Isolation and Characterization of a New Pyrano[4′,3′:6,7]naphtho[1,2-b]xanthene Antibiotic FD-594

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During our screening of microbial metabolites for effective drugs against tumor cell lines, we discovered a new pyrano[4′,3′:6,7]naphtho[1,2-b]xanthene derivative, FD-594 from the fermentation broth of *Streptomyces* sp. TA-0256. FD-594 shows moderate activity against tumor cell lines, comparative to that of adriamycin, as well as antibacterial activity against some Gram-positive bacteria.

In the course of our screening program for low molecular weight antitumor compounds among microbial metabolites guided by cytotoxicity assay using HL-60 cells, we discovered a new antitumor antibiotic FD-594 from the culture broth of *Streptomyces* sp. TA-0256. The structure of FD-594 was determined by extensive spectral analysis as shown in Figure 1[1]. The present paper describes the taxonomy and fermentation of the producing organism and the isolation, physico-chemical properties, and biological activities of FD-594.

Results and Discussion

Taxonomy

The strain TA-0256 was subjected to the standard biological and physiological examinations. Aerial mycelium of this strain developed well on the synthetic and natural agar plates and branched irregularly. The spores were well formed on the inorganic-starch agar and the oatmeal agar. The observation of the morphology on the oatmeal agar plate through a optical microscopy showed that pairs of sporophores were branched from aerial hyphae and 3 to 5 of verticils were formed at equal spaces of 40 to 80 μm. On the tips of these sporophores were

Fig. 1. Structure of FD-594.

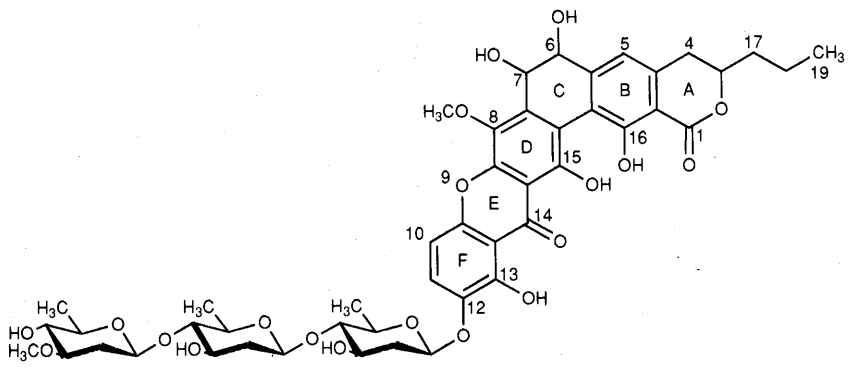


Table 1. Cultural characteristics of the strain TA-0256.

Medium	Growth	Aerial mycelium	Reverse color	Soluble pigment
Sucrose-nitrate agar	Moderate	White (N9. 5)	None	None
Glucose-asparagine agar	Moderate	Very light purplish red (5RP9/2)	Very light purplish red (5RP9/2), deep yellowish red (7. 5R3/10)	None
Glycerol-asparagine agar (ISP-5)	Good	Reddish white (5R9/1), light red (5R8/5)	Very light yellow (5Y9/3), deep yellowish red (7. 5R2/6)	None
Inorganic salts-starch agar (ISP-4)	Good	Reddish white (5R9/1)	Very light yellow (5Y9/3), purplish red (10RP4. 5/12)	None
Tyrosin agar (ISP-7)	Good	Light yellowish red (7. 5TR8/6), very light red (10RP9/2)	Deep purplish red (10RP3/10)	None
Nutrient agar	Moderat	White (N9. 5)	Very light yellow (5Y9/3), dark purplish red (10RP3/10)	None
Yeast extract-malt extract agar (ISP-2)	Good	Very light red (5R9/2), light yellowish red (5YR7. 5/10)	Very light yellow (5YR7. 5/10), dark grayish red (5R3. 5/3)	None
Oatmeal agar (ISP3)	Good	White (N9. 5)	Light purplish red (10RP8/6), purplish red (10RP4. 5/12)	None
Peptone-yeast extract-iron agar (ISP-6)	Moderate	None	Grayish yellow (10YR5/3), dark grayish yellow	None

observed 5 to 10 of chain spores, which consisted of 7 to 10 spores in the length of about 10 μm and were straight to more or less curved. The scanning electron microscopy revealed that the spore chain was twisted and the surface of the spore was smooth. The spores were cylindrical and $1.2 \sim 1.6\,\mu m \times 0.5 \sim 0.7\,\mu m$ in size. The sclerotia and flagellated spores were not observed.

