# STUDIES ON A NEW ANTIVIRAL ANTIBIOTIC, 9-METHYLSTREPTIMIDONE. I

## PHYSICOCHEMICAL AND BIOLOGICAL PROPERTIES

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A new antiviral agent which inhibits the growth of poliovirus, vesicular stomatitis virus (VSV) and Newcastle disease virus (NDV) was isolated from the culture filtrate of a *Streptomyces* and shown to be 3-(5,7-dimethyl-4-oxo-2-hydroxy-6,8-decadienyl)-glutarimide. The actue $LD_{50}$ of the antibiotic in mice by intraperitoneal injection was 280 mg/kg. Serum specimens taken 10 minutes after intraperitoneal injection of 2.5 mg/kg to mice inhibited the growth of poliovirus.

Both *in vitro* and *in vivo* screening for antiviral substances has long been conducted in our laboratory with *Streptomyces* culture filtrates. Myxoviromycin,[1] kikumycin[2] and formycin[3] were discovered and studied in this continuing effort to develop new antiviral agents.[4]

However, with all of these compounds, the concentration in blood after intraperitoneal injection into experimental animals was not high enough to inhibit virus growth *in vitro*, even though a chemotherapeutic effect against certain virus infections *in vivo* can be demonstrated. When the bioretention of the *Streptomyces* culture filtrate reported here was tested by measuring the antiviral effect after intraperitoneal injection into mice, serum specimens obtained after 10 or 30 minutes were strongly inhibitory to the growth of poliovirus and vesicular stomatitis virus. On this basis, the purification and characterization of the antiviral principle in the filtrate were attempted.

### Materials and Methods

Producing organism:

The organism, *Streptomyces* sp. S-885, was isolated from a soil sample collected in Miyagi Prefecture, Japan. The taxonomy will be reported elsewhere.[5] The organism was maintained on KRAINSKY's glucose-asparagine agar slant.

Production of the antibiotic:

For laboratory production of the antibiotic, spore stocks obtained from a 5-day-old KRAINSKY's agar slant were inoculated into 100 ml of a seed medium, consisting of 2 % starch, 1 % dry yeast, 2 % soybean meal, 0.25 % NaCl, 0.2 % $CaCO_3$, 0.005 % $MnSO_4 \cdot 5H_2O$, 0.005 % $CuSO_4 \cdot 5H_2O$ and 0.005 % $MgSO_4 \cdot 7H_2O$ in distilled water (pH 7.0). After 24 hours of culture at 27°C on a reciprocating shaker (4-cm amplitude, 130 strokes per minute), 3 ml of the culture was transferred to 120 ml of production medium, consisting of 1 % glycerin, 3 % starch, 1 % peptone, 0.5 % meat extract, 0.5 % NaCl and 0.2 % $CaCO_3$ (pH 7.0) in 500-ml SAKAGUCHI-flasks. Further incubation was made for 5 days at 27°C with shaking

Growing media for tissue culture cells:

The growth medium for HeLa cells contained 0.5 % lactalbumin hydrolyzate, 0.1 % yeast extract and 10 % unheated bovine serum in EARLE's balanced salt solution with 0.45 % glucose (YLE). The growth medium for primary chick embryo fibroblast (CEF) was HANK's balanced salt solution supplemented with 0.5 % lactalbumin hydrolyzate and 5 % unheated bovine serum (LH).[6] The growth medium for Rabbit kidney (RK-13) and L cells was MEM solution supplemented with 10 % unheated bovine serum.

Virus:

The MAHONEY strain of type 1 poliovirus, Indiana strain of vesicular stomatitis virus (VSV) and MIYADERA strain of Newcastle disease virus (NDV) were used for the antiviral assay.

