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SW-163C and E, Novel Antitumor Depsipeptides Produced by *Streptomyces* sp.

I. Taxonomy, Fermentation, Isolation and Biological Activities

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Novel depsipeptides, SW-163C and E were isolated from the culture broth of an actinomycete strain. The producing organism, designated as SNA15896, was identified as a member of *Streptomyces* from its morphological and cultural characteristics. SW-163C and E exhibited potent antitumor activities against various tumor cell lines *in vitro* and against murine leukemia P388 *in vivo*. The compounds also showed antimicrobial activities.

The development of new antitumor drugs is required for cancer treatment because most human tumors are refractory to current conventional therapy. During our continuing search for new antitumor agents from microbial natural products, we discovered two novel depsipeptides belonging to the quinomycin family designated as SW-163C and E (Fig. 1), from the culture broth of a streptomycete strain SNA15896. This paper deals with the taxonomy of the producing strain, fermentation, isolation and biological activities of the compounds.

Materials and Methods

Microorganism

Strain SNA15896 was isolated from a soil sample collected at Yuuki City, Ibaraki Prefecture, Japan by a dilution agar plating method using Humic acid-vitamin agar[1]. The stock culture was maintained on oatmeal agar (ISP No. 3) slopes or in 20% (W/V) glycerol solution at −80°C.

Taxonomy Studies

The cultural and physiological characteristics of the SNA15896 strain were tested by International *Streptomyces* Project (ISP)[2]. Cultural characteristics were observed on various media at 27°C for 14~21 days. Color of aerial and substrate mycelia and reverse side of colony were determined with the Guide to Color Standard (Nihon, Shikisai Co., Ltd.). Morphological features were observed with a light microscope and a scanning electron microscope (Hitachi, model S-800). Chemical composition of the cell was determined by the methods of HASEGAWA *et al.*[3]. The menaquinones were analyzed by the methods of TAMAOKA *et al.*[4].

Fermentation

A mature slant culture of the strain SNA15896 was inoculated into 500-ml Erlenmeyer flasks, each containing 70 ml of seed medium being composed of $(NH_4)_2SO_4$ 0.14%, KH_2PO_4 0.2%, $CaCl_2$ 0.03%, $MgSO_4 \cdot 7H_2O$ 0.03%, urea 0.03%, Polypepton 0.5%, yeast extract 0.1%, soybean meal 3%, glucose 1%, soluble starch 0.5%, $FeSO_4 \cdot 7H_2O$ 0.0005%, $MnSO_4 \cdot 4{\sim}6H_2O$ 0.00016%, $ZnSO_4 \cdot 7H_2O$ 0.00014% and $CoCl_2$ 0.0002% (pH 7.0 before sterilization). The flasks were shaken at 27°C for 5 days on a rotary shaker (230 rpm). This seed culture (1.2 liters) was transferred into a 200-liter fermentor containing 120 liters of the production medium consisting of soluble starch 1%, glucose 2%, soybean meal 2.5%, dry yeast 0.4%, meat extract 0.1%, K_2HPO_4 0.005% and NaCl 0.2% (pH 7.2 before sterilization). The fermentation was carried out at 27°C for 8 days under aeration of 120 liters/minute and agitation at 200 rpm.

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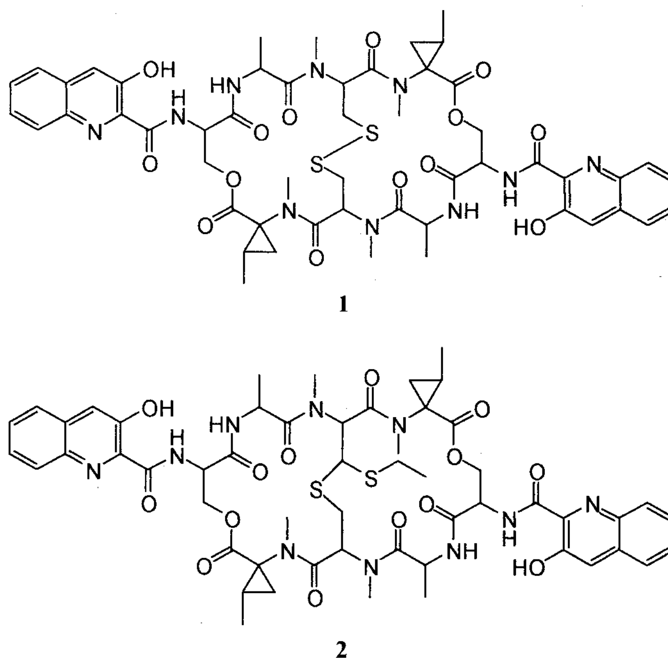
Fig. 1. Structures of SW-163C (**1**) and E (**2**).



