A NEW *MICROMONOSPORA*-PRODUCED MACROLIDE ANTIBIOTIC, ROSAMICIN*

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A new antibiotic, rosamicin, classified as a macrolide, has been isolated from the fermentation broth of a new species of *Micromonospora*, *M. rosaria*. The antibiotic is separated from the broth by a solvent extraction procedure and purified by column chromatography. Chromatographic studies indicate that rosamicin is different from all related macrolides and is a novel antibiotic. It has broad-spectrum activity, although it is more potent against gram-positive organisms. Rosamicin is also active against *Mycoplasma*.

The first macrolide antibiotic to be isolated from a species of *Micromonospora* was megalomicin, produced by *M. megalomicea*.[1] A new macrolide antibiotic unrelated to megalomicin and named rosamicin has now been isolated from the fermentation broth of another new species of the genus *Micromonospora*. This paper presents initial data on the taxonomy of this new organism, and primary data concerning the chemical and biological properties of rosamicin.

Materials and Methods

The organism which produces rosamicin is a new species of *Micromonospora*, named *M. rosaria* and assigned NRRL No. 3718. This culture was isolated from a soil sample obtained from Little Thicket, San Jacinto County, Texas.

The colonial morphology of a 14-day old culture was determined after incubation at 24~26°C on an agar medium consisting of N-Z amine type A, 3%, dextrose, 1%; and agar, 1.5%. Macroscopically, no aerial mycelia are evident; colonies are slightly raised, granular to weakly folded. Growth is fair, glistening, and no diffusible pigment is produced. On an agar medium composed of yeast extract, 1% and glucose 1%, a rose-colored diffusible pigment is produced.

The mycelia are regularly branched averaging 10~20 μ in length and 0.6 μ in diameter. Numerous chlamydospores are produced, up to 2 μ in diameter. Conidia are not observed on the N-Z amine medium.

The laboratory fermentation of *M. rosaria* is carried out in two stages, as follows:

a) Germination stage: A loopful of *M. rosaria* culture from an agar slant is used to inoculate a 300-ml flask containing 100 ml of the following sterile medium: beef extract, 3 g; tryptose, 5 g; yeast extract, 5 g; dextrose, 1 g; potato starch, 24 g, calcium carbonate, 2 g; tap water, 1,000 ml. The flask and its contents are incubated for 72 hours at 35°C on a rotary shaker with 2-inch (5.1 cm) stroke at 280 rpm.

* Formerly named rosaramicin.

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b) Fermentation stage: Five ml of inoculum (from the germination stage) is transferred to a 500-ml Erlenmeyer flask containing 100 ml of the following sterile medium: Amber BYF No. 300, 7 g; fish solubles, 1 g; corn steep liquor solids, 1 g; potato starch, 30 g; calcium carbonate, 1 g; tap water, 1,000 ml. The pH is adjusted to 7.2 before autoclaving. The flask and its contents are incubated for 72~96 hours at 28°C on a rotary shaker as previously described.

The microbiological assay of rosamicin is a cylinder cup assay using *Bacillus subtilis* ATCC 6633 as the test organism.

The physical conditions of the assay consist of a base layer of 21 ml and a seed layer of 4 ml of Antibiotic Medium (Difco) No. 5. A commercial source of *B. subtilis* spores is used as the inoculum. The standard curve used in the assay has the following antibiotic concentrations: 0.64, 0.8, 1.0 (reference point), 1.25 and 1.56 mcg per ml. The diluent used in the preparation of all dilutions is 0.1 M phosphate buffer (pH 8.0).

The reference standard prepared as described has an assigned potency of 1,000 mcg/mg. One mcg of this preparation in 1 ml of 0.1 M phosphate buffer analyzed under the conditions of this assay will elicit a zonal response of 17.8±1.0 mm. The working standard is a preparation having a potency of 775 mcg/mg assayed against the reference standard.

The *in vitro* activity of rosamicin was studied by conventional tube dilution procedures using yeast beef broth at pH 7.4.

The protective activity of rosamicin was tested in male CF-1 mice weighing approximately 20 g each. The antibiotic was given as a suspension or solution in an aqueous vehicle containing 0.5 % carboxy methyl cellulose in two doses; shortly before, and 4 hours after intraperitoneal infection with bacteria. Infected, non-treated mice generally died 18 hours after infection while survivors in treated groups were determined 48 hours after infection.

