METABOLIC PRODUCTS OF MICROORGANISMS 142*

A NEW ANTIBIOTIC DERINAMYCIN, INHIBITOR OF DNA AND RNA SYNTHESIS

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Derinamycin was isolated from the mycelium of Streptomyces venezuelae Tü 1102 and its molecular formula was tentatively assigned as C_{51}H_{93}NO_{23}. The antibiotic inhibits the growth of fungi, gram-positive bacteria and certain gram-negative bacteria but is less active against yeasts. A study of derinamycin action on the macromolecular synthesis of intact Bacillus subtilis revealed that the antibiotic suppressed DNA and RNA syntheses but that protein synthesis was less affected. Derinamycin exerted no selective inhibition between DNA and RNA syntheses in the double-isotope experiment used to assess the relative effects of the antibiotic.

In our search for new antibiotics, various antistreptomycete substances have been examined using Streptomyces cultures in the assays. Rinamycin\(^1\) had previously been found in the mycelium of the strain Tü 1102 of Streptomyces venezuelae which had been isolated from a soil sample collected in India. Rinamycin was subsequently observed to inhibit the growth of rinamycin-sensitive microorganisms by selectively suppressing the RNA synthesis.

We have now discovered another antibiotic, designated derinamycin, in the culture mycelium of the same strain. The physicochemical properties of derinamycin differed significantly from those of rinamycin. This fact tempted us to undertake its biological characterization and subsequently to elucidate its mechanism of action.

Materials and Methods

1. Fermentation process

Streptomyces venezuelae Tü 1102 was isolated from a soil sample collected in India. The antibiotic activity (total activity of derinamycin and rinamycin) was followed by paper disc (diameter: 6mm) method on agar plate using Streptomyces viridochromogenes as test organism. The agar plate was composed of yeast extract 0.4 %, malt extract 1 %, glucose 0.4 % and agar 2 % (pH 7.3). The fermentation medium contained malt extract 2 %, Distiller soluble (Sitos Werke, Minden/Westf.) 2 %, NaCl 0.5 % and NaNO\(_3\) 0.1 % (pH 7.5). S. venezuelae was inoculated into 20 conical flasks, each of which containing 100-ml medium. After 24 hours, the flask cultures were divided and equal amounts placed into 2 jar fermentors previously prepared with 9 liters medium for each jar. The fermentation (aeration, 2 liters/minute) was continued for 20 hours, and the culture broth was introduced into the main fermentor containing 180 liters medium. The fermentation (aeration, 40 liters/minute) was stopped after 29 hours when the pH of the broth reached 7.15. The temperature was 26~28°C throughout the fermentation.

2. Purification procedure

The culture broth was neutralized with hydrochloric acid and filtered with the aid of Celite.

The resultant filter cake was extracted twice with methanol (25 liters each) and the combined extracts were concentrated in vacuo below 35°C to ca. 5 liters. Three volumes of ethanol were added under vigorous stirring. The solution was allowed to stand overnight at 0-4°C and the resultant precipitate was filtered off. The filtrate was concentrated to 800 ml, to which 4 volumes of ethanol was added. The solution was kept overnight at 0-4°C. The resulting supernatant solution, after filtration, was concentrated to 400 ml, which was followed by a third precipitation procedure, yielding 200 ml of concentrated solution. n-Butanol was then added while stirring and a syrupy precipitate formed (ca. 150 ml) which was collected by decanting.

The syrup was diluted to 200 ml with water and subjected to four counter-current distribution (50 ml at a time) in the solvent system n-butanol-water (1:1), utilizing 100 tubes each of which containing 100 ml of total solution. The activity was usually found in the last 60 tubes. The concentration of the recovered active fractions gave a syrup (40 g). This syrup was dissolved in a small amount of methanol and chromatographed with methanol on a column of silicagel (3 kg, Merck Kieselgel 60).

After 3 liters of effluent had been collected, the first active substance (rinamycin) was eluted by the following 4 liters of solvent. The second active substance (derinamycin) was then exhaustively eluted with the same solvent until no more activity could be detected. The concentration of all fractions with derinamycin activity yielded a brown residue (4.8 g).

