

PATENT SPECIFICATION

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DRAWINGS ATTACHED

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(72) Inventors EIJI HIGASHIDE, TORU HASEGAWA,
HIDEO ONO, MITSUKO ASAI, MASAYUKI MUROI
and TOYOKAZU KISHI



(54) NEW ANTIBIOTIC B-5050 AND PRODUCTION THEREOF

(71) We, TAKEDA YAKUHI KOGYO KABUSHIKI KAISHA, also known as TAKEDA CHEMICAL INDUSTRIES LTD., a body corporate organised under the laws of Japan, of, 27, Doshomachi 2-chome, Higashi-ku, Osaka, Japan, 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 novel antibiotic B-5050 and to the production thereof.

We have found that a group of new antibiotic substances is produced by a micro-organism belonging to the genus *Streptomyces* and is accumulated in the culture broth, and that the respective members or components of the new antibiotics are closely related to each other in their properties as well as in their chemical structures and that they can be recovered from the culture broth in a desired degree of purity, as individual members or as a mixture of two or more of them. The group of new antibiotics has been collectively named "Antibiotic B-5050".

It has also been found that there exist, as the active ingredients of the Antibiotic B-5050, those antibiotics which have been named as Antibiotics B-5050-A, B-5050-B, B-5050-C, B-5050-D, B-5050-E and B-5050-F. It is, therefore, to be noted that in the present specification, including the claims, any mixture of two or more of them are collectively referred to as simply "Antibiotic B-5050" unless otherwise noted.

We have sought to provide the new and useful antibiotic B-5050 as individual members or as mixtures of the members.

We have also sought to provide a method for producing the useful antibiotic B-5050 by means of microbial fermentation.

Furthermore, we have sought to provide

a means for isolation of the product, antibiotic B-5050, as individual members or as mixtures of the members.

The present invention provides a method for producing antibiotic B-5050 which comprises culturing *Streptomyces hygroscopicus* No. B-5050, represented by the strain deposited at the Institute for Fermentation, Osaka, Japan, under the accession number IFO-12995, or an antibiotic B-5050-producing mutant or variant thereof in a culture medium containing an assimilable carbon source and a digestible nitrogen source under aerobic conditions until antibiotic B-5050 is accumulated in the culture broth, and recovering the accumulated antibiotic B-5050 therefrom.

The microorganism *Streptomyces hygroscopicus* No. B-5050 was isolated by the present inventors from a soil sample of Chichibu, Saitama, Japan. The microbiological characteristics of this organism are shown below. In the following description, the colour names designated "Rdg." are based on Ridgway's "Color Standards and Color Nomenclature", and the observations are on cultivations at 28°C for 14 days unless otherwise noted. The abbreviations "G", "R", "A" and "SP" mean "growth", "reverse", "aerial mycelium" and "soluble pigment", respectively.

Morphological Characteristics:

Spore bearing hyphae are looped or spiral. Each spore is ellipsoidal or cylindrical, the size being about 0.7 to 1.7 microns by about 0.9 to 1.4 microns, with a smooth surface.

Cultural Characteristics:

- (1) Czapek's agar:
G.: good, colourless to pale yellowish brown.

[Price 25p]

- 5 A.: good, velvety, white to Pale Olive Gray (Rdg. LI, 23¹¹¹¹-f) or Light Olive Gray (Rdg. LI, 23¹¹¹¹-d), with black patches.
R.: Smoke Gray (Rdg. XLVI, 21¹¹¹¹-d).
SP: none.
- (2) *Glucose Czapek's agar*:
G.: moderate, colourless.
10 A.: moderate, white to Pale Drab Gray (Rdg. XLVI, 17¹¹¹¹-f).
R.: colourless to Pale Drab Gray (Rdg. XLVI, 17¹¹¹¹-f).
15 SP: Pale Vinaceous-Fawn (Rdg. XL, 13¹¹¹-f).
- (3) *Glycerin Czapek's agar*:
G.: moderate, colourless.
20 A.: moderate, powdery, white to Pale Drab Gray (Rdg. XLVI, 17¹¹¹¹-f).
R.: Cream colour (Rdg. XVI, 19¹-f).
SP: none.
- (4) *Glucose asparagine agar*:
25 G.: moderate, colourless to pale yellow.
A.: moderate, white to greyish brown, with black patches.
30 R.: Cream Color (Rdg. XVI, 19¹-f) to Smoke Gray (Rdg. XLVI, 21¹¹¹¹-d).
SP: none or pale yellowish brown.
- (5) *Nutrient agar*:
35 G.: moderate, spreading, colourless to faint yellow.
A.: moderate, white.
R.: Cream Color (Rdg. XVI, 19¹-f).
SP: faint yellow.
- (6) *Nutrient broth*:
40 G.: poor to moderate, colourless, sink to the bottom.
A.: none.
SP: none.
- (7) *Glycerin nutrient agar*:
45 G.: good, wrinkled, colourless to faint yellow.
A.: good, white.
R.: Cream Buff (Rdg. XXX, 19¹¹-f).
50 SP: yellow to golden yellow.
- (8) *Glucose nutrient agar*:
55 G.: good, wrinkled, colourless to faint yellow.
A.: good, white to Pallid Mouse Gray (Rdg. LI, 15¹¹¹¹¹-f).
R.: Sorghum Brown (Rdg. XXXIX, 19¹¹¹-j).
SP: Fawn Color (Rdg. XL, 13¹¹¹).
- (9) *Starch agar*:
G.: poor, colourless. 60
A.: none or poor, white.
R.: pale yellow.
SP: none.
- (10) *Yeast extract agar*:
G.: good, wrinkled, colourless to faint yellow. 65
A.: good, white.
R.: Cream Buff (Rdg. XXX, 19¹¹-d).
70 SP: yellow to golden yellow.
- (11) *Egg*: (cultivated at 37°C)
G.: moderate, spreading.
A.: moderate, powdery white.
SP: none.
- (12) *potato plug*: 75
G.: good, wrinkled, raised, pale brown.
A.: poor, white to Pallid Mouse Gray (Rdg. LI, 15¹¹¹¹¹-f).
80 SP: none.
- (13) *Carrot plug*:
G.: good, wrinkled.
A.: moderate, white.
SP: none.
- (14) *Litmus milk*: (cultivated at 37°C) 85
G.: ring formed, later sinks to the bottom.
A.: none.
Peptonisation without coagulation.
- (15) *Löffler's serum*: (cultivated at 37°C) 90
G.: moderate, glistening, colourless.
A.: none or poor, white.
SP: none.
No liquefaction. 95
- (16) *Gelatin stab*: (cultivated at 24°C for 25 days)
G.: poor, restricted, pale yellow.
A.: poor, white.
No liquefaction. 100
- (17) *Cellulose*:
No growth.
- (18) *Calcium malate agar*:
G.: moderate, colourless.
A.: moderate, powdery, white. 105
R.: Buff Pink (Rdg. XXVIII, 11¹¹-d).
SP: Vinaceous - Cinnamon (Rdg. XXIX, 13¹¹-b).
- (19) *Tyrosine agar*: 110
G.: moderate, colourless.
A.: poor, white to Light Drab (Rdg. XLVI, 17¹¹¹¹-b).

R.: Drab Gray (Rdg. XLVI, 17¹¹¹¹-d) to Light Drab (Rdg. XLVI, 17¹¹¹¹-b).

SP: none.

- 5 (20) *Peptone agar*:
G.: poor, colourless.
A.: poor, white.
R.: colourless.
SP: none.

- 10 (21) *Nitrate reduction*:
Positive on Czapek's broth and on peptone medium.

- 15 (22) *Starch hydrolysis*:
Growth zone: 8 to 10 mm.
Enzymatic zone: 27 to 30 mm.

Thus, when this strain is cultured on a synthetic medium, vegetative mycelia are

colourless or pale yellow to pale brown, and soluble pigment is not formed or, when formed, it is Pale Vinaceous-Fawn (Rdg. XL, 13¹¹¹¹-f) or pale purple to pale yellowish brown. Aerial mycelia are firstly white, later coloured Pale Olive Gray (Rdg. LI, 23¹¹¹¹-d) to Pale Drab Gray (Rdg. XLVI, 17¹¹¹¹-f), or in some cases turn to black. When cultured on a protein-containing medium, this strain shows colourless or yellow to Fawn Color (Rdg. XL, 13¹¹¹¹), but does not form melanoid pigment, implying that this strain is a non-chromogenic type.

Utilisation of carbon sources:

Observations according to the method of Pridham and Gottlieb (Journal of Bacteriology, Vol. 59, p. 107 (1948)) on a culture incubated at 28°C for 10 days are shown in Table 1.

TABLE 1

	Carbon Source	Growth	Carbon Source	Growth
40	Erythritol	±	Maltose	+++
85	Adonitol	+++	Sucrose	+++
	D-Sorbitol	+++	Lactose	++
90	i-Inositol	++	Raffinose	+++
	D-Mannitol	+++	Trehalose	+++
45	Dulcitol	±	Salicin	—
95	D-Xylose	+	Esculin	—
	L-Arabinose	+	Inulin	± or +
	L-Sorbose	±	D-Mannose	+++
100	D-Galactose	+++	Starch	+++
50	D-Glucose	+++	Glycerin	+++
	D-Fructose	+++	Na-Acetate	+
105	Rhamnose	±	Na-Succinate	++
	Melibiose	+++	None (control)	—

Key to Table 1:

- 55
+++ : Abundant growth
++ : Good growth
+ : Moderate growth
± : Poor growth
— : No growth

Of course, as is well known, the microbiological characteristics of the organisms belonging to the family *Actinomycetaceae*, particularly of the genus *Streptomyces*, are not always stable and are susceptible to mutations, regardless of whether the mutation is caused spontaneously or artificially, for example with X-rays, ultraviolet rays, or by the action of chemical mutagenic compounds such as nitrogen mustard, nitroso-guanidine or heavy metal salts. *Streptomyces hygroscopicus* No. B-5050 is not an exception, and may give mutants which exhibit a somewhat different appearance from that of the parent strain, e.g. in cultural observations. Any such mutant can be employed in the method of present invention so long as it produces antibiotic B-5050.

