# DIOLMYCINS, NEW ANTICOCCIDIAL AGENTS PRODUCED BY *Streptomyces* sp.

## I. PRODUCTION, ISOLATION AND PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES

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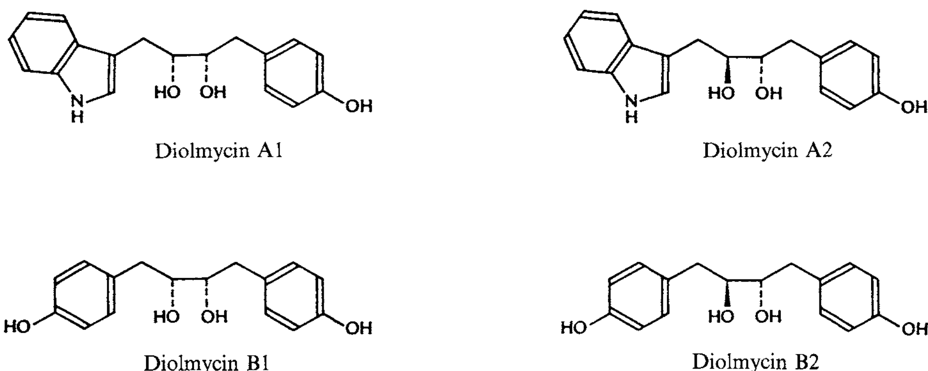
*Streptomyces* sp. WK-2955, a soil isolate, was found to produce a series of new anticoccidial compounds. Four active compounds, designated diolmycins A1, A2, B1 and B2, were isolated from the fermentation broth of the producing strain by solvent extraction, silica gel column chromatography, gel filtration on Sephadex LH-20, and preparative HPLC. Diolmycins inhibited the growth of *Eimeria tenella* in an *in vitro* assay system using BHK-21 cells as a host. No schizont in the cells was observed at concentrations of 0.02∼2.0 μg/ml for diolmycin A1, at 0.2∼2.0 μg/ml for diolmycin A2, and at 20 μg/ml for diolmycins B1 and B2.

Recently, we have reported new anticoccidial agents named xanthoquinodins[1]. From our continuous screening system using BHK-21 cells as a host and monensin-resistant *Eimeria tenella* as a parasitic protozoa, an actinomycetes strain WK-2955 was found to produce a series of new anticoccidial agents. Eventually, four active compounds, termed diolmycins A1, A2, B1 and B2 (Fig. 1), were isolated. In this paper, the taxonomy of the producing strain, fermentation, isolation and physico-chemical and biological properties of diolmycins are described. The structure and synthesis of diolmycin will be presented in the accompanying paper[2].

### Taxonomy of the Producing Strain WK-2955

To investigate the cultural and physiological characteristics, the International Streptomyces Project (ISP) media, recommended by SHIRLING and GOTTLIEB[3] and by WAKSMAN[4], were used. Cultures were observed after incubation at 27°C for 2 weeks. The utilization of carbon sources was tested by growth on

Fig. 1. Structures of diolmycins A1, A2, B1 and B2.



PRIDHAM's medium[5,6] containing 1% carbon source at 27°C. The vegetative mycelia grew abundantly on both synthetic and complex agar media, and did not show fragmentation into coccoid or bacillary elements. The aerial mycelia grew abundantly on yeast extract - malt extract agar and inorganic salts - starch agar. The mature sporophores were of the *Rectiflexibilis* type and had more than 20 spores per chain (Fig. 2). The spores were cylindrical in shape, 1.0 × 0.6 μm in size, and had a smooth surface. Sclerotic granules, sporangia, and flagellated spores were not observed. The cultural characteristics, physiological properties, and utilization of carbon sources are shown in Tables 1, 2 and 3, respectively. The color of vegetative mycelia was pink or wine and that of aerial mycelia was pink or white. Melanin and other pigments were not produced. The type of diaminopimelic acid (DAP) was determined by the method of TAKAHASHI *et al.*[7]. The DAP isomer in cell wall of strain WK-2955 was determined to be

Fig. 2. Scanning electron micrograph of spore chains of strain WK-2955 grown on tyrosin agar for 14 days.

