

PATENT SPECIFICATION

(11) 1274518

DRAWINGS ATTACHED

1274518

- (21) Application No. 31887/69 (22) Filed 24 June 1969
 (31) Convention Application No. 740 742 (32) Filed 27 June 1968
 (31) Convention Application No. 797 304 (32) Filed 16 Dec. 1968 in
 (33) United States of America (US)
 (45) Complete Specification published 17 May 1972
 (51) International Classification C07D 7/46; A61K 21/00; C12D 9/14
 (52) Index at acceptance
 C2A 1B 1C1A 1C1D1 1C1D2 1C2A 1C2B 1C2C 2A3



(54) ANTIBIOTIC 66—40 AND METHODS FOR ITS PRODUCTION

(71) We, SCHERICO LTD., of Topferstrasse 5, Lucerne, Switzerland, a body corporate constituted under the laws of Switzerland, do hereby declare the invention for which we pray that a patent may be granted to us and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to a new broad spectrum antibiotic designated Antibiotic 66—40, having an adverse effect upon the growth of gram positive and gram negative bacteria, to a microbiological method for its production, and to pharmaceutical compositions containing such antibiotic. The antibiotic may be prepared, isolated and used in its free form or in the form of its pharmaceutically acceptable acid addition salts, condensation products with aldehydes and solvates.

Antibiotic 66—40 is a biosynthetic elaborate obtained by cultivating an Antibiotic 66—40 producing strain of *Micromonospora* in an aqueous nutrient medium.

The microorganism used according to this invention for the production of the new antibiotic has been named *Micromonospora inyoensis* (sometimes hereinafter referred to as *M. inyoensis*). This species was isolated from a soil sample taken from the Inyo National Forest in the White Mountains of California. One of its strain characteristics is its ability to produce Antibiotic 66—40. A culture of the living organism has been made a part of the permanent collection of The Northern Utilization and Research Division, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois, where it has been assigned accession number NRRL 3292. Subcultures of *M. inyoensis* NRRL 3292 are readily available to the public from the foregoing Agency upon request. *M. inyoensis* is aerobic and grows well on the surface of a variety of solid and liquid nutrient media. It exhibits especially good growth and anti-

biotic production under submerged aerobic conditions.

M. inyoensis is differentiable from other species of *Micromonospora* by a variety of taxonomical parameters.

After 14 days of incubation at 24—26°C on an agar medium comprising 3% NZ Amine Type A (Sheffield Chemical Company, Norwich, New York), 1% dextrose and 1.5% agar, growth is observed to be only fair to poor. Macroscopically, there is no apparent aerial mycelium. Occasionally a few well developed colonies appear late in the inoculation area. On some plates, a faint reddish brown diffusible pigment associated with the colonies is observed. [In describing the color formations for this observation and others, the following system and references are employed: The color designations consist of two designators. The first is a color name taken from the "Descriptive Color Name Dictionary" by Taylor, Knoche and Granville, published in 1950 by the Container Corporation of America, USA, with a color chip number corresponding to the color name, the chip number being taken from "The Color Harmony Manual", 4th Edition, published 1958 by The Container Corporation of America. The second designator consists of a color name and number which refers to the synonym or near synonym found in the National Bureau of Standards (USA), Circular 553, November 1, 1955.] The colony surface varies in color from Tile Red g5ne—Strong Brown 55 to Brown Mahogany m6pi. Microscopically the mycelium is long, branched, regular and non-septate as observed by phase contrast microscopy. The mycelium has a diameter of approximately 0.5 μ . The spores are borne singly on simple sporophores of 1.0 to 1.5 μ in diameter. The spores are rough walled and ovoid to spherical in shape.

M. inyoensis grows well at 28—37°C; no growth occurs at 50°C. On glucose aspara-

gine-agar medium growth is poor. A growing colony of *M. inyoensis* will hydrolyze gelatin, milk, starch and reduce nitrate to nitrite when such tests are applied as according to Gordon et al. J. Bacteriology 69, 147 (1956)

and 73, 15 (1957). Additionally, sucrose is utilizable as a carbon source.

Additional culture characteristics of *M. inyoensis* are set forth in Table A:

TABLE A

Medium	Characteristics
Bennett's Agar	Growth moderate — Color: rust brown-g5pg; strong brown-55
Emerson's Agar	Growth fair — Color: brick red-g6ng; moderate reddish brown-43
Tomato Paste-Oatmeal Agar	Growth fair
Glucose Asparagine Agar	Growth poor
Glucose Yeast Extract Agar	Growth good — Color: rose brown-g7ni; Dark greyish red-20 (Faint maroon diffusible pigment produced by some colonies.)
Potato slice	Growth poor but improved when reagent grade calcium carbonate is added
Czapek's Agar	Growth fair
Tyrosine Agar	Growth fair, no diffusible pigment
Peptone Iron Agar	Growth fair, no diffusible pigment

M. inyoensis is capable of utilizing a variety of carbon and nitrogen sources. In Table B there are set forth observations on carbohydrate utilization. A visual estimate of

the degree of growth is observed in a medium consisting of 0.5% yeast extract, 1% carbohydrate and 1.5% agar all in distilled water. 15

TABLE B

CARBOHYDRATE UTILIZATION

Carbohydrate in Medium	Growth
control	poor
L-arabinose	poor
D-glucose	good
D-galactose	fair to poor
β -lactose	fair to poor
D-levulose	poor
raffinose	poor
L-rhamnose	poor
starch	good
sucrose	good
D-xylose	fair to poor
inositol	poor
D-mannitol	poor
d(-)-arabinose	poor
dulcitol	poor
D-ribose	fair
α -melibiose	poor
D(+)-melizitose	poor
glycerol	poor

In Table C, nitrogen utilization is set forth as determined by visual estimate of growth on agar plates in a medium consisting of 1%

glucose, 1.5% agar, and a nitrogen source as indicated, all in distilled water. 5

TABLE C

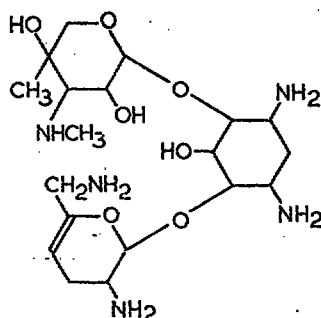
Nitrogen Source	Growth
1% NZ Amine Type A	Fair to moderate
0.5% yeast	good
1% asparagine	poor
1% glutamic acid	poor
1% ammonium nitrate	poor

Antibiotic 66—40, the new compound of this invention, is produced when the elaborating microorganism, *M. inyoensis* is grown in an aqueous nutrient medium under aerobic preferably submerged conditions as described hereinafter.