Analytical Procedure

The amount of the compounds in the fermentation broth and purification steps was monitored by reversed phase HPLC. HPLC was performed under the following conditions: CAPCELL PAK C_{18} UG120 column (4.6×250 mm, Shiseido Co., Ltd.); mobile phase, CH_3CN - H_2O (6 : 4); flow rate, 1.0 ml/minute; detection, UV (220 nm). The retention times of SW-163C and E were 9.6 and 12.4 minutes, respectively.

Antimicrobial Activity

The tests were carried out by the conventional paper-disk method. Bacteria were grown on nutrient agar medium, and fungi and yeasts were grown on Sabouraud agar medium. The compounds were dissolved in MeOH and a paper disk (ADVANTEC, 8 mm in diameter) containing 50 μg of the sample was placed on the agar plates seeded with the respective test organism. Growth inhibition was examined after 24-hour incubation at 37°C for bacteria and after 48-hour incubation at 27°C for fungi and yeasts. The antimicrobial activity was estimated by measuring the diameter of inhibitory zone.

In Vitro Cytotoxicities

The cytotoxicities of the compounds against 2 murine and 3 human tumor cell lines were determined by the MTT colorimetric assay method[5]. P388 (murine leukemia), Colon26 (murine colon carcinoma) and A2780 (human ovarian cancer) cells were kindly provided by Dr. T. TSURUO, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research. HL-60 (human leukemia) and KB (human epidermoid carcinoma, oral) cells were obtained from American Type Culture Collection. The cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco). Cells suspended in 100 μl of the medium ($1 \sim 2 \times 10^4$ cells/ml) were plated in 96-well culture plates and incubated at 37°C in a 5% CO_2 incubator for 24 hours. The test sample, dissolved in dimethyl sulfoxide, was serially diluted with the medium and then added to the wells. The plates were incubated at 37°C in a 5% CO_2 incubator for 48 hours. All experiments were carried out in triplicate. The growth of cells was measured colorimetrically at 550 nm (and 660 nm as a reference) by the MTT method. Cytotoxic activities are expressed as IC_{50} values, the drug concentrations required for 50% inhibition of cell growth.

In Vivo Antitumor Activity

CDF_1 male mice (6 weeks old) were obtained from Charles River Japan Inc. P388 cells for *in vivo* experiments

Table 1. Cultural characteristics of strain SNA15896.

Medium	Growth	Aerial mycelium	Substrate mycelium	Reverse color	Soluble pigment
Yeast extract-malt extract agar (ISP No. 2)	Good	Thick, yellowish white	Dull yellow green	Dark yellow	None
Oatmeal agar (ISP No. 3)	Moderate	Abundant, reddish gray	Dark orange	Dark orange	None
Inorganic salt-starch agar (ISP No. 4)	Good	Abundant, light orangish gray	Dark purple	Dark purple	None
Glycerol-asparagine agar (ISP No. 5)	Poor	Thin, white	Pale yellow	Pale yellow	None
Peptone-yeast extract-iron agar (ISP No. 6)	Poor	Scant, pale reddish yellow	Dull yellow	Dull yellow	None
Tyrosine agar (ISP No. 7)	Poor	Thin, white	Pale yellow	Pale yellow	None

were kept as an ascitic tumor in CDF_1 mice with weekly transplants. The cells (1×10^6) were implanted intraperitoneally (i.p.) into CDF_1 mice (7 weeks old) on day 0. SW-163C and E were suspended in 10% HCO-60 (Nikko Chemicals, Co., Ltd.) solution. Drugs were given i.p. to mice once a day on days 1 and 5. Groups of six animals each were used. Antitumor activity was evaluated by the median survival time of the experimental group and was expressed as the treated/control (T/C) value.