Bar represents 1.0 μm.

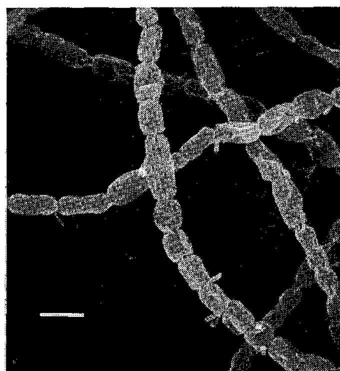


Table 1. Cultural characteristics of strain WK-2955.

| Medium | | Cultural characteristics | Medium | | Cultural characteristics |
|---|---|---|---|---|---|
| Yeast extract - malt extract agar[a] | G: | Good, mapple (4le) | Tyrosine agar[a] | G: | Moderate, cedar (6.5le) |
| | R: | Luggage tan (4ne) | | R: | Ash rose (7ie) |
| | AM: | Abundant, fresh pink (6ca) | | AM: | Moderate, light orchid pink (9ca) |
| | SP: | None | | SP: | None |
| Oatmeal agar[a] | G: | Good, pearl pink (3ca) | Sucrose - nitrate agar[b] | G: | Good, light ivory (2ca) |
| | R: | Pearl pink (3ca) | | R: | Light wheat (2ca) |
| | AM: | Poor, shell pink (5ba) | | AM: | Moderate, shell pink (5ba) |
| | SP: | None | | SP: | None |
| Inorganic salts - starch agar[a] | G: | Good, pearl pink (3ca) | Glucose - nitrate agar[b] | G: | Moderate, fresh pink (4ca) |
| | R: | Salmon pink (5ga) | | R: | Light apricot (4ea) |
| | AM: | Abundant, shell pink (5ba) | | AM: | Moderate, shell pink (5ba) |
| | SP: | None | | SP: | None |
| Glycerol - asparagine agar[a] | G: | Good, pearch (5ga) | Glycerol - calcium malate agar[b] | G: | Good, rose wine (8le) |
| | R: | Copper (5lc) | | R: | Rose wine (8le) |
| | AM: | Moderate, white (a) | | AM: | Moderate, pale pink (8ca) |
| | SP: | None | | SP: | None |
| Glucose - asparagine agar | G: | Good, apricot (4ia) | Glucose - peptone agar[b] | G: | Good, pearl pink (3ca) |
| | R: | Fresh pink (4ca) | | R: | Pearl pink (3ca) |
| | AM: | Moderate, melon yellow (3ia) | | AM: | Moderate, shell pink (5ba) |
| | SP: | None | | SP: | None |
| Peptone - yeast extract - iron agar[a] | G: | Moderate, light ivory (2ca) | Nutrient agar[b] | G: | Moderate, pearl pink (3ca) |
| | R: | Light wheat (2ea) | | R: | Pearl pink (3ca) |
| | AM: | None | | AM: | Moderate, white (a) |
| | SP: | None | | SP: | None |

[a] Medium recommended by International Streptomyces Project.
[b] Medium recommended by S.A. WAKSMAN.
Abbreviation: G, Growth of vegetative mycelium; R, reverse; AM, aerial mycelium; SP, soluble pigment.
( ): Color number designations taken from Color Harmony Manual, 4th Ed., Container Corporation of America, Chicago, Illinois, U.S.A., 1958.

Table 2. Physiological properties of strain WK-2955.

| | |
|---|---|
| Melanin formation | − |
| Tyrosinase reaction | − |
| $H_2S$ production | − |
| Nitrate reduction | − |
| Liquefaction of gelatin (21∼23°C) | − |
| Peptonization of milk (36∼37°C) | − |
| Coagulation of milk (36∼37°C) | − |
| Cellulolytic activity | − |
| Hydrolysis of starch | + |
| Temperature range for growth | 15∼37°C |

+, Active; −, not active.