Antibiotic 66—40 is a basic pseudo-oligosaccharide which is readily distinguished from

other pseudo-oligosaccharides by its biological, physical and chemical properties as set forth herein.

Antibiotic 66—40 in its free form is believed to possess the following gross chemical structure, however no stereochemical assignments are to be implied therefrom:



Thus in accordance with one aspect of the present invention there is provided Antibiotic 66—40 having in its free form a gross structural formula substantially as shown above and being (a) in its free form or (b) in the form of a pharmaceutically acceptable acid addition salt or (c) in the form of a pharmaceutically acceptable condensation product with an aldehyde or mixture of aldehydes or (d) in the

form of a pharmaceutically acceptable solvate of (a), (b) or (c).

Antibiotic 66—40 has a characteristic infrared absorption spectrum in mineral oil (Nujol) as shown by Figure 1. The more significant absorption bands are tabulated in Table D with the following designations: S=strong, M=medium, W=weak, brd=broad, VS=very strong and VW=very weak.

TABLE D

SIGNIFICANT INFRARED ABSORPTION BANDS OF ANTIBIOTIC 66—40

2.98 μ (M—S)	6.82 μ (nujol)	10.46 μ (M—S)
3.05 μ (M—S)	7.25 μ (nujol)	11.97 μ (M)
3.16 μ (M—S)	8.77 μ (M—S)	12.75 μ (VW, brd.)
3.35 μ —3.50 μ (nujol)	9.00 μ (M—S)	13.45—13.90 μ (W, brd)
5.93 μ (W—M)	9.47 μ (S)	
6.25 μ (M)	9.72—10.07 μ (S, brd)	

Antibiotic 66—40 also has a characteristic nuclear magnetic resonance spectrum [NMR spectrum] as shown by Figure 2. The NMR spectrum was obtained by the use of a Varian* A—60—A spectrometer (Varian Associates, 611 Hansen Way, Palo Alto, California) on a solution (about 0.4 ml; concen-

tration about 20 mg/ml) of the antibiotic in deuterium oxide (D_2O). The spectrum is recorded in parts per million (PPM) from 3-(trimethylsilo) - propanesulfonic acid sodium salt, the internal standard.

In Table E is set forth additional data pertaining to qualitative tests on and physical constants of Antibiotic 66—40 (free base).

* "Varian" is a Registered Trade Mark.

TABLE E

a) Color Reactions

Sakaguchi	negative
Starch-Potassium Iodide	positive
Ninhydrin	positive
Stannous Chloride	negative
Molisch	negative
Biuret	positive

b) Physical Constants

$[\alpha]_D^{26}$ (C=0.3% in H_2O)	+ 188.9°
Melting Point (Monohydrate)	185—190°C.
Equivalent Weight	92
PKa	8.0

TABLE E Continued

Elemental Analysis	Found	Calculated for a Monohydrate
C	49.80	49.02
H	8.20	8.44
N	14.95	15.04
O (by difference)	27.05	27.50
Elemental Analysis corresponds to the formula		$C_{19}H_{37}N_5O_7 \cdot H_2O$
Molecular weight as determined by mass spectrometry		447.26

Ultra-violet absorption: transparent in the range between 220—400 mμ.

c) Solubility of Antibiotic 66—40 Base in Various Solvents

Solvent	Solubility*
Methanol	Sparingly soluble
Acetone	Insoluble
Chloroform	Slightly Soluble
Ether	Insoluble
Benzene	Insoluble
Water	Very Soluble

* Terminology is according to U.S. Pharmacopia XVIII, page 8.

- In Table F is set forth the mass spectrum of antibiotic 66—40 free base. In the table the columns headed m/e represents the mass to charge ratio, and the column headed Rel. Int. stands for Relative Intensity and as the name implies sets forth the intensities of the peaks at the various mass to charge ratios (m/e), relative to that of the peak (m/e=118).
- 5 In accordance with another aspect of the present invention there is provided a product comprising Antibiotic 66—40 having in its free form an infrared absorption spectrum in mineral oil substantially as shown in Figure 1 and a nuclear magnetic spectrum in deuterium oxide substantially as shown in Figure 2, and physical data as given in Tables E and F, the solid composition being (a) in its free form or (b) in the form of a pharmaceutically acceptable acid addition salt or (c) in the form of a pharmaceutically acceptable condensation product with an aldehyde or mixture of aldehydes or (d) in the form of a pharmaceutically acceptable solvate of (a), (b) or (c).
- 10 15 20 25

TABLE F
MASS SPECTRUM OF ANTIBIOTIC 65-40 BASE

m/e	Rel. Int.	m/e	Rel. Int.	m/e	Rel. Int.	m/e	Rel. Int.	m/e	Rel. Int.	m/e	Rel. Int.
40	3.0	75	3.0	113	11.0	146	11.0	199	2.0	300	4.0
41	30.0	80	24.0	114	14.0	147	3.5	200	2.0	304	41.0
42	63.0	81	19.5	115	2.5	151	2.0	201	3.0	305	7.0
43	50.0	82	35.5	116	2.5	152	2.5	202	2.5	317	8.5
44	>100%	83	16.5	117	1.0	153	3.0	203	22.0	322	3.0
45	8.0	84	51.0	118	100 (Base Pk)	156	2.0	205	6.0	330	5.0
46	5.0	85	21.0	119	8.0	157	2.5	206	6.0	331	2.0
51	2.5	86	58.0	120	2.0	158	4.5	215	6.0	332	23.0
52	3.5	87	28.0	121	2.0	159	3.5	216	2.0	333	4.0
53	10.0	88	11.0	122	2.0	160	98.0	219	2.0	350	5.0
54	10.0	92	7.0	123	2.0	161	9.5	220	2.0	362	15.0
55	20.0	93	6.0	124	3.0	162	2.0	236	7.0	363	3.0
56	50.0	94	15.0	125	6.0	163	20.0	237	2.0	411	0.5
57	23.5	95	7.0	126	20.0	164	2.0	238	6.0	412	0.6
58	64.0	96	10.0	127	35.0	168	2.0	239	2.0	414	0.5
59	40.0	97	15.0	128	12.0	169	3.5	245	4.0	429	0.9
60	13.5	98	23.0	129	5.0	170	2.5	246	6.0	430	4.5
65	4.0	99	11.0	130	15.0	173	19.0	253	2.5	431	1.0
66	3.5	100	44.0	131	2.5	174	3.0	254	4.5	447	5.0
67	12.0	101	14.0	133	2.0	176	3.0	255	2.5	448	1.3
68	65.5	102	17.0	135	2.0	178	7.0	256	10.0		
69	21.0	107	3.0	138	2.0	185	2.0	270	2.0		
70	19.0	108	7.5	140	3.0	186	2.5	271	11.0		
71	30.0	109	17.0	142	16.0	187	17.0	272	6.0		
72	66.0	110	50.0	143	3.5	188	2.5	282	5.0		
73	66.0	111	13.0	144	6.0	191	32.0	289	4.5		
74	28.0	112	19.0	145	65.0	192	3.5	299	9.0		