Toxicity and antiviral assays on plate:[7,8,9]

Cytotoxicity and antiviral activities of filtrates or extracts were examined by the most sensitive agar plate assay. For the toxicity test, 10 ml of $3\times10^5$ HeLa cells, RK-13 cells or CEF suspension per ml were seeded into a Petri dish of 90-mm diameter, and the plate was incubated in a $CO_2$ incubator at 37°C. After 24 hours, the medium was removed and the plates were overlaid with 5 ml of agar medium containing 1 % agar (Difco, Bacto-agar) and 10 % bovine serum in YLE solution. Filter paper discs, 8 mm in diameter, were immersed in culture filtrate or extracts and 4 discs were placed on each agar plate. After 2 hours at room temperature, the plates were incubated at 37°C for 40 hours. The discs were removed, the plates were washed with PBS (phosphate buffered saline, pH 7.2), and stained with 0.04 % aqueous solution of 2,6-dichlorophenolindophenol for 5 minutes. The diameter of blue toxic zones was measured and minimum inhibitory concentration was determined.

For the antiviral assay, usually poliovirus was used. Ten ml of a suspension containing $6\times10^5$ HeLa S3 cells per ml were seeded into Petri dishes as described above, and the plates were incubated in a $CO_2$ incubator at 37°C. After 24 hours, the plates were washed with HANKS' salt solution and infected with 2 ml of poliovirus at $2\times10^3$ PFU/ml. After incubation at 37°C for 90 minutes for virus adsorption, the plates were washed twice with HANKS' solution, and then overlaid with 5 ml agar medium. The antiviral activity of the filtrate or extracts was determined from the diameter of the plaque-free zone around the paper discs. The minimum inhibitory concentration of the antibiotic obtained by the antiviral test was almost 1/10 of the minimum concentration required to reveal cytotoxicity. When VSV and NDV were used for the antiviral assay, primary culture of CEF was used instead of HeLa S3 cells.

Bioautography of extract:

The ethylacetate extract was chromatographed on a thin-layer plate of silica gel (Merck Kieselgel $GF_{254}$) with ethylacetate. The plates were bioautographed on an agar plate of HeLa S3 cells for the detection of cytotoxic and antiviral spots.

Bioretention test in mice:[10]

Either a filtrate or the purified antibiotic was injected into the *dd* strain of mice weighing an average of 20 g, intraperitoneally. Ten and thirty minutes after the injection, the mice were sacrified to obtain the serum. The concentration of the antibiotic in the serum was determined by plate assay.

## Results

### Isolation and Purification of the Antibiotic

Two liters of filtrate was extracted twice with one liter of ethylacetate at pH 5.0. The ethylacetate layer was washed with one liter of neutral water, dried with anhydrous sodium sulfate, and concentrated *in vacuo*. The dark-brown syrup (239 mg) was dissolved in 10 ml of benzene and chromatographed on a column of silica gel (Merck Kieselgel, 15 g, $22\times90$ mm) which had been prepared as a benzene slurry. It was developed with mixtures of benzene and

ethylacetate. The fractions eluted with benzene-ethylacetate (1 : 1) were combined and evaporated to dryness. The yellowish brown syrup was rechromatographed on silica gel (20 g, 22×120 mm) in the same way. The active preparation was eluted with benzene-ethylacetate (4 : 1) as a yellowish viscous oil (38.5 mg).

The sample obtained after rechromatography was finally purified by preparative silica gel thin-layer chromatography. The active portion with Rf 0.55 (solvent; ethylacetate) was extracted with acetone followed by evaporation. The final product was obtained as a pale yellowish viscous oil; yield 30.8 mg; boiling point (d.p.) 135~140°C (1 mm/Hg).

## Physicochemical Properties of the Antibiotic

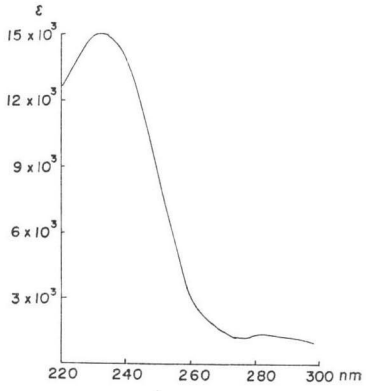
The antibiotic is a weakly acidic substance as shown by paper electrophoresis, and gives positive reactions in the *p*-phenylendiamine test for $\alpha,\beta$-unsaturated aldehyde and ketones, EHRLICH reaction and carbon disulfide test for primary or secondary amine. It gives negative reactions to ninhydrin and TOLLENS reagents. The antibiotic is soluble in chloroform, benzene, acetone, ethylacetate, methanol, ethanol and pyridine but insoluble in water. In an aqueous suspension with 0.001 % DMSO (dimethyl sulfoxide), the antibiotic is stable in the pH range from 7 to 9 and unstable at acidic pH.

