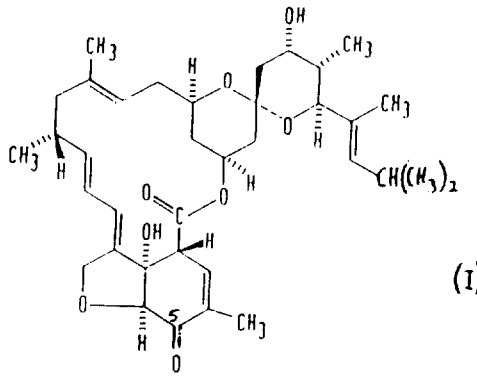
26960

MACROLIDE COMPOUND

ABSTRACT

The compound of formula (I)



(I)

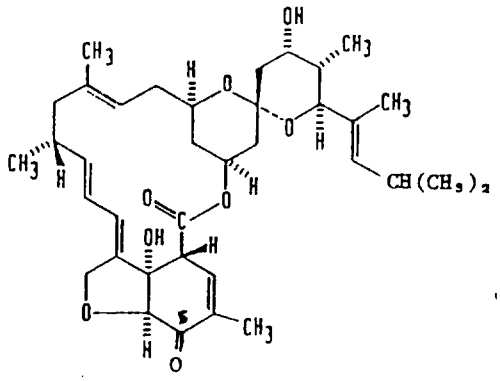
This compound has antiparasitic activity and is a useful intermediate in the preparation of other compounds having antibiotic activity.

The present application is a divisional of application Serial No. 35014 filed on March 11, 1987.

This invention relates to a new antibiotic compound and to processes for its preparation. More particularly it relates to a new antibiotic compound which may be obtained by fermentation of Streptomyces microorganisms.

United Kingdom Patent Specification 2166436 and European Patent Specification No 170006 describe the production of a class of substances, which we have designated Antibiotics S541, which may be isolated from the fermentation products of Streptomyces microorganisms. We have now found a further compound with antibiotic activity which may be prepared by isolation from a culture of a microorganism of the genus Streptomyces or by chemical modification of an Antibiotics S541 compound as described herein.

Thus, according to one aspect of the invention we provide the compound of formula (I)



(I)

A particularly advantageous feature of the compound of the invention is its ability to form crystalline solids and the invention is intended to extend to the compound of formula (I) in crystalline form.

The ability of the compound to crystallise may be made use of as a purification step in its preparation, and by utilising this property

the compound of formula (I) has been obtained as a crystalline solid having a purity in excess of 90%, more particularly in excess of 95%.

In view of its ability to form a crystalline solid having a very high purity, the compound of formula (I) is especially useful as an intermediate in the preparation of other compounds with antibiotic activity.

The compound of formula (I) itself also has antibiotic activity e.g. antihelminthic activity, for example against nematodes, and in particular, anti-endoparasitic and anti-ectoparasitic activity.

The antibiotic activity of the compound of formula (I) may, for example, be demonstrated by its activity in vitro against free living nematodes e.g. Caenorhabiditis elegans.

Ectoparasites and endoparasites infect humans and a variety of animals and are particularly prevalent in farm animals such as pigs, sheep, cattle, goats and poultry (e.g. chickens and turkeys), horses, rabbits, game-birds, caged birds, and domestic animals such as dogs, cats, guinea pigs, gerbils and hamsters. Parasitic infection of livestock, leading to anaemia, malnutrition and weight loss is a major cause of economic loss throughout the world.

Examples of genera of endoparasites infecting such animals and/or humans are Ancylostoma, Ascaridia, Ascaris, Aspicularis, Brugia, Bunostomum, Capillaria, Chabertia, Cooperia, Dictyocaulus, Dirofilaria, Dracunculus, Enterobius, Haemonchus, Heterakis, Loa, Necator, Nematodirus, Nematospiroides (Heligomoroides), Nippostrongylus, Oesophagostomum, Onchocerca, Ostertagia, Oxyuris, Parascaris, Strongylus, Strongyloides, Syphacia, Toxascaris, Toxocara, Trichonema, Trichostrongylus, Trichinella, Trichuris, Triodontophorus. Uncinaria and Wuchereria.

Examples of ectoparasites infecting animals and/or humans are arthropod ectoparasites such as biting insects, blowfly, fleas, lice, mites, sucking insects, ticks and other dipterous pests.

Examples of genera of such ectoparasites infecting animals and/or humans are Ambylomma, Boophilus, Chorioptes, Culliphore, Demodex, Damalinia, Dermatobia, Gastrophilus, Haematobia, Haematopinus, Haemophysalis, Hyaloma, Hypoderma, Ixodes, Linognathus,

Lucilia, Melophagus, Oestrus, Otobius, Otodectes, Psorergates, Psoroptes, Rhipicephalus, Sarcoptes, Stomoxys and Tabanus.

Furthermore, the compound of formula (I) is also of use in combating insect, acarine and nematode pests in agriculture, horticulture, forestry, public health and stored products. Pests of soil and plant crops, including cereals (e.g. wheat, barley, maize and rice) vegetables (e.g. soya), fruit (e.g. apples, vines and citrus) as well as root crops (e.g. sugarbeet, potatoes) may usefully be treated. Particular examples of such pests are fruit mites and aphids such as Aphis fabae, Aulacorthum circumflexum, Myzus persicae, Nephotettix cincticeps, Nilparvata lugens, Panonychus ulmi, Phorodon humuli, Phyllocoptruta oleivora, Tetranychus urticae and members of the genera Trialeuroides; nematodes such as members of the genera Aphelencoides, Globodera, Heterodera, Meloidogyne and Panagrellus; lepidoptera such as Heliothis, Plutella and Spodoptera; grain weevils such as Anthonomus grandis and Sitophilus granarius; flour beetles such as Tribolium castaneum; flies such as Musca domestica; fire ants; leaf miners; Pear psylla; Thrips tabaci; cockroaches such as Blatella germanica and Periplaneta americana and mosquitoes such as Aedes aegypti.