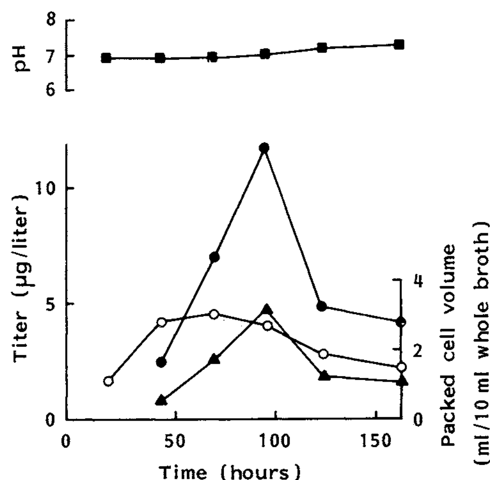
Table 3. Utilization of carbon sources by strain WK-2955.

| | |
|---|---|
| D-Glucose | + |
| D-Fructose | + |
| L-Rhamnose | + |
| D-Mannitol | + |
| L-Arabinose | + |
| *i*-Inositiol | + |
| Raffinose | + |
| D-Xylose | + |
| Sucrose | − |
| Melibiose | + |
| Cellulose | − |

+, Utilized; −, not utilized.

Fig. 3. Time course of diolmycins A1 and A2 production in a 30-liter jar fermentor.

● Diolmycin A1, ▲ diolmycin A2, ○ packed cell volume, ■ pH.




of the LL-type. Based on the taxonomic properties described above, it is reasonable to conclude that strain WK-2955 belongs to the genus *Streptomyces*[8]. The strain was deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, under the name *Streptomyces* sp. WK-2955 and the accession number is FERM P-12318.

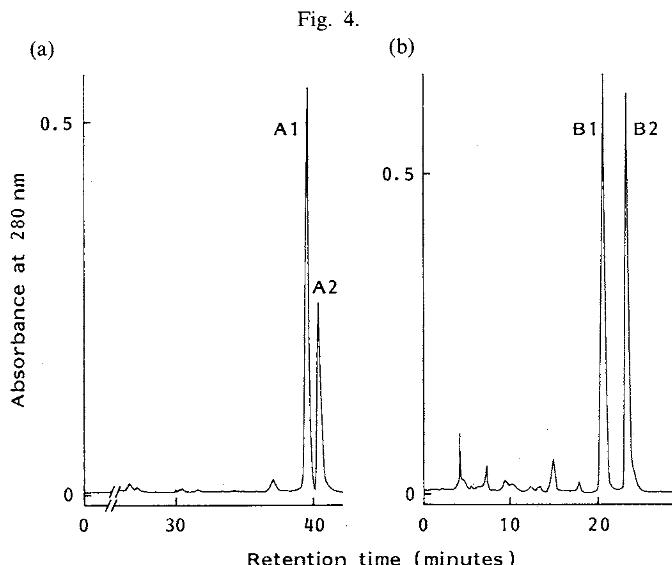
## Fermentation

A slant culture of strain WK-2955 grown on Seino agar (starch 1.0%, N-Z amine 0.3%, yeast extract 0.1%, meat extract 0.1%, $CaCO_3$ 0.3%, agar 1.0%, pH 7.0) was used to inoculate 500-ml Erlenmeyer flasks containing 100 ml of a seed medium (glucose 0.1%, starch 2.4%, peptone 0.3%, meat extract 0.3%, yeast extract 0.5%, $CaCO_3$ 0.4%, pH 7.0). The flasks were shaken on a rotary shaker for 3 days at 27°C. Two hundred ml of the seed culture was transferred into 20 liters of a production medium (glycerol 2.0%, soy bean meal 2.0%, NaCl 0.3%, pH 7.0) in a 30-liter jar fermentor. The fermentation was carried out at 27°C. A typical time course of the fermentation is shown in Fig. 3. The production of diolmycins A1 and A2 was measured by HPLC under the following conditions: column; YMC packed column R-ODS-5 (4.6 × 200 mm), solvent; 40% aq MeOH, detection; UV at 280 nm, flow rate; 0.8 ml/minute. Under these conditions, diolmycin A1 was eluted first with a retention time at 39.0 minutes, followed by diolmycin A2 at 43.7 minutes (Fig. 4a). The concentration of diolmycins A1 and A2 reached a maximum at 96 hours.