The molecular weight, 307, and the molecular formula, $C_{17}H_{25}NO_4$, were determined by mass spectrometry and elemental analysis. Elemental analysis of the antibiotic favored the following composition:

| | |
|---|---|
| Found: | C 65.88, H 8.60, N 4.38 |
| Calcd. for $C_{17}H_{25}NO_4$: | C 66.42, H 8.20, N 4.56. |

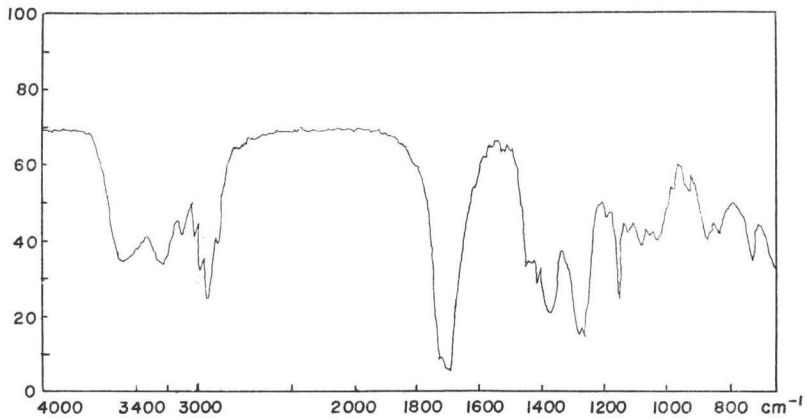
The optical rotation of the antibiotic is $[\alpha]_D^{27}+105°$ (*c* 0.1, $CHCl_3$). The antibiotic exhibits a characteristic ultraviolet absorption spectrum (Fig. 1) in ethanol with maxima at 231.5 nm (ε 15,350), 283 nm (ε 1,260) and 291 nm (ε 1,200).

Fig. 1. UV spectrum of 9-methylstreptimidone in ethanol.




The infrared absorption spectrum in liquid film is presented in Fig. 2. It has characteristic absorption bands

Fig. 2. IR spectrum of 9-methylstreptimidone in liquid film.



at the following frequencies: 3450 ($\nu$ O-H), 3250 ($\nu$ N-H), 3100, 2950, 1725, 1710, 1695, 1638, 1610, 1380, 1270, 1150 and 720 $cm^{-1}$.

The presence of a conjugated double bond is supported by the following evidence: (a) it quickly decolorized bromine solution, (b) it shows an absorption maximum at 231.5 nm ($\varepsilon$ 15,350) in the UV spectrum, (c) it shows absorption bands at 1638 and 1610 $cm^{-1}$ in the IR spectrum.

Further, the antibiotic is shown to contain three

Fig. 3. NMR spectrum (100 MHz) of 9-methylstreptimidone in $CDCl_3$.

