BE-14348 SUBSTANCES, NEW SPECIFIC ESTROGEN-RECEPTOR BINDING INHIBITORS

PRODUCTION, ISOLATION, STRUCTURE DETERMINATION AND BIOLOGICAL PROPERTIES

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Streptomyces graminofaciens BA14348, isolated from a soil sample, was found to produce new specific inhibitors of estrogen binding to its receptor. Five related substances, BE-14348A ~ E, were isolated, and their structures were determined by analyses of spectral properties. Of these substances, A was identical with the known flavanone, naringenin. On the other hand, B, C, D and E were all new compounds; the structure of B was determined to be 2(*S*) : 3(*S*)-3-methyl-4′,5,7-trihydroxyflavanone, C was a racemic mixture of 2(*S*): 3 (*R*) and 2(*R*) : 3(*S*)-3-methyl-4′,5,7-trihydroxyflavanone; D and E were 8-chloro derivatives of B and C, respectively.

Screening of specific hormone-receptor binding inhibitors is one method for finding new antagonists or agonists to be used for the treatment of hormone responsive cancer and other diseases. In the case of estrogen, its antagonists have been clinically used for hormone therapy of breast cancer whose growth is stimulated by estrogen[1]. On the other hand, estrogen agonists can be used for the treatment of estrogen deficient syndromes and prostate cancer[2].

In the course of screening for estrogen-receptor binding inhibitors using a cell-free receptor binding assay, we discovered new flavanones with specific binding-inhibitory activity. They were isolated from the culture broth and mycelium of *Streptomyces graminofaciens* BA14348. In this paper, we describe the production, isolation, structure and biological activities of the inhibitors.

Materials and Methods

Taxonomic Studies

The producing organism, strain BA14348, was isolated from a soil sample collected at Tannan city, Hyogo Prefecture, Japan. The taxonomic studies were carried out by the method of The International Streptomyces Project (ISP)[3], along with several supplementary tests. Cultures were observed after incubation at 27°C for 2 weeks. The utilization of carbon sources was tested by growth on PRIDHAM and GOTTLIEB's medium containing a 1% carbon source at 27°C. The chemical composition of the cell wall was analyzed by the methods BECKER *et al.*[4].

Production of BE-14348 Substances

S. graminofaciens BA14348 was inoculated into 110 ml of a production medium consisting of glucose 0.1%, dextrin 2.0%, corn gluten meal 1.0%, fish meal 0.5%, yeast extract 0.1%, NaCl 0.1%, $MgSO_4 \cdot 7H_2O$

0.05%, $CaCl_2 \cdot 2H_2O$ 0.05%, 3-(*N*-morpholino)propanesulfonic acid (MOPS) 0.5%, $FeSO_4 \cdot 7H_2O$ 0.0002%, $CuCl_2 \cdot 2H_2O$ 0.00004%, $MnCl_2 \cdot 4H_2O$ 0.00004%, $CoCl_2$ 0.00004%, $ZnSO_4 \cdot 7H_2O$ 0.00008%, $Na_2B_4O_7 \cdot 10H_2O$ 0.00008% and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 0.00024% (adjusted to pH 6.8 before sterilization) in a 500-ml Erlenmeyer flask, and cultured at 28°C for 3 days on a rotary shaker (180 rpm). Two ml of the above seed culture was transferred to 110 ml of the same medium in a 500-ml Erlenmeyer flask and cultured for 4 days under the same condition.

Isolation of BE-14348 Substances

BE-14348 substances were produced in both the mycelium and the broth filtrate. The culture broth (10 liters) was filtered and the filtrate was adsorbed on a Diaion HP-20 column (1 liter), which was washed with water and eluted with 3 liters of acetone-H_2O (4:1). The mycelial cake was extracted twice with MeOH (3 liters × 2) and the MeOH extract was concentrated *in vacuo*. The residue was adsorbed on a Diaion HP-20 column (200 ml) and eluted with 1.2 liters of acetone-H_2O (4:1). The active eluates from both columns were combined and concentrated *in vacuo*. The residue (800 ml) was extracted with *n*-hexane (300 ml × 2) to remove nonpolar impurities. BE-14348 substances in the aqueous layer were extracted with EtOAc (400 ml × 2). The EtOAc extract was concentrated *in vacuo* to give an oily residue. This was applied to a silica gel column (42 × 2.2 cm i.d.) and eluted with $CHCl_3$-EtOAc (4:1) to give the BE-14348 mixture. The BE-14348 mixture was then subjected to preparative reversed-phase HPLC on an Inertsil ODS (250 × 7.6 mm i.d., Gasukuro Kogyo) using MeCN-H_2O-AcOH (450:550:2) as a mobile phase at a flow rate of 2.0 ml/minute. The separation was monitored *via* UV at 288 nm. The peak fractions of BE-14348A ~ E were collected and concentrated *in vacuo* to give pure BE-14348A ~ E (hereinafter call A ~ E), respectively.