## Isolation

Four-day cultured broth (20 liters) was extracted with 20 liters of ethyl acetate. The extracts were concentrated *in vacuo* to dryness to yield a red oily material (56.9 g). The material was distributed by *n*-hexane - MeOH - $H_2O$ (40 : 19 : 1). Then the lower layer was concentrated *in vacuo* to dryness to yield a red material (6.44 g). The material was applied on a silica gel column (E. Merck, Kiselgel 60, 500 ml).

Fig. 4.




(a) A chromatographic profile of diolmycins A1 and A2 separated by analytical HPLC. Column: YMC-Packed column R-ODS-5 (4.6 × 200 mm); mobile phase: 40% aq MeOH; flow rate: 0.8 ml/minute; detection: 280 nm.

(b) A chromatographic profile of diolmycins B1 and B2 separated by analytical HPLC. Column: YMC-Packed column R-ODS-5 (4.6 × 200 mm); mobile phase: 35% aq $CH_3CN$; flow rate: 0.7 ml/minute; detection: 280 nm.

The column was washed with 1.5 liters of chloroform and 2.0 liters of chloroform - methanol (50 : 1). The active components were eluted with 2.0 liters of chloroform - methanol (20 : 1), and each 60 ml was successively collected. The 9th to 31st fractions enriched with diolmycins A1, A2, B1 and B2 were concentrated *in vacuo* to give a red material (270 mg). Further purification of diolmycins was carried out on gel filtration by Sephadex LH-20 (2 × 120 cm, MeOH, 0.6 ml/minute). The active components were eluted with 500 ml of methanol, and each 8.5 ml was successively collected. The 33rd to 37th fractions enriched with diolmycins A1 and A2 and the 30th to 32nd fractions with diolmycins B1 and B2 were evaporated *in vacuo* to give colorless materials (18.0 and 6.5 mg, respectively). Diolmycins A1 and A2 were finally purified by preparative HPLC (column; YMC pack D-ODS-5, 20 × 250 mm, 40% aq MeOH, UV at 280 nm, 6.0 ml/minute). The active fractions were concentrated and extracted with ethyl acetate to give pure diolmycins A1 (9.76 mg) and A2 (5.33 mg) as colorless materials. Diolmycins B1 and B2 were also purified by HPLC (column; YMC packed column R-ODS-5, 4.6 × 200 mm, 35% aq $CH_3CN$, UV at 280 nm, 0.7 ml/minute) (Fig. 4b). The active fractions were also concentrated and extracted with ethyl acetate to give pure diolmycins B1 (1.83 mg) and B2 (0.54 mg) as colorless materials.

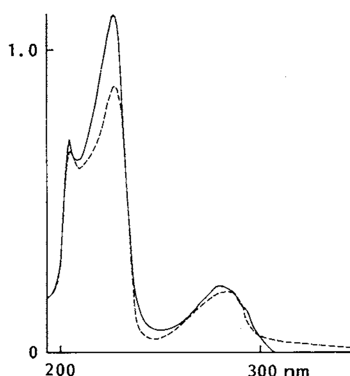
## Physico-chemical Properties

The physico-chemical properties of diolmycins A1, A2, B1 and B2 are summarized in Table 4. The molecular formula of diolmycins A1 and A2 were determined both to be $C_{18}H_{19}NO_3$ on the basis of HREI-MS. Similar UV spectra of diolmycins A1 and A2 were observed with three maxima at 222, 280 and 290 nm in MeOH (Fig. 5). The molecular formula of diolmycins B1 and B2 were also determined both to be $C_{16}H_{18}O_4$. UV spectra of diolmycins B1 and B2 showed three maxima at 223, 277 and 285 nm in MeOH (Fig. 5). These data indicate that diolmycins A1, A2, B1 and B2 are new compounds. Their

Table 4. Physico-chemical properties of diolmycins A1, A2, B1 and B2.

