

AFRICAN REGIONAL INDUSTRIAL PROPERTY
ORGANIZATION (ARIPO)

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<p>(21) Application Number: AP/P/88/00088</p> <p>(22) Filing Date: 28.04.88</p> <p>(24) Date of Grant & (45) Publication 15.12.89</p>	<p>(73) Applicant(s): Pfizer Inc. 235 East 42nd Street, New York USA</p>
<p>(30) Priority Data:</p> <p>(33) Country: US</p> <p>(31) Number: 126,650</p> <p>(32) Date: 01.12.87</p>	<p>(72) Inventor(s): Edmund William Hafner 12 Ferro Court, East Lyme, Connecticut, USA (See Overleaf)</p>
<p>(84) Designated States: BW GM GH KE MW SD UG ZM ZW</p>	<p>(74) Representative: Galloway and Company P.O. Box 2609 HARARE Zimbabwe</p>

(51) International Patent Classification Int. Cl.⁴ C12N 1/20

(54) Title: "Process for Production of B Avermectins and Cultures Therefor"

(57) Abstract:

Streptomyces avermitilis lacking branched-chain 2-oxo acid dehydrogenase activity and avermectin B O-methyltransferase activity, method for preparation thereof, and use thereof to produce natural and non-natural B avermectins useful as parasiticides.

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(56) Documents cited: No Citation could be determined.

PROCESS FOR PRODUCTION OF B AVERMECTINS
AND CULTURES THEREFOR

5 This invention relates to Streptomyces avermitilis
lacking avermectin B O-methyltransferase activity and
branched-chain 2-oxo acid dehydrogenase activity, to
methods for producing said S. avermitilis and to the
use of S. avermitilis to produce natural and
non-natural B avermectins.

10 U.S. Patents 4,310,519 and 4,429,042 describe the
avermectins, a complex of related agents having potent
antiparasitic activity, and their production by aerobic
fermentation of strains of Streptomyces avermitilis;
namely, S. avermitilis ATCC Nos. 31267, 31271 and
15 31272. The last two strains cited represent a frozen
vial and a lyophilized tube, respectively of a culture
obtained by ultraviolet irradiation of S. avermitilis
ATCC 31267.

20 EP 214,731, published March 18, 1987, the counter-
part of U.S. Patent Application Serial No. 886,867,
filed July 16, 1986, discloses a number of compounds
(referred to herein as non-natural avermectins) related
to the natural or known avermectins but having a novel
substituent group at the 25-position, and a process for
25 their preparation by fermentation of an avermectin
producing organism in the presence of certain specified
carboxylic acids, or derivatives or precursors thereof.
The S. avermitilis organisms used to produce the said

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novel C-25 substituted avermectins are S. avermitilis ATCC 31267, 31271, 31272 and NCIB 12121. The latter organism, described in EP 214,731, is derived from S. avermitilis ATCC 31271. It gives improved yields of the novel C-25 substituted avermectins when it is cultured in a semi-defined medium. Each of ATCC 31267, 31271, 31272 and NCIB 12121 may also produce, in addition to the novel C-25 substituted derivative, varying amounts of the known, or natural, avermectins wherein the 25-substituent is isopropyl or (S)-sec-butyl (1-methylpropyl).

The carbon skeleton of the avermectins (depicted in formula (I) below) is derived from acetates and propionates and the C-25 substituent of natural avermectins from L-isoleucine (R=(S)-sec-butyl) or L-valine (R=isopropyl) [Fisher and Mrozik, "Macrolide Antibiotics", Academic Press (1984) Ch. 14].

By "known" or "natural" avermectins is meant those avermectins produced by S. avermitilis ATCC 31267, ATCC 31271 and ATCC 31272 wherein the 25-position substituent is either isopropyl or (S)-sec-butyl(1-methylpropyl). Avermectins wherein the 25-position substituent is other than isopropyl or sec-butyl (S-form) are referred to herein as novel or non-natural avermectins.

The strains of S. avermitilis cited in the above-mentioned U.S. patents produce a class of substances described generically therein as C-076. The class comprises eight distinct but closely related compounds described as C-076 A1a, A1b, A2a, A2b, B1a, B1b, B2a and B2b. The "a" series of compounds refers to the natural avermectins wherein the 25-substituent is (S)- sec-butyl and the "b" series to those wherein

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the 25-substituent is isopropyl. The designations "A" and "B" refer to avermectins wherein the 5-substituent is methoxy or hydroxy, respectively. Lastly, the numeral "1" refers to avermectins wherein a double bond is present at the 22-23 position; and numeral "2" to avermectins having a hydrogen at the 22-position and hydroxy at the 23 position.

In this application no such identifiers are used as regards the 25-substituent of the non-natural avermectins. Identifiers A1, A2, B1 and B2 have been retained to refer to non-natural avermectins having the structural features corresponding to those of the natural avermectins as noted above.

Generation of mutants devoid of branched-chain 2-oxo acid dehydrogenase activity has been reported for Bacillus subtilis, Willecke and Pardee, J. Biol. Chem. 246, 5264-72 (1971) and Pseudomonas putida, Martin et al., J. Bacteriology, 115 198-204 (1973), but not for Streptomyces.

S. avermitilis Agly-1, a mutant strain which produces virtually only avermectin aglycones Ala and A2a is reported by Schulman et al. J. Antibiot. 38(11), 1494-1498 (1985). Also reported is the fermentation of S. avermitilis Agly-1 in the presence of sinefungin which caused increased production of avermectin aglycone B components. Likewise, S. avermitilis O8, a high producing strain for avermectins, when fermented in the presence of sinefungin as inhibitor of O-methyl transferases, resulted in production of avermectins lacking O-methyl groups on the aglycone at C-5 and in the oleandrose disaccharide moiety.

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U.S. Patent 4,378,353 describes C-076 related compounds and their preparation by cultivation of MA-5218, a mutant strain of S. avermitilis ATCC 31272, obtained therefrom by ultraviolet irradiation. The mutant is identified as ATCC 31780. The C-076 related compounds produced by said mutant lack the C-076 furan ring. Additionally, in certain of the compounds reported, one or both of the oleandrose sugar moieties have been cleaved while in others the 5-position group was oxidized to a keto group.

Three classes of O-methyltransferase mutants of S. avermitilis that produce avermectins lacking O-methyl groups have been reported by Ruby et al., 6th International Symposium on the "Biology of Actinomycetes", Debrecen, Hungary, August 26-30 (1985) and by Schulman et al., Antimicrobial Agents and Chemotherapy 31, 744-7 (1987). The first class produces primarily B avermectins due to their inability to methylate the C-5 hydroxyl of the macrocyclic lactone ring. The second class produces 3'-O, 3"-O-bis-demethylavermectins (avermectins lacking the O-methyl substituent at the 3 position of both oleandrose monosaccharide residues), and which are referred to as demethylavermectins. The third class is unable to methylate at any position.