Results and Discussion

Isolation and Characterization

Rosamicin is isolated from the fermented broth by a solvent extraction procedure. A 60-liter fermentation is adjusted to a pH of 9.5 with sodium hydroxide and is extracted two times using 2 volumes of ethyl acetate, each time, for each volume of whole broth. After separation, the solvent phase is concentrated to a volume of 1.5 liters. The biological activity of a typical concentrate utilizing a disc 6.35 mm in diameter gives a zone diameter of 40 mm against *Staphylococcus aureus* and 22 mm against *Pseudomonas aeruginosa*. The active substance is separated from this concentrate with three 600 ml extractions of 0.1 N H_2SO_4. The aqueous phases are pooled and adjusted to a pH of 9.5 with sodium hydroxide and extracted two times in small segments with equal volumes of ethyl acetate. The solvent phase is separated and concentrated to a volume of about 70 ml. This concentrate is slowly added to a mixture of ether-hexane (3:2) with stirring. The resulting precipitate is filtered, discarded and the mother liquor evaporated to dryness. This residue is redissolved in 100 ml of ethyl acetate and extracted three times with 25 ml of water. The solvent phase is separated, dried over sodium sulfate, filtered and concentrated to dryness.

This material is now dissolved in 20 ml of ethyl ether and insolubles if any, filtered and discarded. The ether solution is added to about 100 ml of petroleum ether (b.p. 30~60°C) and the resulting buff-colored precipitate filtered and dried under vacuum. The mother liquor is concentrated to a smaller volume affording additional precipitate which is also filtered, dried and combined with the initial

precipitate. The weight of the combined precipitate is about 1.5 g.

Material produced in this way has been assigned a working potency of about 700 mcg/mg of rosamicin complex according to the bioassay procedure previously described.

Characterization and Properties of the Antibiotic

Chromatography in a variety of systems differentiated rosamicin from all other groups of compounds except the macrolide group of antibiotics. Chromatography on thin-layer plates using a solvent mixture consisting of chloroform - methanol - 17 % ammonia, 2 : 1 : 1 (lower phase), shows the similarity of rosamicin with other macrolide antibiotics (Table 1). Solvent mixtures consisting of butanol - water - acetic acid, 3 : 1 : 1; and chloroform - methanol, 3 : 2 (Table 1) differentiated the antibiotic from a number of important macrolides except for cirramycin A_1. Cirramycin A_1 was differentiated from rosamicin chromatographically and by the comparison of several additional characteristics which are summarized in Table 2.

Bioautography following chromatography on thin-layer silica gel plates using a

Table 1. Comparative thin-layer chromatography of rosamicin with some macrolide antibiotics

System	Antibiotic	Rf and color by H_2SO_4 spray*	Rf of inhibition zone**
Chloroform-methanol-17 % ammonia, 2 : 1 : 1	Rosamicin	0.82 0.98 Tan	0.98
	Megalomicin	0.97 Black	0.97
	Oleandomycin	0.78 0.95 Black	0.95
	Erythromycin	0.94 Brown	0.94
	Spiramycin	0.95 Red-brown	0.95
	Carbomycin	0.96 Purple	0.96
Butanol-acetic acid-water, 3 : 1 : 1	Rosamicin	0.31, 0.37 0.44 Tan	0.37
	Megalomicin	0.21 Purple	0.21
	Erythromycin	0.38 ~ 0.45 Brown	0.40
	Spiramycin	0.16, 0.31 0.35, 0.45 Red-brown	0.16
	Oleandomycin	0.14, 0.36 Black	0.36
	Carbomycin	0.58 Purple	0.58
Chloroform-methanol, 3 : 2	Rosamicin	—	0.48
	Oleandomycin	—	0.28
	Erythromycin	—	0.29

* Plate heated at 100℃ and sprayed with H_2SO_4 in methanol (1 : 1).
** Plated against *Sarcina lutea*.

Table 2. Comparative data; rosamicin and cirramycin A_1

	Rosamicin	Cirramycin A_1
Chromatography Silica gel G Plates, chloroform-methanol (4 : 1), plated against *Sarcina lutea*	Rf 0.51	Rf 0.44
Melting point	119~122℃*	124~128℃
pKa	8.7	8.0
Optical rotation	−33.4°	−28.0°

* From REIMANN *et al.*[3]

Table 3. Chemical and physical properties of rosamicin

Elemental analysis (average of 2 determinations)	Carbon	63.18
	Hydrogen	8.86
	Nitrogen	2.29
	Oxygen (by difference)	25.67
Optical rotation		$[\alpha]_D^{25}$ −33.4° (*c* 0.3)
pKa		8.7
Neutralization equivalent		599
Ultra-violet spectrum (methanol)		$E_{1cm}^{1\%}$=238 (240 nm)

Table 4. Solubility of rosamicin free base

Solvent	Solubility*
Water	Slightly soluble
Methanol	Very soluble
Acetone	Very soluble
Chloroform	Very soluble
Benzene	Very soluble
Ether	Sparingly soluble

* According to U.S. Pharmacopeia, 17th revision (1965) p. 8.