Results and Discussion

Taxonomic Characterization of the Producing Strain

The cultural and taxonomic characteristics of strain SNA15896 are summarized in Tables 1 and 2, respectively. The substrate mycelia were well developed, branched, and did not fragment into bacillary elements or coccoid forms. Mature aerial mycelia corresponded to the gray color-series and formed long chains of spores. Thick masses of spores were formed in ISP media No. 2 and No. 3. The spore chains were Spirales type, tightly coiled or slightly open or coiled into an irregular mass with 2 to 5 turns per chain, and each had 20 to 50 spores per chain (Photo 1a). The sporophores were monopodially branched and the spores were short-rod shaped to rod shaped, $0.5\sim0.7\times0.7\sim0.9$ μm in size. Their surfaces were rugose (Photo 1b). No synnemata, sclerotia or sporangia were observed. Reverse

Table 2. Taxonomic characteristics of strain SNA15896.

Spore chain morphology	Spirales
Spore surface	Rugose
Spore dimensions (μm)	(0.5~0.7) x (0.7~0.9)
Temperature for growth (℃)	17~42
Soluble pigment	Negative
Formation of melanoid pigment	Negative
Liquefaction of gelatin	Positive
Coagulation of milk	Negative
Peptonization of milk	Positive
Hydrolysis of starch	Positive
Utilization of	
L-Arabinose	−
D-Xylose	+
L-Rhamnose	−
D-Glucose	+
D-Galactose	+
D-Fructose	+
Sucrose	+
Raffinose	+
Inositol	+
D-Mannitol	+
Mannose	+

+: Utilization, −: no utilization.

mycelial pigment was not pH sensitive. Melanoid and other soluble pigments were not produced. Permissive temperatures for growth ranged from 17 to 42°C, with the optimal temperature at 30°C. Liquefaction of gelatin, peptonization of milk and hydrolysis of starch were

Photo. 1. Scanning electron micrographs (a and b) of strain SNA15896 grown on ISP-2 agar at 27°C for 14 days.

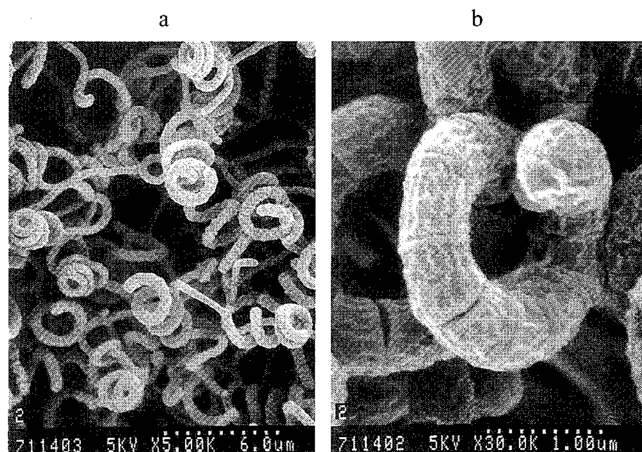
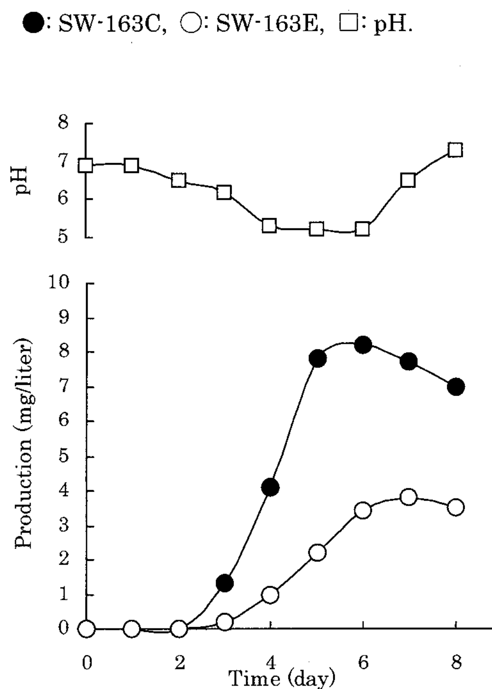


Fig. 2. Time course of SW-163C and E production in a 200-liter jar fermenter.