Schulman et al., Fed. Proc. 44, 931 (1985) disclose increased production of B avermectins by fermenting S. avermitilis in the presence of substances such as sinefungin, S-adenosylethionine and S-adenosylhomocysteine which inhibit the methylation of the C-5 hydroxy group of the aglycone moiety by the enzyme avermectin B-O-methyltransferase. Streptomyces avermitilis mutants which lack O-methyltransferase

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The natural avermectins, as noted, are produced as a complex mixture of eight distinct but closely related compounds; formula (I), R=isopropyl and (S)-sec-butyl. While they have been recovered in substantially pure form (see U.S. 4,429,042), the methodology is, at best, laborious. The B avermectins generally exhibit greater anthelmintic activity than do the corresponding A avermectins. The production of non-natural avermectins (A and B components) according to the process described in EP 214,731 may also produce some of the natural avermectins in varying amounts due to the presence of the branched-chain 2-oxo acid dehydrogenase and the amino acids L-valine and L-isoleucine in the cell of the S. avermitilis microorganisms and in the medium used in their production.

The ability to produce only the more bioeffective B components of either natural or non-natural avermectins, to minimize the number and complexity of the products, and by so doing to increase the purity of a chosen avermectin, and thereby to simplify separation procedures, is a desirable goal.

S. avermitilis strains lacking branched-chain 2-oxo acid dehydrogenase activity are produced by mutation of avermectin producing strains of S. avermitilis and especially by mutation of S. avermitilis ATCC 31267, ATCC 31271, ATCC 31272 or NCIB 12121. The mutants are unable to synthesize the natural avermectins except where the fatty acid, or a precursor thereto, bearing the isopropyl or sec-butyl (S-form) group is added to the medium in which the mutants are fermented. They are capable of producing natural and non-natural avermectins when fermented

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under aqueous aerobic conditions in a nutrient medium containing an appropriate primer acid or compound convertible thereto in the fermentation process.

5 Those mutants characterized by their lack of branched-chain 2-oxo acid dehydrogenase activity, are isolated from the mutagenized colonies on the basis of a $^{14}\text{CO}_2$ assay. In this procedure the absence of $^{14}\text{CO}_2$ evolution by permeabilized cells from a substrate of
10 [^{14}C -1]-2-oxoisocaproic acid or [^{14}C -1]-2-oxo-3-methylvaleric acid or [^{14}C -1]-2-oxo-3-methylbutyric acid indicates absence of branched-chain 2-oxo acid dehydrogenase activity.

15 It was surprising and unexpected that the herein-described mutants lacking branched-chain 2-oxo acid dehydrogenase activity retained the ability to produce avermectins, especially non-natural avermectins. The inability of the mutants to produce the natural fatty acyl coenzyme A derivatives when
20 grown on a conventional medium could have been a lethal mutation if membrane integrity depended upon said derivatives or if 2-oxo acid accumulation by the former mutant led to cytotoxicity. Furthermore, the mutants were not expected to be able to synthesize acetyl CoA
25 and propionyl CoA from L-isoleucine and L-valine degradative metabolism as this requires the enzyme activities that the mutants are missing. The requirement for these acyl CoA derivatives for avermectin biosynthesis, noted above, led to the
30 expectation that the mutants might be severely impaired in non-natural avermectin production, which, surprisingly, was not the case.

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The lack of branched-chain 2-oxo acid dehydrogenase activity in the mutants described herein results in the prevention of branched-chain fatty acyl CoA synthesis from the degradation of L-isoleucine, L-leucine and L-valine and, thereby, the synthesis of the natural avermectins, except when acid R-COOH (wherein R is (S) sec-butyl or isopropyl), or a precursor therefore, is added to the fermentation medium.

Further mutation of the branched-chain 2-oxo acid dehydrogenase activity deficient mutants produces mutants which are, additionally, deficient in avermectin B O-methyltransferase activity. Mutants lacking avermectin B O-methyltransferase activity are unable to methylate the C-5 oxygen of the aglycone moiety of avermectins. Mutants lacking such activity produce essentially only B avermectins by preventing preparation of A avermectins.

The present invention also includes any organism, regardless of its appearance or physiological behavior, that may be developed by means of transformation, transduction, genetic recombination or some other genetical procedure, using a nucleic acid or an equivalent material from the herein described species, whereby it has acquired the characteristics of the herein described mutants.

The terms "avermectin" or "avermectins" as used herein refers to compounds having formula (I) below but wherein the 25-substituent (R) can be any group assimilable at said position by the S. avermitilis of this invention.

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The herein described mutants are highly valuable for producing non-natural B avermectins by the processes disclosed and exemplified herein. They are especially valuable for production of preferred avermectins, i.e., compounds wherein the C-25 substituent is C₄-C₆ cycloalkyl or cycloalkenyl, optionally substituted by C₁-C₄ alkyl group; 1-methylthioethyl, or a 5- or 6-membered oxygen or sulfur heterocyclic group, especially 3-thienyl or 3-furyl.

Mutation of an avermectin producing member of the species Streptomyces avermitilis is carried out according to known procedures using any of a variety of mutating agents including ultraviolet irradiation, X-ray irradiation, N-methyl-N'-nitro-N-nitrosoguanidine, ethylmethane sulfonate, nitrous acid and nitrogen mustards, e.g., N-methylbis(2-chloroethyl)amine, or like treatments. The mutagenesis can be conducted on spores or on a vegetative culture of S. avermitilis capable of producing natural avermectins, e.g., S. avermitilis ATCC 31272.

Following procedures well known to those skilled in the art, mutagenized colonies are selected for lack of branched-chain 2-oxo acid dehydrogenase on the basis of a biochemical assay method which permits screening of large numbers of randomly mutagenized bacterial colonies for ¹⁴CO₂ production from selected [¹⁴C-1]-2-oxo branched-chain acids (Tabor et al., J. Bact. 128, 485-486, 1976).

The methodology comprises growing the mutant colonies in the wells of a microtiter plate on a suitable nutrient medium, permeabilizing the cells with toluene followed by adding the [¹⁴C-1]-2-oxo acid (e.g.



2-oxoisocaproic acid) to each well and checking the atmosphere above the fermentation for $^{14}\text{CO}_2$. Alternatively, [^{14}C -1]-2-oxo-3-methylvaleric acid, or
5 [^{14}C -1]-2-oxo-3-methylbutyric acid can be used in place of [^{14}C -1]-2-oxo-isocaproic acid. Production of $^{14}\text{CO}_2$ is conveniently checked for by placing moist $\text{Ba}(\text{OH})_2$ -saturated filter paper above the individual wells to trap any $^{14}\text{CO}_2$ released and detection of $\text{Ba}^{14}\text{CO}_3$, if
10 any, by autoradiography. Mutants which lack branched-chain 2-oxo acid dehydrogenase activity give autoradiograms approximating those of blank (uninoculated) controls; i.e., no additional $\text{Ba}^{14}\text{CO}_3$ is produced by the mutants.

