Avermectins, New Family of Potent Anthelmintic Agents: Producing Organism and Fermentation

RICHARD W. BURG,† BRINTON M. MILLER,† EDWARD E. BAKER,† JEROME BIRNBAUM,† SARA A. CURRIE,† ROBERT HARTMAN,† YU-LIN KONG,† RICHARD L. MONAGHAN,† GEORGE OLSON,† IRVING PUTTER,† JOSEFINO B. TUNAC,‡ HYMAN WALLICK,‡ EDWARD O. STAPLEY,† RUIKO ŌIWA,‡ AND SATOSHI ŌMURA‡

Merck Sharp & Dohme Research Laboratories, Rahway, New Jersey 07065,† and The Kitasato Institute and Kitasato University, Shirokane, Minato-ku, Tokyo 108, Japan‡

Received for publication 15 November 1978

The avermectins are a complex of chemically related agents which exhibit extraordinarily potent anthelmintic activity. They are produced by a novel species of actinomycete, NRRL 8165, which we have named Streptomyces avermitilis. The morphological and cultural characteristics which differentiate the producing organism from other species are described. The avermectins have been identified as a series of macrocyclic lactone derivatives which, in contrast to the macrolide or polyene antibiotics, lack significant antibacterial or antifungal activity. The avermectin complex is fully active against the gastrointestinal nematode Nematospioroides dubius when fed to infected mice for 6 days at 0.0002% of the diet. Fermentation development, including medium modification and strain selection, resulted in increasing the broth yields from 9 to 500 μg/ml.

Of the several thousand microbial fermentation products which have been described, only a few have been reported to have anthelmintic activity. Of those which do, many belong to the aminoglycoside family of antibiotics. Hygromycin B (6, 10) and antibiotic G-418 (11) are active against both nematodes and cestodes. The desmycins (12, 16) are active only against nematodes, whereas paromomycin (22) and antibiotic complex S15-1 (1) are cestocidal. Antibiotics of other structural classes which are active against nematodes include the netropsin relative anthelvencin (15) and two cytosine-containing antibiotics, aspiculamycin (9) and anthelmycin (8). Three other fermentation products, myxin (7), the thaimycins (3), and the axenomycins (4), are cestocidal.

We have found a group of anthelmintic agents unrelated to any of the above compounds. They are produced by an actinomycete which was isolated at Kitasato Institute from a soil sample collected at Kawana, Ito City, Shizuoka Prefecture, Japan. It had been sent to Merck Sharp & Dohme Research Laboratories for testing in the various screening programs there. Early tests indicated that whole broth was active against Nematospioroides dubius in mice over at least an eightfold range without notable toxicity. Subsequently, the anthelmintic activity was isolated and identified as a family of closely related compounds whose structures are shown in Fig. 1.

The complex, which has been named avermectin, is highly active against a variety of nematodes. The complex is fully active against N. dubius when fed to infected mice for 6 days at 0.0002% of the diet. The individual components, although differing in their potencies, are active against other nematode species when administered orally or parenterally at 0.1 mg/kg or less; e.g., avermectin B₁₅ is greater than 98% effective against gastrointestinal nematodes and lungworms of cattle at a single oral dose of 0.1 mg/kg or against hookworms of dogs at 0.005 mg/kg (5).

In this paper, we describe the characteristics of the producing culture Streptomyces avermitilis MA-4680 (NRRL 8165) and the production of avermectin by fermentation. Subsequent papers will describe the isolation and chromatographic properties (13) and the anthelmintic activity (5). Details of the elucidation of the structures are reported elsewhere (G. Albers-Schönberg, B. H. Arison, J. C. Chabala, A. W. Douglas, P. Eskola, M. H. Fisher, A. Lusi, H. Mrozik, J. L. Smith, and R. L. Tolman, J. Am. Chem. Soc., in press).

(This paper was presented in part at the 18th Interscience Conference on Antimicrobial Agents and Chemotherapy, 1-4 October 1978, Atlanta, Ga.)