According to the invention we therefore provide the compound of formula (I) as defined above, which may be used as an antibiotic. In particular, it may be used in the treatment of animals and humans with endoparasitic, ectoparasitic and/or fungal infections and in agriculture, horticulture, or forestry as a pesticide to combat insect, acarine and nematode pests. It may also be used generally as a pesticide to combat or control pests in other circumstances, e.g. in stores, buildings or other public places or location of the pests. In general the compound may be applied either to the host (animal or human or plants or other vegetation) or to the pests themselves or a locus thereof.

The compound of the invention may be formulated for administration in any convenient way for use in veterinary or human medicine and the invention therefore includes within its scope pharmaceutical compositions comprising a compound in accordance with the invention adapted for use in veterinary or human medicine. Such

compositions may be presented for use in conventional manner with the aid of one or more suitable carriers or excipients. The compositions of the invention include those in a form especially formulated for parenteral (including intramammary administration), oral, rectal, topical, implant, ophthalmic, nasal or genito-urinary use.

The compound of formula (I) may be formulated for use in veterinary or human medicine according to the general methods described in UK Patent Specification 2166436.

The total daily dosages of the compound of the invention employed in both veterinary and human medicine will suitably be in the range 1-2000µg/kg bodyweight, preferably from 50-1000µg/kg and these may be given in divided doses, e.g. 1-4 times per day.

The compound according to the invention may be formulated in any convenient way for horticultural or agricultural use and the invention therefore includes within its scope compositions comprising the compound according to the invention adapted for horticultural or agricultural use. Such formulations include dry or liquid types, for example dusts, including dust bases or concentrates, powders, including soluble or wettable powders, granulates, including microgranules and dispersible granules, pellets, flowables, emulsions such as dilute emulsions or emulsifiable concentrates, dips such as root dips and seed dips, seed dressings, seed pellets, oil concentrates, oil solutions, injections e.g. stem injections, sprays, smokes and mists.

Generally such formulations will include the compound in association with a suitable carrier or diluent. Such carriers and diluents are as described in UK Patent Specification 2166436.

In the formulations, the concentration of active material is generally from 0.01 to 99% and more preferably between 0.01% and 40% by weight.

Commercial products are generally provided as concentrated compositions to be diluted to an appropriate concentration, for example from 0.001 to 0.0001% by weight, for use.

The rate at which the compound is applied depends upon a number of factors including the type of pest involved and the degree of infestation. However, in general, an application rate of 10g/h to

10kg/h will be suitable; preferably from 10g/h to 1kg/h for control of mites and insects and from 50g/h to 10kg/h for control of nematodes.

For use in veterinary medicine or for horticultural and agricultural use it may be desirable to use the whole fermentation broth, as a source of the active compound. It may also be suitable to use dried broth (containing mycelia) or to use mycelia separated from the broth and pasteurised or more preferably, dried e.g. by spray-, freeze-, or roller drying. If desired the broth or mycelia may be formulated into compositions including conventional inert carriers, excipients or diluents as described above.

The antibiotic compound of the invention may be administered or used in combination with other active ingredients.

In particular, the antibiotic compound of the invention may be used together with Antibiotics S541 compounds or with other antibiotic compounds. This may occur, for example, where whole fermentation broth is used without prior separation of the compound of the invention or where crude fermentation products are reacted according to a process of the invention without prior or subsequent separation; this may be preferable for example in agricultural use of the compound, where it is important to maintain low production costs.

The compound of the invention may be prepared by the processes discussed below.

Thus according to a further aspect of the invention we provide a process for the production of the compound of formula (I) which comprises the step of cultivating a microorganism of the genus Streptomyces capable of producing the compound of formula (I), and if desired isolating said compound therefrom.

Microorganisms capable of producing the compounds of the invention may readily be identified by using a small scale test and analysing a test sample obtained from fermentation of the microorganism by high performance liquid chromatography.

In general the microorganism for use in the process according to the invention will be a strain of the genus Streptomyces capable of producing the compound of formula (I) and may be for example a microorganism of the species Streptomyces thermoarchaensis or Streptomyces cyaneogriseus noncyanogenus. Particular examples of

suitable strains include Streptomyces thermoarchaensis NCIB 12015 [deposited 10th September 1984] Streptomyces thermoarchaensis NCIB 12111, NCIB 12112, NCIB 12113 and NCIB 12114 [all deposited 26th June, 1985] Streptomyces cyaneogriseus noncyanogenus NRRL 15773 [deposited 3rd May 1984] and mutants of all these strains.

Mutants of the above strains may arise spontaneously or may be produced by a variety of methods including those outlined in Techniques for the Development of Micro-organisms by H. I. Adler in 'Radiation and Radioisotopes for Industrial Microorganisms', Proceedings of the Symposium, Vienna 1973, p241, International Atomic Energy Authority. Such methods include ionising radiation, chemical methods e.g. treatment with N-methyl-N'-nitro-N-nitrosoguanidine (NTG), heat, genetic techniques, such as recombination, transduction, transformation, lysogenisation and lysogenic conversion, and selective techniques for spontaneous mutants.

Mutants which are particularly suitable for use in the process according to the invention are those mutants which do not produce any substantial amounts of the 5β-hydroxy or 5β-methoxy Antibiotics S541 compounds described in UK Patent Specification No 2166436. Mutants with impaired ability to form Antibiotics S541 compounds form a further aspect of the present invention.

Mutants capable of producing the compound of formula (I) but with impaired ability to form the Antibiotics S541 compounds described in UK Patent Specification No. 2166436 may readily be identified by using a small scale test and analysing a test sample obtained from the fermentation of the microorganism by high performance liquid chromatography.

According to a further aspect of the invention we provide the genetic materials of mutants with impaired ability to form Antibiotics S541 compounds that participate in the synthesis of the compound of formula (I). Such material may be obtained using conventional genetic engineering techniques as described just below.

A particularly preferred strain capable of producing the compound of formula (I) with impaired ability to form Antibiotics S541 compounds is Streptomyces thermoarchaensis NCIB 12334 [deposited 15th September 1986 in the permanent culture collection of the National

Collections of Industrial and Marine Bacteria, Torry Research Station, Aberdeen, United Kingdom] and mutants thereof. Streptomyces thermoarchaensis NCIB 12334 and mutants thereof form a further aspect of the invention. Streptomyces theromoarchaensis NCIB 12334 has substantially similar essential characteristics to those described for Streptomyces thermoarchaensis NCIB 12015 in UK Patent Specification 2166436. However, NCIB 12334 can be distinguished from NCIB 12015 in that it produces little or none of the Antibiotics S541 compounds described in UK Patent Specification 2166436 under the fermentation conditions described therein. Mutants of Streptomyces thermoarchaensis NCIB 12334 may arise spontaneously or may be prepared by the methods described just above.

