

RS-22A, B and C: New Macrolide Antibiotics from *Streptomyces violaceusniger*

## I. Taxonomy, Fermentation, Isolation and Biological Activities

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(Received for publication October 26, 1994)

Three novel 36-membered macrolide antibiotics, RS-22A, B and C produced by *Streptomyces violaceusniger* have been isolated. These antibiotics were purified from an acetone extract of the mycelia followed by butanol extraction, centrifugal partition chromatography and HPLC. RS-22A, B, C showed antimicrobial activity against fungi and Gram-positive bacteria.

In the course of a screening program for new biologically active compounds, we isolated three new macrolide antibiotics, RS-22A, B and C (Fig. 1) from the mycelia of *Streptomyces violaceusniger*. The strain RS-22, (FERM P-14441) was isolated from a soil sample collected in Wuhan city, China. These antibiotics have antimicrobial activities against fungi and Gram-positive bacteria. In this paper, we report the taxonomy, production, isolation and biological activities. Physico-chemical properties and the structural elucidation of these compounds are reported in the following paper.<sup>1)</sup>

### Materials and Methods

#### Taxonomic Study

Cultural, physiological, and biochemical characteristics of strain RS-22 were examined by the method of SHIRLING and GOTTLIEB.<sup>2)</sup> Morphology on yeast-starch agar (containing soluble starch 1%, yeast extract 0.2%, agar 1.5%, pH 7.3) was observed after incubation at 28°C for 10 days. The Color Harmony Manual<sup>3)</sup> was used to identify the color of mycelia and soluble pigments. A scanning electron microscope (model S-430; Hitachi Ltd., Tokyo, Japan) was used to study the morphology of the spore chains. A sample for scanning electron microscopy was prepared as follows: cutting an agar block,

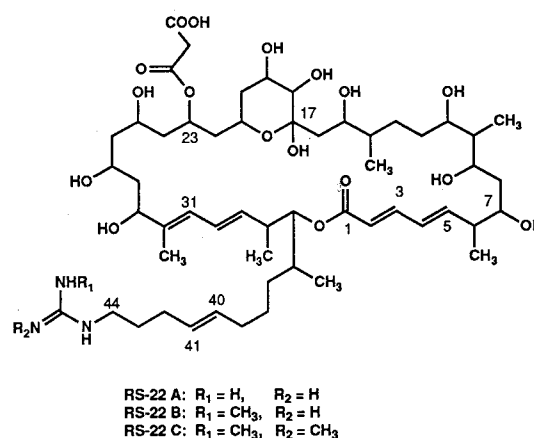
air-drying it, and sputter-coating it with gold under a vacuum.

The temperature range for growth was determined on yeast-starch agar by using a temperature gradient incubator (model TN-3; Advantec Toyo, Tokyo, Japan). Diaminopimelic acid ( $A_2$  pm) isomers in the cell wall were analyzed by the method of STANECK and ROBERTS.<sup>4)</sup>

#### Bacterial Strain

The following type strains were used for comparison with strain RS-22: *Streptomyces antimycoticus* JCM 4228, *S. cuspidosporus* JCM 4316, *S. hygroscopicus* subsp.

Fig. 1. Structure of RS-22A, B and C.



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*hygroscopicus* JCM 4772, *S. melanosporofaciens* JCM 4495, *S. sparsogenes* JCM 4517, and *S. violaceusniger* JCM 4850.

The producing strain RS-22 has been deposited in the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, with the accession No. FERM P-14441.

#### Antimicrobial Activities

The minimal inhibitory concentrations (MIC) of RS-22A, B and C against bacteria and fungi were determined by the agar dilution method. Sensitivity disk agar-N (Nissui Pharmaceutical Co., Ltd., Japan) and Sabouraud agar medium were used for bacteria and fungi, respectively.

### Results

#### Taxonomy

Strain RS-22 produced well-developed, branched, and non-fragmented vegetative hyphae. The aerial hyphae were branched monopodially, and spore chains with relatively tight or compact spirals were formed on the aerial hyphae. Each spore was characterized by its rugose surface (Fig. 2).

Cultural characteristics of strain RS-22 on various agar media are summarized in Table 1. The vegetative mycelia were yellowish brown to brown, and no distinctive

pigments were produced. The aerial mycelia were colored with a shade of gray, and the color became black in a culture incubated on oatmeal agar (International *Streptomyces* Project<sup>2)</sup> [ISP] medium No. 3) and inorganic salts-starch agar (ISP No. 4) for more than 2 weeks. Melanin-like pigments or other significant diffusible pigments were not produced in any agar medium tested. Utilization of carbon sources on strain RS-22 is shown in Table 2, and growth occurred at temperatures between 12 and 42°C. Whole-cell hydrolysate of strain RS-22 contained LL-A<sub>2</sub>pm, and this indicates the strain has a type I cell wall of *LECHEVALIER*

Fig. 2. Scanning electron micrograph of spore chains of strain RS-22 grown on yeast-starch agar for 10 days.

Bar represents 100 nm.