The crude antibiotic was triturated with methanol at room temperature. After filtration, the soluble part was divided into 3 parts and each part was successively chromatographed on Sephadex LH 20 column (800 ml) using methanol as solvent. The combined active fractions of the three columns were concentrated and applied to another column. Throughout the chromatography, the first 300 ml of methanol eluate contained no antibiotic. The following 150 ml eluted the antibiotic fractions. The antibiotic was detected by the inhibition assay and its ultraviolet absorption. The concentration of the recovered active fractions gave a slightly brown powder (1.03 g).

3. Physicochemical analysis

The paper electrophoresis was carried out on a Whatman No. 1 paper for 3 hours at 250 V/19 cm x 5 cm, in the following buffers: acetate buffer (pH 4.0, 0.1 m), phosphate buffer (pH 7.0, 0.1 m) and veronal buffer (pH 8.6, 0.1 m). The ascending paper chromatography was done on a Whatman No. 1 paper.

The elementary analysis and the thermoelectric analysis for molecular weight were performed by the Microanalysis Service of Federal Institut of Technology, Zürich.

Molecular sieve chromatography was done on a column of Sephadex LH 20 (800 ml) in methanol. Running, simultaneously with the antibiotic, was one of polyethyleneglycols (MW 600, 1,000 and 1,500) as marker. The elution of the antibiotic was detected by U.V. absorption and that of polyethyleneglycols by iodine coloration.

The ultraviolet spectrum was measured in methanol by a Beckman Spectrophotometer and the infrared spectrum in KBr pellet by a Perkin-Elmer Spectrophotometer. The p.m.r. spectra were registered in methanol-d4 and in benzene-d6 (for the acetyl derivative) at 100 MHz by a Varian NMR Spectrometer.

4. Acetylation

The antibiotic (30 mg) was acetylated overnight in pyridine (2 ml)-acetic anhydride (2 ml) mixture at room temperature and the product was dried by repeated evaporation with chloroform. The resultant residue (52 mg) was chromatographed on a column of silicagel (5 g, Merck Kieselgel 60), using the solvent system; benzene-methanol (95:5). Fractions containing the principal product were detected by the ultraviolet absorption and the sulfuric acid coloration on a silicagel chromatogram (Merck Kieselgel 60, DC-Fertigplatten) done in the same solvent system. The evaporation of solvent of these fractions gave an acetyl derivative (33 mg).

5. Biological characterization

The antibiotic activity was determined by the paper disc method at a concentration of 1
mg/ml, measuring the diameter of the zone of inhibition on agar plates. Media used in this experiment were as follows: *Arthrobacter*, *Chromobacterium* and *Corynebacterium*: nutrient broth 0.8% and agar 1.5%, pH 6.8; *Achromobacter*, *Agrobacterium*, *Bacillus*, *Escherichia*, *Lactobacillus*, *Micrococcus*, *Pseudomonas* and *Staphyloccocus*; meat extract 0.3%, peptone 0.5%, NaCl 0.25% and agar 1.5%, pH 7.2; *Propionibacteria*: meat extract 0.3%, malt extract 0.3%, yeast extract 0.3%, peptone 2%, glucose 0.5%, ascorbic acid 0.02% and agar 0.1%, pH 7.0.

For all the other organisms, the medium was yeast extract 0.4%, malt extract 1%, glucose 0.4% and agar 2%, pH 7.3.

The minimal inhibitory concentrations (MIC), tested for certain organisms in liquid media, were determined by a Jouan Biophotometer. The liquid media were prepared similar to the agar media *loc. cit.* for appropriate organisms, but without agar. The method is as follows: When turbidity of culture reached 70% transmission, the antibiotic in DMSO solution was added to the culture. The concentration that stopped the turbidity growth as long as 10 hours was considered to be MIC. The DMSO concentration to be added was less than 1% of the culture, this concentration being harmless for the growth of microorganisms.

Throughout the tests, the organisms were grown at 27°C except *Bacillus subtilis* (37°C), *Streptomyces viridochromogenes* (37°C) and *Botrytis cinerea* (24°C).