†Present address: Parke, Davis & Co., Detroit, MI 48231.
MATERIALS AND METHODS

Taxonomic studies. The morphological and cultural characteristics of the microorganism which produces avermectin were determined by use of the media and methods described by Shirling and Gottlieb (17). Previously described taxonomic keys and cultural descriptions (2, 18-21, 23) were used to compare the culture with recognized genera and species.

Fermentation studies. Fermentations were performed in 250-ml Erlenmeyer flasks incubated at 28°C on a rotary shaker (2-inch [ca. 5.08-cm] throw, 220 rpm). Vegetative growth stored in a liquid nitrogen refrigerator in seed medium containing 20% glyceral was used as the culture source for the experiments described here. Inocula were grown for 1 or 2 days in baffled flasks containing 50 ml of seed medium. Seed medium I contained (in grams per 1,000 ml of water) the following: cerelose, 1.0; soluble starch, 10.0; beef extract, 3.0; Ardamine pH (Yeast Products, Inc.), 5.0; N-Z-Amine E (Sheffield Chemical), 5.0; MgSO4·7H2O, 0.05; KH2PO4, 0.182; Na2HPO4, 0.190; and CaCO3, 0.5. The pH was adjusted to 7.0 before the addition of the CaCO3. In later experiments, seed medium II, consisting of the following (in grams per 1,000 ml of water), was used: lactose, 20.0; distillers solubles (Brown-Forman Distillers' Corp.), 15.0; and Ardamine pH, 5.0. The pH was adjusted to 7.0 before autoclaving. All media were sterilized by autoclaving at 120°C for 20 min. Production flasks, containing 40 ml of medium, were inoculated with 2 ml of the seed culture.

Reducing sugar was determined with a Technicon Autoanalyzer by a modification of the ferricyanide method of Park and Johnson (14). Growth was estimated by measuring the packed mycelial volume from 10 ml of broth centrifuged in a 15-ml graduated centrifuge tube.

Mouse assay. Anthelmintic assays were performed in mice infected with N. dubius. Male Charles River CD-1 white mice (15 to 20 g) were infected by gavage with larvae of N. dubius. After inoculation, they were fed for 6 days with milled Purina Lab Chow in which the test material had been mixed. The mice were returned to the normal diet and, at 14 days postinfection, examined for the production of eggs and the presence or absence of worms. The results of the assay were scored subjectively as fully active (no eggs, no worms), moderately active, slightly active, and inactive.

TLC assay. For the thin-layer chromatography (TLC) densitometric assay, mycelia from 10 ml of broth were removed by centrifugation. The mycelial cake was mixed with 10 ml of acetone-1 NHCl (100:1, vol/vol) and shaken for about 2 min. A 5-ml amount of CHCl3 was added, and the mixture was again shaken. The mixture was centrifuged, and the upper (mycelial) layer was discarded. The lower phase (~8
ml) was removed and evaporated to dryness under vacuum. The residue was taken up in 0.1 ml of CHCl₃.

A 2-µl sample was applied to the origin of a silica gel 60–F254 plate (E. M. Laboratories, Inc.). The plate was developed for 30 to 40 min with chloroform–ethyl acetate–methanol–methylene chloride (9:9:1:2). The avermectins can be detected under short-wave ultraviolet light or by spraying with 1% ceric sulfate in 10% sulfuric acid followed by heating at 100°C for about 10 min.

For a quantitative assay, the plate was scanned at 245 nm with a double-beam densitometer (Shoefield Instrument Corp.). The peak heights were compared with those of a standard extract which had been calibrated by high-pressure liquid chromatography (HPLC) assay (13). A scan of this standard is shown in Fig. 2. Only the four major components, avermectins A₁₆, A₂₆, B₁₆, and B₂₆, were detected since the b components were present in only trace amounts. Total avermectin was determined as the sum of the four individual components.

Isolation of S. avermitilis MA-4848. A suspension of spores of S. avermitilis MA-4860 was irradiated with ultraviolet light and plated on a dextrose-mineral salts medium supplemented with 1 g of tryptophan per liter. The colonies which grew were replicated onto plates of the same medium lacking tryptophan. “Auxotrophs” were picked and tested for production of avermectin. MA-4848 was the highest producing isolate. However, subsequent tests demonstrated that this culture does not require tryptophan, and it has no known difference from MA-4860 other than much greater ability to produce avermectin. It has been deposited with the American Type Culture Collection as ATCC 31271.