15 The mutants thus obtained are subjected to further mutagenesis using any of the above-mentioned mutating agents. Mutagenized colonies are assayed for lack of avermectin B O-methyltransferase activity by chromatography (thin layer or high performance liquid
20 chromatography) after fermentation in the presence of added precursor (e.g. 2-methylbutyric acid). Avermectin A compounds are essentially absent from fermentation broths of such mutants.

In addition to production of desired alleles of a
25 given strain of microorganism by mutagenesis, protoplast fusion permits introduction of desirable alleles produced/identified in one strain into the chromosome of another strain. For example, a strain of S. avermitilis deficient in branched-chain 2-oxo acid
30 dehydrogenase and avermectin B O-methyltransferase can, by protoplast fusion with a S. avermitilis strain having the aforementioned activities, produce a strain of S. avermitilis deficient only in avermectin B

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O-methyltransferase activity. As those skilled in the art recognize, protoplast fusion technology enables combination of desirable alleles from divergent lines of selection into a single strain.

The morphological and cultural characteristics of the mutants of this invention are generally as described in U.S. Patent 4,429,042. The distinguishing characteristic of the mutants of this invention is their lack of branched-chain 2-oxo acid dehydrogenase activity and of avermectin B O-methyltransferase activity which characteristics are determined as described herein. The lack of said activities results in the failure of the mutants to produce the natural B avermectins when grown on a defined medium substantially free of fatty acids RCOOH wherein R is isopropyl or (S)-sec-butyl, or compounds convertible to said RCOOH during fermentation. A taxonomic investigation conducted by the American Type Culture Collection, confirmed that the characteristics of mutant strain I-3, selected by the above $^{14}\text{CO}_2$ assay, bears a close relationship to those of the parental ATCC 31272 strain described in U.S. 4,429,042, but with certain exceptions. Thus, mutant strain I-3 (ATCC 53567) forms significantly fewer spore chains than does ATCC 31272. In experiments by applicants, raffinose did not appear to support the growth of I-3. In contrast to the description given for ATCC 31272 in U.S. 4,429,042, we are unable to detect growth of the mutant or of ATCC 31272 with sucrose as sole carbon source. Mutant I-3 is deficient in branched-chain 2-oxo acid dehydrogenase activity. The doubly

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deficient mutant of this invention, S. avermitilis
7881, which lacks branched-chain 2-oxo acid
dehydrogenase activity and avermectin B O-methyl-
5 transferase activity, produced by further mutagenesis
of mutant I-3 (ATCC 53567), bears a similar taxonomic
relation to ATCC 31272 as does mutant strain I-3.

Streptomyces avermitilis I-3 and 7881 have been
deposited under the terms of the Budapest Treaty in the
10 American Type Culture Collection, Rockville, Maryland,
a recognized depository affording permanence of the
deposits and ready accessibility thereto by the public
if a patent is granted on this application. They have
been given the designation Streptomyces avermitilis
15 ATCC 53567 and ATCC 53692, respectively. The deposits
are available during pendency of this application to
one determined by the Commissioner of the United States
Patent and Trademark Office to be entitled thereto
under 37 CFR 1.14 and 35 USC 122, and in accordance
20 with foreign patent laws in countries wherein
counterparts of this application, or its progeny, are
filed. All restrictions on the availability to the
public of the microorganisms deposited will be
irrevocably removed upon granting of the patent.

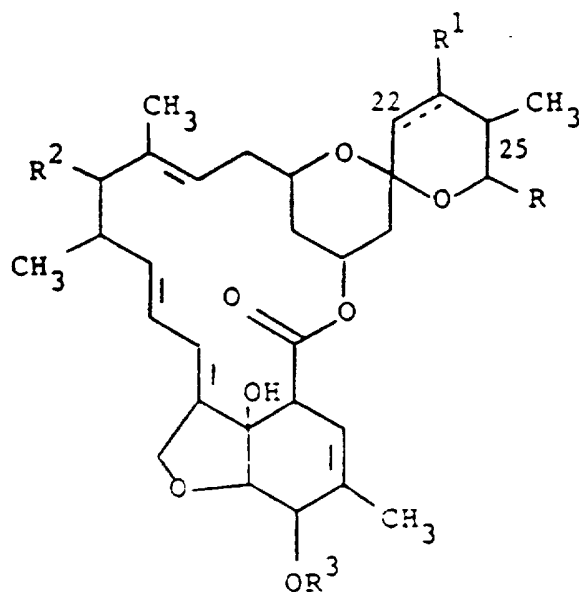
25 Each of S. avermitilis ATCC 31267, ATCC 31271,
ATCC 31272 and NCIB 12121 produces the natural
avermectins, formula (I) compounds

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(I)

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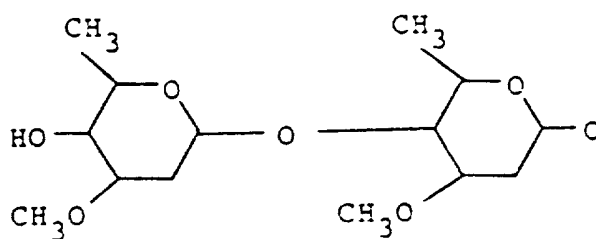
wherein the broken line at the 22-23 position represents an optional double bond;

R^1 is hydroxy and is present only when the double bond is absent;

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R^2 is 4'-(α -L-oleandrosyl)- α -L-oleandrosyloxy of the formula

25



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R^3 is hydrogen or methyl; and



R is isopropyl or (S)-sec-butyl. U.S. Patent 4,285,963 describes an avermectin of formula (I) wherein the 25-position is substituted with a methyl and an ethyl group; R¹ is hydroxy and R³ is methyl.

In the non-natural avermectins referred to herein R is a substituent other than isopropyl or (S)-sec-butyl and is as defined below.

The compounds essential for utilization in the biosynthesis of formula (I) compounds occur in the cell of S. avermitilis and in the medium. These compounds arise from degradation of L-valine and L-isoleucine or from their corresponding 2-oxo acids via decarboxylation of the 2-oxo acid by branched-chain 2-oxo acid dehydrogenase, concomitant with coupling the product with coenzyme A. Their presence accounts for the concurrent production of both the isopropyl and (S)-sec-butyl compounds of formula (I). This, of course, gives rise to problems in separating the isopropyl from the (S)-sec-butyl derivatives.