The culture medium used in the method according to the invention must contain at least one assimilable carbon source and at least one digestible nitrogen source and optionally other nutrients. The culture medium can be liquid or solid, but a liquid medium is more convenient. In the medium are incorporated one or more assimilable carbon sources such as glucose, sucrose, maltose, dextrin, glycerin and starch syrup, and one or more digestible nitrogen sources such as peptone, soybean meal, cornsteep liquor, meat extracts, rice bran, wheat bran, urea and various ammonium salts (e.g. NH_4Cl , NH_4NO_3 , $(\text{NH}_4)_2\text{SO}_4$), as well as small amounts of inorganic salts which are conventionally used for cultivating microorganisms, such as sodium chloride, phosphates or particularly metal salts of calcium, magnesium, zinc, manganese or iron. In addition, growth promoting material may suitably be added, and an antifoaming agent such as fat, oil or mineral oil may be added as conditions demand.

The antibiotic B-5050-producing strain often produces other antibiotic substances together with antibiotic B-5050 under the conventional conditions. In order to produce antibiotic B-5050 advantageously, it is recommended to select a particular culture medium. Thus, for example a preferable culture medium contains 3 to 5% of the carbon source and 1 to 2% of the organic nitrogen source.

The method according to the invention can produce a single antibiotic B-5050 or a mixture of at least two of the antibiotics: B-5050-A, B-5050-B, B-5050-C, B-5050-D, B-5050-E and B-5050-F. In particular, the antibiotic mixture may be a mixture of antibiotics B-5050-A and B-5050-B.

The cultivation may be carried out by stationary culture but in general a submerged culture using aerated agitation is more advantageous. In the latter case, the pH of the culture medium is prefer-

ably about neutral, i.e. pH 6 to 8, more preferably 6.8 to 7.4, and the cultivation temperature lies in the range of from 20° to 43°, particularly in the range of 23° to 32°C.

The fermentation broth thus produced contains the components of antibiotic B-5050. To isolate this group of antibiotics, use may be made of conventional means for recovering the metabolites of a microorganism from its fermentation broth. Thus, by taking advantage of the fact that these antibiotics consists of weakly basic, fat-soluble substances, they may be separated by techniques utilising these properties.

Since antibiotic B-5050 is accumulated predominantly in the liquid phase of the fermentation broth, the broth may first be filtered to eliminate the mycelium, and the active ingredients may then be extracted from the filtered broth at a weakly basic pH with a water-immiscible organic solvent. For example, the filtered broth is adjusted to a pH of 7 to 10, and is extracted with an organic solvent which is immiscible, or not completely miscible, with water such as a lower fatty acid ester (e.g. ethyl acetate and *n*-butyl acetate), a halogenated aliphatic hydrocarbon (e.g. chloroform and ethylene chloride), a ketone (e.g. methyl ethyl ketone and methyl isobutyl ketone), an aromatic hydrocarbon (e.g. benzene and toluene), an alcohol (e.g. *n*-butanol), or a mixture of two or more of these solvents.

The portion of the antibiotics contained in the mycelium can be extracted therefrom, after the filtration of the broth, with a water-miscible organic solvent, such as acetone or methanol or a dilute aqueous acid solution. From the organic solvent extracts, the active ingredients are recovered by filtration, concentration *in vacuo* and extracted with a water immiscible organic solvent just described. From the aqueous acid solution, they can be recovered by filtration, adjusting to a neutral or weakly basic pH and extraction with the water-immiscible organic solvent.

The active ingredients in the fermentation may be adsorbed on an adsorbent such as activated charcoal and white carbon (or colloidal silica). The adsorbed active ingredients may then be eluted with a suitable developer or eluant, such as an aqueous alcohol (e.g. aqueous methanol and aqueous ethanol), aqueous acetone, a mixture of such aqueous solution plus an acid and an organic solvent (e.g. ethyl acetate and chloroform).

Alternatively, by taking advantage of the basic property of antibiotic B-5050, the active ingredients can be adsorbed on a cation exchange resin such as "Amberlite (Registered Trade Mark) IRC-50", "Amberlite IR-120" (both produced by Rohm

& Haas Co., U.S.A.) or "Dowex (Registered Trade Mark) 50" (produced by Dow Chemical, U.S.A.). The active ingredients adsorbed on such a resin can then be eluted with a suitable eluant such as a dilute aqueous acid solution, a dilute aqueous basic solution, an aqueous acid methanol solution, an aqueous basic methanol solution, an aqueous acid acetone solution or an aqueous basic acetone solution.

The organic extract thus obtained is further transferred into an aqueous acid solution, preferably after washing with water. The acid solution for this purpose may be a dilute aqueous acid solution or an acid buffer solution. The aqueous extract thus obtained is then adjusted to a neutral or weakly basic pH, and may again be extracted with a water-immiscible organic solvent as just detailed.

When the content of the active ingredients in the organic solvent extract thus obtained is relatively high, antibiotic B-5050 may crystallise solely by concentrating the extract *in vacuo*. When this is not the case, a nonpolar organic solvent such as petroleum ether or *n*-hexane is added to the concentrate, whereupon crude powdery antibiotic B-5050 separates. The powdery product can be crystallised from an organic solvent such as benzene, diethyl ether, ethyl acetate, or a mixture of ethyl acetate-*n*-hexane, acetone-*n*-hexane, acetone:water or ethanol:water.

When the crude powder includes some coloured impurities, they can be removed by the use of activated charcoal.

In a particular case where antibiotic B-5050 cannot be crystallised even by such a treatment, further purification may be applied thereto by means of such adsorbents as silica gel or alumina. The adsorbed ingredients can be eluted with a fatty acid ester (e.g. methyl acetate, ethyl acetate or *n*-butyl acetate, or a mixed solvent (e.g. benzene:acetone, benzene:ethyl acetate, benzene-methanol or chloroform:methanol).

Antibiotic B-5050 mixture, as well as its respective components, is a basic substance, and can form salts with a water-soluble acid such as hydrochloric acid, sulphuric acid, tartaric acid or nicotinic acid.

The following are to show the general properties of antibiotic B-5050 mixture, but it is to be noted that the specific values were obtained by the measurement on a sample of antibiotic B-5050 mixture produced in the manner described in Example 7, and there may naturally be some deviations from the specific values in cases of other samples of antibiotic B-5050.

(1) Melting point: 137° to 141°C.

(2) Elementary analysis:

Recrystallised from benzene:

C: 58.14 ± 0.5; H: 7.88 ± 0.3;

N: 1.73 ± 0.3; O: 31.25 ± 1.0.

Recrystallised from ethyl acetate:

C: 57.83 ± 0.5; H: 8.14 ± 0.3;

N: 1.33 ± 0.3; O: 31.70 ± 1.0.

(3) Molecular weight measured by vapour pressure osmosis:

880 ± 90 (in ethyl acetate)

(4) Specific rotation:

(α)²⁴_D: -776.4° ± 8° (c = 1, ethanol)

(5) Colour reactions:

Dragendorff reaction:

positive

Erythromycin test:

positive exhibiting reddish purple, the chloroform layer being pale blue.

Carbomycin test:

negative

(6) Solubilities:

Easily soluble in methanol, ethanol, acetone, ethyl acetate, methyl ethyl ketone, chloroform, and an aqueous acid solution;

Soluble in benzene and diethyl ether; Hardly soluble in *n*-hexane and neutral water.

(7) Ultraviolet absorption:

No significant maximum in methanol.

(8) Infra-red absorption:

As shown in Figure 1 (KBr disc), the main absorption bands (cm⁻¹) being as follows:

3448(s), 2924(m), 1736(s), 1453(m), 1001376(m), 1295(m), 1239(m), 1167(s), 1081(s), 1050(s), 968(m), 912(m), 861(w), 840(w).

Remark:

The parenthesised abbreviations "vs", "s", "m", "sh" and "w" mean "very strong", "strong", "medium", "shoulder" and "weak", respectively, and the same applies to the following description on infra-red spectra.

As just mentioned, the instant antibiotic consists of several components, and there have so far been found at least six components, which are closely related to each other in their chemical and biological properties. This fact is easily confirmed by subjecting the antibiotic mixture to paper chromatography or thin layer chromatography. The following are the examples of such identification of the respective ingredients.

(1) Paper Chromatography:

Paper:

Toyoroshi No. 50, impregnated with a 2% liquid paraffin solution in ligroin.

Developer:

M/15-phosphate buffer solution of pH 8.0 saturated with *n*-butyl acetate.

Detection:

Bioautography employing *Bacillus subtilis* as the test organism.

