FORM 1

609172

SPRUSON & FERGUSON

COMMONWEALTH OF AUSTRALIA

PATENTS ACT 1952

APPLICATION FOR A STANDARD PATENT

Eli Lilly and Company, a corporation organised and existing under the laws of the State of Indiana, of Lilly Corporate Center, Indianapolis, Indiana, 46285, UNITED STATES OF AMERICA, hereby apply for the grant of a standard patent for an invention entitled:

Antibiotic A80577 and Process for its Production

which is described in the accompanying complete specification.

Details of basic application(s):-

Basic Applic. No:	Country:	Application Date:
085,039	US	13 August 1987

The address for service is:-

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Level 33 St Martins Tower
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Sydney New South Wales Australia

DATED this EIGHTH day of AUGUST 1988

Eli Lilly and Company

By: M.J. Anderson

Registered Patent Attorney

TO: THE COMMISSIONER OF PATENTS
OUR REF: 62443
S&F CODE: 53710

APPLICATION ACCEPTED AND AMENDMENTS ALLOWED 2.2.91

REPRINT OF RECEIPT
5845/2

5001679 11/08/88

~~CASE: X-7343~~

SPRUSON & FERGUSON

COMMONWEALTH OF AUSTRALIA

PATENTS ACT 1952

DECLARATION IN SUPPORT OF A
CONVENTION APPLICATION FOR A PATENT

In support of the Convention Application made for a patent for an invention entitled:

ANTIBIOTIC A80577 AND PROCESS FOR ITS PRODUCTION

I, Mary Ann Tucker, Lilly Corporate Center, City of Indianapolis, State of Indiana 46285, United States of America do solemnly and sincerely declare as follows:

1. I am authorized by ELI LILLY AND COMPANY the applicant for the patent to make this declaration on its behalf.

2. The basic application as defined by Section 141 of the Act was/~~were~~ made in United States of America

on 13 August 1987

by Robert L. Hamill and Raymond Che-Fong Yao

3. Robert L. Hamill and Raymond Che-Fong Yao of 617 Brookview Drive, Greenwood, Indiana 46142 and 987 Hawthorne Drive, Carmel, Indiana 46032 respectively both in United States of America

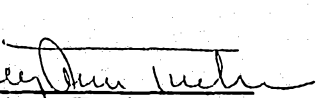
are the actual inventors of the invention and the facts upon which the applicant is entitled to make the application are as follows:

The said applicant is the assignee of the actual inventors.

4. The basic application~~(s)~~ referred to in paragraph 2 of this Declaration was/~~were~~ the first application~~(s)~~ made in a Convention country in respect of the invention~~(s)~~ the subject of the said application.

DECLARED at Indianapolis, Indiana
this 9TH day of JUNE , 1988

ELI LILLY AND COMPANY

By 
Mary Ann Tucker
Assistant Patent Counsel

TO: THE COMMISSIONER OF PATENTS
AUSTRALIA

(12) PATENT ABRIDGMENT (11) Document No. AU-B-20965/88
(19) AUSTRALIAN PATENT OFFICE (10) Acceptance No. 609172

(54) Title
ANTIBIOTIC A80577 AND PROCESS FOR ITS PRODUCTION

International Patent Classification(s)
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(51)[4] **A61K 031/35** **C07H 007/06** **C12P 001/06**

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085039 **13.08.87** **US UNITED STATES OF AMERICA**

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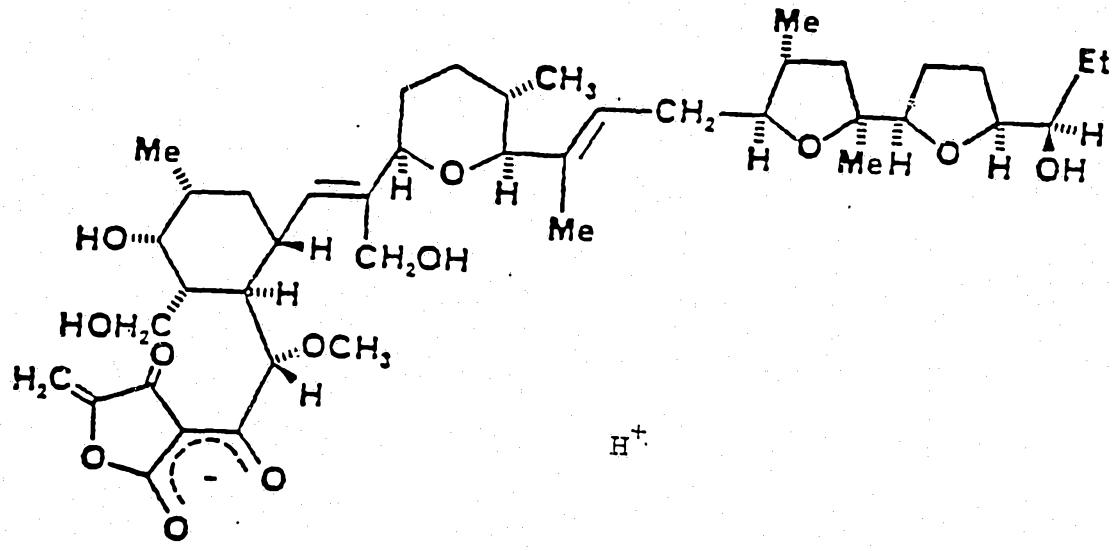
(57) Claim

1. Antibiotic A80577 which has the Formula 1



or an acyl ester, as herein defined, or alkyl ether, as herein defined, derivative of A80577, or a salt of A80577 or of the ester or ether derivative.

12. A process for increasing feed-utilization efficiency in ruminant animals which comprises orally administering to the animal an effective propionate-increasing amount of a compound of Claim 2.

S & F Ref: 62443

FORM 10

COMMONWEALTH OF AUSTRALIA

PATENTS ACT 1952

609172

COMPLETE SPECIFICATION

This document contains the amendments made under Section 49 and is correct for printing.

(ORIGINAL)

FOR OFFICE USE:

Class Int Class

Complete Specification Lodged:
Accepted:
Published:

Priority:

Related Art:

Name and Address of Applicant: Eli Lilly and Company
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Indianapolis Indiana 46285
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Level 33 St Martins Tower, 31 Market Street
Sydney, New South Wales, 2000, Australia

Complete Specification for the invention entitled:

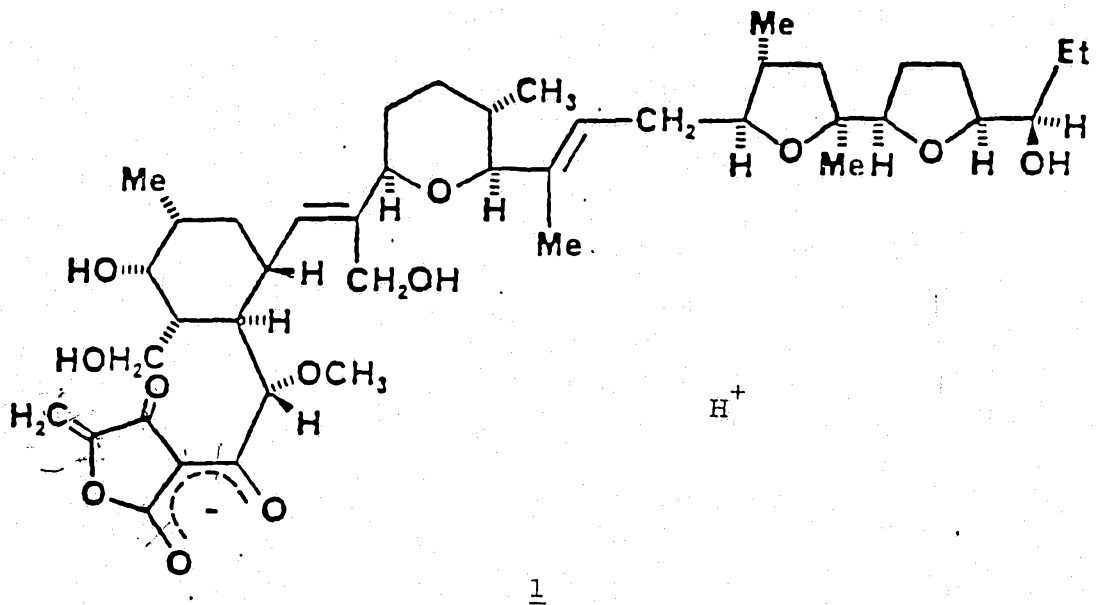
Antibiotic A80577 and Process for its Production

The following statement is a full description of this invention, including the best method of performing it known to me/us

5845/3

ANTIBIOTIC A80577 AND PROCESS FOR ITS PRODUCTION

This invention relates to the new polyether antibiotic A80577 and to a new strain of Actinomadura verrucosospora, NRRL 18236, which produces this antibiotic. The structure of A80577 is shown in Formula 1:



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This invention also relates to acyl ester and alkyl ether derivatives of A80577 and to the salts of A80577 and of the derivatives.

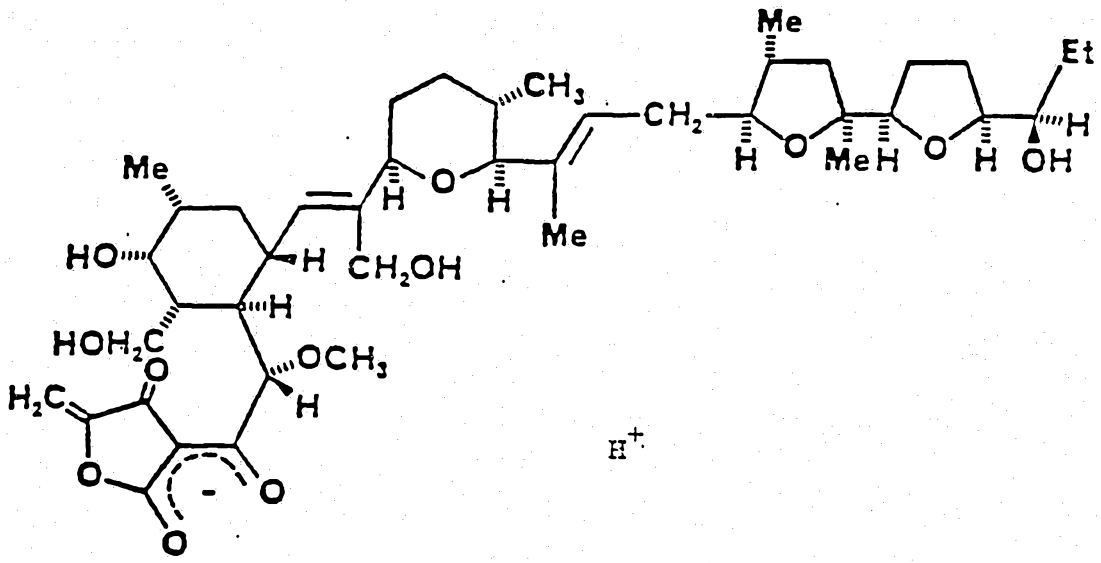
Another aspect of this invention is a process for producing antibiotic A80577 by cultivating Actinomadura verrucosospora, NRRL 18236, or an A80577-producing

variant or mutant thereof, in a culture medium containing assimilable sources of carbon, nitrogen and inorganic salts under submerged aerobic fermentation conditions until antibiotic A80577 is produced. A80577 is extracted from the fermentation broth and from the mycelium with organic solvents. A80577 is separated and further purified by techniques such as column chromatography.

Because Actinomadura verrucosospora, NRRL 18236, is a newly discovered strain, this application describes a biologically pure culture of this microorganism or a mutant, variant or recombinant thereof which produces antibiotic A80577.

A80577 is a useful antibacterial agent. It improves feed-utilization efficiency in ruminants and acts as a growth promotant in ruminants and in monogastric animals. In addition, A80577 has parasiticidal activity and is useful as an ionophore. Therefore, the invention also relates to a feed composition for increasing feed utilization efficiency in ruminant animals comprising animal feed and an effective amount of antibiotic A80577, its acyl ester or alkyl ether derivatives, or a salt of A80577 or of the derivatives; and to a process for increasing feed utilization efficiency which comprises orally administering to an animal an effective amount of these compounds.

According to a first embodiment of this invention there is provided antibiotic A80577 which has the Formula 1



or an acyl ester, as herein defined, or alkyl ether, as herein defined, derivative of A80577, or a salt of A80577 or of the ester or ether derivative.

According to a second embodiment of this invention there is provided a process for producing antibiotic A80577 which comprises cultivating Actinomadura verrucosospora, NRRL 18236, or an A80577-producing variant or mutant thereof, in a culture medium containing assimilable sources of carbon, nitrogen, and inorganic salts under submerged aerobic fermentation conditions until antibiotic A80577 is produced.

According to a third embodiment of this invention there is provided a process for increasing feed-utilization efficiency in ruminant animals which comprises orally administering to the animal an effective propionate-increasing amount of a compound of the first embodiment.

According to a fourth embodiment of this invention there is provided a feed composition for increasing feed utilization efficiency in ruminant animals comprising animal feed and an effective amount of a compound of the first embodiment.

The drawings show the following infrared absorption spectra in chloroform:

Figure 1 A80577 (Na Salt)
Figure 2 A80577 (K Salt)

Improved antibiotics continue to be needed in the veterinary field. Enhancing growth promotion in animals is one desired feature of such antibiotics. Growth promotion can be achieved by reducing disease and by increasing feed-utilization efficiency. Of particular interest is growth promotion in ruminants, such as cattle.

The mechanism for utilizing the major nutritive portion (carbohydrates) of ruminant feeds is well known. Microorganisms in the rumen of the animal degrade carbohydrates to produce monosaccharides and then convert these monosaccharides to pyruvate compounds. Pyruvates are metabolized by microbiological processes to form acetates, butyrates or propionates, collectively known as volatile fatty acids (VFA).

The relative efficiency of VFA utilization is connected to overall efficiency. Thus, although acetates and butyrates are used, propionates are used with greater efficiency. Also, the fermentation efficiency of propionate production is greater than that of butyrate or acetate. This is in addition to the utilization efficiency. A beneficial compound, therefore, stimulates animals to produce a higher proportion of propionates from carbohydrates, thereby increasing carbohydrate-utilization efficiency.

A80577 is a new member of the group of polyether antibiotics. Westley (John W. Westley, "Polyether

Antibiotics: Naturally Occurring Acid Ionophores, Vol. 2, Chemistry," Marcel Dekker, New York, 1983; Journal of Natural Products, 49(1), 35 [1986]) has separated existing polyethers by class and type. Using Westley's system, A80577 is a new member of the Class 4 group of polyethers. This group includes tetronomycin, which is described in J. Antibiotics, 142 (1982), and M139603, which is described in J. Chem. Soc., Chem. Comm., 1073 (1981) and U.S. Patent No. 4,279,849.

