ISOLATION AND CHARACTERIZATION OF A NEW 12-MEMBERED MACROLIDE FD-895

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During the course of our screening program for natural product drugs effective against multidrug resistant cells by using adriamycin resistant HL-60 cells, we have discovered a new 12 membered macrolide FD-895 in the fermentation broth of *Streptomyces hygroscopicus* A-9561 isolated from a soil sample collected at Iriomote Island, Okinawa prefecture, Japan. FD-895 showed stronger cytocidal activities against *in vitro* tumor cell lines than adriamycin. FD-895 had the same IC_{50} values against parent and adriamycin resistant HL-60 cells.

A major drawback to cancer chemotherapy is that many tumors are either intrinsically resistant to the compound or develop resistance over the course of treatment. Treatment with chemotherapeutic agents generally results only in temporary remission of tumor disease in the clinic.

It is also well known experimentally[1,2] that mammalian cells selected for resistance to a single cytotoxic natural product drug can become not only resistant to the agent used but also cross-resistant to a wide range of structurally and functionally unrelated antibiotics and alkaloids.

For these reasons the development of chemotherapeutic agents equally effective against malignant and resistant cells has been desired world wide for overcoming tumor disease. From this standpoint, we have explored natural product drugs effective against multidrug resistant cells.

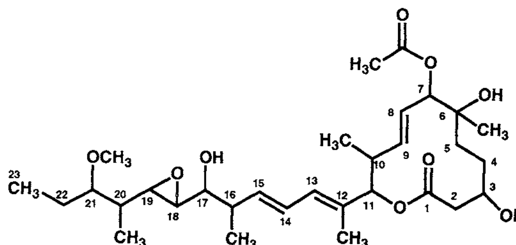
In the course of our screening program using adriamycin resistant HL-60 cells to discover low molecular compounds produced in microbial fermentation broths and capable of circumventing multidrug resistance, we have discovered a new 12-membered macrolide FD-895 (Fig. 1) from the cultured broth of *Streptomyces hygroscopicus* A-9561. The present paper describes the taxonomy, fermentation of *Streptomyces hygroscopicus* A-9561 and the isolation, physico-chemical properties and biological activities of FD-895.

Results

Taxonomy

Strain A-9561 was isolated from a soil sample collected at Iriomote Island, Okinawa prefecture, Japan. The vegetative mycelium of this strain developed well on synthetic agar plate and diverged irregularly. Septum was not observed. The spores were well formed on the tip of aerial branches from

Fig. 1. Structure of FD-895.



the vegetative mycelium on inorganic salts-starch agar and oatmeal agar. Observation of the micromorphology was performed through light microscopy. The aerial mycelium simply branched and no whirl formation was observed, and spore chains were spirally formed on the tip. Strain A-9561 formed more than ten spores per chain and had a rugose surface. The spores were cylindrical and $1.0\sim1.2\times0.8\sim0.9$ mm in size. Sclerotia, sporangia or fladellated were not observed. The cultural characteristics of strain A-9561 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 light yellow to brown color as described in Table 1. Moist, black and hygroscopic areas were found in the aerial mycelium on oat meal agar and inorganic salts-starch agar in 12 to 14 days. Soluble pigments were not produced on all agar media. Strain A-9561 grew well in the range of 24.5 to 30°C on yeast extract-malt extract agar, but not below 15°C or over 38°C. Liquefaction of gelatin, peptonization of skim milk and hydrolysis of starch were positive, but coagulation of skim milk and production of melanoid pigment were negative as

Table 1. Cultural characteristics of strain A-9561.

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	Good	Moderate, Grayish white	Light brown	None
Inorganic salts-starch agar	Good	Moderate, Grayish white	Light yellow	None
Tyrosine agar	Good	Poor, Grayish white	Brown	None
Nutrient agar	Moderate	None	Light yellow	None
Yeast extract-malt extract agar	Moderate	Moderate, Grayish white	Light yellow	None
Oat meal agar	Good	Good, Grayish white	Light yellow	None
Peptone-yeast extract-iron agar	Good	None	Light yellow	None

Table 2. Comparison of taxonomic characteristics of strain A-9561 with those *S. hygroscopicus*.