As mentioned before, this invention also relates to certain pharmaceutically acceptable derivatives of Antibiotic 66—40, these being its solvates (e.g. hydrates), salts and condensation products with aldehydes. It is to be understood that the antibiotic can be subjected to more than one after-treatment; for example, solvates of salts of Antibiotic 66—40 are also comprised by the invention.

Examples of these derivatives are described in detail below:

Antibiotic 66—40 being basic readily forms non-toxic salts with organic and inorganic acids, such as for example hydrochloric, sulfuric, phosphoric, acetic, stearic, propionic, tartaric, maleic and benzoic acid. Also partly neutralized (e.g. neutralized with an inorganic base such as, for example, NaOH or an organic base) polybasic acids can be used. In general, the mineral acid salts, such as those formed with hydrochloric acid, sulfuric acid or phosphoric acid, are water soluble and may be obtained by concentration or lyophilization of an aqueous solution thereof or by precipitation with a water miscible organic non-solvent preferably a lower aliphatic alcohol or ketone. It is to be noted, however, that hydrochlorides of Antibiotic 66—40 exhibit substantial solubility in

methanol and are, therefore, atypical. The hydrochlorides may be precipitated from an aqueous solution by the addition of a lower alkyl ketone, such as acetone. By titrating an aqueous solution of Antibiotic 66—40 with less than a stoichiometric amount of acid, it is possible to form partial acid addition salts. As used herein the term "acid addition salt" embraces all such compounds.

The above-described non-toxic acid addition salts exhibit substantially the same antibiotic spectrum as does the free nitrogen base, however, they do differ in solubility characteristics.

The new antibiotic and its acid addition salts form hydrates with water and other solvates with organic solvents. It becomes obvious, therefore, that in the isolation procedures described herein the antibiotic is normally obtained as a hydrate and its acid addition salts are normally obtained as solvates of lower aliphatic alcohols or ketones. These hydrates and solvates are relatively stable, therefore, the isolated products contain water or solvent, usually about one mole per mole of antibiotic.

Physical data of some derivatives of this sort are collected in Table G.

TABLE G

PHYSICAL CONSTANTS OF ANTIBIOTIC 66—40 SALTS
ANTIBIOTIC 66—40 HYDROCHLORIDE AS METHANOL SOLVATE

$[\alpha]^{25}_D$ (C=1% in H_2O) +112.2°

Elemental Analysis	Found	Calculated
C	37.70	36.29
H	7.15	7.00
N	10.61	10.58
Cl	25.60	26.78

Analysis corresponds to $C_{16}H_{37}N_5O_7 \cdot 5HCl \cdot CH_3OH$

Ultra-violet absorption: transparent in the range between 220—400m μ

TABLE G Continued

ANTIBIOTIC 66—40 SULFATE AS METHANOL SOLVATE

[α]_D²⁵ (C=1% in H₂O) +105.1°

Elemental Analysis	Found	Calculated
C	32.52	33.14
H	6.33	6.39
N	9.32	9.66
SO ₄	33.90	33.13

Analysis corresponds to (C₁₉H₃₇N₅O₇)₂·5H₂SO₄·2CH₃OHUltra-violet absorption: transparent in the range between 220—400m μ .

- As mentioned before, Antibiotic 66—40 also forms non-toxic condensation products with aldehydes by procedures that are known in the art. The preferred products, probably Schiff bases, contemplated herein are those prepared by condensing the antibiotic with aldehydes having up to 12 carbon atoms. Included among such aldehydes are aliphatic, alicyclic, aromatic and heterocyclic compounds. Further, it is to be understood that the aldehydes having a closed ring structure may bear substituents such as hydroxy, halogeno, nitro, alkoxy and alkanoyloxy, the alkoxy and alkanoyloxy substituents having up to 8 carbon atoms. Solely for illustration and without limitation, the following are among the aldehydes contemplated herein: acetaldehyde, crotonaldehyde, furfural, cyclopentyl-acetaldehyde, vanillin, veratraldehyde, benzaldehyde, salicylaldehyde and pyridoxal.

It is to be noted that these condensation products are not stable in the presence of water.

- As mentioned before, Antibiotic 66—40 is produced when the elaborating microorganism, *M. inyoensis* is grown in an aqueous nutrient medium under aerobic, preferably submerged conditions.

- Thus in accordance with a further aspect of the present invention, a process for the production of an antibiotically active product comprising Antibiotic 66—40 in any of forms (a), (b), (c) and (d) referred to above, comprises incubating a microorganism of the species *Micromonospora inyoensis* in an aqueous nutrient medium containing assimilable sources of at least nitrogen and carbon under aerobic, preferably submerged conditions until substantial antibacterial activity is imparted to said medium and isolating the said antibiotically active product therefrom.

For limited amounts of antibiotic, surface culture in bottles or shake flasks can be employed instead of submerged conditions. The organism is grown in a nutrient medium containing a carbon source, for example, an assimilable carbohydrate. An assimilable nitrogen compound or proteinaceous material is also required. Preferred carbon sources include glucose, maltose, mannose, sucrose, starch and corn starch. Preferred nitrogen sources include corn steep liquor, yeast extracts, soybean meal, meat peptones, casein hydrolysates and beef extracts. Combinations of these carbon and nitrogen sources can be used advantageously. It generally is not necessary to add trace elements since tap water is used as the formulation medium; however, addition of cobalt salts has been found to be advantageous.

Production of Antibiotic 66—40 can be effected at any temperature conducive to satisfactory growth of the microorganism, for example between 20° and 40°C, preferably 25—35°C.

