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Ramycin: A New Antibiotic

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SUMMARY: A strain of *Mucor ramannianus* from Campine forest soil was found to secrete an antibiotic, for which the name Ramycin is proposed. It is especially active against Gram-positive micro-organisms and *Mycobacterium tuberculosis* H37Rv, but sensitive organisms quickly acquire resistance to it. Although it is not very toxic, it possesses a necrotic action on tissues. Ramycin can protect mice against streptococcal and staphylococcal infections but not against H37Rv.

Ramycin crystallizes in orthorhombic plates. The new antibiotic is an optically-inactive unsaturated hydroxy acid, containing C, H and O only.

In general, it is considered that the Phycomycetes build up relatively simple metabolic products only and that they are not capable of synthesizing the complex molecules characteristic of so many antibiotics (Brian, 1951). In fact, the only reports of antibiotic activity from this group which we have found are those of Jefferys, Brian, Hemming & Lowe (1953) and of Miller & Porter (1956-7). The last authors reported that the action was due to common organic acids such as fumaric and lactic acid.

In a study of the microbial population of soils from Campine in Northern Belgium we isolated a strain of *Mucor ramannianus* which forms a new antibiotic. This has been isolated in crystalline form and the name Ramycin is proposed for it. This report presents some biological and chemical characteristics of this new product.

RESULTS

Cultural characteristics of the fungus

On potato agar (potato scrapings 200 g., glucose 10 g., agar 15 g., tap water 1 l.) the fungus grows as an interlacing network of white sterile filaments lying loosely on the surface of the medium, with a pink fluff of aerial hyphae never more than 3 mm. high. The underside of the mycelium remains uncoloured. On media predominantly mineral, the pink colour is replaced by white and the colony becomes more filamentous through the overgrowth of sterile filaments.

The hyphae are not septate, except some branches of older cultures, especially where sporangiophores arise. The latter vary in length and the sporangia vary in size. Microscopically they appear as brown spheres with spores moving inside them. The columella, which is not visible until after the disruption of the sporangium wall at maturity, is round, and sometimes surrounded by a short collar, which is a remnant of the sporangium wall. The spores are round and very uniform in size, of diameter 1.5 to 2 μ . Rhizoids are absent. Chlamydospores are present but zygosporangia most probably are not formed. Sometimes we have encountered oval or round spheres surrounded by three or four appendages, yet we have never seen suspensors. The mycelium

seems to be homothallic. In submerged cultures the hyphae form rounded buds of irregular size, in yeast-like fashion.

The foregoing properties correspond to those of *Mucor ramannianus* (Naumov, Buchet & Mouraviev, 1939). Robbins *et al.* (1945) found no antibiotic production in this species and we have often isolated antibiotically inactive *M. ramannianus*. We propose the varietal name 'keerbergensis' after the village where the soil sample was collected.

Production of antibiotic

The fungus was maintained on the above-mentioned potato agar. An inoculum was grown at 26°–28° in rotary shakers on the following medium: corn-steep solids 3 g., skim milk powder 3 g., dried brewer's yeast 3 g., NaCl 5 g., glucose 20 g., tap water 1 l., pH adjusted to 7 with 10 % NaOH. After 24 hr. it was transferred to the same medium but containing lactose instead of glucose, in either rotary shakers or fermentation tanks. In rotary shakers at 27°–28°, the maximum antibiotic yield (up to 120 µg./ml.) was obtained after 52 hr. After this the antibiotic activity decreased, and autolysis of the mycelium started at 80 hr. The pH fell at the beginning and rose after about 10 hr.; later it fell again. We did not observe a correlation between antibiotic yield and sugar utilization, soluble organic-N, mycelium weight or pH. As in the penicillin fermentation lactose, which is less readily metabolized than glucose, gives higher yields of antibiotic. Starch also gives satisfactory yields. To obtain a homogenous growth on rotary shakers we had to inoculate with a heavy spore suspension and use a large quantity of antifoam. In fermentation tanks growth was more homogenous, probably due to the agitation of the impellers.