	A-9561	*S. hygroscopicus*		A-9561	*S. hygroscopicus*
Spore chain morphology	Spirals	Spirals	Temperature range for growth	15~38°C	N.D.
Spore surface	Rugose	Rugose	Carbon utilization		
Aerial mass color	Gray color series	Gray color series	D-Glucose	+	+
Melanoid formation			D-Xylose	+	±
Tryptone-yeast extract broth (ISP-1)	N.D.	−	L-Arabinose	+	±
			L-Rhamnose	+	+
Peptone-yeast extract iron agar (ISP-6)	−	−	D-Fructose	+	+
			Raffinose	+	−
Tyrosine agar (ISP-7)	−	−	D-Mannitol	+	+
Coagulation of milk	−	N.D.	Inositol	+	±
Peptonization of milk	+	N.D.	Sucrose	+	−
Liquefaction of gelatin	+	N.D.	D-Galactose	−	N.D.
Hydrolysis of starch	+	N.D.	Salicin	−	N.D.

Data of *S. hygroscopicus* are cited from ref. 4.
N.D.: not determined.

Fig. 2. Scanning electron micrograph of *Streptomyces hygroscopicus* A-9561 grown on oat meal agar medium.

Bar represents 2.5 μm.

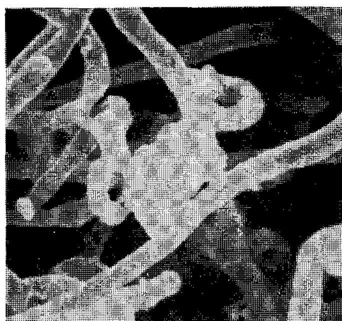
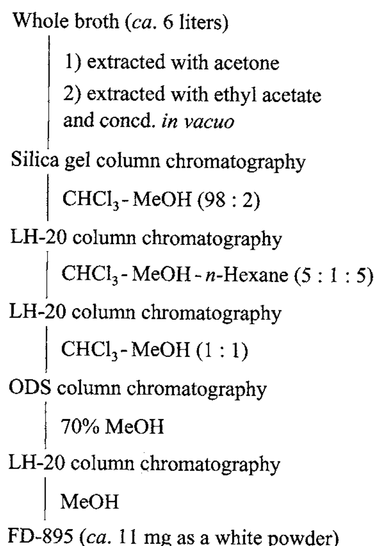


Fig. 3. Isolation procedure of FD-895.



shown in Table 2. The carbon source utilization patterns of strain A-9561 are depicted in Table 2. Analysis of hydrolyzed whole-cell of strain A-9561 showed the presence of LL-diaminopimelic acid. Accordingly, the cell wall of strain A-9561 was determined to be Type I. On the basis of on the taxonomic properties described above, strain A-9561 was classified as belonging to the genus *Streptomyces*. Comparison of characteristics of strain A-9561 with those of *Streptomyces* species described in ISP[3], BERGEY's Manual[4], and the Actinomyces[5] written by WAKSMAN indicated that strain A-9561 closely resembled *Streptomyces hygroscopicus*. On the basis of these results, strain A-9561 was identified as *Streptomyces hygroscopicus* A-9561. The scanning electron micrograph of strain A-9561 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-12223.

Fermentation and Isolation

A loopful of *Streptomyces hygroscopicus* A-9561 on oatmeal agar slant was inoculated in two 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%, $MnCl_2 \cdot 5H_2O$ 0.04% and Na_2CO_3 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 liters jar fermenters containing 3 liters of the same medium as in the seed culture. The fermentation was carried out at 28°C for 96 hours under aeration 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 growth inhibition of adriamycin resistant 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.* 2.2 g of yellow syrup. The resultant material was loaded on a silica gel column charged with chloroform and eluted with $CHCl_3$ - MeOH by 0.5% of stepwise increase of MeOH concentration from 0~4.0%. The active majority was eluted with 2.5% of MeOH in $CHCl_3$. The active fraction was concentrated *in vacuo* and subjected to Sephadex LH-20 column chromatography with $CHCl_3$ - MeOH - *n*-hexane (5 : 1 : 5). The active fractions were collected and concentrated *in vacuo* to obtain 400 mg of yellow syrup. This material was furthermore chromatographed

over Sephadex LH 20 in MeOH. The active fractions were collected and concentrated to obtain 39 mg of white powder. The white powder dissolved in 2 ml of 70% of MeOH was chromatographed on an ODS column in 70% MeOH. The active fractions were concentrated under reduced pressure to yield 11 mg of FD-895 as a white powder.