RESULTS AND DISCUSSION

Taxonomy. Taxonomic studies of culture MA-4680 revealed it to be a Streptomyces species with sporo-

phores forming spirals as side branches on aerial my-

celia. The spirals are compact but become more open as the culture ages (Fig. 3A). Spores were in chains of more than 15 and were usually spherical to oval. The spore surface was smooth as revealed by electron microscopy (Fig. 3B). Sporulation occurred on oatmeal agar, glycerol–asparagine agar, inorganic salts–starch agar, and egg albumin agar. The culture grew well at 28 and 37°C but not at 50°C. These characteristics, plus those summarized in Tables 1 and 2, were compared with those of known species in the classical references (2, 18–22). No Streptomyces species is described as having the brownish-gray spore mass color, smooth spore surface, spiral sporophore structure, production of melanoid pigments, and cultural and carbon utilization patterns that together comprise the distinctive species S. avermitilis.

Assays. In early studies, avermectin was determined by its anthelmintic activity since it lacked detectable antimicrobial activity. The anthelmintic activity was associated with the mycelia and absent from the filtrate. Chromatographic assays were developed once the identity of the anthelmintic product was known. The TLC assay was used for much of the work presented here. It offered the advantages of sensitivity and rapidity. The four major components were resolved adequately although there was some interference by extraneous ultraviolet-absorbing materials (peaks C and D in Fig. 2). Peak C is an artifact derived from the medium, and peak D is a fermentation product unrelated to the avermectins.

The HPLC assay (13) is more precise and capable of resolving all of the anthelmintic components. At times it was used to confirm the results of the TLC assays. In one experiment, a comparison of the anthelmintic assay with the HPLC assay gave the following correlation of total avermectin complex to anthelmintic activity: full activity, 54 µg/25 g of feed; moderate activity, 34 µg/25 g of feed; slight activity, 26 µg/25 g of feed.

Fermentation. Numerous complex media were screened for their ability to support the production of avermectin. Two media which gave high yields are described in Table 3. Whole broth from S. avermitilis MA-4680 grown on medium A was fully active in the mouse assay at 6 ml/25 g of feed and slightly to moderately active at 3 ml/25 g of feed, indicating a yield of about 9 µg/ml.

Medium A served as the basis for initial medium development. Suitable substitutes for Amidex were dextrin 600, soluble starch, corn starch, mannose, and dextrose. Glycerol, blackstrap molasses, lactose, N-acetylg glucosamine, sucrose, glucoamine, inositol, xylose, raffinose, and dextrin 100C gave reduced yields of avermectin.

Suitable substitutes for distillers’ solubles in me-
dium A were cornmeal, cottonseed meal, peanut meal, whole egg powder, wheat gluten, and soybean meal. Primary yeast, corn steep, and whole milk powder were less effective substitutes. Elimination of distillers’ solubles from medium A without substitution only slightly reduced avermectin production.

Autolyzed yeast (Ardamine pH) was required for avermectin production in medium A. A vitamin mix, yeast nucleic acids, monosodium glutamate, or a trace
element mix could not replace Ardamine. Elimination of CoCl$_2$·6H$_2$O from medium A slightly stimulated avermectin production.

The effects of various additions to medium A were determined by the anthelmintic assay. Of 12 vitamins tested at 2.5 µg/ml, only biotin stimulated the production of avermectin. The vitamins without effect were folic acid, thiamine hydrochloride, riboflavin, pyridoxine hydrochloride, niacin, inositol, $p$-aminobenzoic acid, ascorbic acid, calcium pantothenate, alpha-tocopherol, and lipoic acid. Vitamin K$_5$ at 10 or 50 µg/ml was also without effect. Eighteen amino acids were tested at 0.5 and 2.5 mg/ml. Of these, only DL-tryptophan (0.5 mg/ml) and possibly DL-methionine (2.5 mg/ml) stimulated production. The amino acids without effect were glycine, L-cystine, L-cysteine hydro-