6. Radioactive incorporation

*Bacillus subtilis* ATCC 6051 was inoculated into the synthetic medium consisting of KH$_2$PO$_4$ 0.3%, K$_2$HPO$_4$ 0.7%, sodium citrate 0.05%, (NH$_4$)$_2$SO$_4$ 0.1%, MgSO$_4$ 0.01% and glucose 0.2%. After 20 hours at 37°C the cell suspension formed was introduced into a fresh medium of the same composition, to give a turbidity of 95% transmission at 578 nm. The culture was continued until 80% transmission and then distributed to cuvettes by 7 ml. When turbidities of cuvette cultures approached 70% transmission, thymidine-2-C$^{14}$ (1 µCi, 60 mCi/mmol), uracil-2-C$^{14}$ (1.5 µCi, 61 mCi/mmol) or L-isoleucine-C$^{14}$ (U) (2.5 µCi, 10 mCi/mmol) together with 10 µg L-isoleucine in this last case was added per cuvette, and after 10 minutes the antibiotic in DMSO was added to make final concentrations of 35 µg/ml or 25 µg/ml. Control experiments were carried out without antibiotic for each radioactive incorporation. An aliquot (200 µl) of the cultures was sampled and added to 7% trichloroacetic acid (1 ml) on ice. After 30 minutes the precipitate which formed was filtered on a membrane filter (pore size 0.45 µ), washed four times by cold trichloroacetic acid (5%, 5ml for each wash) and dried. The precipitate and filter were placed in a scintillation vial and mixed with toluene containing 0.4% diphenyloxazole and the radioactivity was counted by a Nuclear Chicago (Mark II) Liquid Scintillation Counter.

The double-isotope experiment to examine the relative effects of the antibiotic on DNA versus RNA synthesis was carried out by adding concurrently the antibiotic (the final concentration 25 µg/ml), thymidine-(methyl-H$^3$)-5'-monophosphate (5 µCi, 1 Ci/mmol) and uracil-2-C$^{14}$ (1 µCi, 61 mCi/mmol) to the same culture, at the cell turbidity of 70% transmission. Control experiment contained no antibiotic.

Results and Discussion

The time courses of the antibiotic activity (total of rinamycin and derinamycin), pH and sediment during the main fermentation are summarized in Fig. 1. After the isolation, the approximate ratio of rinamycin versus derinamycin produced in the mycelium was 20:1. Because the rinamycin activity is dominant in this production, it is difficult to point out the optimal condition for derinamycin production.

Derinamycin is soluble in methanol, but much less soluble in ethanol, n-butanol, acetone, and practically insoluble in the less polar solvents and in water. The antibiotic is ninhydrin-positive and can also be detected by the sulfuric acid and iodine coloration.
It appears neutral on the paper electrophoreses at pH 4.0, 7.0 and 8.6, presumably because of the poor solubility in water.

Fig. 2. summarizes the paper-chromatographic behaviours in the solvents frequently used for identification purpose, suggesting the hydrophobic but not quite lipophilic nature of the antibiotic.

The antibiotic melted at 142~145°C. The microanalysis data are as follows:

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Rt Value</th>
<th>Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>A: 20% NH₄Cl</td>
<td></td>
<td>ninhydrin</td>
</tr>
<tr>
<td>B: Benzene-methanol (4:1)</td>
<td></td>
<td>bioautograph</td>
</tr>
<tr>
<td>C: n-Butanol satd. with water</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D: Ethylacetate-pyridine-water (2:1:2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E: n-Butanol-acetic acid-water (4:1:2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F: n-Butanol-methanol-water (4:1:2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G: Acetone-water (1:1)</td>
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</tbody>
</table>

It appears neutral on the paper electrophoreses at pH 4.0, 7.0 and 8.6, presumably because of the poor solubility in water.

Fig. 2. Paper chromatogram; detected by ninhydrin and bioautograph against Ps. saccharophila
Found: C 56.16, H 8.54, N 1.19
Calcd. for C₅₁H₉₃NO₂₃ (MW1087): C 56.30, H 8.56, N 1.29

The molecular sieve chromatography suggested that the molecular weight of derinamycin might be around 1,000.

The ultraviolet spectrum is shown in Fig. 3. It has a maximal absorption at λₓ(max) 209 (E₁% 1cm 156) and an inflexion at λₓ(sh) 216~223 (E₁% 145).

The infrared spectrum given in Fig. 4 demonstrates a large absorption of OH group (3400 cm⁻¹) and relatively small peaks in the regions of carbonyl (1700 cm⁻¹) and alkene or amino (1600~1640 cm⁻¹) absorptions.