Ordinarily optimum production is obtained in 3—7 days. The pH of the medium usually stays fairly close to 7 during the fermentation. The final pH is in part dependent on the buffers present, if any, and is advantageously adjusted to about 8.0 prior to sterilization. When growth is carried out in large vessels and tanks, it is desirable to produce a vegetative inoculum in a nutrient broth by inoculating the broth culture with a soil or slant culture or a lyophilized culture of the organism. When an active inoculum has been so obtained, it is transferred aseptically to larger vessels or tanks. The medium in which the vegetative inoculum is produced can be the same as or different from that utilized for the production of the antibiotic in tanks as

long as good growth of the microorganism is obtained.

After completion of the fermentation, the medium may, for instance, be worked up as follows: The whole broth is adjusted to a pH of about 2 with mineral acid, preferably aqueous sulfuric acid, whereby the basic water-soluble antibiotic is released from the mycelium and dissolved in the aqueous fermentation medium. The entire mixture is filtered so as to remove the mycelium, and the filtrate is neutralized, followed by addition of oxalic acid to precipitate calcium ions. After an additional filtration and adjustment to neutrality, preferably with ammonium hydroxide, the clear neutralized filtrate is passed through an ion exchange resin, preferably of the IRC-50 Amberlite type in the ammonium form. ("AMBERLITE" is a Registered Trade Mark). Examples of the Amberlite type resins employed herein, both for anionic and cationic exchange, are found in the Handbook of Chemistry and Physics, 42nd Edition, Chemical Rubber Publishing Company, Cleveland, Ohio (1960). The spent broth is discarded, and the antibiotic is eluted from the resin with ammonium hydroxide. The eluate is concentrated and evaporated to a residue consisting of crude Antibiotic 66-40 having a potency of about 500 mcg/mg according to the assay procedure hereinafter described.

Purification of the crude antibiotic may be effected by utilization of an anionic exchange resin, preferably Dowex 1X2 or an Amberlite IRA 401S type. ("DOWEX" is a Registered Trade Mark). The crude antibiotic may be adsorbed from an aqueous solution onto the column and eluted therefrom with distilled water. Material obtained in this manner assays approximately 900 mcg/mg. Alternatively, for instance, the crude antibiotic is purifiable by column chromatography on cellulose utilizing a solvent system of chloroform:methanol:17% ammonium hydroxide (2:1:1); the upper phase of the solvent system is used to first "wet" the column, and the lower phase is used for elution purposes. The antibiotics may be placed on the column by adsorption from a concentrated solution in the upper phase of the aforementioned solvent system.

In order to assay the various preparations for potency in terms of micrograms per milligrams of the antibiotic, the standard cylinder cup assay method is usually employed using *Staphylococcus aureus* ATCC 6538P (ATCC=American Type Culture Collection) as test organism. This method is completely analogous to that described by Oden et al. In Antimicrobial Agents and Chemotherapy (1963): One microgram of antibiotic activity of the antibiotic is the amount of material which produces a zonal response of 16.3 ± 0.9

mm under the conditions of the assay method and is expressed as mcg/mg.

The following examples are illustrative of fermentation and isolation procedures yielding Antibiotic 66-40:

EXAMPLE 1.

Tank Fermentation of *Micromonospora inyoensis*.

Germination Stage 1:

Under aseptic conditions, add a lyophilized culture (or cells obtained from a slant culture) of *M. inyoensis* to a 300 ml shake flask containing 100 ml of the following sterile medium:

Beef extract	3 g	
Tryptone	5 g	80
Yeast extract	5 g	
Dextrose	1 g	20
Starch	24 g	
Calcium carbonate	2 g	
Tap water	1000 ml	85

Incubate the flask and its contents for five days at 35°C on a rotary shaker (280 rotations per minute, [r.p.m.], 50.8 mm stroke).

Germination Stage 2:

Aseptically transfer 25 ml of the fermentation medium of germination stage 1 to a two-liter shake flask containing 500 ml of the aforescribed sterile germination medium. Incubate the flask and its contents for three days at 28°C on a rotary shaker (280 r.p.m., 50.8 mm stroke).

Fermentation stage:

Aseptically transfer 500 ml of the medium obtained from germination stage 2 to a 14-liters fermentation tank containing 9.5 liters of the following sterile medium:

Dextrin	50 g	
Dextrose	5 g	
Soybean meal	35 g	
Calcium carbonate	7 g	105
Cobalt chloride	10^{-6} molar	
Tap water	1000 ml	
Antifoam (GE60=silicone emulsion)	10 ml	50

Prior to sterilizing the aforescribed medium, adjust the pH to 8. Aerobically ferment for 66-90 hours while stirring at 250 r.p.m. with air input at 4.5 liters per liter/minute and 22.5 at. The potency of the antibiotic produced at the end of this period reaches a peak of 150-225 mcg/ml and remains relatively constant. The pH of the fermentation medium changes slightly during the antibiotic production varying in the range of 6.8-7.3.

EXAMPLE 2.

Isolation of Antibiotic 66—40.

- The whole broth from Example 1 is adjusted to pH 2 with 6N sulfuric acid. (For the purpose of this example, quantities are given in terms of 170 liters of fermentation broth obtained by pooling acidified broths from 17 batches obtained according to the procedure of Example 1). The acidified broth is stirred for about 15 minutes and then filtered. Wash the mycelium with water and combine the washings with the filtrate. Adjust the pH of the filtrate to 7 with 6N ammonium hydroxide. To the neutralized filtrate, add sufficient oxalic acid to precipitate calcium and filter. RENEUTRALIZE the filtrate with ammonium hydroxide. Charge the filtrate onto a cationic exchange adsorption column containing 1500—2000 g of IRC—50 Amberlite in its ammonium form. Discard the eluate, wash the resin with water, and elute with 2N ammonium hydroxide. Collect 400 ml fractions and monitor by disc testing with *S. aureus* ATCC 6538P.
- Combine active fractions and evaporate to dryness under vacuum obtaining about 28 g of crude Antibiotic 66—40 having an activity of about 500 mcg/g.

EXAMPLE 3.

Purification of Antibiotic 66—40.

- Dissolve 28 g of crude Antibiotic 66—40 obtained in Example 2 in 100 ml of distilled water and charge to an anion exchange adsorption column (Dowex 1×2) in the hydroxyl form. Slurry 2000 g of the resin in water into a column 63.5 mm in diameter and 914.4 mm high. Elute the column with distilled water at a rate of about 23 ml/min collecting 100 ml fractions and monitor with a conductivity meter and by disc testing against *Staphylococcus aureus*. The disc testing provides a gross separation of antibiotic-containing eluate fractions from those devoid of antibiotic. To insure that the fractions are properly combined, a portion of each fraction is paper chromatographed using the lower phase of a chloroform:methanol:17% ammonium hydroxide system (2:1:1). Each paper is sprayed with ninhydrin and the eluates containing the required material are combined and lyophilized yielding about 5.7 g of Antibiotic 66—40 assaying about 900 mcg/mg.

