

# PATENT SPECIFICATION

880,035



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International Classification:—C12d.

## COMPLETE SPECIFICATION

### DRAWINGS ATTACHED

#### New Antibiotic



We, SOCIETA FARMACEUTICI ITALIA, a  
Body Corporate organised and existing  
under the laws of Italy, formerly of 18 Via  
Filippo Turati, Milan, Italy, now at:  
5 Societa Farmaceutici Italia, Viale Bezzi 24  
Milan Italy, 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 des-  
10 cribed in and by the following statement:—

The present invention relates to a new and  
therapeutically useful broad spectrum anti-  
biotic product, to its production by fermen-  
tation and a process for its recovery.

15 We have discovered that, using a new  
species of streptomycetes, a high concentra-  
tion of an antibiotic substance different  
from those known before is formed in a  
fermentation broth, that this product can be  
20 recovered under proper process conditions  
(to be described hereinafter) and further that  
this substance has properties which mark it  
as a new and useful therapeutic product.  
The new antibiotic provided by the present  
25 invention is designated by us as F.I. 1600 or  
amminosidin and we include in that denomi-  
nation also the free base or its salts and  
solutions thereof. The new organism which  
permits the production of the antibiotic F.I.  
30 1600 is now designated by us as *Strepto-  
myces Krestomyceticus* (n.sp.). *Streptomyces*

*Krestomyceticus* has been deposited with the  
National Collection of Industrial Bacteria  
and received the index number of N.C.I.B.  
8995. *S.Krestomyceticus* has also been de- 35  
posited at the Commonwealth Mycological  
Institute under the number 79589. The pro-  
cess of the present invention therefore is one  
for producing the antibiotic substance F.I.  
40 1600 which comprises the cultivation of *Stre-  
ptomyces Krestomyceticus*, N.C.I.B. 8995 and  
Commonwealth Mycological Institute No.  
79589, as herein described, in an aqueous.  
nutrient medium containing a source of car- 45  
bon, nitrogen and mineral salts under aerobic  
conditions until substantial anti-bacterial  
activity is imparted to the medium and then  
recovering F.I. 1600 from the medium.

The strain *Streptomyces Krestomyceticus*  
has been found in a soil sample obtained at 50  
Massa Marittima (Grosseto) isolated and  
grown on synthetic as well as organic media  
within the range of 28-37°C. Under the elec-  
tron microscope *S.Krestomyceticus* shows  
55 smooth spores rather regular/cylindrical or  
oval in shape/somewhat transparent. The  
hyphae are usually straight but sometimes  
hooks and spirals are observed. The strain  
of *S.Krestomyceticus* has been investigated  
60 for its classification Table I sets forth its  
cultural characteristics.

[Price 3s. 6d.]



*Streptomyces*

12

12



TABLE I

|                                                                    |                                                                                                                                                                                       |                                                                                                                                                                                                                                                                                               |                                                                                                                                   |     |
|--------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------|-----|
| Agar glycerin glycine                                              | Good growth colourless with abundant production of spores. Aerial hyphae and spores white.                                                                                            | meal agar                                                                                                                                                                                                                                                                                     | splits. Yellowish vegetative mycelium covered by a very short whitish aerial mycelium.                                            |     |
| 5 Soil-Extract Agar                                                | Good colourless growth, covered with moderate formation of whitish aerial mycelium.                                                                                                   | Soyabean meal glycerin agar                                                                                                                                                                                                                                                                   | Flat, opaque and brownish colonies with very short aerial mycelium on the bottom of the slants.                                   | 70  |
| 10 Czapek Agar                                                     | Colourless growth very superficial. Good white sporulation.                                                                                                                           | Corn steep agar                                                                                                                                                                                                                                                                               | Similar to soyabean meal agar.                                                                                                    | 75  |
| YED salts Agar (Yeast extract, and salts)                          | Bulky yellowish growth not very deep into the substrate; moderate white sporulation.                                                                                                  | Peptone Corn steep agar                                                                                                                                                                                                                                                                       | Flat and yellowish growth with few splits. Short aerial mycelium, abundant, more evident on the borders of the culture.           | 80  |
| 15 Peptone and salts agar                                          | Good growth, colourless to yellowish, with good white sporulation; very short hyphae.                                                                                                 | Anderson's sporulation medium                                                                                                                                                                                                                                                                 | Yellowish growth generally flat, showing splits where agar is thicker. Some aerial mycelium on the folds.                         | 85  |
| 20 Glycerin agar                                                   | Bulky nearly colourless growth; scarce formation of aerial mycelium.                                                                                                                  | Tryptone agar                                                                                                                                                                                                                                                                                 | Similar to the Anderson's medium, but with colourless vegetative mycelium; abundant aerial mycelium.                              | 90  |
| 25 Asparagine dextrose agar                                        | Yellowish small colonies, opaque, flat and waxy, not very evident, without aerial mycelium.                                                                                           | NZ Amine asparagine agar                                                                                                                                                                                                                                                                      | Flat, yellow colonies with small tufts of white aerial mycelium.                                                                  | 95  |
| Potato dextrose agar                                               | Not very voluminous colourless growth with sparse whitish aerial mycelium. Typical concentric splits or crackings on the bottom of the culture slants.                                | Pridham's medium with starch and inorganic salts                                                                                                                                                                                                                                              | Flat yellowish colonies which are more wrinkled where the substrate is thicker.                                                   | 100 |
| 30 Casein hydrolysate agar, supplemented with salts and aminoacids | Small scanty colourless growth. No aerial mycelium.                                                                                                                                   | Oats meal agar                                                                                                                                                                                                                                                                                | Flat yellowish colonies with colourless borders. Aerial mycelium generally absent, which only appears occasionally on some folds. | 105 |
| 35 PYS agar (peptone yeast salts)                                  | Abundant, colourless growth flat, with scarce aerial mycelium, superficial splits.                                                                                                    | Saubouraud agar                                                                                                                                                                                                                                                                               | Good colourless or cream-coloured growth covered by an abundant white aerial mycelium.                                            | 110 |
| 40 Potato plugs                                                    | Excellent growth throughout the plug, completely covered with abundant and aerial mycelium well sporulated.                                                                           | Yeast Broth                                                                                                                                                                                                                                                                                   | Superficial bulky and yellowish growth covered with white aerial mycelium.                                                        | 115 |
| 45 Emerson's agar                                                  | Yellowish growth with undulated surface showing many splits. The whole surface is covered by a very short whitish aerial mycelium more evident where the growth is thicker.           | <i>S. Krestomyceticus</i> hydrolyses both starch and gelatin, and decomposes tyrosine within 6 days.                                                                                                                                                                                          |                                                                                                                                   |     |
| 50 Casein hydrolysate agar                                         | Very abundant and thick growth not very deep into substrate. Evident splits with reversed rims. Uniformly distributed short aerial mycelium but evident. The reverse is sharp yellow. | Acids are produced using the following substances as sole source of carbon: inulin, threose, levulose, sorbitol, mannose, galactose, lactose, adonitol, mannitol, maltose, glycerol, dextrose, dextrin.                                                                                       |                                                                                                                                   |     |
| 55 Soyabean                                                        | Undulated growth with                                                                                                                                                                 | Milk is only slightly coagulated after 13 days. Neutral reaction, no peptonisation.                                                                                                                                                                                                           |                                                                                                                                   |     |
|                                                                    |                                                                                                                                                                                       | The cultural characteristics of the F.I. 1600 producing strain have been compared with those of species already described.                                                                                                                                                                    |                                                                                                                                   |     |
|                                                                    |                                                                                                                                                                                       | Among these species, <i>S. Virgatus</i> and <i>S. flocculus</i> differ from our strain because their vegetative mycelium is greenish and the former also produces a brown pigment on some media. Besides <i>S. flocculus</i> has its surface covered by a velvety or cottony aerial mycelium. |                                                                                                                                   |     |
|                                                                    |                                                                                                                                                                                       | <i>S. thermophilus</i> presents a deep growth into the medium and whitish aerial my-                                                                                                                                                                                                          |                                                                                                                                   |     |



celium.

Further it has a growth optimum at 50°C. The F.I. 1600 producing strain shows a very good growth at 37°C but very small amounts of antibiotic are produced at 33°C or more.

*S. Kanamyceticus* shows a deep growth into the substrate and sometimes greenish or rosy shades in the vegetative mycelium. Sometimes it also produces a brown pigment. No one of the aforesaid species show the typical splits on the surface of the culture, which are the most peculiar characteristics of the F.I. 1600 producing strain. On the basis of those characteristics, which do not correspond to those of any known species, *S. Krestomyceticus* has been considered a new species. The production of the antibiotic F.I. 1600 has been carried out by fermentation in submerged aerial cultures in an aqueous nutrient solution containing a source of nitrogen, a source of carbon and mineral salts.

As a nitrogen source, the best results are obtained by using protein containing material such as barley malt extract, corn malt extract, corn steep liquor, enzymatic casein hydrolysate, peanut meal, soyabean meal, cotton bean meal, meat extract.

As a source of assimilable nitrogen the inorganic ammonium salts are suitable. Among these are ammonium sulphate and ammonium phosphate. It is also possible to use organic sources of nitrogen such as the amino acids and various proteinaceous natural materials such as distillery residues.

