VOL. 52 NO. 12, DEC. 1999 THE JOURNAL OF ANTIBIOTICS pp. 1101 — 1107

Ferroverdins, Inhibitors of Cholesteryl Ester Transfer Protein Produced by *Streptomyces* sp. WK-5344

I. Production, Isolation and Biological Properties

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(Received for publication July 9, 1999)

Streptomyces sp. WK-5344, a soil isolate, was found to produce structurally related inhibitors of cholesteryl ester transfer protein (CETP). New active compounds, designated ferroverdins B and C, were isolated along with known ferroverdin A from the fermentation broth by solvent extraction, ODS column chromatography and silica gel column chromatography. All ferroverdins showed a dose-dependent inhibitory activity against human CETP. The IC_{50} values were 21, 0.62 and 2.2 μM for ferroverdins A, B and C, respectively, indicating that ferroverdin B is one of the most potent CETP inhibitors of microbial origin.

During our screening for cholesteryl ester transfer protein (CETP) inhibitors of microbial origin, novel erabulenols were isolated from the culture broth of *Penicillium* sp. FO-5637[1,2]. Recently, another actinomycete strain WK-5344 was found to produce a series of CETP inhibitors. Three structurally related active compounds were isolated from the fermentation broth of the producer. One compound was identified as ferroverdin (newly designated ferroverdin A in this paper), which was previously reported as a green pigment[3,4], but the others named ferroverdins B and C (Fig. 1) were found to be new. In this paper, the taxonomy of the producing strain, fermentation, isolation and biological properties of ferroverdins are described. The structure elucidation of ferroverdins B and C will be described in the accompanying paper[5].

Materials and Methods

General Experimental Procedures

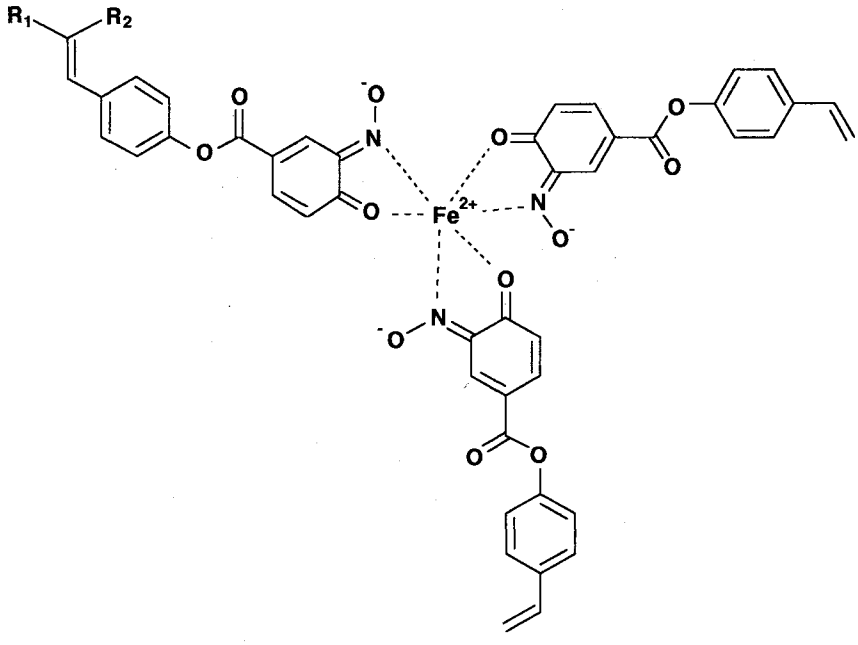
The strain WK-5344 was isolated from a soil sample, and was used for production of ferroverdins. ODS gel (SS 1020T, Senshu Sci. Co.) and Kieselgel 60 (E. Merck) were used for column chromatographies. HPLC was carried out using the Waters (600E) system.

Taxonomic Studies

The morphological properties were observed with a scanning electron microscope (model JSM-5600, JEOL Ltd.).