EXAMPLE 4.

Preparation of Antibiotic 66—40 sulfate.

- Dissolve 3.9 g of Antibiotic 66—40 base prepared as described in Example 3 in 60 ml of water and adjust the pH to 4.5 with 6N sulfuric acid. Stir the solution with decolorizing charcoal for about ½ hour and filter. Add the filtrate to about 1 liter of methanol. Filter and dry obtaining 4.8 g of the sulfate salt; assay about 640 mcg/mg.

EXAMPLE 5.

Alternate Purification of Antibiotic 66—40 via its Sulfate.

200 g of Whatman No. 1 cellulose powder ("WHATMAN" is a Registered Trade Mark) are mixed with 20 ml of the top phase of a solvent system composed of chloroform:methanol:17% ammonium hydroxide (2:1:1) and packed in small segments in a column having an inner diameter of 6.35 mm and a height of 508 mm. The lower phase of the solvent system is run through the column until a yellow band of impurities emerges. Two grams of Antibiotic 66—40 sulfate prepared as described in Example 4 are dissolved in about 3 ml of the upper solvent phase, mixed with some cellulose powder, dried under vacuum and packed atop the cellulose column. Lower phase is allowed to run through the column at the rate of 1 ml per minute collecting 5 ml fractions every 15 minutes.

Aliquots of each fraction were spotted on filter paper and tested with ninhydrin reagent to determine the presence or absence of antibiotic. Paper chromatography of the antibiotic-containing fractions established that the desired material was located between fractions 121 to 190.

Fractions 121 to 190 are combined, evaporated to dryness, redissolved in water, and passed through IRA 401S (an anion exchange resin) in the hydroxyl form. The pH of the eluate is adjusted to 4.5 with sulfuric acid; the eluate is treated with charcoal, filtered and concentrated to a smaller volume. The concentrate is added to an excess amount of methanol and the white precipitate that is formed is separated by filtration. The precipitate is dissolved in water and passed through an IRA 401S resin column in the hydroxyl form. The effluent is collected, concentrated and lyophilized, yielding about 300 mg of Antibiotic 66—40 base having an assay of about 1000 mcg/mg.

EXAMPLE 6.

Preparation of Antibiotic 66—40 Hydrochloride.

Dissolve 104.7 mg of Antibiotic 66—40 base, prepared as described in Example 2 in 4 ml of water and adjust the pH to 4.5 with hydrochloric acid. Evaporate the solution to dryness and redissolve the residue in methanol. Add this solution to excess acetone and filter the resulting precipitate obtaining 130 mg of Antibiotic 66—40 hydrochloride; assaying at 753 mcg/mg.

EXAMPLE 7.

Preparation of Crystalline Antibiotic 66—40 Monohydrate.

Prepare a (26×2.5 cm) silica gel chromatographic column using the lower (organic) phase of a solvent mixture consisting of iso-

propanol:CHCl₃:ammonium hydroxide in the volume ratio of 1:2:1 as the developer/eluent. Dissolve 1.0 gram of Antibiotic 66—40 base in 5.0 ml of solvent mixture. Adsorb the antibiotic solution to the silica gel and chromatograph. Collect 5.0 ml fractions and determine the location of the desired fractions by thin-layer chromatography on silica gel plates. Combine and evaporate the appropriate (44—78) fractions *in vacuo* and obtain thereby a pale yellow syrup which upon azeotropic distillation with ethanol crystallizes as pale yellow rosettes. The product obtained in this manner is Antibiotic 66—40 monohydrate which melts at about 185—190°C. The yield is about 650 mg.

EXAMPLE 8.

Preparation of Benzaldehyde Condensation Product of Antibiotic 66—40.

5.0 g of Antibiotic 66—40 in 60 ml absolute ethanol is treated with 5.9 g benzaldehyde (slight excess over 5 equivalents) and refluxed for 1 hour. The solution is cooled and filtered affording 7.0 g of a white crystalline solid, m.p. 123—126°C. $[\alpha]_D^{26} = +43.2^\circ$ (C=0.3% in CHCl₃). Elemental analysis indicates five benzaldehyde residues.

Similarly, by replacing the benzaldehyde reactant in the foregoing example with an equivalent quantity of any one of the following aldehydes

acetaldehyde,
furfural,
cyclopentylacetaldehyde,
crotonaldehyde,
salicylaldehyde,
vanillin,
veratraldehyde or
pyridoxal

and by following substantially the procedure set forth in the example, the corresponding aldehyde condensation products (probably Schiff's Bases in terms of chemical structure) are obtained.

Antibiotic 66—40 and its pharmaceutically acceptable functional derivatives possess a broad antibacterial spectrum. The antibiotic has the property of adversely affecting the growth of gram positive and gram negative bacteria and thus can be used alone or in combination with other antibiotic agents to prevent the growth of or reduce the number of bacteria in various environments. It may be used, for example, to disinfect laboratory glassware, dental and medical equipment contaminated with *Staphylococcus aureus* or other bacteria whose growth is adversely affected by Antibiotic 66—40. Because of its particularly effective activity against gram negative bacteria, it is useful in combatting infections caused by such gram negative organisms.

The *in vitro* activity of Antibiotic 66—40 against a variety of gram positive and gram negative bacteria is set forth in Table H. The minimal inhibitory concentration (MIC) was determined utilizing yeast beef broth as the test medium. A two-fold serial dilution technique was employed. The MIC is the midpoint between the last clear tube and the first turbid tube determined by visual observation. Determinations were made using a 10⁻³ dilution of a 24 hour broth culture of the test bacterial. All tubes were incubated for 18 hours at 37°C. In the table, Antibiotic 66—40 having a potency of 1000 mcg/mg was used for the study.

