R1128 SUBSTANCES†, NOVEL NON-STEROIDAL ESTROGEN-RECEPTOR ANTAGONISTS PRODUCED BY A *Streptomyces*

I. TAXONOMY, FERMENTATION, ISOLATION AND BIOLOGICAL PROPERTIES

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New non-steroidal estrogen-receptor antagonists, R1128 A, B, C and D, were isolated from the cultured broth of *Streptomyces* sp. No. 1128 by solvent extraction, silica gel chromatography, reverse phase chromatography and preparative HPLC. These compounds inhibited estrogen binding to its receptor. The IC_{50} values of R1128 A, B, C and D for partially purified rat uterine cytosol receptor were 1.1×10^{-7} M, 1.2×10^{-7} M, 2.6×10^{-7} M and 2.7×10^{-7} M, respectively.

Non-steroidal estrogen-receptor antagonists, exemplified by tamoxifen, have been used successfully in the therapy of advanced breast cancer, especially estrogen-receptor positive breast cancer[1]. Although this therapy results in remarkable improvement in breast cancer patients, the development of resistance to tamoxifen frequently occurs and most patients eventually relapse. One potential method to overcome the resistance is the use of new chemical-structure estrogen-receptor antagonists different from tamoxifen and related compounds with the triphenylethylene moiety.

Consideration outlined above led us to screen microbial products for new non-steroidal estrogen-receptor antagonists without the triphenylethylene moiety.

As a result of the screening, we discovered new non-steroidal estrogen-receptor antagonists, R1128 A, B, C and D, from the cultured broth of *Streptomyces* sp. No. 1128 (Fig. 1). In this paper, we describe the taxonomy of the producing strain and the fermentation, isolation and biological properties of R1128 A, B, C and D. The physico-chemical properties, structure determination, pharmacological properties and antitumor activities of R1128 A, B, C and D are reported in the following papers[2,3].

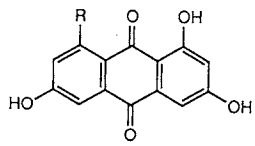
Materials and Methods

Taxonomic Studies

The method described by SHIRLING and GOTTLIEB[4] were employed for the taxonomic studies. Morphological observations were made with a light microscope and a scanning electron microscope from the cultures grown at 30°C for 2 to 4 weeks on yeast extract-malt extract agar and potato-dextrose agar.

Cultural and physiological properties were

Fig. 1. Structures of R1128 A, B, C and D.



R1128 A: $R=CH_2CH_2CH_3$
R1128 B: $R=CH_2CH_2CH_2CH_3$
R1128 C: $R=CH_2CH_2CH(CH_3)_2$
R1128 D: $R=CH_2CH_2CH_2CH_2CH_3$

† R1128 substances were called WS1128 substances in Jpn. Kokai 7244 ('91), January 14, 1991.

observed on several media described by SHIRLING and GOTTLIEB[4], and WAKSMAN[5]. Each culture was incubated at 30°C for 2 to 4 weeks. The color names used in these studies were taken from Methuen Handbook of Colour[6].

Cell wall analysis was perfomed by the method of BECKER *et al.*[7] and whole cell analysis was performed by the method of LECHEVALIER and LECHEVALIER[8,9]. Lipid composition was determined by the method of LECHEVALIER and LECHEVALIER[9]. Glycolate test was performed by the method of UCHIDA and AIDA[10].

The temperature range for growth was determined on yeast extract-malt extract agar using a temperature gradient incubator (Advantec Toyo Co., Ltd., Tokyo, Japan). Utilization of carbon sources was examined according to the method of PRIDHAM and GOTTLIEB[11].