Extraction

The substance is acidic and can be extracted and concentrated by solvent transfer in water-immiscible solvents. The culture fluid was adjusted to pH 7.5 and filtered on Buchner funnels through Whatman no. 1 filter-paper, covered with a thin layer of infusorial earth. One fifth of its volume of benzene or toluene was added to the filtrate, whose pH was adjusted to 3 and the mixture was shaken. Practically all the Ramycin passed into the solvent, from which it could be re-extracted into a smaller volume of water brought to a final pH of 8. This was adjusted to pH 3 and the product extracted again into ether or chloroform. The extract was concentrated *in vacuo* to about 1 % of the original volume. Ramycin was then precipitated by five volumes of petroleum ether and separated.

Chemical and physical properties

The colourless orthorhombic plates in which Ramycin crystallized melted at 158°–160° (decomp.). In the acidic form the antibiotic was very soluble in most organic solvents with the exception of light petroleum. In water at room temperature, about 100 µg./ml. dissolved. The antibiotic was optically inactive (1 % in ethanol). No specific absorption in the ultraviolet was found.

The infrared spectrum exhibited peaks at 3435, 1727, 1688, 1439, 1380, 1258, 1032 and 973 $m\mu$. The absorption at 3435 indicates probably an alcoholic OH. The titration curve indicates a single acidic group (pK 4.6) and an equivalent weight of 478.

The stability of Ramycin depends principally on the temperature. After 3 min. boiling at pH 2, 70% activity was destroyed, at pH 6, 20% and at pH 8, 80%. No loss of activity was noted after 24 hr. at 37° at pH 2 or 9.5.

The following reactions on active functions were negative: the reactions for amines, amides (boiling with 10% NaOH); the reaction for aldehydes and carbonyl function (Schiff test, reduction of AgNO_3 , temperature elevation with phenylhydrazine, reaction with *o*-dianisidine); the reaction for sugars (tests with α -naphthol, anthrone and resorcinol); the reaction for phenol having a free OH in ortho position (Millon's test); the reaction for phenol and enol (no colour with FeCl_3); the reaction for pyrrol (test with *p*-aminobenzaldehyde); Fehling's solution was not reduced; the biuret reaction was negative.

Positive results were obtained for the following functions: the reaction for $\text{CH}_3\text{-CHOH-R}$ or $\text{CH}_3\text{-COR}$ (iodoform test), the test for acids (with Na_2CO_3) and hydroxy acids (with *s*-diphenylcarbazide); test for double bond (bromine fixation in CCl_4). From these tests we may conclude that Ramycin is a hydroxy acid with one or more double bonds.

Ramycin contains only C, H and O, and yields the following data on elementary analysis: C, 69.7%; H, 9.32%; O, 19.53%. Since S is absent and there is no colour with FeCl_3 we suppose the acidity is due to a carboxyl group.

Antibacterial action

The sensitivity of various organisms is listed in Table 1. Ramycin is especially active against Gram-positive micro-organisms and against *Mycobacterium tuberculosis* H37Rv. Micrococci are more sensitive than streptococci.

Table 1. *Inhibitory concentrations of Ramycin against different micro-organisms*

Organism	Minimum inhibitory concentration of Ramycin ($\mu\text{g./ml.}$)
<i>Streptococcus</i> ATCC 8668	1
<i>Micrococcus pyogenes aureus</i> ATCC 6538 P	0.05
<i>M. pyogenes aureus</i> resistant to penicillin, streptomycin, tetracyclines, erythromycin and chloramphenicol	0.05
<i>Sarcina lutea</i>	0.05
<i>Salmonella typhi</i>	> 500
<i>Brucella abortus</i>	500
<i>Corynebacterium diphtheriae</i> ATCC 9059	0.5
<i>Bacillus mycoides</i>	2
<i>Mycobacterium tuberculosis</i> H37Rv	0.1
<i>M. tuberculosis</i> (clinical case)	0.3
<i>M. lacticola</i>	100
<i>Candida albicans</i>	> 500

The organisms were streaked out from 24 hr. old cultures, upon the surface of Trypticase Soy agar (Baltimore Biological Laboratory) wherein the antibiotic was incorporated at 45°. The inhibition was noted after 24 hr. incubation at 37°. The mycobacteria were tested in Dubos medium (Difco) and results read after 7 days at 37°.

We did not find cross resistance to streptomycin, penicillin, the tetracyclines, bacitracin, erythromycin, novobiocin or Staphylomycin (a trade name for antibiotic number 899, De Somer & Van Dijck, 1955). Sensitive micrococci quickly acquired resistance to Ramycin, in a pattern similar to that shown with streptomycin. The antibiotic is not active against poliomyelitis virus type I, II or III.