According to a still further aspect of the invention we provide the genetic material of Streptomyces thermoarchaensis NCIB 12334 and mutants thereof that participates in the synthesis of the compound of formula (I). Such material may be obtained using conventional genetic engineering techniques including those outlined by D A Hopwood in 'Cloning genes for Antibiotic Biosynthesis in Streptomyces Spp.: Production of a hybrid antibiotic' p409-413 in Microbiology 1985, Ed. L. Lieve, American Society of Microbiology, Washington D.C. 1985. Such techniques may be used in a similar manner to that described previously for cloning antibiotic biosynthetic genes, including the biosynthetic genes for actinorhodin (Malpartida, F. and Hopwood, D. A. 1984, Nature 309, p462-464), erythromycin (Stanzak, R. et al, 1986, Biotechnology, 4, p229-232) and an important enzyme involved in penicillin and cephalosporin production in Acremonium chrysogenum (Sansom, S.M. et al, 1985, Nature, 318, p191-194). The Streptomyces thermoarchaensis genetic material so obtained may be used, for example, for strain improvement, for production of biosynthetic enzymes for in vitro applications, or for generating novel antibiotics by introduction of such material into organisms other than Streptomyces thermoarchaensis.

The production of the compound of the invention by fermentation of a suitable Streptomyces organism may be effected by conventional means i.e. by culturing the Streptomyces organism in the presence of assimilable sources of carbon, nitrogen and mineral salts.

Assimilable sources of carbon, nitrogen and minerals may be provided by either simple or complex nutrients. Sources of carbon will generally include glucose, maltose, starch, glycerol, molasses, dextrin, lactose, sucrose, fructose, carboxylic acids, amino acids, glycerides, alcohols, alkanes and vegetable oils. Sources of carbon will generally comprise from 0.5 to 10% by weight of the fermentation medium.

Sources of nitrogen will generally include soya bean meal, corn steep liquors, distillers solubles, yeast extracts, cottonseed meal, peptones, ground nut meal, malt extract, molasses, casein, amino acid mixtures, ammonia (gas or solution), ammonium salts or nitrates. Urea and other amides may also be used. Sources of nitrogen will generally comprise from 0.1 to 10% by weight of the fermentation medium.

Nutrient mineral salts which may be incorporated into the culture medium include the generally used salts capable of yielding sodium, potassium, ammonium, iron, magnesium, zinc, nickel, cobalt manganese, vanadium, chromium, calcium, copper, molybdenum, boron, phosphate, sulphate, chloride and carbonate ions.

An antifoam may be present to control excessive foaming and added at intervals as required.

Cultivation of the Streptomyces organism will generally be effected at a temperature of from 20 to 50^{0}C preferably from 25 to 40^{0}C, especially around 34^{0}C, and will desirably take place with aeration and agitation e.g. by shaking or stirring. The medium may initially be inoculated with a small quantity of a suspension of the sporulated microorganism but in order to avoid a growth lag a vegetative inoculum of the organism may be prepared by inoculating a small quantity of the culture medium with the spore form of the organism, and the vegetative inoculum obtained may be transferred to the fermentation medium, or, more preferably to one or more seed stages where further growth takes place before transfer to the principal fermentation medium. The fermentation will generally be carried out in the pH range 5.5 to 8.5, preferably 5.5 to 7.5. It may be necessary to add acid to the fermentation medium to keep the pH within the desired range. Suitable acids which may be added include

aqueous acids such as sulphuric acid or fatty acids such as valeric acid or isobutyric acid or mixtures thereof.

The fermentation may be carried out for a period of 2-10 days, e.g. about 5 days.

Where it is desired to separate the compound of the invention from the whole fermentation this may be carried out by conventional isolation and separation techniques. The compound according to the invention is predominantly contained in the mycelia of the cells, but may also be found in the fermentation broth and the isolation techniques may also be applied to the fermentation broth either before or after clarification. It will be appreciated that the choice of isolation techniques may be varied widely.

The compound of the invention may be isolated and separated by a variety of fractionation techniques, for example adsorption-elution, precipitation, fractional crystallisation, solvent extraction and liquid-liquid partition, which may be combined in various ways.

Solvent extraction, partition between two solvents which are immiscible or only partially miscible with each other, and chromatography have been found to be particularly suitable for isolating and separating the compound of the invention.

Following the fermentation, the mycelia may be harvested (optionally after treatment with a flocculant or with an acid until the pH of the fermentation medium is below pH 6 or after heating or, preferably heating and acid addition) using conventional techniques, for example, filtration or centrifugation. Thereafter, for example, the compound of the invention may be extracted from the mycelia with an appropriate organic solvent such as a ketone. e.g. acetone, methylethyl ketone or methylisobutyl ketone; a hydrocarbon, e.g. hexane; a halogenated hydrocarbon e.g. chloroform, carbon tetrachloride or methylene chloride; an alcohol, e.g. methanol or propan-2-ol; a diol, e.g. propan-1,2-diol or butan-1,3-diol; an ester, e.g. methyl acetate or ethyl acetate or mixtures thereof. It will be appreciated that if the mycelia contain significant amounts of water, it will be preferable to use a water-miscible solvent such as methanol or acetone.

Generally, more than one extraction of the mycelia will be needed to achieve optimum recovery. The compound of the invention may be recovered from the solvent extracts as a crude preparation by removal of the solvent e.g. by evaporation or by precipitation, for example, by running the solvent extract into water. Alternatively the initial solvent extract after reduction of solvent volume, for example by evaporation, may itself be extracted with a second solvent which is immiscible or only partially miscible with the first. If the initial extraction of mycelia is with a water miscible solvent such as methanol or propan-1,2-diol, then the second solvent may suitably be a water immiscible solvent such as hexane, chloroform, dichloromethane, ethyl acetate or petroleum ether or mixtures thereof, sufficient water being added to the initial extract to ensure immiscibility with the second solvent and to control the partition of the antibiotic compound between the phases. By suitable choice of water miscible and water immiscible solvents, and by manipulation of the water content of the water miscible solvent, a number of transfers of the antibiotic compound from water miscible to water immiscible solvent and vice versa are possible. Alternatively, the initial solvent extract may be passed over a bed of an ion exchange resin such as IRA 68 or a non-functional resin such as XAD-1180 (Rohm and Haas) to further purify the extract.