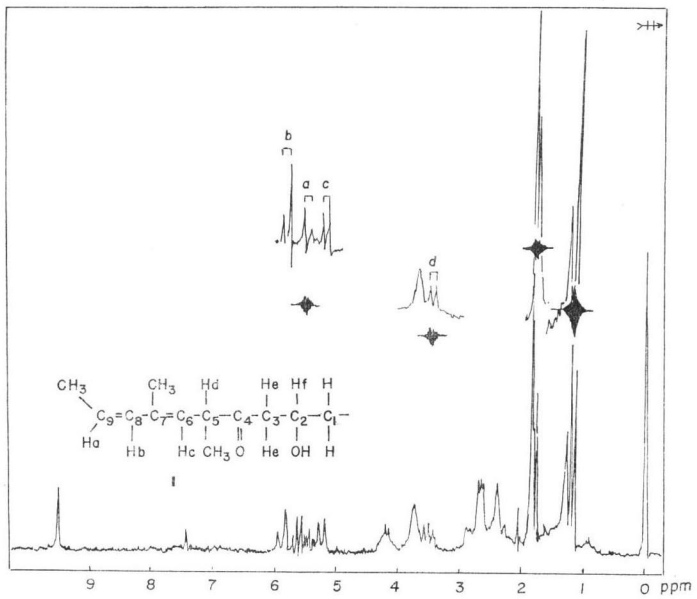
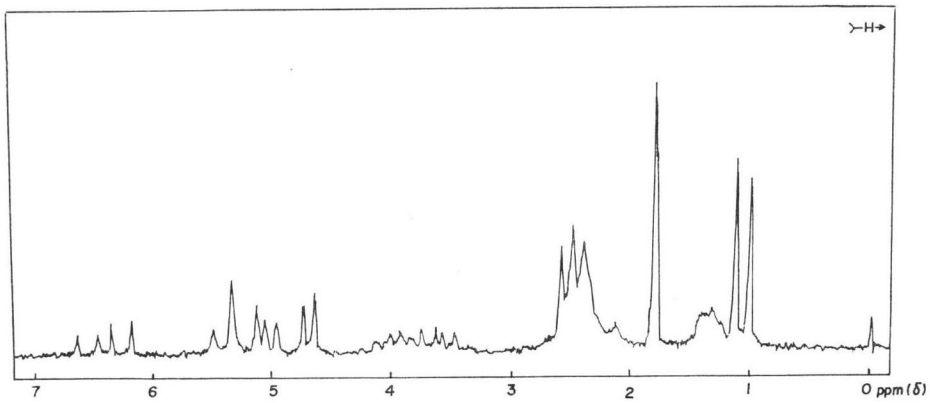



Fig. 4. NMR spectrum (100 MHz) of streptimidone in DMSO.




carbonyl functions. One is an isolated ketone (IR: 1725 $cm^{-1}$, UV: 283 nm ($\varepsilon$ 1,260)). The other two are present as an imide function in the glutarimide ring (IR: 3250, 1710 and 1695 $cm^{-1}$).

The results obtained suggest that this antibiotic is similar to streptimidone.[11] The NMR spectra (100 MHz) of the antibiotic and streptimidone are shown in Figs. 3 and 4, respectively. In the NMR spectrum of the antibiotic, the doublet signal (J=7.3 Hz) centered at $\delta$ 1.18 can be assigned to the C-5 methyl protons and the doublet signal (J=1.5 Hz) centered at $\delta$ 1.8 to the C-7 metyl protons by comparison with the spectrum of streptimidone. Irradiation at $\delta$ 1.78 (3H, J=8.6 Hz) and at $\delta$ 1.18 (C-5 methyl protons) in the spectrum of this antibiotic converted the multiplet signal ($\delta$ 5.4~5.7) of Ha to a doublet signal (J=12.0 Hz), and the multiplet signal ($\delta$ 3.4~3.6) of Hd to a doublet signal (J=10.6 Hz), respectively (Fig. 3). On the other hand, irradiation at the Hd and Ha signals converted the doublets centered at $\delta$ 1.18 and $\delta$ 1.78 to

singlets. Thus, the doublet signal (J=8.6 Hz) centered at $\delta$ 1.78 can be assigned to the C-9 methyl protons, Ha is coupled to Hb (Ja, b=12.0 Hz) centered at $\delta$ 5.9, Hc is coupled to Hd (Jc, d=10.6 Hz) centered at $\delta$ 2.3, and the signal at $\delta$ 2.3 can be assigned to the Hd proton. Moreover, these observations indicate that the C-5 methyl group and Hd proton are located on the same carbon which is attached to a carbonyl group. This assignment is based on the fact that (1) signals of the C-5 methyl and Hd protons are shifted to somewhat low field ($\delta$ 1.18 and $\delta$ 3.5 respectively), (2) the multiplet signal of Hd proton was changed by irradiation of the C-5 methyl protons to a doublet which is coupled to only the Hc proton.