Physico-chemical Properties of FD-895

The physico-chemical properties of FD-895 are described in Table 3.

FD-895 is lipophilic, neutral in nature and gave positive color response to iodine, H_2SO_4 and vanillin-H_2SO_4, but negative to ninhydrin. The maximum absorption at 238 nm indicated the existence of a diene system in the molecule. The bands at 3450 cm^{-1} and 1725 cm^{-1} in the IR spectrum were ascribed to a hydroxyl group and an ester carbonyl, respectively, the latter being confirmed by the signal at 168.9 ppm in the ^{13}C NMR spectrum. The positive and negative FAB mass spectra showed pseudo-molecular ions at 567 $(M+H)^+$ and 565 $(M-H)^-$, respectively. The molecular weight was determined to be 566 by the observation of its molecular ion in the EI mass spectrum. The molecular formula of FD-895 was established as $C_{31}H_{50}O_9$ by its molecular ion measurement $(M)^+$ at m/z 566.3465 (calcd. 566.3455 for $C_{31}H_{50}O_9$) in the high resolution EI mass spectrum. The degree of unsaturation was estimated to be 7 by its molecular formula. Three unsaturations were assigned to three double bonds and two to two carbonyl groups, leaving the final two unsaturations to accommodate two rings. The ^{1}H and ^{13}C NMR spectra of FD-895 are shown in Figs. 3 and 4. The functionalities of the carbon signals of FD-895 were determined by the DEPT spectra. Consistent with its molecular formula, the ^{13}C NMR spectrum of FD-211 gave 13 lines, which were classified into $CH_3 \times 7$, $-CH2 \times 4$, $>CH- \times 3$, $OCH_3 \times 1$, $-CHO- \times 7$, $>C<$ $O \times 1$, $-CH= \times 5$, $>C= \times 1$ and $>C=O \times 2$. $CH_3 \times 7$,

Table 3. Physico-chemical properties of FD-895.

Appearance	White powder
MP (°C)	72~76
$[\alpha]_D^{26}$	+20.0° (*c* 0.01, MeOH)
EI-MS	m/z 566 (M^+)
FAB-MS (+)	m/z 567 $(M+H)^+$
FAB-MS (−)	m/z 565 $(M-H)^-$
HREI-MS	m/z 566.3465 (found) m/z 566.3455 calcd. for $C_{31}H_{50}O_9$
Molecular formula	$C_{31}H_{50}O_9$
MW	566
UV λ_{max}^{MeOH} nm (ε)	238 (24,400)
IR ν_{max} (KBr) cm^{-1}	3450, 1725

Fig. 4. The ^{1}H NMR spectrum of FD-895 measured in $CDCl_3$.

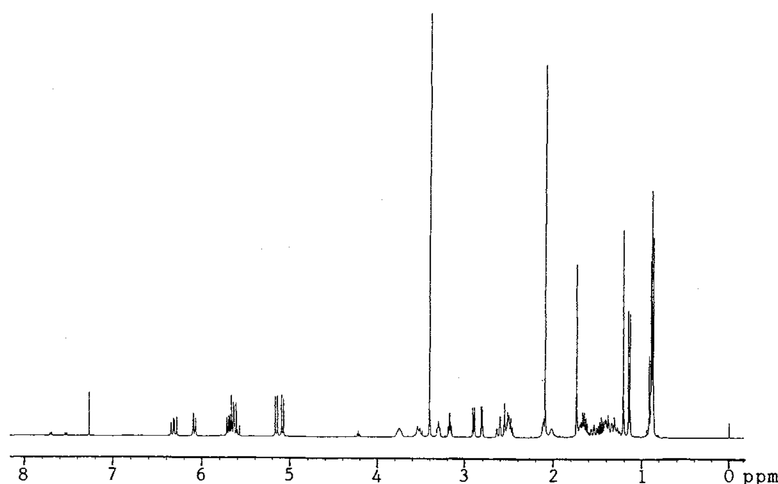


Fig. 5. The ^{13}C NMR spectrum of FD-895 measured in $CDCl_3$.