The antibiotic compound may be recovered from the final solvent solution by evaporation of the solvent, by precipitation or by adsorption onto a suitable solid support, depending on the nature of the final solvent.

Purification and/or separation of the compound of the invention may be further effected by conventional techniques such as for example, chromatography (including high performance liquid chromatography) on a suitable support such as silica; a non-functional macroreticular adsorption resin for example cross linked styrene divinyl benzene polymer resins such as Amberlite XAD-2, XAD-4, XAD-16 or XAD-1180 resins (Rohm & Haas Ltd) or Kastell S112 (Montedison); a substituted styrene-divinyl benzene polymer, for example a halogenated (e.g. brominated) styrene-divinyl benzene polymer such as Diaion SP207 (Mitsubishi); an organic solvent-compatible cross-linked dextran

such as Sephadex LH20 (Pharmacia UK Ltd), or on reverse phase supports such as hydrocarbon linked silica e.g. C_{18}-linked silica. The support may be in the form of a bed, or more preferably packed in a column.

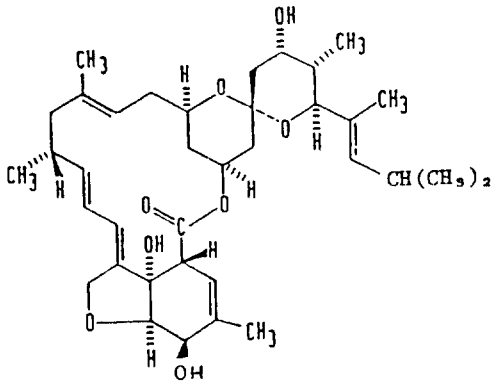
A solution of the compound of formula (I) in a suitable solvent, e.g. dichloromethane, tetrahydrofuran, petroleum ether, acetonitrile, chloroform, ethyl acetate or mixtures thereof will generally be loaded on to the chromatography column, e.g. a silica or Sephadex column, if desired after first reducing the volume of solvent. The column may optionally be washed and then eluted with a solvent of suitable polarity for example alcohols, such as methanol; hydrocarbons eg hexane or petroleum ether; esters, such as ethyl acetate; ketones, such as acetone; ethers, such as diethyl ether; acetonitrile; or halogenated hydrocarbons such as dichloromethane or chloroform. Combinations of such solvents or combinations with a polar solvent, e.g. water, may also be used.

The presence of the compound of the invention during the extraction/isolation procedures may be monitored by conventional techniques such as high performance liquid chromatography or UV spectroscopy or by utilising the properties of the compound described hereinafter.

Where the compound of the invention is obtained in the form of a solution in an organic solvent, for example after purification by chromatography, the solvent may be removed by conventional procedures, e.g. by evaporation, to yield the compound in a solid or crystalline form.

By a suitable combination of the foregoing procedures the compound of the invention has been isolated as a solid. It will be appreciated that the order in which the above purification steps are carried out and the choice of those which are used may be varied widely.

In a further process according to the invention the compound of formula (I) may be prepared by oxidation of a compound of formula (II)



(II)

The reaction may be effected with an oxidising agent serving to convert an allylic secondary hydroxyl group to an oxo group, whereby the compound of formula (I) is produced.

Suitable oxidising agents include, for example, transition metal oxides such as manganese dioxide, and atmospheric oxygen in the presence of a suitable catalyst such as a finely divided metal e.g. platinum.

The oxidising agent will generally be used in excess over the stoichiometric quantity.

The reaction may conveniently be effected in a suitable solvent which may be selected from a ketone, e.g. acetone; an ether, e.g. diethyl ether, dioxan or tetrahydrofuran; a hydrocarbon, e.g. hexane; a halogenated hydrocarbon e.g. chloroform or methylene chloride; or an ester, e.g. ethyl acetate. Combinations of such solvents either alone or with water may also be used.

The reaction may be carried out at a temperature of from -50 ^{0}C to +50 ^{0}C, preferably from 0 ^{0} to 30 ^{0}C.

The intermediate compound of formula (II) may be prepared as described in UK Patent Specification 2166436.

The compound of formula (I), prepared by fermentation or from a compound of formula (II) according to the aforementioned procedures either as a solution or a crude solid, may be obtained in crystalline

form using conventional methods. Thus, for example, crystallisation may be achieved from a solution of the compound e.g. by standing in a suitable solvent (eg an alcohol such as methanol or propan-2-ol; acetonitrile; a hydrocarbon such as hexane; or an ether such as diethylether, isopropylether or petroleum ether) optionally combined with water.

The following Examples illustrate the invention. All temperatures are in ^{0}C. 'L' refers to litre.

Factor A is the compound of formula (II). Factor A may be prepared as described in UK Patent Specification 2166436.

Example 1

Factor A (250 mg) in ether (30 ml) was stirred with active manganese dioxide (1.0 g) for 2.75 hr. The mixture was filtered through a Kieselguhr pad, and the filtrate evaporated to give the compound of the invention as a foam (240 mg). A portion was crystallised from petroleum-ether to give microcrystals, λ_{max} (EtOH) 240.5 nm (E_1^1 495); δ ($CDCl_3$) values 6.58 (s; 1H), 2.60 (m; 1H) 1.89 (s; 3H), 1.62 (s; 3H), 1.53 (s; 3H), 1.05 (d 6; 3H), 1.00 (d 6; 3H), 0.96 (d 6; 3H) and 0.80 (d 6; 3H); m/z include 610, 592, 574, 441 265, 247, 237, 219 and 151.

Example 2

Spores of Streptomyces thermoarchaensis NCIB 12015 were inoculated onto agar slants made up of the following ingredients

	gL^{-1}
Yeast extract (Oxoid L21)	0.5
Malt extract (Oxoid L39)	30.0
Mycological peptone (Oxoid L40)	5.0
Agar No. 3 (Oxoid L13)	15.0
Distilled water to 1 litre	
pH ~ 5.4	

and incubated at 28 ^{0}C for 10 days.