When fermented in a nutrient medium containing the appropriate primer compound the mutants of this invention produce a compound of formula (I) or, as is more usually the case, a mixture of two compounds of formula (I) in which R corresponds to the primer compound used. Up to two products, conveniently and trivially referred to as R-avermectin B1 and B2, according to the designations used in U.S. 4,429,042, can be produced. The "R-" group, of course, refers to the C-25 substituent. For example, when R is cyclopentyl the two possible avermectins are:

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described in European Patent Application Publication No. 0001689, and its counterpart U.S. 4,199,569, issued April 22, 1980.

5 The compounds of formula (I) wherein R^2 is H are prepared from the corresponding compounds wherein R^2 is 4'-(alpha-L-oleandrosyl)-alpha-L-oleandrosyloxy by removing the 4'-(alpha-L-oleandrosyl)-alpha-L-
10 oleandrose group by mild hydrolysis with an acid in an aqueous organic solvent to yield the aglycone having a hydroxy group at the 13-position; this is then halogenated, for example by reaction with a benzene sulfonyl halide, to yield the 13-deoxy-13-halo derivative which is finally selectively reduced, for
15 example using tributyltin hydride. In order to avoid unwanted side reactions it is desirable to protect any other hydroxy groups which may be present, for example using a tert-butyldimethylsilyl group. This is then readily removed after the halogenation or reduction
20 step by treatment with methanol containing a trace of acid. All these steps together with appropriate reagents and reaction conditions for their performance are described in European Patent Application Publication No. 0002615.

25 The compounds capable of utilization by the S. avermitilis of this invention for the biosynthesis of avermectins, natural and non-natural, are compounds of formula (II-A)



30 including compounds convertible to (II-A) during the fermentation process. Said compounds are referred to herein as "primer compounds". In formula (II-A), R is an alpha-branched-chain group, the carbon atom thereof

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to which is attached the -COOH group is also attached to at least two other atoms or groups other than hydrogen. This definition, of course, embraces saturated and unsaturated acyclic and cyclic groups, including those optionally bearing a sulfur or oxygen heteroatom as a member of the acyclic chain or cyclic ring.

More specifically, R, which becomes the C-25 substituent, can be an alpha-branched C₃-C₈ alkyl, alkenyl, alkynyl, alkoxyalkyl or alkylthioalkyl group; a C₅-C₈ cycloalkylalkyl group wherein the alkyl group is an alpha-branched C₂-C₅ alkyl group; a C₃-C₈ cycloalkyl or C₅-C₈ cycloalkenyl group, either of which may optionally be substituted by methylene or one or more C₁-C₄ alkyl groups or halo atoms (fluoro, chloro, iodo or bromo); or a 3 to 6 membered oxygen or sulfur containing heterocyclic ring which may be saturated, or fully or partially unsaturated and which may optionally be substituted by one or more C₁-C₄ alkyl groups or halo atoms.

Compounds convertible to RCOOH; i.e., precursors, in the fermentation process are compounds of formulae (II-B) wherein R is as defined above:



n is 0, 2, 4 or 6; and Z is -CH₂OH, -CHO, -CH₂NH₂, -COOR⁴ or -CONHR⁵ wherein R⁴ is H or (C₁₋₆)alkyl; R⁵ is hydrogen, (C₁₋₄)alkyl, or the residue of an amino acid, especially of aspartic acid, glutamic acid and methionine, e.g., -CH(COOH)CH₂COOH, -CH(COOH)(CH₂)₂COOH and -CH(COOH)(CH₂)₂SCH₃, respectively.



Also included in this invention are the isomeric forms of formula (II-A) compounds, and compounds convertible thereto during the fermentation process, and the isomeric avermectins at C-25 resulting from their use in the herein described process.

The process of this invention is carried out by aerobic fermentation with a strain of S. avermitilis which lacks branched-chain 2-oxo acid dehydrogenase activity and avermectin B-O-methyltransferase activity in an aqueous nutrient medium comprising an assimilable source of nitrogen, carbon, inorganic salts and a compound of formula RCOOH, or a compound convertible to said compound (i.e., a precursor) during the fermentation. The acid, or compound convertible thereto, is added to the fermentation either at the time of inoculation or at intervals during the fermentation. Production of the avermectin products may be monitored by removing samples from the fermentation, extracting with an organic solvent and following the appearance of the product by chromatography, for example using high pressure liquid chromatography. Incubation is continued until the yield of the product has been maximized, generally for a period of from 4 to 15 days.

A preferred level of each addition of the primer compounds (carboxylic acid or compound convertible thereto) is between 0.05 and 3.0 grams per liter. The primer compound can be added continuously, intermittently or all at once to the fermentation. The acid (RCOOH) is added as such or as a salt, such as the sodium, lithium or ammonium salt, or as a compound convertible to the acid as defined above. The acid, if a solid, is preferably dissolved in a suitable solvent such as water or (C₁₋₄) alcohols.

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The media used for the fermentation can, especially when the C-25 substituent is to be isopropyl or (S)-sec-butyl, be conventional media containing assimilable sources of carbon, nitrogen and trace elements. When the C-25 substituent is to be a non-natural group; i.e., it is not isopropyl or (S)-sec-butyl, the fermentation medium is one in which the chosen ingredients lack, or contain only minimal amounts of primer compounds wherein the R moiety is isopropyl or (S)-sec-butyl.

After fermentation for a period of several days at a temperature preferably in the range of 24 to 33°C., the fermentation broth is centrifuged or filtered and the mycelial cake is extracted with preferably acetone or methanol. The solvent extract is concentrated and the desired product is then extracted into a water-immiscible organic solvent, such as methylene chloride, ethyl acetate, chloroform, butanol or methyl isobutyl ketone. The solvent extract is concentrated and the crude product is further purified as necessary by chromatography, for example using preparative reverse phase, high pressure liquid chromatography.

The product is generally obtained as a mixture of the compounds of formula (I) wherein R^2 is 4'-(alpha-L-oleandrosyl)-alpha-L-oleandrosyloxy, R^1 is OH and the double bond absent or R^1 is absent and the double bond is present and wherein R^3 is H. Compounds wherein R^3 is CH_3 are essentially absent. However, the proportions of each compound can vary depending on the particular mutant and primer compound employed and the conditions used.



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The source of the R group; i.e., whether it comes directly from R-COOH or is produced from one of the above precursors, or from any precursor, is immaterial to the production of the avermectins. The critical requirement of the process of this invention for their production is that the desired R group be made available to the S. avermitilis strains of this invention in the fermentation process.