Fermentation

A loopful of slant culture of strain No. 1128 was inoculated into each of four 500-ml flasks containing 160 ml of a sterile seed medium consisting of glucose 1%, modified starch 1%, meat meal 0.3%, yeast extract 0.5%, Bacto Tryptone (pancreatic digest of casein, Difco Laboratories, Detroit, MI, U.S.A.) 0.5% and $CaCO_3$ 0.2% (pH 7.0). The flasks were shaken on a rotary shaker (220 rpm, 7.5-cm throw) at 30°C for 4 days. The resultant seed culture was inoculated into a 30-liter jar fermentor containing 20 liters of a sterile production medium consisting of modified starch 1%, glycerin 2%, peanut powder 1%, chicken meat-bone meal 1%, $CaCO_3$ 0.2%, Adekanol (LG109, Asahi Denka Kogyo Co., Ltd., Tokyo, Japan) 0.05% and Silicone (KM-70, Shin-etu Kagaku Kogyo Co., Ltd., Tokyo, Japan) 0.05%. The fermentation was carried out at 30°C under aeration of 20 liters/minute and agitation of 200 rpm for 4 days.

Assay for R1128 B Production

The amount of R1128 B in the fermentation broth was determined by HPLC using a Hibar LiChrosorb RP-18 (5 μm, 4 × 200 mm, E. Merck, Darmstadt, F.R.G.) with 67% aqueous CH_3CN containing 0.1% TFA, detected by UV absorption at 210 nm and 280 nm. The sample for the HPLC assay was prepared as follows: After 2 ml of acetone was added to 1 ml of the fermentation broth, the mixture was stirred vigorously for 1 minute and centrifuged at 3,000 rpm for 5 minutes. Packed mycelium volume was measured by centrifuging the broth at 2,000 rpm for 10 minutes.

Drugs

Tamoxifen (citrate salt) and 17β-estradiol were purchased from Sigma Chemicals Co. (MO, U.S.A.). For *in vitro* experiments, R1128 substances, 17β-estradiol and tamoxifen were dissolved in ethanol and further diluted in buffer or medium.

Animals

Specific pathogen free (SPF) DBA/2 mice (female, 6~7 weeks old) and SPF Sprague-Dawley rats (female, 3 weeks old and 7~8 weeks old) were purchased from Japan S.L.C. (Shizuoka, Japan). BDF_1 mice (C57BL/6 × DBA/2) (female, 6~7 weeks old) were purchased from Charles River Japan Inc. (Kanagawa, Japan).

Preparation of Estrogen Receptors

Cytosol from rat uteri was used as estrogen receptors. Mature female Sprague-Dawley rats (7~8 weeks old) were ovariectomized and 2 days later, the rats were sacrificed.

Unless specified, all the following procedures were carried out at 0~4°C. Uteri taken from the rats were homogenized with a Polytron homogenizer in buffer (1 g of tissue/4 ml of buffer) consisting of 5 mM KH_2PO_4 (pH 7.4), 10% glycerol and 0.1% α-thioglycerol. Cytosol was obtained by centrifuging the homogenate at 108,000 × g for 30 minutes. Cytosol was stored at −80°C until used. Under these conditions, estrogen-receptor binding activity in the preparation was stable for several months. The protein concentration of the cytosol was determined using BioRad Protein Assay Kit (Bio-Rad Laboratories, Richmond, CA, U.S.A.).

Estrogen-receptor Binding Assay

The reaction mixture containing 10 μl of a test sample, 100 μl of 2,4,6,7-[^{3}H]estradiol (4.26 TBq/mmol,

New England Nuclear, Wilmington, DE, U.S.A., 14.8 KBq/ml) and 100 μl of rat uterus cytosol (1~2 mg protein/ml) was incubated in a test tube at 0°C for 1 hour. After incubation, 125 μl of dextran-coated charcoal solution consisting of 0.5% Norit A (Nakarai Chemicals, Ltd., Kyoto, Japan) and 0.05% Dextran T-70 (Pharmacia Fine Chemicals, Ltd., Uppsala, Sweden) were added to the incubation mixture and further incubated at 0°C for 10 minutes. The mixture was then centrifuged at 3,000 rpm for 5 minutes. The radioactivity in 50 μl of the supernatant was counted in 8 ml of Aquazol-2 (New England Nuclear) with a liquid scintillation counter. The specific binding was calculated by subtracting the non-specific binding of [^{3}H]estradiol in the presence of 200-fold molar excess of unlabeled estradiol from the total binding.

