ISOLATION AND CHARACTERIZATION OF NEW 18-MEMBERED MACROLIDES FD-891 AND FD-892

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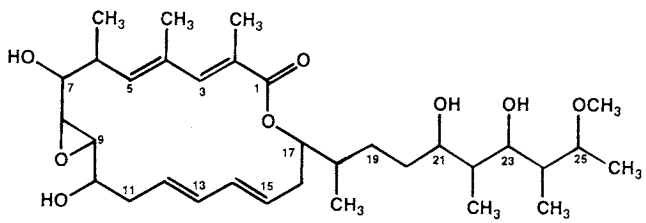
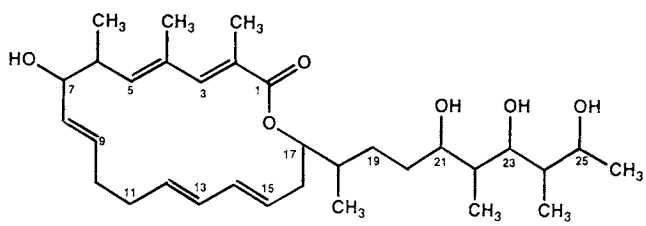
New 18-membered macrolides FD-891 and FD-892 were discovered from the fermentation broth of *Streptomyces graminofaciens* A-8890 isolated from a soil sample collected at Yamanashi prefecture, Japan. They induce morophological changes of HL-60 cells at low concentration below IC_{50}s and have cytocidal activity against *in vitro* tumor cell lines. FD-891 showed 2~7 times stronger activity than doxorubicin whereas FD-892 was 20~100 fold weaker than FD-891 and doxorubicin.

It is well known that human promyelocytic leukemia (HL-60) cells are differentiated to macrophage or monogranucyte by DMSO, vitamin D_3, retinoids, teleocidin and TPA[1,2] and we have recently found out that their morphological changes are related to their mode of action of such biologically active compounds as brefledin, cytochlasine, vincristine, concanamycins and actinomycins (K. MIZOUE and T. OKAZAKI, unpublished data). So, biologically active compounds screened by using HL-60 cells can be distinguished into their mode of action by the observation of morphological changes of HL-60 cells under the microscope. In the course of our screening program for low molecular compounds inducing morphological changes of HL-60 cells in the microbial fermentation broth, we have discovered new 18-membered macrolides FD-891 and FD-892 from the cultured broth of *Streptomyces graminofaciens* A-8890 as shown in Fig. 1. Such 18-membered antibiotics as concanamycin 1[3,4] and virustomycin[5,6] are

Fig. 1. Structures of FD-891 and FD-892.



FD-891



FD-892

of considerable interest because of their various biological properties; for example, antifungal[7,8], antiviral[5], antiprozoal[5], antitumor activities[9], inhibitory activity of V-ATPase[10~12] and blocking activity of intracellular translocation of viral-envelope glycoproteins[11,12]. The present paper describes taxonomy, fermentation, isolation, physico-chemical properties and biological activities.

Results

Taxonomy

Strain A-8890 was isolated from a soil sample collected at Nirasaki City, Yamanashi prefecture, Japan. The vegetative mycelium of this strain developed well on the synthetic agar plate and branched irregularly. The septum was not observed. The spores were well formed on the tip of aerial hyphae on the inorganic-starch agar and the oatmeal agar. The observation of the morphology through light microscopy showed that divergence of the mycelium forming spores was simple and spores were spirally formed on the tip of aerial mycelium. Strain A-8890 formed more than ten spores per chain with warty surface. The spores were cylindrical and $1.3 \sim 1.7 \times 0.7 \sim 0.8\ \mu m$ in size. The sclerotia, sporangium and flagellated spores were not observed. The cultural characteristics of strain A-8890 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 yellowish white to brownish white color. Soluble pigments were not produced on all agar media. Strain A-8890 grew well at the range of 20 to 33°C on the yeast extract-malt extract agar, but not below 14°C and over 39°C. Liquefaction of gelatin, coagulation of skim milk, peptonization of skim milk, hydrolysis of starch were positive, but production of melanoid pigment was negative as shown in Table 2. The carbon source utilization patterns of strain A-8890 are shown in Table 2. The whole cell analysis of strain A-8890 showed the presence of LL-diaminopimelic acid, revealing that it was classified as cell wall type I and as belonging to the genus *Streptomyces*. From taxonomical characteristics mentioned above, strain A-8890 was estimated to belong to the genus *Streptomyces*. Comparison of characteristics of strain A-8890 with those of *Streptomyces* species described by SHIRLING[13], BERGEY[14], and WAKSMAN[15] indicated that strain A-8890 closely resembled *Streptomyces graminofaciens*. On the basis of these results, strain A-8890 was identified as *Streptomyces graminofaciens* A-8890. The scanning

