A NEW ANTIBIOTIC, C-9154

Sir:

In the course of studies on antibiotics, it was found that a new species of *Streptomyces*, *Streptomyces ishigakiensis* produces a novel antibiotic C-9154. The present communication deals with the taxonomy of *S. ishigakiensis*, isolation of the antibiotic C-9154 and definition of its physico-chemical and biological properties, and chemical structure.

1. Taxonomy

Streptomyces sp. strain No. C-9154 was isolated from a soil sample collected in Kawahira, Ishigakijima, Okinawa Prefecture. The taxonomic characteristics of strain No. C-9154 are summarized as follows: it is a chromogenic type of *Streptomyces*; it forms many kinds of sporophores including straight, flexuous, imperfect spirals and true spirals but not whorls, and moreover forms sclerotic granules (5~15 μ in diameter) which originate from the aerial mycelium; the surface of the spores is smooth; the strain generally forms a colorless to pale brown growth with white to gray aerial mycelium. Cell wall preparations of the strain contain the LL isomer of diaminopimelic acid with alanine, glutamic acid and glycine as major components.

Among the known species of *Streptomyces*, *S. griseosporeus*[1~3] was found to be similar to the new strain, especially, with respect to its aerial mass color and chromogenicity. However, *S. griseosporeus* differs from strain No. C-9154 in the following characters: *S. griseosporeus* forms neither true spiral nor sclerotic granules; it exhibits little aerial mycelium when grown on glucose asparagine agar and starch inorganic salts agar, and it does not produce a soluble pigment on glucose asparagine agar. Carbon sources such as D-xylose, fructose, lactose, rhamnose, D-sorbitol, D-mannitol and *i*-inositol are moderately utilyzed for growth. Differences between the taxonomic properties given above and those of recognized species in the genus *Streptomyces* are considered significant enough to warrant the assignment of a new species name, *S. ishigakiensis* sp. nov. to strain No. C-9154. Toxonomic details will be presented elsewhere in near future.

2. Production and Isolation

Submerged culture fermentations were conducted for 66 hours at 24°C in a medium consisting of 2 % glucose, 3 % soluble starch, 1 % corn steep liquor, 1 % soybean flour, 0.5 % peptone, 0.3 % NaCl and 0.5 % $CaCO_3$. The pH was adjusted to 7.0 prior to sterilization.

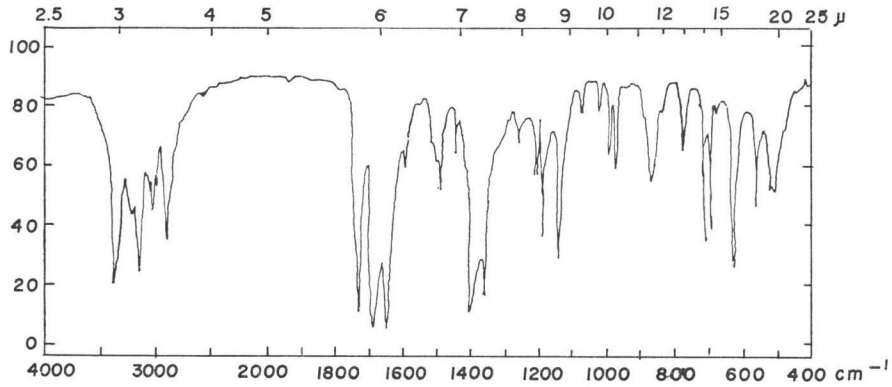
The culture broth (20 liters) was diluted with an equal volume of acetone followed by filtration. Concentration of the filtrate was followed by extraction with ethyl acetate. The ethyl acetate extract was concentrated to give crude crystals of C-9154 (4.5 g). Alternatively, the concentrated aqueous acetone filtrate was mixed with an equal volume of methanol, applied to a column of Amberlite XAD-2, which was developed with 50 % aqueous methanol. The active fraction was then adsorbed on an active carbon column, and the column washed with water and 50 % aqueous methanol. The active substance was eluted with a mixture of methanol-acetone-water (2:2:1) and then concentrated to yield crude crystals (4.5 g). Recrystallization of the crude crystals was carried out using a mixture of methanol-acetone-water (2:2:1) to give pure compound C-9154 as colorless plates or prisms.