Antimicrobial Activity

Antimicrobial activity was determined by a serial broth dilution method in Nutrient Broth (Kyokuto Seiyaku Kogyo Co., Ltd., Tokyo, Japan) for Gram-positive and Gram-negative bacteria and in Sabouraud Broth (Difco Laboratories) for fungi and yeast. The inoculum was adjusted to 5×10^5 cfu/ml for bacteria and 1×10^6 cfu/ml for fungi and yeast. Minimum inhibitory concentration (MIC) is expressed in terms of μg/ml after 18 hours at 37°C for bacteria and 48~72 hours incubation at 28°C for fungi and yeast.

Cytotoxic Activity

Human lung adenocarcinoma A549 cells, human mammary adenocarcinoma MCF-7 cells, mouse lymphocytic leukemia P388 cells and mouse bone marrow cells were used for the experiments.

Unless otherwise indicated, complete medium comprised DULBECCO's modified EAGLE's medium (DMEM) (Flow Laboratories, Rockville, MD, U.S.A.) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY, U.S.A.), 50 U/ml benzylpenicillin and 50 μg/ml streptomycin.

A549 cells and MCF-7 cells were maintained *in vitro* by serial passage in each medium. DMEM complete medium was used for A549 cells and DMEM complete medium supplemented with 4 μg/ml insulin and 10^{-8} M 17β-estradiol for MCF-7 cells. P388 were maintained *in vivo* by serial passage in DBA/2 mice intraperitoneally once a week. P388 cells obtained from the tumor-bearing mice were suspended and cultured in DMEM complete medium supplemented with 5×10^{-5} M 2-mercaptoethanol. Bone marrow cells were obtained from femurs of mice, filtered though nylon mesh and cultured in DMEM complete medium supplemented with 5×10^{-5} M 2-mercaptoethanol and 10% L-929 cell's supernatant as colony-stimulating factors (L-929 cells were maintained in DMEM complete medium). All cell lines were cultured at 37°C in a humidified atmosphere of 5% CO_2-95% air.

In vitro cytotoxic activity was tested in 96-well microtiter plates, with each well containing 2×10^3 A549 cells, 4×10^3 MCF-7 cells, 2×10^4 P388 cells or 1×10^5 bone marrow cells in 100 μl of medium. The cells were incubated at 37°C for 4 days and growth was measured by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay described by MOSMANN[12]. Briefly, MTT was dissolved in DULBECCO's phosphate buffered saline (pH 7.4) at 5 mg/ml and filtered to sterilize and remove a small amount of insoluble residue. On the fourth day of the culture, the MTT solution (10 μl per 100 μl medium) was added to all wells, and the plates were further incubated at 37°C for 4 hours. After removal of the medium from all wells, 2-propanol was added and mixed thoroughly to dissolve the dark blue crystals. After all crystals were dissolved, the plates were measured on a two-wavelength microplate photometer (Model MTP-100; Corona Electric Co., Ltd., Ibaraki, Japan) at 550 nm with a reference wavelength at 660 nm.

Results

Taxonomic Studies on Strain No. 1128

Strain No. 1128 was isolated from a soil sample collected at Iwaki City, Fukushima Prefecture, Japan.

The substrate mycelium was well developed and irregularly branched but not fragmented. The aerial hyphae on the substrate mycelium were straight and sometimes branched. Most of the aerial mycelia were fragmented and formed spore chains with 10 to 50 spores per chain. The spores had smooth surface and were cylindrical with a size of 0.4~0.6 × 1.3~1.9 μm. Sporangia, sclerotia and zoo-spores were not observed (Fig. 2).