Rf values:

B-5050-A	$= 0.32 \pm 0.05$
B-5050-B	$= 0.38 \pm 0.05$
B-5050-C	$= 0.66 \pm 0.05$
B-5050-D	$= 0.72 \pm 0.05$
B-5050-E	$= 0.74 \pm 0.05$
B-5050-F	$= 0.82 \pm 0.05$

(b) silica gel ("Kieselgel G" manufactured by Merck A.G. Darmstadt, W. Germany)

(c) the same as (b)

Developer:

- (a) benzene:acetone (3:2 by volume)
 (b) benzene:acetone (3:2 by volume)
 (c) benzene:methanol (10:1 by volume), but the development was repeated three times.

Detection:

- (a) concentrated H_2SO_4 sprayed.
 (b) and (c) treated as follows:

- 1) 5% phosphomolybdate solution in ethanol was sprayed and dried at $110^\circ C$ for 5 minutes;
- 2) 5% cerium sulphate solution in NH_4SO_4 was sprayed and dried at $110^\circ C$ for 5 minutes;
- 3) 10% aqueous H_2SO_4 solution was sprayed and dried at $110^\circ C$ for 5 minutes; and
- 4) Conc. H_2SO_4 or 5% I_2 solution in chloroform was sprayed.

(2) Thin Layer Chromatography:

Thin layer:

- (a) silica gel ("Spotfilm f" manufactured by Tokyo Kasei K.K., Japan) preheated at $105^\circ C$ for 1 hour.

Rf Values:

	(a)	(b)	(c)
B-5050-A	0.48 ± 0.05	0.68 ± 0.05	0.71 ± 0.05
B-5050-B	0.42 ± 0.05	0.63 ± 0.05	0.66 ± 0.05
B-5050-C	0.37 ± 0.05	0.57 ± 0.05	0.61 ± 0.05
B-5050-D	0.32 ± 0.05	0.53 ± 0.05	0.55 ± 0.05
B-5050-E	0.30 ± 0.05	0.50 ± 0.05	0.52 ± 0.05
B-5050-F	0.27 ± 0.05	0.43 ± 0.05	0.48 ± 0.05

In order to separate or isolate these ingredients in an industrial scale, it is recommended to employ techniques of adsorption chromatography, partition chromatography or countercurrent distribution.

A suitable adsorbent for the adsorption chromatography is silica gel or alumina. When silica gel is employed, for example, the elution may be made with a mixture of a nonpolar organic solvent and a polar organic solvent, whereby the respective ingredients are fractionally eluted in the order of affinity to the nonpolar solvent, i.e. antibiotics B-5050-A, -B -C, -D, -E and -F. A mixed solvent suitable as the eluant may be exemplified by benzene-ethyl acetate, benzene-acetone, *n*-hexane-methanol and chloroform-methanol.

When using methods based on the difference in distribution between two liquid phases, the combination of a lipophilic organic solvent (e.g. ethyl acetate) and an aqueous buffer solution is recommended. By

using a buffer of pH 5.5, antibiotic B-5050-D, -E and -F can be transferred into the buffer, while B-5050-A, -B and -C remain in the organic solvent phase. From the latter solvent phase, B-5050-C can be extracted with a buffer of pH 4.9. With a buffer of pH 4.4, B-5050-B and -C can be extracted from the organic layer mentioned above, while B-5050-A remains in the organic solvent phase. In this manner, respective ingredients can finally be isolated.

Partition chromatography is another good means by separating the ingredients. There may be used, as the carrier of the stationary phase, such conventionally used materials as cellulose powder or diatomaceous earth (e.g. "Celite" (Registered Trade Mark) or "Hyflo (Registered Trade Mark) Super Cel", distributed by Johns Manville Sales Corp., U.S.A.).

The respective characteristics and properties of the active ingredients of antibiotic

B-5050 are summarised in the following.

- (1) *Melting point* (with decomposition):
Measured for samples recrystallised
from a mixture of acetone and *n*-
hexane (1:3 by volume)

B-5050-A: 129° to 132°C
B-5050-B: 134° to 136°C
B-5050-C: 135° to 138°C
B-5050-D: 143° to 146°C
B-5050-E: 144° to 149°C
B-5050-F: 149° to 154°C

(2) *Elementary analysis*:

B-5050-A: C 59.40 ± 0.5; H 8.15 ± 0.3;
(C₄₃H₇₁NO₁₆) N 1.40 ± 0.3; O 31.33 ± 1.0

B-5050-B: C 58.67 ± 1.0; H 8.02 ± 0.5;
(C₄₂H₆₉NO₁₆) N 1.86 ± 0.5; O 31.18 ± 1.0

B-5050-C: C 57.93 ± 1.0; H 8.18 ± 0.5;
(C₄₁H₆₇NO₁₆) N 1.69 ± 0.5; O 31.86 ± 1.0

B-5050-D: C 57.48 ± 1.0; H 8.17 ± 0.5;
(C₄₀H₆₅NO₁₆) N 1.64 ± 0.5; O 32.90 ± 1.0

B-5050-E: C 57.34 ± 1.0; H 8.25 ± 0.5;
(C₄₀H₆₅NO₁₆) N 1.34 ± 0.5; O 32.48 ± 1.0

B-5050-F: C 58.59 ± 0.5; H 7.82 ± 0.3;
(C₃₉H₆₃NO₁₆) N 1.62 ± 0.3; O 32.38 ± 1.0

(3) *Molecular weight*:

All ingredients have a molecular weight of from 800 to 900, measured by vapour
pressure osmosis method in ethyl acetate.

(4) *Specific rotation*:

B-5050-A: α^{23}_D : -72.3° ± 7° (c = 1, ethanol)
B-5050-B: α^{23}_D : -71.9° ± 7° (c = 1, ethanol)
B-5050-C: α^{23}_D : -76.0° ± 8° (c = 1, ethanol)
B-5050-D: α^{23}_D : -76.2° ± 8° (c = 1, ethanol)
B-5050-E: α^{23}_D : -73.6° ± 7° (c = 1, ethanol)
B-5050-F: α^{23}_D : -77.7° ± 8° (c = 1, ethanol)

(5) *Colour reactions*:

All ingredients are positive to Dragen-
dorff reaction and Erythromycin test,
but negative to Carbomycin test.

(6) *Solubilities*:

All ingredients are soluble in methanol,
ethanol, acetone, methyl ethyl ketone,
ethyl acetate, benzene, diethyl ether,
chloroform and an aqueous acid solu-
tion, but hardly soluble in *n*-hexane
and neutral water.

(7) *Ultraviolet absorption*:

All ingredients exhibit no significant
maximum absorption.

(8) *Infra-red absorptions* (KBr disc):

B-5050-A:
As shown in Figure 2, the main
absorption bands in wave number
(cm⁻¹) being as follows:
3448(m), 2941(m), 1739(s), 1458(w),
1374(m), 1295(m), 1188(s), 1167(s),
1120(m), 1086(s), 1053(s), 1033(s),
971(m), 917(w), 862(m).

B-5050-B:

As shown in Figure 3, the main
absorption bands in wave number
(cm⁻¹) being as follows:
3500(s), 2990(s), 2960(s), 1740(vs),
1454(m), 1370(m), 1296(m), 1233(s),
1188(sh), 1166(vs), 1120(s), 1080(s),
1052(vs), 1025(vs), 968(m), 916(w),
858(w), 842(w).

B-5050-C:

As shown in Figure 4, the main
absorption bands in wave number
(cm⁻¹) being as follows:
3460(s), 2980(s), 2940(s), 1735(vs),
1455(m), 1375(s), 1357(s), 1295(s),
1270(s), 1240(m), 1183(sh), 1162(vs),
1080(vs), 1050(vs), 1028(sh), 1018(sh),
972(m), 910(m), 862(w), 840(w).

B-5050-D:

As shown in Figure 5, significant
absorption bands in wave number
(cm⁻¹) being as follows:
3440(s), 2946(m), 1735(vs), 1450(m),
1373(m), 1357(sh), 1296(m), 1274(m),
1237(s), 1163(s), 1127(m), 1083(s),
1047(vs), 1031(sh), 1012(sh), 972(m).

913(m), 862(w), 843(w).

B-5050-E:

As shown in Figure 6, significant absorption bands in wave number (cm^{-1}) being as follows:

3430(s), 2920(m), 1732(vs), 1450(m), 1373(m), 1354(m), 1296(m), 1239(s), 1163(s), 1127(m), 1084(s), 1048(vs), 1032(sh), 1011(sh), 970(m), 914(m), 862(w), 840(w).

B-5050-F:

As shown in Figure 7, significant absorption bands in wave number (cm^{-1}) being as follows:

3425(s), 2994(m), 1745(s), 1730(s).

1460(w), 1379(m), 1326(m), 1300(m), 1242(s), 1166(s), 1133(m), 1089(m), 1053(s), 1031(m), 973(w), 919(w), 864(w).

Antimicrobial spectra of the antibiotic B-5050 mixture thus recovered and its respective ingredients (i.e. B-5050-A, -B, -C, -D, -E and -F) are shown in Table 4. For the measurements, common bacteria were cultured on a nutrient agar medium, while *Mycobacterium* species were cultured on a glycerin nutrient agar medium. Yeasts or other fungi were cultured on a glucose nutrient agar medium.