The isomer of diaminopimelic acid (DAP) was determined by the method of BECKER *et al.*[6] Major menaquinones were extracted and purified by the method of COLLINS *et al.*[7] and analyzed by HPLC [column, Capcell Pak C18 SG (4.6×150 mm); solvent, methanol - 2-propanol (7 : 3); detection, UV at 270 nm; flow rate, 1.0 ml/minute]. To investigate the cultural characteristics and physiological properties, the International Streptomyces Project (ISP) media recommended by SHIRLING and GOTTLIEB[8] and media recommended by WAKSMAN[9] were used. Cultures were observed after incubation at 27°C for two weeks. Color Harmony Manual, 4th Ed., 1958 (Container Corporation of America, Chicago)[10] was used for color names and hue numbers. The utilization of carbon sources was tested by growth on PRIDHAM and GOTTLIEB's medi-

Fig. 1. Structures of ferroverdins A, B and C.



Ferroverdin	R_1	R_2
A	H	H
B	OH	H
C	H	COOH

um[11] containing 1% carbon sources.

Assay for CETP Activity

The assay for CETP activity was carried out according to our established method[12].

Antimicrobial Activity

Antimicrobial activity was tested using paper disks (6 mm, ADVANTEC). Bacteria were grown on Mueller-Hinton agar medium (Difco), and fungi and yeasts were grown on potato broth agar medium. Antimicrobial activity was observed after a 24-hour incubation at 37°C for bacteria and after a 48-hour incubation at 27°C for fungi and yeasts.

Results

Taxonomy of the Producing Strain WK-5344

Morphological Properties

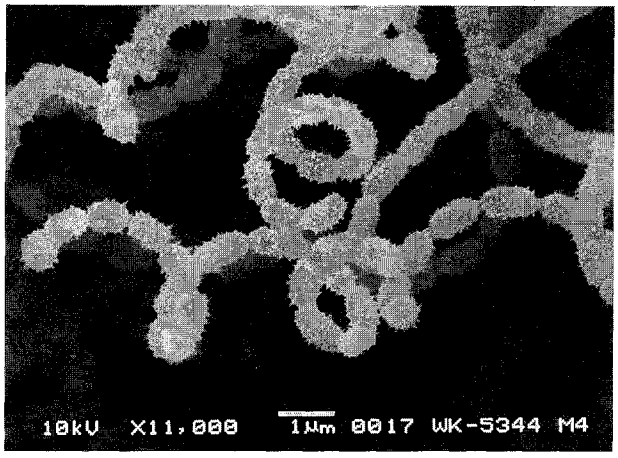
The vegetative mycelia grew abundantly on both synthetic and complex media, and did not show fragmentation into coccoid or bacillary elements. The aerial mycelia grew abundantly on oatmeal agar and inorganic salts - starch agar. The spore chains were spiral type and each had more than 20 spores per chain. The spores were oval in shape, 1.0×0.5 μm in size, and had a spiny surface (Fig. 2). Whirls, sclerotic granules, sporangia and flagellated spores were not observed.

Chemical Composition

The DAP isomer in whole cells of the strain was determined to be LL-type. Major menaquinones were MK-9(H_6) and MK-9(H_8).

Fig. 2. Scanning electron micrograph of spore chains of strain WK-5344 grown on inorganic salts - starch agar for 14 days.

Bar represents 1.0 μm.



Cultural Characteristics and Physiological Properties

The cultural characteristics and the physiological properties are shown in Tables 1 and 2. The vegetative mycelia showed beige to brown color on various media. The aerial mass color showed white to gray. Soluble pigment was produced on yeast extract - malt extract agar, oatmeal agar, glucose - asparagine agar, glucose - nitrate agar and glycerol - calcium malate agar.

The utilization of carbon sources is shown in Table 3.

Based on the taxonomic properties described above, the strain WK-5344 is considered to belong to the genus *Streptomyces*[13]. The strain was deposited in the National Institute of Bioscience and Human-Technology, Japan, under the name *Streptomyces* sp. WK-5344 and the accession No. is FERM BP-6668.