The cultural properties of strain No. 1128 are summarized in Table 1. Growth of substrate mycelium was very flat and often penetrative into agar medium. The color of substrate mycelium was dark violet to blackish blue on many media used. The formation of aerial mycelium was not observed on most of media used, but was slight on yeast extract - malt extract agar, BENNETT's agar and potato - dextrose agar. The color of aerial mycelium was white. Melanoid pigments were not produced. Pale violet soluble pigments were produced on BENNETT's agar and potato - dextrose agar. The pigments in mycelium and medium were not pH sensitive.

The physiological properties of strain No. 1128 are summarized in Table 2.

Utilization of carbon sources by strain No. 1128 is shown in Table 3.

Cell wall hydrolysates of strain No. 1128 contained L,L-diaminopimelic acid, glycine and galactose with a trace of D,L-diaminopimelic acid. Whole cells contained galactose and arabinose, phospholipid type was type PII and the acyl type of cell wall was acetyl type.

Cell wall type and whole cell sugar pattern of strain No. 1128 were not identical to any known type, but the cell wall type was considered to be related to type I due to the predominant presence of

Table 1. Cultural properties of strain No. 1128.

Medium	Cultural properties	Medium	Cultural properties
Yeast extract - malt extract agar	G: Moderate, flat A: Thin, white R: Blackish blue (20F6) S: None	Tyrosine agar	G: Moderate, flat A: None R: Yellowish white (3A2) S: None
Oatmeal agar	G: Moderate, flat A: None R: Dull violet (16D3) S: None	BENNETT's agar	G: Moderate, flat A: Thin, white R: Blackish blue (19F5) S: Pale violet
Glycerin - asparagine agar	G: Moderate, flat A: None R: Yellowish white (3A2) S: None	Sucrose - nitrate agar	G: Poor, flat A: None R: Dark blue (21F4) S: None
Peptone - yeast extract - iron agar	G: Moderate, flat A: None R: Pale yellow (3A3) S: None	Potato - dextrose agar	G: Moderate, flat A: Thin, white to lilac gray (16B2) R: Dark violet (16F5) S: Pale violet

G: growth, A: aerial mycelium, R: reverse side color, S: soluble pigment.

Fig. 2. Scanning electron micrograph of spore chains of strain No. 1128 on potato - dextrose agar.

Bar represents 5 μm.

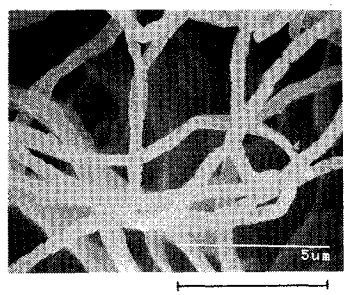


Table 2. Physiological properties of strain No. 1128.

Conditions	Characteristics
Temperature range for growth	12~34°C
Optimum temperature	30°C
Gelatin liquefaction	Weakly positive
Milk coagulation	Positive
Milk peptonization	Positive
Cellulose decomposition	Negative
Starch hydrolysis	Negative
Production of melanoid pigment	Negative
H_2S production	Negative

L,L-diaminopimelic acid.

From these taxonomic studies, it is concluded that strain No. 1128 belongs to the genus *Streptomyces*. Therefore, this strain is designated *Streptomyces* sp. No. 1128.