The activity of the product is not influenced by variation of the pH between 6.5 and 8.

Ramycin is reversibly inactivated *in vitro* by blood serum. When tested in media containing different amounts of bovine serum ranging from 5 to 50 %, 0.5 ml. serum could inactivate 1.4 μ g. Ramycin. The activity could be recovered again by extraction with organic solvents.

The action of Ramycin is only bacteriostatic. Synergism is observed in combination with staphylomycin. The antibiotics in common clinical use (penicillin, the tetracyclines, bacitracin, streptomycin, chloramphenicol and erythromycin) exhibit only an additive effect against micrococci; similar results were obtained against H37Rv when combined with streptomycin, *p*-aminosalicylic acid and isonicotinic acid hydrazide.

Toxicity. The acute LD 50 for intraperitoneal administration to mice was found to be 165 mg./kg. The animals die 3–4 days after the injection of the Ramycin, without distinct symptoms. Autopsy and histological examination of the organs revealed no abnormalities.

For studying chronic toxicity we injected white mice on 10 consecutive days with 1 mg. intraperitoneally. No important deviations were found in the weight curve or in the blood picture, apart from an increase of 12.9 % in the leucocyte count over that in the control animals. Histological examination showed no lesions in the liver, spleen, brain, kidneys or intestine. Intradermal injections of 500 μ g. into rabbits caused a little topical necrosis of about 1 mm. diameter, which disappeared after 3–4 days. However, a red irritation zone such as formed by other irritating products was not seen. A solution of 1 mg./ml. in physiological saline caused no irritation to the eye of rabbits.

An intramuscular injection in the thigh of guinea-pigs caused local oedema, subsequently involving the entire limb and leading to progressive necrosis. The administration of Ramycin by this route is thus excluded.

Animal protection tests

The effect of Ramycin given by various routes on mice infected with *Streptococcus pyogenes* or with *Staphylococcus aureus* Strain 663 (kindly supplied by Glaxo Laboratories Ltd.) is summarized in Table 2. Subcutaneous and oral administration gave the best results. However, a survival of 100 % was never attained.

The activity *in vivo* against H37Rv was tested in guinea-pigs, which were infected subcutaneously over the sternum with 0.1 ml. of a 5-day-old culture on Dubos medium. The treatment was started 10 days after the infection and continued for a month after which the animals were killed.

Table 3 summarizes the autopsy findings in treated and control animals.

Table 2. *Chemotherapeutic effectiveness of Ramycin against experimental infections in mice*

	Route of administration				Not treated
	Intra-peritoneal	Intra-muscular	Oral	Sub-cutaneous	
Against <i>Streptococcus pyogenes</i> ATCC 8668					
Survival time (hr.)	74	74.4	85	90.7	28.2
Survivals % after 10 days	0	0	20	20	0
Against <i>Staphylococcus aureus</i> strain 663					
Survival time (hr.)	49	56	90.8	72	20
Survivals % after 10 days	0	0	10	40	0

The dosage of Ramycin was 1 mg. at 6 and 18 hr. after infection. Groups of 15 animals were used for each treatment. They were infected intraperitoneally with about 10^8 organisms.

Table 3. *Macroscopic involvement of the organs of guinea-pigs infected with Mycobacterium tuberculosis strain H37Rv and treated with Ramycin*

Daily treatment	Lungs	Spleen	Liver	Axillary lymph nodes	Abcess
10 mg./kg. <i>per os</i>	+	+++	+++	⊥	+
50 mg./kg. <i>per os</i>	+	++	++	⊥	⊥
10 mg./kg. subcutaneously	⊥	+	+	⊥	+
50 mg./kg. subcutaneously	—	⊥	—	—	+
(killed after 15 days)					
Untreated	+	+++	+++	+	+

The symbols represent the extent of the tuberculous lesions seen at necropsy (summarized) after 1 months treatment: ⊥: rare or questionable foci of involvement; +: moderate number of tubercles; ++: many tubercles; +++: extensive involvement, nearly confluent tubercles; —: no pathogenic involvement.

It is obvious that Ramycin did not influence the evolution of tuberculosis in these animals when oral doses of 50 mg./kg. were given. When 10 mg./kg. were administered subcutaneously, a partial protection was noted, but the antibiotic could hardly be considered active *in vivo*. In control experiments using streptomycin (10 mg./kg.) no lesions were found and the weight curve increased regularly.

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