A80577 (in its sodium salt form) has the following characteristics:

State: white crystals (from acetone-water)

mp: (Na Salt) 276-278°C.
(K Salt) 270-272°C.

Molecular weight: 782 by field desorption mass spectrometry (FDMS); 760 for acid form.

$[\alpha]^{25}D$: -89.03 (c 5, MeOH)

No titratable groups (66% DMF)

Empirical formula: $C_{42}H_{63}O_{12}Na$

UV max: Ethanol neutral 253 nm (ε = 17,735), 298 nm (ε = 10,003)

acidic 250 nm (ε = 14,715), 289 nm (ε = 7,971)

basic 208 nm (ε = 192,222), 248 nm (ε = 15,968), 286 nm (ε = 9,665)

IR (Na salt, $CHCl_3$): 3018, 2966, 2931, 1750, 1611, 1453, 1439, 1219, 1022 and 1012 cm^{-1} (see Fig. 1)

IR (K salt, $CHCl_3$): 3023, 3019, 2967, 2964, 2930, 2874, 1750, 1650, 1451, 1437, 1224, 1214, 1210, 1079, 1059, 1022 and 1012 cm^{-1} (see Fig. 2)

Solubility: Not very soluble in water; soluble in dimethyl sulfoxide, dimethylformamide, lower alcohols such as methanol, ketones such as acetone, esters such as ethyl acetate, halogenated hydrocarbons such as chloroform and hydrocarbons such as diethyl ether, benezene, toluene and warm hexane.

Based on its physical characteristics A80577 is believed to have the structure shown in formula 1. As is apparent from its structure, A80577 is a charged molecule and is capable of forming salts. A80577 also has up to four hydroxyl groups which can be esterified or which can form ether derivatives. The acyl ester and the alkyl ether derivatives of A80577, and the pharmaceutically-acceptable salts of A80577 and of these derivatives are also useful as antibiotics and as agents which increase feed-utilization efficiency.

The term "an A80577 compound" is used herein to designate antibiotic A80577 (formula 1), an acyl ester or an alkyl ether derivative of antibiotic A80577 or a pharmaceutically acceptable salt of antibiotic A80577 or of its acyl ester or alkyl ether derivatives.

Antibiotic A80577 is produced by a culture of an A80577-producing strain of Actinomadura verrucosospora,

NRRL 18236, as described herein. The antibiotic is produced under submerged aerobic conditions in a suitable culture medium; it can be recovered from the culture medium by using various isolation and purification procedures understood in the art.

A culture of the A80577-producing organism was deposited on 2 July 1987 and made part of the stock culture collection of the Midwest Area Northern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, 1815 North University Street, Peoria, Illinois 61604, from which it is available to the public under the accession number NRRL 18236.

Taxonomic studies of this organism were carried out by Frederick P. Mertz of the Lilly Research Laboratories. Based on these studies, the organism belongs to the genus Actinomadura [Lechevalier and Lechevalier, 1970. In Prouser, H. (ed.). The Actinomycetales. VEB Gustav Fischer Verlag, Jena, pp. 393-405].

The methods recommended by the International Streptomyces Project (ISP) for the characterization of Streptomyces species [E. B. Shirling and D. Gottlieb, "Methods for Characterization of Streptomyces Species", Int. J. Syst. Bacteriol. 16(3), 313-340 (1966)] have been followed.

Melanoid pigment production (chromogenicity) was determined with ISP No. 1 (tryptone-yeast extract broth), ISP No. 6 (peptone-yeast extract iron agar), and ISP No. 7 (tyrosine agar).

Starch hydrolysis was determined by testing for the presence of starch with iodine on ISP No. 4 (inorganic salts-starch agar) plates.

Morphology was studied using an optical light microscope. A scanning electron microscope (SEM) was used to study the spore surface ornamentation.

ICSS-NBS Centroid Color Charts, standard sample No. 2106 (National Bureau of Standards, 1958, U. S. Department of Commerce, Washington, D.C.) and the Color Harmony Manual (4th ed., Color Standards Department, Container Corporation of America, Chicago, Illinois, 1958) were used to assign color names to the reverse side and aerial spore mass respectively.

The isomers of diaminopimelic acid (DAP) and the carbohydrates in hydrolysates of whole cells were established by the chromatographic methods of Becker et al. [B. Becker, M. P. Lechevalier, R. E. Gordon, and H. A. Lechevalier, "Rapid Differentiation between Nocardia and Streptomyces by Paper Chromatography of Whole-cell Hydrolysates", Appl. Microbiol. 12, 421-423 (1964)] and of Lechevalier [M P. Lechevalier, and H. Lechevalier, "Chemical Composition as a Criterion in the Classification of Aerobic Actinomycetes", Int. J. Syst. Bacteriol. 20, 435-443 (1970].

Resistance to antibiotics was measured by padding antibiotic sensitivity discs onto the surface of seeded ISP No. 2 agar plates.

Cultural Characteristics

Culture A80577 grew well on both complex and defined media. The aerial spore mass was moderately formed, and was white. The reverse side was yellowish white to pale yellow. No distinctive pigments were observed. No soluble pigments were observed, except for a light yellow brown pigment in several media. These cultural characteristics are given in Table I.

Table I

Cultural Characteristics of A80577 at 30°C 18 days incubation

ISP No. 2	G: Abundant R: 88.d.Y C: Trace: a White Sp: None to 1.y Br		Czapek's	G: Fair R: 92.y White Am: Fair: a White Sp: None	
ISP No. 3	G: Fair R: 92.y White Am: Fair: a White Sp: None		Tomato Paste Oatmeal Agar	G: Poor R: 88.d.Y Am: Poor: a White Sp: None	
ISP No. 4	G: Good R: 92.y White Am: Good a White Sp: None		Potato Carrot Agar	G: Good R: 89.p.Y Am: Good: a White Sp: None	
ISP No. 5	G: Abundant R: 89.p.Y Am: Good: a White Sp: None		Jensen's Agar	G: Poor. R: 92.y White Am: Poor: a White Sp: None	
ISP No. 7	G: Good R: 90.gy.Y Am: None Sp: None		Glucose Asparagine	G: Good R: 89.p.Y Am: Good: a White Sp: None	
No. 172	G: Good R: 89.p.Y Am: Good: a White Sp: None		Glycerol Glycine	G: Trace only R: - Am: - Sp: -	
Tap Water Agar	G: Not grown R: - Am: - Sp: -		Yeast Dextrose Agar	G: Abundant R: 87.m.Y Am: Fair: a White Sp: None to 1.yBr	

Table I (cont.)

	G:	Abundant
Emerson's	R:	87.m.Y
agar	Am:	Trace: a White
	Sp:	None to 1.yBr

G = Growth; R = Reverse; Am = Aerial mycelium; Sp = Soluble Pigment

Morphological Characteristics

Culture A80577 produces an extensive substrate mycelium. Moderately formed aerial hyphae produce clusters of tightly packed short chains, arranged in Rectus-flexibilis (RF) morphology. This morphology is typical of the genus Actinomadura. The spore shape is spherical, spore size averages 0.8 μm, and the spore surface has a distinctive granular appearance. The spore chain contains less than 10 spores per chain.