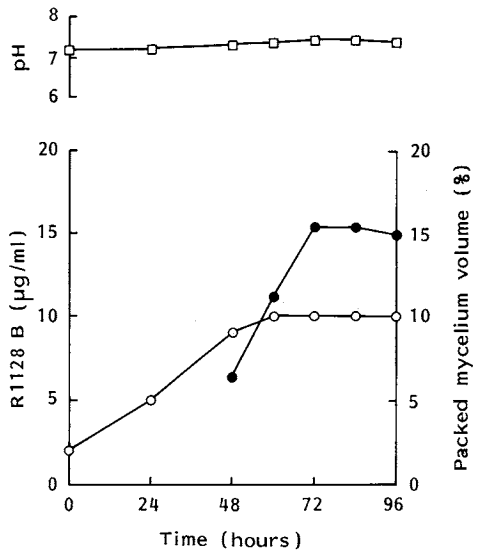
The strain has been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, under the accession number FERM BP-2398.

Fermentation

Fig. 3 presents the data from a typical example of the time course of 30-liter jar fermentation. The pH of the culture maintained steadily from a starting value of 7.2 through the course of the fermentation. The amount of R1128 B, the most abundant component, in the culture broth reached about 15 μg/ml at 72 hours and was maintained up to 96 hours.

Fig. 3. Time course of R1128 B production in a 30-liter jar fermentor.

□ pH, ○ packed mycelium volume, ● potency.



Isolation

The isolation scheme is shown in Fig. 4.

The cultured broth from 4 jar fermentors was filtered with the aid of diatomaceous earth (4 kg). The filtrate (60 liters) was adjusted to pH 4.0 with 6 N HCl and extracted with 60 liters of EtOAc. The extraction procedure was carried out twice and the extracts were combined. Additionally, the mycelial cake was extracted with 60 liters of acetone. The acetone extract was concentrated under reduced pressure to give 5 liters of an aqueous solution, adjusted to pH 4.0 with 6 N HCl and extracted with 5 liters of EtOAc. The extraction procedure was carried out twice and the extracts were combined. The EtOAc extracts from filtrate and mycelial cake were combined and concentrated under reduced pressure to give one liter of EtOAc solution. After dehydration with Na_2SO_4, the EtOAc solution was further concentrated under reduced pressure and the oily residue was mixed with 150 ml of Silica gel 60 (70~230 mesh, E. Merck) and slurried in EtOAc. After evaporating the solvent, the resulting dry powder was added to the top of a silica gel column (450 ml) which was pre-packed with *n*-hexane. The column was developed with *n*-hexane (1 liter), *n*-hexane - EtOAc (9 : 1, 1 liter), *n*-hexane - EtOAc (4 : 1, 1 liter) and *n*-hexane - EtOAc (2 : 1, 1 liter). R1128 substances were eluted with *n*-hexane - EtOAc (4 : 1) and *n*-hexane - EtOAc (2 : 1). The fractions containing R1128 substances were combined and concentrated under reduced pressure to give an oily residue. The oily residue was dissolved in 30 ml of a mixed solution of *n*-hexane - acetone (4 : 1) and added to the top of a silica gel column (500 ml) packed with the same solvent system. The column was washed with 1,700 ml of *n*-hexane - acetone (4 : 1) and then eluted with 800 ml of the same solvent. The fractions containing the objective compounds were combined and concentrated under reduced pressure to give an oily residue. This oily residue was mixed with 50 ml of YMC gel ODS-A60 (60/200 mesh, YMC Co., Ltd., Kyoto, Japan) and slurried in MeOH. After evaporating the solvent, the resulting dry powder was added to the top of ODS column (450 ml) which was pre-packed with 80% aqueous MeOH. After washing of

Fig. 4. Isolation procedure for R1128 substances.

