Studies on the Antibiotic Substances from Actinomyces
(6th Report)
On the Properties of 5 Kinds of Streptothricin-like Substances

By
Masahiko Kuroya, Nakao Ishida, Ken Katagiri, Jiro Konno,
Masami Kikuchi and Ryutaro Mizuguchi

(From the Department of Bacteriology, Faculty of Medicine,
Tohoku University, Sendai)

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In the research for antagonistic streptomycetes, we designated the strains as 1st group actinomyces,\(^1\) which represented antibacterial activity against several gram positive, gram negative and acid-fast bacteria, on the cross-streak test. Further, this 1st group actinomyces were classified\(^2\) into basic and nonbasic groups according to the activity of their filtrates in alkaline or acidic test media and many basic antibiotic producing strains were obtained. Except streptomycin, almost all of these basic compounds were anticipated to be streptothricin-like substances according to their bacteriostatic nature. Further, on the account of the different diffusibility of their active agents on assay agar and several other properties, streptothricin type 1 and 2 were differentiated, the former exhibited good, whereas the latter not so good diffusibility. Now, 5 of these representative strains which appeared to produce streptothricin type 1 and 2, viz, Nos. 0-36, 0-24, 39, 259 and 120 (Seki) were selected, cultivated and chemical purifications from their filtrates were performed respectively.

All of these summarized experimental results will be reported here, and concerning with each strain further report will be made elsewhere.

**Experimental**

**Strains tested**

As streptothricin type 1 producing strains Nos. 0-36, 0-24 and 39 and as type 2 producing strains, 259 and 120 (Seki) were employed. Taxonomic studies proved that all of these strains resembled to *Str. roseochromogenus*. Only No. 24 could grow on 10 µg/cc streptomycin containing agar and became to grow abundantly on the agar containing
800 μg/cc of streptomycin after successive transfers on the agar containing gradually increasing amounts of streptomycin. After this procedure, however, there were noticed neither morphological nor biochemical changes as well as no tendency to producing streptomycin itself.

**Antibacterial spectra of the strains**

As shown in Table I, all 5 strains showed several properties, characteristic to streptothricin producing strains in the following respects. (1) They inhibited *Sta. aureus* (Terashima strain) stronger than *Sta. aureus* (209-P), (2) *B. subtilis* stronger than *B. anthracis*, (3) normal *E. coli* stronger than streptothricin resistant *E. coli*, while they inhibited *E. coli* in a similar degree as streptomycin resistant *E. coli*.

**Table I**

Antibacterial Spectra of the Five Strains of *Str. roseochromogenus*
(Cross-streak method)

<table>
<thead>
<tr>
<th>Producing strains</th>
<th><em>Sta. aureus</em> (Terashima)</th>
<th><em>Sta. aureus</em> (F.D.A.209-P)</th>
<th><em>B. agri</em></th>
<th><em>B. subtilis</em> (NRRRL-258)</th>
<th><em>E. coli</em></th>
<th>SM* Resistant <em>E. coli</em></th>
<th>0-36* Resistant <em>E. coli</em></th>
<th>0-259* Resistant <em>E. coli</em></th>
<th>0-20* Resistant <em>E. coli</em></th>
<th>Seki* Resistant <em>E. coli</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>No. 39</td>
<td>17.0</td>
<td>13.1</td>
<td>11.1</td>
<td>5.4</td>
<td>14.7</td>
<td>14.0</td>
<td>14.4</td>
<td>1.1</td>
<td>7.1</td>
<td>3.0</td>
</tr>
<tr>
<td>No. 24</td>
<td>9.0</td>
<td>7.0</td>
<td>3.0</td>
<td>4.0</td>
<td>7.0</td>
<td>5.0</td>
<td>7.0</td>
<td>0.4</td>
<td>1.0</td>
<td>0.8</td>
</tr>
<tr>
<td>No. 39</td>
<td>21.0</td>
<td>20.4</td>
<td>6.4</td>
<td>9.1</td>
<td>17.5</td>
<td>14.9</td>
<td>15.8</td>
<td>3.8</td>
<td>6.2</td>
<td>2.0</td>
</tr>
<tr>
<td>No. 259</td>
<td>12.0</td>
<td>9.0</td>
<td>7.0</td>
<td>7.2</td>
<td>10.9</td>
<td>8.4</td>
<td>11.0</td>
<td>8.7</td>
<td>8.4</td>
<td>5.1</td>
</tr>
<tr>
<td>No. 120 (Seki)</td>
<td>13.2</td>
<td>12.8</td>
<td>7.2</td>
<td>8.9</td>
<td>12.4</td>
<td>11.2</td>
<td>11.4</td>
<td>8.7</td>
<td>9.1</td>
<td>5.1</td>
</tr>
</tbody>
</table>

* Means *E. coli* resistant against streptomycin and against final substances obtained from the cultures of Nos. 0-36, 259, 0-20 and 120 (Seki) respectively.