Table 1. Cultural characteristics of strain A-8890.

Medium	Growth	Aerial mycelium	Reverse color	Soluble pigment
Sucrose-nitrate agar	Good	None	Light yellow	None
Glucose-asparagine agar	Moderate	None	Light yellow	None
Glycerol-asparagine agar (ISP-5)	Good	Moderate Grayish white	Light brown	None
Inorganic salts-starch agar (ISP-4)	Good	Good Grayish brown	Yellowish brown	None
Tyrosine agar (ISP-7)	Moderate	Moderate	Yellowish white	None
Nutrient agar	Moderate	None	Yellowish white	None
Yeast extract-malt extract agar (ISP-2)	Good	Moderate Brownish white	Light brown	
Oatmeal agar (ISP-3)	Good	Good Grayish white	Light yellow	None
Peptone-yeast extract-iron agar (ISP-6)	Moderate	None	Light brown	None

Table 2. Comparison of taxonomic characteristics strain A-8890 with *S. graminofaciens*.

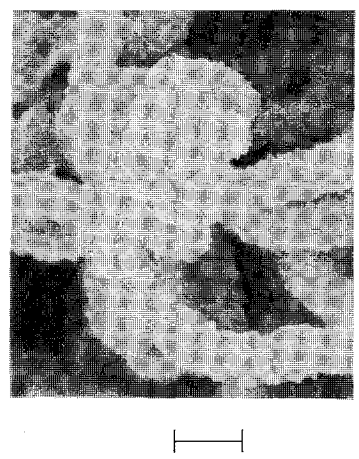
	A-8890	*S. graminofacience*
Spore chain morphology	*Spirals*	*Spirals*
Spore surface	Warty	Warty
Aerial mass color	Gray color series	Gray or white
Melanoid formation		
Tryptone-yeast broth (ISP-1)	−	−
Peptone-yeast extract-iron agar (ISP-6)	−	−
Tyrosine agar (ISP-7)	−	−
Coagulation of milk	+	N.D.
Peptonization of milk	+	N.D.
Liquefaction of gelatin	+	N.D.
Hydrolysis of starch	+	N.D.
Temperature range for growth	17~38°C	N.D.
Carbon utilization		
D-Glucose	+	+
D-Xylose	+	+
L-Arabinose	+	+
L-Rhamnose	+	+
D-Fructose	+	+
Raffinose	+	+
D-Mannitol	+	+
i-Inositol	+	+
Sucrose	+	+
D-Galactose	−	N.D.
Salicin	−	N.D.

Data of *S. graminofaciens* are cited from ref. 14.
N.D.: Not described.

electron micrograph of strain A-8890 is shown in Fig. 2. This strain 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-11504.

Fig. 2. Scanning electron micrograph of spore chains of *S. graminofaciens* A-8890 grown on oatmeal agar medium.

Bar represents 1.4 μm.