3. Physical and Chemical Properties and Structural Determination

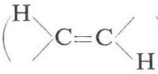
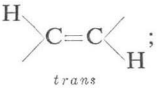
The antibiotic was obtained as colorless crystals, mp 219°C (decomp.), $[\alpha]_D^{22}$ 0° (*c* 0.5, dimethyl sulfoxide). The molecular formula $C_{12}H_{12}N_2O_3$ was established by elemental analysis (Found: C, 61.77; H, 5.16; N, 11.93; O, 22.08. Calcd. for $C_{12}H_{12}N_2O_3$ (M.W. 232): C, 62.06; H, 5.21; N, 12.06; O, 20.67) and mass spectrometry ($M^+ = m/e$ 232). Antibiotic C-9154 shows positive color reactions with a peptide-detection reagent,[4] potassium permanganate, but exhibits negative ninhydrin, FEHLING, EHRLICH, and SAKAGUCHI tests. It is soluble in dimethyl sulfoxide, dimethylformamide, pyridine and a mixture of acetone-methanol-water (2:2:1); sparingly soluble in acetone, methanol, ethyl acetate and chloroform; and

After we had completed this study, we read the Abstracts of Paper of 95th Meeting of Pharmaceutical Society of Japan presented by MARUYAMA *et al.* and SUHARA on the same compound (Ro 09-0049) which is produced by *Streptomyces kurssanovii* (Abs. II, 273 (1975): III, 53 (1975)).

Fig. 1. IR spectrum of C-9154 (KBr).

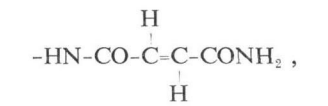


almost insoluble in diethyl ether, benzene and water.

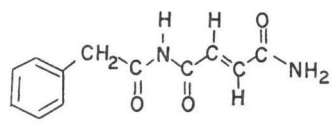
Compound C-9154 has an ultraviolet absorption maximum at 225 nm (ε 27840) in methanol. The IR spectrum (Fig. 1) exhibits characteristic absorption bands at about 3400, 3220 (NH, NH_2), 1738 (C=O), 1695, and 1667 cm^{-1} (conjugated C=O and amide C=O). The NMR spectrum in dimethyl sulfoxide (100 MHz, tetramethylsilane as the internal reference) shows signals at δ 3.87 ppm (s, 2H) ($-CH_2-$); 6.96 ppm (d, 1H, J=15 Hz); 7.17 ppm (d, 1H, J=15 Hz)  ; 7.23 ppm (s, 5H) (aromatic ring protons); 7.37 ppm (s, 1H, disappears with D_2O); 7.83 ppm (s, 1H, disappears with D_2O; and 11.11 ppm (s, 1H, disappears with D_2O). On thin-layer chromatography using silica gel (Spot Film F, Tokyo Kasei) C-9154 gives a single spot at Rf 0.6 with chloroform - methanol (5:1, v/v) and at Rf 0.3 with ethyl acetate - acetone (3:1, v/v). C-9154 was not acetylated with acetic anhydride and pyridine. Consequently, the oxygens in the molecule were attributed to three carbonyl residues. The NMR signals at δ 6.96 and 7.13 ppm were assigned to isolated *trans* vinyl protons. The signals which disappeared with D_2O were assigned as follows: δ 11.11 ppm (s, 1H) is NH, and δ 7.37 ppm (1H) and 7.83 ppm (1H) are due to NH_2 protons, because they appeared at 7.3 ppm as a broad signal when measured at 60°C. These observations indicated the partial structures; C_6H_5-; $-CH_2-$; 3  ;  ; =NH; and $-NH_2$. The UV

trans

spectrum of C-9154 clearly indicates that the phenyl group and the double bond are not conjugated, therefore, the NMR signal at δ 3.87 ppm was assigned to the $C_6H_5-C\underline{H}_2-CO-$ group. The other functional groups described above may be combined to form the structure



and the structure of C-9154 is therefore assumed to be **I**.