In this antibacterial spectrum, however, it was scarcely possible to differentiate both streptothricin types 1 and 2 producing strains from one another. There are, however, inconformities between the antibacterial spectra of these strains and those of their final products, and will be discussed later.

**Cultural condition**

All of these strains were cultivated using modified Vander Brook's medium (added with 0.1 to 1% peptone instead of curbay B. G.) in shaking condition at 28°C.

**Purification of the active substance**

After the adjustment of the filtrate to pH 3.5, mycelium was removed
by filtration and the active agent was further purified by adsorption to and elution from carbon as already described concerning with streptomycin and streptothricin. By being treated with 0.5% charcoal and stirred for 15 minutes at acidic pH (pH 2.0), the filtrate was freed from some impurities and decolorized. Clarified filtrate, thus obtained, was further adsorbed with 1 to 2% charcoal at neutral pH, and the charcoal was washed with water and neutral 10% acetone several times. The carbon cake was then eluted with acidic 10% acetone (pH 2.0, 1/10 to 1/20 volume of the filtrate) 2 or 3 times. Almost all the activity was recovered in such a manner. These eluates were combined and adjusted to pH 5.0 with NaOH and occuring flocculous precipitate was removed. After the concentration of the solution in vacuo at pH 5.0 the residue was desiccated on P₂O₅, and redissolved in 1/10 N anhydrous HCl-methanol in order to remove inorganic salts. The active agent was precipitated with 10 vol. of acetone. This final procedure was repeated 2 times and the crude hydrochloride was obtained in a white amorphous powder.

The crude hydrochloride was purified by the chromatographic method using Al₂O₃ as adsorbent and further precipitated with methylorange. The induced crystalline helianthate was converted again into the hydrochloride.

In this purification course noticeable points are as follows:

1) The selection of culture media is an important problem. If the medium rich in organic materials such as Waksman’s was used, crude hydrochloride was obtained in recovery of 1 mg/cc from the broth with contamination of some brownish pigments and other impurities, which was rather difficult to be removed. When Vander Brook’s semi-synthetic medium which was composed chiefly of inorganic materials was used it was easier to obtain a purified hydrochloride in much more amounts and to induce them into crystalline reineckate immediately.

2) The decolorization and removal of impurities using charcoal at acidic pH must be carried out precautiously, and if more charcoal was used some losses of activity were unavoidable. In order to obtain a highly purified preparation, however, such a procedure was necessary to be taken.

3) The elution from charcoal was usually made with 10% acetone or sometimes with 80% methanol at acidic pH. Concerning with the above antibiotics, almost all of the activities were recovered by this procedure, except No. 39 which showed always about 60% recovery, even if strongly acidic acetone was used.

4) The procedure of chromatographic separation was conducted according to Cartor’s description, using Al₂O₃ (Japan Alum. Co. Ltd. A.P.6) as adsorbent, and 20 to 60% recovery was obtained, and the ac-
tivity of purified hydrochloride, thus obtained, reached about 2 to 8 times of starting material.

5) For the recrystallization of helianthate 50 to 100% methanol was the most suitable solvent. The helianthate was slightly soluble in ethanol too, whereas hardly soluble in propyl-, isopropyl-, butyl-, amyl-alcohol, chloroform and ether.

*The biological and chemical nature of the purified antibiotics*

Concerning with these 5 kinds of purified hydrochloride, which were able to be induced to crystalline reineckates, some biological and chemical natures were compared with those of pure streptomycin (Merck) and with each other. All these results are shown in Table II.

Antibacterial properties of these compounds were tested both with cup and dilution assay method. Cup assay was conducted on nutrient agar plate for common bacteria and on 4% glycerol nutrient agar for acid-fast bacteria. A penicillinlinder was set at the center of each plate, being filled with each of 1 mg/cc hydrochloride solutions. They were allowed to stay overnight in an ice box. Concerning with the substance No. 259, the agar plate was stored for 48 hrs. for its low diffusibility. Next morning all of the test organisms, including normal *E. coli*, streptomycin-, No. 0-36-, No. 259-, No. 0-20 (ST 1)-, and No. 120-resistant strains of *E. coli*, were streaked radially around the cup.

The resistant *E. coli* strains toward streptomycin and each of 4 streptothricin-like substances respectively were obtained by the successive transplantations through the broth, containing highest concentrations of each of corresponding antibiotics, which permit the growth of *E. coli*.