The carbon source may be either a soluble carbohydrate such as dextrose or an insoluble one such as starch, for example corn starch. A good yield of antibiotic is obtained also with maltose, dextrin/lactose and other sugars. The mineral salts which are necessary for the fermentation medium are preferably phosphates, which may be present either as ammonium phosphate or as metallic phosphate such as potassium phosphate, chlorides such as NaCl and sulphates such as ammonium or magnesium sulphate.

Suitable buffering agents are calcium carbonate and salts of organic acids such as citrates, acetates and lactates which are useful in maintaining the pH within the proper range.

It is necessary, sometimes, to provide a source of heavy metals which may be present as impurities or added; among these are copper, zinc, manganese, iron and chromium.

The use of a defoaming agent is desirable in large scale fermenters, even though in this fermentation foaming is not a particularly difficult problem and is readily controlled by the use of conventional defoaming agents such as octadecanol in lard oil or other suitable commercial defoamers such as silicones.

The relative ratio of the fermentation medium ingredients has been quantitatively established as follows:

|                   |                |    |
|-------------------|----------------|----|
| Nitrogen source   | 0.5-4%         |    |
| Carbon source     | 1-5%           | 70 |
| Phosphates        | 0.01-0.3%      |    |
| Sulphates         | 0.05-0.25%     |    |
| CaCO <sub>3</sub> | 0.1-0.7%       |    |
| Chlorides         | 0.1-0.3%       |    |
| Trace elements    | 0.0001-0.0005% | 75 |

The exact amounts of all the substances are particularly indicated in the examples. The fermentation is carried out at a temperature of from 24°C to 32°C for a period of from two days to one week at a pH of 6.3-7.8. The initial pH which ensures the most favourable fermentation process is 6.4-6.7; it increases to 7.6-7.8 towards the end of the fermentation.

Aeration is also necessary for a good yield. The ratio between the amount of air which must be introduced into the fermentation tank and the volume of the culture liquid varies through the fermentation phases from 0.1 to 0.8.

F.I. 1600 may be recovered as a hydrochloride as well as a sulphate from the fermentation broth, by adopting a recovery process which we have devised, which has proved to be highly effective from the standpoint of recovering a product free of inactive impurities, and of other antibiotic substances simultaneously formed in the broth. This method is exemplified below. Basically, it involves the following steps:

- Precipitation of Ca<sup>++</sup> with oxalic acid and filtration of mycelium and calcium oxalate after adjusting the pH to 7-7.5.
- Absorption of the antibiotic on a cationic exchange resin and elution with dilute acids, such as 1N HCl.
- Neutralisation of the eluted solution with an anionic exchange resin, in hydroxylate phase, until the pH rises to 7-7.5 and subsequently filtering.
- Concentration of the filtered solution to dryness under vacuum.
- Dissolution of the antibiotic in a suitable organic solvent, such as methanol.
- Precipitation of the F.I. 1600 hydrochloride by addition of acetone to the organic solution.

The filtered F.I. 1600 hydrochloride is dissolved in water and the aqueous filtered solution, after concentration to a small volume under vacuum, is treated with triethylamine sulphate.

After filtering, the solution is added to methanol with stirring. The F.I. 1600 sulphate is filtered, washed with methanol and dried *in vacuo* over P<sub>2</sub>O<sub>5</sub> at 50°C.

It has been found that the antibiotic F.I. 1600 can also be recovered by absorption and elution from carbon. More specifically,



we have found that F.I. 1600 may be removed either from its aqueous solutions or from cultural broths by means of carbon, preferably at a pH higher than 7.

5 F.I. 1600 may be eluted from carbon either by means of aqueous solution or by means of alcoholic solutions of inorganic or organic acids.

10 F.I. 1600 is a basic substance and is water soluble.

F.I. 1600 in the form of the sulphate or hydrochloride, is a white powder, soluble in water and insoluble in almost all organic solvent. The F.I. 1600 hydrochloride is 15 soluble in methanol.

F.I. 1600 may be finally crystallized from a mixture of methanol and acetone.

The ultra-violet spectrum shows no absorption within the range of 220-400 m $\mu$ .

20 Infra-red spectrum of the F.I. 1600 sulphate in KBr (5%), which is reported in the draw-

ing accompanying Provisional Specification 10858/59 shows pronounced peaks at 2.94 $\mu$ , 3.41 $\mu$ , 6.15 $\mu$ , 6.55 $\mu$ , 9.00 $\mu$  and 9.52 $\mu$ , weak bands at 6.82 $\mu$ , 7.08 $\mu$ , 7.28 $\mu$ , 7.46 $\mu$ , 10.70 $\mu$ , 25 11.17 $\mu$ , 11.62 $\mu$ , 13.08 $\mu$  and inflections at 3.09 $\mu$  and 10.23 $\mu$  attributed to the following groups:

OH, NH, CH, CH<sub>2</sub>, NH<sub>4</sub><sup>+</sup> ion, C-O-C.

There is no evidence of carbonyl groups 30 in keto, amide or ester form.

The optical rotations of highly pure F.I. 1600, hydrochloride and sulphate in water were determined.

Hydrochloride 35

( $\alpha$ )25° = +55° (c=1% in water)

D

Sulphate ( $\alpha$ )20° = +51° (c=1% in water)

D

Table II sets forth the elementary analysis 40 of the F.I. 1600 salts.

| F.I. 1600 Salts                                                    | C%             | H%            | N%            | S%            | Cl%   |
|--------------------------------------------------------------------|----------------|---------------|---------------|---------------|-------|
| Hydrochloride                                                      | 33.79          | 6.76          | 8.17          | —             | 21.05 |
| Sulphate                                                           | 30.57-<br>30.8 | 6.44-<br>7.00 | 7.19-<br>7.38 | 8.99-<br>9.08 | —     |
| 45 <i>p</i> -( <i>p</i> -hydroxy-phenylazo)-<br>benzene-sulphonate | 50.58          | 5.25          | 10.27         | 7.72          | —     |
| Helyanthine salt                                                   | 51.29          | 6.20          | 12.23         | 7.50          | —     |

By analysis of F.I. 1600 sulphate and its degradation products it has 50 been determined that F.I. 1600 has the probable empirical formula C<sub>23</sub>H<sub>45</sub>N<sub>5</sub>O<sub>14</sub> and that of the sulphate should be C<sub>23</sub>H<sub>45</sub>N<sub>5</sub>O<sub>14</sub>·2.5H<sub>2</sub>SO<sub>4</sub>·H<sub>2</sub>O (calculated: C 30.80, H 6.03, N 7.81, S 8.93). F.I. 1600 55 gives penta-acyl derivatives by acylation; by this way penta-benzoyl- and penta-acetyl-derivatives have been obtained. It has been found that one mole of penta-acetyl-F.I. 1600, in 0.02 M alkaline periodate solution, 60 consumes 2.95 mols of periodate at room temperature.

On the basis of elementary analysis, the basic properties of the product, the fact that all nitrogen is basic, its U.V. and I.R. 65 spectra, we exclude any polypeptide type structure and we attribute to F.I. 1600 a

structure similar to that one of the group of oligosaccharide basic water soluble antibiotics, such as streptomycin and neomycin.

Chromatographically, F.I. 1600 may be 70 distinguished from streptomycin, dihydro-streptomycin, neomycin, viomycin and other glycopeptidic basic substances such as streptothricin and the geomycins.

Prolonged acid hydrolysis of Neomycins 75 or of Catenulin (J. W. Davisson et al. Antibiot. and Chem. 2, 1952, p.460) yields a microbiologically active fragment, identified by paper chromatography as neamine.

Acid hydrolysis of F.I. 1600 with 6N HCl 80 yields no active fragment.

Table III shows the differences between F.I. 1600 and the other antibiotics of the same group.



*viomycin*



TABLE III

| Antibiotic               | Organism                                   | Analysis of the sulphate                                                                                                                          | ( $\alpha$ )D of the sulphate | Other properties and differences                                                                                                                                                                                                              |
|--------------------------|--------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 5 F.I. 1600 sulphate     | <i>Strept. Krestomyceticus</i>             | C = 30.8-30.57<br>H = 7.00-6.44<br>N = 7.19-7.38<br>S = 8.99-9.08<br>Empirical formula:<br>$C_{25}H_{45}N_5O_{14} \cdot 2.5 H_2SO_4 \cdot 2 H_2O$ | +51                           | By acid hydrolysis no formation of microbiologically active fragment.                                                                                                                                                                         |
| 10 Neomycins sulphate    | <i>Strept. fradiae</i>                     | C = 29.35<br>B H = 6.86<br>N = 9.21<br>(1) $SO_4 = 28.3$<br>C = 31.18<br>C H = 6.14<br>N = 8.93<br>(2) S = 9.90                                   | B = +56(2)<br>C = +82(2)      | a) By acid hydrolysis formation of active fragment (neamine).<br>b) Infra-red spectrum is different from that of F.I. 1600.<br>c) It is chromatographically different from F.I. 1600.                                                         |
| 20 Streptomycin sulphate | <i>Strept. griseus</i>                     | C = 34.62<br>H = 5.77<br>N = 13.45<br>S = 6.61                                                                                                    | -73°(3)                       | a) Infra-red spectrum is different from that of F.I. 1600.<br>b) It is chromatographically different from F.I. 1600.                                                                                                                          |
| 25 Catenulin sulphate    | Not described                              | C = 31.53<br>31.58<br>H = 5.29<br>5.26 (4)<br>N = 7.92<br>7.93<br>$SO_4 = 28.11$<br>28.13                                                         | +51.9(4)                      | a) Infra-red absorption (not reported) is typical of a polypeptide spectrum (4).<br>b) By acid hydrolysis formation of active fragment (neamine) (4).<br>c) It shows crossed resistance with neomycin (6).                                    |
| 30 Kanamycin sulphate    | <i>Strept. Kanamyceticus</i>               | C = 37.3<br>37.4<br>H = 6.8<br>6.3 (5)<br>N = 9.3<br>9.6<br>S = 5.5                                                                               | +146°(5)                      | Infra-red spectrum is different from that of F.I. 1600.                                                                                                                                                                                       |
| 40 Hydroxymycin          | <i>Strept. paucisporogenes</i>             | N = 6.2 (7)<br>Probable empirical formula reported (7) of the base:<br>$C_{25}H_{47}N_5O_{12}$                                                    | +51(7)                        | Its pentabenzate, soluble in 5 parts of hot methanol, melts at 233°C with dec. and its ( $\alpha$ ) is +36: these properties are different from those of F.I. 1600 pentabenzate. The formula of the base is different from that of F.I. 1600. |
| 45 Paromomycin sulphate  | <i>Strept. rimosus forma paromomycinus</i> | Probable empirical formula reported (8):<br>$(C_{16}H_{18-22}N_2O_6 \cdot H_2SO_4)_x$                                                             | +50.5(8)                      | Infra-red spectrum is different from that of F.I. 1600 (absence of some absorption bands) and also the empirical formula is different.                                                                                                        |