- 10 Suitable compounds include the following:
 2,3-dimethylbutyric acid
 2-methylhexanoic acid
 2-methylpent-4-enoic acid
 2-cyclopropyl propionic acid
15 4,4-difluorocyclohexane carboxylic acid Lithium
 salt
 4-methylenecyclohexane carboxylic acid
 3-methylcyclohexane carboxylic acid (cis/trans)
 1-cyclopentene carboxylic acid
20 1-cyclohexene carboxylic acid
 tetrahydropyran-4-carboxylic acid
 thiophene-2-carboxylic acid
 3-furoic acid
 2-chlorothiophene-4-carboxylic acid
25 cyclobutane carboxylic acid
 cyclopentane carboxylic acid
 cyclohexane carboxylic acid
 cycloheptane carboxylic acid
 2-methylcyclopropane carboxylic acid
30 3-cyclohexene-1-carboxylic acid
 2-methylthiopropionic acid
 2-methyl-4-methoxybutyric acid
 thiophene-3-carboxylic acid
 hydroxymethylcyclopentane

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3-thiophene carboxaldehyde
3-cyclohexylpropionic acid
3-cyclopentylpropionic acid
5 hydroxymethylcyclobutane
tetrahydrothiophene-3-carboxylic acid
3-cyclopentyl-1-propanol
3-methylcyclobutane carboxylic acid Lithium salt
3-fluorocyclobutane carboxylic acid
10 3-methylenecyclobutane carboxylic acid Lithium
salt
2-methyl-4-methylthiobutyric acid
tetrahydrothiopyran-4-carboxylic acid
cyclobutylmethylamine
15 ethyl cyclobutanecarboxylate
4-hydroxymethylcyclopentene
2-(3-thiophenecarbonyl)propionic acid ethyl ester
S-2-methylpentanoic acid
R-2-methylpentanoic acid

20 Three classes of O-methyltransferase mutants can
be obtained from the herein-described branched-chain
2-oxo acid dehydrogenase negative mutants. Mutants in
which a mutation in active branched-chain 2-oxo acid
dehydrogenase activity is combined with one or more of
25 the O-methyltransferase mutations, to yield strains of
S. avermitilis that will, when fed RCOOH compounds or
compounds convertible to RCOOH during the fermentation
process, produce primarily B avermectins, demethyl-
30 methylated at all. Said mutants are obtained by
mutagenesis of the herein described mutants which lack
branched-chain 2-oxo acid dehydrogenase activity by
means of ultraviolet light and/or chemical mutagens
such as N-methyl-N-nitrosourethan, nitrosoguanidine or

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other agent such as those enumerated above. Alternatively, branched-chain 2-oxo acid dehydrogenase positive mutants which lack one or more of the O-methyltransferases can be mutated by treatment with UV light or a mutagenizing agent to produce the branched-chain 2-oxo acid dehydrogenase negative mutants.

The non-natural avermectins produced by such mutants are characterized by the presence of hydroxy groups at the C-5 position of the aglycone moiety and/or the C-3' and/or C-3" positions of the oleandrose moieties.

The above-described mutants are identified according to the methodology described by Schulman et al. Antimicrobial Agents and Chemotherapy, 29, 620-624 (1986). They are useful for the same purposes and in the same way as are the known avermectins.

Alternatively, increased amounts of the B avermectins, including those lacking methyl groups on the oleandrose disaccharide moiety, are produced by fermenting the mutants of this invention, which lack active branched-chain 2-oxo acid dehydrogenase, in the presence of a substance such as sinefungin, S-adenosylethionine or S-adenosylhomocysteine which inhibit O-methyltransferase activity.

The compounds of the invention are highly active antiparasitic agents having particular utility as anthelmintics, ectoparasiticides, insecticides and acaricides.

Thus the compounds are effective in treating a variety of conditions caused by endoparasites including, in particular, helminthiasis which is most frequently caused by a group of parasitic worms

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administered by injection or as an implant. Such formulations are prepared in a conventional manner in accordance with standard veterinary practice. Thus capsules, boluses or tablets may be prepared by mixing the active ingredient with a suitable finely divided diluent or carrier additionally containing a disintegrating agent and/or binder such as starch, lactose, talc, magnesium stearate etc. A drench formulation may be prepared by dispersing the active ingredient in an aqueous solution together with dispersing or wetting agents, etc., and injectable formulations may be prepared in the form of a sterile solution which may contain other substances, for example, enough salts or glucose to make the solution isotonic with blood. These formulations will vary with regard to the weight of active compound depending on the species of host animal to be treated, the severity and type of infection and the body weight of the host. Generally for oral administration a dose of from about 0.001 to 10 mg per kg of animal body weight given as a single dose or in divided doses for a period of from 1 to 5 days will be satisfactory, but, of course, there can be instances where higher or lower dosage ranges are indicated and such are within the scope of this invention.

As an alternative the compounds may be administered with the animal feedstuff and for this purpose a concentrated feed additive or premix may be prepared for mixing with the normal animal feed.

For use as an insecticide and for treating agricultural pests the compounds are applied as sprays, dusts, emulsions and the like in accordance with standard agricultural practice.



Production of S. avermitilis I-3 (ATCC 53567)

Step 1. S. avermitilis ATCC 31272 was grown as a
confluent lawn on New Patch Agar Medium for 12 days at
30°C. The medium comprised

5

	V-8 Juice*	200 ml
	CaCO ₃	3 grams
	Agar	15 grams
10	H ₂ O	to 1000 ml
	Nutrient broth	1.0 grams/L
	sodium acetate.3H ₂ O	1.4 grams/L
	isovaleric acid	50 mg/L
	isobutyric acid	50 mg/L
15	methylbutyric acid	50 mg/L
	isoleucine	250 mg/L
	leucine	250 mg/L
	valine	250 mg/L
	trace elements solution**	1 ml/L

20

*A mixture of 8 vegetable juices (tomato, carrots, celery, beets, parsley, lettuce, watercress and spinach) plus salt, ascorbic and citric acids and natural flavors. Available from Campbell Soup Company, Camden, NJ.

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**Composition of Trace elements solution:

	FeCl ₃ .6H ₂ O	2.7 g
	MnSO ₄ .H ₂ O	4.2
5	CuSO ₄ .5H ₂ O	0.5
	CaCl ₂	11.0
	H ₃ BO ₃	0.62
	CoCl ₂ .6H ₂ O	0.24
	ZnCl ₂	0.68
10	Na ₂ MoO ₄	0.24

Dissolve the above in 1 liter of 0.1N HCl.

Spores were harvested from 3 such plates and
15 suspended in 20 ml. of 0.05M tris-maleic acid buffer,
pH 9.0.

Step 2. 10 ml of the spore suspension was added to a
vial containing 10 mg of N-methyl-N'-nitro-N-nitroso-
guanidine (NTG). The vial was incubated and shaken at
20 28°C. for 60 minutes and the spores then washed pro-
fusely with 1% NaCl solution.

Step 3. The washed spores were suspended in 1% NaCl
and mixed with an equal volume of 80% ethylene glycol.
This suspension was preserved at -20°C. and used as a
25 source of cells to be screened for mutants. It gave
approximately 10⁴ colonies/ml when germinated.

