponding  $\alpha$ -keto acids. In our laboratory S. N. Sehgal and A. Kudelski (unpublished data) added  $^{14}\text{C}$ -shikimic acid to the organism growing in synthetic medium, isolated antimycin A and degraded it according to the method of van Tamelen *et al*<sup>31</sup>. All the radioactivity was found in 3-amino-salicylic acid. When  $^{14}\text{C}$ -threonine was added, all radioactivity was recovered in the threonine residue which is part of the dilactone. These preliminary results indicate that the salicylic acid residue is derived from shikimic acid, whereas threonine would be incorporated as such. When threonine was added to the synthetic medium, no increase in yield was observed. The role of myristic acid is still unknown.

### Mechanism of action of antimycin A

The mode of action of antimycin A was recently reviewed by Rieske<sup>42</sup> and is summarized in Figure 6. Antimycin A specifically blocks the passage of

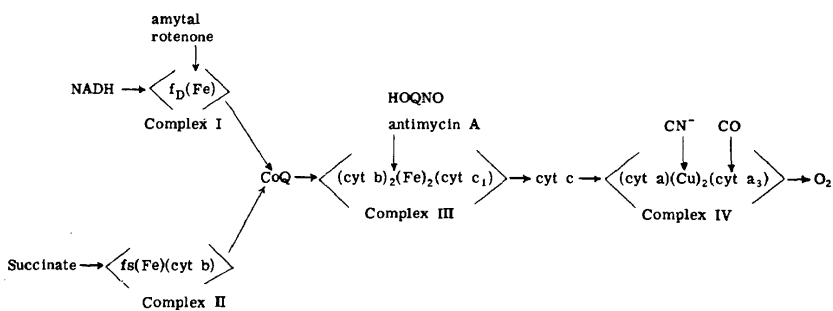


Figure 6. Components and sequence as proposed respiratory chain of mammalian mitochondria. Abbreviations:  $f_D$ , reduced nicotinamide adenine dinucleotide-dehydrogenase flavoprotein;  $f_S$ , succinate-dehydrogenase flavoprotein; CoQ, coenzyme Q or ubiquinone. Data of Rieske<sup>42</sup>. Reproduced with the permission of Springer-Verlag, New York.

electrons at a site between cytochrome b and cytochrome c. The antimycin A-sensitive site is located in complex III of the mitochondrial respiratory chain. Most bacteria either have no antimycin A-sensitive site or have an alternate electron transfer pathway, and are resistant to antimycin A. At the other extreme, fish is extremely sensitive; as a gilled animal, it is separated from its aquatic environment by a membrane only one cell layer thick<sup>43</sup>; this may account for the higher toxicity of antimycin A against fish as compared with mammals. Schoettger and Svendsen<sup>44</sup> also reported recently that the tissues of highly sensitive fish, such as trout, had higher rates of respiration than did catfish tissues.

### ACKNOWLEDGEMENTS

The author wishes to acknowledge the information supplied by Professors H. S. Goldberg and R. N. Goodman, University of Missouri, D. Pramer, Rutgers University and G. Rangaswami, University of Agricultural Sciences (Bangalore, India), and Drs V. Carroll, Charles Pfizer Co., R. F. Elliott, Allied

Chemical Corporation, J. S. Kiser, American Cyanamic Co., A. D. Rankin, Merck, Sharp & Dohme, and Y. Yagisawa, Japan Antibiotics Research Association.

The author is also indebted to his colleagues who have contributed results on antimycin A. Their names appear in the figure legend or in the text. He also wishes to express his appreciation to Miss Cécile Bolduc, who isolated most of the high-producing strains, Mr René Saucier, who ran the pilot plant fermentations, Dr Gordon Myers and Mr Paul Duchesne, who contributed to the extraction of this antibiotic.

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