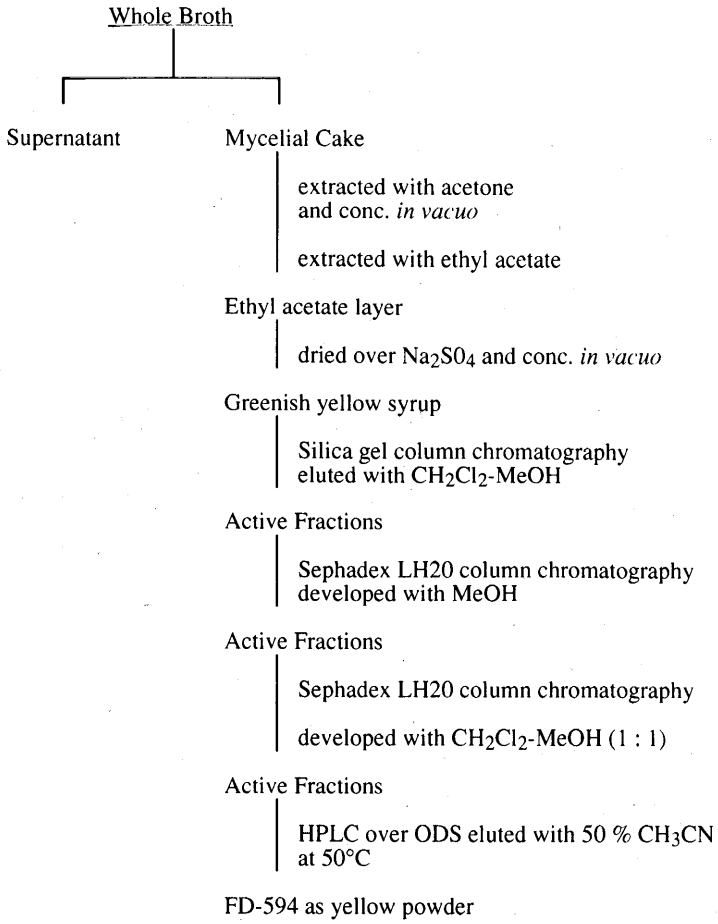
The cultural characteristics of the strain TA-0256 grown on various media at 28°C for 14 days are shown in Table 1. The growth was good on various agar media and the reverse side of colonies showed colors as depicted in Table 1. Soluble pigments were not produced on all agar media. The strain TA-0256 grew in yeast extract-malt extract liquid medium at the range of 10 to 49°C, and the optimum temperature for growth was 33°C to 37°C. Coagulation of skim milk, peptonization of skim milk, and hydrolysis of starch was positive, but liquefaction of gelatin was pseudo-positive. Production of melanoid pigment was found in peptone-yeast extract-iron agar.

The carbon source utilization patterns of the strain are shown in Table 2. The whole cell analysis showed the presence of LL-diaminopimelic acid, which assigned the strain TA-0256 to the Type I cell wall group. The major menaquinone type was identified as MK-9 (H6) and MK-9 (H8). From taxonomical characteristics mentioned above, the strain TA-0256 was determined to belong

Table 2. Physiological properties of the strain TA-0256.

Characteristics	
Temperature range for growth	10~49°C
Optimum temperature range	33~39°C
Liquefaction of gelatin	Pseudo positive
Hydrolysis of starch	Positive
Coagulation of milk	Positive
Peptonization of milk	Positive
Formation of melanin	Positive (ISP-6)
Carbon utilization	
D-Glucose	Positive
Inositol	Only little
D-Fructose	Negative
D-Xylose	Negative
Sucrose	Negative
D-Mannitol	Negative
L-Arabinose	Negative
L-Rhamnose	Negative
Raffinose	Negative

Fig. 2. Isolation procedure of FD-594.



to the genus of *Streptomyces*. Comparison of characteristics of the strain TA-0256 with those of *Streptomyces* species described by SHIRLING[2], BERGEY[3], and WAKSMAN[4] strongly suggested that the strain TA-0256 closely resembled *Streptomyces baldaccii*. The strain TA-0256 was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Tsukuba-shi, Japan as the accession No. FERM P-14406.