TABLE H

IN VITRO ACTIVITY OF ANTIBIOTIC 66-40

Microorganisms Used	MIC
Gram Positive Bacteria	mcg/ml
<i>Diplococcus pneumoniae</i> DA 150 ¹	3.0
<i>Enterococcus</i> sp. DA 800	2.25
<i>Enterococcus</i> sp. DA 801	2.25
<i>Enterococcus</i> sp. DA 802	2.25
<i>Staphylococcus aureus</i> ATCC 6538P	0.23
<i>Staphylococcus aureus</i> ATCC 11631	0.21
<i>Staphylococcus aureus</i> Gray	0.05
<i>Staphylococcus aureus</i> DA 2033	0.08
<i>Streptococcus faecalis</i> ATCC 10541	3.0
<i>Streptococcus pyogenes</i> DA 1	3.0
<i>Streptococcus pyogenes</i> DA 21	3.7
<i>Streptococcus pyogenes</i> DA 15	3.7
Gram Negative Bacteria	
<i>Escherichia coli</i> ATCC 10536	0.6
<i>Escherichia coli</i> DA 3	0.3
<i>Escherichia coli</i> DA 4	0.6
<i>Escherichia coli</i> DA 1	0.6
<i>Klebsiella pneumoniae</i> DA 20	0.23
<i>Klebsiella pneumoniae</i> ATCC 10031	3.0
<i>Proteus vulgaris</i> DA 121	0.6
<i>Proteus vulgaris</i> ATCC 9921	0.6
<i>Proteus vulgaris</i> DA 13	0.3
<i>Proteus vulgaris</i> DA 12	3.0
<i>Pseudomonas aeruginosa</i> ATCC 8709	0.45
<i>Pseudomonas aeruginosa</i> ATCC 8689	0.6
<i>Pseudomonas aeruginosa</i> ATCC 9027	0.6
<i>Salmonella schottmuelleri</i> DA 10	0.53
<i>Salmonella</i> sp. DA 101	0.3
<i>Salmonella</i> sp. DA 102	0.3
<i>Aerobacter</i> sp. DA 3a	0.3

¹DA refers to Schering Corporation collection number.

The acute toxicity of Antibiotic 66—40 in mice weighing 18—20 grams. The toxicity data given in Table J is expressed in terms of the free base.

TABLE J

ACUTE TOXICITY OF ANTIBIOTIC 66—40

Mode of Administration	LD ₅₀ (mg/kg)
subcutaneous	288
intraperitoneal	221
intravenous	34

Antibiotic 66—40 exhibits an antibacterial action against pathogenic bacterial infections induced in laboratory animals and in particular in the mouse. To determine the *in vivo* protective activity of Antibiotic 66—40 against infections of pathogenic bacterial origin in mice, mice were dosed twice with the antibiotic, once immediately before an intraperi-

toneal injection of the infecting bacteria and once 4 hours after such injection. The number of survivors was determined 48 hours after infection and that data analyzed by standard probit procedures to determine PD₅₀ values with 95% confidence limits. Table K sets forth the protective activity of Antibiotic 66—40 against various pathogenic bacteria.

TABLE K

PROTECTIVE ACTIVITY OF ANTIBIOTIC 66—40 IN MICE

Organism	Route of Administration	PD ₅₀ (mg/kg)
<i>Staphylococcus aureus</i> Gray DC 445	subcutaneous	0.12
	oral	25.0
<i>Streptococcus pyogenes</i> C DC 28	subcutaneous	0.87
<i>Klebsiella pneumoniae</i> DC 801	subcutaneous	0.70
	oral	50.0
<i>Pseudomonas aeruginosa</i> ATCC 8709	subcutaneous	1.12
<i>Salmonella paratyphi</i> B DC 837	subcutaneous	1.91

It is evident from Table K that the therapeutic index (LD₅₀/PD₅₀) via the subcutaneous route ranges from 330 to 2400 with regard to the gram positive organisms and 150 to 410 with regard to the gram negative organisms.

In addition, mice infected intraperitoneally with eight LD₅₀ doses of *Rickettsia akaria* were afforded 100% protection by the subcutaneous administration of 2 mg of Anti-

biotic 66—40 administered once a day for four days.

In view of the foregoing *in vivo* data and especially in view of the favorable therapeutic index exhibited by Antibiotic 66—40, it is evident that the antibiotic may be used to control and treat a variety of infections in mammalian hosts. Among such infections are those caused by species of such organisms as *Streptococcus*, *Staphylococcus*, *Streptococcus*,

5 *Escherichia*, *Salmonella*, *Klebsiella*, *Proteus*,
10 *Pseudomonas* and the like. The foregoing
organisms cause or are suspected of causing
bovine mastitis, urinary tract infections and
diarrhea. Species of the same organisms are
suspected of causing skin and upper respira-
tory diseases or of aggravating pre-existing
manifestations of such diseases in mammals.
Antibiotic 66—40 and its derivatives, there-
fore, provide a potent weapon for combatting
such organisms and disease states caused
thereby.

15 Antibiotic 66—40 and its derivatives may
be applied topically in the form of ointments,
both hydrophilic and hydrophobic, in the form
of lotions which may be aqueous, non-aqueous
or of the emulsion type or in the form of
creams. Pharmaceutical carriers useful in the
preparation of such formulations will include,
20 for example, such substances as water, oils,
greases, polyesters and polyols.

25 In general, the topical preparations will con-
tain from about 0.1 to about 3.0 g of the
antibiotic per 100 g of ointment, cream or
lotion. The topical preparations are usually
applied gently to lesions from about 2 to
about 5 times a day.

30 The antibiotics of the instant invention may
be utilized in liquid form such as solutions
or suspension for otic and optic use and may
also be administered parenterally via intra-
muscular injection. The injectable solution or

suspension will usually be administered at
from about 1 mg to about 5 mg of antibiotic
per kilogram of body weight per day divided
into about 2 to about 4 doses. The precise
dose depends on the stage and severity of the
infection, the susceptibility of the infecting
organism to the antibiotic and the individual
characteristics of the mammals being treated.

Thus in accordance with a further aspect
of the present invention there is provided a
method of preparing a therapeutical composi-
tion having antibiotic activity, characterised
in that an antibiologically active product com-
prising Antibiotic 66—40 in any of forms
(a), (b), (c) and (d) specified herein, as an
active ingredient is brought into a form suit-
able for therapeutical administration, for ex-
ample by mixing with a suitable pharmaceu-
tical carrier. The invention also provides,
according to a further aspect thereof, anti-
biotically active compositions characterised by
an antimicrobially effective content of an anti-
biotically active product comprising Antibiotic
66—40 in any of forms (a), (b), (c) and (d)
specified herein as an active ingredient in
admixture with a suitable pharmaceutical car-
rier. The compositions may be in the form
of a shaped composition of matter, such as a
tablet or capsule.