This spore stock was spread on YPD plates to yield
approximately 100 colonies per plate (YPD medium
comprises 10 g/l of each of yeast extract, Bacto
30 peptone* and dextrose; and 15 g/l of Bacto agar*,
adjusted to pH 6.9 before autoclaving). Ingredients
marked with an asterisk are available from Difco
Laboratories, Detroit, Michigan 48238.

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5 Step 4. Single colonies were picked from plates after 2-3 weeks of growth at 28°C. and placed in individual wells of a standard 96 well microtiter plate. Also, a small quantity of the colony was patched onto a fresh agar medium to serve as a source of viable cells when mutants are identified.

10 Step 5. To each well was added approximately 75 microliters of a liquid M9 salts medium containing 1% glucose, 0.1% casamino acids, and 0.01% of each of isovaleric, isobutyric and 2-methylbutyric acids. After several days of incubation at 28°C., the cells were assayed for the presence of branched-chain 2-oxo acid dehydrogenase. (Each liter of M9 salts medium
15 comprises 6g Na₂HPO₄, 3g KH₂PO₄, 0.5g NaCl and 1 g of NH₄Cl. The medium is autoclaved and then 1 ml of each of sterilized 1M MgSO₄ and 0.1M CaCl₂ are added aseptically).

20 Step 6. A microsuspension of 5% toluene in M9 salts medium was prepared by a brief sonication of the immiscible mixture. To 25 ml of this suspension was added 1.2 ml of a solution containing [¹⁴C-1]-2-oxo-isocaproic acid, 2.5 microcurie/ml and 10.0 microcurie/micromole. 50 Microliters of this overall
25 mixture was added to each of the wells of the microtiter plates containing the colonies to be assayed.

30 Step 7. The ¹⁴CO₂ produced from each well was trapped and visualized by the procedure described by Tabor et al., J. Bacteriol. 128 485-486 (1976) entitled "Convenient Method for Detecting ¹⁴CO₂ in Multiple Samples: Application to Rapid Screening for Mutants". Mutants lacking active branched-chain 2-oxo acid dehydrogenase produce no Ba¹⁴CO₃ beyond that observed for the controls.



5 A more refined method which improves the contrast between a positive assay for $^{14}\text{CO}_2$, indicated by a dark spot on the autoradiogram as a result of $\text{Ba}^{14}\text{CO}_3$ formation, and a negative assay indicated by no spot or a very light spot, comprises the following modified screen.

10 Single colonies (see Step 4 above) were picked from the agar medium after 7-14 days of growth (rather than 2-3 weeks and assayed directly by steps 6 and 7 above). Step 5 of the above procedure is omitted.

15 An even more refined assay method which is quantitative in nature as regards $^{14}\text{CO}_2$ release comprises growing the mutants detected by the above screens on a suitable medium comprising M9 salts medium with glucose, 1% and "Syncasa-bcaa", 0.1% (a synthetic mixture of L-amino acids with the approximate composition of commercial casamino acids, but without the presence of L-valine, L-isoleucine and L-leucine, see below).

20 After growth to high cell density, the cells were washed in M9 salts medium and resuspended in cold M9 salts medium containing 1% toluene which had been sonicated to produce a milky white dispersion of the toluene. The cell/buffer/toluene suspension was 25 incubated for 40 minutes at 30°C. in order to permeabilize the cells. The permeabilized cells were then washed in M9 medium salts and finally resuspended in one-fifth the original volume of M9 medium buffer. 30 180 Microliters of this suspension were used per assay.

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5 A reaction volume of 300 microliters contained the
toluenized cells, thiamine pyrophosphate (TPP), 0.4 mM;
coenzyme A (CoA), 0.11 mM; nicotinamide adenine
dinucleotide (NAD), 0.68 mM, dithiothreitol (DTT), 2.6
mM; MgCl₂, 4.1 mM; Tris-HCl, 60 mM; Tris-HCl, 60 mM, pH
7.5; and [¹⁴C-1]-2-oxoisocaproate, 6,000 cpm,
microcurie per micromole. The efficiency of counting
was 73%. The reaction was carried out in 15 ml
10 scintillation vials containing a 2 x 2 cm Whatman #4
paper square pressed into the screw cap of the vial.
The paper contains 30 microliters of 1M Hyamine
Hydroxide (1M solution of methylbenzethonime hydroxide
in methanol; available from Sigma Chemical Co.,
15 St. Louis, MO 63178), which traps ¹⁴CO₂ evolved in the
reaction. After incubation for 2 hours, the papers are
immersed in 10 ml of Beckman Aquasol II (Universal LSC
(liquid scintillation counter) available from New
England Nuclear Research Products, Boston, MA 02118)
20 and the radioactivity measured in a liquid
scintillation counter after equilibration in this
solvent for 4 hours or more. A blank control reaction
(i.e. - no cells) gives ca. 50-300 cpm.

25 Mutant I-3 and others like it gave counts that
were less than or equal to the blank control reaction,
whereas the parent strain gave counts several fold
higher than blank control value.

30



Composition of "Syncasa - bccaa", 100 fold Concentrate

		<u>grams/liter</u>
	L-alanine	3
5	L-arginine	4
	L-aspartic acid	6
	L-cystine	1
	L-glutamic acid	20
	glycine	1
10	L-histidine	2
	L-lysine	7
	L-methionine	3
	L-phenylalanine	6
	L-proline	10
15	L-serine	6
	L-threonine	4
	L-tyrosine	4
	L-tryptophan	1

20 The mixture is adjusted to pH 7 and filter sterilized.
One volume of concentrate is added to 99 volumes of
medium to achieve standard use concentrations.

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Production of Doubly Blocked Mutants of
S. avermitilis 7881 (ATCC 53692)
Deficient in Branched-Chain 2-Oxo Acid
Dehydrogenase and in Avermectin B
O-Methyltransferase

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Step 1. S. avermitilis ATCC 53567 was grown as a confluent lawn on New Patch Agar Medium for 12 days at 30°C.

10 Spores were harvested from 3 such plates and suspended in 20 ml. of 0.05M tris-maleic acid buffer, pH 9.0.

Step 2. 10 ml of the spore suspension was added to a vial containing 10 mg of N-methyl-N'-nitro-N-nitrosoguanidine (NTG). The vial was incubated and 15 shaken at 28°C. for 60 minutes and the spores then washed profusely with 1% NaCl solution.

Step 3. The washed spores were suspended in 1% NaCl and mixed with an equal volume of 80% ethylene glycol. This suspension was preserved at -20°C. and 20 used as a source of cells to be screened for mutants.