When the antibiotic was treated with acetic anhydride in pyridine at 0~4°C for 20 hours, it gave a mono-acetate.

In the NMR spectrum of the acetate of this antibiotic, the Hf signal at $\delta$ 4.2 shifted to $\delta$ 5.4 (Fig. 5). Thus the Hf proton is located on a carbon atom bearing a hydroxyl group.

Fig. 5. NMR spectrum (60 MHz) of acetate of 9-methylstreptimidone in $CDCl_3$.

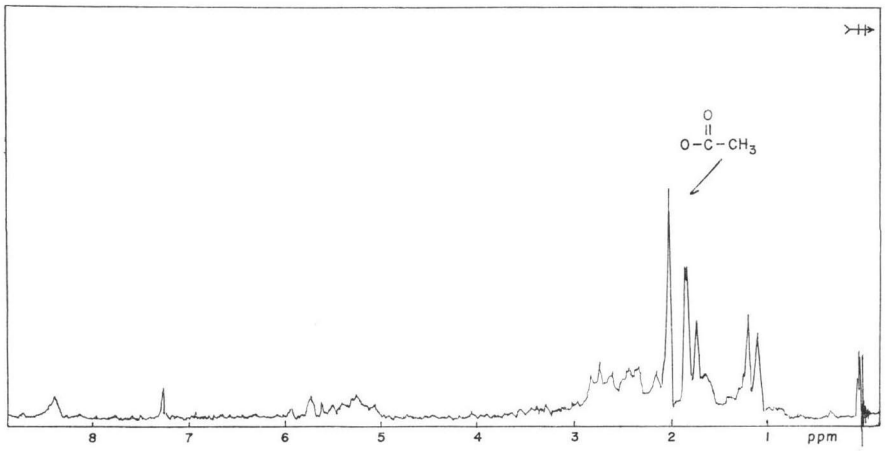



Fig. 6. NMR spectrum (60 MHz) of 9-methylstreptimidone in $CDCl_3$.

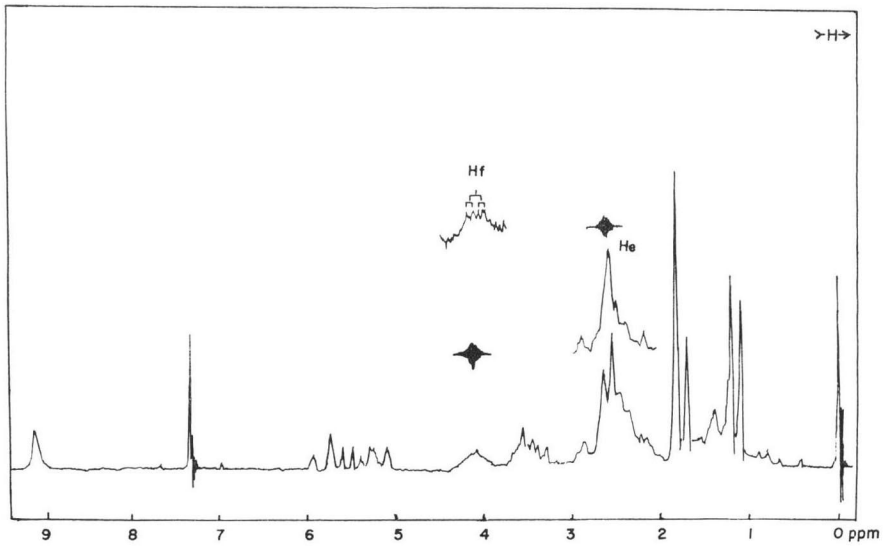


Fig. 7. Mass spectrum of 9-methylstreptimidone.