---

**Fig. 3.** (A) Scanning electron micrograph of *S. avermitilis* MA-4680 (NRRL 8165) showing sporophores forming compact to slightly open spirals; (B) transmission electron micrograph showing the smooth surface of spherical to oval spores.
chloride, L- and DL-aspartic acid, L-saparaginase • H2O, L-arginine, L-alanine, L-hydroxyproline, DL-isoleucine, DL-leucine, L-glutamine, DL-serine, L-tyrosine, DL-phenylalanine, DL-histidine, and DL-homoserine. The addition of 0.5 mg of KH2PO4 per ml or 0.25 mg of MgSO4 • 7H2O per ml to medium A inhibited production, whereas sodium chloride (1 mg/ml) was without effect. A mixture of trace elements stimulated production although this effect was not always reproducible. The element or elements responsible have not been identified.

The components of medium B were determined by

### Table 1. Cultural characteristics of S. avermitilis MA-4680 (NRRL 8165)

<table>
<thead>
<tr>
<th>Medium</th>
<th>Vegetative growth</th>
<th>Aerial mycelia</th>
<th>Soluble pigment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oatmeal agar (ISP medium 3)*</td>
<td>Reverse--very dark brown</td>
<td>Powdery, brownish gray (41i)* mixed with white</td>
<td>Brown</td>
</tr>
<tr>
<td>Caspek-Dox agar</td>
<td>Poor, colorless</td>
<td>Scant, grayish</td>
<td>Light grayish brown</td>
</tr>
<tr>
<td>Egg albumin agar</td>
<td>Tan</td>
<td>Moderate, light grayish yellow-brown (5ge)* mixed with white</td>
<td>Light yellowish brown</td>
</tr>
<tr>
<td>Glycerol-saparaginase agar (ISP medium 5)*</td>
<td>Reverse--yellowish brown</td>
<td>Powdery, brownish gray (41i)*</td>
<td>Light yellowish brown</td>
</tr>
<tr>
<td>Inorganic salts-starch agar (ISP medium 4)*</td>
<td>Reverse-grayish-yellowish brown</td>
<td>Powdery, light brownish gray (41i)* edged with darker brownish gray (41i)*</td>
<td>Light yellowish brown</td>
</tr>
<tr>
<td>Yeast extract--dextrose-salts agar</td>
<td>Reverse--dark brown</td>
<td>Moderate, brownish white</td>
<td>Brown</td>
</tr>
<tr>
<td>Yeast extract--malt extract agar (ISP medium 2)*</td>
<td>Reverse--dark brown</td>
<td>Moderate, brownish white</td>
<td>Brown</td>
</tr>
<tr>
<td>Peptone-iron--yeast extract agar</td>
<td>Dark brown</td>
<td>None</td>
<td>Dark brown to black</td>
</tr>
<tr>
<td>Nutrient agar</td>
<td>Tan</td>
<td>Sparse, grayish</td>
<td>Light brown</td>
</tr>
<tr>
<td>Nutrient starch agar</td>
<td>Tan</td>
<td>Sparse, grayish white</td>
<td>Light brown</td>
</tr>
<tr>
<td>Nutrient gelatin agar</td>
<td>Tan</td>
<td>Sparse, grayish white</td>
<td>Light brown</td>
</tr>
<tr>
<td>Gelatin stab</td>
<td>Brown ring</td>
<td>None</td>
<td>Greenish brown</td>
</tr>
<tr>
<td>Skim milk agar</td>
<td>Dark brown</td>
<td>None</td>
<td>Dark brown</td>
</tr>
<tr>
<td>Littmus milk</td>
<td>Dark brown growth ring</td>
<td>None</td>
<td>Dark brown</td>
</tr>
<tr>
<td>Skim milk</td>
<td>Dark brown growth ring</td>
<td>None</td>
<td>Dark brown</td>
</tr>
<tr>
<td>Potato plug</td>
<td>Tan</td>
<td>Brown mixed with grayish white</td>
<td>Grayish brown</td>
</tr>
<tr>
<td>Loeffler blood serum</td>
<td>Grayish tan</td>
<td>None</td>
<td>Some browning of medium</td>
</tr>
<tr>
<td>Nutrient tyrosine agar</td>
<td>Reverse--dark brown to black</td>
<td>Sparse, grayish</td>
<td>Dark brown</td>
</tr>
</tbody>
</table>

* All readings were taken after 3 weeks at 28°C.