TABLE 4
Minimum Inhibitory Concentrations (mcg/ml)

Test Organism	Antibiotic B-5050						
	Mixture	A	B	C	D	E	F
<i>Bacillus subtilis</i> ...	0.5	0.5	0.2	0.5	1	2	2
<i>Bacillus cereus</i> ...	1	0.5	0.5	1	2	5	5
<i>Bacillus brevis</i> ...	0.5	0.2	0.2	0.5	5	10	10
<i>Bacillus megaterium</i> ...	1	0.5	0.5	0.5	1	2	2
<i>Staphylococcus aureus</i> ...	1	0.5	0.5	1	2	5	5
<i>Staphylococcus aureus</i> (*1) ...	50	20	20	50	>100	>100	>100
<i>Staphylococcus aureus</i> (*2) ...	50	20	20	50	>100	>100	>100
<i>Sarcina lutea</i> ...	0.2	0.1	0.1	0.2	0.5	0.5	0.5
<i>Micrococcus flavus</i> ...	0.2	0.1	0.1	0.2	0.5	0.5	0.5
<i>Serratia marcescens</i> ...	>100	>100	>100	>100	>100	>100	>100
<i>Escherichia coli</i> ...	>100	>100	>100	>100	>100	>100	>100
<i>Proteus vulgaris</i> ...	>100	>100	>100	>100	>100	>100	>100
<i>Pseudomonas aeruginosa</i> ...	>100	>100	>100	>100	>100	>100	>100
<i>Mycobacterium avium</i> ...	50	50	100	>100	>100	>100	>100

TABLE 4 (continued)
Minimum Inhibitory Concentrations (mcg/ml)

Test Organism	Mixture	Antibiotic B-5050					
		A	B	C	D	E	F
5 <i>Mycobacterium avium</i> (*3) ...	50	50	100	>100	>100	>100	>100
<i>Mycobacterium avium</i> (*4) ...	50	50	100	>100	>100	>100	>100
10 <i>Mycobacterium smegmatis</i> ...	50	50	100	>100	>100	>100	>100
<i>Mycobacterium phlei</i> ...	50	50	100	>100	>100	>100	>100
<i>Mycobacterium</i> 607 ...	50	50	100	>100	>100	>100	>100
15 <i>Candida albicans</i> ...	>100	—	—	—	—	—	—
<i>Saccharomyces cerevisiae</i> ...	>100	—	—	—	—	—	—
20 <i>Penicillium chrysogenum</i> ...	>100	—	—	—	—	—	—
<i>Aspergillus niger</i> ...	>100	—	—	—	—	—	—
<i>Trichophyton mentagrophytes</i> ...	100	—	—	—	—	—	—

25 Key to Table 4:

(*1): Strain resistant to Oleandomycin and Erythromycin.

(*2): Strain resistant to Streptomycin, Chloramphenicol, Tetracycline, Oleandomycin and Erythromycin.

(*3): Strain resistant to Streptomycin.

30 (*4): Strain resistant to Dextromycin.

—: Not tested.

35 Antibiotic B-5050 is concluded as belonging to the so-called "macrolide antibiotics" in view of its properties such as colour reactions (e.g. Erythromycin Test), solubilities (e.g. lipophilic and basic), infra-red spectra (e.g. the values of V_{max} or V_{min}), and antimicrobial spectra (e.g. differences in the minimum inhibitory concentrations between an Erythromycin- and Oleandomycin-resistant strain and a susceptible strain of *Staphylococcus aureus*).

40 Among the macrolide antibiotics, antibiotic B-5050 differentiates itself from those which show maximal absorption at a wavelength of 225, 230, 240 or 280 to 290 millimicrons. Erythromycin and Oleandomycin have relatively weak maximal absorption in a range of 280 to 290 millimicrons, but
50 they differ from antibiotic B-5050 in their infra-red absorption spectra, molecular

weights and melting points, for example.

It is known that the macrolide antibiotics Carbomycin, Relomycin and Tylosin may be produced by *Streptomyces hygroscopicus*. However, they have maximum absorptions at the wavelength of 238, 282 and 289 millimicrons, respectively, and hence clearly differ from antibiotic B-5050. Thus, it is concluded that antibiotic B-5050 consists of a new group of antibiotics, each of which is also novel.

Antibiotic B-5050, regardless of whether it is a mixture of active ingredients or an isolated single active ingredient as just described, has a strong antimicrobial activity, particularly against Grampositive bacteria, and is characterised by an unusually low toxicity against warm-blooded animals including mankind. For example, the mean effective dose (ED_{50}) of antibiotic B-5050

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mixture in mice infected with *Staphylococcus aureus* was 640 mg/kg when administered orally. Nevertheless, the mean lethal dose (LD₅₀) of the same in mice could not be determined due to its low toxicity and can only be expressed as "higher than 10,000 mg/kg" when administered orally. Furthermore, rats were daily given 1 g/kg. of antibiotic B-5050 mixture orally for 1 month. During the observation period, no appreciable difference from a normal control group was noted, and the subsequently conducted autopsy showed no substantial change in the organs and viscera of the tested rats.

Staphylococci are pyogenic or pus-forming bacteria. They tend to produce circumscribed lesions, e.g. in the form of abscesses, which often occur in the skin. *Staphylococci* are the cause of furuncles and of carbuncles and other common wound infections. The new products of the present invention are useful in topical preparations for the treatment of this type of infection in mammals (dogs, cats and humans). Thus, a useful preparation for topical application to an infection due to *Staphylococcus aureus* is as follows:

Into 1 gram of wool fat is uniformly incorporated one of the following itemised components:

- (1) 20 mg of antibiotic B-5050-A,
- (2) 20 mg of antibiotic B-5050-B,
- (3) 20 mg of antibiotic B-5050-C,
- (4) 50 mg of antibiotic B-5050-D,
- (5) 50 mg of antibiotic B-5050-E,
- (6) 50 mg of antibiotic B-5050-F,
- (7) 20 mg of the crystalline antibiotic B-5050 mixture as obtained in Example 1, or
- (8) 20 mg of Crystal II as obtained in Example 2, and the mixture is then admixed uniformly with sufficient white petrolatum to make 10 grams of ointment.

This is topically applied in an amount sufficient to cover the wound being treated, with gentle rubbing in, the application being made at least once daily and being repeated several times daily, if necessary or desired.

Due to the disclosed bactericidal and bacteriostatic properties of the new products of the invention, they are useful e.g. to disinfect hospital apparatus, which are generally exposed to pathogenic Gram-positive bacteria of the type which are sensitive to such products, as aforementioned. Disinfection is effected by application or spraying of a solution (e.g. methanolic or ethanolic) containing one of the following itemised components:

- (1) 20 mcg/ml of antibiotic B-5050-A,
- (2) 20 mcg/ml of antibiotic B-5050-B,
- (3) 20 mcg/ml of antibiotic B-5050-C,
- (4) 50 mcg/ml of antibiotic B-5050-D,
- (5) 50 mcg/ml of antimiotic B-5050-E,
- (6) 50 mcg/ml of antibiotic B-5050-F,
- (7) 20 mcg/ml of the crystalline antibiotic B-5050 mixture as obtained in Example 1, or
- (8) 20 mcg/ml of Crystal II as obtained in Example 2.

The following examples further illustrate the invention. In these examples, as well as the foregoing, the abbreviations "ml", "l", "mm", "cm", "mcg", "mg", "g", "kg", "rpm", "N" and "M" mean "millilitre(s)", "litres(s)", "millimetre(s)", "centimetre(s)", "microgram(s)", "milligram(s)", "gram(s)", "kilogram(s)", "revolutions (or reciprocations as the case may be) per minute", "normal concentration" and "mole concentration", respectively. As for the composition of the culture media, percentages are on a weight per volume basis, i.e. grams per 100 millilitres. All percentages in other respects are on the weight basis unless otherwise stated.

EXAMPLE 1

500 ml each of a preseed culture medium (pH 7.0) consisting of glucose (2.0%), soluble starch (3.0%), soybean flour (1.0%), cornsteep liquor (1.0%), "Polypepton" trade name of a casein hydrolysate, produced by Daigo Nutritive Chemicals, Ltd. Osaka, Japan) (0.5%), sodium chloride (0.3%), calcium carbonate (0.3%) and water is charged in 2000 ml-Sakaguchi flasks, and sterilised. The sterilised medium is inoculated with a loopful of *Streptomyces hygroscopicus* (IFO-12995) from an agar slant culture which has been cultivated for at least 4 days, and incubated at 28°C on a reciprocal shaker of 115 rpm with 10 cm stroke for 48 hours. The resulting broth in an amount of 1000 ml is used as the preseed culture.

The preseed culture is inoculated into 100 litres of a culture medium (pH 7.0) charged and sterilised in a 200 litre stainless steel tank, the medium consisting of glucose (3.0%), cornsteep liquor (0.5%), defatted soybean meal (1.0%), sodium chloride (0.5%), magnesium sulphate (0.05%), calcium carbonate (0.3%) and water. Incubation is conducted at 28°C with aeration of 100 litres per minute and agitation of 200 rpm for 24 hours.

100 litres of the resulting seed culture is transferred into 1000 litres of a sterilised main culture medium of the same composition as that of the culture medium for seed culture, charged in a 2000 litre stainless steel tank. Incubation is continued at

28°C with aeration of 1000 litres per minute and agitation by impeller of 120 rpm for 66 hours. During the incubation, "Actocol" (trade name of a polyoxypropylene triol mixture of OH number 56, sold by Takeda Chemical Industries, Ltd., Osaka, Japan) is added as an antifoaming agent at a concentration of about 0.05% relative to the whole medium. The culture broth thus obtained exhibits an antimicrobial potency of 350 units in terms of dilution units against *Bacillus subtilis* as the assay micro-organism.