Interconversion of B to Its Stereoisomers

Sixteen mg of B was dissolved in pyridine (1.5 ml) and H_2O (13.5 ml) and heated at 75°C for 2 hours. After solvent removal *in vacuo*, the residue was subjected to preparative HPLC on an Inertsil ODS, and racemics B and C were obtained. For the optical resolution of the racemates, preparative HPLC was carried out using a Chiralcel OC (250 × 4.6 mm i.d., Daicel Chemical Ind., Ltd.) using *n*-hexane-2-PrOH (75:25) for the separation of B and its enantiomer, and *n*-hexane-2-PrOH (65:35) for the separation of racemic C.

Hormone Receptors

Cytosol from hog uterus cells was used as estrogen and progesteron receptors[5]. Uteri taken from hogs were frozen within 2 hours after sacrifice and kept at −80°C. The frozen tissue was cut into about 1 cm cubes with bone scissors and homogenized in a TED buffer consisting of 10 mM Tris-HCl (pH 7.4), 1 mM EDTA and 1 mM dithiothreitol (DTT) with a Waring blender and a Polytrone homogenizer. Cytosol was obtained by centrifuging the homogenate at 100,000 × *g* for 1 hour, and stocked at −80°C. The protein concentration of the cytosol was determined using a BioRad Protein Assay Kit.

Determination of Inhibitory Effect on Estrogen Binding in Cytosol

Estrogen is bound to the receptor in TED buffer. An aliquot of the cytosol (0.1 mg as protein) and 5 μl of a test sample were placed in wells of 96-well test plates and incubated with 3 nM of [^{125}I] 17β-estradiol (Ohtsuka Assay Co.) in a final volume of 0.1 ml at 4°C for 2 hours. At the end of the incubation period 0.2 ml of dextran-coated charcoal (DCC, 0.01% dextran T70 and 1% NoritA) was added to the incubation mixture and left at 4°C for 20 minutes. Then the mixture was centrifuged at 2,000 rpm for 10 minutes and the radioactivity in the supernatant was counted with a γ counter. The specific binding was calculated by subtracting the non-specific binding estimated in the presence of 1,000-fold molar excess of unlabeled diethylstilbestrol from the total binding.

Determination of Inhibitory Effect of Progesterone Binding in Cytosol

Assay procedures were almost the same as those for estrogen binding. Cytosol concentration was 2 mg protein/ml and [^{3}H]promegestone (New England Nuclear) was 4 nM. Cold promegestone (R5020) or medroxyprogesterone acetate was employed to measure the non-specific binding.

Effect of BE-14348B and Its Stereoisomers on MCF-7 Cell Proliferation

An estrogen-responsive cell line of human breast carcinoma MCF-7 cells was grown in plastic flasks in phenol red-free DULBECCO's modified minimum essential medium supplemented with L-glutamate, nonessential amino acids and 10% dextran-coated charcoal-treated fetal calf serum[6]. Cells were maintained in a humidified atmosphere of 95% air-5% CO_2 at 37°C.

The cells harvested from the plastic flasks with trypsin-EDTA solution were suspended in the medium described above at a density of 4×10^4 cells/ml, and 0.25 ml of the suspension was plated in each well of 48-well test plates. Then, a total of 0.25 ml of the medium containing various concentrations of B and its stereoisomers and 1 nM of 17β-estradiol was added. After 4 days incubation, the cell number was estimated with a Coulter Counter.

Results and Discussion

Taxonomic Studies

Microscopic studies showed that aerial mycelia were formed from the substrate mycelia grown in various agar media. Mature spore-chains were *Rectinaculiaperti*, and consisted of less than 20 spores per chain. Most of the spores were cylindrical ($0.8 \sim 1.2 \times 0.8 \sim 1.6\ \mu m$) and possessed a rugose

Fig. 1. Scanning electron micrograph of the spores of strain BA14348.

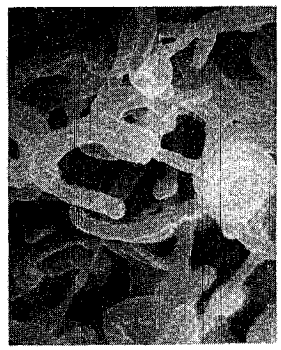


Table 1. Cultural characteristics of strain BA14348.