Fermentation

A loopful of the strain TA-0256 on a oatmeal agar slant was inoculated in nine 500 ml Erlenmeyer flasks each containing 100 ml of the medium consisting of oatmeal 2%, glucose 2%, NaCl 0.3%, $Fe_2(SO_4)_3$ 0.04%, $MnCl_2 \cdot 4H_2O$ 0.04% and $CaCO_3$ 0.3%. The inoculated flasks were cultured at 28°C for 72 hours on the rotary shaker. Three hundred ml of the cultured broth was transferred into three 50 liter jar fermenter containing 30 liters of the same medium as in the seed culture. The fermentation was carried out at 28°C for 72 hours under aeration of 30 liters per minute and agitation speed 200 rpm.

Isolation and Purification

FD-594 was isolated from cells as outlined in Figure 2. The whole fractionation was guided by a bioassay of growth inhibition of HL-60 cells. The cultured broth was separated into supernatant and mycelium by centrifugation. The cell mass was extracted twice with 20 liters of acetone and the combined extract was concentrated *in vacuo*. The residual aqueous layer was further extracted twice with a half volume of ethyl acetate. The ethyl acetate layer was dried over anhydrous sodium sulfate and concentrated *in vacuo* to obtain the extract (50.6 g). The combined extract was subjected to a silica gel column chromatography charged with CH_2Cl_2, and then the column was eluted with CH_2Cl_2-MeOH by stepwise increase of MeOH concentration from 0~60%. A major fraction was eluted with 50% MeOH in CH_2Cl_2. The active fraction was concentrated *in vacuo* to give 1.28 g

of greenish yellow powder. All of this powder were dissolved in a little volume of dimethylsulfoxide, which was subjected to Sephadex LH-20 column chromatography. The column was developed with MeOH. The active fractions were collected and dried to yield 412 mg of greenish yellow syrup. Further purification of this material was performed on a Sephadex LH-20 column eluted with CH_2Cl_2-MeOH (1 : 1). The active fractions were combined and evaporated to give 78 mg of yellow powders. The active powders were dissolved in a little volume of dimethylsulfoxide and purified by HPLC over ODS eluted with 50% aqueous acetonitrile. The active fractions at the retention time of about 10 minute were collected and concentrated *in vacuo* to obtain 71 mg of homogeneous FD-594.

Physico-chemical Properties

The physico-chemical properties of FD-594 are summarized in Table 3. FD-594 was isolated as a yellow powder. It is sparingly soluble in most organic solvents, slightly in methanol, and easily soluble only in dimethylsulfoxide and pyridine. The UV spectrum showed absorptions characteristic to an anthraquinone chromophore as described in Table 3. The bathochromic shift by addition of an alkali solution suggested the presence of a phenolic group. In the IR spectrum, the bands at 1667 and 1624 cm^{-1} indicated the existence of an ester carbonyl and a carbonyl forming a hydrogen bond to a hydroxyl group. This was confirmed by the signals at 170.3 ppm and 186.2 ppm in the ^{13}C NMR spectrum and three lines at 11.8, 11.9 and 12.3 ppm due to a hydroxyl group in the ^{1}H NMR spectrum. The molecular formula of FD-594 was determined to be $C_{47}H_{56}O_{20}$ by the observation of its psuedo-molecular ion $(M+K)^+$ at m/z 979.3016 (calcd. for $C_{47}H_{56}O_{20}K$; 979.3007) in the high-resolution FAB-MS and secondary ion MS data. The ^{13}C and ^{1}H NMR spectra were shown in Figures 3 and 4. The functionalities of the carbon signals were determined with the DEPT spectra. By compiling the ^{13}C NMR spectra taken in various solvents, 47 lines were identified ($CH_3 \times 4$, $-CH_2- \times 6$, $-OCH_3 \times 2$, $-OCH- \times$

Table 3. Physico-chemical properties of FD-594.