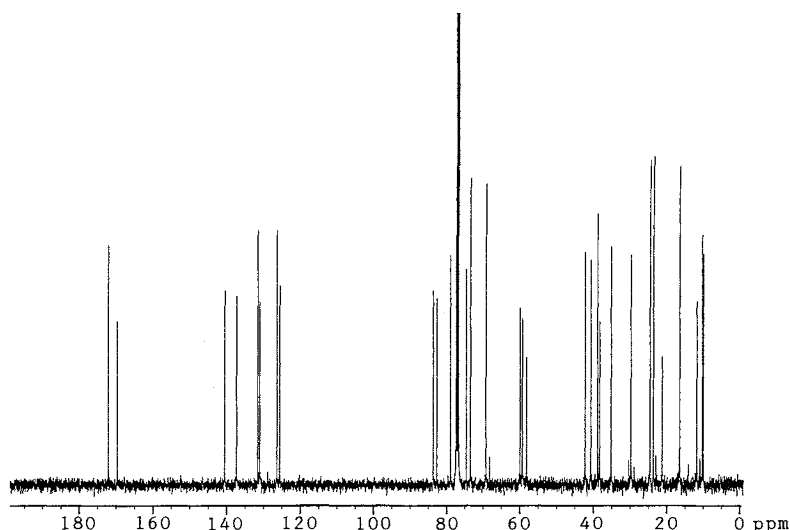


Table 4. Activities of FD-895 and adriamycin against various cultured tumor cells.

	IC_{50} (ng/ml)	
	FD-895	Adriamycin
HL-60/ADR[a]	2.0	2,000
HL-60	2.0	20.0
P388	4.0	32.0
L1210	4.0	32.0
HeLa	4.0	80.0
A549	8.0	80.0

[a] Adriamycin resistant cells.

Table 5. Inhibition of macromolecule biosynthesis by FD-895 in Hella cells.

IC_{50} (ng/ml)		
DNA	RNA	Protein
0.34	0.34	0.34

Table 6. Effects of FD-895 and concanamycin A on H^+-ATPases.

	IC_{50} (μg/ml)		
	V-type *S. cerevisiae* vacuol	F-type *S. cerevisiae* mitochondria	P-type *porcine* cerebral cortex
FD-895	>40	>40	>40
Concanamycin A	0.008	>20	>20

$-CH_2\times4$, $>CH-\times3$, $OCH_3\times1$, $-CHO-\times7$, $>C<O$, $-CH=\times5$, $>C=\times1$ and $>C=O\times2$. Also it was revealed that 46 protons were bonded to 26 carbons with the existence of three hydroxyl groups in the molecule. The plain structure of FD-895 was elucidated by extensive NMR experiments as shown in Fig.1. The structure determination of FD-895 will be described in detail elsewhere.

Biological Activities of FD-895

As shown in Table 4, FD-895 exerted eight to ten times stronger cytocidal activities against various cultured cell lines than adriamycin. FD-895 showed the same 50% inhibitory concentration against parent and resistant cells of HL-60. FD-895 inhibited the incorporation of thymidine, uridine and leucine labeled with ^{14}C into the macromolecules, however little differences among them were observed as depicted in Table 5. Unexpectedly FD-895 did not induce prolongation of survival time of mice transplanted with P388 leukemia cells (data not shown). FD-895 showed no activities against fungi, gram positive and negative bacteria (data not shown). Concanamycins and bafilomycins, 18- and 16- membered macrolides,

are specific inhibitors of V-ATPases and affect cell surface expression of viral glycoproteins[6~9]. As FD-895 is a 12 membered macrolide, we examined it for similar activities to those of 16- and 18-membered macrolides. FD-895 had no inhibitory effect against V-ATPase as shown in Table 6.