Physiological Characteristics

Analysis of hydrolyzed whole cells indicates the presence of meso-diaminopimelic acid. Madurose was detected in the whole cell extracts. Galactose, glucose, mannose and ribose were also detected. The cell wall is type III and the sugar pattern is type B. Galactose is not typical of type B sugar pattern.

Culture A80577 utilized the following carbohydrates: adonitol, D and L-arabinose, cellobiose, dulcitol, ethanol, i-erythritol, D-fructose,

D-galactose, glucose, glycerol, glycogen, i-inositol, D-mannitol, D-mannose, D-melizitose, D-melibiose, L-rhamnose, D-ribose, sucrose, D-trehalose D-xylose and sodium butyrate. It was unable to utilize: cellulose, dextrin, inulin, D-lactose, D-maltose, α-methyl-D-glucoside, D-raffinose, salicin, sorbitol, L-sorbose, and xylitol. Control plates with no carbohydrates supported a minimal growth.

Culture A80577 grew in a temperature range of 15-45°C, optimum growth appeared to be between 30 and 37°C. A80577 tolerated up to 4% NaCl, produced catalase and reduced nitrates. It did not hydrolyze starch.

A80577 was resistant to: bacitracin 10 units, cephalothin 30 µg, lincomycin 2 µg, oleandomycin 15 µg, penicillin G 10 units, rifampin 5 µg, and tetracycline 30 µg. It was sensitive to: gentamicin 10 µg, neomycin 30 µg, streptomycin 10 µg, tobramycin 10 µg and vancomycin 30 µg.

The chemotaxonomic properties and the general cultural and morphological characteristics of A80577 support the assignment of this strain to the genus Actinomadura and the species verrucosospora. Therefore this strain is classified as verrucosospora A80577.

As is the case with other organisms, the characteristics of the A80577-producing culture Actinomadura verrucosospora, NRRL 12836, are subject to variation. Recombinants, mutants or variants of the strain may be obtained by methods known in the art. For example, mutants can be obtained by treatment with various known

physical and chemical mutagens such as ultraviolet light, X rays, gamma rays and chemicals such as N-methyl-N'-nitro-N-nitrosoguanidine. All natural and induced variants, mutants and recombinants of this Actinomadrua verrucosospora strain which retain the characteristic of A80577 production are part of this invention.

The culture medium used to grow Actinomadura verrucosospora, NRRL 18236, can be any one of a number of media. For economy in production, optimal yield, and ease of product isolation, however, certain culture media are preferred. For example, a preferred carbohydrate source in large-scale fermentation is glucose, although blackstrap molasses, starch and the like can also be used.

A preferred nitrogen source is enzyme-hydrolyzed casein, although other nitrogen sources should also be useful.

Among the nutrient inorganic salts which may advantageously be incorporated in the culture media are the customary soluble salts capable of yielding zinc, sodium, magnesium, calcium, ammonium, chloride, carbonate, sulfate, nitrate and like ions.

Essential trace elements necessary for the growth and development of the organism should also be included in the culture medium. Such trace elements commonly occur as impurities in other substituents of the medium in amounts sufficient to meet the growth requirements of the organism. If foaming is a problem, small amounts (i.e. 0.2 ml/L) of an antifoam agent such as polypropylene glycol may be added to large scale fermentation media if needed.

For production of substantial quantities of antibiotic A80577, submerged aerobic fermentation in tanks is preferred. Small quantities of A80577 may be obtained by shake-flask culture. Because of the time lag in antibiotic production commonly associated with inoculation of large tanks with the spore form of the organism, it is preferable to use a vegetative inoculum. The vegetative inoculum is prepared by inoculating a small volume of culture medium with the spore form or mycelial fragments of the organism to obtain a fresh, actively growing culture of the organism. The vegetative inoculum is then transferred to a larger tank. The vegetative inoculum medium can be the same as that used for larger fermentations, but other media are also suitable.

A80577 is produced by Actinomadura verrucosospora when grown at temperatures between about 25° and about 37°C. A good temperature for A80577 production appears to be about 30°C.

As is customary in submerged aerobic culture processes, sterile air is blown into the vessel from the bottom while the medium is stirred with conventional turbine impellors. In general, the aeration rate and agitation rate should be sufficient to maintain the level of dissolved oxygen at or above 30% of saturation.

Production of antibiotic A80577 can be followed during the fermentation by testing samples of the broth for antibiotic activity against organisms known to be sensitive to the antibiotic. One assay organism useful in testing A80577 is Bacillus subtilis ATCC 6633.

The bioassay is conveniently performed by the agar-well plate test.

Following its production under submerged aerobic fermentation conditions, A80577 can be recovered from the fermentation medium by methods used in the fermentation art. The antibiotic activity produced during fermentation of the A80577-producing organism occurs both in the mycelia and the broth. Maximum recovery of A80577 is accomplished, therefore, by initially filtering the medium to separate the broth from the mycelial mass. The filtered broth and the mycelial mass can then be purified separately to give their respective portion of A80577. A variety of techniques may be used in this purification.

A preferred technique for purification of the filtered broth involves adjusting it to a pH of about 9 and extracting with a suitable solvent such as, for example, ethyl acetate. The extracting solvent can then be evaporated under vacuum to give the broth portion of A80577.

A preferred method of purifying the mycelial mass is to extract the separated mycelial filter cake with a suitable solvent such as, for example, acetone. The extracting solvent is then evaporated under vacuum to give a concentrated aqueous solution. This aqueous solution is then adjusted to a pH of about 9 and is extracted with a suitable solvent such as, for example, ethyl acetate. The extracting solvent is then concentrated under vacuum to give the mycelial portion of A80577.

The broth and mycelial portions of the A80577 complex are further purified by similar procedures. A preferred procedure involves silica gel chromatography.

Separation of antibiotic A80577 can be followed by thin-layer chromatography (TLC) or high performance liquid chromatography (HPLC). Convenient silica gel TLC solvent systems are ethyl acetate, ethyl acetate:acetonitrile:ammonium hydroxide and toluene: acetonitrile:acetic acid. Preferred silica gel solvent systems are ethyl acetate, ethyl acetate:acetonitrile: ammonia (90:9:1) and toluene:acetonitrile:acetic acid (40:59:1). Polyamide plates also may be conveniently used with an acetone:water:ammonia solvent system, preferably in the ratio 30:69:1. The antibiotic can be detected by bioautography using, for example, Bacillus subtilis or by other methods such as, for example, vanillin-sulfuric acid spray reagent.

Alternatively, the culture solids, including medium constituents and mycelium can be used without extraction or separation, but preferably after removal of water, as a source of A80577. For example, after production of A80577, the whole fermentation broth can be dried by lyophilization, by drum-drying, or by azeotropic distillation and drying. The dried broth is then mixed directly into feed premix.

The salts of A80577 and of its derivatives are useful for separating and purifying the antibiotics. The pharmaceutically-acceptable salts are particularly useful. Examples of salts are the alkali-metal and

alkaline-earth-metal salts of A80577 and of its derivatives.

Representative and suitable alkali-metal and alkaline-earth metal salts of A80577 include the sodium, potassium, lithium, cesium, rubidium, barium, calcium and magnesium salts.

It is well known in the veterinary pharmaceutical art that the form of an antibiotic is not ordinarily of great significance when treating an animal with the antibiotic. In most cases, conditions within the animal change the drug to a form other than that in which it was administered. The salt form in which it may be administered is, therefore, not of great significance. The salt form may, however, be chosen for reasons of economy, convenience, and toxicity.