Fermentation

A slant culture of the strain WK-5344 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 a 50-ml test tube containing 10 ml of the 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 tube was shaken on a reciprocal shaker for 4 days at 27°C. One ml of the seed culture was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of the same medium. After a 72-hour incubation, 1% of the second seed culture was inoculated into a 30-liter jar fermentor charged to 20 liters of a production medium (soluble starch 4.0%, hypro - toast meal (nonfat soybean meal) 2.0%, Na_2SO_4 (1/10 N) 32 ml/liter, $FeSO_4 \cdot 7H_2O$ 0.05%, KH_2PO_4 0.05%, KCl 0.03%, pH 6.5). The fermentation was carried out at 27°C. The production of ferroverdins was measured by HPLC under the following conditions: column, PEGASIL ODS 3 μ (4.6×50 mm); a linear gradient from 30% CH_3CN to 65% CH_3CN for 30 minutes; detection, UV at 280 nm; flow rate, 1.5 ml/minute. Ferroverdins A, B and C were eluted as peaks with retention times of 26.0, 10.8 and 19.3 minutes, respectively. The production of ferroverdins was observed at day 2 after inoculation, and reached a maximum at day 3 for ferroverdin A and at day 4 for ferroverdins B and C (Fig. 3).

Isolation

The 4-day old culture broth (20 liters) was centrifuged to obtain the mycelium, which was extracted with 6.0 liters of acetone. After filtration, the extract was concentrated to remove acetone, and the resulting aqueous solution was adjusted to pH 5.0 with H_3PO_4 and extracted with ethyl acetate (10 liters). The organic layer was dried over Na_2SO_4 and concentrated *in vacuo* to dryness to give a green oil (9.20 g, IC_{50} 90 μg/ml). A portion of the oil (4.16 g) was distributed in a solution of *n*-hexane - methanol - H_2O (900 ml, 40 : 19 : 1, v/v). Then the lower layers was concentrated *in vacuo* to dryness to give a dark green powder (1.29 g, IC_{50} 45 μg/ml).

The powder was subjected to an ODS column (Senshu SSC-ODS-7515-12, 200 ml), which was eluted by a linear gradient from 30% CH_3CN in 0.05% H_3PO_4 (480 ml) to 100% CH_3CN (580 ml), and each 12 ml of the elution was successively collected. The 39th to 46th fractions containing ferroverdins were concentrated and extracted with ethyl acetate (100 ml). The organic layer was dried over Na_2SO_4 and concentrated *in vacuo* to dryness to give a green powder (157 mg, IC_{50} 8.0 μg/ml).

The powder was dissolved in chloroform and applied on a silica gel column (Kieselgel 60, 126 ml, 4.0×10 cm) previously equilibrated with chloroform. The materials were eluted stepwise with chloroform - methanol solutions (400 ml of 100 : 0, 200 ml of 96 : 4, 100 ml of 90 : 10 and 400 ml of 75 : 25, v/v), and fractions (8 ml each) were collected. Ferroverdins A, B and C were eluted in the 68th~73rd, 91st~95th and 80th~83rd fractions, respectively. They were concentrated *in vacuo* to give pure ferroverdin A (2.63 mg, IC_{50} 18 μg/ml), B (1.69 mg, IC_{50} 0.54 μg/ml) and C (1.12 mg, IC_{50} 2.0 μg/ml) as green powders.

Table 1. Cultural characteristics of strain WK-5344.

Medium	Cultural characteristics
Yeast extract-malt extract agar [a]	G: Good, bamboo (2gc) R: Mustard (2le) AM: Abundant, pearl gray (13cb) SP: Olive yellow (1le)
Oatmeal agar [a]	G: Moderate, bamboo~golden yellow (2fb~2kb) R: Bamboo~covert brown (2fb~2li) AM: Moderate, ashes (5fe) SP: Citron (1gc)
Inorganic salts-starch agar [a]	G: Good, bamboo (2gc) R: Camel (3ie) AM: Abundant, ashes (5fe) SP: None
Glycerol-asparagine agar [a]	G: Good, biscuit (2ec) R: Bamboo (2gc) AM: Abundant, alabaster tint~ashes (13ba~5fe) SP: Pastel yellow (1db)
Glucose-asparagine agar	G: Good, bamboo(2gc) R: Bamboo (2gc) AM: Abundant, alabaster tint~ashes (13ba~5fe) SP: Pastel yellow (1db)
Peptone-yeast extract-iron agar [a]	G: Moderate, dull gold (2ng) R: Light mustard tan (2ie) AM: Poor, white (a) SP: None
Tyrosine agar [a]	G: Good, bamboo (2gc) R: Light mustard tan~moss green (2ie~24lg) AM: Abundant, alabaster tint (13ba) SP: None
Sucrose-nitrate agar [b]	G: Good, light mustard tan (2ie) R: Light mustard tan~moss green (2ie~24lg) AM: Abundant, ashes (5fe) SP: None
Glucose-nitrate agar [b]	G: Moderate, light mustard tan (2ie) R: Mustard (2le) AM: Moderate, white (a) SP: Gold ($1^1/_2$lc)
Glycerol-calcium malate agar [b]	G: Good, bamboo (2gc) R: Light wheat~bamboo (2ea~2gc) AM: Abundant, pearl (3ba) SP: Citron (1gc)
Glucose-peptone agar [b]	G: Moderate, bamboo (2gc) R: Light wheat~golden yellow (2ea~2kb) AM: Poor, white (a) SP: None
Nutrient agar [b]	G: Moderate, bamboo (2gc) R: Nugget gold (2ne) AM: Moderate, ashes (5fe) SP: None