## References:

- 1) Regna et al. J. Chem. Soc. 1950, p. 1045
- 2) Ford et al. J. Chem. Soc. 1955, p. 5311
- 3) Regna et al. J. Biol. Chem. 165, 1946, p. 621
- 4) Davisson et al. Antibiotic and Chem. 2, 1952, p. 460
- 5) Cron et al. J. Chem. Soc. 80, 1958, p. 752
- 6) Szybalsky et al. Am. Rev. Tuberc. 69, 1954, p. 267
- 7) G. Hagemann et al. Pharm. Franc. 16, 1958, p. 585
- 8) B.P. 797,568

Therefore it appears that F.I. 1600 provided by the present invention, is a new component of the group of basic, water soluble oligosaccharide type antibiotics.

## Pharmacological properties

F.I. 1600 shows the following pharmacological properties:

- a) Broad antibiotic spectrum which comprises gram-positive, gram-negative and acid resistant bacteria.
- b) Inhibitory actions against the afore-said organisms at very low concentration.
- c) Activity against antibiotic resistant strains.
- d) Very low toxicity.

Table IV shows the minimum dose in mcg/ml (MID) of F.I. 1600 sulphate inhibiting the growth of the under mentioned micro-organisms in Yeast-extract broth.

TABLE IV

| Test organism            | After 24 hours of incubation | After 48 hours of incubation |
|--------------------------|------------------------------|------------------------------|
| <i>M.pyogenes aureus</i> | 1                            | 1                            |
| <i>B.subtilis</i>        | 1                            | 1                            |
| <i>S.faecalis</i>        | 1                            | 1                            |
| <i>Mycobacterium 607</i> | 1                            | 5                            |
| <i>M.phlei</i>           | 1                            | 5                            |
| <i>A.bostromi A</i>      | 1                            | 1                            |
| <i>E.coli</i>            | 5                            | 5                            |
| <i>K.friedlanderi</i>    | 5                            | 25                           |
| <i>P.vulgaris</i>        | 25                           | 25                           |
| <i>P.aeruginosa</i>      | 100                          | >100                         |
| <i>S.flexneri Y</i>      | 25                           | 25                           |
| <i>O.albicans</i>        | >100                         | >100                         |
| <i>G.graphii</i>         | >100                         | >100                         |
| <i>T.mentagrophytes</i>  | >100                         | >100                         |

The same concentrations of the antibiotic inhibit the growth of *M.tbc* or *M.607* and that of many antibiotic resistant strains of the same species-(see Table V).

TABLE V

| Test organism                     | MID mcg/ml liquid Dubos medium |
|-----------------------------------|--------------------------------|
| <i>M.Tuberculosis H 37 Rv</i>     | 1                              |
| <i>M.tbc. - isoniazide res.</i>   | 2.5                            |
| <i>Mycobacterium sp. ATCC 607</i> | 2.5                            |
| <i>M. 607 Strept. resistant</i>   | 2.5                            |
| " " <i>Chlortetracycl</i> "       | 2.5                            |
| " " <i>Oxytetracycl</i> "         | 2.5                            |
| " " <i>Neom.</i> "                | 5.0                            |
| " " <i>Viom.</i> "                | 2.5                            |
| " " <i>Oxam.</i> "                | 2.5                            |
| " " <i>Eritrom.</i> "             | 5.0                            |
| " " <i>Vancom.</i> "              | 5.0                            |
| <i>M.phlei</i>                    | 0.98                           |
| <i>M.para-smegmatis</i>           | 0.124                          |

Table VI shows the relative amounts in mcg/ml (MID) of F.I. 1600 sulphate required to inhibit a number of representative organisms over different cultures.

TABLE VI

In the table are reported the values of bacteriostatic and bactericidal concentrations of F.I. 1600 in different culture media.

|    | Test Organism                 | MID in mcg/ml               |                   |                                               |                                                     |
|----|-------------------------------|-----------------------------|-------------------|-----------------------------------------------|-----------------------------------------------------|
|    |                               | Yeast<br>broth              | Nutrient<br>broth | Penassay<br>broth<br>Difco<br>(Regd.<br>T.M.) | Brain<br>heart infusion<br>Difco<br>(Regd.<br>T.M.) |
| 5  |                               |                             |                   |                                               |                                                     |
| 10 |                               |                             |                   |                                               |                                                     |
|    |                               | Bacteriostatic              |                   |                                               |                                                     |
|    | <i>Staphyl. aureus</i> 114    | 0.35                        | 0.6               | 1.6                                           | 3.8                                                 |
|    | <i>Staphyl. aureus</i> 209 P  | 0.62                        | 1.05              | 4.0                                           | 12.0                                                |
|    | <i>Staphyl. aureus</i> 503 MB | 0.075                       | 0.31              | 0.13                                          | 0.35                                                |
| 15 | <i>Staphyl. aureus</i> CAMP   | —                           | 0.15              | 1.45                                          | 4.8                                                 |
|    | <i>Staphyl. Londres</i>       | 0.15                        | 0.45              | 3.5                                           | 6.0                                                 |
|    |                               | Bactericidal after 24 hours |                   |                                               |                                                     |
|    | <i>Staphyl. aureus</i> 114    | 2.5                         | 2.5               | 10                                            | >20                                                 |
|    | <i>Staphyl. aureus</i> 209 P  | 10                          | 20                | 20                                            | >20                                                 |
| 20 | <i>Staphyl. aureus</i> 503 MB | 0.3                         | 1.25              | 20                                            | 5                                                   |
|    | <i>Staphyl. aureus</i> CAMP   | 5                           | 20                | 5                                             | 10                                                  |
|    | <i>Staphyl. Londres</i>       | 5                           | 5                 | 10                                            | 20                                                  |
|    |                               | Bactericidal after 48 hours |                   |                                               |                                                     |
|    | <i>Staphyl. aureus</i> 114    | 2.5                         | 2.5               | 10                                            | >20                                                 |
| 25 | <i>Staphyl. aureus</i> 209 P  | 10.0                        | 20                | 20                                            | >20                                                 |
|    | <i>Staphyl. aureus</i> 503 MB | 0.3                         | 1.25              | 20                                            | 5                                                   |
|    | <i>Staphyl. aureus</i> CAMP   | 5.0                         | 20                | 5                                             | 10                                                  |
|    | <i>Staphyl. Londres</i>       | 5                           | 5                 | 10                                            | 20                                                  |

The following Examples are given to illustrate the production and the recovery of F.I. 1600 by fermentation under submerged aerobic conditions. The same media may be used for fermentation in shaker flasks on a smaller scale or in tanks on a larger scale.

#### EXAMPLE 1

300 ml flasks containing 60 ml of an aqueous medium having the following composition:

|    |                   |      |
|----|-------------------|------|
| 40 | Soyabean flour    | 2%   |
|    | NaCl              | 2.5% |
|    | Dextrose          | 3%   |
|    | Corn-steep        | 1.5% |
|    | CaCO <sub>3</sub> | 0.7% |

are sterilized at 120°C for 20 minutes and inoculated each with 0.25 ml of a suspension of spores of *S.Krestomyceticus*, the medium being in continuous contact with air.

The flasks are incubated at 28°C on a shaking machine. A good growth is obtained within 7-8 days and about 500 mcg/ml of 50 antibiotic are produced.

#### EXAMPLE 2

A spore suspension of *S.Krestomyceticus* is inoculated into 2000 ml Erlenmeyer flasks containing 500 ml of a suitable aqueous medium which has the following composition:

|                                                 |         |
|-------------------------------------------------|---------|
| Dextrin                                         | 2%      |
| Casein                                          | 0.5%    |
| CaCO <sub>3</sub>                               | 0.4%    |
| MgSO <sub>4</sub>                               | 0.1%    |
| (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> | 0.1%    |
| ZnSO <sub>4</sub> · 7H <sub>2</sub> O           | 0.0002% |

After 48 hours of stirring at 28°C, 0.4% of the culture is transferred into a 10 litre tank containing the following medium:

- 5
- |                                                 |       |
|-------------------------------------------------|-------|
| Dextrin                                         | 2%    |
| Casein                                          | 0.5%  |
| CaCO <sub>3</sub>                               | 0.4%  |
| (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> | 0.1%  |
| K <sub>2</sub> HPO <sub>4</sub>                 | 0.01% |
| Corn steep                                      | 0.5%  |
- (pH after sterilisation=6.6)

After 7 hours of fermentation with stirring at 28°C under aerobic conditions (1.1 lt/minute) the culture has its maximum growth.