●: SW-163C, ○: SW-163E, □: pH.



positive, but coagulation of milk was negative. Xylose, glucose, galactose, fructose, sucrose, raffinose, inositol, mannitol and mannose were utilized as a sole carbon source but not arabinose and rhamnose. Analysis of whole-cell hydrolysate of the strain showed the presence of LL-diaminopimelic acid, indicating that the cell wall belongs to type I. The major components of menaquinones were MK-9 (H_6) and MK-9 (H_8).

Based on the taxonomic properties described above, strain SNA15896 was considered to belong to the genus *Streptomyces*. We searched the data of known *Streptomyces* species in the ISP descriptions of SHIRLING's reports[6~9], and the ACTINOBASE[10]. From the search, we learned that strain SNA15896 was related to *Streptomyces nigrescens*[7] and *Streptomyces braegensis*[11], although it could not be identified as any of the type strains. Further taxonomic study will be undertaken for the final identification of this strain. Therefore, it was designated as *Streptomyces* sp. SNA15896. Strain SNA15896 has been deposited in the National Institute of Bioscience and Human-Technology, the Agency of Industrial Science and Technology, Tsukuba City, Ibaraki Prefecture, Japan, with an accession number of FERM BP-6735 under the Budapest treaty.

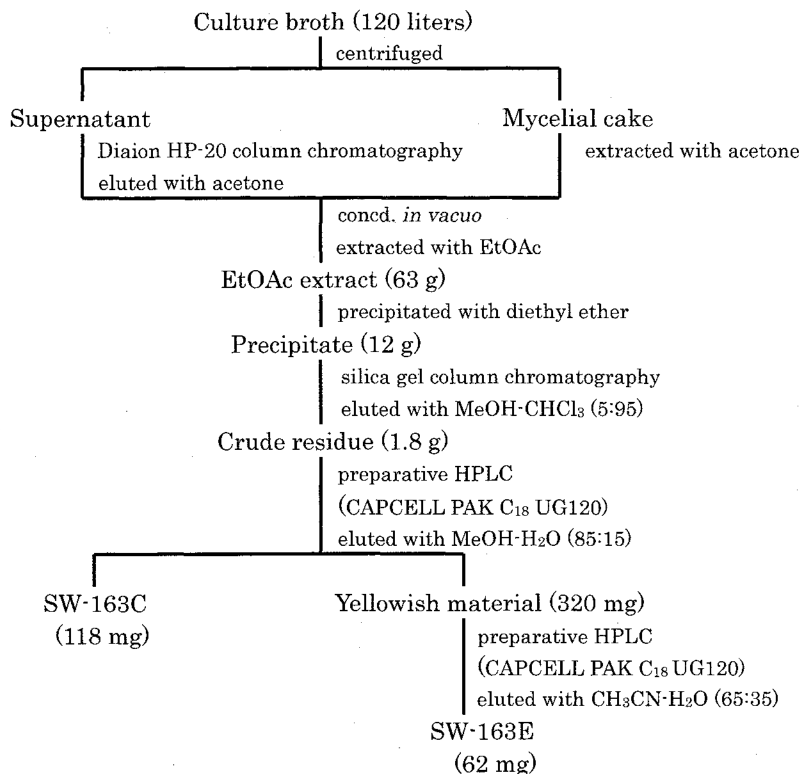
Fermentation

A typical time course of fermentation by *Streptomyces* sp. SNA15896 in a 200-liter fermenter is shown in Fig. 2. The pH of the culture broth gradually decreased from the beginning of fermentation, reached a minimum at 4 days, and showed a gradual increase after 6 days of cultivation. The production of SW-163C and E began after 3 days of fermentation and reached a maximum after 6 and 7 days, respectively. The concentration of accumulated SW-163C (8.3 mg/liter) was higher than that of SW-163E (4.0 mg/liter).