Table 5. Comparative stability study; rosamicin and erythromycin

pH*	Minutes at 100°C	Potency (mcg/ml)	
		Rosamicin	Erythromycin
2.2	0	242	< 10
	30	121	< 10
4.0	0	218	249
	30	240	< 10
6.0	0	210	257
	30	180	207
7.0	0	224	254
	30	234	242
8.0	0	250	259
	30	206	250
9.0	0	228	257
	30	146	164
10.0	0	236	246
	30	64	170

* McIlvaine's buffers from pH 2.2~8.0; borate buffer at pH 9.0~10.0.

solvent system consisting of chloroform - methanol (4 : 1) indicates that rosamicin consists of one major antibiotic component (Rf 0.51) and at least two minor active components (Rf's 0.16, 0.31).

Rosamicin complex gives a positive color reaction in the Molisch, Starch-KI and Elson-Morgan tests and a negative color reaction in the biuret, ninhydrin and Sakaguchi tests. The ultraviolet absorption spectrum in 95% methanol displays a peak ($E^{1\%}_{1cm}$ 238) at 240 nm.

Separation of the major component is achieved by dissolving rosamicin complex in a mixture of chloroform - methanol (9 : 1) and adsorbing this solution at the top of a silicic acid column. The column is eluted with chloroform - methanol (4 : 1) and fractions are collected. The column is monitored by disc testing each fraction against *S. aureus*. The fractions are chromatographed on silica gel thin-layer plates, developed for 1 hour in a chloroform - methanol (4 : 1) solvent system. The patterns of the antibiotic fractions are determined by chromatography and detected by bioautography and sulfuric acid treatment of the plates. Also, by viewing the sulfuric acid sprayed plates under a U. V. source after heating some biologically inactive materials fluorescence under U. V. light and therefore become apparent. Fractions are combined according to their chromatographic patterns and are concentrated.

By this method, rosamicin was separated and isolated after pooling similar active fractions, drying, dissolving in acetone and precipitating with ethyl ether. The mother liquor is now evaporated to dryness yielding the antibiotic as a white powder. Rosamicin at this stage exhibits a defined potency of 1,000 mcg/mg.

The chemical and physical properties of rosamicin are summarized in Table 3 and solubility characteristics are shown in Table 4. Desosamine was found to be present (procedure of Flynn *et al.*[2]) and identical to an authentic sample by paper chromatographic comparison using a solvent mixture of butanol - pyridine - acetic acid - water (6 : 4 : 1 : 3). Additional chemical studies and elucidation of the structure of rosamicin are given in Reimann *et al.*[3]

A stability study comparing rosamicin base with erythromycin base is shown in Table 5. Buffered solutions containing approximately 250 mcg/ml of each antibiotic were made up in a range of pH 2.2~10. Samples were taken at 0 and 30 minutes

after heating in closed tubes at 100°C in a boiling water bath, cooled rapidly and submitted for microbiological assay. As is illustrated in Table 5, rosamicin is much more stable than erythromycin under acid conditions, has a similar pattern at mid-range through pH 9 and is somewhat less stable at pH 10.

The antibiotic has been tested for stability against trypsin, chymotrypsin, pepsin, and α-amylase at the optimal condition for activity of each enzyme. The antibiotic was stable to all of the enzymes tested for up to 24 hours at 37°C.

In Vitro Antimicrobial Activity of Rosamicin

The results of *in vitro* tests in which rosamicin was compared with erythromycin and megalomicin A are shown in Table 6 and demonstrate that it has a broad antibacterial spectrum *in vitro* with greatest activity against gram-positive bacteria. Its activity against gram-negative bacteria appears to be considerably greater than that evidenced by erythromycin.