The alkali-metal and alkaline-earth-metal cationic salts of A80577 are prepared according to procedures commonly used for the preparation of cationic salts. For example, the free acid form of A80577 is dissolved in a suitable solvent such as acetone; about ½ volume of water is added and this solution is adjusted to a pH of about 9 to 10 with the base of the desired cationic salt (e.g. NaOH, KOH). The salt thus formed can be isolated by routine methods, such as filtration or evaporation of the solvent.

A preferred method of forming salts is to dissolve A80577 (acid form) in a solvent such as tetrahydrofuran; add an equal volume of water; adjust the mixture to pH 10 with the corresponding cationic base (e.g. NaOH, KOH, etc.); and extract with a water

immiscible solvent such as diethyl ether or ethyl acetate. The separated organic phase is washed with water and concentrated to dryness. The residue is lyophilized from dioxane. The salt can be crystallized from an appropriate solvent, such as acetone.

The alkyl ether derivatives of A80577 are those compounds wherein one or more of the hydroxyl groups has been replaced by a YR group wherein:

Y represents O or S; and

R represents C_1-C_6-alkyl,
C_1-C_4-alkoxy-C_2-C_5-alkyl,
C_1-C_4-alkoxycarbonyl-C_2-C_5-alkyl,
amino-C_2-C_5-alkyl,
mercapto-C_2-C_5-alkyl,
hydroxyalkyl,
haloalkyl, or
$(R')_m$-phenyl$(CH_2)_n$-,

wherein R' represents C_1-C_4-alkyl, C_1-C_4-alkoxy, or hydroxy
m represents 0-2; and
n represents 0-3.

The term "alkyl" means a C_1 to C_7 straight or branched chain hydrocarbon, preferably a C_1 to C_4 hydrocarbon, e.g., methyl, ethyl, propyl, isopropyl, n-butyl, etc.

The term "alkoxy" means a C_1 to C_7 lower alkyl group having an oxygen function substituted therein, such as methoxy, ethoxy, propoxy and the like.

The term "hydroxyalkyl" refers either to a monohydroxy-C_1-C_5-alkyl moiety or, when Y is O, to the 2,3-dihydroxyprop-1-yl moiety.

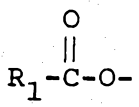
The term "haloalkyl" refers to a C_2-C_5-alkyl moiety having from one to three halogen substituents, selected from the group consisting of bromine, chlorine, and fluorine. When the alkyl moiety is dihalo- or trihalo-substituted, the halo-substituents must be the same halogen moiety.

Preferred A80577 ether derivatives are those compounds wherein Y represents O and R represents C_1-C_6-alkyl. The ether derivatives are prepared by reacting A80577, or a salt thereof, with a corresponding primary alcohol or thiol.

With some of the starting alcohols or thiols it may be necessary to add an acid catalyst to the reaction. Suitable catalysts include hydrochloric acid, sulfuric acid, perchloric acid, methanesulfonic acid, benzenesulfonic acid, toluenesulfonic acid, selenium dioxide, and boron trifluoride.

A solvent such as, for example, water, acetone, benzene, ether, tetrahydrofuran, or dioxane may be added to facilitate the reaction. Reactions generally occur at room temperature, although higher temperatures may be used.

The acyl ester derivatives of A80577 are those compounds wherein one or more of the hydroxyl groups has been replaced by a radical of the formula



wherein R_1 is C_1 to C_6-alkyl or hydrogen.

A80577 acyl ester derivatives are prepared by treating A80577 with a corresponding acid anhydride or acid chloride. Esterification occurs at one of the A80577 hydroxyl groups. Such esters are typically prepared by reacting A80577 with, for example, the corresponding acid anhydride at room temperature.

Although ordinary reaction work-up procedures are sometimes sufficient, additional purification may be required to obtain the compounds of this invention. Such purification may be accomplished by well-known methods, such as, for example, column chromatography, thin-layer chromatography, fractional crystallization and the like.

The A80577 compounds inhibit the growth of bacteria which are pathogenic to animal life. For example, Table III shows the minimal inhibitory concentration (MIC) at which A80577 inhibits certain organisms. The MIC's in Table III were determined by conventional agar-dilution assays.

Table III: Antibacterial Activity of A80577 (Na salt)

Test Organism	MIC (mcg/mL)
Staphylococcus aureus X1.1	0.5
" " V41	0.5
" " X400	0.5
" " S13E	0.5
Staphylococcus epidermidis EPI1	0.5
" " 222	0.5
Streptococcus pyogenes C203	0.125
" pneumoniae Park1	0.125
" " X66	0.5
" " 2041	0.5
Haemophilus influenzae	32-128
Other Gram-negative bacteria tested	>128
Veterinary Organisms	
Staphylococcus sp.	<0.05
Streptococcus sp.	<0.05
Pasteurella multocida	1.56
Pasteurella hemolytica	> 50
Bordetella bronchiseptica	> 50
Mycoplasma gallisepticum	0.78
Mycoplasma hyopneumoniae	1.56
Escherichia coli	> 50
Salmonella typhimurium	> 50

The A80577 compounds also are active against anaerobic bacteria. Table IV shows the MIC's at which A80577 inhibits various anaerobic bacteria, as determined by standard agar-dilution assay. End points were read after 24-hour incubation.

Table IV: Susceptibility of Anaerobic Bacterial Isolates to A80577 (Na salt)

Anaerobic Bacteria	MIC (mcg/mL)
Clostridium difficile 2994	<0.5
Clostridium perfringens 81	<0.5
Clostridium septicum 1128	<0.5
Eubacterium aerofaciens 1235	<0.5
Peptococcus asaccharolyticus 1302	<0.5
Peptococcus prevoti 1281	<0.5
Peptostreptococcus anaerobius 1428	<0.5
Peptostreptococcus intermedius 1624	<0.5
Propionibacterium acnes 79	<0.5
Bacteroides fragilis 111	>128
Bacteroides fragilis 1877	>128
Bacteroides fragilis 1936B	>128
Bacteroides thetaiotaomicron 1438	4
Bacteroides melaninogenicus 1856/28	1.0
Bacteroides melaninogenicus 2736	16
Bacteroides vulgatis 1211	4
Bacteroides corrodens 1874	>128
Fusobacterium symbiosum 1470	<0.5
Fusobacterium necrophorum 6054A	<0.5

The acute toxicity of A80577 in mice, when administered by intraperitaneal injection and expressed as LD_{50}, was 53.3 mg/kg.

Another important property of the A80577 compounds is the ability to improve feed-utilization efficiency in animals. For example, the A80577 compounds improve feed-utilization efficiency in ruminants which have a developed rumen function.

The efficiency of feed use can be monitored by observing the production and concentration of propionate compounds in the rumen using the method described by Arthur P. Raun in U.S. Patent 3,794,732 (see especially Example 5). Table V shows the effect of compound A80577 on ruminant feed-utilization efficiency. Table VA. shows the relationship between dosage of A80577 and propionate production. Table VB. shows the ratio of volatile-fatty-acid (VFA) concentrations in A80577-treated flasks to concentrations in control flasks in this test.

Table V: Effect of A80577 (Na Salt) on Ruminant Feed-Utilization Efficiency

A.