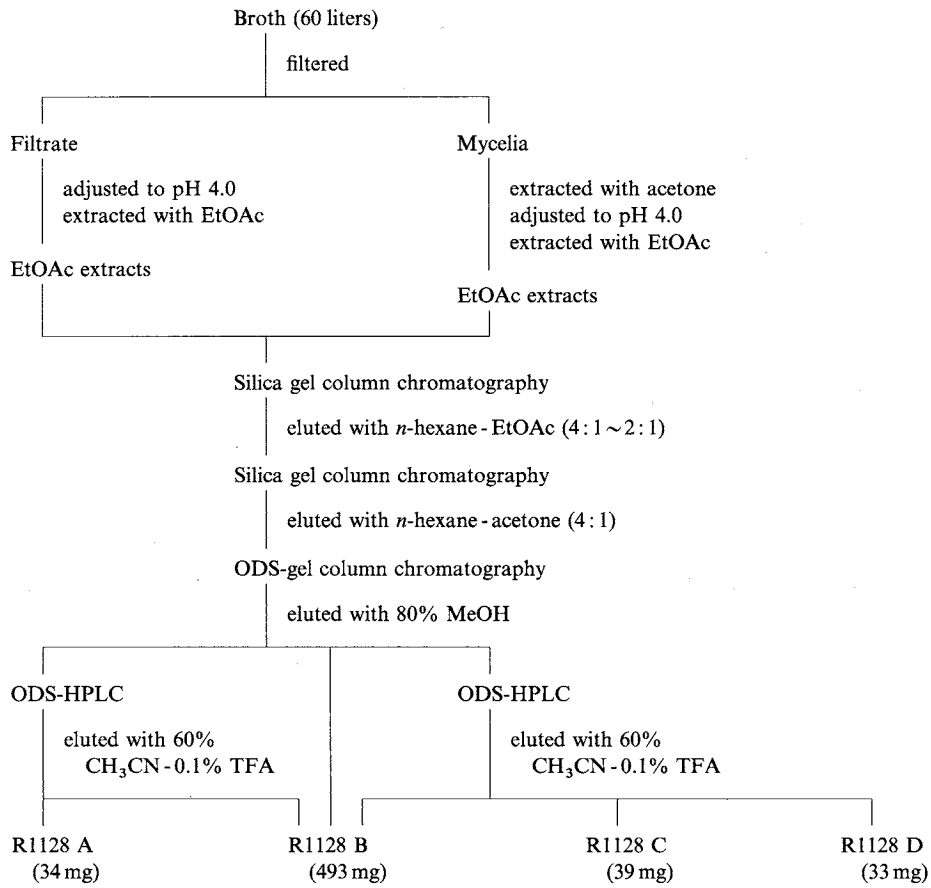


Table 3. Carbon utilization of strain No. 1128.

Compounds	Growth
D-Glucose	+
Sucrose	−
D-Xylose	−
D-Fructose	+
L-Rhamnose	+
Raffinose	−
L-Arabinose	−
myo-Inositol	−
Mannitol	−

−: No growth, +: good growth.

Table 4. Inhibition of estrogen-receptor binding by R1128 substances and tamoxifen.

Drugs	IC_{50} (M)
R1128 A	1.1×10^{-7}
R1128 B	1.2×10^{-7}
R1128 C	2.6×10^{-7}
R1128 D	2.7×10^{-7}
Tamoxifen	1.6×10^{-8}

the column with 350 ml of 80% aqueous MeOH, the mixture of R1128 A and B was eluted with 850 ml of 80% aqueous MeOH, then R1128 B was eluted with 600 ml of 80% aqueous MeOH and finally the mixture of R1128 B, C and D was eluted with 1,600 ml of 80% aqueous MeOH. The mixture of R1128 A and B and the mixture of R1128 B, C and D were effectively separated from each other by preparative HPLC using an YMC gel pre-packed column (D-ODS-15-B, S-15 120A ODS, 30 × 250 mm, YMC Co., Ltd.) with 60% aqueous CH_3CN containing 0.1% TFA at a flow rate of 9.9 ml/minute. The retention times of R1128 A, B, C and D were 8, 11, 15

Table 5. Antimicrobial activities of R1128 substances.