Example 9 below sets forth the ingredients
and the process for making an injectable solu-
tion.

EXAMPLE 9

INJECTABLE SOLUTION

	Per 2.0 ml Vial	Per 50 liters
	*	*
Antibiotic 66—40 sulfate	84.0 mg	2100.0 g
p-Hydroxybenzoic acid methyl ester, USP** (=Methyl paraben)	3.6 mg	90.0 g
p-Hydroxybenzoic acid propyl ester, USP** (=Propylparaben)	0.4 mg	10.0 g
Sodium bisulfite, USP**	6.4 mg	160 g
Disodium Ethylenediamino- tetraacetate Dihydrate, R.G.	0.2 mg	5.0 g
Water for injection, USP** q.s. ad	2.0 ml	50.0 liters

*Includes a 5% manufacturing overcharge

** US-Pharmacopoeia

Procedure: For a 50.0 liter batch.

Charge approximately 35 liters of water for injection to a suitable stainless steel jacketed vessel and heat to about 70°C. Charge the methylparaben and propylparaben to the heated water for injection and dissolve with agitation. When the parabens are completely dissolved, cool the contents of the tank to 25–30°C by circulating cold water through the tank jacket. Sparge the solution with nitrogen gas for at least 10 minutes and keep covered with nitrogen during subsequent processing. Charge and dissolve the disodium ethylene diamine-tetra acetate dihydrate and sodium bisulfite. Charge and dissolve the Antibiotic 66–40 sulfate. Bring the batch volume up to 50.0 liters with water for injection and agitate until homogenous.

Under sterile conditions, filter the solution through a suitable bacteria retentive filter collecting the filtrate in a filling tank.

Fill the product aseptically into sterile pyrogen-free multiple dose vials, stopper and seal.

EXAMPLE 10.

Antibiotic Ointment.

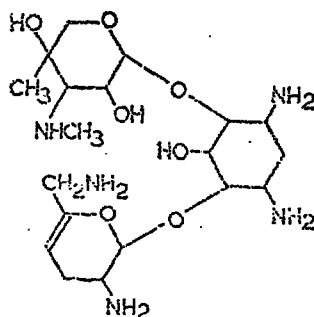
Antibiotic 66–40 base	10 g
Petrolatum	990 g

Procedure

- (1) Melt the petrolatum;
- (2) Admix Antibiotic 66–40 base with about 10% of the molten petrolatum;
- (3) Pass the antibiotic–petrolatum mixture through a colloid mill;
- (4) Add in the remainder of the petrolatum and cool the mixture until it becomes semi-solid. At this stage the product may be put into suitable containers.

WHAT WE CLAIM IS:—

1. Antibiotic 66–40 having in its free form a gross structural formula substantially as follows:—



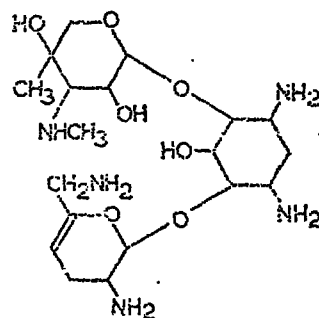
- and being (a) in its free form or (b) in the form of a pharmaceutically acceptable acid addition salt or (c) in the form of a pharmaceutically acceptable condensation product

with an aldehyde or mixture of aldehydes or (d) in the form of a pharmaceutically acceptable solvate of (a), (b) or (c).

2. A product comprising Antibiotic 66–40 having in its free form an infrared absorption spectrum in mineral oil substantially as shown in Figure 1 and a nuclear magnetic spectrum in deuterium oxide substantially as shown in Figure 2, and physical data as given in Tables B and F herein, the Antibiotic being (a) in its free form or (b) in the form of a pharmaceutically acceptable acid addition salt or (c) in the form of a pharmaceutically acceptable condensation product with an aldehyde or (d) in the form of a pharmaceutically acceptable solvate of (a) or (b).

3. A product comprising Antibiotic 66–40 having in its free form an infrared absorption spectrum in mineral oil substantially as shown in Figure 1 and a nuclear magnetic spectrum in deuterium oxide substantially as shown in Figure 2, and physical data as given in Tables E and F herein, the Antibiotic being in the form of a pharmaceutically acceptable condensation product with a mixture of aldehydes or in the form of a pharmaceutically acceptable solvate of a pharmaceutically acceptable condensation product with an aldehyde or mixture of aldehydes.

4. A product according to claim 2, having in its free form a gross structural formula substantially as follows:



5. A solvate according to any preceding claim.

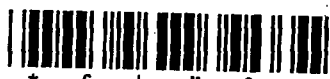
6. A product according to claim 5, wherein the solvate is a hydrate.

7. A product according to claim 6, said product being Antibiotic 66–40 monohydrate in substantially pure crystalline form.

8. A product according to any one of claims 1 to 6, said product being an acid addition salt.

9. A product according to claim 8, said product being an acid addition salt, which when derived from a polybasic acid may contain free acid groups and/or acid groups neutralized by other bases.

10. A product according to claim 9, containing acid moieties of at least one of the



Procedure: For a 50.0 liter batch.

Charge approximately 35 liters of water for injection to a suitable stainless steel jacketed vessel and heat to about 70°C. Charge the methylparaben and propylparaben to the heated water for injection and dissolve with agitation. When the parabens are completely dissolved, cool the contents of the tank to 25—30°C by circulating cold water through the tank jacket. Sparge the solution with nitrogen gas for at least 10 minutes and keep covered with nitrogen during subsequent processing. Charge and dissolve the disodium ethylene diamine-tetra acetate dihydrate and sodium bisulfite. Charge and dissolve the Antibiotic 66—40 sulfate. Bring the batch volume up to 50.0 liters with water for injection and agitate until homogenous.

Under sterile conditions, filter the solution through a suitable bacteria retentive filter collecting the filtrate in a filling tank.

Fill the product aseptically into sterile pyrogen-free multiple dose vials, stopper and seal.

EXAMPLE 10.

Antibiotic Ointment.

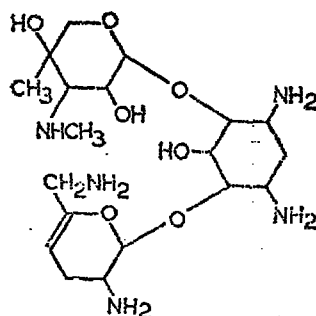
Antibiotic 66—40 base 10 g
Petrolatum 990 g

Procedure

- (1) Melt the petrolatum;
- (2) Admix Antibiotic 66—40 base with about 10% of the molten petrolatum;
- (3) Pass the antibiotic—petrolatum mixture through a colloid mill;
- (4) Add in the remainder of the petrolatum and cool the mixture until it becomes semi-solid. At this stage the product may be put into suitable containers.