Agar medium	Growth	Aerial mycelium	Reverse	Soluble pigment
Yeast extract-malt extract agar (ISP-2)	Good spreading	Scant (light gray)	Pale yellow orange ~colorless	Yellow (faint)
Oatmeal agar (ISP-3)	Good spreading	Scant (light gray)	Pale yellow orange ~colorless	None
Inorganic salts-starch agar (ISP-4)	Good	Scant (light gray)	Pale yellow orange ~colorless	None
Peptone-yeast extract-iron agar (ISP-6)	Moderate spreading	None	Pale yellow orange ~colorless	None
Tyrosine agar (ISP-7)	Good spreading	Scant (light gray)	Pale brown	None
Sucrose-nitrate agar	Moderate spreading	None	Pale grayish white ~colorless	None
Water agar	Very poor	None	Colorless	None

Table 2. Physiological characteristics of strain BA14348.

Temperature range for growth	12~37°C
Optimum temperature for growth	20~28°C
Liquefaction of gelatin	+
Coagulation of milk	−
Peptonization of milk	+
Hydrolysis of starch	+
Melanoid production	−
NaCl tolerance	2%

+: Positive, −: negative.

Table 3. Utilization of carbon sources by strain BA14348.

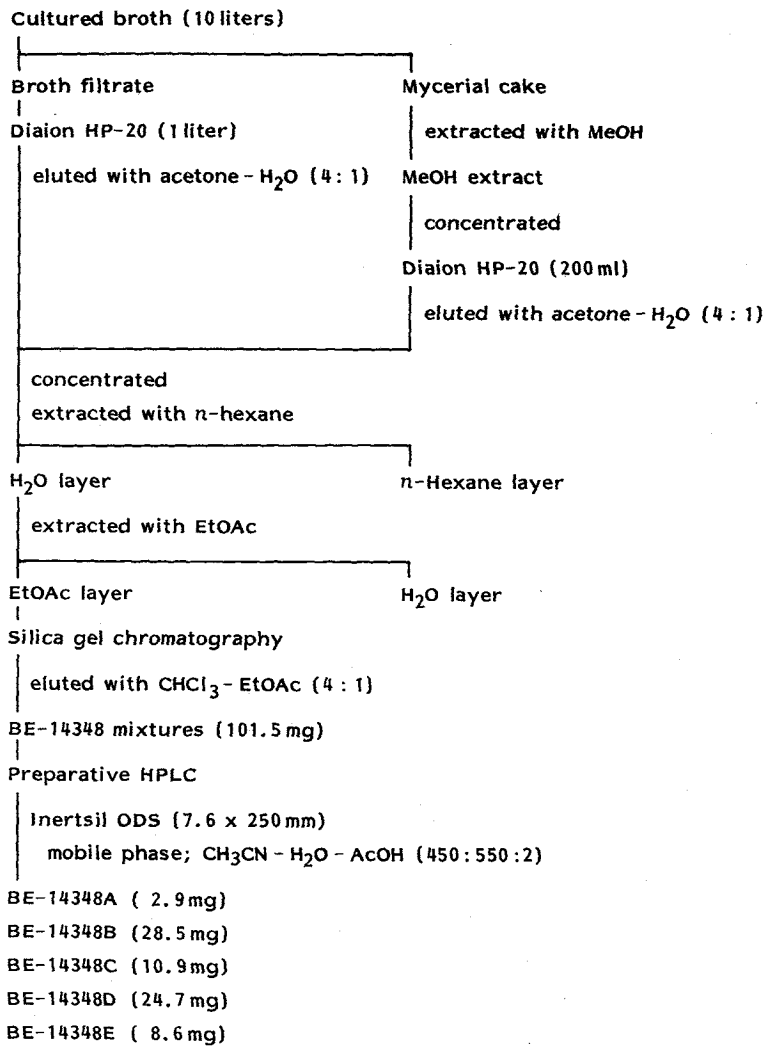
D-Glucose	+
D-Xylose	+
L-Arabinose	+
D-Galactose	+
D-Fructose	+
L-Rhamnose	+
Raffinose	+
D-Mannitol	+
i-Inositol	+
Sucrose	+
Melibiose	+

+: Utilized.

surface (Fig. 1). Typical verticillate aerial mycelia and the other special morphology were not observed. The cultural characteristics and the utilization of carbon sources of strain BA14348 are shown in Tables 1, 2 and 3, respectively. On most media, the aerial mycelia was scant and the aerial mass color was light gray. Melanoid and other soluble pigments were not produced. Hydrolyzed cell wall of strain BA14348 contained LL-diaminopimelic acid and glycine.