[a] Medium recommended by ISP.
[b] Medium recommended by S. A. Waksman.
Abbreviations: G, growth of vegetative mycelium; R, reverse side color; AM, aerial mycelium; SP, soluble pigment

Table 2. Physiological properties of strain WK-5344.

Melanin formation	
Tyrosine agar	–
Peptone-yeast extract-iron agar	–
Tryptone-yeast extract broth	–
Reduction of nitrate	+
Liquefaction of gelatin (21~23°C)	–
Hydrolysis of starch	+
Coagulation of milk	–
Cellulolytic activity	–
Peptonization of milk	+
Decomposition of cellulose	–
Temperature range for growth	9~37°C

+: Positive, –: Negative.

Table 3. Utilization of carbon sources by strain WK-5344.

Utilized: D-Glucose, L-Arabinose, D-Xylose, D-Fructose, D-Mannitol, Melibiose, L-Rhamnose, i -Inositol
Weakly utilized: Raffinose, Sucrose

Biological Properties

Effect of Ferroverdins on CETP Activity *In Vitro*

As shown in Fig. 4, all ferroverdins inhibited CETP activity dose-dependently in the *in vitro* assay. Ferroverdin B showed the most potent inhibitory activity with an IC_{50} value of 0.62 μM, followed by ferroverdin C (2.2 μM) and

Fig. 3. Time course of ferroverdins production in a 500-ml Erlenmeyer flask.

● Ferroverdin A, ◐ ferroverdin B, ○ ferroverdin C, ■ packed cell volume, and □ pH.

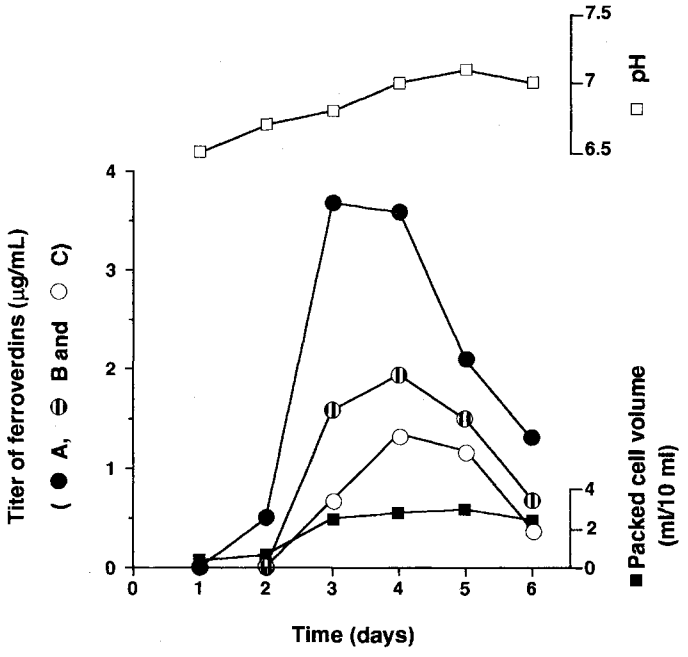
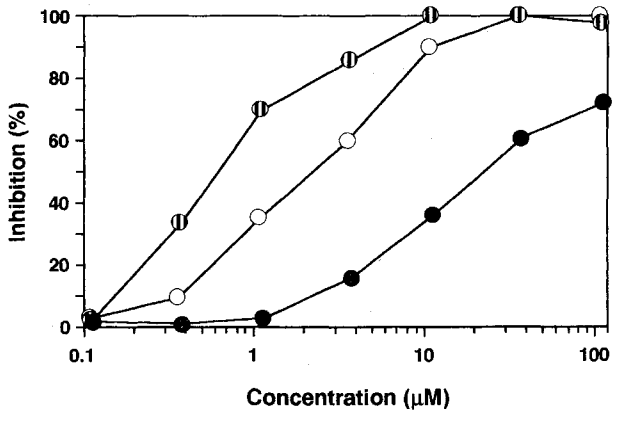