There was seen, however, a remarkable difference between the acquired resistances toward 4 streptothricines and that toward streptomycin. A strain of *E. coli*, which was inhibited initially in a concentration of 0.6 µg/cc of streptomycin became resistant toward a concentration of 65 mg/cc of the same (24,000 fold compared with initial resistance) by the procedures mentioned above and it seemed to be possible to elevate the resistance further to a higher degree. In the case of streptothricines, however, it was not so easy to elevate the resistance of the same strain of *E. coli*, viz., the resistance toward No. 36-substance was brought up to the height of 80 fold (0.5 mg/cc) of initial resistance, those toward No. 259-, No. 0-20- and No. 120-substances were elevated to the heights of 80 (0.1 mg/cc), of 200 (0.5 mg/cc) and of 200 (0.25 mg/cc) fold respectively by the same techniques and they were coming to a stationary condition in these degrees of resistance, because it was no more possible to elevate their resistance by repeating the transfers.

The results of the antibacterial cup assays were summarized as follows:
### TABLE II

Biological Properties of Streptomycin and Five Streptothricin-like Substances

<table>
<thead>
<tr>
<th>HCl salt of antibiotic substances</th>
<th>Antibacterial spectra in cup assays (Inhibition zones are measured in mm)</th>
<th>Dilution unit (x1000)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Sta. aureus</strong></td>
<td><strong>B. agricola</strong></td>
</tr>
<tr>
<td>----------------------------------</td>
<td>----------------</td>
<td>----------------</td>
</tr>
<tr>
<td>Terahsima</td>
<td>19.4</td>
<td>19.3</td>
</tr>
<tr>
<td>Basic antibiotic No. 36</td>
<td>14.7</td>
<td>13.8</td>
</tr>
<tr>
<td>Basic antibiotic No. 24</td>
<td>11.4</td>
<td>10.8</td>
</tr>
<tr>
<td>Basic antibiotic No. 39</td>
<td>13.1</td>
<td>13.2</td>
</tr>
<tr>
<td>Basic antibiotic No. 259</td>
<td>11.9</td>
<td>11.7</td>
</tr>
<tr>
<td>Basic antibiotic No. 120 (Seki)</td>
<td>7.2</td>
<td>6.8</td>
</tr>
</tbody>
</table>

Properties of Streptothricin-like Substances

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1) All 5 kinds of substances inhibited *B. subtilis* more strongly than *B. anthracis* on the agar plate, while streptomycin inhibited the latter stronger than the former. This relationship was useful to divide these antibiotics into streptomycin and streptothricin-like groups, besides the use of drug-resistant bacteria. Streptomycin resistant *E. coli* was only resistant to streptomycin itself and rather more susceptible to the 5 substances than normal *E. coli*. On the contrary, the resistant *E. coli* to each of 4 streptothricins respectively were resistant to all of these 5 substances and to streptomycin too, even if the resistance toward the latter was only in a limited extent.

2) *B. agri*, confirmed by Prof. Hosoya to be susceptible toward streptothricins and not susceptible to streptomycin, like Bodenheim's organism, was not inhibited by 2 out of 5 substance belonging to streptothricin type 1 (Nos. 36 and 24), whereas it was somewhat susceptible against other 3 kinds of streptothricins (39, 259 and 120).

Using *B. agri* as test organism, therefore, it was possible to differentiate streptothricin type 1 from type 2, while it was not able to distinguish streptomycin from streptothricin type 1. It was revealed further, that either streptothricin type 1 or type 2 producing strains inhibited *B. agri*, though the activity of the former was rather lower than the latter as shown in Table I. In this respects it was able to find a discomformity between both antibacterial spectra, one produced by cross streak test and the other showed by purified antibiotics. Another discomformity was found in the respect, that all streptothricin producing strains could inhibit all of the streptothricin resistant *E. coli* strains more or less, whereas none of the purified streptothricins was able to do so. With regards to these repects, the report will be made elsewhere.

3) When these cup assay titers were compared with those of dilution assay titers, both streptothricin type 2 substances (Nos. 259 and 120) appeared to be low potent due to their low diffusibilities.

Two-fold serial dilution assay was performed using less nutrient broth (meat extract and peptone 0.5% each without NaCl, pH 7.5), against *Sta. aureus* Terashima, *Sta. aureus* 209-P, and *E. coli*. Twenty four hrs. broth cultures of these bacteria were used as the inoculum, 0.05 cc of which being added to 5 cc of assay broth, and cultivated at 37°C for 18 hrs. These data coincided good with that of culture filtrates (as described in 3rd report2), and this fact indicates that the chief antibiotics contained in the filtrates were extracted and purified to these hydrochlorides.