Table 1. Cultural characteristics of strain RS-22 on various media.

	Aerial mycelium	Vegetative mycelium, Reverse color	Soluble pigment
Yeast extract - malt extract agar (ISP-2)	Good, Pussywillow gray (5dc)	Good, Cinnamon(3le)	Pale brown
Oatmeal agar (ISP-3)	Good, Beige gray (3ih)	Good, Tan (3ie)	None
Inorganic salts - starch agar (ISP-4)	Good, Lead gray (5ih)	Good, Mustard tan (2lg)	None
Glycerol - asparagine agar (ISP-5)	Good, Taupe gray (7ih)	Good, Beige brown (3ig)	None
Peptone - yeast extract - iron agar (ISP-6)	None	Good, Bamboo (2gc)	None
Tyrosine agar (ISP-7)	Good, Lead gray (5ih)	Good, Cinnamon brown (3lg)	Pale brown

Table 2. Comparison of carbon utilization among strain RS-22, *Streptomyces antimycoticus* JCM 4228, *S. cuspidosporus* JCM 4316, *S. hygroscopicus* subsp. *hygroscopicus* JCM 4772, *S. melanosporofaciens* JCM 4495, *S. sparsogenes* JCM 4517, and *S. violaceusniger* JCM 4850.

	RS-22	JCM4495	4772	4850	4517	4228	4316
L-Arabinose	+	+	+	+	+	+	+
D-Xylose	+	+	+	+	+	+	+
D-Glucose	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+	+
Sucrose	+	+	-	+	+	+	±
<i>i</i> -Inositol	+	-	-	+	-	+	+
L-Rhamnose	+	+	+	+	+	+	+
Raffinose	+	+	-	+	+	+	+
D-Mannitol	+	+	+	+	+	+	+
D-Galactose	+	+	+	+	+	+	+

JCM4495: *S. melanosporofaciens* JCM 4495; 4772: *S. hygroscopicus* subsp. *hygroscopicus* JCM 4772; 4850: *S. violaceusniger* JCM 4850; 4517: *S. sparsogenes* JCM 4517; 4228: *S. antimycoticus* JCM 4228; 4316: *S. cuspidosporus* JCM 4316.

and LECHEVALIER.<sup>5)</sup> On the basis of these morphological and chemical characteristics, it was concluded that strain RS-22 be classified in the genus *Streptomyces*. In comparison to previously described members of this genus<sup>6~10)</sup>, strain RS-22 was considered to resemble the following six species: *S. antimycoticus*, *S. cuspidosporus*, *S. hygrosopicus* subsp. *hygrosopicus*, *S. melanosporofaciens*, *S. sparsogenes*, and *S. violaceusniger*. Strain RS-22 was therefore compared directly with representative strains of these species for morphological and cultural characteristics and utilization of carbon sources. The aerial mycelia of all the strains tested were gray and hygroscopic, and spiral spore chains with rugose surfaces were formed in common. Comparison of carbon utilization among the strains tested (Table 2) indicated that strain RS-22 was closely related to *S. violaceusniger* and *S. antimycoticus*. As these two species are not distinguishable by the phenotypic characteristics in the ISP system, strain RS-22 was identified as *S. violaceusniger* that has nomenclatural priority over *S. antimycoticus*.

#### Fermentation and Isolation

Fermentations for the production of RS-22A, B and C were carried out as follows. The strain RS-22 on agar slants was inoculated into twenty-four 500 ml cylindrical flasks containing each 70 ml of an autoclaved medium consisting of glucose 2%, soluble starch 1%, meat extract 0.1%, dry yeast 0.4%, soybean flour 2.5%, NaCl 0.2% and K<sub>2</sub>HPO<sub>4</sub> 0.005%. The medium was adjusted to pH 7.0 prior to sterilization. Culturing was performed on a rotary shaker at 300 rpm for 96 hours at 28°C.

The isolation of RS22-A, B and C was monitored by antimicrobial activity against *Candida albicans* and the procedure is illustrated in Fig. 3. The fermentation broth (1.6 liters) was centrifuged at 5°C for 20 minutes at 6,000 rpm. The inactive supernatant fluid was discarded and the mycelial cake was extracted with 1 liter of 80% acetone. The extract was concentrated to 150 ml and the aqueous solution was extracted with 120 ml of *n*-butanol. The organic layer was concentrated *in vacuo* to give 2.2 g of crude oil. The crude material was triturated with chloroform to give 1 g of a chloroform insoluble fraction, which was further partitioned on a centrifugal partition chromatograph (Sanki Model CPC-LLN, Japan) employing the following conditions: BuOH-EtOH-H<sub>2</sub>O (upper phase stationary, 10:2.5:10), 3 ml/minute in the ascending mode, at 1,000 rpm. The active fractions were combined and concentrated *in vacuo* to give 725 mg of a crude powder. Ninety eight mg of this powder were

Fig. 3. A diagram of isolation procedure.