Based on the taxonomic properties described above, strain BA14348 was classified in the genus *Streptomyces*. The strain was compared with *Streptomyces* species described in the literature[7,8], and the results showed that strain BA14348 closely resembles *S. graminofaciens*. Then strain BA14348 was directly compared with *S. graminofaciens* JCM4157. It was found that the properties of the two strains were almost identical except for NaCl tolerance (data not shown). Therefore, strain BA14348 was identified as a strain of *S. graminofaciens*. This organism was deposited at the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan under the accession No. FERM P-10491.

Fig. 2. Isolation and purification of BE-14348 substances.



Production and Isolation

The strain of *S. graminofaciens* BA14348 was cultured in Erlenmeyer flasks at 28°C for 4 days. The isolation procedure is outlined in Fig. 2. BE-14348A~E were hardly distinguishable by silica gel column chromatography or TLC, but easily separated by reversed-phase HPLC. The yields of pure A~E from 10 liters of whole broth were 2.9 mg, 28.5 mg, 10.9 mg, 24.7 mg and 8.6 mg, respectively.

Physico-chemical Properties

The physico-chemical properties of A~E are summarized in Table 4. All five compounds are soluble

Table 4. Physico-chemical properties of BE-14348A~E.

	BE-14348A	BE-14348B	BE-14348C	BE-14348D	BE-14348E
Appearance	Pale yellow powder	Pale yellow powder	Pale yellow powder	Pale yellow powder	Pale yellow powder
Molecular formula	$C_{15}H_{12}O_5$	$C_{16}H_{14}O_5$	$C_{16}H_{14}O_5$	$C_{16}H_{13}O_5Cl$	$C_{16}H_{13}O_5Cl$
$[\alpha]_D^{20}$ (MeOH)	NT	−93.5° (*c* 0.99)	+1.22°[c] (*c* 0.98)	−117.7° (*c* 0.99)	+1.7°[c] (*c* 0.93)
HRFAB-MS (positive)					
Calcd:	273.0763	287.0919	287.0919	321.0530	321.0530
Found:	273.0777	287.0948	287.0937	321.0508	321.0558
UV $\lambda_{max}^{MeOH-HCl}$ nm ($E_{1cm}^{1\%}$)	225 (760), 287 (530), 330 (sh, 120)	226 (764), 286 (546), 330 (sh, 103)	225 (801), 286 (560), 330 (sh, 106)	226 (682), 287 (417), 330 (sh, 96)	225 (714), 287 (407), 330 (sh, 93)
$\lambda_{max}^{MeOH-NaOH}$ nm ($E_{1cm}^{1\%}$)	245 (499), 323 (689)	245 (561), 323 (806)	245 (607), 323 (852)	245 (505), 325 (628)	245 (489), 325 (601)
TLC (Rf)[a]	0.15	0.25	0.25	0.31	0.31
HPLC (Rt, minutes)[b]	9.68	12.47	13.79	15.63	16.70

[a] Silica gel $60F_{254}$ plate (Merck). Solvent system: $CHCl_3$-MeOH-AcOH (50:1:1).
[b] Column: Inertsil ODS (250×4.6 mm i.d.), mobile phase, CH_3CN-H_2O-AcOH (450:550:2); flow rate, 1 ml/minute; detection, UV 288 nm.
[c] BE-14348C and E were racemic mixture.
NT: Not tested.

Table 5. ^1H NMR data for BE-14348A~E in acetone-d_6[a].

Proton	BE-14348A	BE-14348B	BE-14348C	BE-14348D	BE-14348E
3-CH_3	—	0.96 (3H, d, *J*=7.4)	0.95 (3H, d, *J*=6.9)	0.99 (3H, d, *J*=7.4)	1.00 (3H, d, *J*=7.0)
3-H	2.74 (1H, dd, *J*=3.0, 17.1), 3.19 (1H, dd, *J*=12.9, 17.1)	2.74 (1H, dq, *J*=3.1, 7.4)	3.15 (1H, dq, *J*=12.0, 6.9)	2.90 (1H, dq, *J*=3.2, 7.4)	3.24 (1H, dq, *J*=12.0, 7.0)
2-H	5.46 (1H, dd, *J*=3.0, 12.9)	5.58 (1H, d, *J*=3.1)	5.05 (1H, d, *J*=12.0)	5.73 (1H, d, *J*=3.2)	5.19 (1H, d, *J*=12.0)
6-H	5.95 (1H, d, *J*=2.2)	5.98 (1H, d, *J*=1.6)	5.92 (1H, d, *J*=2.2)	6.19 (1H, s)	6.15 (1H, s)
8-H	5.96 (1H, d, *J*=2.2)	6.04 (1H, d, *J*=1.6)	5.96 (1H, d, *J*=2.2)	—	—
3′,5′-H	6.90 (2H, d, *J*=8.6)	6.88 (2H, d, *J*=8.4)	6.92 (2H, d, *J*=8.6)	6.93 (2H, d, *J*=8.6)	6.93 (2H, d, *J*=8.6)
2′,6′-H	7.41 (2H, d, *J*=8.6)	7.33 (2H, d, *J*=8.4)	7.40 (2H, d, *J*=8.6)	7.40 (2H, d, *J*=8.6)	7.42 (2H, d, *J*=8.6)
4′,7′-OH[b]	8.50 (1H, br s), 9.55 (1H, br s)	8.45 (1H, br s), 9.60 (1H, br s)	8.60 (1H, br s), 9.60 (1H, br s)	8.2~8.8 (2H, br)	8.2~9.8 (2H, br)
5-OH	12.18 (1H, s)	12.18 (1H, s)	12.28 (1H, s)	12.12 (1H, s)	12.23 (1H, s)