The mature slant was then covered with 6ml of a 10% glycerol solution and scraped with a sterile tool to loosen the spores and mycelium. 0.4ml aliquots of the resulting spore suspension were transferred to sterile polypropylene straws which were then heat-sealed and stored in liquid nitrogen vapour until required.

Two 250ml Erlenmeyer flasks containing 50ml of seed medium made up as follows:

	gL^{-1}
D-Glucose	15.0
Glycerol	15.0
Soya peptone	15.0
NaCl	3.0
$CaCO_3$	1.0

Distilled water to 1 L

[The unadjusted pH of the medium was 6.7 which was adjusted to pH 7.0 with aqueous sodium hydroxide before autoclaving. The pH of the medium after autoclaving was 7.3]

were each inoculated with 0.2ml of the spore suspension taken from a straw.

The flasks were incubated at 28^0 for 2 days on a shaker rotating at 250rpm with a 50mm diameter orbital motion.

The contents of both flasks were used to inoculate a 70 L fermenter vessel containing 40 L of the same seed medium supplemented with polypropylene glycol 2000 (0.06% v/v). Polypropylene glycol 2000 was added as required throughout the fermentation to control foaming. The fermentation was carried out at 28^0, with agitation and aeration sufficient to maintain a dissolved oxygen level of greater than 30% saturation. After 24 hours of fermentation, a 9 L portion of broth was transferred to a 700 L fermenter containing 450 litres of medium made up as follows:

	gL^{-1}
D-glucose	2.8
Malt Dextrin (MD30E)	27.8
Arkasoy 50	13.9
Molasses	1.7
K_2HPO_4	0.14
$CaCO_3$	1.39
Silicone 525 (Dow Corning)	0.06% (v/v)

Adjusted to pH 6.5 before sterilisation.

The fermentation was carried out at 28^0 with agitation and aeration. Polypropylene glycol 2000 antifoam was added as required and the pH was kept down to pH 7.2 by the addition of H_2SO_4 until harvest. The fermentation was harvested after 5 days.

The broth (450 L) was clarified on a Westfalia KA 25 centrifuge and the residual supernatant was displaced with water (20 L). The recovered cells (25.5 kg) were stirred for 1 hour with a Silverson mixer model BX in sufficient methanol to give a total volume of 75 L. The suspension was filtered and the solid residue was re-extracted with methanol (35 L) and filtered. The combined filtrate (87 L) was diluted with water (40 L) and extracted with 60^0-80^0 petroleum ether (30 L). After 30 min. the phases were separated on a Westfalia MEM 1256 centrifuge and the lower methanol phase was re-extracted with 60^0-80^0 petroleum ether (30 L) after the addition of water (40 L). After separation the lower phase was again extracted with 60^0-80^0 petroleum ether (30 L). The combined petroleum ether phases (85 L) were concentrated by three passes through a Pfaudler 8.8-12v-27 wiped-film evaporator (vapour pressure 0.1 bar, vapour temperature 20^0, steam temperature 127^0). The concentrate (9 L) was dried with sodium sulphate (2 kg) and further concentrated under reduced pressure at 40^0 in a rotary film evaporator.

The oily residue (130g) was dissolved in chloroform to give 190ml and this was applied to a column of Merck 7734 silica 60 (200x4cm) packed in chloroform. The column was washed with chloroform

(500ml) and eluted with chloroform:ethyl acetate (3:1) and fractions of approximately 40ml were collected after a forerun of 1,400ml.

Fractions 32-46 were combined and evaporated to yield an oil (21.2g). Fractions 47-93 were combined and evaporated to give an oil (20.1g) which was dissolved in chloroform:ethyl acetate (3:1) to 50ml, and applied to a column of Merck 7734 silica 60 (200x4cm) packed in chloroform:ethyl acetate (3:1), and fractions of approximately 40mls were collected after a forerun of 1,400 ml. Fractions 22-36 were combined and evaporated to give an oil (3.1g) which was added to the oil obtained from fractions 32-46 from the first column. The combined oils were dissolved in boiling methanol (4ml) which was then added to hot propan-2-ol (20ml) and allowed to crystallise.

Mother liquor after crystallisation was evaporated to yield an oil which was dissolved in an equal volume of dichloromethane and loaded onto a column (30x2.2cm) of Merck Kieselgel 60 (70-230 mesh ASTM, Art. No. 7734) packed in dichloromethane. The bed was washed with dichloromethane (2 bed volumes) and eluted with chloroform:ethyl acetate (3:1) (2 bed volumes). Evaporation of the eluate yielded an oil which was dissolved in methanol and subjected to preparative HPLC on Spherisorb S5 ODS-2 (250mmx20mm, Phase Sep.Ltd.). Portions of the sample (5ml) were pumped onto the column over a period of 1 minute and the column was eluted with acetonitrile:water (7:3) under the following conditions:

Time (mins)	Flow (ml/min)	
0.00	0.00	) Injection
1.00	0.00	) time
1.10	30.00	
39.90	30.00	
40.00	35.00	
75.00	35.00	

Material eluting from the hplc column was monitored by uv spectroscopy at 238nm.

Evaporation of the combined fractions with peaks eluting at 33.4 minutes yielded the compound of the invention (34mg).

E.I. mass spectroscopy yielded a molecular ion at 610 and gave characteristic fragments at : 592
574
556
422
259
241

Example 3

Streptomyces thermoarchaensis NCIB 12334 was stored as spore suspensions in 20% glycerol at -70^0 in a manner similar to that described for Streptomyces thermoarchaensis NCIB 12015 in Example 2. Spores (1ml) were thawed and used to inoculate a 250ml Erlenmyer flask containing 50ml of Medium A:

Medium A

	gL^{-1}
D-glucose	2.5
Malto Dextrin MD30E	25.0
Arkasoy 50	12.5
Beet Molasses	1.5
K_2HPO_4	0.125
$CaCO_3$	1.25
MOPS [3-(N-morpholino)propanesulphonic acid]	21.0

Distilled water to 1L pH adjusted to 6.5 prior to autoclaving (15 min at 121^0).