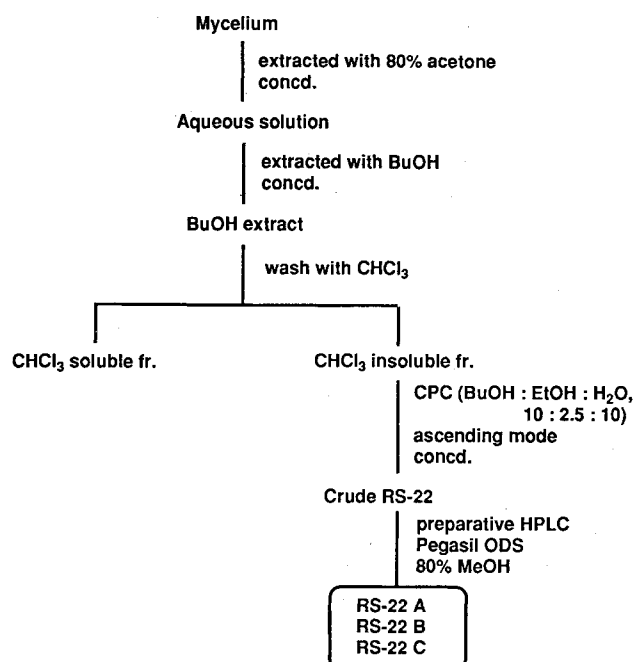


Table 3. Antimicrobial spectra of RS-22A, B and C.

Test organism	MIC(μg/ml)		
	RS-22A	RS-22B	RS-22C
<i>Staphylococcus aureus</i> FDA209P	6.25	6.25	12.5
<i>S. aureus</i> Smith	12.5	12.5	25
<i>S. pyogenes</i> Cook	25	12.5	25
<i>Escherichia coli</i> NIHJ JC-2	> 50	> 50	> 50
<i>Pseudomonas aeruginosa</i> PAO-1	> 50	> 50	> 50
<i>Cryptococcus neoformans</i> KC-201	3.13	3.13	3.13
<i>Candida albicans</i> KC-07	3.13	3.13	6.25
<i>C. tropicalis</i> KC-104	1.56	3.13	3.13
<i>C. prarsilosis</i> KC-110	6.25	6.25	6.25
<i>C. glabrata</i> KC-308	6.25	6.25	6.25
<i>Aspergillus fumigatus</i> KA-01	12.5	12.5	25
<i>A. flavus</i> KA-06	12.5	12.5	12.5
<i>Trichophyton mentagrophytes</i> KD-114	25	25	25
<i>T. rubrum</i> KD-114	12.5	12.5	12.5
<i>Microsporum canis</i> KD-305	12.5	12.5	12.5
<i>M. gypsum</i> KD-318	12.5	12.5	12.5

applied to preparative HPLC (Senshu-Pak, Pegasil-ODS, 20 × 250 mm; mobile phase: 80% methanol, flow rate 9.9 ml/minute; UV at 225 nm) to give 14.5 mg of RS-22A, 27 mg of RS-22B and 28.7 mg of RS-22C. Under these conditions, RS-22A eluted firstly with a retention time at 20.8 minutes, followed by RS-22B at 21.4 minutes and RS-22C at 24 minutes.

Detailed physico-chemical properties and the structures of RS-22A, B and C are described in the succeeding paper.<sup>1)</sup>

#### Biological Activities

The minimal inhibitory concentrations (MIC) of RS-22A, B and C are shown in Table 3. RS-22A, B and C were active against Gram-positive bacteria and fungi including yeast. The acute toxicity (LD<sub>50</sub>) of a mixture of RS-22A, B and C was 25 mg/kg in intraperitoneal injection (Jcl/ICR mice).

#### Discussion

New 36-membered macrolide antibiotics RS-22A, B and C were isolated from the culture broth of *Streptomyces violaceusniger* RS-22. They are related to azalomycins,<sup>11)</sup> scopafungin<sup>12)</sup> (niphimycin), copiamycin,<sup>13)</sup> guanidylfungins,<sup>14)</sup> amycins,<sup>15)</sup> RP63834,<sup>16)</sup> malolactomycin A<sup>17)</sup> and shurimycins.<sup>18)</sup> Most of the producing strains of these antibiotics are *Streptomyces hygroscopicus* or unidentified *Streptomyces* species. Although FIEDLER *et al.*<sup>19)</sup> pointed out that "*Streptomyces violaceoniger*", which should be changed to *Streptomyces violaceusniger*,<sup>20)</sup> produced 32-membered macrolide antibiotics, namely, niphithricins, both the producing strains of the closely related 36-membered macrolides, azalomycins and shurimycins were identified as *Streptomyces hygroscopicus*. In the present taxonomic study, it was found that the producing strain of RS-22A, B and C is *Streptomyces violaceusniger*. RS-22A, B and C showed antimicrobial activities against Gram-positive bacteria, filamentous fungi and yeast *in vitro*.

#### Acknowledgments

We are grateful to Kaken Pharmaceutical Co., Ltd. for measurements of MIC and the toxicity test.

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