Isolation and Purification

The isolation procedure for SW-163C and E is schematically shown in Fig. 3. The culture broth of SNA15896 (120 liters) was centrifuged to separate supernatant and mycelial cake. The supernatant was applied to a Diaion HP-20 (Mitsubishi Chemical Ind. Co., Ltd.) column (15 liters) and the active substances were eluted from the column with acetone after washing with water. The active substances in the mycelial cake were extracted with acetone. The HP-20 eluate and the mycelium extract were combined, concentrated *in vacuo* to remove acetone and the resultant aqueous layer was extracted with EtOAc. The organic layer was evaporated to dryness *in vacuo* and then treated with diethyl ether to precipitate the active compounds. The precipitate was dissolved in chloroform and applied to a silica gel column (Merck, Kieselgel 60, 5

Fig. 3. Isolation of SW-163C and E.



liters). The active compounds were eluted from the column with MeOH - $CHCl_3$ (5 : 95) and the active fraction was evaporated to obtain a brownish residue. This was dissolved in a small volume of MeCN and subjected to preparative HPLC (mobile phase: MeOH - H_2O 85 : 15, flow rate: 100 ml/minute) on an ODS column (CAPCELL PAK C_{18} UG120, Shiseido Co. Ltd., 10×50 cm). The peak of SW-163C was eluted at a retention time of 51.1 minutes, followed by the peak of SW-163E at 68.5 minutes. The former peak was evaporated to remove the solvent and 118 mg of pure SW-163C was obtained as pale yellow crystalline powder. On the other hand, the latter peak contained impurities. Therefore, SW-163E was further purified by preparative HPLC under the same conditions described above except for the mobile phase system, MeCN - H_2O (65 : 35) at a flow rate of 200 ml/minute. The peak eluted at 57.0 minutes was concentrated *in vacuo* to give 62 mg of pure SW-163E as pale yellow needles.

The structures of SW-163C and E are shown in Fig. 1. The studies on the structural elucidation of these compounds will be reported in an accompanying paper.

Biological Activities

Antimicrobial Activity

The antimicrobial activities of SW-163C and E are summarized in Table 3. These compounds had potent antimicrobial activities against an extensive range of organisms and showed few differences in antifungal spectrum. They were predominantly active against Gram-positive bacteria such as *Staphylococcus aureus* including the drug resistant strain (R-209), *Bacillus subtilis*, *Micrococcus luteus* and *Mycobacterium phlei*. The activity of SW-163E was more potent than that of SW-163C.

Antitumor Activity (*In Vitro*)

In vitro cytotoxicities of SW-163C and E are shown in Table 4. Both compounds were potent cytotoxic agents and strongly suppressed the growth of all tested tumor cell lines. SW-163C and E showed cytotoxicity against the cell lines with IC_{50} values of 17~140 nM and 0.2~1.6 nM, respectively. The antitumor activities assessed by IC_{50} values of SW-163E were about 100-fold more potent than those of SW-163C.

Table 3. Antimicrobial activities of SW-163C and E.

Organisms	Diameter of inhibition zone (mm)	
	SW-163C	SW-163E
Escherichia coli AB 1157	0	0
Salmonella typhimurium TV 119	0	0
Pseudomonas aeruginosa IFO 13130	0	0
Xanthomonas oryzae IFO3312	15	15
Staphylococcus aureus IFO12732	15	20
Staphylococcus aureus R-209	14	20
Bacillus subtilis H17 Rec$^+$	15	24
Micrococcus luteus IFO 12708	12	20
Mycobacterium phlei IFO 3158	15	23
Alternaria mali IFO 8984	15	20
Botryotinia fuckeliana IFO 5365	12	33
Glomerella lagenaria IFO 7513	0	12
Pyricularia oryzae IFO 5994	11	12
Fusarium oxysporum IFO 9761	0	0
Trichophyton rubrum IFO 6203	0	0
Aspergillus fumigatus IFO 9733	0	15
Candida albicans IFO 1594	0	0
Schizosaccharomyces pombe IFO 0638	0	12

Table 4. Cytotoxicities of SW-163C and E.