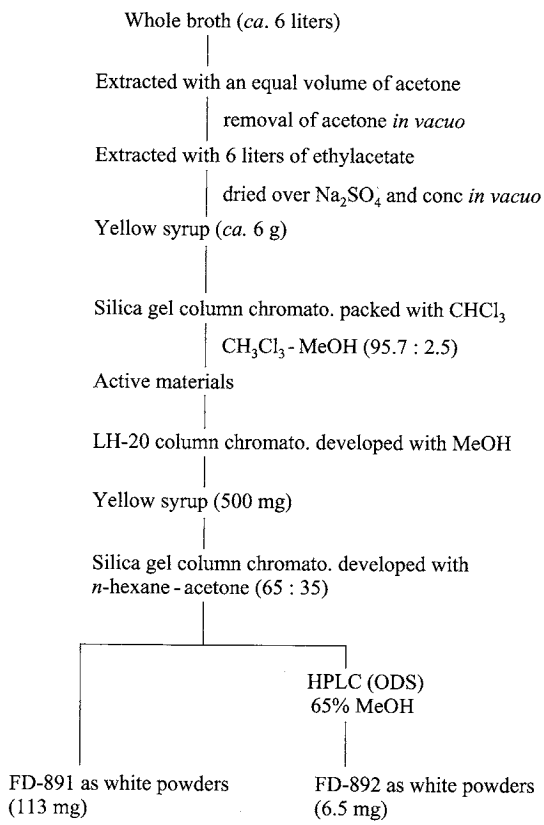
Fermentation and Isolation

A loopful of *Streptomyces graminofaciens* A-8890 on oatmeal agar slant was inoculated in two 500 ml Erlenmyer flasks each containing 100 ml of the medium consisting of oatmeal 2%, glucose 2%, NaCl 0.3%, $Fe_2(SO_4)_3$ 0.04%, $MnCl \cdot 5H_2O$ 0.04% and Na_2CO 0.3%. The inoculated flasks were cultured at 28°C for 96 hours on the rotary shaker. Each of 100 ml of the cultured broth was transferred into two 5-liter jar fermentors containing 3 liters of the same medium as in the seed culture. The fermentation was carried out at 28°C for 96 hours under airation of 3 liters per minute and agitation speed 150 rpm.

The isolation procedure is shown in Fig. 3. The whole fractionation was guided by bioassay of

morphological changes of HL-60 cells. The cultured broth was extracted with 6 liters of acetone. After removal of acetone *in vacuo*, the aqueous layer was extracted with 6 liters of ethyl acetate. The ethyl acetate layer was dried over Na_2SO_4 and concentrated *in vacuo* to give *ca.* 2 g of yellow syrup. The resultant material was subjected to a silica gel column chromatography charged with chloroform and eluted with $CHCl_3$ - MeOH by 0.5% of stepwise increase of MeOH concentration from 0~3.0%. The active majority was eluted with 2.5% of MeOH in $CHCl_3$. The active fraction was concentrated *in vacuo* followed by Sephadex LH-20 column chromatography prepared with MeOH. The column chromatography was developed with MeOH. The active fractions were collected and concentrated *in vacuo* to obtain 500 mg of yellow syrup. This material was applied to a silica gel column chromatography charged with *n*-hexane - acetone (65 : 35) and eluted with the same solvent. Two active fractions were obtained. One was concentrated under reduced pressure to yield 113 mg of FD-891 as a white powder, while the other was further purified by HPLC. As a result, 6.5 mg of FD-892 was obtained as a white powder.

Fig. 3. Isolation procedure of FD-891 and FD-892.