The broth is filtered. The filtered broth (950 l) is adjusted to pH 8.5 with a 2N-aqueous NaOH solution, and extracted with ethyl acetate (300 l). The ethyl acetate layer is separated, washed with water and extracted twice with 70 l each time with 0.005N HCl. The aqueous extracts are combined, adjusted to pH 8.5 with dilute aqueous ammonia and again extracted twice with 45 l each time with ethyl acetate. The extracts are washed with water, dehydrated and concentrated *in vacuo* to 50 ml. To the concentrate is added 750 ml of *n*-hexane to yield 95 g of crude powdery antibiotic B-5050 mixture.

The powdery product is dissolved in 450 ml of benzene with warming. After the addition of 2 g of activated carbon to the solution while warming, the mixture is filtered, and the filtrate is allowed to cool on standing to separate 19.0 g of antibiotic B-5050 mixture as colourless needles.

10 g of the crystalline antibiotic B-5050 mixture is dissolved in 50 ml of ethyl acetate and is subjected to a column chromatography on silica gel (450 g, produced by Merck A.G., Germany, the granular size ranging from 0.05 to 0.2 mm). The charged materials are developed and eluted with a mixture of ethyl acetate and benzene (2:1 by volume). The first 600 ml of the eluate showing antimicrobial activity is concentrated to dryness and the residue is dissolved in hot benzene. On standing, the solution separates 1 g of colourless crystals which consist mainly of antibiotic B-5050-A.

After the column has been eluted with 4 l of the ethyl acetate-benzene mixture, it is further eluted with 3 l of ethyl acetate to collect the eluates which are then concentrated *in vacuo* to leave a residue. The residue is dissolved in hot benzene. On standing, the solution separates 550 mg of colourless crystals which consist substantially of antibiotic B-5050-F.

500 mg of the crystals consisting substantially of antibiotic B-5050-A are dissolved in 1.5 ml of ethyl acetate, and again chromatographed on a column of silica gel (50 g, see above). The charged material is eluted with a mixture of ethyl acetate and benzene (1:1 by volume) to give fractions. Each

fraction is tested for the active ingredients contained, by means of thin layer chromatography on silica gel (sold by Merck A.G., Germany, under the name of "HF₂₅₄") with the use of a mixture of benzene and acetone (3:2 by volume) as the developer. The fractions which contain solely antibiotic B-5050-A as the active ingredient are collected, and concentrated *in vacuo* to leave a residue. On crystallisation of the residue from benzene, 88.5 mg of antibiotic B-5050-A is obtained as colourless prisms.

350 mg of the crystals consisting substantially of antibiotic B-5050-F are subjected to column chromatography on the silica gel (50 g) in the same manner as above. After the column has been eluted with 700 ml of ethyl acetate-benzene (2:1 by volume), it is further eluted with a mixture of ethyl acetate and benzene (3:1 by volume), followed by the tests by thin layer chromatography for the active ingredients on each fraction. The fractions which contain solely antibiotic B-5050-F as the active ingredient are collected, and concentrated *in vacuo* to leave a residue. The residue is crystallised to yield 75.6 mg of B-5050-F as colourless prisms.

EXAMPLE 2

In 2 litre-Sakaguchi flasks, 500 ml each of a sterilised preseed culture medium of the same composition as used in Example 1 is inoculated with *Streptomyces hygroscopicus* (IFO-12995), and the inoculated flask is incubated in the same manner as in Example 1 to prepare a preseed culture.

In a 500 litre stainless steel tank, there is prepared 200 l of a culture medium of the same composition as used in Example 1 for the seed culture. After being sterilised at 121°C for 20 minutes, the culture is inoculated with 2 l of the preseed culture thus obtained. Incubation is effected at 28°C with aeration of 200 l per minute and agitation of 200 rpm for 24 hours to prepare the seed culture for main fermentation.

200 l of the seed culture thus prepared is transferred into 4000 l of a main culture medium of the same composition as just described, charged in a 6000 l stainless steel tank, and the cultivation is conducted at 28°C with aeration of 2400 l per minute and agitation by impeller of 180 rpm for 48 hours. During the incubation, "Actocol" (see above) is used as an antifoaming agent.

The culture broth is filtered. The filtered broth (4000 l) is adjusted to pH 9 with 2N NaOH and extracted with 1330 l of ethyl acetate, followed by washing with water to obtain 1052 l of ethyl acetate extract. The ethyl acetate solution is concentrated *in vacuo* to 100 l. The concentrated solution is extracted twice with 50 l

each time of a M/1.5 aqueous KH_2PO_4 solution previously adjusted to pH 4.0 with phosphoric acid. The extracts are combined, adjusted to pH 8.5 with a dilute aqueous ammonia solution, and extracted with 50 l of ethyl acetate. The ethyl acetate solution is washed with water, dehydrated and concentrated *in vacuo* to leave a syrup. Addition of 3 l of *n*-hexane to the syrup gives 330 g of crude powder. 300 g of the powder is dissolved in 1.1 litres of benzene while warming, and the solution is allowed to cool on standing to give 117 g of crystals, which consist substantially of antibiotics B-5050-C, -D and -E (crystal I).

The ethyl acetate layer which was extracted with the phosphate solution is further extracted twice with 50 l each of N/200 H_2SO_4 , and the aqueous layers are combined, adjusted to pH 8 with a dilute aqueous ammonia solution and extracted three times with 30 l each of ethyl acetate. The ethyl acetate extracts are combined, washed with water, dehydrated and concentrated *in vacuo* to leave a syrup. Addition of 1.5 l of *n*-hexane to the syrup gives 160 g of a crude powder. 150 g of the powder is crystallised from hot benzene to give 72.5 g of crystals, which consist mainly of antibiotic B-5050-A and -B together with a small amount of antibiotic B-5050-C (Crystal II). On thin layer chromatography under the same conditions as 2(a) or 2(c) described above with regard to the antibiotic B-5050 mixture of the six ingredients, Crystal II gives two spots, i.e. at the R_f values of 0.48 and 0.42 under conditions (a) or the R_f values of 0.66 and 0.71 under conditions (c).

2.0 g of Crystal II is dissolved in 20 ml of ethyl acetate, followed by the addition of 14 ml of benzene. The solution is subjected to column chromatography on silica gel (produced by Merck A.G., Germany, the granular size ranging from 0.05 to 0.2 mm) which has been filled with an equi-volume mixture of benzene and ethyl acetate. The chromatogram is developed firstly with ethyl acetate-benzene (1:1 by volume) to collect 200 ml of the eluate, then with ethyl acetate-benzene (3:2 by volume) to give fractions exhibiting antimicrobial activity against *Bacillus subtilis*. The fractions of the same ingredient are respectively combined, concentrated and crystallised from benzene to give 435 mg of crystals of antibiotic B-5050-A and 185 mg of crystals of antibiotic B-5050-B.

10 g of Crystal I is dissolved in 50 ml of ethyl acetate, followed by the addition of 25 ml of benzene. The solution is chromatographed on silica gel (400 g) in the same manner as just explained. The column is eluted firstly with 3 l of ethyl acetate-benzene (1:1 by volume), then with ethyl

acetate-benzene (2:1 by volume). The eluate is fractionated in 500 ml portions. The fractions obtained by the 1:1 mixture contain antibiotics B-5050-A and -B. The first fraction obtained by the 2:1 mixture contains antibiotics B-5050-B and -C, the second fraction antibiotics B-5050-C and -D, the third fraction antibiotics B-5050-D and -E, and the last fraction antibiotics B-5050-E and -F, respectively.

2 g of the crystals obtained from the fraction containing antibiotics B-5050-B and -C is again dissolved in 15 ml of ethyl acetate, followed by the addition of 10 ml of benzene. The solution is subjected to column chromatography with 250 g of silica gel, and the column is eluted with ethyl acetate-benzene (3:2 by volume). The eluate is fractionated into 20 ml portions. The fractions of the same component are combined, concentrated and crystallised from benzene or a mixture of ethyl acetate and *n*-hexane to give 83 mg of crystals of antibiotic B-5050-B, 940 mg of crystals of antibiotic B-5050-C and 75 mg of crystals of antibiotic B-5050-D.

In the same way, 1.2 g of the fraction containing antibiotics B-5050-D and -E is subjected to column chromatography on silica gel, to give 600 mg of crystals of antibiotic B-5050-E.

The fraction obtained subsequent to the fraction containing antibiotics B-5050-B and -C contains antibiotic B-5050-C as the main ingredient. 770 mg of the resultant crude crystals are again dissolved in 5 ml of acetone, the solution is subjected to column chromatography with 100 g of silica gel in a similar way to the above, whereupon 220 mg of antibiotic B-5050-C is obtained as crystals.

EXAMPLE 3

4000 l of the filtered broth obtained by the main fermentation as described in Example 2 is adjusted to pH 8.0, and is extracted with one third of its volume of ethyl acetate. The ethyl acetate extract is concentrated to about 100 litres. The concentrate is washed with 50 l of water, and extracted three times with 50 l each time of an M/3 aqueous KH_2PO_4 solution previously adjusted to pH 3.0 with phosphoric acid. The aqueous extracts are combined, adjusted to a pH of 9 to 10 with an 8N aqueous ammonia solution, and extracted with 75 l of ethyl acetate. The ethyl acetate extract is washed three times with 25 l each time with water and then concentrated to about 1.5 litres. Addition of about 30 l of *n*-hexane to the concentrate gives 522 g of crude powder. Crystallisation of the powder from benzene gives 272 g of crystals of the antibiotic B-5050 mixture.