Cells	IC_{50}(nM)	
	SW-163C	SW-163E
P388	38	0.3
Colon26	140	1.6
A2780	17	0.2
HL-60	110	1.3
KB	20	0.4

Table 5. Antitumor effects of SW-163C and E against P388 leukemia.

Compound	Dose (mg/kg)	Survival days (Median)	T/C (%)	B. W. [a] Change (g)	D/T [b]
SW-163C	1	14.0	139	0.5	0/6
	10	15.8	156	0	0/6
SW-163E	0.01	14.3	142	0.1	0/6
	0.1	12.0	119	-1.3	0/6
Vehicle (10% HCO60)	-	10.1	100	0.7	0/6

[a] Body weight change in the period from day 1 to 5.

[b] No. of toxic death mice/No. of mice tested.

Criterion: T/C >120 considered significant antitumor activity.

Antitumor Activity (*In Vivo*)

The antitumor effects of SW-163C and E against P388 leukemia implanted i.p. in mice are shown in Table 5. SW-163C showed a significant prolongation of survival day at doses of 1 and 10 mg/kg, giving T/C value of 139 and 156%, respectively. On the other hand, SW-163E was remarkable for the clear increase in life span at a dose of 0.01 mg/kg (T/C value of 142%) but was inactive at a dose of 0.1 mg/kg because of the toxicity. The acute toxicity (LD_{50}) of SW-163C and E were >100 mg/kg and 0.6 mg/kg, respectively, when administered intraperitoneally to mice. It appeared that SW-163C is relatively less toxic than SW-163E.

Through a series of the examination, novel depsipeptide antibiotics belonging to the quinomycin family, SW-163C and E were found to possess antitumor activity in a P388 murine tumor model. In conclusion, the results of this study suggest that SW-163C and E might be worthwhile for further evaluation of the antitumor effects and toxic profiles. Detailed evaluation of their antitumor activity will be published elsewhere.

References

1) HAYAKAWA, M. & H. NONOMURA: Humic acid-vitamin agar, a new medium for the selective isolation of soil actinomycetes. J. Ferment. Technol. 65: 501~509, 1987
2) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
3) HASEGAWA, T.; M. TAKIZAWA & S. TANIDA: A rapid analysis for chemical grouping of aerobic actinomycetes. J. Gen. Appl. Microbiol. 29: 319~322, 1983
4) TAMAOKA, J.; Y. KATAYAMA-FUJIMURA & H. KURAISHI: Analysis of bacterial menaquinone mixtures by high performance liquid chromatography. J. Appl. Bacteriol. 54: 31~36, 1983
5) MOSMANN, T.: Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J. Immuno. Methods 65: 55~63, 1983
6) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type cultures of *Streptomyces*. II. Species descriptions from first study. Int. J. Syst. Bacteriol. 18: 69~189, 1968
7) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type cultures of *Streptomyces*. III. Additional species descriptions from first and second studies. Int. J. Syst. Bacteriol. 18: 279~392, 1968
8) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type cultures of *Streptomyces*. IV. Species descriptions from the second, third and fourth studies. Int. J. Syst. Bacteriol. 19: 391~512, 1969
9) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type cultures of *Streptomyces*. V. Additional descriptions. Int. J. Syst. Bacteriol. 22: 265~394, 1972
10) UGAWA, Y.; K. SUGAWA, T. KUDO, H. SUGAWARA, Y. TATENO & A. SEINO: ACTINOBASE: An image database for identification of actinomycetes. Trends in actinomycetology in Japan, pp. 17~19, Actinomycetologica forum 1989. Society for Actinomycetes, Japan, 1989
11) DOLAK, L. A.; F. REUSSER, T. M. CASTLE, B. R. HANNON, A. L. LABORDE & C. K. MARSCHKE (Upjohn): Antibiotic U-64846 prepared from *Streptomyces braegensis* NRRL 12567, active against gram positive bacteria including *Staphylococcus aureus*. U.S. 4,404,190, Sep. 13, 1983