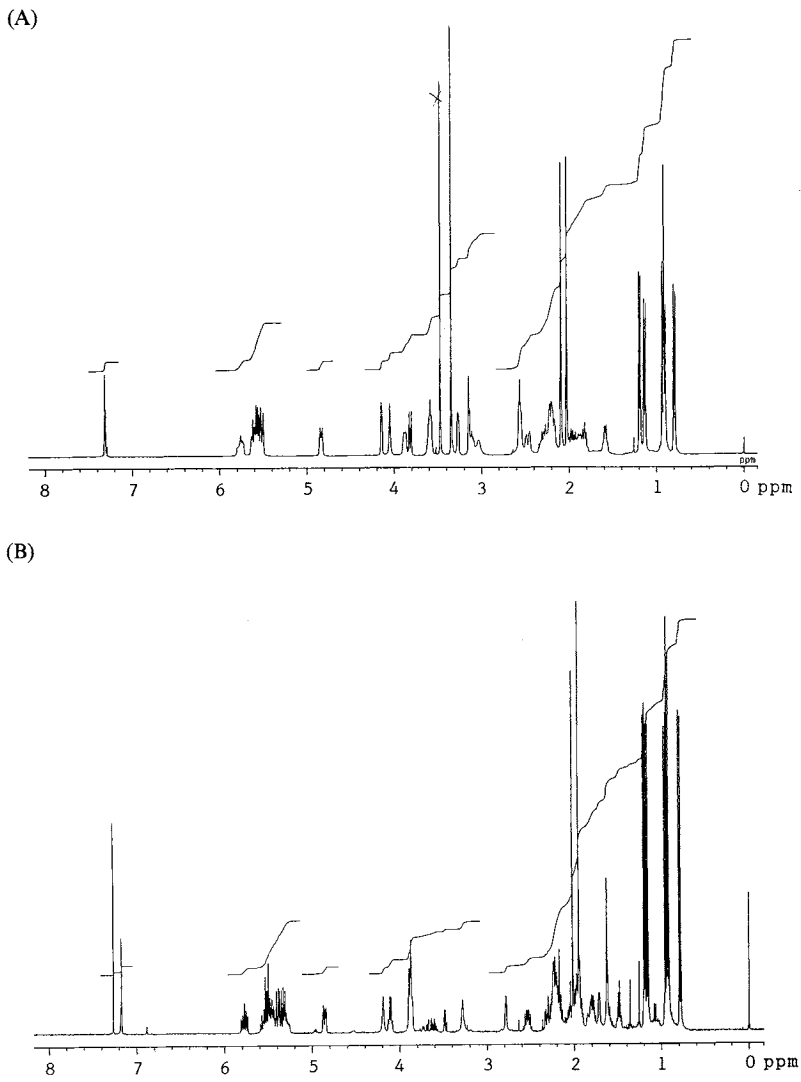
Physico-chemical Properties of FD-891 and FD-892

The physico-chemical properties of FD-891 and FD-892 are described in Table 3. Both FD-891 and FD-892 are lipophilic, neutral in nature and gave positive color response to iodine, H_2SO_4 and vanillin-H_2SO_4, but negative to ninhydrin. The UV and the IR spectra of FD-891 and FD-892 showed similar spectral patterns to each other. The maximum absorptions at 270 nm of FD-891and at 275 nm of FD-892 due to an α, β, γ, δ diene system conjugated with a carbonyl group were observed in their UV spectra. The bands at 1710 cm^{-1} (FD-891) and at 1705 cm^{-1} (FD-892) in their IR spectra

Table 3. Physico-chemical properties of FD-891 and FD-892.