WHAT WE CLAIM IS:—

1. Antibiotic 66—40 having in its free form a gross structural formula substantially as follows:—



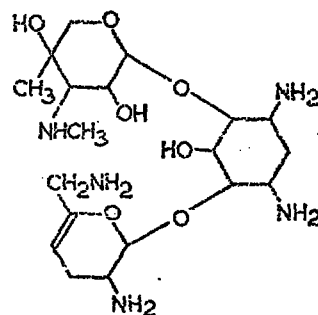
- and being (a) in its free form or (b) in the form of a pharmaceutically acceptable acid addition salt or (c) in the form of a pharmaceutically acceptable condensation product

with an aldehyde or mixture of aldehydes or (d) in the form of a pharmaceutically acceptable solvate of (a), (b) or (c).

2. A product comprising Antibiotic 66—40 having in its free form an infrared absorption spectrum in mineral oil substantially as shown in Figure 1 and a nuclear magnetic spectrum in deuterium oxide substantially as shown in Figure 2, and physical data as given in Tables E and F herein, the Antibiotic being (a) in its free form or (b) in the form of a pharmaceutically acceptable acid addition salt or (c) in the form of a pharmaceutically acceptable condensation product with an aldehyde or (d) in the form of a pharmaceutically acceptable solvate of (a) or (b).

3. A product comprising Antibiotic 66—40 having in its free form an infrared absorption spectrum in mineral oil substantially as shown in Figure 1 and a nuclear magnetic spectrum in deuterium oxide substantially as shown in Figure 2, and physical data as given in Tables E and F herein, the Antibiotic being in the form of a pharmaceutically acceptable condensation product with a mixture of aldehydes or in the form of a pharmaceutically acceptable solvate of a pharmaceutically acceptable condensation product with an aldehyde or mixture of aldehydes.

4. A product according to claim 2, having in its free form a gross structural formula substantially as follows:



5. A solvate according to any preceding claim.

6. A product according to claim 5, wherein the solvate is a hydrate.

7. A product according to claim 6, said product being Antibiotic 66—40 monohydrate in substantially pure crystalline form.

8. A product according to any one of claims 1 to 6, said product being an acid addition salt.

9. A product according to claim 8, said product being an acid addition salt, which when derived from a polybasic acid may contain free acid groups and/or acid groups neutralized by other bases.

10. A product according to claim 9, containing acid moieties of at least one of the



following acids: hydrochloric, sulfuric, phosphoric, acetic, stearic, propionic, tartaric, maleic and benzoic acid.

11. A product according to any one of claims 1 to 6, said product being a condensation product with an aldehyde or mixture of aldehydes.

12. A product according to claim 11, wherein the aldehyde moiety has up to 12 carbon atoms.

13. A product according to claim 12, wherein the aldehyde is benzaldehyde.

14. Antibiotic 66—40 in any of forms (a), (b), (c) and (d) specified in claim 1 and substantially as hereinbefore described.

15. Process for the production of an antibiotically active product comprising Antibiotic 66—40, in any of forms (a), (b), (c) and (d) specified in claim 1 which comprises incubating a microorganism of the species *Micromonospora inyoensis* in an aqueous nutrient medium containing assimilable sources of at least nitrogen and carbon under aerobic conditions until substantial antibacterial activity is imparted to said medium and isolating the said antibiotically active product therefrom.

16. Process according to claim 15, wherein the incubation is performed under submerged conditions.

17. Process according to claim 15 or 16, wherein the microorganism incubated is *Micromonospora inyoensis* NRRL 3292 or an Antibiotic 66—40 producing mutant or variant thereof, possessing substantially the same biological activity.

18. Process according to any one of claims 15 to 17, wherein the microorganism is incubated at a temperature from about 20 to 40°C, and at a pH from 7 to 8.

19. Process according to claim 18 where the microorganism is incubated at a temperature from 25 to 35°C.

20. Process according to any one of claims 15 to 19 wherein the antibiotically active product is recovered by: freeing the antibiotically active product from the mycelium, adsorbing the antibiotically active product on an ion-exchange resin, washing the resin adsorbate with water, eluting the antibiotically active product from the resin, concentrating the eluate, and isolating the antibiotically active product from the concentrate.

21. Process according to any one of claims 15 to 20, wherein chromatographic methods are used for the purification of the antibiotically active product.

22. Process according to any one of claims 15 to 21, which includes at least one of the following after-treatments, carried out after formation of the antibiotically active product: (a) precipitation as a solvate out of an organic or aqueous solution by adding a solvent wherein the antibiotically active product is not or only slightly soluble; (b) addition of an acid or acid derivative so as to obtain an acid addition salt, which when derived from a polybasic acid may contain free acid groups and/or acid groups neutralized by other bases; (c) condensation with an aldehyde or mixture of aldehydes.

23. Process according to claim 22 in which the or each aldehyde has up to 12 carbon atoms.

24. Process for the production of an antibiotically active product comprising Antibiotic 66—40, in any of forms (a), (b), (c) and (d) specified in claim 1, substantially as described herein.

25. Antibiotic 66—40 in any of forms (a), (b), (c) and (d) when obtained by the process of any one of claims 15 to 24.

26. Method for the preparation of a therapeutical composition having antibiotic activity, characterized in that an antibiotically active product comprising Antibiotic 66—40 in any of forms (a), (b), (c) and (d) specified in claim 1, as an active ingredient is brought into a form suitable for therapeutical administration.

27. A method according to claim 26 which comprises mixing the antibiotically active product with a suitable pharmaceutical carrier.

28. Method for the preparation of a therapeutical composition, according to claim 26 and substantially as hereinbefore described.

29. Antibiotically active composition characterized by an antimicrobially effective content of an antibiotically active product comprising Antibiotic 66—40 in any of forms (a), (b), (c) and (d) specified in claim 1, as an active ingredient in admixture with a suitable pharmaceutical carrier.

30. Composition according to claim 29, characterized in that it is a shaped composition of matter, such as a tablet or capsule.

31. Composition according to claim 29, substantially as described and exemplified herein.

MATHYS & SQUIRE,
Chartered Patent Agents,
10 Fleet Street, London, E.C.4,
Agents for the Applicants.