Table 6. *In vitro* activity of rosamicin

Organism	No. Strains	MIC (mcg/ml)		
		Rosamicin	Megalomicin A base*	Erythromycin base*
Staphylococcus aureus	6	0.03~3.0	0.3	0.01~0.75
Streptococcus pyogenes	7	0.75~3.0	0.3 ~7.5	0.3 ~0.75
Enterococcus sp.	2	0.03~0.08	0.75~3.0	0.3
Diplococcus pneumoniae	3	0.03~0.08	3.0	3.0
Escherichia coli	3	3.0	0.3 ~7.5	7.5
Klebsiella pneumoniae	4	0.3 ~3.0	0.75~>25	7.5
Aerobacter aerogenes	3	0.75~7.5	7.5 ~>25	—
Proteus sp.	3	0.3	0.3 ~0.75	7.5
Pseudomonas aeruginosa	4	0.75~3.0	0.75~7.5	12.5
Salmonella schottmuelleri	3	0.3 ~3.0	0.3 ~>25	7.5
Mycoplasma gallisepticum	1**	0.05	0.05	—

Medium: Yeast beef broth pH 7.4. Incubated 18 hours at 37°C.
* Not performed at the same time with same strains.
** PPLO broth and agar.

Table 7. Effect of pH on *in vitro* activity of rosamicin

Organism		MIC (mcg/ml)				
		pH 6.6	pH 7.0	pH 7.4	pH 7.7	pH8.0
Staphylococcus aureus	2933	0.6	0.3	0.3	0.15	0.15
" "	209P	0.6	0.3	0.15	0.075	<0.05
" "	Gray	1.2	0.6	0.6	0.3	0.3
Streptococcus pyogenes	C	1.2	0.6	0.3	0.3	0.3
Escherichia coli	10536	>6.4	>6.4	>6.4	6.4	4.8

Medium: Yest beef broth

Table 8. Effect of rosamicin against erythromycin-resistant strains

Organism		MIC (mcg/ml)			
		Rosamicin		Erythromycin	
		pH 7	pH 8	pH 7	pH 8
Staphylococcus aureus	2033	1.2	0.4	25	18
" "	303	>50	4.9	62	18
" "	388	>50	4.9	62	18
Streptococcus pyogenes	33	>50	50	62	62

Medium: Yest beef broth

The effect of pH on the *in vitro* activity of rosamicin was tested against a select group of organisms (Table 7). As with other macrolides, rosamicin has greater activity at higher pH levels. Of interest is the fact that the greatest shift in activity for the antibiotic occurs around pH 7.5, while the shift for erythromycin occurs at pH 7.7 and for megalomicin A it occurs at pH 7.8~8.0. Rosamicin thus has less diminution of activity in the physiological pH range.

The antibiotic was studied *in vitro* against a limited number of erythromycin-resistant *S. aureus* and *S. pyogenes* strains (Table 8). It was effective against one, and moderately active against 2 of the 3 resistant *Staphylococcus* strains studied.

It is also effective against *Mycoplasma* species, which are detailed by WAITZ *et al.*[4]

In Vivo Activity of Rosamicin

The results of limited protection tests with rosamicin in comparison with erythromycin and megalomicin A are shown in Table 9. This preparation was active when given parenterally and orally against lethal infections of *Staphylococcus aureus* and *Streptococcus pyogenes*. Further tests, particularly with gram-negative infections, are described by WAITZ *et al.*[4]

Table 9. *In vivo* activity of rosamicin; protective activity in mice

	Route	PD_{50} (mg/kg)		
		Rosamicin	Megalomicin A base*	Erythromycin base*
Staphylococcus aureus Gray	S. C.	50	20	30
	Oral	200	300	106
Streptococcus pyogenes C	S. C.	50	134	90
	Oral	250	300	180
		LD_{50} (mg/kg)		
Acute Toxicity	I. P.	350	350	500
	S. C.	625	7,000	8,000
	I. V.	155		

* Not done at same time.

The intraperitoneal acute toxicity of the antibiotic (LD_{50}) was 350 mg/kg (Table 5), 625 mg/kg when given subcutaneously, and 155 mg/kg intravenously.

The intraperitoneal LD_{50} for rosamicin is similar to erythromycin and megalomicin A, but it is more toxic than these two antibiotics when given subcutaneously. This may be due to the aqueous solubility and improved absorption of rosamicin compared to the low solubility of the base forms of megalomicin A and erythromycin.

Based on the biological data available, rosamicin appears to have *in vitro* and *in vivo* properties typical of other macrolides with better gram-negative activity and activity against some erythromycin-resistant strains. These data, along with its apparent stability, suggest that further investigation of rosamicin is warranted.

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