This spore stock was spread on YPD plates to yield approximately 100 colonies per plate. Colonies of the mutagenized population (nitroso-guanidine treated) of Streptomyces avermitilis strain 25 I-3 (ATCC 53567) are picked, and spread as patches on an agar medium prepared as follows (grams per liter): thinned starch, 80; K_2HPO_4 , 1; $MgSO_4 \cdot 7H_2O$, 1; ardamine PH, 5; $CaCO_3$, 5; P-2000, 1 ml; $FeSO_4 \cdot 7H_2O$, 0.01; 30 $MnCl_2 \cdot 4H_2O$, 0.001; $ZnSO_4 \cdot 7H_2O$, 0.001; Bacto agar, 17; distilled H_2O to 980 ml. The pH is adjusted to 7.0 with NaOH prior to autoclaving at 121°C for 20 minutes. After autoclaving, 20 ml of a sterile 5% stock solution of (+)-2-methylbutyric acid, pH 7.0 is added.



The agar cultures are incubated 8 to 12 days at 28°C. Cells (mycelia) are removed from the agar surface, and put into 250 microliters of acetone.

5 Twenty-five (25) microliters of the acetone extracts are then spotted on Analtech Silica Gel GF precoated thin layer chromatography plates. The chromatogram is run for 30 to 40 minutes with ethyl acetate as solvent, then dried, and sprayed with 3% vanillin in ethanol.

10 The plates are placed in a 100°C oven for 1 to 3 minutes, then sprayed with 3% sulfuric acid in ethanol, and again placed in a 100°C oven for 10 to 15 minutes. Mutants deficient in avermectin B O-methyltransferase are identified by a change in the chromatographic

15 pattern; i.e., the spots corresponding to avermectin B components (Rf ca 0.54, 0.42 for B1 and B2, respectively) are still present, but the spots corresponding to avermectin A components (Rf ca 0.69, 0.58 for A1 and A2, respectively) are missing.

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General High Performance Liquid
Chromatography (HPLC) Procedures

Mobile Phase:

- 5 150 ml of water
 70 ml of acetonitrile
 bring to 1 liter with methanol

Column:

- 10 Ultrasphere ODS 25 cm (Beckman Instruments,
 Fullerton, CA 92634-3100)
 flow: 0.75 ml/minute
 detection: UV a 240 nm
 attenuation: near 6

Sample diluent (D):

- 15 35 ml acetonitrile plus 390 ml methanol

Standards:

1. weigh 0.5 mg avermectin A2A into 10 ml
 flask and bring to volume with methanol
2. weigh 0.5 mg test product into 10 ml flask
20 and bring to volume with methanol

1 and 2 are standard stock solutions; for standard
solution to run:

take 100 ul (1) and 100 ul (2) into a vial
add 800 ul mobile phase

25 Samples:

1. Take 1 ml of well shaken broth; spin down
2. Remove as much supernatant as possible
without

disturbing pellet

- 30 3. Add 100 ul of HPLC water to the pellet and
 vortex mix to disperse
4. Add 2 ml diluent (D) and mix well
5. Filter the same and run on HPLC.



The natural and non-natural avermectins described herein were subjected to this HPLC chromatographic procedure and the retention time of the peaks of the individual avermectins divided by the retention time observed for the oligomycin A present and which serves as internal standard for a given HPLC determination. Oligomycin A is almost always observed by HPLC as by-product of S. avermitilis fermentations and is the only product seen on HPLC produced by the mutants described herein when they are cultured in a medium free of acids RCOOH wherein R is as defined herein or in medium free of compounds convertible to acids of the formula RCOOH wherein R is as defined herein. Typically, oligomycin A retention time is 12.5-14 minutes. The ratio of the retention times (RT) affords a more significant basis for comparing the identity and yields of avermectin products. The general order of appearance of the avermectin products on HPLC is B2, A2, B1 and A1.

	<u>Natural Avermectin</u>	<u>RT/RT (oligomycin A)</u>
	B2b	0.70
	B2a	0.84
25	A2b	0.90
	A2a	1.09
	B1b	1.40
	B1a	1.83
	Alb	1.83
30	Ala	2.42

Note that B1a and Alb are unresolved.

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	<u>Non-Natural Avermectin</u>	<u>RT/RT (oligomycin A)</u>
	cyclopentyl B2	0.94
5	cyclopentyl A2	1.23
	cyclopentyl B1	1.99
	cyclopentyl A1	2.62

10 Retention times vary 1-2 minutes on different
days, with oligomycin A generally appearing near
12.5-14 minutes.

15 In the following examples the avermectins were
determined by the above described HPLC procedure,
except where noted.

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The compositions of media used in the following examples are presented below.

	<u>AS-7 Medium</u>	
5		g/l
	thinned starch ^a	20
	Ardamine pH ^b	5
	Pharmamedia ^c	15
10	CaCO ₃	2

^a Prepared by hydrolysis of starch by alpha-amylase from Bacillus licheniformis (available from Novo Enzymes, Wilton, CT and sold under the trademark "Termamyl") to a dextrose equivalent of 40% ± 5%.

^b From Yeast Products, Inc., Clifton, NJ 07012

^c From Traders Protein., Memphis, TN 38108

Adjust pH to 7.2 with NaOH.

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AP-5 Medium

	<u>g/l</u>
5	thinned starch 80
	Ardamine pH 5
	K ₂ HPO ₄ 1
	MgSO ₄ ·7H ₂ O 1
	NaCl 1
	CaCO ₃ 7
10	FeSO ₄ ·7H ₂ O 0.01
	MnCl ₂ ·7H ₂ O 0.001
	ZnSO ₄ ·7H ₂ O 0.001
	P-2000 (antifoam) ^a 1 ml/l

15 ^a From The Dow Chemical Co., Midland, Michigan 48640
Adjust pH to 6.9 with 25% NaOH.

EXAMPLES 1-4

Avermectins from Streptomyces avermitilis
I-3 (ATCC 53567)

20 S. avermitilis I-3 (ATCC 53567) from a sporulated
V-8 plate as inoculated into 80 ml of AS-7 medium in a
500 ml triple-baffled flask. The flask was incubated
on a rotary shaker with agitation at 200 rpm at
28-30°C. After 24 hours of incubation, 1 ml of whole
25 broth was inoculated into 300 ml flasks containing
40 ml AP-5 medium. Duplicate fermentations were
performed at 28-30°C in the presence of 400 ppm of each
of the primer compounds listed below, added after 24
hours.

30 After 312 hours, 2 ml samples of whole broth were
mixed with 8 ml of methanol:acetonitrile (390:35).
After filtration, 50 ul samples were injected onto a
Beckman Ultrasphere ODS column (3.9 x 250 mm). The
column was eluted with methanol:acetonitrile:water

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(89:14:7) at 0.8 ml/min and the eluate monitored with uv detection at 240 nm. The retention times of the novel avermectins are shown below.