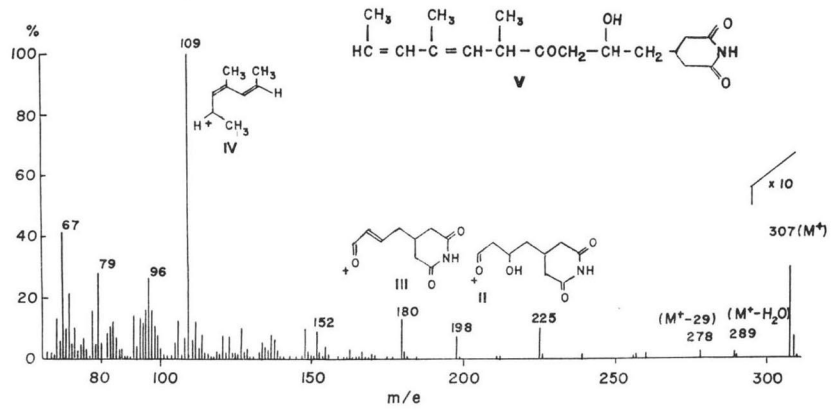
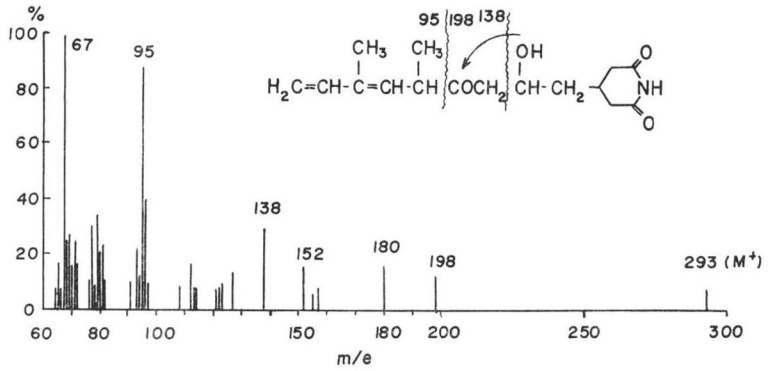



Fig. 8. Mass spectrum of streptimidone.




In Fig. 6, irradiation at the Hf signal changed the doublet signal (J=6.0 Hz) of He centered at $\delta$ 2.7 to a singlet, while irradiation at the He converted the multiplet signal of Hf centered at $\delta$ 4.2 to a double doublet signal (J=4.5, 3.8 Hz). These results indicate the presence of the partial structure I in this antibiotic (Fig. 3).

The mass spectra of the antibiotic and streptimidone are shown in Fig. 7 and Fig. 8. The molecular ion peak, *m/e* 307, of the new antibiotic was fourteen mass units more than that of streptimidone,[12] *m/e* 293. The base peak appeared at *m/e* 109 which was also fourteen mass units ($-CH_2$) more than the fragment *m/e* 95 of streptimidone. Three peaks, *m/e* 198, *m/e* 180 and *m/e* 152, were common to two antibiotics. The fragments of *m/e* 198, *m/e* 180 and *m/e* 109 can be assigned to **II**, **III** and **IV** respectively (Fig. 7).

Thus, from these physicochemical studies, the structure of this antibiotic was determined to be 3-(5,7-dimethyl-4-oxo-2-hydroxy-6,8-decadienyl)-glutarimide (**V**), *i.e.*, 9-methylstreptimidone.

## Biological Properties of 9-Methylstreptimidone

The antibiotic had no antibacterial or antimycoplasmal activity, but was inhibitory for some yeasts, as shown in Table 1. It inhibits the growth of *Saccharomyces sake* HUT 7119, *Cryptococcus albidus* IFO 0378 and *Torula rubra* var. *alpa* at a concentration of 4 mcg/ml.

When the inhibitory effect of the antibiotic was tested on several tissue culture cells by the agar plate method,[7] HeLa S3 cells, Rabbit Kidney cells, L cells and CEF were found to be sensitive at 0.2~0.5 mcg/ml concentration (Table 2).