Dosage, ppm	Observations	Propionate Production, mM/d Mean	Std. Dev.
0	26	9.6	1.4
0.04	14	14.3	1.7
0.2	14	14.8	1.4
1.0	14	22.3	3.4
5.0	14	21.8	2.7

B.

	Ratio of Treated to Control Values			
Dosage mcg/mL	Molar % Propionate	Molar % Acetate	Molar % Butyrate	Total VFA mM/L
1.0	1.532	0.845	0.754	0.858
2.5	1.459	0.851	0.827	0.823
5.0	1.562	0.799	0.839	0.773
10.0	1.709	0.772	0.728	0.876

LSD; two-tailed t-test; significant at $P<0.01$; C_3 >99 percent upper confidence limit

The A80577 compounds are typically effective in increasing propionate and, thereby, improve the efficiency of feed utilization when administered to ruminants orally at rates of from about 0.01 mg/kg/day to about 1.0 mg/kg/day. Preferable rates of administration are from about 0.06 mg/kg/day to about 0.35 mg/kg/day. A preferred method of administration is to mix the compound with the animals' feed. Feed compositions adapted to increase feed utilization in ruminant animals typically comprise a feed ration and from 0.5 to 50 grams of an A80577 compound per ton of feed, preferably 2 to 15 grams per ton.

As described supra, A80577 compounds are active against anaerobic bacteria, including Clostridium perfringens. A80577 compounds should, therefore, be beneficial in the treatment of (which includes prevention of) enteritis in chickens, swine, cattle and sheep. A80577 compounds should also be useful in the treatment of enterotoxemia in ruminants.

The A80577 compounds can be administered to animals orally or parenterally. The most practical way to administer the A80577 compounds is by formulation into the feed supply. A variety of feeds, including the common dry feeds, liquid feeds, and pelleted feeds, may be used. Although the preferred method of administration is by mixing it with the animals' feed, it can also be administered in other ways, for example, tablets, drenches, boluses, or capsules. Each individual dosage unit should contain a quantity of A80577 compound

directly related to the proper daily dose for the animal to be treated.

The methods of formulating drugs into animal feeds are well known. A preferred method is to make a concentrated drug premix which in turn is used to prepare medicated feeds. Typical premixes may contain from about 1 to about 200 grams of drug per pound of premix. Premixes may be either liquid or solid preparations.

The final formulation of feeds for animals will depend upon the amount of drug to be administered. The common methods of formulating, mixing, and pelleting feeds may be used to prepare feeds containing an A80577 compound.

The A80577 compounds may be formulated for parenteral administration by methods recognized in the veterinary pharmaceutical art. Effective injectable compositions containing the A80577 compounds may be in either suspension or solution form. In the solution form, the A80577 compound is dissolved in a physiologically acceptable carrier. Such carriers comprise a suitable solvent, preservatives such as benzyl alcohol, if needed, and buffers. Useful solvents include, for example, alcohols, glycols, or inert oils such as vegetable oils or highly refined mineral oils.

Injectable suspension compositions are prepared using a nonsolvent for the compound with adjuvants, as a carrier. The nonsolvent can be, for example, water or a glycol such as polyethylene glycol.

Suitable physiologically-acceptable adjuvants are necessary to keep the compound suspended in suspension compositions. The adjuvants may be chosen from among thickeners such as carboxymethylcellulose, polyvinylpyrrolidone, gelatin, and the alginates. Many surfactants are also useful for suspending the compounds. Lecithin, alkylphenol polyethylene oxide adducts, naphthalenesulfonates, alkylbenzenesulfonates, and the polyoxyethylene sorbitan esters are useful suspending agents in liquid nonsolvents.

Many substances which affect the hydrophilicity, density, and surface tension of the liquid nonsolvent can assist in making injectable suspensions in individual cases. For example, silicone antifoams, glycols, sorbitol, and sugars can be useful suspending agents.

In order to illustrate more fully the operation of this invention, the following examples are provided:

Example 1

Preparation of Antibiotic A80577 Using *Actinomadura verrucosospora*,

A. Shake-flask Fermentation of *Actinomadura verrucosospora*,

The culture *Actinomadura verrucosospora*, either as a lyophilized pellet or as a suspension maintained in liquid nitrogen, is used to inoculate a vegetative medium having the following composition:

Vegetative or Seed Medium

Ingredient	Amount (g/L)
Glucose	10.0
Soluble starch	20.0
Enzyme-hydrolyzed casein*	5.0
Yeast extract	5.0
$CaCO_3$	1.0
Deionized water	q.s. 1 liter

*N-Z Amine A, Humko-Sheffield Chemical, Norwich, NJ.

Slants or plates are prepared by adding 2.5% agar to the seed medium. The inoculated slant is incubated at 30°C. for from about 10 to about 14 days. The mature slant culture is scraped with a sterile tool to loosen the spores and remove and macerate the mycelial mat. About one-fourth of the loosened spores and culture growth thus obtained is used to inoculate 50 mL of a first-stage seed medium.

The inoculated first-stage medium is incubated in a 250-mL Erlenmeyer flask at 30°C. for about 72 hours on a shaker orbiting in a two-inch (5.08 cm) circle at 250 rpm.

This incubated first-stage medium (1.00 mL) is used to inoculate 50 mL of a production medium having the following composition:

Ingredient	Amount (g/L)
Glucose	25.0
Blackstrap molasses	15.0
Yeast extract	5.0
Enzyme-hydrolyzed casein*	3.0
$MgSO_4$ (anhydrous)	1.0
$CaCO_3$	2.0
Tap water	q.s. 1 liter

*N-Z Amine A.

The inoculated production medium is incubated in a 250-mL wide-mouth Erlenmeyer flask at 30°C. for 8

to 10 days on a shaker orbiting in a two-inch circle at 250 rpm.

B. Tank Fermentation of Actinomadura verrucosospora

In order to provide a large volume of inoculum, 10 mL of incubated first-stage medium, prepared as described in Section A, is used to inoculate 400 mL of a second-stage growth medium having the same composition as that of the first-stage medium. This second-stage vegetative medium is incubated in a two-liter wide-mouth Erlenmeyer flask for about 48 hours at 30°C. on a shaker orbiting in a two-inch circle at 250 rpm.

Incubated second-stage vegetative medium (400 mL) thus prepared is used to inoculated 100 liters of sterile production medium, prepared as described in Section A except that P-2000 (0.1 ml/L) and Sag 471 (0.2 g/L) antifoam agents are added. The inoculated production medium is allowed to ferment in a 165-liter stirred fermentation tank for 4 to 5 days at a temperature of 30°C. The airflow (0.5-0.6 v/v/m) in the stirred vessel (200-250 rpm) is adjusted to maintain a dissolved oxygen level above 30% of air saturation.

Example 2

Isolation of A80577 Sodium Salt

Combined fermentation broth (187 L) from two 100 L fermentations was filtered with the aid of 3% Hyflo Supercel. The mycelial filter cake was extracted twice with 40 L acetone. The acetone extracts were combined and concentrated *in vacuo* to remove the acetone. The concentrate (20 L) was combined with the broth filtrate (162 L), the solution was adjusted to pH 9.0 with 50% NaOH and extracted with 125 L ethyl acetate. The ethyl acetate extract was concentrated *in vacuo* to a residue which crystallized upon standing at 5°C. The crystals were washed sequentially with pentane, acetonitrile and diethyl ether and dried *in vacuo*. A yield of 36.4 grams of white crystalline A80577 sodium salt (m.p. 275-278°C) was obtained.