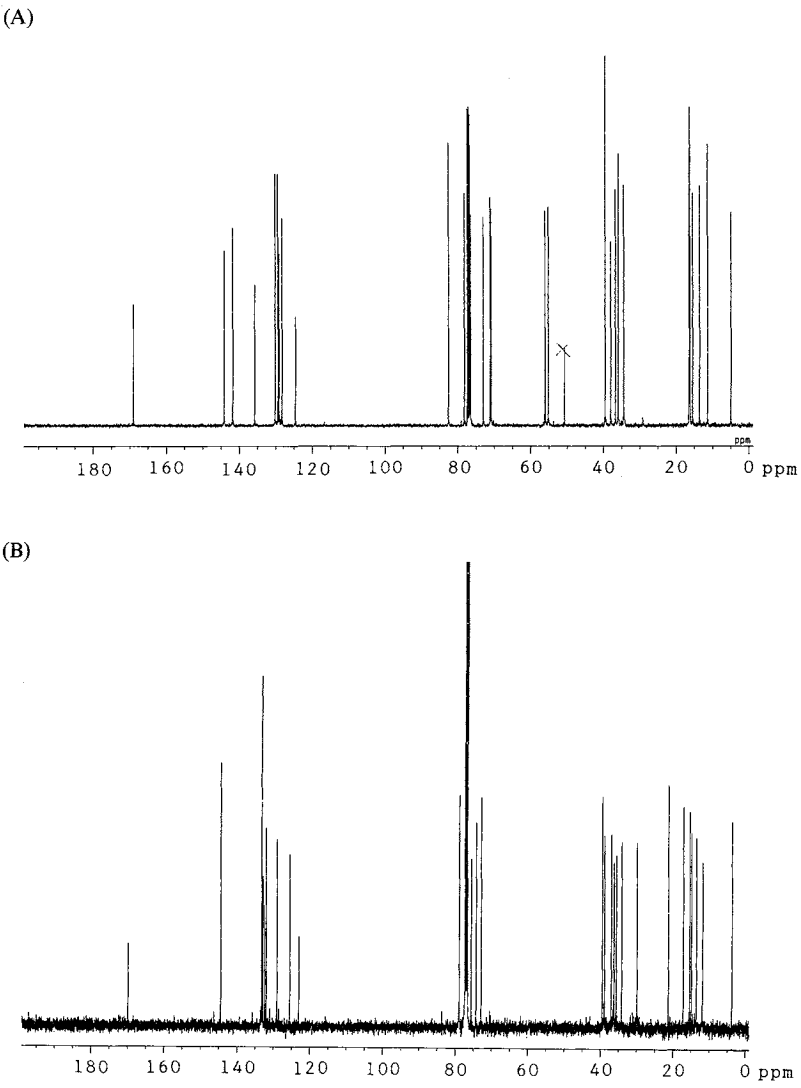
	FD-891	FD-892
Appearance	White powder	White powder
MP (°C)	68.5~72	55~62
$[\alpha]_D$	+14.0° (*c* 0.1, MeOH)	+48.0° (*c* 0.05, MeOH)
FAB-MS (*m/z*)	579 $(M+H)^+$	533 $(M+H)^+$
HREI-MS (*m/z*)		
Found:	578.3827	532.3759
Calcd:	578.3819	532.3764
	for $C_{33}H_{54}O_8$	for $C_{32}H_{52}O_6$
UV λ_{max}^{MeOH} (ε) nm	208 (5,060), 270 (12,480)	207 (7,710), 275 (14,040)
IR ν_{max}^{KBr} cm^{-1}	3400, 1710	3400, 1705

Fig. 4. The ^1H NMR spectra of FD-891 (A) and FD-892 (B) measured in $CDCl_3$.



indicated the existence of an ester carbonyl group, which was confirmed by the signals at 168.9 ppm (FD-891) and at 169.8 ppm (FD-892) due to carbonyl groups in their ^{13}C NMR spectra. Their molecular formula were established as $C_{33}H_{54}O_8$ (FD-891) and $C_{32}H_{52}O_6$ (FD-892), respectively, by their pseudo molecular ion measurements $(M+H)^+$ at *m/z* 579 and at *m/z* 533 in the FAB mass spectra and by the observation of their molecular ions at *m/z* 578.3827 (calcd. 578.3819 for $C_{33}H_{54}O_8$) and at *m/z* 532.3759 (calcd. 532.3764 for $C_{32}H_{52}O_6$), respectively. As shown in Figs. 4 and 5, their ^1H and ^{13}C NMR spectra resembled each other except for some regions, indicative of structurally similar compounds . The functionalities of the carbon signals of FD-891 and FD-892 were determined by the DEPT spectra. Consistent with their molecular formula, the ^{13}C NMR spectrum of FD-891 gave 33 lines ($CH_3 \times 7$, $-CH_2 \times 4$, $>CH- \times 4$, $OCH_3 \times 1$, $-CHO- \times 8$, $-CH= \times 6$, $>C= \times 2$ and $>C=O \times 1$) revealing that 46 protons were bonded to 26 carbons with the existence of four hydroxyl groups in the molecule, whereas the

Fig. 5. The ^{13}C NMR spectra of FD-891 (A) and FD-892 (B) measured in $CDCl_3$.



^{13}C NMR spectrum of FD-892 showed 32 carbon signals ($CH_3 \times 7$, $-CH_2- \times 5$, $-CHO- \times 5$, $>CH- \times 4$, $-CH= \times 8$, $>C= \times 2$ and $>C=O \times 1$) indicating that 29 carbons attached a total of 46 protons and FD-892 was shown to possess three hydroxyl groups by taking into consideration its molecular formula. The structures of FD-891 and FD-892 as shown in Fig.1 were elucidated by their extensive NMR experiments. Their structural determination will be described in the accompanying paper[16].