#### EXAMPLE 3

A 2000 ml flask containing 500 ml of an aqueous medium having the following composition:

- 15
- |                    |      |
|--------------------|------|
| Casein hydrolysate | 1%   |
| NaCl               | 0.2% |
- is sterilized at 120°C for 20 minutes and inoculated with a suspension of spores of *S.Krestomyceticus* (cultivated on potato-agar medium). The flask is incubated at 28°C for 48 hours with stirring.

After 48 hours the culture is transferred into a 100 litre tank containing the following aqueous medium:

- 25
- |                   |      |
|-------------------|------|
| Soyabean flour    | 2%   |
| Dextrin           | 2.0% |
| CaCO <sub>3</sub> | 0.3% |
- (pH=6.16)

After 36 hours of fermentation with stirring at 28°C under aerobic conditions, about 15% of the culture is transferred into a 1000 litre tank containing a medium of the following composition:

- 35
- |                     |       |
|---------------------|-------|
| Soyabean flour      | 2.5%  |
| Dextrin             | 2.5%  |
| Distillery residues | 0.5%  |
| NaCl                | 0.25% |
| Soyabean oil        | 0.03% |
| CaCO <sub>3</sub>   | 0.03% |
- 40 (pH=6.4-6.5)

The fermentation is continued for 80-90 hours at 28°C with stirring and under aerobic conditions.

#### EXAMPLE 4

45 To about 500 litres of fermentation broth obtained by the process described in Example 3 is added with stirring oxalic acid (correspondent to Ca<sup>++</sup> contained in the broth), with subsequent adjustment of the pH to 7-7.5 with NaOH. The stirring is continued for 30 minutes and the suspension is filtered. The cake is washed with water.

The filtered solution is absorbed over 5 litres of Amberlite (Registered Trade Mark) 55 IRC 50 (sodium phase).

By elution with 1N HCl several separated fractions are obtained.

The active fractions are neutralised with anionic exchange resins concentrated to dryness under vacuum and the residue is treated, 2-3 times, with 1 litre of methanol with stirring for 30 minutes. Each time the suspension is filtered. The F.I. 1600 hydrochloride is obtained by adding the 65 methanolic solution to acetone (2-3

volumes); the white flocced product is filtered, washed with acetone and dried: yield=80-90 g.

#### EXAMPLE 5

The F.I. 1600 hydrochloride is dissolved 70 in water and the solution is filtered. The filtrate is concentrated to a small volume under vacuum, then triethylamine sulphate is added. After cooling in a refrigerator, the isolated calcium sulphate is eliminated by 75 filtration. The filtered solution is added to methanol with stirring. When the addition is finished, the stirring is continued for 30 minutes and the F.I. 1600 sulphate is filtered and washed with methanol. The product is dried at 50° in vacuo over P<sub>2</sub>O<sub>5</sub>. The raw sulphate is purified by chromatography on carbon and celite (Regd. Trade Mark) and elution with diluted H<sub>2</sub>SO<sub>4</sub>.

#### EXAMPLE 6

85 1 g of the sulphate dissolved in 50 ml of water is treated with a slight excess of sodium *p*-hydroxy-phenylazobenzene-sulphonate. The precipitated dye-salt is filtered and crystallised several times from hot water. This salt is suitable for preparing a highly purified sulphate in the following way:

The dye-salt, suspended in methanol, is treated with dried HCl. From the resulting 95 solution hydrochloride is precipitated with acetone (yield: 0.6 g). The sulphate is obtained by dissolving the hydrochloride in water and adding 3 ml of triethylamine sulphate solution (containing 0.25 g of SO<sub>4</sub><sup>2-</sup> 100 per ml). The product is collected on a filter, washed with acetone and dried. Yield: 0.65 g, of highly purified F.I. 1600 sulphate. By analysis it contains 30.57% carbon, 6.44% hydrogen, 7.38% nitrogen and 9.08% 105 sulphur. Infra-red spectrum of F.I. 1600 sulphate in KBr (5%) shows strong absorption maxima at 2.94μ, 3.41μ, 6.15μ, 6.55μ, 9.00μ, 9.52μ, weak bands at 6.82μ, 7.08μ, 7.28μ, 7.46μ, 10.70μ, 11.17μ, 11.62μ, 13.08μ and 110 inflections at 3.09μ and 10.23μ.

#### Assay of F.I. 1600

A microbiological method of assay of F.I. 1600 is based on measurement of the inhibition zones around the agar wells containing solutions of various aerial dilutions 115 of the antibiotic against agar medium seeded with *B.subtilis* ATCC 6633. An aqueous solution of F.I. 1600 sulphate is used. 120

#### Toxicity - Toxicity per mouse:

LD 50 (intravenously administered)= 110 mg/Kg

LD 50 (subcutaneously administered)= 1.062 g/Kg 125

LD 50 (orally administered)= 25 g/Kg.

Toxicity per dog: The administration of a dose of 20 mg/Kg (intravenously) to a healthy dog causes a slight astenic syndrome. 130

Toxicity per rabbit: The administration of a dose of 2.5 mg (intracisternal) shows no signs of toxicity.

Mice are found to survive injections of 200 mg/Kg (per day) for two months without ill-effect.

Rats are not affected by subcutaneous injections of 200 mg/Kg (per day) for two months.

10 Cats receiving daily intramuscular injections of 50 mg/Kg show no neutrotoxic symptoms.

#### EXAMPLE 7

1.4 g of F.I. 1600 sulphate and 5 g of potassium bicarbonate are dissolved in 20 ml of water. 3 ml of benzoyl chloride are added to the above solution at 0°C and the mixture is stirred for 1 hour at 0°C, then allowed to stand for a night between 0° and +5°C, and at last at room temperature for some minutes. The precipitate is filtered, washed with an aqueous solution of sodium bicarbonate, water and at last with ether. The product is crystallised from 40 parts of hot methanol. 0.34 g of F.I. 1600 pentabenzate, as white crystals melting at 208-211°C without decomposition, are obtained. After several crystallisations from methanol, the m.p. does not rise above 208-211°C. By analysis it contains 60.60% carbon, 6.14% hydrogen, 6.05% nitrogen. It has the probable empirical formula  $C_{58}H_{65}N_5O_{19} \cdot CH_3OH$  (calcd: C 60.66; H 5.95; N 6.01).  $(\alpha)_D^{20} = +26^\circ \pm 2$  (C = 0.3% in methanol).

#### EXAMPLE 8

A suspension of 0.70 g of F.I. 1600 in 30 ml of methanol and 5 ml of acetic anhydride is allowed to stand for a night at room temperature. Three volumes of acetone are added and the precipitate is dissolved in aqueous methanol and then precipitated with acetone. 0.31 g of penta-acetate of F.I. 1600, as amorphous product melting at 198-204°C are obtained.  $(\alpha)_D^{20} = +62.6^\circ \pm 2$  (C = 1% in methanol).

By analysis it contains 43.23% carbon, 7.34% hydrogen and 7.45% nitrogen. It has the probable empirical formula

50  $C_{33}H_{35}N_5O_{19.5} \cdot 5 H_2O$  (calcd: C 43.27; H 7.15; N 7.65).

One mole of penta-acetyl-F.I. 1600, in 0.02 M. periodate solution, consumes 2.95 moles of periodate at room temperature (this consumption has reached after 18 hours and remains constant also after 42 hours).

#### EXAMPLE 9

In a cultural broth containing 266  $\gamma$ /ml of the antibiotic F.I. 1600 (volume 4000 ml; total content : 1.06 g), the pH is adjusted to 8.5 and the mixture is stirred twice with 40 g of carbon (Darco (Regd. Trade Mark) G 60) at room temperature for 1 hour.

65 The two filtered carbon cakes are stirred

for 1 hour in 600 ml of 50% aqueous solution adjusted to about pH 3 by adding aqueous HCl.

The mixture is filtered and the filtrate is neutralised with aqueous NaOH and concentrated to 210 ml (containing 2100  $\gamma$ /ml of F.I. 1600). The solution is evaporated to dryness, the residue is treated with methanol and 3 volumes of acetone are added to the filtered methanolic solution. The product is filtered and dried. Yield : 1.06 g containing 33% of antibiotic.

#### WHAT WE CLAIM IS:

1. A process for producing the antibiotic substance F.I. 1600 which comprises the cultivation of *Streptomyces Krestomyceticus* N.C.I.B. 8995 and Commonwealth Mycological Institute No. 79589, as herein described in an aqueous nutrient medium containing a source of carbon, nitrogen and mineral salts under aerobic conditions until substantial anti-bacterial activity is imparted to the medium and then recovering F.I. 1600 from the medium.

2. A process according to Claim 1, in which a heavy metal is present in the nutrient medium.

3. A process according to Claim 2, in which the heavy metal is copper, zinc, manganese, iron or chromium.

4. A process according to any one of the preceding claims in which the cultivation is carried out at a temperature of from 24°C to 32°C for a period of from two days to one week at a pH of from 6.3 to 7.8.