Appearance	Yellow powder
Melting point	199~202°C
Molecular formula	$C_{47}H_{56}O_{20}$
Cl-MS (m/z)	941 $(M+H)^+$
HR FAB-MS (m/z)	
Found	979.3016 $(M+K)^+$
Calcd.	979.3007 for $C_{47}H_{56}O_{20}K$
$[\alpha]_D^{26}$	−215° (c 0.58, $CHCl_3$)
UV λ_{max}/nm (ε)	
in MeOH	420 (3200), 363 (12600), 276 (27500), 233 (23200), 214 (24800)
in 0.1 M NaOH/MeOH	440 sh (5200), 379 (11700), 277 (21400), 232 (26300)
IR ν_{max}/cm^{-1} (KBr)	3436, 1667, 1624, 1575, 1462, 1229, 1061

Fig. 3. ^{13}C NMR spectrum of FD-594.

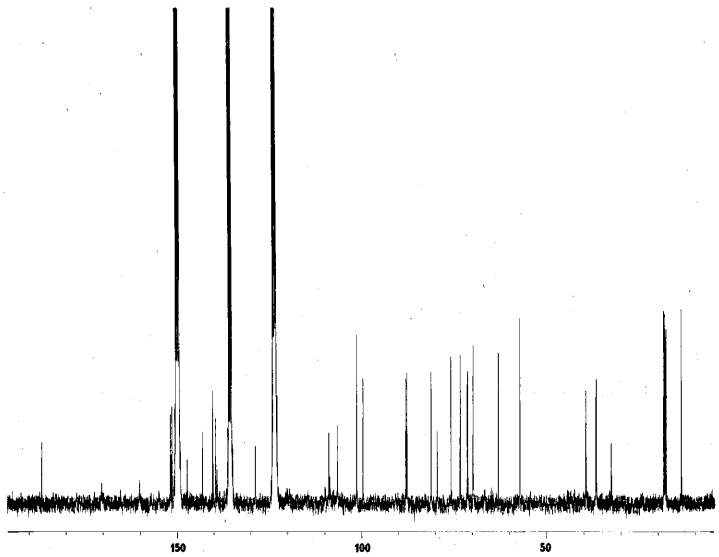
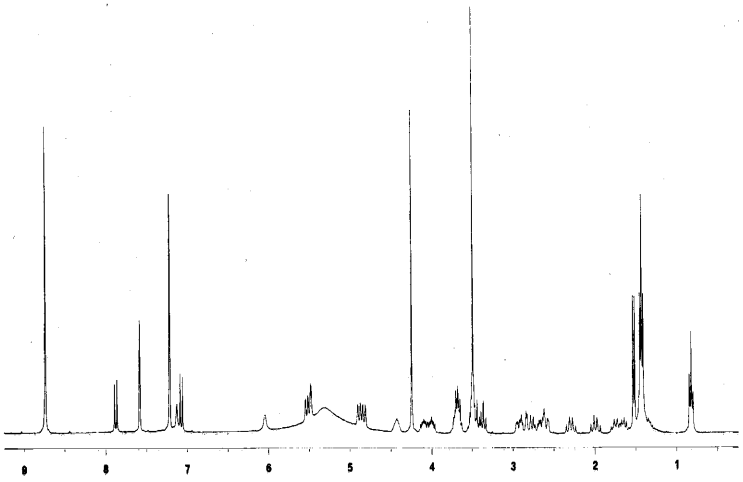


Fig. 4. ^{1}H NMR spectrum of FD-594.



15 including three anomeric carbon, $-CH=\times 3$, $>C=\times 15$ and $>C=O\times 2$). The ^{1}H NMR spectrum showed three proton signals at 6.96, 7.09 and 7.56 ppm due to an aromatic ring. Three anomeric protons were observed at 4.50, 4.51 and 5.14 ppm, suggesting the existence of three sugar moieties in the molecule. These physicochemical properties suggested similarity to BE-13793X[5] and MSO901890[6]. However, the structural novelty of FD-594 was unequivocally elucidated by the extensive spectral and biosynthetic experiments as described in the accompanying paper[1].