Fig. 4. CETP inhibition by ferroverdins A, B and C in an *in vitro* assay system in the presence of 200 μM BSA.

● Ferroverdin A, ◐ ferroverdin B, ○ ferroverdin C.



ferroverdin A (21 μM).

Other Biological Activities

Antimicrobial activity of the drugs at 1 mg/ml was tested against *Bacillus subtilis*, *Staphylococcus aureus*, *Micrococcus luteus*, *Mycobacterium smegmatis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Xanthomonas campestris pv. oryzae*, *Bacteroides fragilis*, *Acholeplasma laidlawii*, *Pyricularia oryzae*, *Aspergillus niger*, *Mucor racemosus*, *Candida albicans*, and *Saccharomyces cerevisiae* by paper disk (i.d. 6 mm) method. Ferroverdins B and C showed no antimicrobial activity. Ferroverdin A showed very weak activity only against *A. laidlawii* (trace inhibition).

Discussion

Purification of ferroverdins from the culture broth of *Streptomyces* sp. WK-5344 is summarized in Table 4. Silica gel chromatography gave poor recovery because only fractions containing highly pure ferroverdins were pooled. To improve the total recovery, purification by preparative HPLC using an ODS column is now in progress instead of the silica gel column chromatography.

Ferroverdin A was originally isolated as a green pigment produced by a streptomycete[3,4]. In this paper, we discovered structurally related new components B and C,

Table 4. Isolation of ferroverdins A, B and C from culture broth of *Streptomyces* sp. WK-5344.

Step	Weight (mg)	Activity (IC_{50}: μg/ml)	Total activity (Weight/Activity)	Yield (%)
Extraction	4160	90	46.2	100
Distribution	1290	45	28.7	62
ODS column	157	8.0	19.6	42
Silica gel column			3.84	8.3
Ferroverdin A	2.63	18	0.15	(0.3)
Ferroverdin B	1.69	0.54	3.13	(6.8)
Ferroverdin C	1.12	2.0	0.56	(1.2)

Starting from 10 liters of culture broth.

along with ferroverdin A, as CETP inhibitors. All ferroverdins are composed of iron and three ligands as described in the accompanying paper[5]. Addition of iron ion to the fermentation medium is essential for ferroverdin production, since no ferroverdins were detected in the mycelium when the producer was cultured in the medium without $FeSO_4$ (data not shown).

Several iron complexes with *p*-substituted *O*-nitrosophenols such as viridomycins[14~18], actinoviridin[19], 4-hydroxy-3-nitrosobenzamide ferrous chelate[20] and ferroverdin A[3,4] were isolated as green pigments from actinomycetes. This series of compounds have been reported to show no significant biological activities; viridomycin A exhibited only weak antibacterial activity[14] and viridomycin F showed weak insecticidal and nematocidal activities[18]. As described in this paper, ferroverdins showed no antimicrobial and no insecticidal activities even at 200 μg/ml, but they were found to show potent CETP inhibition. Especially ferroverdin B is one of the most potent CETP inhibitors of microbial origin. It might be worth testing whether or not other iron complexes show CETP inhibition.

Acknowledgments

This research was supported in part by Grant-in Aid for Scientific Research (B) from the Ministry of Education, Science, Sports and Culture of Japan (09480147), and from Japan Keirin Association.

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