Test strains	MIC (μg/ml)			
	R1128 A	R1128 B	R1128 C	R1128 D
Staphylococcus aureus 209P JC-1	10	10	3	3
Bacillus subtilis ATCC 6633	10	10	10	10
Escherichia coli NIHJ JC-2	>100	>100	>100	>100
Candida albicans	>100	>100	>100	>100
Aspergillus niger	>100	>100	>100	>100

and 16 minutes, respectively. From 60 liters of the cultured broth of *Streptomyces* sp. No. 1128, 34 mg of R1128 A, 493 mg of R1128 B, 39 mg of R1128 C and 33 mg of R1128 D were obtained, all as orange powders.

Table 6. Cytotoxic activities of R1128 substances.

Test cells	IC_{50} (μg/ml)			
	R1128 A	R1128 B	R1128 C	R1128 D
A549	18.0	9.5	6.2	8.3
MCF-7	2.6	5.1	4.4	5.7
P388	1.0	3.8	2.6	4.8
BM	17	15	10	10

BM: bone marrow.

Biological Properties

The assay of estrogen-receptor binding was examined using rat uterine cytosol. R1128 substances were potent estrogen-receptor binding inhibitors. The IC_{50} values of R1128 A, B, C and D were 1.1×10^{-7} M, 1.2×10^{-7} M, 2.6×10^{-7} M and 2.7×10^{-7} M, respectively (Table 4). The inhibitory activities of R1128 A, B, C and D were less potent than that of tamoxifen used as the positive control.

Antimicrobial activities of R1128 substances were evaluated by a serial broth dilution method. R1128 A, B, C and D exhibited weak antimicrobial activities against *Bacillus subtilis* and *Staphylococcus aureus*, but were not effective against *Escherichia coli*, *Candida albicans* and *Aspergillus niger* at 100 μg/ml (Table 5).

Cytotoxic activities of R1128 substances by MTT assay are shown in Table 6. R1128 A, B, C and D had weak cytotoxic activities against human lung adenocarcinoma A549 cells, human mammary adenocarcinoma MCF-7 cells, mouse lymphocytic leukemia P388 cells and mouse bone marrow cells. Tamoxifen also showed weak cytotoxic activity activity against MCF-7 cells (IC_{50}: 3,5 μg/ml).

Acute toxicities of R1128 B were examined in both BDF_1 mice (female, 7 weeks old) by a single intraperitoneal injection and Sprague-Dawley rats (female, 3 weeks old) by a single subcutaneous injection. R1128 B had very low toxicity; neither the mice nor the rats died after the injections of 500 mg/kg of R1128 B.

Discussion

A large number of enzyme inhibitors of microbial origin have been isolated as pharmacological agents. Recently, several receptor-binding inhibitors have been also discovered from microbial products[13~16].

We have screened for new compounds of microbial origin which have inhibitory activities against estrogen-receptor binding. As a result of the screening, we isolated novel non-steroidal estrogen-receptor binding inhibitors, R1128 A, B, C and D, from the cultured broth of *Streptomyces* sp. No. 1128. They were isolated as orange powders which were soluble in organic solvents and were found to have novel structures as shown in Fig. 1. Details on the physico-chemical properties and structure elucidation are reported in the following paper[2].

R1128 substances showed potent inhibitory activities against estrogen-receptor binding. In addition, R1128 substances had weak antimicrobial activities and weak *in vitro* cytotoxic activities. R1128 B also

showed very low *in vivo* toxicity. Thus, R1128 substances were expected to be potent, specific and very low toxic estrogen-receptor binding inhibitors.

Although several estrogen-receptor binding inhibitors of microbial origin have been isolated, all of them were estrogen-receptor agonists[17~19]. In this paper, we described the inhibitory activities of R1128 A, B, C and D against estrogen-receptor binding. Based on the experiments measuring the colony formation of estrogen-responsive human mammary adenocarcinoma MCF-7 cells in soft agar, it was concluded that R1128 substances were estrogen-receptor antagonists. Detailed studies on the pharmacological properties including the experiments mentioned above and antitumor activities of R1128 substances are reported in the following paper[3].

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