The p.m.r. spectrum is shown in Fig. 5 suggesting the presence of methyl (~1 ppm) and methylene (~1.7 ppm) protons but not of aromatic protons.

In order to survey the molecule more closely, a peracetyl derivative was made, the p.m.r. spectrum of which is demonstrated in Fig. 6. Though not sufficiently resolved, peaks at 1.7~2.1 ppm imply 8~11 acetyl groups incorporated into OH or NH₂ functions of the molecule. The molecular weight of this derivative was determined to be 1347 by the thermoelectric method performed in ethyl acetate. On an assumption that the acetylation introduced 8~11 acetyl groups, the molecular weight of derinamycin could be calculated to be 885~1011, which is approximately in accord with the results obtained by the molecular sieve method and the micro-analysis.

With these results on derinamycin, we compared the known antibiotics in two ways: comparison of ultraviolet absorptions and of molecular weights together with the nitrogen content.

Derinamycin possesses a characteristic ultraviolet absorption which can easily be differentiated from the known antibiotics. Although some of peptide, depsipeptide and macrolide antibiotics bear a certain resemblance of absorption pattern to derinamycin, in that they exhibit...
Table 1. Antimicrobial spectrum of derinamycin

<table>
<thead>
<tr>
<th>Strain</th>
<th>M.I.C. in liquid medium (µg/ml)</th>
<th>Inhibition zone (mm). Antibiotic concn.: 1mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinomycetales</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Nocardia brasiliensis</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Streptomyces collinus</em></td>
<td>11.5</td>
<td></td>
</tr>
<tr>
<td><em>Streptomyces fradiae</em></td>
<td>11.5</td>
<td></td>
</tr>
<tr>
<td><em>Streptomyces glaucescens</em></td>
<td>11.5</td>
<td></td>
</tr>
<tr>
<td><em>Streptomyces griseoviridis</em></td>
<td>27.5</td>
<td></td>
</tr>
<tr>
<td><em>Streptomyces griseus</em></td>
<td>18.0</td>
<td></td>
</tr>
<tr>
<td><em>Streptomyces prasinos</em></td>
<td>20.5</td>
<td></td>
</tr>
<tr>
<td><em>Streptomyces ramulosus</em></td>
<td>15.5</td>
<td></td>
</tr>
<tr>
<td><em>Streptomyces viridochromogenes</em></td>
<td>40</td>
<td>16.0</td>
</tr>
<tr>
<td><em>Streptosporangium roseum</em></td>
<td></td>
<td>25.0</td>
</tr>
<tr>
<td>Eubacteriales</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Achromobacter geminiani</em></td>
<td>10</td>
<td>26.0</td>
</tr>
<tr>
<td><em>Agrobacterium tumefaciens</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Arthrobacter aurescens</em></td>
<td>10</td>
<td>31.0</td>
</tr>
<tr>
<td><em>Arthrobacter pascens</em></td>
<td></td>
<td>18.0</td>
</tr>
<tr>
<td><em>Arthrobacter simplex</em></td>
<td></td>
<td>10.5</td>
</tr>
<tr>
<td><em>Bacillus brevis</em></td>
<td></td>
<td>12.0</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>35</td>
<td>17.0</td>
</tr>
<tr>
<td><em>Chromobacterium violaceum</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Corynebacterium rathayi</em></td>
<td>10</td>
<td>20.0</td>
</tr>
<tr>
<td><em>Escherichia coli K12</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Lactobacillus casei</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Micrococcus luteus</em></td>
<td>25</td>
<td>19.5</td>
</tr>
<tr>
<td><em>Propionibacterium freudenreichii</em></td>
<td></td>
<td>15.5</td>
</tr>
<tr>
<td><em>Propionibacterium shermanii</em></td>
<td></td>
<td>14.0</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td></td>
<td>11.5</td>
</tr>
<tr>
<td>Pseudomonadales</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas saccharophila</em></td>
<td>10</td>
<td>22.5</td>
</tr>
<tr>
<td>Yeasts</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>50</td>
<td></td>
</tr>
<tr>
<td><em>Candida lipolytica</em></td>
<td></td>
<td></td>
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<tr>
<td><em>Saccharomyces cerevisiae</em></td>
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<td></td>
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<tr>
<td>Fungi</td>
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<tr>
<td><em>Aspergillus fumigatus</em></td>
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<tr>
<td><em>Aspergillus niger</em></td>
<td>6.5</td>
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<tr>
<td><em>Botrytis cinerea</em></td>
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<td></td>
</tr>
<tr>
<td><em>Fusarium larvarum</em></td>
<td>9.5</td>
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</tr>
<tr>
<td><em>Fusarium solani</em></td>
<td>14.0</td>
<td></td>
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<tr>
<td><em>Mucor miehei</em></td>
<td>8.0</td>
<td></td>
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<tr>
<td><em>Mucor mucedo</em></td>
<td>12.0</td>
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<tr>
<td><em>Paecilomyces varioti</em></td>
<td>8.0</td>
<td></td>
</tr>
<tr>
<td><em>Penicillium sp.</em></td>
<td>7.5</td>
<td></td>
</tr>
<tr>
<td><em>Trichophyton mentagrophytes</em></td>
<td></td>
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</tbody>
</table>
a maximum in the low wavelength-region and an inflexion in the higher area, the former two groups have a much higher content of nitrogen and a characteristic carbonyl NH absorption at \( \sim 1530 \text{ cm}^{-1} \) in the infrared spectrum, while the macrolides having a strong absorption of carbonyl group at \( \sim 1730 \text{ cm}^{-1} \) and rather weak or no absorption of hydroxyl group, which is not the case of derinamycin.