Biological Activities of FD-891 and FD-892

As shown in Table 4, FD-891 showed cytocidal activities against human and mouse leukemia cells two to seven times stronger than doxorubicin, whereas the activities of FD-892 was very weak compared with those of FD-891 and doxorubicin. When FD-891 and FD-892 were applied to HL-60 cells at the concentration below IC_{50}, they induced the same morphological change of round cells into swelling cells as concanamycins. FD-891 and FD-892 showed activity only against *Saccharomyces sake* but no activities

Table 4. Cytocidal activities of FD-891 and FD-892.

	IC_{50} (μg/ml)			
	FD-891	FD-892	Doxorubicin	Concanamycin A
HL-60	0.008	1.0	0.02	0.0032
P388	0.016	2.0	0.03	0.0032
L-1210	0.008	1.0	0.06	0.0032

against other fungi, Gram-positive and -negative bacteria (data not shown). Concanamycins are specific inhibitors of V-ATPases[10~12] and affect cell surface expression of viral glycoproteins[11,12]. As FD-891 and FD-892 belong to concanamycin-type 18-membered macrolides, they are expected to have similar activities to those of concanamycins. We have recently discovered[17] that FD-891 and FD-892 exert similar activities against V-ATPase and exocytosis of viral glycoproteins.

Discussion

We have isolated new biologically active compounds FD-891 and FD-892 from the fermentation broth of *Streptomyces graminofaciens* A-8890. FD-891 and FD-892 belong to such 18-membered antibiotics as concanamycins[3,4] and virustomycin[5,6], and showed strong cytocidal activities against human and mice leukemia cells HL-60, P388 and L1210, but not antibacterial activities against Gram-positive and Gram-negative bacteria. At the concentration below IC_{50} they induced the same morphological change of swelling cells into round cells as concanamycins. Concanamycins and bafilomycins, 18- and 16-membered macrolide antibiotics, respectively, are specific inhibitors of V-ATPase[10~12,18]. FD-891 and FD-892 also specifically inhibit V-ATPase[17]. This specific inhibition of V-ATPase activity may be common to those 16- and 18-membered bafilomycin and concanamycin-type macrolides. But significant differences are observed with the degree of their biological activities. The result presented in Table 4 indicates the significant decrease of cytocidal activities due to the substitution of a double bond with an epoxide ring.

It was suggested that the epoxide ring at C-8 and C-9 in the macrolide ring is estimated to play an important rule in their cytocidal activities.

Experimental

General

Melting points were determined with a Yanagimoto micro-melting point apparatus and were uncorrected. Optical rotations were measured on a Jasco DIP-360 polarimeter in 10 cm tube. IR spectra were recorded on a Perkin-Elmer 1760 FI-IR spectrophotometer. UV spectra were measured on a Hitachi 220A spectrophotometer. EI-MS and FAB-MS spectra were obtained with a JEOL JMX-SX 102 mass spectrometer. NMR spectra were measured on a JEOL JMN-GX 400 spectrometer at ambient temperature at 400 MHz (^1H) and 100 MHz (^{13}C) using the solvent peaks as internal references downfield of TMS at 0 ppm.

HPLC

Preparative HPLC separations were performed using a Senshu-Pack ODS column (ODS-4251-N, 10 mm × 25 cm) with a Waters Model 600E system, maintained at 50°C and developed with 60% methanol solution at a flow rate of 5 ml/minute, monitoring the absorbance at 215 nm.

Taxonomic Studies

To investigate the morphological properties of the strain, the International Streptomyces Project (ISP)

media recommended by SHIRLING and GOTTLIEB were employed[19]. Cultures were observed after 2 weeks at 28°C. The color index is in accordance with the ISCC-NBS Color-name chart. The utilization of carbon sources was tested by growth on PRIDHAM and GOTTLIEB's medium[21].

Biological Activity

For the evaluation of *in vitro* cytocidal activity, a microculture tetrazolium assay (MTT assay)[21] method was used. Cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum. The IC_{50} value was calculated with PROBIT's method.

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