4) Streptothricin type 1 showed the same Terashima: *E. coli* unit ratio and Terashima: F.D.A. 209-P unit ratio with those of streptomycin, while streptothricin type 2 showed remarkably higher ratios in both cases.
5) The toxicity test was carried out using the mice weighing 15 g on average. Each required amount of the pure hydrochloride was dissolved in 0.3 cc physiological saline and injected both intravenously and intramuscularly. Concerning with some antibiotics, maximal tolerated dosage was not determined. However, by the injection of 1/10 of lethal dosage as indicated in the Table III no toxicity was shown.

The lowest toxicity was shown by the antibiotic No. 36 and in this respect it was more preferable than streptomycin. The other substances showed a so-called “delayed toxicity” and some lung-, and liver hemorrhage on autopsy, both of which were characteristic properties of streptothricin groups.

6) All of these crystalline reineckates and helianthates were obtained in a platelet form, in contrast with streptomycin, which obtained in needles. The decomposition points of these crystals were found to be in the vicinities of that of streptothricin, whereas the reineckate of the antibiotic No. 36 showed a characteristic low decomposition point (114°C). On the other hand the antibiotic No. 39 resembled most closely Waksman’s streptothricin (reineckate: d.p. 192°–194°C, helianthate d.p. 225°–230°C).

7) Some chemical reactions, using 0.25% aqueous solution of each antibiotics, were examined. Streptomycin is characteristic in positive Sakaguchi and maltol tests while 5 streptothricin substances were negative in them. Elson-Morgan’s glucosamine test was positive both, in these 5 substances and streptomycin, so no unique reaction especially positive in these 5 substances and streptomycin were not found yet.

**SUMMARY**

1) Five kinds of basic antagonistic substances, which resembled streptothricin in nature, were obtained in a form of hydrochloride, crystalline helianthate and reineckate, and the identification between these substances was performed.

2) All of these substances had rather lower potencies than streptomycin, in a dilution titer against *E. coli*, while the substances No. 259 and No. 120 (Seki) were higher inhibitory against *Staphylococcus* (Terashima strain) than streptomycin.

3) Six substances, including streptomycin, were arranged in the order of increasing toxicities as follows:

36, streptomycin, 120 (Seki), 24, 39, and 259. The substance No. 36 was able to be injected both, intravenously and intramuscularly into mice in an amount of 20 mg without showing any toxic signs. In this respect, the substance No. 36 has advantages over streptomycin.

4) The decomposition points of helianthates or reineckates of 4 streptothricin-like substances were found to be in the vicinities of that of
<table>
<thead>
<tr>
<th>HCl salt of antibiotic substances</th>
<th>Toxicity</th>
<th>D.p. of crystals</th>
<th>Chemical reactions of HCl-salt (10 mg/40 cc in distilled water)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Helianthate</td>
<td>Reineckate</td>
</tr>
<tr>
<td><strong>Streptomycin</strong></td>
<td>intraven. 5 mg dead</td>
<td>220-221°</td>
<td>161-163°</td>
</tr>
<tr>
<td></td>
<td>intramus. 20 mg alive</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Basic antibiotic No. 36</strong></td>
<td>intraven. 20 mg alive</td>
<td>211-216°</td>
<td>114°</td>
</tr>
<tr>
<td></td>
<td>intramus. 20 mg alive</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Basic antibiotic No. 24</strong></td>
<td>intraven. 5 mg dead (12 days)</td>
<td>275-285°</td>
<td>185-201°</td>
</tr>
<tr>
<td><strong>Basic antibiotic No. 39</strong></td>
<td>intraven. 5 mg dead (6 days)</td>
<td>225-230°</td>
<td>198-201°</td>
</tr>
<tr>
<td><strong>Basic antibiotic No. 259</strong></td>
<td>intraven. 1.5 mg dead (6 days)</td>
<td>244°</td>
<td>193-196°</td>
</tr>
<tr>
<td><strong>Basic antibiotic No. 120 (Seki)</strong></td>
<td>intraven. 10 mg alive</td>
<td>224-230°</td>
<td>184-185°</td>
</tr>
</tbody>
</table>
streptothricin except No. 36 (Roseomycin). In reference to the other biological and chemical properties, however, the 5 substances seem quite different from streptomycin and from each other.

5) Maltol and Sakaguchi reactions were found to be positive in streptomycin alone, while glucosamine and other sugar reactions were positive in both streptomycin and streptothricin-like 5 substances. The chemical reactions positive in streptothricin group only, were not found yet.

6) A strain of *E. coli*, which was made resistant to the hydrochlorides of 4 substances (36, 20, 259, and 120) respectively was resistant to each of 5 substances and somewhat less resistant to streptomycin too, while the same strain of *E. coli*, which was made resistant to streptomycin, was not resistant to each of 5 substances. In this respect it seems very probable, that each of 5 substances resembles closely in their mechanisms of the antibiotic functions.

**References**