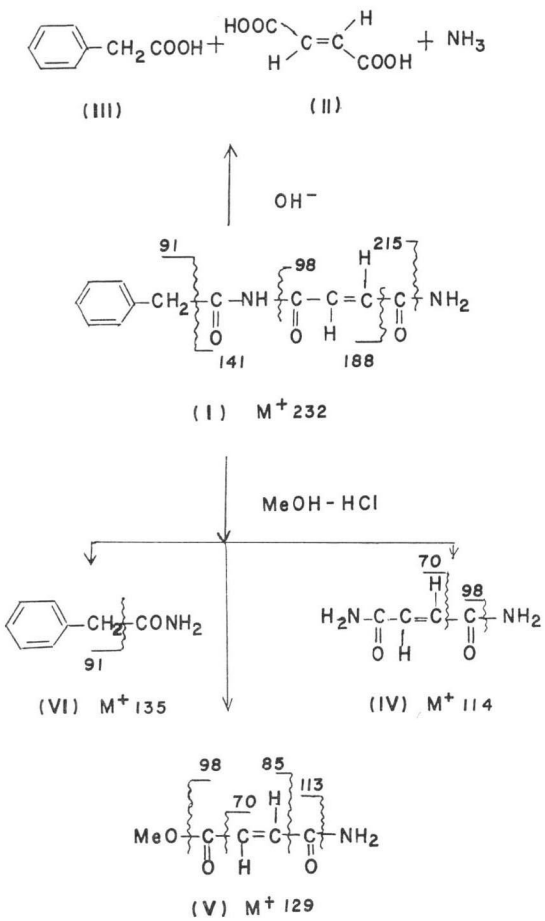


I

Degradative studies of C-9154 also supported the proposed structure (Fig. 2). Hydrolysis of C-9154 with 1N NaOH at 37°C for 2 hours yielded NH_3, fumaric acid ($C_4H_4O_4$) (**II**) and phenylacetic acid ($C_8H_8O_2$) (**III**). These products were identical with authentic samples (mp, IR, UV). Hydrolysis of C-9154 with methanol-HCl at 70°C for 2 hours gave compounds **IV**, **V**, and **VI**. Compound **IV**, mp 200°C, analyzed for $C_4H_6N_2O_2$, and the IR spectrum indicated carbonyl (1690 cm^{-1}) and NH_2 (3300, 3150 cm^{-1}) groups. The NMR (100 MHz, dimethyl sulfoxide) spectrum of **IV** exhibited signals which disappeared in D_2O at δ 6.72 ppm (s, 2H) (vinyl proton); 7.2 ppm (s, 2H) (NH_2); and 7.7 ppm (s, 2H) (NH_2). From these results the structure of **IV** was established as fumaramide.

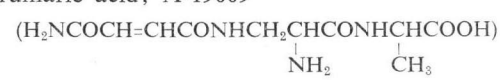
Substance **V**, mp 160~162°C (decomp.), analyzed for $C_5H_7NO_3$ and its mass spectrum

Fig. 2.

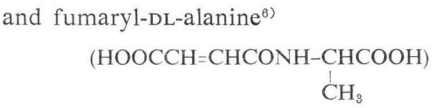


showed *m/e* 129 (M^+), 113, 98, 85 and 70. The IR spectrum indicated an ester-carbonyl (1720 cm^{-1}), conjugated carbonyl (1685 cm^{-1}) and NH_2 (3400 cm^{-1}) functions. The NMR (100 MHz, dimethyl sulfoxide) spectrum of **V** exhibited signals at δ 3.70 ppm (s, 3H) (OCH_3); 6.54 ppm (d, 1H, J=15 Hz); 6.96 ppm (d, 1H, J=15 Hz) (*trans* vinyl proton) and 7.42, 7.9 ppm (s, each 1H, disappear with D_2O) (NH_2). These results indicated that the structure of **V** is fumaramic acid methyl ester. Product **VI**, mp 155°C, analyzed for C_8H_9NO and its mass spectrum showed *m/e* 135 (M^+), 91. The NMR (100 MHz, $CDCl_3$) spectrum of **VI** exhibited signals at δ 3.22 ppm (s, 2H) ($-CH_2-$); 7.22 ppm (s, 5H) (aromatic ring protons) and 6.84 ppm, 7.4 (s, each 1H, disappear with D_2O) (NH_2). Substance **VI** was confirmed to be phenylacetamide, because it showed no depression of the melting point on admixture with an authentic sample and the IR, UV and mass spectra were identical. Consequently, the chemical structure of C-9154 was defined as N-(phenylacetyl)-fumaramide (**I**).

Other antibiotics which are derivatives of fumaric acid; A-19009[5]



and fumaryl-DL-alanine[6]



are already known. They have only a simple amide structure.

4. Biological Activities

As indicated in the Table 1, C-9154 shows broad spectrum, antimicrobial activity against gram-positive and gram-negative bacteria. The intraperitoneal acute toxicity in mice is LD_{50} 75 mg/kg.

Table 1. Antimicrobial spectrum of antibiotic C-9154

Test organisms	MIC (mcg/ml)
Escherichia coli K 12	50
Escherichia coli NIHJ	100
Proteus vulgaris IFO 3045	20
Proteus morganii IFO 3168	50
Proteus mirabilis IFO 3849	10
Pseudomonas aeruginosa IFO 3080	>100
Salmonella typhimurium IFO 12529	100
Salmonella enteritidis IFO 3313	>100
Alcaligenes faecalis IFO 13111	20
Aerobacter cloacae IFO 12009	>100
Serratia marcescens IFO 3046	100
Bacillus subtilis PCI 219	50
Bacillus subtilis ATCC 6633	50
Bacillus cereus IFO 3514	50
Bacillus pumilus IFO 3813	100
Bacillus megaterium IFO 12108	50
Staphylococcus aureus 209P	20
Mycobacterium sp. ATCC 607	>100
Mycobacterium phlei IFO 3158	>100
Candida albicans IFO 0583	>100
Saccharomyces cerevisiae IFO 0209	>100
Aspergillus niger IFO 4066	>100
Penicillium chrysogenum IFO 4626	>100

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