The antiviral activity was examined using the same agar plate method. The antibiotic

Table 1. Antimicrobial spectrum of 9-methylstreptimidone

| Test organisms | Media | MIC (mcg/ml) |
|---|---|---|
| *Staphylococcus aureus* FDA 209P | N. A | >100 |
| *Sarcina lutea* 1001 | P. A | >100 |
| *Bacillus subtilis* PCI 219 | P. A | >100 |
| *Escherichia coli* NIHJ | N. A | >100 |
| *Proteus mirabilis* 129 | N. A | >100 |
| *Pseudomonas aeruginosa* | N. A | >100 |
| *Streptococcus hemolyticus* | B. A | >100 |
| *Trichophyton rubrum* | S. A | >100 |
| *Candida albicans* | S. A | >100 |
| *Candida utilis* IFO 0396 | S. A | 100 |
| *Candida parapsilosis* IFO 0708 | M. A | >100 |
| *Candida tropicalis* | M. A | 100 |
| *Hansenula anomala* IFO 0118 | M. A | 20 |
| *Hansenula capsulate* IFO 0378 | M. A | 20 |
| *Cryptococcus albidus* IFO 0378 | M. A | 4 |
| *Brettanomyces anomalus* IFO 0648 | M. A | >100 |
| *Rhodotorula qulutinis* var. *aurantica* IFO 0754 | M. A | 100 |
| *Rhodotorula rubra* IFO 0001 | M. A | 20 |
| *Torula rubra* var. *alpa* | M. A | 4 |
| *Saccharomyces sake* HUT 7119 | M. A | 4 |
| *Saccharomyces fragilis* IAM 7160 | M. A | 20 |
| *Saccharomyces rose* AHU 3174 | M. A | 100 |

Determined by the agar dilution method.
N. A: Nutrient agar P. A: Peptone agar
B. A: Blood agar S. A: SABOURAUD agar
M. A: Malt agar

inhibited plaque formation of poliovirus on HeLa cells, VSV and NDV on chick embryo fibroblasts at a dose of 0.02 mcg/ml (Table 3).

The intraperitoneal $LD_{50}$ in mice (*dd* strain) is

Table 2. Toxicity of 9-methylstreptimidone on mammalian cells

| Cells | Medium | MIC (mcg/ml) |
|---|---|---|
| HeLa S3 | YLE | 0.2 |
| RK-13 | MEM | 0.25 |
| Chick embryo | LH | 0.2 |
| L | MEM | 0.5 |

Agar method using cylinder was used.
Number of cells seeded: $3\times10^5$ cells/ml.
Detection: 0.04 % of 2,6-dichlorophenol-indophenol solution.

Table 3. Antiviral activity of 9-methylstreptimidone using agar plate method

| Virus/Cells | MIC (mcg/ml) |
|---|---|
| Polio/HeLa S3 | 0.02 |
| VSV/CEF | 0.02 |
| NDV/CEF | 0.02 |

Number of cells seeded: $6\times10^5$ cells/ml.
Detection: 0.00005 % neutral red solution was used.

Table 4. Bioretention test of 9-methylstreptimidone in mice

| Dose (mg/kg) | Dilution titer against Polio/HeLa | |
|---|---|---|
| | 10 min. | 30 min. |
| 25 | 1/125 | 1/120 |
| 2.5 | 1/90 | 1/29 |

9-Methylstreptimidone was injected into the mice, intraperitoneally. Ten and thirty minutes after injection, the mice were sacrified to obtain the serum. The concentration of the antibiotic in the serum was determined by a plate assay with poliovirus.

Table 5. Comparison of antimicrobial activity of 9-methylstreptimidone, streptimidone and protomycin

| Test organisms | MIC (mcg/ml) | | |
|---|---|---|---|
| | 9-Methylstreptimidone | Streptimidone* | Protomycin** |
| *Staphylococcus aureus* | >500 | >100 | >100 |
| *Sarcina lutea* | >500 | 100 | >100 |
| *Streptococcus hemolyticus* | >500 | N. D | 50 |
| *Escherichia coli* | >500 | >100 | 100 |
| *Candida tropicalis* | >500 | 25 | >100 |
| *Saccharomyces sake* | 4 | N. D | 1 |

* Ref. (14); ** Ref. (15).