Table 4. Activities of FD-594 and adriamycin against various tumor cell lines.

	IC_{50} (μg/ml)	
	FD-594	Adriamycin
HL-60	0.10	0.02
P388	0.25	0.032
L1210	0.25	0.032
HeLa	0.10	0.08
A549	1.00	0.08

Biological Activity

As shown in Table 4, FD-594 exerted similar to weaker cytocidal activities against various cultured cell lines than those of adriamycin. As to the antimicrobial activities, FD-594 showed moderate activity against some Gram-positive bacteria (Table 5).

Experimental

General

Melting point was determined with a Yanagimoto micro-melting point apparatus and was uncorrected. The IR spectra were recorded on a Perkin-Elmer 1760 FT-IR spectrophotometer. The UV spectra were measured on a Hitachi 220A spectrophotometer. The EI-MS and FAB-MS spectra were obtained with a JEOL JMX-SX 102 mass spectrometer. The SI-MS spectrum was recorded with a Shimadzu Profile HV300. The NMR spectra were measured on a JEOL-A 500 spectrometer at ambient temperature at 500 MHz (^{1}H) and 125 MHz (^{13}C) using the solvent peaks as internal references.

HPLC

Preparative HPLC separations were performed by monitoring the absorbance at 215 nm using a Senshu-Pack ODS column (ODS-4251-N, 10 mm × 25 cm) with a Waters Model 600E system, maintained at 50°C and with 50% acetonitrile solution at a flow rate of 4.5 ml/minute.

Taxonomy

The strain TA-0256 was isolated from a soil sample collected at Urawa city, Saitama prefecture in Japan. The cultural characteristics and physiological properties of the strain were investigated by the method of the International Streptomyces Project (ISP)[2,7]. To evaluate the cultural characteristics, the strain was incubated for 21 days. Color names and hue numbers are adopted from the Book of JIS (Japanese Industrial Standard) Color

Table 5. Antimicrobial activities of FD-594, amphotericin B and clarithromycin.

	MIC (μg/ml)		
	FD-594	Amphotericin B	Clarithromycin
Candida albicans TMM0239	>100	0.39	N.D.
Cryptococcus neoformans TIMM0354	>100	0.78	N.D.
Aspergillus fumigatus TIM0063	>100	3.13	N.D.
Trichophyton mentagrophytes #81028	>100	1.56	N.D.
Staphylococcus aureus 209P-J	0.10	N.D.	0.10
Staphylococcus epidermidis	0.39	N.D.	0.10
Bacillus subtilis ATCC 6633	0.39	N.D.	0.05
Escherichia coli NIHJ JC-2	>100	N.D.	>100
Pseudomonas aeruginosa P-32	>100	N.D.	>100

Standards (JISZ 8721). The temperature ranges for growth of the strain were determined after submerge cultivation in ISP No. 2 medium for 7 days. Diaminopimelic acid was analyzed on the hydrolysate of aerial mycelia grown on ISP No. 4 agar[8].

Antitumor Activity *In Vitro*

HL-60, P388 and L1210 cells were maintained in PRMI-1640 medium supplemented with 10% fetal bovine serum. HeLa and A549 cells were grown in DULBECCO's modified EAGLE's medium supplemented with 10% calf serum. All cells were maintained at 37°C in a humidified 5% CO_2 atmosphere. Cells were seeded into 96-well microtiter plates (2×10^4 cells/well) and incubated for 24 hours. The test sample, dissolved in DMSO, was added in serial dilution. After addition, the plates were incubated for 72 hours. For the evaluation of *in vitro* antitumor activity, a microculture tetrazolium assay (MTT) method was used. The IC_{50} was calculated with PROBOT's method.

Antimicrobial Activities

The MIC values of antimicrobial activities against several yeast, fungi and bacteria were determined by an agar dilution method. Mueller-Hinton agar was used for the test at 37°C for 18 hours. SABOURAUD agar was used for the evaluation of antifungal activities. The test organisms and the temperature used are; *C. albicans* and *C. neoformans* 37°C for 48 hours; *A. fumigatus* and *T. mentagrophytes*, 26°C for 72 hours.

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