The seed flask was incubated at 28^0 for 2 days at which time 1ml was asepticially transferred into each of four similar flasks (each containing 50ml Medium A). Each flask was incubated at 28^0 for a 2 day period with agitation on a rotary shaker (250 rev/min) with a 50mm diameter throw. Two 7L seed fermenters were then inoculated with about 80ml [2% (v/v)] of the bulked shake flask inoculum. Each 7L fermenter contained 4L of Medium B:

Medium B

	gL^{-1}
Meritose	45.0
Arkasoy 50	18.0
Beet Molasses	2.2
K_2HPO_4	0.18
$CaCO_3$ (Analar)	1.8
Silcone 1520 (Dow Corning)	2.5ml in 4L

Distilled water to 4L with pH adjusted to 6.5 prior to autoclaving (45 min at 124^0). The fermentation was carried out at 28^0 with agitation and aeration.

A 700L fermenter was inoculated with 5.4 litres transferred aseptically from the seed vessels at 24h. The fermenter contained 450L of Medium B in which $CaCO_3$ (Analar) was replaced by Sturcal chalk and 250ml of silicone 1520 was batched in. The pH was adjusted to 6.5 using concentrated H_2SO_4 prior to in situ sterilization for 30 min at 121^0. In addition three 70L fermenters were inoculated with 800ml (2% v/v) transferred aseptically from the seed vessel at 24h. Each vessel contained 35L of Medium B as shown above with 25ml of silicone 1520 added prior to in situ sterilization (30 min 121^0).

The subsequent fermentations were carried out at 34^0 with agitation and aeration. Polypropylene glycol 2000 antifoam was added as required throughout the fermentations, and the pH was kept down to pH 7.4 by addition of 75:25 valeric acid/isobutyric acid until harvest.

After 120h the contents of the fermenters were bulked (600L), mixed with Dicalite (3kg) and filtered through cellulose (24kg Rettenmaier BE00) on a rotary vacuum filter. The recovered cell paste (45kg) was stored at -20^0.

The frozen cell paste was suspended in methanol (55L) and allowed to thaw. After 30 min at 5^0 the suspension was filtered through a cellulose bed. The residue was resuspended in methanol (50L), filtered through the cellulose bed and washed with methanol (10L).

The combined filtrates and washes (116L) were diluted with water (22L) and extracted with 60^0-80^0 petroleum ether (100L). The petroleum

ether extract was concentrated at low pressure (approx. 0.15 bar) in a wiped-film evaporator.

The concentrate (8.4L) was extracted three times with propan-1,2-diol (3x4.2L). The combined extracts (13.5L) were stirred with ethyl acetate (22L) and water (12L), and were then left to settle and the phases separated. The upper phase (17.5L) was washed twice with water (12L and 10L) and the remaining upper organic phase was dried to an oil (112g).

The oil was dissolved in dichloromethane (500mℓ) and chromatographed on a column (425mm x 45mm diam) of Merck Kieselgel 60 (type 7734) packed in the same solvent. The column was washed with dichloromethane (500mℓ) and was then eluted with a mixture of dichloromethane and diethyl ether (400mℓ of 9:1 v/v respectively). The eluate was dried to a pale yellow tar (54g).

(a) A portion (1.2g) of the tar was dissolved in acetonitrile (5mℓ) and chromatographed on a column (340mm x 25mm diam) of Sephadex LH20 which was packed and eluted with acetonitrile. The fraction eluting between 110mℓ and 130mℓ was dried and redissolved in acetonitrile (2.5mℓ). A portion (2mℓ) of this solution was diluted with 70% aqueous acetonitrile (2mℓ containing 1.4mℓ acetonitrile). This solution was then chromatographed in two equal parts on a column (250mm x 25mm diam) of Spherisorb S5 ODS2 with 70% aqueous acetonitrile as the eluting solvent. The material eluting between 0.8L and 0.95L was collected from the two runs and was diluted with water (about 0.9L). This solution was pumped back onto the same column of S5 ODS2 and the column was eluted with acetonitrile. The acetonitrile was removed by evaporation and the sample was dried azeotropically from a mixture of acetone and cyclohexane to yield the compound of the invention (33mg) as a solid. The nmr, uv and mass spectra of the solid were compatible with those of the product of Example 1.

(b) A portion of the tar (4.32g) dissolved in diethyl ether was dried to a solid, and redissolved in acetonitrile at a concentration of 300mg/mL. This solution (8.5mL) was mixed with an equal volume of 75% v/v acetonitrile in water, and was chromatographed in nine portions on a column of Spherisorb ODS-2 (250mmx20mm). The several components were eluted (25mL/min) with 75% acetonitrile. The peak

eluting between 0.51L and 0.61L was collected from each run and bulked. The bulk was mixed with an equal volume of water and was readsorbed onto the same Spherisorb column. The column was eluted with acetonitrile. The eluate was warmed to 45^0, and water was added until the eluate just went cloudy. This was cooled to 4^0 and was left to crystallise. The crystals were filtered off, and dried to yield the compound of the invention (0.26g). Analysis of the solid by reverse phase analytical high performance liquid chromatography showed the presence of 4% impurities based on the total absorption at 238nm.

^{1}H NMR SPECTRUM

A 200 MH_z ^{1}H nmr spectrum of a solution in deuterochloroform includes signals at about the following positions :

δ 6.55 (broadened s; 1H)	1.88 (broadened s; 3H)
5.86 (d15; 1H)	1.60 (s; 3H)
5.72 (dd 15,11;1H)	1.52 (s; 3H)
	1.03 (d7; 3H)
	1.00 (d7; 3H)
	0.95 (d7; 3H)
	0.79 (d6; 3H)

^{13}C NMR SPECTRUM

A 25.05 MH_z ^{13}C nmr spectrum of a solution in deuterochloroform has signals at about the following positions

δ 192.2 (s)	130.7 (s)
171.9 (s)	123.4 (d)
143.9 (d)	121.9 (d)
138.4 (d)	120.4 (d)
137.7 (s)	99.8 (s)
137.4 (s)	82.0 (s)
137.3 (d)	81.0 (d)
136.7 (s)	76.8 (d)

69.9 (t)	34.8 (t)
69.3 (d)	26.9 (d)
68.6 (d)	22.9 (q)
48.4 (t)	22.2 (q)
46.6 (d)	15.6 (q)
41.1 (t)	14.0 (q)
40.8 (t)	11.0 (q)
36.1 (?)	