[a] 300 MHz. Chemical shifts in ppm, coupling constants in Hz.
[b] The chemical shifts were varied by sample concentration.

Table 6. ^{13}C NMR data for BE-14348A~E in acetone-d_6[a].

Carbon	BE-14348A	BE-14348B	BE-14348C	BE-14348D	BE-14348E
C-2	79.7 (d)	81.7 (d)	85.6 (d)	82.3 (d)	86.2 (d)
C-3	43.5 (t)	46.3 (d)	45.5 (d)	45.8 (d)	45.3 (d)
C-4	197.1 (s)	201.6 (s)	199.5 (s)	201.6 (s)	199.7 (s)
C-4a	103.2 (s)	101.7 (s)	102.5 (s)	102.2 (s)	102.9 (s)
C-5	165.2 (s)	165.7 (s)	165.3 (s)	163.3 (s)	162.9 (s)
C-6	96.8 (d)	97.1 (d)	96.8 (d)	97.4 (d)	97.1 (d)
C-7	167.2 (s)	167.2 (s)	167.3 (s)	162.6 (s)	162.6 (s)
C-8	95.8 (d)	95.7 (d)	95.6 (d)	100.0 (s)	99.8 (s)
C-8a	164.3 (s)	163.8 (s)	164.1 (s)	158.6 (s)	159.0 (s)
C-1′	130.7 (s)	129.0 (s)	130.0 (s)	128.5 (s)	129.5 (s)
C-2′,6′	128.9 (d)	128.1 (d)	129.9 (d)	128.0 (d)	130.0 (d)
C-3′,5′	116.1 (d)	116.0 (d)	116.2 (d)	116.1 (d)	116.2 (d)
C-4′	158.6 (s)	158.0 (s)	158.8 (s)	158.1 (s)	159.0 (s)
3-CH_3	—	10.3 (q)	10.3 (q)	10.2 (q)	10.3 (q)

[a] 75 MHz. Chemical shifts in ppm.

in methanol, ethanol, acetone and ethyl acetate, but insoluble in water and *n*-hexane. The molecular formula was determined by HRFAB-MS. The ^1H and ^{13}C NMR data of A~E are presented in Tables 5 and 6, respectively.

Structure Elucidation

The physico-chemical properties of A~E are quite similar to each other, so these five compounds seemed to be analogues. The structure of A was suggested to be naringenin from the ^1H and ^{13}C NMR data and characteristic UV spectrum (red shift nature of alkaline solution). By a direct comparison with an authentic sample, A was identified as naringenin (4′,5,7-trihydroxyflavanone). Comparison of the ^1H NMR data of A and B, the 3-methylene proton signals (δ 2.74 and 3.19) of A were converted to a methine proton signal (δ 2.74) and one additional methyl proton (δ 0.96) appeared in B. The data for the other protons of A and B were nearly identical. Similar correlation to that cited above was observed in carbon chemical shifts of A and B. The 3-methylene carbon signal (δ 43.5, t) of A disappeared, and a methine carbon signal (δ 46.3, d) and an additional methyl carbon signal (δ 10.3, q) were observed in B. From these data and the molecular formula of B ($C_{16}H_{14}O_5$), the structure of B was elucidated to be the 3-methyl derivative of A, and which was confirmed by long range selective proton decoupling (LSPD) experiments. The coupling constant (J=3.1 Hz) between 2-H and 3-H of B indicated a *cis* configuration. The CD spectrum of B showed a positive Cotton effect at 325 nm and a negative one at 287 nm, which indicated a 2-*S* configuration[9,10]. Thus, the structure of B was assigned as 2(*S*):3(*S*)-3-methyl-4′,5,7-trihydroxyflavanone.