5	Primer Compound	Avermectin Retention Time (Minutes)			
		B2	A2	B1	A1
	1) ± 2-methyl butyric acid	*11.60, 12.40	*14.75, 16.10	23.1	29.82
10	2) cyclopentane-carboxylic acid	12.32	15.86	25.28	32.96
	3) cyclohexane-carboxylic acid	14.84	19.26	31.46	41.14
	4) +2 methyl-butyrlic acid	11.60	14.75	23.1	29.82

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*Both +methylbutyric acid and -methylbutyric acid are incorporated into avermectins. Under the chromatography conditions adopted, resolution of the + and - avermectins is achieved only with B2 and A2.

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EXAMPLE 5

Cyclohexyl Avermectins from Streptomyces avermitilis 7881 (ATCC 53692)

A frozen vial of culture S. avermitilis 7881 (ATCC 53692) was inoculated into 100 ml of AS-7 medium in a 500 ml triple-baffled flask. The flask was incubated on a rotary shaker with agitation at 200 rpm at 28-30°C. After 28 hours of incubation, 5 ml of the whole broth was inoculated into another 100 ml of AS-7 medium or a 500 ml triple-baffled flask. The flask was again incubated on a rotary shaker with agitation at 200 rpm at 28-30°C. After 24 hours of incubation, 1 ml of whole broth was inoculated into a 300 ml flask containing 40 ml of AP-5 medium. The flasks were

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incubated at 28-30°C at 200 rpm. After 24 hours,
400 ppm of cyclohexane carboxylic acid were added,
after 312 hours 2 ml samples were taken and subjected
5 to high performance liquid chromatography as described
in Example 1. Under these conditions, the only
avermectin components detected in the fermentation were
eluted at 14.84 (54.9 mg/l) and 31.46 (32.1 mg/l)
minutes corresponding to cyclohexyl B2 and B1,
10 respectively.

EXAMPLE 6

Sec-Butyl Avermectins from *Streptomyces avermitilis*
7881 (ATCC 53692)

The procedure of Example 5 was repeated but using
15 400 ppm of \pm 2-methylbutyric acid in place of
cyclohexanecarboxylic acid, all other conditions were
the same as those described in Example 2. Only
sec-butyl avermectin B2 (11.60, 12.40 minutes, 63.5,
42.4 mg/l), and sec-butyl avermectin, B1 (23.1 minutes,
20 105.5 mg/l) were detected in the fermentation.

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CLAIMS

1. Streptomyces avermitilis lacking branched-chain 2-oxo acid dehydrogenase activity and avermectin B O-methyltransferase activity.
2. Streptomyces avermitilis lacking branched-chain 2-oxo acid dehydrogenase activity and avermectin B O-methyltransferase activity characterized by the inability of permeabilized cells thereof to produce $^{14}\text{CO}_2$ from added [^{14}C -1]-2-oxoisocaproic acid.
3. Streptomyces avermitilis having the identifying characteristics of ATCC 53692.
4. Streptomyces avermitilis ATCC 53692.
5. A process for preparation of a B avermectin which comprises aerobically fermenting with a strain of Streptomyces avermitilis which lacks branched-chain 2-oxo acid dehydrogenase activity and avermectin 5-O-methyltransferase activity, an aqueous nutrient medium comprising an assimilable source of nitrogen, carbon and inorganic salts and a compound capable of utilization in the biosynthesis of an avermectin.
6. A process according to claim 5 wherein the S. avermitilis has the identifying characteristics of ATCC 53692.
7. A process according to claim 5 wherein the compound is of the formula



wherein R is an alpha-branched-chain group, the carbon atom thereof to which is attached the -COOH group is also attached to at least two other atoms or groups other than hydrogen;

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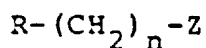
- 1-cyclohexenyl
- 3-methylcyclohexyl (cis/trans)
- 4-methylenecyclohexyl
- 5 3-methylcyclobutyl
- 3-methylenecyclobutyl
- 3-cyclopentenyl
- 1-cyclopropylethyl
- 3-fluorocyclobutyl
- 10 4,4-difluorocyclohexyl
- isopropyl
- sec-butyl
- 2-pentyl
- 2,3-dimethylpropyl
- 15 2-hexyl
- 2-pent-4-enyl
- 2-methylthioethyl
- S-2-pentyl
- R-2-pentyl
- 20 2-thienyl
- 3-thienyl
- 4-tetrahydropyranyl
- 3-furyl
- 2-chlorothienyl
- 25 3-tetrahydrothienyl
- 4-methylthio-2-butyl
- 4-tetrahydrothiopyranyl
- 4-methoxy-2-butyl or
- 4-methylthio-2-butyl.

30 11. A process according to claim 10 wherein R is derived from a carboxylic acid of formula



12. A process according to claim 11 wherein R is cyclopentyl, cyclohexyl or thienyl.

13. A process according to claim 10 wherein R is derived from a compound convertible to R-COOH during the fermentation, said compound being of the formula



wherein R is a previously defined; n is 0, 2, 4 or 6; Z is $-CH_2OH$, $-CHO$, $-COOR^4$, $-CH_2NH_2$ or $-CONHR^5$ wherein R^4 is (C_{1-6}) alkyl; R^5 is hydrogen, (C_{1-4}) alkyl, $-CH(COOH)CH_2COOH$, $-CH(COOH)(CH_2)_2COOH$ or $-CH(COOH)(CH_2)_2SCH_3$.

14. A process according to claim 8 wherein when R is an alpha-branched C_3-C_8 alkyl group, it is not isopropyl or (S)-sec-butyl.

15. The process according to claim 8 wherein the strain of S. avermitilis is S. avermitilis ATCC 53692.

16. A novel strain belonging to the genus Streptomyces which produces substantially no C-076 avermectins when fermented in an aqueous nutrient medium comprising an assimilable source of carbon, nitrogen and inorganic salts under aerobic conditions, said medium being substantially free of an acid of the formula



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or a compound convertible to said acid by S.
avermertilis, wherein R is isopropyl or (S)-sec-butyl,
but being capable of producing substantially only B
5 avermectins upon fermentation in said medium when said
medium contains an acid of the formula RCOOH, or a
compound convertible to said acid by S. avermertilis,
wherein R is isopropyl or (S)-sec-butyl, wherein said
strain is obtained by a process which comprises
10 mutating Streptomyces avermertilis ATCC 53567.

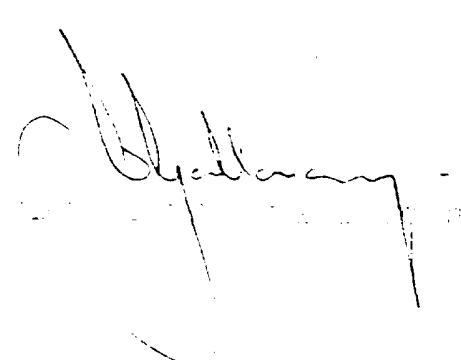
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