The δ 36.1 peak arises from several superimposed signals and its multiplicity is therefore unclear.

U.V. SPECTRUM

A U.V. spectrum in methanol (c=0.001%) has a λ_{max} of about 240.4nm E_1^1 509.

I.R. SPECTRUM

An ir spectrum of a bromoform solution (c=1%) includes bands at about 3500 (OH), 1710 (ester), 1678 (conjugated ketone) and 996 cm^{-1} (C-O).

MASS SPECTRUM

A low resolution E.I. mass spectrum gave a molecular ion at m/z 610 and fragment ions at m/z 592, 574, 480, 441, 440, 423, 422, 265, 259, 247, 241, 237, 219 and 151.

MICROANALYSIS

Microanalysis gave C 70.95%, H 8.33%. (predicted C 70.82%, H 8,20%)

Example 4

A 1ml spore suspension of Streptomyces thermoarchaensis NCIB 12334 (prepared according to Example 3) was used to inoculate a 250ml shake flask containing Medium A (50ml, pH adjusted to 6.5 prior to autoclaving). The flask was incubated at 28^0 for 2 days with

agitation on a rotary shaker (250rev/min) with a 50mm diameter throw. 1ml Portions of the two day developed inoculum were transferred to each of six similar flasks containing Medium A (50mℓ) and fermented in the same manner for two days. A fermenter (20L) containing Medium B (12L, pH adjusted to 6.5 prior to sterilization at 121° for 30 min) was then inoculated with about 300ml [2% (v/v)] of the bulked shake flask inoculum. The fermentation was carried out at 28° with agitation and aeration for 24h.

A fermenter (700L) containing Medium B (450L, pH adjusted to 6.5 prior to sterilization at 121° for 30 min) was inoculated with 12L of the 24h developed inoculum. The fermentation was carried out at 34° with agitation and aeration and the pH controlled down to pH 7.4 with 10% H_2SO_4.

After 90h the harvest broth (400L) was filtered through cellulose (Rettenmaier BE00) with a mixture of Dicalite (2kg) and cellulose (2kg) as filter aid. The recovered cell paste was suspended in methanol (about 100L) and filtered after about 45min. The cells were reextracted with methanol (about 50L). The combined extracts (145L) were diluted with water (35L) and extracted with hexane on a Robatel LX204, adding water at the third stage of extraction. The hexane extract (145L) was concentrated.

The concentrate (2L) was extracted three times with 1L portions of propan-1,2-diol. The extracts were bulked (3.16L) and mixed with ethyl acetate (3.34L) and water (3.0L). The upper layer of ethyl acetate was recovered and washed with water. The ethyl acetate was removed and the residual oil azeotroped with acetone to a dry foam (71g).

The solid was dissolved in methanol (300ml) and water (32ml) and applied to a column (4.6L, 12.8x36cm) of Whatman ODS3 Prep 40 reverse phase silica. The column was eluted with methanol/water (9:1) and fractions eluting between 8.6L and 10.2L were bulked and concentrated until a precipitate began to form. After standing overnight at 4° the solid was filtered off and dried in vacuo.

A portion of the solid (8.5g) was dissolved in a mixture of acetonitrile (140ml), tetrahydrofuran (17ml) and water (13ml). Portions (5.0ml) of this solution were then chromatographed on a

column (25x2.1cm) of Spherisorb ODS2 (5μ) using acetonitrile/water (4:1) as the eluting solvent added at a rate of 25ml/min. The peaks emerging between 9.8 min and 13.6 min were bulked (2.75L), diluted with water (1.5L) and the solution split into three portions. Each portion was pumped back onto the column and eluted with acetonitrile. The three samples were then bulked, evaporated to a solid and recrystallised from acetonitrile to give crystals of the compound of the invention (4.8g). Analysis of the solid by normal and reverse phase analytical high performance liquid chromatography showed the presence of <2% impurities.

Example 5

Harvest broth (25L, prepared according to the method of Example 3) was acidified to pH 3 with 15% v/v sulphuric acid and then heated at 90^0 for 15 min. before filtration through a hyflo bed. The cell paste (1556g) was stirred with methanol (3.9L) for 30min, the slurry filtered and the solid residue resuspended in methanol (3.9L) and refiltered. The combined filtrate was adjusted to pH 4 with 15% v/v sulphuric acid and passed over a bed of IRA 68 resin (acetate cycle, 750ml) at 3750ml/h. The column was washed with methanol and the combined methanol percolates were concentrated to 3.5L and added simultaneously with 0.1% v/v sulphuric acid (10.5L) to cold (ca. 5^0) stirred 0.1% sulphuric acid (50ml). The slurry was stirred for 10min, filtered and the solid slurry washed with cold water before drying. The dry solid contained 12.9g of the compound of the invention by comparison upon high performance liquid chromatography with an authentic sample.

Example 6

Harvest broth (25L, prepared according to the method of Example 3) was acidified to pH 3 with 15% v/v sulphuric acid and then heated to 90^0 for 15 minutes before filtration through a hyfo bed. A portion of the cell paste (500g) was stirred with methanol (750ml) for 30min, the slurry filtered and the solid residue resuspended in methanol (750ml) and filtered. The combined filtrate (1.5L) was diluted with water (617ml) and extracted with three aliquots of hexane

(706ml). The combined hexane extracts were bulked (2790ml) and a portion (1390ml) was concentrated to 250ml and passed over a bed of XAD 1180 resin (10ml), prepared in hexane, at 50ml/h. The resin was washed with hexane (10ml) and the bulked hexane concentrated to 20ml. A portion (10.2ml) of the concentrated hexane was loaded on to a column (100ml) of Crossfield SD 210 silica gel prepared in hexane. The bed was washed with 5% v/v acetone/hexane (700ml) at 100ml/h and eluted with 10% v/v acetone/hexane at 100ml/h. The eluate was monitored by high performance liquid chromatography and those fractions containing the compound of the invention bulked and evaporated to an oil.

The oil was dissolved in methanol (150ml) and water (70ml), followed by evaporation of the methanol in vacuo to leave a solid which was filtered and dried. The dry solid contained 0.21g of the compound of the invention by comparison upon high performance liquid chromatography with an authentic sample.