The molecular formula of C was the same as that of B. Comparison of the ^1H NMR data of B and C showed that the chemical shifts and the coupling constants of 2- and 3-methine protons (δ 5.05 and 3.15, respectively) of C were changed, but the other proton signals were almost identical to each other. From these data and the coupling constant (J=12.0 Hz) between 2-H and 3-H, it was indicated that C was a *trans* isomer of B. The optical resolution of C on a Chiralcel OC revealed that it was a racemic mixture. So, it was determined that C was a racemic mixture of 2(*S*):3(*R*) and 2(*R*):3(*S*)-3-methyl-4′,5,7-trihydroxyflavanone.

HRFAB-MS of D and E showed a characteristic isotope peak due to chlorine. The molecular formu-

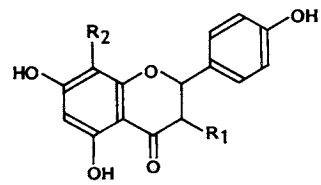
lae of D and E were identical and determined to be $C_{16}H_{13}O_5Cl$. The ^1H and ^{13}C NMR data of D and E were compared to those of B and C. In the ^1H NMR spectra of D and E, the lack of the 8-H proton signal of B and C (δ 6.04 and 5.96, respectively) was observed. In addition, the carbon chemical shifts for C-8 of D and E (δ 100.0 s, and 99.8 s, respectively) were observed at lower fields compared to those of B and C (δ 95.7 d, and 95.6 d, respectively). The chemical shifts for C-7 and C-8a of D (δ 162.6 and 158.6) and E (δ 162.6 and 159.0) were observed at higher fields compared to those of B (δ 167.2 and 163.8) and C (δ 167.3 and 164.1). From these data and hydrogen bonding phenolic OH (δ 12.12) of D and E coupled with C-4a, C-5 and C-6, the structures of D and E were suggested to be 8-chloro derivatives of B and C, respectively, and confirmed by LSPD experiments. Considering the interconversion of B to its stereoisomers, C and E might be artifacts derived from B and D during isolation, respectively. The structures of A~E are shown in Fig. 3.

Almost all 3-substituted flavanones, which have been isolated from natural products or synthesized chemically, have *trans* configurations, and the preparation of *cis*-3-substituted flavanones is difficult since acid epimerization readily gives a mixture of *cis* and *trans* isomers[10,11]. This time, we isolated *cis* 3-methyl and 3-methyl-8-chloro-flavanone as the main products, that may be caused by metabolic differences between microbes and plants.

Interconversion of B to Its Stereoisomers

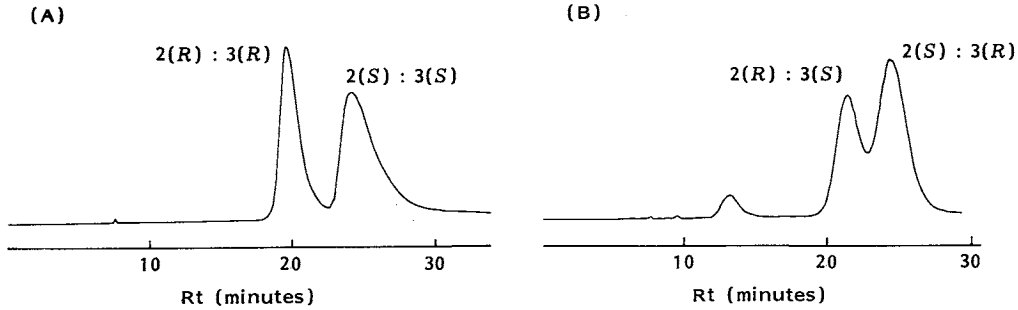
The isomerization of 3-*O*-glycosyl derivatives of 3-hydroxyflavanones proceeds *via* chalcones as intermediates when they are heated in aqueous pyridine[10]. By heating B at 75°C in H_2O-pyridine (9:1) for 2 hours, four isomers of B were produced. The analysis of reversed phase HPLC and optical resolution on a Chiralcel OC revealed that the reaction products comprised 22% of *cis* isomers (B, 13.6% and B enantiomer, 8.4%) and 78% of *trans* isomers (2(*S*):3(*R*), 51.7% and 2(*R*):3(*S*), 26.3%) as shown in Fig. 4. These results suggested that these isomers could be formed by ring opening with chalcone formation and followed by recyclization.

Fig. 3. Structures of BE-14348A~E.