The crystals were recrystallized by dissolving 1.0 g in 100 ml acetone, adding 100 ml H_2O and allowing to stand at 5°C for 72 hours to crystallize. Crystallization was completed with the further addition of 100 ml of water:acetone (3:1). The crystals were filtered off and dried to yield 788 mg (m.p. 276-278°C).

Further recovery of A80577 sodium salt from the combined above washes and mother liquor was achieved by concentrating the solution to dryness, dissolving in 250 ml toluene and applying to a column containing 2 L of silica gel (Grace Grade 62) packed in toluene. The column was washed with 10 L toluene and then developed sequentially with 10 L toluene:ethanol (98:2) and 10 L toluene:ethanol (96:4) collecting 1 L fractions. The

elution was followed by TLC and bioassay using Bacillus subtilis. Fractions 13-18 containing A80577 were combined and concentrated in vacuo to a residue which was washed with diethyl ether and dried to yield 2.3 g of amorphous A80577 Na.

Example 3

Preparation of A80577 Free Acid

A80577 sodium salt (1 g) was dissolved in 100 ml acetone and 0.1N HCl (100 ml) was added. The solution was stirred for 15 minutes. The solution was extracted twice with ethyl acetate (100 mL each). The ethyl acetate extracts were combined and concentrated in vacuo to an oily residue. The residue was dissolved in dioxane (50 mL) and freeze-dried to yield 0.9 g of A80577 acid.

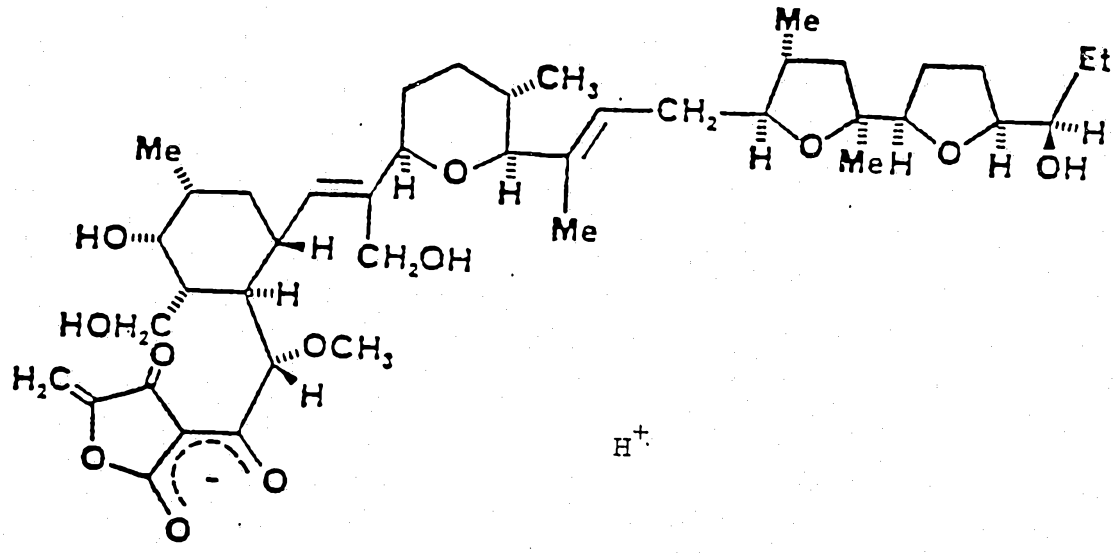
Example 4

Preparation of A80577 Potassium Salt

A80577 (acid form, 100 mg) was dissolved in tetrahydrofuran (20 mL). Water (3 mL) and 2N KOH (6 mL) were added and the mixture was stirred for 15 minutes. Water (30 mL) was added and the solution was extracted twice with diethyl ether (60 mL each). The combined extracts were evaporated under vacuum to dryness. The residue was dissolved in dioxane and freeze-dried to yield 100 mg of A80577 as the potassium salt (m.p. 270-272°C).

The claims defining the invention are as follows:

1. Antibiotic A80577 which has the Formula 1



or an acyl ester, as herein defined, or alkyl ether, as herein defined, derivative of A80577, or a salt of A80577 or of the ester or ether derivative.

2. A compound of Claim 1 which is A80577, an acyl ester or alkyl ether derivative of A80577 or a pharmaceutically acceptable salt of A80577 or of the derivatives.

3. A compound of Claim 1 or 2 which is antibiotic A80577 or a salt of A80577.

4. A compound of any one of Claims 1 to 3 which is the sodium salt of A80577.

5. A compound of any one of Claims 1 to 3 which is the potassium salt of A80577.

6. A compound of Claim 1 or 2 which is a C_1-C_7-acyl ester derivative of A80577 or a salt of this compound.

7. The compound of Claim 1, 2 or 6 which is the acetyl derivative of A80577.

8. The compound of Claim 1, 2 or 6 which is the propionyl derivative of A80577.

9. A process for producing antibiotic A80577 which comprises cultivating Actinomadura verrucosospora, NRRL 18236, or an A80577-producing

variant or mutant thereof, in a culture medium containing assimilable sources of carbon, nitrogen, and inorganic salts under submerged aerobic fermentation conditions until antibiotic A80577 is produced.

10. The process of Claim 9 which includes the additional step of separating A80577 from the culture medium.

11. The process of Claim 9 or 10 wherein Actinomadura verrucosospora, NRRL 18236, is used.

12. A process for increasing feed-utilization efficiency in ruminant animals which comprises orally administering to the animal an effective propionate-increasing amount of a compound of Claim 2.

13. The process of Claim 12 wherein the compound is A80577 or a pharmaceutically acceptable salt of A80577.

14. The process of Claim 12 wherein the compound is a C_1-C_7-acyl ester derivative of A80577 or a pharmaceutically acceptable salt of the derivative.

15. The process of Claim 12 wherein the compound is a C_1-C_4-alkyl ether derivative of A80577 or a pharmaceutically acceptable salt of the derivative.

16. A feed composition for increasing feed utilization efficiency in ruminant animals comprising animal feed and an effective amount of a compound of Claim 2.

17. Antibiotic A80577 according to Claim 1 and as herein described with reference to any one of Examples 2 to 4.

18. A process for producing antibiotic A80577 substantially as herein described with reference to any one of Examples 1 to 4.