In the above procedures, the use of 0.2N aqueous acetic acid solution in place of the KH_2PO_4 solution gives substantially the same result.

- 5 19 g of the antibiotic B-5050 mixture thus obtained is dissolved in 75 ml of acetone. The solution is subjected to column chromatography with 800 g of silica gel. The column is eluted with 3000 ml of ethyl acetate-benzene (1:1 by volume) to give 7.25 g of crystals consisting substantially of antibiotics B-5050-A and -B. Then, the column is eluted with ethyl acetate-benzene (2:1 by volume). The first fraction 10 eluted with 1100 ml of the developer is similarly treated to give 2.22 g of crystals consisting mainly of antibiotics B-5050-B and -C. The fraction obtained by the subsequent 200 ml gives 0.90 g of crystals which consist mainly of antibiotic B-5050-C. From the further subsequent 300 ml is obtained 1.4 g of a mixture consisting mainly of antibiotics B-5050-C and -D. From the last 600 ml fraction is obtained 1.99 g of crystals consisting mainly of antibiotics B-5050-E and -D. The column is then eluted with 600 ml of ethyl acetate-benzene (3:1 by volume) to give 0.645 g of crystals consisting mainly of antibiotics B-5050-D and -E. From the last fraction eluted with 1600 ml of ethyl acetate is obtained 2.09 g of crystals consisting substantially of antibiotic B-5050-F.

- 35 The above obtained antibiotics B-5050-A and -B crystals (7.25 g) are dissolved in 40 ml of acetone, and subjected to column chromatography with 400 g of silica gel, followed by elution with ethyl acetate-benzene (1:1 by volume), whereby 1.5 g of crystals of antibiotic B-5050-A and 0.65 g of crystals of antibiotic B-5050-B are obtained.

- 45 The afore-obtained antibiotics B-5050-B and -C crystals (2.22 g) are dissolved in 20 ml of acetone, and column chromatographed on 300 g of silica gel, followed by elution with benzene-acetone (3:1 by volume) to give 0.398 g of crystals of B-5050-B. Further elution with benzene-acetone (2:1 by volume) gives 0.269 g of crystals of antibiotic B-5050-C.

- 50 The crystals consisting mainly of antibiotic B-5050-C (0.90 g) are dissolved in 5 ml of acetone, and column chromatographed on 100 g of silica gel, followed by elution with ethyl acetate-benzene (2:1 by volume), to give 0.258 g of crystals of antibiotic B-5050-C.

- 60 The antibiotics B-5050-C and -D crystals (1.40 g) are dissolved in 10 ml of acetone, and chromatographed on a column packed with 150 g of silica gel, followed by elution with benzene-acetone (2:1 by volume), to give 0.596 g of antibiotic B-5050-C and 65 0.187 g of crystals of antibiotic B-5050-D.

The antibiotics B-5050-D and -E crystals (1.99 g) are dissolved in 30 ml of acetone, and column chromatographed on 300 g of silica gel, followed by elution with benzene-acetone (2:1 by volume), to give 0.304 g of crystals of antibiotic B-5050-D and 0.187 g of crystals of antibiotic B-5050-E.

The crystals consisting mainly of antibiotics B-5050-F (2.09 g) are dissolved in 15 ml of acetone, and column chromatographed on 250 g of silica gel, followed by elution with ethyl acetate-benzene (3:1 by volume), to give 0.585 g of crystals of antibiotic B-5050-F.

EXAMPLE 4

A mixture of 120 ml of *n*-hexane, 80 ml of ethylene dichloride, 30 ml of methanol and 6 ml of water is well agitated and then allowed to stand, whereby it forms two layers. The lower layer is separated from the upper layer. 1 mg of chlorphenol red is dissolved in 6 ml of the lower layer and the latter is dissolved in the former. To the solution are added 5 g. of "Celite" (trade name of processed diatomaceous earth distributed by Johns Manville Sales Corp., U.S.A.; "Celite 535" is particularly preferable) and 100 ml of the above separated upper layer. The mixture is vigorously agitated, and then hydrogen chloride is introduced into the mixture until the violet colour of the indicator has turned to yellow. This suspension is allowed to flow on to a glass column little by little to prepare a uniform Celite column.

25 mg of crystals of the antibiotic B-5050 mixture obtained in Example 3 is dissolved in 0.4 ml of the above separated upper layer, and the solution is subjected to chromatography with the above prepared Celite column. The column is developed with the upper layer, whereby active ingredients are separated and observed as respective violet bands. Further elution gives the respective fractions of antibiotics B-5050-A, -B, -C, -D, -E and -F in this order. Thus, 4 mg of B-5050-A, 3 mg of B-5050-B, 5 mg of B-5050-C, 4 mg of a mixture of B-5050-D and -E, and 2 mg of B-5050-F are obtained as crystals, respectively.

EXAMPLE 5

In the same manner as in Example 1, a seed culture of *Streptomyces hygroscopicus* (IFO-12995) is prepared.

2 ml of the seed culture is inoculated in 50 ml each of a main culture medium charged and sterilised in 200 ml Erlenmeyer flasks, said medium consisting of glucose (3.0%), cornsteep liquor (0.5%), defatted soybean meal (1.0%), sodium chloride (0.5%), magnesium sulphate (0.05%), dipotassium hydrogen phosphate (0.1%),

potassium chloride (0.05%), calcium carbonate (0.3%) and water, and is adjusted to pH 7.0. Fermentation is conducted on a rotary shaker at 28°C for 96 hours. The filtered broth thus obtained exhibits an antimicrobial potency of 350 dilution units against *Bacillus subtilis*.

1.6 Litres of the filtered broth is adjusted to pH 7.8, and is extracted first with 800 ml and then with 600 ml of ethyl acetate. The combined extracts are washed with water, and extracted twice with 600 ml each of N/200 aqueous HCl solution. The aqueous layers are combined, neutralised to pH 7 to 8 with N/10 aqueous NaOH solution, and extracted twice with 500 ml each time of ethyl acetate. The ethyl acetate extracts are washed with water, dehydrated with anhydrous sodium sulphate, and concentrated *in vacuo*. To the concentrate is added 30 ml of *n*-hexane to give 59 mg of crude powder. The powder is dissolved in hot benzene and the solution is allowed to stand, whereby 15 mg of antibiotic B-5050 mixture is obtained as colourless needles.

EXAMPLE 6

2 ml of a seed culture obtained in the same way as in Example 1 is inoculated in 50 ml each of a main culture medium in 200 ml Erlenmeyer flasks, the medium being of the same composition as that of the main culture medium used in Example 1. Cultivation is carried out at 28°C on a rotary shaker for 96 hours. The filtered broth thus obtained exhibits an antimicrobial potency of 350 dilution units against *Bacillus subtilis*.

1.8 Litres of the filtered broth is adjusted to pH 8.0, and extracted twice each time with 400 ml of ethyl acetate. After washing with water and dehydration, the ethyl acetate solution is concentrated *in vacuo*, and to the concentrate is added 30 ml of *n*-hexane to give 210 mg of crude powder. The powder is dissolved in 15 ml of benzene while warming, and the solution is left standing, whereby 59 mg of antibiotic B-5050 mixture is obtained as crystals.

EXAMPLE 7

900 Litres of a filtered broth obtained in the same manner as in Example 1 is treated in a similar way as described in Example 1, to give 70 g of a crude antibiotic B-5050 mixture.

The crude sample is dissolved in a mixture of ethyl acetate and benzene (2:1 by volume), and is chromatographed on a column packed with 2.1 kg of silica gel. The column is eluted with ethyl acetate-benzene (2:1 by volume), and colourless fractions are collected and concentrated to dryness. The residue is dissolved in 350 ml of hot benzene, and the solution is left

standing to give 7.0 g of colourless needles of antibiotic B-5050 mixture.

EXAMPLE 8

800 ml of a filtered broth obtained in the same manner as in Example 6 is adjusted to pH 7.6, and is allowed to pass through a column packed with 40 ml of "Amberlite XAD-2" (trade name of a molecular sieve distributed by Rohm & Haas Co., U.S.A.). The column is washed with 200 ml of distilled water, and then eluted with 200 ml of a 30% aqueous methanol solution to give a brown eluate which shows substantially no antimicrobial activity. Elution is continued with 500 ml of a 80% aqueous methanol solution to give the fractions containing most of the active ingredients. The fractions are concentrated *in vacuo* until methanol has been eliminated. The concentrate is adjusted to a pH of 7.5 to 8.5, and extracted twice each time with 100 ml of ethyl acetate. The ethyl acetate extracts are combined, washed with water, dehydrated and concentrated *in vacuo*. To the concentrate is added *n*-hexane to give 53 mg of a crude powder of antibiotic B-5050 mixture. The powder is dissolved in hot benzene, and the solution is left standing to give 17 mg of crystals.

EXAMPLE 9

1.0 g of Crystal I obtained in Example 2 is dissolved in 100 ml of ethyl acetate. The solution is extracted three times each with 100 ml portions of Sorensen's buffer solutions of pH 5.5, pH 4.9 and pH 4.4 (containing M/10 disodium hydrogen citrate) in this order. Each fraction is adjusted to pH 9.5 with a dilute aqueous ammonia, and is extracted with half its volume of ethyl acetate. The ethyl acetate layer is washed with water, dehydrated and concentrated *in vacuo* to crystallise. Thus, from the fraction extracted with the buffer solution of pH 4.9, 365 mg of antibiotic B-5050-C is obtained.