	R_1	R_2	
BE-14348A	H	H	
BE-14348B	CH_3	H	2(*S*):3(*S*)
BE-14348C	CH_3	H	Racemic mixture of 2(*S*):3(*R*) and 2(*R*):3(*S*)
BE-14348D	CH_3	Cl	2(*S*):3(*S*)
BE-14348E	CH_3	Cl	Racemic mixture of 2(*S*):3(*R*) and 2(*R*):3(*S*)

Fig. 4. Optical resolution of racemates of BE-14348B and its stereoisomers.



Column: Chiralcel OC (250 × 4.6 mm i.d.); flow rate, 0.5 ml/minute; detection, UV 288 nm; mobile phase, (A) *n*-hexane-2-PrOH (75:25) and (B) *n*-hexane-2-PrOH (65:35).

Biological Activities

The inhibitory activities of A, B, B stereoisomers (2(*R*):3(*R*), 2(*S*):3(*R*) and 2(*R*):3(*S*)), D, E, flavanone, flavones and isoflavones on estrogen-receptor binding were examined. The IC_{50} values of B stereoisomers 2(*R*):3(*R*), 2(*S*):3(*R*) and 2(*R*):3(*S*) were 10-time, 36-time and 33-time larger than that of B, respectively (Table 7). These data indicated that the absolute configuration of 2-proton and 3-methyl group played a very important role in the inhibition of estrogen-receptor binding. Flavanone is obviously less active than A, suggesting that some hydroxyl groups were also necessary. On the other hand, introduction of chlorine at position 8 markedly reduced the activity. Among flavones and isoflavones, genistein showed activity almost equal to that of B.

Of these BE-14348 compounds, B was the most active estrogen binding inhibitor, so the effect of B on estrogen binding was investigated along with those of tamoxifen and diethylstilbestrol. Tamoxifen is an estrogen antagonist and has been used clinically for the hormone therapy of breast cancer[1]. Diethylstilbestrol is an estrogen agonist and has been used for prostate cancer[2]. Under experimental conditions where the IC_{50} of radioinert estradiol against the binding of radioactive estradiol was 0.7 nM, the IC_{50} of B was 34 nM. B inhibited receptor binding a little more potently than diethylstilbestrol, and much more potently than tamoxifen (Fig. 5).

Therefore, it was determined by measuring the effects on the growth of estrogen-responsive MCF-7 cells whether B and its stereoisomers were antagonists or agonists. As shown in Fig. 6, B and its stereoisomers stimulated the growth of MCF-7 cells dose-dependently as well as estrogen. At a higher concentration the effect was reduced, perhaps due to its non specific cytotoxicity. Based

Table 7. Inhibitory effect of BE-14348 substances and other related compounds on estrogen-receptor binding.

Compound	IC_{50} (nM)
BE-14348A (naringenin)	10,500
BE-14348B 2(*S*):3(*S*)	34
BE-14348B enantiomer 2(*R*):3(*R*)	315
BE-14348B diastereomer 2(*S*):3(*R*)	1,224
BE-14348B diastereomer 2(*R*):3(*S*)	1,136
BE-14348D 2(*S*):3(*S*)	12,600
BE-14348E (racemic mixture)	7,000
Flavanone	136,000
Quercetin	15,700
Apigenin	1,400
Genistein	42
Daidzein	840
Biochanin A	9,800

Fig. 5. Effect of BE-14348B, diethylstilbestrol and tamoxifen on estrogen-receptor binding.
● BE-14348B, ○ 17β-estradiol, △ diethylstilbestrol, ▲ tamoxifen.