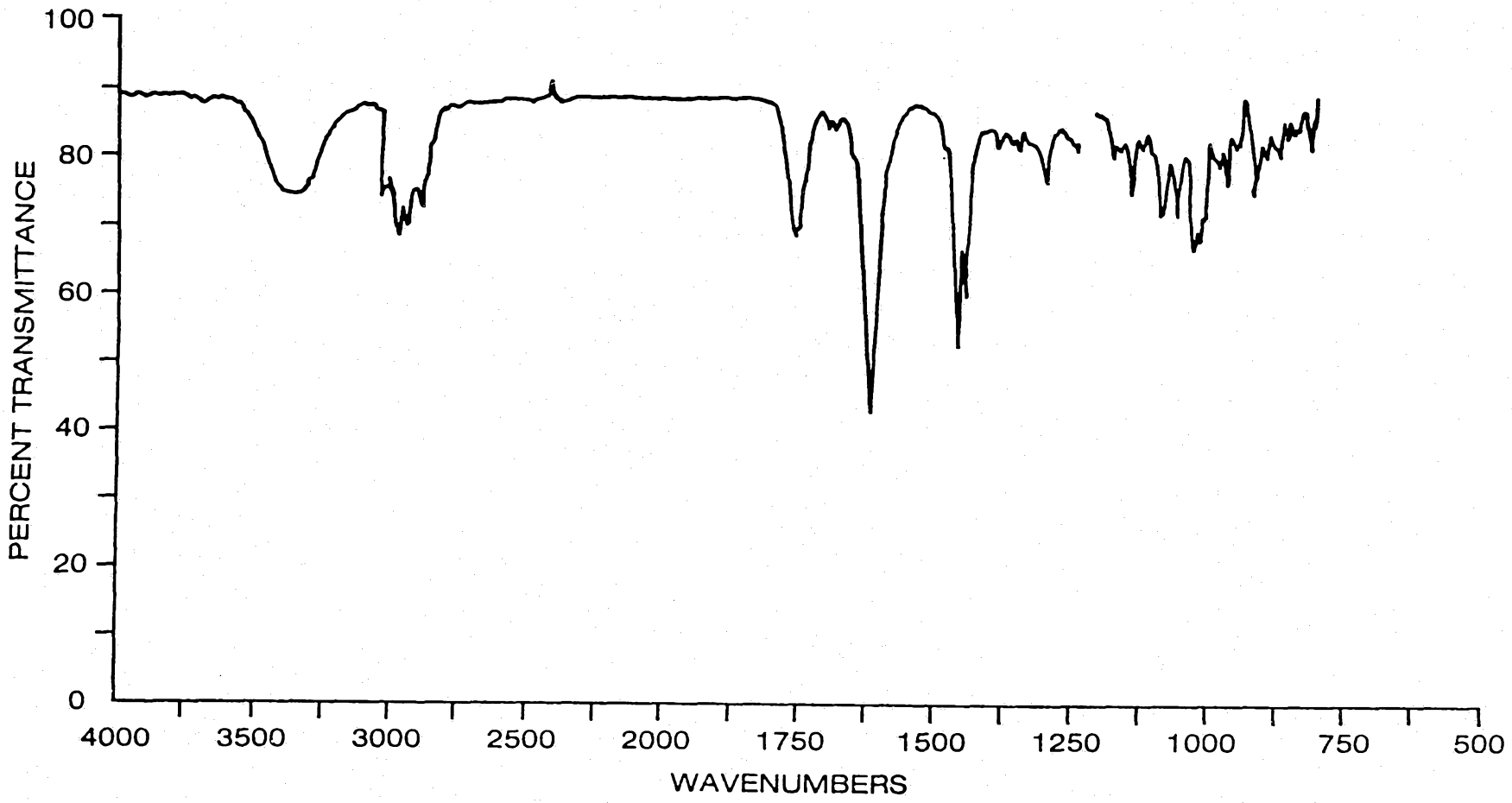
19. A feed composition for increasing feed-utilization efficiency in a ruminant animal comprising animal feed and an effective amount of a compound as defined in Claim 17.

20. A method for increasing feed-utilization efficiency in a ruminant animal comprising orally administering to said animal an effective propionate-increasing amount of a compound of Claim 17 and/or a feed composition of Claim 19.

DATED this TWENTY-FIFTH day of JANUARY 1991
Eli Lilly and Company

Patent Attorneys for the Applicant
SPRUSON & FERGUSON

FIG. 1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60
40
20
0
PERCENT TRANSMITTANCE
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3500
3000
2500
2000
1750
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1250
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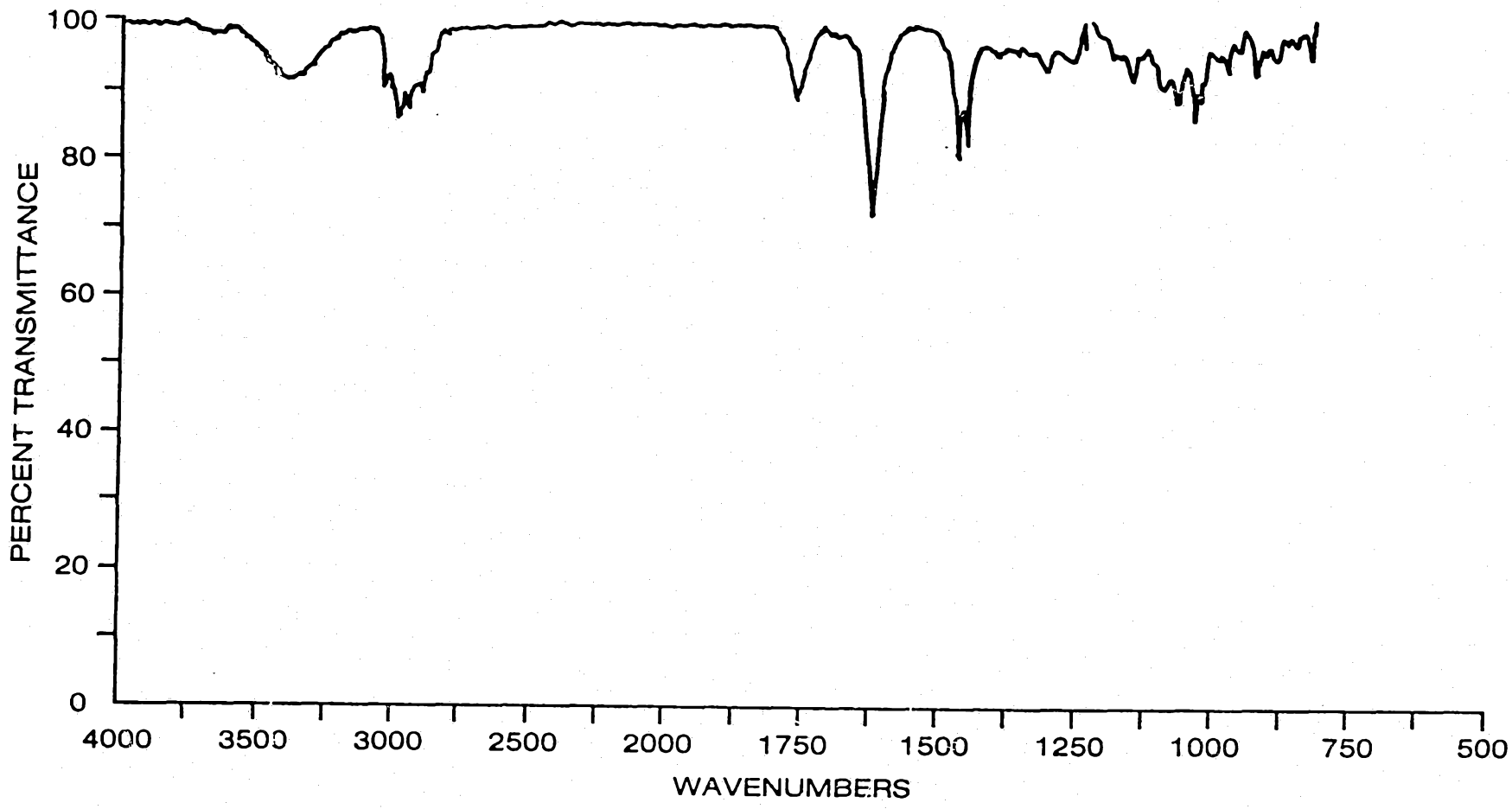
WAVENUMBERS

FIG.2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