5. A process according to Claim 4, in which the initial pH is from 6.4 to 6.7 and the final pH is from 7.6 to 7.8.

6. A process according to any one of the preceding claims, in which the cultivation is carried out by submerged fermentation with aeration and agitation of the medium.

7. A process according to Claim 6, in which the ratio between the amount of air which must be introduced into the fermentation tank and the volume of the culture liquid varies through the fermentation phase from 0.1 to 0.8.

8. A process according to any one of the preceding claims, in which the F.I. 1600 is recovered from the medium by absorption onto a solid absorbant from which it is subsequently eluted.

9. A process according to claim 8 in which the solid absorbant is carbon.

10. A process according to Claim 8, in which the F.I. 1600 is recovered by absorption on an ion exchange resin.

11. A process according to Claim 10 in which the F.I. 1600 is recovered from the resin by elution, subsequently filtered and the filtered solution evaporated to dryness under vacuum.

12. A process according to any one of



Toxicity per rabbit: The administration of a dose of 2.5 mg (intracisternal) shows no signs of toxicity.

Mice are found to survive injections of 200 mg/Kg (per day) for two months without ill effect.

Rats are not affected by subcutaneous injections of 200 mg/Kg (per day) for two months.

10 Cats receiving daily intramuscular injections of 50 mg/Kg show no neurotoxic symptoms.

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#### EXAMPLE 8

A suspension of 0.70 g of F.I. 1600 in 30 ml of methanol and 5 ml of acetic anhydride is allowed to stand for a night at room temperature. Three volumes of acetone are added and the precipitate is dissolved in aqueous methanol and then precipitated with acetone. 0.31 g of penta-acetate of F.I. 1600, as amorphous product melting at 198-204°C are obtained.  $(\alpha)_D^{25} = +62.6^\circ \pm 2$  (C=1% in methanol).

By analysis it contains 43.23% carbon, 7.34% hydrogen and 7.45% nitrogen. It has the probable empirical formula 50  $C_{33}H_{55}N_5O_{19.5} \cdot 5 H_2O$  (calcd: C 43.27; H 7.15; N 7.65).

One mole of penta-acetyl-F.I. 1600, in 0.02 M. periodate solution, consumes 2.95 moles of periodate at room temperature (this consumption has reached after 18 hours and remains constant also after 42 hours).

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65 The two filtered carbon cakes are stirred

for 1 hour in 600 ml of 50% aqueous solution adjusted to about pH 3 by adding aqueous HCl.

The mixture is filtered and the filtrate is neutralised with aqueous NaOH and concentrated to 210 ml (containing 2100 γ/ml of F.I. 1600). The solution is evaporated to dryness, the residue is treated with methanol and 3 volumes of acetone are added to the filtered methanolic solution. The product is filtered and dried. Yield : 1.06 g containing 33% of antibiotic.

#### WHAT WE CLAIM IS:

1. A process for producing the antibiotic substance F.I. 1600 which comprises the cultivation of *Streptomyces Krestomyceticus* N.C.I.B. 8995 and Commonwealth Mycological Institute No. 79589, as herein described in an aqueous nutrient medium containing a source of carbon, nitrogen and mineral salts under aerobic conditions until substantial anti-bacterial activity is imparted to the medium and then recovering F.I. 1600 from the medium.

2. A process according to Claim 1, in which a heavy metal is present in the nutrient medium.

3. A process according to Claim 2, in which the heavy metal is copper, zinc, manganese, iron or chromium.

4. A process according to any one of the preceding claims in which the cultivation is carried out at a temperature of from 24°C to 32°C for a period of from two days to one week at a pH of from 6.3 to 7.8.

5. A process according to Claim 4, in which the initial pH is from 6.4 to 6.7 and the final pH is from 7.6 to 7.8.

6. A process according to any one of the preceding claims, in which the cultivation is carried out by submerged fermentation with aeration and agitation of the medium.

7. A process according to Claim 6, in which the ratio between the amount of air which must be introduced into the fermentation tank and the volume of the culture liquid varies through the fermentation phase from 0.1 to 0.8.

8. A process according to any one of the preceding claims, in which the F.I. 1600 is recovered from the medium by absorption onto a solid absorbant from which it is subsequently eluted.

9. A process according to claim 8 in which the solid absorbant is carbon.

10. A process according to Claim 8, in which the F.I. 1600 is recovered by absorption on an ion exchange resin.

11. A process according to Claim 10 in which the F.I. 1600 is recovered from the resin by elution, subsequently filtered and the filtered solution evaporated to dryness under vacuum.

12. A process according to any one of



75 methanolic



Claims 8 to 11 in which the F.I. 1600 is finally crystallised from a mixture of methanol and acetone.

13. A process according to any one of the preceding claims, in which the F.I. 1600 is further converted into the sulphate by treating the solution of the hydrochloride with triethylamine sulphate.

14. A process for producing F.I. 1600 or its salts substantially as hereinbefore described in Examples 1 to 6.

15. A process for producing F.I. 1600 or its salts substantially as hereinbefore described in Examples 7 to 9.

16. F.I. 1600 or its salts when produced by a process as claimed in any one of Claims 1 to 14.

17. An antibiotic F.I. 1600 as hereinbefore defined.

18. An antibiotic F.I. 1600 effective in inhibiting the growth of pathogenic micro-organisms or its salts which has a basic character, contains carbon, hydrogen, nitrogen and oxygen and gives penta-acyl derivatives by acylation, has a sulphate which is a white powder soluble in water and insoluble in almost all organic solvents, con-

taining 30.8-30.57% carbon, 7.00-6.44% hydrogen, 7.19-7.38% nitrogen and 9.08-8.99% sulphur and having characteristic absorption in the infra-red region when suspended in potassium bromide solution with pronounced peaks at the following frequencies (in  $\mu$ ) 2.94, 3.41, 6.15, 6.55, 9.00, and 9.52, weak bands at 6.82, 7.08, 7.28, 7.46, 10.70, 11.17, 11.62 and 13.08 and inflections at 3.09 and 10.23, e.g. aqueous solutions (C=1% of sulphate of  $20^\circ = +51^\circ$ , a hydrochloride which is a white powder insoluble in most organic solvents except methanol, e.g. aqueous solutions (C=1%) of hydrochloride of  $25^\circ = +55^\circ$

D and by analysis it contains 33.79 C, 6.76 H, 8.17 N and Cl-21.05%, a *p*(*p*-hydroxy-phenylazo)-benzene sulphonate, containing 50.58% C, 5.25% H, 10.27% N and 7.72% S and a helyanthine salt containing 51.29% C, 6.20% H, 12.23% N and 7.5% S.

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317 High Holborn,  
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#### PROVISIONAL SPECIFICATION

#### New Antibiotic

We, SOCIETA FARMACEUTICI ITALIA, a Body Corporate organised and existing under the laws of Italy, of 18 Via Filippo Turati, Milan, Italy, do hereby declare the invention to be described in the following statement:—

The present invention relates to a new and therapeutically useful broad spectrum antibiotic product, to its production by fermentation and a process for its recovery.

We have discovered that, using a new species of streptomyces, a high concentration of an antibiotic substance different from those known before is formed in a fermentation broth, that this product can be recovered under proper process conditions (to be described hereinafter) and further that this substance has properties which mark it as a new and useful therapeutic product. The new antibiotic provided by the present invention is designated by us as F.I. 1600, and we include in that denomination also the free base or its salts and solutions thereof. The new organism which permits the production of the antibiotic F.I. 1600 is now designated by us as *Streptomyces Krestomyceticus* (n.sp.).

This strain has been found in a soil sample obtained at Massa Marittima (Grosseto), isolated and grown on synthetic as well as organic media within the range of 28-37°C. Under the electron microscope *S. Krestomyceticus* shows smooth spores rather regular, cylindrical or oval in shape, somewhat transparent. The hyphae are usually straight but sometimes hooks and spirals are observed. The strain of *S. Kresto-*

*myceticus* has been investigated for its classification. Table I sets forth its cultural characteristics.