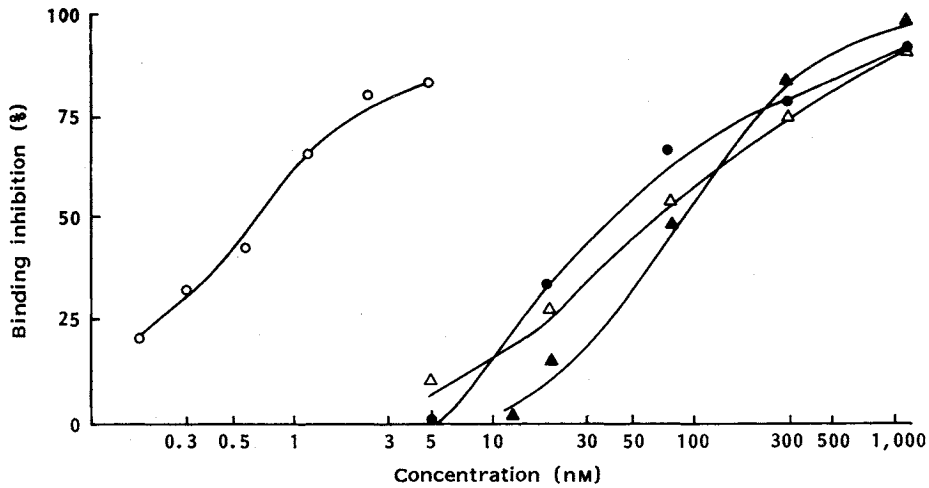
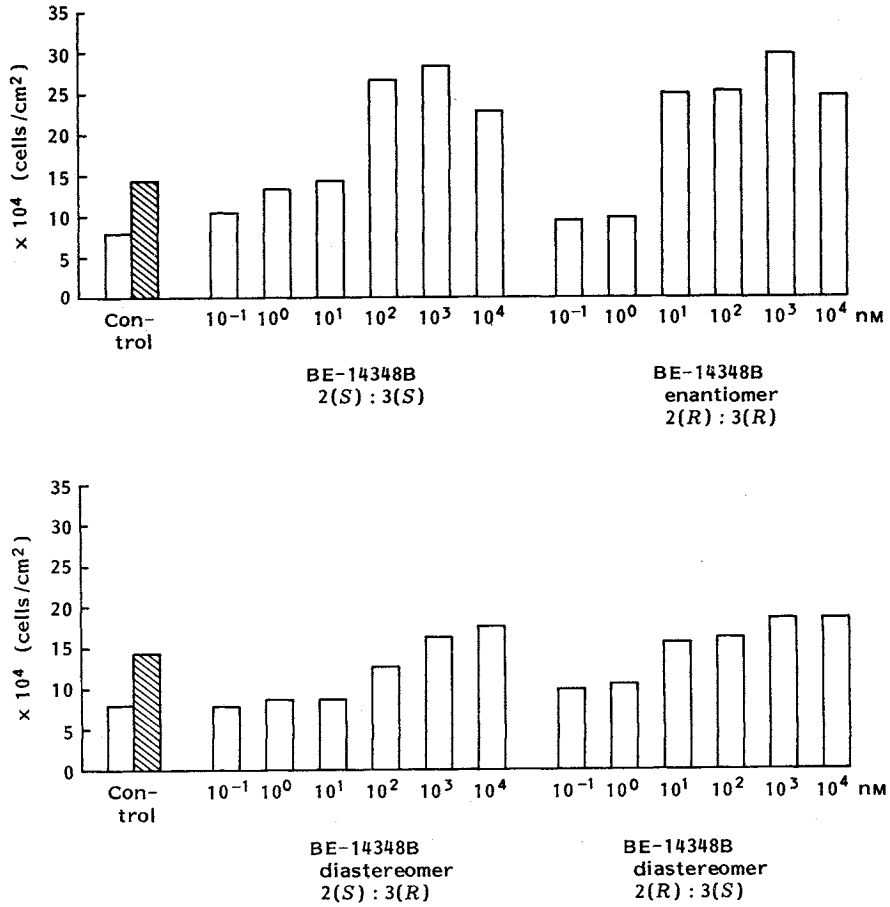


Fig. 6. Agonistic activity of BE-14348B and its stereoisomers in MCF-7 estrogen-responsive cells.



Determined after cultured with (closed bar) or without (open bar) 1 nM 17β-estradiol for 5 days.

Table 8. Biological activities of BE-14348B, diethylstilbestrol and tamoxifen.

Activity	IC_{50} (μM)		
	BE-14348B	Diethylstilbestrol	Tamoxifen
Estrogen-receptor binding	0.034	0.051	0.091
Progesteron-receptor binding	94	4	Not tested
Estrogen-dependent growth of MCF-7	0.001 ~ 10 (agonistic)	0.001 ~ 10 (agonistic)	0.1 ~ 1 (antagonistic)
Cytotoxicity against MCF-7	140	39	9
Cytotoxicity against HeLa	91	5	4
Toxicity in female mice[a]	500 (ip)	445 (iv)	575 (ip)

[a] LD_{50} (mg/kg).

on these experiments, it was concluded that B and its stereoisomers are estrogen agonists[6,12].

The summary of biological activities of B, diethylstilbestrol and tamoxifen is shown in Table 8. The IC_{50} value of B against progesterone-receptor binding was about 3,000-time greater than that against estrogen binding. Cytotoxicity of B against MCF-7 and HeLa cells was very weak. It had low toxicity;

no mice were dead after the intraperitoneal injection of 500 mg/kg.

Since B showed low toxicity and cytotoxicity, the possibility of the use of B as an estrogen agonist has to be studied.

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