By a similar procedure, the ethyl acetate layer remaining after the extraction with the buffer solutions gives 110 mg of crystals consisting substantially of antibiotics B-5050-A and -B; the fraction extracted with the buffer solution of pH 4.4 gives 165 mg of crystals consisting mainly of antibiotics B-5050-B and -C; and the fraction extracted with the buffer solution of pH 5.5 gives 320 mg of crystals which consist substantially of antibiotics B-5050-C, -D, -E and -F.

EXAMPLE 10

10 g of Crystal II obtained in Example 2 is dissolved in 1 litre of ethyl acetate. The solution is extracted twice with 800 ml portions, and four times with 1 litre por-

tions of Sorensen's buffer solutions of pH 4.9 and pH 4.4 in this order. Each fraction is adjusted to pH 9.5 with dilute aqueous ammonia, and is extracted with a half volume of ethyl acetate. The ethyl acetate layer is washed with water, dehydrated and concentrated *in vacuo* to crystallise. The respective fractions are treated in the same manner as in Example 9. The ethyl acetate layer remaining after the extraction with the buffer solutions gives 4.2 g of crystals consisting mainly of antibiotic B-5050-A. The fractions extracted with the buffer solution of pH 4.4 gives 3.8 g of crystals consisting substantially of antibiotics B-5050-B. The fractions extracted with the buffer solution of pH 4.9 gives 1.3 g of crystals consisting substantially of antibiotics B-5050-B and -C.

20 WHAT WE CLAIM IS:—

1. A method for producing antibiotic B-5050 which comprises culturing *Streptomyces hygroscopicus* No. B-5050, as hereinbefore identified, represented by the strain deposited at the Institute for Fermentation, Osaka, Japan, under the accession number IFO-12995, or an antibiotic B-5050-producing mutant or variant thereof in a culture medium containing an assimilable carbon source and a digestible nitrogen source under aerobic conditions until antibiotic B-5050 is accumulated in the culture broth, and recovering the accumulated antibiotic B-5050 therefrom.

2. A method according to claim 1 wherein the recovered antibiotic B-5050 is substantially antibiotic B-5050-A.

3. A method according to claim 1 wherein the recovered antibiotic B-5050 is substantially antibiotic B-5050-B.

4. A method according to claim 1 wherein the recovered antibiotic B-5050 is substantially antibiotic B-5050-C.

5. A method according to claim 1 wherein the recovered antibiotic B-5050 is substantially antibiotic B-5050-D.

6. A method according to claim 1 wherein the recovered antibiotic B-5050 is substantially antibiotic B-5050-E.

7. A method according to claim 1 wherein the recovered antibiotic B-5050 is substantially antibiotic B-5050-F.

8. A method according to claim 1 wherein the recovered antibiotic B-5050 is a mixture of at least two of: B-5050-A, B-5050-B, B-5050-C, B-5050-D, B-5050-E and B-5050-F.

9. A method according to any of claims 1 to 8 wherein submerged cultivation with aeration and agitation is used.

10. A method according to claim 9 wherein the pH of the culture medium is from 6 to 8.

11. A method according to claim 10

wherein the pH of the culture medium is from 6.8 to 7.4.

12. A method according to any of claims 9 to 11 wherein the temperature of the culture is from 20 to 43°C.

13. A method according to claim 12 wherein the temperature of the culture is from 23° to 32°C.

14. A method as claimed in any of claims 1 to 13 which further comprises the step of separating antibiotic B-5050 into at least one of its components.

15. Antibiotic B-5050-A characterised by the following properties:

- (1) its melting point is from 129° to 132°C (recrystallised from acetone: *n*-hexane (1:3 by volume) mixture: 80
- (2) its molecular formula is $C_{43}H_{71}NO_{16}$ and its elementary analysis is:

C	59.40 ± 0.5;	H	8.15 ± 0.3;	85
N	1.4 ± 0.3;	O	31.33 ± 1.0;	

- (3) its molecular weight is 800—900, measured by vapour pressure osmosis in ethyl acetate;
- (4) its specific rotation is $[\alpha]^{25}_D$: $-72.3^\circ \pm 7^\circ$ ($c=1$, in ethanol); 90
- (5) it is positive to the Dragendorff reaction and the Erythromycin test, but negative to the Carbomycin test;
- (6) it is soluble in methanol, ethanol, acetone, methyl ethyl ketone, ethyl acetate, benzene, diethyl ether, chloroform and an aqueous acid solution, but hardly soluble in *n*-hexane and neutral water; 95
- (7) it has no significant maximum absorption in the ultraviolet wavelength range; 100
- (8) it has significant infra-red absorption bands at the wave numbers (cm^{-1}):

3448,	2941,	1739,	1458,	1374,	1295,	105
1188,	1167,	1120,	1086,	1053,	1033,	
971,	917,	862.				

16. Antibiotic B-5050-B, characterised by the following properties:

- (1) its melting point (recrystallised from a mixture of acetone and *n*-hexane) is from 134° to 136°C; 110
- (2) its molecular formula is $C_{42}H_{69}NO_{16}$ and its elementary analysis is:

C	58.67 ± 1.0;	H	8.02 ± 0.5;	115
N	1.86 ± 0.5;	O	31.18 ± 1.0;	

- (3) its molecular weight is 800—900, measured by vapour pressure osmosis in ethyl acetate;
- (4) its specific rotation is $[\alpha]^{25}_D$: $-71.9^\circ \pm 7^\circ$ ($c=1$, in ethanol); 120



tions of Sorensen's buffer solutions of pH 4.9 and pH 4.4 in this order. Each fraction is adjusted to pH 9.5 with dilute aqueous ammonia, and is extracted with a half volume of ethyl acetate. The ethyl acetate layer is washed with water, dehydrated and concentrated *in vacuo* to crystallise. The respective fractions are treated in the same manner as in Example 9. The ethyl acetate layer remaining after the extraction with the buffer solutions gives 4.2 g of crystals consisting mainly of antibiotic B-5050-A. The fractions extracted with the buffer solution of pH 4.4 gives 3.8 g of crystals consisting substantially of antibiotics B-5050-B. The fractions extracted with the buffer solution of pH 4.9 gives 1.3 g of crystals consisting substantially of antibiotics B-5050-B and -C.

20 WHAT WE CLAIM IS:—

1. A method for producing antibiotic B-5050 which comprises culturing *Streptomyces hygroscopicus* No. B-5050, as hereinbefore identified, represented by the strain deposited at the Institute for Fermentation, Osaka, Japan, under the accession number IFO-12995, or an antibiotic B-5050-producing mutant or variant thereof in a culture medium containing an assimilable carbon source and a digestible nitrogen source under aerobic conditions until antibiotic B-5050 is accumulated in the culture broth, and recovering the accumulated antibiotic B-5050 therefrom.

2. A method according to claim 1 wherein the recovered antibiotic B-5050 is substantially antibiotic B-5050-A.

3. A method according to claim 1 wherein the recovered antibiotic B-5050 is substantially antibiotic B-5050-B.

4. A method according to claim 1 wherein the recovered antibiotic B-5050 is substantially antibiotic B-5050-C.

5. A method according to claim 1 wherein the recovered antibiotic B-5050 is substantially antibiotic B-5050-D.

6. A method according to claim 1 wherein the recovered antibiotic B-5050 is substantially antibiotic B-5050-E.

7. A method according to claim 1 wherein the recovered antibiotic B-5050 is substantially antibiotic B-5050-F.

8. A method according to claim 1 wherein the recovered antibiotic B-5050 is a mixture of at least two of: B-5050-A, B-5050-B, B-5050-C, B-5050-D, B-5050-E and B-5050-F.

9. A method according to any of claims 1 to 8 wherein submerged cultivation with aeration and agitation is used.

10. A method according to claim 9 wherein the pH of the culture medium is from 6 to 8.

11. A method according to claim 10

wherein the pH of the culture medium is from 6.8 to 7.4.

12. A method according to any of claims 9 to 11 wherein the temperature of the culture is from 20 to 43°C.

13. A method according to claim 12 wherein the temperature of the culture is from 23° to 32°C.

14. A method as claimed in any of claims 1 to 13 which further comprises the step of separating antibiotic B-5050 into at least one of its components.

15. Antibiotic B-5050-A characterised by the following properties:

- (1) its melting point is from 129° to 132°C (recrystallised from acetone: *n*-hexane (1:3 by volume) mixture);
- (2) its molecular formula is $C_{43}H_{71}NO_{16}$ and its elementary analysis is:

C 59.40 ± 0.5; H 8.15 ± 0.3;
N 1.4 ± 0.3; O 31.33 ± 1.0;

(3) its molecular weight is 800—900, measured by vapour pressure osmosis in ethyl acetate;

(4) its specific rotation is $[\alpha]^{25}_D$: -72.3° ± 7° (c=1, in ethanol);

(5) it is positive to the Dragendorff reaction and the Erythromycin test, but negative to the Carbomycin test;

(6) it is soluble in methanol, ethanol, acetone, methyl ethyl ketone, ethyl acetate, benzene, diethyl ether, chloroform and an aqueous acid solution, but hardly soluble in *n*-hexane and neutral water;

(7) it has no significant maximum absorption in the ultraviolet wavelength range;

(8) it has significant infra-red absorption bands at the wave numbers (cm⁻¹):

3448, 2941, 1739, 1458, 1374, 1295, 105
1188, 1167, 1120, 1086, 1053, 1033,
971, 917, 862.