#### TABLE I

|                                           |                                                                                                                                                        |        |
|-------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------|--------|
| Agar glycerin glycine                     | Good growth colourless with abundant production of spores. Aerial hyphae and spores white.                                                             | 90     |
| Soil-Extract Agar                         | Good colourless growth, covered with moderate formation of whitish aerial mycelium.                                                                    | 95     |
| Czapek Agar                               | Colourless growth very superficial. Good white sporulation.                                                                                            | 100    |
| YED salts Agar (Yeast extract, and salts) | Bulky yellowish growth not very deep into the substrate; moderate white sporulation.                                                                   | 105    |
| Peptone and salts agar                    | Good growth, colourless to yellowish, with good white sporulation; very short hyphae.                                                                  | 110    |
| Glycerin agar                             | Bulky nearly colourless growth; sparse formation of aerial mycelium.                                                                                   | scarce |
| Asparagine dextrose agar                  | Yellowish small colonies, opaque, flat and waxy, not very evident, without aerial mycelium.                                                            | 115    |
| Potato dextrose agar                      | Not very voluminous colourless growth with sparse whitish aerial mycelium. Typical concentric splits or crackings on the bottom of the culture slants. | 120    |



|    |                                                                 |                                                                                                                                                                                       |                           |                                                                                                                                                                                                                                                                                                                                                                                |     |
|----|-----------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
|    | Casein hydrolysate agar, supplemented with salts and aminoacids | Small scanty colourless growth. No aerial mycelium.                                                                                                                                   | Saubouraud agar           | which only appears occasionally on some folds. Good colourless or cream-coloured growth covered by an abundant white aerial mycelium.                                                                                                                                                                                                                                          | 70  |
| 5  | PYS agar (peptone yeast salts)                                  | Abundant, colourless growth flat, with scarce aerial mycelium, superficial splits.                                                                                                    | Yeast Broth               | Superficial bulky and yellowish growth covered with white aerial mycelium.                                                                                                                                                                                                                                                                                                     |     |
| 10 | Potato plugs                                                    | Excellent growth throughout the plug, completely covered with abundant and aerial mycelium well sporulated.                                                                           | <i>S. Krestomyceticus</i> | hydrolyses both starch and gelatin, and decomposes tyrosine within 6 days.                                                                                                                                                                                                                                                                                                     | 75  |
| 15 | Emerson's agar                                                  | Yellowish growth with undulated surface showing many splits. The whole surface is covered by a very short whitish aerial mycelium more evident where the growth is thicker.           |                           | Acids are produced using the following substances as sole source of carbon: inulin, threulose, levulose, sorbitol, mannose, galactose, lactose, adonitol, mannitol, maltose, glycerol, dextrose, dextrin.                                                                                                                                                                      | 80  |
| 20 |                                                                 |                                                                                                                                                                                       |                           | Milk is only slightly coagulated after 13 days. Neutral reaction, no peptonisation.                                                                                                                                                                                                                                                                                            |     |
|    | Casein hydrolysate agar                                         | Very abundant and thick growth not very deep into substrate. Evident splits with reversed rims. Uniformly distributed short aerial mycelium but evident. The reverse is sharp yellow. |                           | The cultural characteristics of the F.I. 1600 producing strain have been compared with those of species already described.                                                                                                                                                                                                                                                     | 85  |
| 25 |                                                                 |                                                                                                                                                                                       |                           | Among these species, <i>S. Virgatus</i> and <i>S. flocculus</i> differ from our strain because their vegetative mycelium is greenish and the former also produces a brown pigment on some media. Besides <i>S. flocculus</i> has its surface covered by a velvety or cottony aerial mycelium.                                                                                  | 90  |
| 30 | Soyabean meal agar                                              | Undulated growth with splits. Yellowish vegetative mycelium covered by a very short whitish aerial mycelium.                                                                          | <i>S. termophilus</i>     | presents a deep growth into the medium and a whitish aerial mycelium.                                                                                                                                                                                                                                                                                                          | 95  |
| 35 | Soyabean meal glycerin agar                                     | Flat, opaque and brownish colonies with very short aerial mycelium on the bottom of the slants.                                                                                       |                           | Further it has a growth optimum at 50°C. The F.I. 1600 producing strain shows a very good growth at 37°C but very small amounts of antibiotic are produced at 33°C or more.                                                                                                                                                                                                    | 100 |
| 40 | Corn steep agar                                                 | Similar to soyabean meal agar.                                                                                                                                                        | <i>S. Kanamyceticus</i>   | shows a deep growth into the substrate and sometimes greenish or rosy shades in the vegetative mycelium. Sometimes it also produces a brown pigment. No one of the aforesaid species show the typical splits on the surface of the culture, which are the most peculiar characteristics of the F.I. 1600 producing strain.                                                     | 105 |
| 45 | Peptone Corn steep agar                                         | Flat and yellowish growth with few splits. Short aerial mycelium, abundant, more evident on the borders of the culture.                                                               |                           | On the basis of those characteristics, which do not correspond to those of any known species, <i>S. Krestomyceticus</i> has been considered a new species. The production of the antibiotic F.I. 1600 has been carried out by fermentation in submerged aerial cultures in an aqueous nutrient solution containing a source of nitrogen, a source of carbon and mineral salts. | 110 |
|    | Anderson's sporulation medium                                   | Yellowish growth generally flat, showing splits where the agar is thicker. Some aerial mycelium on the folds.                                                                         |                           |                                                                                                                                                                                                                                                                                                                                                                                | 115 |
| 50 | Tryptone agar                                                   | Similar to the Anderson's medium, but with colourless vegetative mycelium; abundant aerial mycelium.                                                                                  |                           | As a nitrogen source, the best results are obtained by using protein containing material such as barley malt extract, corn malt extract, corn steep liquor, enzymatic casein hydrolysate, peanut meal, soyabean meal, cotton bean meal, meat extract.                                                                                                                          | 120 |
| 55 | NZ Amine asparagine agar                                        | Flat, yellow colonies with small tufts of white aerial mycelium.                                                                                                                      |                           | As a source of assimilable nitrogen the inorganic ammonium salts are suitable. Among these are ammonium sulphate and ammonium phosphate. It is also possible to use organic sources of nitrogen such as the amino acids and various proteinaceous natural materials.                                                                                                           | 125 |
| 60 | Pridham's medium with starch and inorganic salts                | Flat yellowish colonies which are more wrinkled where the substrate is thicker.                                                                                                       |                           |                                                                                                                                                                                                                                                                                                                                                                                | 130 |
| 65 | Oats meal agar                                                  | Flat yellowish colonies with colourless borders. Aerial mycelium generally absent,                                                                                                    |                           |                                                                                                                                                                                                                                                                                                                                                                                |     |



The carbon source may be either a soluble carbohydrate such as dextrose or an insoluble one such as starch, for example corn starch. A good yield of antibiotic is obtained also with maltose, dextrin, lactose and other sugars. The mineral salts which are necessary for the fermentation medium are preferably phosphates, which may be present either as ammonium phosphate or as metallic phosphate such as potassium phosphate, chlorides such as NaCl and sulphates such as ammonium or magnesium sulphate.

Suitable buffering agents are calcium carbonate and salts of organic acids such as citrates, acetates and lactates which are useful in maintaining the pH within the proper range.

It is necessary, sometimes, to provide a source of heavy metals which may be present as impurities or added; among these are copper, zinc, manganese, iron and chromium.

The use of a defoaming agent is desirable in large scale fermentors, even though in this fermentation foaming is not a particularly difficult problem and is readily controlled by the use of conventional defoaming agents such as octadecanol in lard oil or other suitable commercial defoamers such as silicons.

The relative ratio of the fermentation medium ingredients has been quantitatively established as follows:

|    |                   |                |
|----|-------------------|----------------|
| 35 | Nitrogen source   | 0.5-4%         |
|    | Carbon source     | 1-5%           |
|    | Phosphates        | 0.01-0.3%      |
|    | Sulphates         | 0.05-0.25%     |
|    | CaCO <sub>3</sub> | 0.1-0.7%       |
| 40 | Chlorides         | 0.1-0.3%       |
|    | Trace elements    | 0.0001-0.0005% |

The extract amounts of all the substances are particularly indicated in the examples. The fermentation is carried out at a temperature of from 24°C to 32°C for a period of from two days to one week at a pH of 6.3-7.8. The initial pH which ensures the most favourable fermentation process is 6.4-6.7; it increases to 7.6-7.8 towards the end of the fermentation.

Aeration is also necessary for a good yield. The ratio between the amount of air which must be introduced into the fermentation tank and the volume of the culture liquid varies through the fermentation phases between 0.1 and 0.8.

F.I. 1600 may be recovered as a hydrochloride as well as a sulphate from the fermentation broth, by adopting a recovery

process which we have devised, which has proved to be highly effective from the standpoint of recovering a product free of inactive impurities, and of other antibiotic substances simultaneously formed in the broth. This method is exemplified below. Basically, it involves the following steps:

a) Precipitation of Ca<sup>++</sup> with oxalic acid and filtration of mycelium and calcium oxalate after adjusting the pH to 7-7.5.

b) Absorption of the antibiotic on a cationic exchange resin and elution with dilute acids, such as 1N HCl.

c) Neutralisation of the eluted solution with an anionic exchange resin, in hydroxylate phase, until the pH rises up to 7-7.5 and subsequently filtering.

d) Concentration of the filtered solution to dryness under vacuum.

e) Dissolution of the antibiotic in a suitable organic solvent, such as methanol.

f) Precipitation of the F.I. 1600 hydrochloride by addition of acetone to the organic solution.

g) The filtered F.I. 1600 hydrochloride is dissolved in water and the aqueous filtered solution, after concentration to a small volume under vacuum, is treated with triethylamine sulphate. After filtering, the solution is added to methanol with stirring. The F.I. 16000 sulphate is filtered, washed with methanol and dried *in vacuo* over P<sub>2</sub>O<sub>5</sub> at 50°C.

F.I. 1600 is a basic substance and is water soluble.

F.I. 1600 in the form of the sulphate or hydrochloride, is a white powder, soluble in water and insoluble in almost all organic solvents. The F.I. 1600 hydrochloride is soluble in methanol.

The ultra-violet spectrum shows no absorption within the range of 220-400μm. Infra-red spectrum of the F.I. 1600 sulphate is KBr (5%), which is reported in the accompanying drawing, shows absorption maxima attributed to the following groups: OH, NH, CH, CH<sub>2</sub>, NH<sub>3</sub><sup>+</sup> ion, C-O-C.

There is no evidence of carbonyl groups in keto, amide or ester form.

The optical rotations of highly pure F.I. 1600, hydrochloride and sulphate in water were determined.