| | Diolmycin A1 | Diolmycin A2 | Diolmycin B1 | Diolmycin B2 |
|---|---|---|---|---|
| Appearance | Colorless powder | Colorless powder | Colorless powder | Colorless powder |
| $[\alpha]_D^{25}$ (*c* 0.1, MeOH) | −8.0° | −12.0° | −6.0° | −6.7° |
| Molecular formula | $C_{18}H_{19}NO_3$ | $C_{18}H_{19}NO_3$ | $C_{16}H_{18}O_4$ | $C_{16}H_{18}O_4$ |
| HREI-MS (*m/z*) | | | | |
| Calcd: | 297.1364 | 297.1364 | 274.1204 | 274.1204 |
| Found: | 297.1363 | 297.1361 | 274.1207 | 274.1187 |
| UV $\lambda_{max}^{MeOH}$ (nm) | 222 (33,300), 280 (5,900), 290 (4,500) | 222 (33,700), 280 (6,200), 290 (4,500) | 223 (24,000), 277 (5,500), 285 (4,600) | 223 (24,900), 277 (5,700), 285 (4,700) |
| Solubility | | | | |
| Soluble: | MeOH, DMSO | MeOH, DMSO | MeOH, DMSO | MeOH, DMSO |
| Insoluble: | $CHCl_3$ | $CHCl_3$ | $CHCl_3$ | $CHCl_3$ |
| Color reaction | | | | |
| Positive | 50% $H_2SO_4$ | 50% $H_2SO_4$ | 50% $H_2SO_4$ | 50% $H_2SO_4$ |
| Negative | Ninhydrin reagent | Ninhydrin reagent | Ninhydrin reagent | Ninhydrin reagent |

Fig. 5. UV spectra of diolmycin A1 (—) and B1 (---) (in MeOH).




structures will be reported in the following paper[2].

Biological Properties

Effect on Anticoccidial Activity in an *In Vitro* System

Anticoccidial activity *in vitro* was assayed as reported previously[1]. Diolmycin A1 showed anticoccidial activity at concentrations ranging above 0.02 μg/ml (Table 5), indicating the highest anticoccidial potency among diolmycins. Diolmycins B1 and B2 showed poor anticoccidial activity at concentrations above 20 μg/ml (Table 5).

Table 5. Anticoccidial activity of diolmycins A1, A2, B1 and B2 in an *in vitro* assay.

| Compounds | Minimum effective concentration (μg/ml) | |
|---|---|---|
| | Anticoccidial activity[a] | Cytotoxicity[b] |
| Diolmycin A1 | 0.02 | 0.2 |
| Diolmycin A2 | 0.2 | 2.0 |
| Diolmycin B1 | 20 | NT[d] |
| Diolmycin B2 | 20 | NT[d] |
| Monensin | —[c] | 0.02 |

BHK-21 cells stained with hematoxylin solution was microscopically observed. In control experiments (no drug) infected sporocysts grew in the cells to form mature shizonts.

[a] No mature shizonts observed in the cells when the drug was added to the culture medium at the indicated concentrations.

[b] No BHK-21 cells observed when the drug was added to the culture medium at the indicated concentrations.

[c] No anticoccidial activity.

[d] NT: Not tested higher than concentration of 20 μg/ml.

Antimicrobial Activities

Diolmycins A1, A2, B1 and B2 showed no antimicrobial activity *in vitro* at a concentration of 1 mg/ml against *Staphylococcus aureus* FDA 209P, *Micrococcus luteus* PCI 1001, *Bacillus subtilis* PCI 219, *Mycobacterium smegmatis* ATCC 607, *Escherichia coli* NIHJ, *Escherichia coli* NIHJ JC-2 IFO 12734,

*Pseudomonas aeruginosa* P-3, *Xanthomonas oryzae*, *Bacteroides fragilis* ATCC 23745, *Acholeplasma laidlawii* PG8, *Candida albicans*, *Saccharomyces sake*, *Aspergillus niger* ATCC 6275, *Pyricularia oryzae*, and *Mucor racemosus* IFO 4581.

## Discussion

The diolmycin producer, *Streptomyces* sp. WK-2955 co-produced a great amount of red materials, presumably cytotoxic anthracyclines, which interfered with the isolation of diolmycins and the *in vitro* anticoccidial assay. Eventually, the final yields of diolmycins were quite low. Improvement of fermentation conditions to increase diolmycin production and to reduce red material production will be required.

The evaluation of diolmycin A1 in an *in vivo* system is being investigated.

Acknowledgment

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