Table 6. Comparison of physicochemical properties of 9-methylstreptimidone, streptimidone and protomycin

| Properties | 9-Methylstreptimidone | Streptimidone* | Protomycin** |
|---|---|---|---|
| Form | pale yellowish oil | colorless needle | white crystals |
| M.P. (B.P.) | 135~140°C/1 mmHg | 72~73°C | 58~61°C |
| $[\alpha]_D$ | +105° (*c* 0.1, $CHCl_3$) | +238° (*c* 0.5, $CHCl_3$) | +126° (*c* 1.08, $CHCl_3$) |
| Mol. Formula | $C_{17}H_{25}NO_4$ | $C_{16}H_{23}NO_4$ | $C_{19}H_{29}NO_5$ |
| Mol. Wt. | 307 | 293 | 351 |
| $\lambda_{max}$ : | 231.5 (15,350) | 232 (23,100) | 232.5 (24,300) |
| nm ($\varepsilon$) | 283.0 (1,260)<br>291.0 (1,230) | 291 (790) | 287 (1,440) |
| IR: $cm^{-1}$ | 3475, 3225, 1725, 1710, 1650, 720 | 3575, 3425, 1710, 1700, 1680 | 3700, 3550, 1740, 1700, 1640 |

* Ref. (11); ** Ref. (15).

280 mg/kg and the intraveneous $LD_{50}$ is 210 mg/kg. When 100 mg/kg/day of this antibiotic was given to mice intraperitoneally for six days, all mice survived.

The result of bioretention experiment is summarized in Table 4. When a single dose of 25 mg/kg of this antibiotic, approximately 1/10 of the $LD_{50}$ in mice, was injected into mice, serum specimens collected ten minutes after injection inhibited the growth of poliovirus at 1/125 dilution. With a dose of 2.5 mg/kg, which corresponds to 1/100 of the $LD_{50}$, serum specimens obtained after 10 minutes inhibited the growth of poliovirus at 1/90 dilution.

## Discussion

A new antiviral agent, 9-methylstreptimidone, was isolated from a culture filtrate of a *Streptomyces* sp. S-885. Although the taxonomic characterization of the producing organism is not completed, the strain is very similar to *Streptomyces pluricolorescens*,[13] if not identical.

Initially, physicochemical properties of the antibiotic and inhibitory activity on the growth of *Saccharomyces* suggested that the antibiotic was related to streptimidone[14] and protomycin,[15] which belong to the glutarimide antibiotics. However, the difference between these 3 antibiotics was first apparent in their antimicrobial spectra as illustrated in Table 5.

As shown in Table 5, streptimidone inhibited the growth of *Sarcina lutea* at 100 mcg/ml, and protomycin inhibited the growth of *Streptococcus hemolyticus* at 50 mcg/ml, whereas the isolated antibiotic was not active against these 2 organisms at 500 mcg/ml. Although the UV and IR spectra of these antibiotics were very similar (Table 6), the molecular weights were significantly different. The molecular weight of the new antibiotic was found to be 307, whereas that of streptimidone[11] and protomycin[15] are 293 and 351, respectively. Further structural analysis revealed that the antibiotic is a new antibiotic, 9-methylstreptimidone.

For the assay of this antiviral and cytotoxic agent, the agar plate method was used. The assay itself could be routinely carried out as are antibacterial diffusion assays, dealing with many test specimens in limited period. The cytotoxicity and antiviral effect could be determined and compared under the same conditions. Bioautograms of the antibiotic were easily made with the same plate. Moreover, the minimum inhibitory concentration or toxic concentration obtained by this procedure was twice as sensitive as that from the tube culture method. For these reasons, paper disc agar plate assay was employed throughout the experiment.

The antibiotic was found to be active against poliovirus, VSV and NDV. Further tests with other viruses are now in progress. A preliminary *in vivo* antiviral test with this antibiotic has established an effect against influenza A2 infection in mice. These antiviral effects of the antibiotic will be reported elsewhere.

Acknowledgement

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