### Table 2. Physiological properties of S. avermitilis MA-4680 (NRRL 8165)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Observation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrate utilization*</td>
<td>Good growth: glucose, arabinose, fructose, inositol, lactose, maltose, mannositol, mannose, raffinose, rhamnose, sucrose, xylose</td>
</tr>
<tr>
<td>Melanin production*</td>
<td>Positive</td>
</tr>
<tr>
<td>H2S production*</td>
<td>Positive</td>
</tr>
<tr>
<td>Hydrolysis of starch*</td>
<td>Good</td>
</tr>
<tr>
<td>Liquefaction of gelatin*</td>
<td>Good</td>
</tr>
<tr>
<td>Hydrolysis of casein*</td>
<td>Good</td>
</tr>
<tr>
<td>Peptonization of milk*</td>
<td>Complete, becoming alkaline (pH 8)</td>
</tr>
<tr>
<td>Liquefaction of Loeffler blood serum</td>
<td>None</td>
</tr>
<tr>
<td>Decomposition of tyrosine*</td>
<td>None</td>
</tr>
</tbody>
</table>

* All readings were taken after 3 weeks at 28°C.

Pridham-Gottlieb basal medium plus 1% carbon source (17).

Nutrient iron--yeast extract agar (17).

Nutrient starch agar (23).

Nutrient gelatin agar; gelatin stab (23).

Skim milk agar (23).

Litmus milk; skim milk (23).

Nutrient tyrosine agar (23).
substitution in a basic medium consisting of 3% carbon and energy source, 1.5% nitrogen source, and 0.15% vitamin source. It was determined that dextrose was superior to dextrin, sucrose, or lactose as a carbon and energy source for the production of avermectin. Peptonized milk nutrient was superior to Ferm-Amine I, Sheftene WP 100, and prime meat peptone as the nitrogen source. Autolyzed yeast (Ardamine pH) was the best vitamin source. The addition of polyglycol P-2000 stimulated the production of avermectin threefold from 17 to 53 µg/ml, as determined by the TLC assay. This is probably the single most effective modification of the medium which was made.

**Table 3. Complex media for the production of avermectin**

<table>
<thead>
<tr>
<th>Medium</th>
<th>Component and amt</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Amidex(^a), 40 g; distillers' solubles(^b), 7 g; Ardamine pH(^c), 5 g; CoCl(_2)-6H(_2)O, 0.05 g; distilled water, pH 7.3, 1 liter</td>
</tr>
<tr>
<td>B</td>
<td>Cerelose(^d), 45 g; peptonized milk nutrient(^e), 24 g; Ardamine pH(^f), 2.5 g; polyglycol P-2000(^g), 2.5 ml; distilled water, pH 7.0, 1 liter</td>
</tr>
</tbody>
</table>

\(^a\) Corn Products Refining Co.  
\(^b\) Brown-Forman Distillers' Corp.  
\(^c\) Yeast Products, Inc.  
\(^d\) Corn Products Refining Co.  
\(^e\) Humko Sheffield Chemical Co.  
\(^f\) The Dow Chemical Co.
The levels of each of the three components (Cere-
lose, peptonized milk nutrient, and Ardamine pH)
were varied in a three-factor medium optimization
study. Two successive studies, each followed by com-
puter analysis of the data, led to the final optimum
composition of medium B given in Table 3.

Figure 4 compares the time course of the avermectin
fermentation on medium B by S. avermitilis MA-4680
and a high-producing isolate, MA-4848 (ATCC 31271).
Typical tropohypho-idiophase kinetics were exhibited.
Both cultures began producing avermectin sometime
between 2 and 3 days when growth had ceased. The
production by MA-4680 proceeded at a slower rate
and was essentially complete by 5 days when the
concentration reached 120 μg/ml. The production by
MA-4848 proceeded much more rapidly and at a nearly
linear rate until the concentration reached 500 μg/ml
at 6 days.