Hydrochloride

(α)<sub>D</sub><sup>25</sup> = +55° (c=1% in water)

D

Sulphate (α)<sub>D</sub><sup>20</sup> = +51° (c=1% in water)

D

Table II sets forth the elementary analysis of the F.I. 1600 salts.





| <i>F.I. 1600 Salts</i>                              | <i>C%</i> | <i>H%</i> | <i>N%</i> | <i>S%</i> | <i>Cl%</i> |
|-----------------------------------------------------|-----------|-----------|-----------|-----------|------------|
| Hydrochloride                                       | 33.79     | 6.76      | 8.17      | —         | 21.05      |
| Sulphate                                            | 30.8      | 7.00      | 7.19      | 8.99      | —          |
| 5 <i>p.(p.hydroxy-phenylazo)-benzene-sulphonate</i> | 58.58     | 5.25      | 10.27     | 7.72      | —          |
| Helyanthine salt                                    | 51.29     | 6.20      | 12.23     | 7.50      | —          |

On the basis of elementary analysis, the basic properties of the product, the fact that all nitrogen is basic, its U.V. and I.R. spectra, we exclude any polypeptide type structure and we attribute to F.I. 1600 a structure similar to that one of the group of oligosaccharide basic water soluble antibiotics, such as streptomycin and neomycin. Chromatographically, F.I. 1600 may be distinguished from streptomycin, dihydro-streptomycin, neomycin, viomycin and other glycopeptidic basic substances such as

viomycin



streptothricin and the geomycins.

Prolonged acid hydrolysis of Neomycins or of Catenulin (J. W. Davisson et al. Antibiot. and Chem. 2, 1952, p.460) yields a microbiologically active fragment, identified by paper chromatography as neamine.

Acid hydrolysis of F.I. 1600 with 6N HCl yields no active fragment.

Table III shows the differences between F.I. 1600 and the other antibiotics of the same group.

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25

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TABLE III

| Antibiotic           | Organism                        | Analysis                                                                                                                 | ( $\alpha$ ) <sub>D</sub> of sulphate | Other properties                                                                                                                                                                                              |
|----------------------|---------------------------------|--------------------------------------------------------------------------------------------------------------------------|---------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 5 F.I. 1600 sulphate | <i>Strept. Krestomy-ceticus</i> | C = 30.8<br>H = 7.00<br>N = 7.19<br>S = 8.99                                                                             | +51                                   | a) By acid hydrolysis no formation of microbio-logically active fragment.<br>b) No cross-resistance with neomycin (see below).                                                                                |
| 10 Neomycin          | <i>Strept. fradiae</i>          | B C = 29.35<br>H = 6.86<br>N = 9.21<br>(1) SO <sub>4</sub> = 28.3<br>C = 31.18<br>C H = 6.14<br>(2) N = 8.93<br>S = 9.90 | B = +56(2)<br>C = +82(2)              | a) By acid hydrolysis for-mation of active frag-ment (neamine).<br>b) Infra-red spectrum is dif-ferent from that of F.I. 1600.<br>c) It is chromatographically different from F.I. 1600.                      |
| 15 Strepto-mycin     | <i>Strept. griseus</i>          | C = 34.62<br>H = 5.77<br>N = 13.45<br>S = 6.61                                                                           | -79°(3)                               | a) Infra-red spectrum is dif-ferent from that of F.I. 1600.<br>b) It is chromatographically different from F.I. 1600.                                                                                         |
| 20 Catenulin         | Not des-cribed                  | C = 31.53<br>31.58<br>H = 5.29<br>5.26 (4)<br>N = 7.92<br>7.93<br>SO <sub>4</sub> = 28.11<br>28.13                       | =51.9(4)                              | a) Infra-red absorption (not reported) is typical of a polypeptide spectrum (4).<br>b) By acid hydrolysis for-mation of active frag-ment (neamine) (4).<br>c) It shows crossed resist-ance with neomycin (6). |
| 25 Kana-mycin        | <i>Strept. Kanamy-ceticus</i>   | C = 37.3<br>37.4<br>H = 6.8<br>6.3 (5)<br>N = 9.3<br>9.6<br>S = 5.5                                                      | +146°(5)                              | Infra-red spectrum is dif-ferent from that of F.I. 1600.                                                                                                                                                      |

## References:

- 1) Regna et al. J. Chem. Soc. 1950, p. 1045
- 40 2) Ford et al. J. Chem. Soc. 1955, p. 5311
- 3) Regna et al. J. Biol. Chem. 165, 1946, p. 621
- 4) Davisson et al. Antibiotic and Chem. 2, 1952, p. 460
- 45 5) Cron et al. J. Chem. Soc. 80, 1958 p. 752
- 6) Szybalsky et al. Am. Rev. Tuberc. 69, 1954, p. 267.
- 50 Therefore it appears that F.I. 1600 provided by the present invention, is a new component of the group of basic, water soluble oligosaccharide type antibiotics.

## Pharmacological properties

F.I. 1600 shows the following pharmaco- 55 logical properties:

- a) Broad antibiotic spectrum which com-prises gram-positive, gram-negative and acid resistant bacteria.
- b) Inhibitory actions against the afore- 60 said organisms at very low concen-tration.
- c) Activity against antibiotic resistant strains.
- 4) Very low toxicity. 65

Table IV shows the minimum dose in mcg/ml (MID) of F.I. 1600 sulphate inhibit-ing the growth of the under mentioned micro-organisms in Yeast-extract broth.



\* C L M S \*



TABLE IV

| Test organism            | After 24<br>hours of<br>incubation | After 48<br>hours of<br>incubation |
|--------------------------|------------------------------------|------------------------------------|
| <i>M.pyogenes aureus</i> | 1                                  | 1                                  |
| <i>B.subtilis</i>        | 1                                  | 1                                  |
| <i>S.faecalis</i>        | 1                                  | 1                                  |
| <i>Mycobacterium 607</i> | 1                                  | 5                                  |
| <i>M.phlei</i>           | 1                                  | 5                                  |
| <i>A.bostromi A</i>      | 1                                  | 1                                  |
| <i>E.coli</i>            | 5                                  | 5                                  |
| <i>K.friedlanderi</i>    | 5                                  | 25                                 |
| <i>P.vulgaris</i>        | 25                                 | 25                                 |
| <i>P.aeruginosa</i>      | 100                                | >100                               |
| <i>S.flexneri Y</i>      | 25                                 | 25                                 |
| <i>O.albicans</i>        | >100                               | >100                               |
| <i>G.graphii</i>         | >100                               | >100                               |
| <i>T.mentagrophytes</i>  | >100                               | >100                               |

TABLE V

| Test organism                     | MID<br>mcg/ml<br>liquid<br>Dubos<br>medium |    |
|-----------------------------------|--------------------------------------------|----|
| <i>M.Tuberculosis H 37 Rv</i>     | 1                                          | 25 |
| <i>M.tbc. - isoniazide res.</i>   | 2.5                                        | 30 |
| <i>Mycobacterium sp. ATCC 607</i> | 2.5                                        |    |
| <i>M. 607 Strept. resistant</i>   | 2.5                                        |    |
| " " <i>Chlortetracycl</i> "       | 2.5                                        | 35 |
| " " <i>Oxytetracycl</i> "         | 2.5                                        |    |
| " " <i>Neom.</i> "                | 5.0                                        |    |
| " " <i>Viom.</i> "                | 2.5                                        |    |
| " " <i>Oxam.</i> "                | 2.5                                        |    |
| " " <i>Eritrom.</i> "             | 5.0                                        |    |
| " " <i>Vancom.</i> "              | 5.0                                        | 40 |
| <i>M.phlei</i>                    | 0.98                                       |    |
| <i>M.para-smegmatis</i>           | 0.124                                      |    |

20 The same concentrations of the antibiotic inhibit the growth of *M.tbc* or *M.607* and that of many antibiotic resistant strains of the same species-(see Table V).

Table VI shows the relative amounts in mcg/ml (MID) of F.I. 1600 sulphate required to inhibit a number of representative 45 organisms over different cultures.

TABLE VI

In the table are reported the values of bacteriostatic and bactericidal concentrations of F.I. 1600 in different culture media.

| Test Organism |                               | MID in mcg/ml               |                   |                            |                                       |
|---------------|-------------------------------|-----------------------------|-------------------|----------------------------|---------------------------------------|
|               |                               | Yeast<br>broth              | Nutrient<br>broth | Penessay<br>broth<br>Difco | Brain<br>heart in-<br>fusion<br>Difco |
| 5             |                               |                             |                   |                            |                                       |
| 10            |                               |                             |                   |                            |                                       |
| 15            |                               | Bacteriostatic              |                   |                            |                                       |
|               | <i>Staphyl. aureus</i> 114    | 0.35                        | 0.6               | 1.6                        | 3.8                                   |
|               | <i>Staphyl. aureus</i> 209 P  | 0.62                        | 1.05              | 4.0                        | 12.0                                  |
|               | <i>Staphyl. aureus</i> 503 MB | 0.075                       | 0.31              | 0.13                       | 0.35                                  |
|               | <i>Staphyl. aureus</i> CAMP   | —                           | 0.15              | 1.45                       | 4.8                                   |
|               | <i>Staphyl. Londres</i>       | 0.15                        | 0.45              | 3.5                        | 6.0                                   |
| 20            |                               | Bactericidal after 24 hours |                   |                            |                                       |
|               | <i>Staphyl. aureus</i> 114    | 2.5                         | 2.5               | 10                         | >20                                   |
|               | <i>Staphyl. aureus</i> 209 P  | 10                          | 20                | 20                         | >20                                   |
|               | <i>Staphyl. aureus</i> 503 MB | 0.3                         | 1.25              | 20                         | 5                                     |
|               | <i>Staphyl. aureus</i> CAMP   | 5                           | 20                | 5                          | 10                                    |
|               | <i>Staphyl. Londres</i>       | 5                           | 5                 | 10                         | 20                                    |
| 25            |                               | Bactericidal after 48 hours |                   |                            |                                       |
|               | <i>Staphyl. aureus</i> 114    | 2.5                         | 2.5               | 10                         | >20                                   |
|               | <i>Staphyl. aureus</i> 209 P  | 10.0                        | 20                | 20                         | >20                                   |
|               | <i>Staphyl. aureus</i> 503 MB | 0.3                         | 1.25              | 20                         | 5                                     |
|               | <i>Staphyl. aureus</i> CAMP   | 5.0                         | 20                | 5                          | 10                                    |
|               | <i>Staphyl. Londres</i>       | 5                           | 5                 | 10                         | 20                                    |

The following Examples are given to illustrate the production and the recovery of F.I. 1600 by fermentation under submerged aerobic conditions. The same media may be used for fermentation in shaker flasks on a smaller scale or in tanks on a larger scale.