The following are examples of formulations according to the invention. The term 'Active Ingredient' as used hereinafter means the compound of the invention.

Multidose parenteral injection

		% w/v	Range
Active Ingredient		4.0	0.1 - 7.5% w/v
Benzyl alcohol		2.0	
Glyceryl triacetate		30.0	
Propylene glycol	to	100.0	

Dissolve the active ingredient in the benzyl alcohol and glyceryl triacetate. Add propylene glycol and make up to volume. Sterilise the product by conventional pharmaceutical methods, for example sterile filtration or by heating in an autoclave and package aseptically.

Aerosol spray

	% w/w	Range
Active Ingredient	0.1	0.01 - 2.0% w/w
Trichloroethane	29.9	
Trichlorofluoromethane	35.0	
Dichlorodifluoromethane	35.0	

Mix the Active Ingredient with trichloroethane and fill into the aerosol container. Purge the headspace with the gaseous propellant and crimp the valve into position. Fill the required weight of liquid propellant under pressure through the valve. Fit with actuators and dust-caps.

Tablet

Method of manufacture - wet granulation

	mg
Active Ingredient	250.0
Magnesium stearate	4.5
Maize starch	22.5
Sodium starch glycolate	9.0
Sodium lauryl sulphate	4.5
Microcrystalline cellulose	to tablet core weight of 450mg

Add sufficient quantity of a 10% starch paste to the active ingredient to produce a suitable wet mass for granulation. Prepare the granules and dry using a tray or fluid-bed drier. Sift through a seive, add the remaining ingredients and compress into tablets.

If required, film coat the tablet cores using hydroxypropylmethyl cellulose or other similar film-forming material using either an aqueous or non-aqueous solvent system. A plasticizer and suitable colour may be included in the film-coating solution.

Veterinary tablet for small/domestic animal use

Method of manufacture - dry granulation

	mg
Active Ingredient	50.0
Magnesium stearate	7.5
Microcrystalline cellulose to tablet core weight of	75.0

Blend the active ingredient with the magnesium stearate and microcrystallise cellulose. Compact the blend into slugs. Break down the slugs by passing through a rotary granulator to produce free-flowing granules. Compress into tablets.

The tablet cores can then be film-coated, if desired, as described above.

Veterinary intramammary injection

		mg/dose	Range
Active Ingredient		150mg	0.05 - 1.0g
Polysorbate 60	3.0% w/w)	)	
White Beeswax	6.0% w/w)	to 3g)	to 3 or 15g
Arachis oil	91.0% w/w)	)	

Heat the arachis oil, white beeswax and polysorbate 60 to 160°C with stirring. Maintain at 160°C for two hours and then cool to room temperature with stirring. Aseptically add the active ingredient to the vehicle and disperse using a high speed mixer. Refine by passing through a colloid mill. Aseptically fill the product into sterile plastic syringes.

Veterinary oral drench

	% w/v	Range
Active Ingredient	0.35	0.01 - 2% w/v
Polysorbate 85	5.0	
Benzyl alcohol	3.0	
Propylene glycol	30.0	
Phosphate buffer	as pH 6.0 - 6.5	
Water	to 100.0	

Dissolve the active ingredient in the Polysorbate 85, benzyl alcohol and the propylene glycol. Add a proportion of the water and adjust the pH to 6.0 - 6.5 with phosphate buffer, if necessary. Make up to final volume with the water. Fill the product into the drench container.

Veterinary oral paste

	% w/w	Range
Active Ingredient	7.5	1 - 30% w/w
Saccharin	25.0	
Polysorbate 85	3.0	
Aluminium distearate	5.0	
Fractionated coconut oil	to 100.0	

Disperse the aluminium distearate in the fractionated coconut oil and polysorbate 85 by heating. Cool to room temperature and disperse the saccharin in the oily vehicle. Dispense the active ingredient in the base. Fill into plastic syringes.

Granules for veterinary in-feed administration

	% w/w	Range
Active Ingredient	2.5	0.05-5% w/w
Calcium sulphate, hemi-hydrate	to 100.0	

Blend the Active Ingredient with the calcium sulphate. Prepare the granules using a wet granulation process. Dry using a tray or fluid-bed drier. Fill into the appropriate container.

Emulsifiable Concentrate

Active ingredient	50g
Anionic emulsifier	40g
(e.g. Phenyl sulphonate CALX)	
Non-ionic emulsifier	60g
(e.g. Syperonic NP13)	

Aromatic solvent (e.g. Solvesso 100) to 1 litre.

Mix all ingredients, stir until dissolved.

Granules

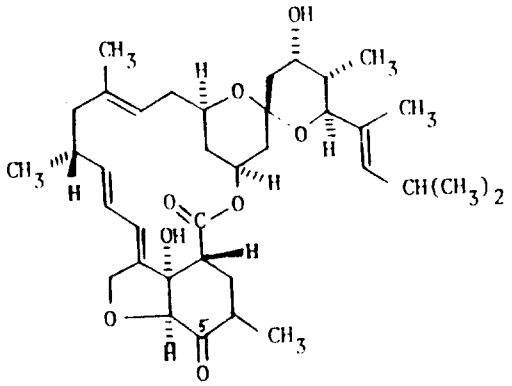
(a) Active ingredient 50g
Wood resin 40g
Gypsum granules (20-60 mesh) to 1kg
(e.g. Agsorb 100A)

(b) Active ingredient 50g
Syperonic NP13 40g
Gypsum granules (20-60 mesh) to 1kg.

Dissolve all ingredients in a volatile solvent e.g. methylene chloride, add to granules tumbling in mixer. Dry to remove solvent.

CLAIMS:

1. A process for the preparation of the compound of formula I



(I)

which comprises cultivating a microorganism of the genus Streptomyces and, if desired, isolating said compound therefrom; the microorganism used being a strain of Streptomyces thermoarchaensis or Streptomyces cyaneogriseus noncyanogenus capable of producing the compound of formula (I) which does not produce any substantial amount of 5β-hydroxy or 5β-methoxy antibiotics S541 compounds; and the cultivation being carried out at 20-50° C at pH5.5-8.5 in an aqueous medium.

2. A process according to claim 1 in which the microorganism is Streptomyces thermoarchaensis NCIB 12334.

3. Streptomyces thermoarchaensis NCIB 12334.

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