Figure 5 compares the production of the four major
components of the avermectin complex by S. avermi-
tilis MA-4680 (Fig. 5A) and MA-4848 (Fig. 5B) as
determined by HPLC assay. Although there was some
variability in the relative proportions of the four com-
ponents, A1 was usually produced in the least amount
(about 10 to 15% of the total), whereas A2 and B1 were
produced in larger amounts (each about 30 to 40% of the
total).

From the structures (Fig. 1), one could infer a pre-
cursor-product relationship among the various compo-
nents. For example, the A components could be
produced by methylation of the B components, and A1
and B1 could be produced by dehydration of A2 and
B2. However, the data shown in Fig. 5 do not indicate
any such relationship. All four of the major compo-
nents appeared to be produced concomitantly al-
though at different rates.

ACKNOWLEDGMENTS

We thank Ellen P. Lesniak for technical assistance in
the fermentations and culture isolations and George Wilks for
technical assistance in the mouse assays.

LITERATURE CITED

and K. Arima. 1977. Tseniical activity of strepto-
30:886-889.
gey’s manual of determinative bacteriology, 8th ed. The
Williams & Wilkins Co., Baltimore.
3. Cassinelli, G., E. Cotta, G. D’Amico, C. Della Brusa,
A. Grein, R. Mazzoleni, M. L. Ricciardi, and R.
Tintielli. 1970. Thaimycins, new anthelmintic and
antiprotozoal antibiotics produced by Streptomyces mi-
chiagenensis var. amyloyticus var. nova. Arch. Mikro-
1973. Axenomycins, new cestoidal antibiotics. Antimi-
D. Suhayda, S. Cifelli, R. F. Riek, and W. C. Camp-
bell. 1979. Avermectins, new family of potent anthel-
mintic agents: efficacy of the B4 component. Antimi-
7. Green, E. J., J. Beggs, G. B. Corlind, R. Cleeeland, H.
N. Prince, and E. Titworth. 1967. Studies on the in
vivo and in vitro chemotherapeutic properties of the
new antibiotic with anthelmintic properties. J. Antibi-
ott. Ser. A 17:100-103.
1974. Aspiculamycin, a new cytosine nucleoside anti-
biotic. III. Biological activities, in vitro and in vivo. J.
Antibiot. 27:339-342.
10. Kelley, S. W., L. Harris, M. A. Alexander, and L. S.
Olsen. 1960. Hygromycin B for removing Thesanosoma
actinoides, fringed tapeworms from feedlot lambs. J.
Antibiotic G-418, a new Micromonospora-produced
aminoglycoside with activity against protozoa and hel-
Chemother. 7:811-815.
ness of destonate-20 against nematodes in pigs and
Flor, R. T. Goegevelan, V. P. Gullo, H. Joshua, A.
J. Kemph, W. R. Krellwitz, R. L. Monaghan, R. E.
Ormond, K. E. Wilson, G. Albers-Schonberg, and I.
Putter. 1979. Avermectins, new family of potent
anthelmintic agents: isolation and chromatographic
14. Park, J. T., and M. J. Johnson. 1949. A submicrodeter-
Anthelvincins, new antibiotics with anthelmintic prop-
16. Shimura, M., Y. Sekizawa, K. Inuma, H. Naganawa,
and S. Kondo. 1975. Destomycin C, a new member of
destomycin family antibiotics. J. Antibiot. 28:83-84.
acterization of Streptomycetes species. Int. J. Syst.
description of type cultures of Streptomycetes. II. Species
descriptions from first study. Int. J. Syst. Bacteriol. 18:
69-189.
description of type cultures of Streptomycetes. III. Addi-
tional species descriptions from first and second studies.
description of type cultures of Streptomycetes. IV. Species
descriptions from second, third and fourth studies. Int.
description of type cultures of Streptomycetes. V. Addi-
in the treatment of Diphyldobothrium latum infection.
Williams & Wilkins Co., Baltimore.