However the invention is not limited by the details set forth herein. It will be apparent to those skilled in the art that numerous modifications in materials and methods can be practiced without departing from the spirit of the invention.

#### EXAMPLE 1

300 ml flasks containing 60 ml of a medium having the following composition:

|                   |      |
|-------------------|------|
| Soyabean flour    | 2%   |
| NaCl              | 2.5% |
| Dextrose          | 3%   |
| Corn-steep        | 1.5% |
| CaCO <sub>3</sub> | 0.7% |

are sterilized at 120°C for 20 minutes and

inoculated each with 0.25 ml of a suspension of spores of *S. Krestomyceticus*.

The flasks are incubated at 28°C on a shaking machine. A good growth is obtained within 7-8 days and about 500 mcg/ml of antibiotic are produced.

#### EXAMPLE 2

A spore suspension of *S. Krestomyceticus* is inoculated into 2000 ml Erlenmeyer flasks containing 500 ml of a suitable medium which has the following composition:

|                                                 |         |
|-------------------------------------------------|---------|
| Dextrin                                         | 2%      |
| Casein                                          | 0.5%    |
| CaCO <sub>3</sub>                               | 0.4%    |
| MgSO <sub>4</sub>                               | 0.1%    |
| (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> | 0.1%    |
| ZnSO <sub>4</sub> · 7H <sub>2</sub> O           | 0.0002% |

After 48 hours of stirring at 28°C, 0.4% of the culture is transferred into a 10 litre tank containing the following medium:



55

60

65





|                                                 |       |
|-------------------------------------------------|-------|
| Dextrin                                         | 2%    |
| Casein                                          | 0.5%  |
| CaCO <sub>3</sub>                               | 0.4%  |
| (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> | 0.1%  |
| K <sub>2</sub> HPO <sub>4</sub>                 | 0.01% |
| Corn steep                                      | 0.5%  |
| (pH after sterilisation=6.6)                    |       |

After 7 hours of fermentation with stirring at 28°C under aerobic conditions (1.1 l/m) the culture has its maximum growth.

#### EXAMPLE 3

A 2000 ml flask containing 500 ml of a medium having the following composition:

|                    |      |
|--------------------|------|
| Casein hydrolysate | 1%   |
| NaCl               | 0.2% |

is sterilized at 120°C for 20 minutes and inoculated with a suspension of spores of *S. Krestomyceticus* (cultivated on potato-agar medium). The flask is incubated at 28°C for 48 hours with stirring.

After 48 hours the culture is transferred into a 100 litre tank containing the following medium:

|                   |      |
|-------------------|------|
| Soyabean flour    | 2%   |
| Dextrin           | 2.0% |
| CaCO <sub>3</sub> | 0.3% |
| (pH=6.16)         |      |

After 36 hours of fermentation with stirring at 28°C under aerobic conditions, about 15% of the culture is transferred into a 1000 litre tank containing a medium of the following composition:

|                   |       |
|-------------------|-------|
| Soyabean flour    | 2.5%  |
| Dextrin           | 2.5%  |
| Distiller's       | 0.5%  |
| NaCl              | 0.25% |
| Soyabean oil      | 0.03% |
| CaCO <sub>3</sub> | 0.03% |
| (pH=6.4-6.5)      |       |

The fermentation is continued for 80-90 hours at 28°C with stirring and under aerobic conditions.

#### EXAMPLE 4

To about 500 litres of fermentation broth is added with stirring oxalic acid (correspondent to Ca<sup>++</sup> contained in the broth), with subsequent adjustment of the pH to 7-7.5 with NaOH. The stirring is continued for 30 minutes and the suspension is filtered.

The cake is washed with water.

The filtered solution is absorbed over 5 litres of IRC 50 (sodium phase).

By elution with 1N HCl several separated fractions are obtained.

The active fractions are neutralised with anionic exchange resins concentrated to dryness under vacuum and the residue is treated, 2-3 times, with 1 litre of methanol with stirring for 30 minutes. Each time the suspension is filtered. The F.I. 1600 hydrochloride is obtained by adding the methanolic solution to acetone (2-3 volumes); the white flocced product is filtered, washed with acetone and dried; yield=80-90 g.

#### EXAMPLE 5

The F.I. 1600 hydrochloride is dissolved

in water and the solution is filtered. The filtrate is concentrated to a small volume under vacuum, then triethylamine sulphate is added. After cooling in a refrigerator, the isolated calcium sulphate is eliminated by filtration. The filtered solution is added to methanol with stirring. When the addition is finished, the stirring is continued for 30 minutes and the F.I. 1600 sulphate is filtered and washed with methanol. The product is dried at 50° *in vacuo* over P<sub>2</sub>O<sub>5</sub>. The raw sulphate is purified by chromatography on carbon and celite and elution with diluted H<sub>2</sub>SO<sub>4</sub>.

#### EXAMPLE 6

1 g of the sulphate dissolved in 50 ml of water is treated with a slight excess of sodium *p*-hydroxy-phenylazobenzene-sulphonate. The precipitated dye-salt is filtered and crystallised several times from hot water. This salt is suitable for preparing a highly purified sulphate in the following way:

The dye-salt, suspended in methanol, is treated with dried HCl. From the resulting solution hydrochloride is precipitated with acetone (yield: 0.6 g). The sulphate is obtained by dissolving the hydrochloride in water and adding 3 ml of triethylamine sulphate solution (containing 0.25 g of SO<sub>4</sub><sup>2-</sup> per ml). The product is collected on a filter, washed with acetone and dried.

Yield: 0.65 g.

#### Assay of F.I. 1600

A microbiological method of assay of F.I. 1600 is based on measurement of the inhibition zones around the agar wells containing solutions of various serial dilutions of the antibiotic against agar medium seeded with *B. subtilis* ATCC 6633. An aqueous solution of F.I. 1600 sulphate is used.

Toxicity - Toxicity per mouse:

LD 50 (intravenously administered)= 110 mg/Kg

LD 50 (subcutaneously administered) = 1.062 g/Kg

LD 50 (orally administered)= 25 g/Kg.

Toxicity per dog: The administration of a dose of 20 mg/Kg (intravenously) to a healthy dog causes a slight, astenic syndrome only.

Toxicity per rabbit: The administration of a dose of 2.5 mg (intracisternal) shows no signs of toxicity.

Mice are found to survive injections of 200 mg/Kg (per day) for two months without ill effect.

Rats are not affected by subcutaneous injections of 200 mg/Kg (per day) for two months.

Cats receiving daily intramuscular injections of 50 mg/Kg show no neurotoxic symptoms.

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## PROVISIONAL SPECIFICATION

## Improvements relating to Antibiotics

We, SOCIETA FARMACEUTICI ITALIA, a Body Corporate organised and existing under the laws of Italy, of 18 Via Filippo Turati, Milan, Italy, do hereby declare this invention to be described in the following statement:—

The invention relates to antibiotics.

In the Specification of our copending Application No. 108589 there is described a new antibiotic designated F.I. 1600 and methods for its recovery.

We have now found that the antibiotic F.I. 1600 can also be recovered by absorption and elution from carbon. More specifically we have found that F.I. 1600 may be removed either from its aqueous solutions or from cultural broths by carbon, preferably at a pH higher than 7. F.I. 1600 may be eluted from carbon either by means of an aqueous solution or by means of alcoholic solutions of inorganic or organic acids.

The following Example is given to illustrate the invention.

## EXAMPLE

In a cultural broth, containing 2667/ml

of the antibiotic F.I. 1600 (volume 4000 ml; total content : 1.06 g), the pH is adjusted to 8.5 and the mixture is stirred twice with 40 g of carbon (Darcó G 60) at room temperature for 1 hour.

The two filtered carbon cakes are stirred for 1 hour in 600 ml of 50% aqueous solution adjusted to a pH of about 3 by adding aqueous HCl.

The mixture is filtered and the filtrate is neutralized with aqueous NaOH and concentrated to 210 ml (containing 2100  $\gamma$ /ml of F.I. 1600). The solution is evaporated to dryness, the residue is treated with methanol and 3 volumes of acetone are added to the filtered methanolic solution. The product is filtered and dried. Yield: 1.06 g containing 33% of antibiotic.

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