16. Antibiotic B-5050-B, characterised by the following properties:

(1) its melting point (recrystallised from a mixture of acetone and *n*-hexane) is from 134° to 136°C;

(2) its molecular formula is $C_{42}H_{69}NO_{16}$ and its elementary analysis is:

C 58.67 ± 1.0; H 8.02 ± 0.5;
N 1.86 ± 0.5; O 31.18 ± 1.0;

(3) its molecular weight is 800—900, measured by vapour pressure osmosis in ethyl acetate;

(4) its specific rotation is $[\alpha]^{25}_D$: -71.9°C ± 7° (c=1, in ethanol);



* C L M S *

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120

(5) it is positive to the Dragendorff reaction and the Erythromycin test, but it is negative to the Carbomycin test;

5 (6) it is soluble in methanol, ethanol, acetone, methyl ethyl ketone, ethyl acetate, benzene, diethyl ether, chloroform and an aqueous acid solution, but hardly soluble in *n*-hexane and neutral water;

10 (7) it has no significant maximum absorption in the ultraviolet wavelength range;

(8) it has significant infra-red absorption bands at the wave numbers (cm^{-1}):

15 3500, 2990, 2960, 1740, 1454, 1370, 1296, 1233, 1188, 1166, 1120, 1080, 1052, 1025, 968, 916, 858, 842.

19. Antibiotic B-5050-C characterised by the following properties:

20 (1) its melting point (recrystallised from a mixture of acetone and *n*-hexane) is from 135° to 138°C ;

(2) its molecular formula is $\text{C}_{41}\text{H}_{67}\text{NO}_{16}$ and its elementary analysis is:

25 C 57.93 ± 1.0 ; H 8.18 ± 0.5 ;
N 1.69 ± 0.5 ; O 31.86 ± 1.0 ;

(3) its molecular weight is from 800 to 900, measured by vapour pressure osmosis in ethyl acetate;

30 (4) its specific rotation is $[\alpha]^{25}_{\text{D}}$: $-76.0^{\circ} \pm 8^{\circ}$ ($c=1$, in ethanol);

(5) it is positive to the Dragendorff reaction and the Erythromycin test, but is negative to the Carbomycin test;

35 (6) it is soluble in methanol, ethanol, acetone, methyl ethyl ketone, ethyl acetate, benzene, diethyl ether, chloroform and an aqueous acid solution, but hardly soluble in *n*-hexane and neutral water;

40 (7) it has no significant maximum absorption in the ultraviolet wavelength range;

45 (8) it has significant infra-red absorption bands at the wave numbers (cm^{-1}):

3460, 2980, 2940, 1735, 1455, 1375, 1357, 1295, 1270, 1240, 1183, 1162, 1080, 1050, 1028, 1018, 972, 910, 862, 840.

50 18. Antibiotic B-5050-D characterised by the following properties:

(1) its melting point (recrystallised from a mixture of acetone and *n*-hexane) is from 143° to 146°C ;

55 (2) its molecular formula is $\text{C}_{40}\text{H}_{65}\text{NO}_{16}$ and its elementary analysis is:

C 57.48 ± 1.0 ; H 8.17 ± 0.5 ;
N 1.64 ± 0.5 ; O 32.90 ± 1.0 ;

(3) its molecular weight is from 800 to 900, measured by vapour pressure osmosis in ethyl acetate;

(4) its specific rotation is $[\alpha]^{25}_{\text{D}}$: $-76.2^{\circ} \pm 8^{\circ}$ ($c=1$, in ethanol);

(5) it is positive to the Dragendorff reaction and the Erythromycin test, but it is negative to the Carbomycin test;

(6) it is soluble in methanol, ethanol, acetone, methyl ethyl ketone, ethyl acetate, benzene, diethyl ether, chloroform and an aqueous acid solution, but hardly soluble in *n*-hexane and neutral water;

(7) it has no significant maximum absorption in the ultraviolet wavelength range;

(8) it has significant infra-red absorption bands at the wave numbers (cm^{-1}):

3440, 2946, 1735, 1450, 1373, 1357, 1296, 1274, 1237, 1163, 1127, 1083, 1047, 1031, 1012, 972, 913, 862, 843.

19. Antibiotic B-5050-E characterised by the following properties:

(1) its melting point (recrystallised from a mixture of acetone and *n*-hexane) is from 144° to 149°C ;

(2) its molecular formula is $\text{C}_{40}\text{H}_{65}\text{NO}_{16}$ and its elementary analysis is:

C 57.34 ± 1.0 ; H 8.25 ± 0.5 ;
N 1.34 ± 0.5 ; O 32.48 ± 1.0 ;

(3) its molecular weight is from 800 to 900, measured by vapour pressure osmosis in ethyl acetate;

(4) its specific rotation is $[\alpha]^{25}_{\text{D}}$: $-73.6^{\circ} \pm 7^{\circ}$ ($c=1$, in ethanol);

(5) it is positive to the Dragendorff reaction and the Erythromycin test, but it is negative to the Carbomycin test;

(6) it is soluble in methanol, ethanol, acetone, methyl ethyl ketone, ethyl acetate, benzene, diethyl ether, chloroform and an aqueous acid solution, but hardly soluble in *n*-hexane and neutral water;

(7) it has no significant maximum absorption in the ultraviolet wavelength range;

(8) it has significant infra-red absorption bands at the wave numbers (cm^{-1}):

3430, 2920, 1732, 1450, 1373, 1354, 1296, 1239, 1163, 1127, 1084, 1048, 1032, 1011, 970, 914, 862, 840.

20. Antibiotic B-5050-F characterised by the following properties:

(1) its melting point (recrystallised from



* C L M S *

- a mixture of acetone and *n*-hexane) is from 149° to 154°C;
- (2) its molecular formula is $C_{33}H_{63}NO_{16}$ and its elementary analysis is:
- 5 C 58.59 ± 0.5 ; H 7.82 ± 0.3 ;
 N 1.62 ± 0.3 ; O 32.38 ± 1.0 ;
- (3) its molecular weight is from 800 to 900, measured by vapour pressure osmosis in ethyl acetate;
- 10 (4) its specific rotation is $[\alpha]^{25}_D: -77.7^\circ \pm 8^\circ$ ($c=1$, in ethanol);
- (5) it is positive to the Dragendorff reaction and the Erythromycin test, but it is negative to the Carbomycin test;
- 15 (6) it is soluble in methanol, ethanol, acetone, methyl ethyl ketone, ethyl acetate, benzene, diethyl ether, chloroform and an aqueous acid solution, but hardly soluble in *n*-hexane and neutral water;
- 20 (7) it has no significant maximum absorption in the ultraviolet wavelength range;
- (8) it has significant infra-red absorption bands at the wave numbers (cm^{-1}):
- 25 3425, 2994, 1745, 1730, 1460, 1379, 1362, 1300, 1242, 1166, 1133, 1089, 1053, 1031, 973, 919, 864.
21. An antibiotic B-5050 which is a mixture of at least two of the antibiotics: B-5050-A, B-5050-B, B-5050-C, B-5050-D, B-5050-E and B-5050-F, as defined in claims 15, 16, 17, 18, 19 and 20, respectively.
22. An antibiotic B-5050 mixture as claimed in claim 21 characterised by the following properties:
- 35 (1) its melting point is from 137° to 141°C;
- (2) its elementary analysis when recrystallised from benzene is:
- C 58.14 ± 0.5 ; H 7.88 ± 0.3 ; 40
N 1.73 ± 0.3 ; O 32.15 ± 1.0 ;
- (3) its molecular weight is 880 ± 90 measured by vapour pressure osmosis in ethyl acetate;
- (4) its specific rotation is $[\alpha]^{24}_D: -76.4^\circ \pm 8^\circ$ ($c=1$, in ethanol);
- (5) it is positive to the Dragendorff reaction and the Erythromycin test, but is negative to the Carbomycin test;
- (6) it is soluble in methanol, ethanol, acetone, ethyl acetate, methyl ethyl ketone, chloroform, benzene, diethyl ether and an aqueous acid solution, but hardly soluble in *n*-hexane and neutral water;
- (7) it has no significant maximum absorption in the ultraviolet wavelength when measured in methanol; and
- (8) it has significant infra-red absorption bands at the wave numbers (cm^{-1}):
- 3448, 2924, 1736, 1453, 1376, 1295, 1239, 1167, 1081, 1050, 968, 912, 861, 840.
23. An antibiotic mixture as claimed in claim 21 which is substantially a mixture of antibiotic B-5050-A and antibiotic B-5050-B.
24. A method of producing antibiotic B-5050 according to claim 1 and substantially as herein described with reference to any of the specific Examples.
25. Antibiotic B-5050 when produced by a method according to any of claims 1 to 14 and 24.
- 65 23. An antibiotic mixture as claimed in claim 21 which is substantially a mixture of antibiotic B-5050-A and antibiotic B-5050-B.
- 70 24. A method of producing antibiotic B-5050 according to claim 1 and substantially as herein described with reference to any of the specific Examples.
25. Antibiotic B-5050 when produced by a method according to any of claims 1 to 14 and 24.

ELKINGTON & FIFE,
Chartered Patent Agents,
High Holborn House,
52/54 High Holborn,
London, W.C.1.
Agents for the Applicants.