On the other hand, the large molecular weight and the small nitrogen content of derinamycin enabled us point to only a few number of antibiotics which possess figures comparable to derinamycin. Those antibiotics are macrolides such as angolamycin, shincomycin and leucomycin or such antibiotics as monazomycin, ossamycin and takacidin which also show a macroclide-like absorption at \( \sim 1730 \text{ cm}^{-1} \) in the infrared spectrum, they can thus be differentiated from derinamycin.

Derinamycin was differentiated from an authentic sample of monazomycin* by comparisons of paperchromatographic behaviours.

From these data, we concluded that derinamycin was a new antibiotic.

The antimicrobial activity of derinamycin by the agar-diffusion method and the minimal inhibitory concentrations for certain organisms in liquid media are given in Table 1. When tested by the agar-diffusion method, all the organisms tabulated were insensitive to the antibiotic at the concentration of 100 \( \mu \text{g/ml} \), apparently because of the poor diffusibility of the antibiotic. The table indicates that derinamycin inhibits the growth of fungi, gram-positive bacteria and certain gram-negative bacteria but that yeasts are less affected by the antibiotic.

Studies of the antibiotic effect on the incorporation of radioactive precursors into macromolecules, using intact cells of *Bacillus subtilis*, are shown in Fig. 8a, b and c.

The figure indicates that derinamycin can suppress DNA, RNA and protein syntheses at the minimal concentration for inhibition (35 \( \mu \text{g/ml} \)).

An antibiotic concentration of 25 \( \mu \text{g/ml} \) also significantly inhibited DNA and RNA syntheses while having relatively little effect on protein synthesis. This concentration of antibiotic produced only a slight deceleration effect on the cell proliferation, as could be observed by the

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* We thank Prof. H. YONEHARA, Institute of applied Microbiology, University of Tokyo, for the monazomycin sample.
biophotometer. Because the degree of antibiotic effect on DNA and RNA syntheses could not be compared by this method, we initiated a double-isotope experiment in order to define more accurately the relative effect on DNA and RNA syntheses.

Fig. 9 demonstrates the experiment in which thymidine-(methyl-H3)-5'-phosphate and uracil-2-C14 were used simultaneously as precursors. Derinamycin exhibited a somewhat greater inhibition in the outset of radioactive incorporation of uracil than that of thymidine. The overall inhibition pattern, however, was quite similar for both incorporations. This difference of inhibition at this early stage of incorporation would not be significant enough to determine whether there was selective inhibition of RNA synthesis because of uncertainties such as possible differences in precursor pool size. The overall inhibition pattern indicates that the both syntheses are simultaneously affected.

Antibiotics which interfere with DNA primer function, are apt to inhibit both DNA and RNA syntheses. Our current efforts are directed to examine this possibility.

Acknowledgement

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References