Discussion

We have isolated a new biologically active compound FD-895 from the fermentation broth of *Streptomyces hygroscopicus* A-9561. FD-895 is a 12-membered macrolide antibiotic and showed strong cytocidal activities against various cultured cancer cell lines including adriamycin resistant HL-60. Other 12-membered macrolides include methymycin[10], patulolides[11,12] and lactimidomycin[13]. In contrast to these 12-membered macrolides, FD-895 has no antimicrobial activities against fungi, Gram-positive and Gram-negative bacteria, suggesting that the mode of action of FD-895 is different from those of other 12-membered macrolides. No significant differences were observed with inhibition of incorporation of the labeled precursors into the macromolecules. FD-895 did not show any antitumor effect on P388 leukemia in mice. These results suggested that FD-895 has some action on cell membranes and this is consistent with the fact that FD-895 is equally effective against parent and adriamycin resistant HL-60 cells. The mode of action of FD-895 is under study in detail.

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 (^{1}H) and 100 MHz (^{13}C) using the solvent peaks as internal references downfield of TMS at 0 ppm.

Taxonomic Studies

To investigate the morphological properties of the strain,the International *Streptomyces* Project (ISP) media recommended by Shirling and Gottlieb were employed[14]. Diaminopimelic acid in the whole cell hydrolysates was analyzed by the method of Y. Takahashi *et al.*[15]. Cultures were observed after 2 weeks at 28°C. The color index in accordance with the ISCC-NBS Color-name chart. The utilization of carbon sources was tested by growth on Pridham and Gottlieb's medium[16].

Isolation and Maintenance of Adriamycin Resistant HL-60 Cell

Adriamycin resistant cells were isolated by stepwise selection in increasing concentrations of adriamicin starting with $2 \times IC_{50}$ (0.02 μg/ml). Cells were grown and maintained in RPMI-1640 medium containing 10% fetal bovine serum and 1 μg/ml of adriamycin.

Cytocidal Activity

HL-60, HL-60/ADR, P388 and L1210 were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum. A549 lung adenocarcinoma, Hela and T-24 renal carcinoma 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 atomosher. Cells were seeded into 96-well microtiter plates (2×10^4 cells/well) and incubated for 24 hours. The test sample, dissolved in MeOH, was added in serial dilutions. After addition, the plates were incubated for 72 hours. For the evaluation of *in vitro* cytocidal activity, a microculture tetrazolium assay (MTT assay)[17] method was used. The IC_{50} value was calculated with Probit's method.

Inhibition of macromolecule biosynthesis in the cultured Hela cells

Hela cells grown in RPMI-160 medium containing 10% fetal bovine serum for 24 hours at 37°C in

CO_2 incubator were collected by centrifugation at 1,000 rpm for 2 minutes. The collected cells were resuspended in a warm fresh medium (5×10^4 cells/ml) and 0.8 ml of the cell suspension was incubated with 0.1 ml of the medium containing the test compound at various concentrations at 37°C for 15 minutes prior to the addition of 0.1 ml of ^{14}C-labeled uridine, thymidine and leucine (0.005 μCi/ml). After incubation for 48 hours, cells were harvested. 1.0 ml of cold trichloroacetic acid (TCA) was added and centrifuged at 2,000 rpm for 5 minutes. The radioactivity of the acid insoluble fraction was determined with a Pharmacia Betaplate liquid scintillation counter.

ATPase Assay

Vacuole and mitochondrial membrane fractions of *Saccharomyces cerevisiae* X-2180 1A were prepared as previously reported[7,8], respectively, and porcine cerebral cortex obtained from Sigma. Effect of ATPase activity was examined following the reported method[7,8]. Briefly, the membrane fraction or ATPase was pretreated for 30 minutes at 35°C with designated concentrations of FD-895 or concanamycin A, and then added with ATP to start enzyme reaction. ATPase activity was determined by quantifying liberated inorganic phosphate after 30 minutes of incubation at 35°C.

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