Estrogenic and Anti-estrogenic Activities of *Cassia tora* Phenolic Constituents

Ali Mahmoud EL-HALAWANY,[a] Mi Hwa CHUNG,[a] Norio NAKAMURA,[b] Chao-Mei MA,[a] Tsutomu NISHIHARA,[c] and Masao HATTORI*,[a]

[a] *Institute of Natural Medicine, University of Toyama; 2630 Sugitani, Toyama 930–0194, Japan:* [b] *Faculty of Pharmaceutical Sciences, Doshisha Women's College of Liberal Arts; Kodo, Kyotanabe, Kyoto 610–0395, Japan: and* [c] *Faculty of Pharmaceutical Sciences, Hyogo College of Medicine; 1–3–6 Minatojima, Chuo-ku, Kobe 650–0045, Japan.*
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Through an estrogenic activity bioassay-guided fractionation of the 70% ethanolic extract of *Cassia tora* seeds two new phenolic triglucosides, torachrysone 8-*O*-[β-D-glucopyranosyl(1→3)-*O*-β-D-glucopyranosyl(1→6)-*O*-β-D-glucopyranoside] (1) and toralactone 9-*O*-[β-D-glucopyranosyl-(1→3)-*O*-β-D-glucopyranosyl-(1→6)-*O*-β-D-glucopyranoside] (2), along with seven known compounds were isolated. The structures of the new compounds were elucidated on the basis of spectroscopic and chemical evidence. The estrogenic activity of the fractions and the isolated compounds were investigated using the estrogen-dependent proliferation of MCF-7 cells. In addition, the yeast two hybrid assay expressing estrogen receptor α (ERα) and β (ERβ) and the ERα competitor screening assay (ligand binding screen) were used to verify the binding affinities of the isolated compounds to ER. Furthermore, a naringinase pre-treatment of the 70% alcoholic extract of *Cassia tora* seeds resulted in a significant increase in its estrogenic activity. From the naringinase pre-treated extract six compounds were isolated, among which 6-hydroxymusizin and aurantio-obtusin showed the most potent estrogenic activity, while torachrysone, rubrofusarin and toralactone showed a significant anti-estrogenic activity. Finally, the structure requirements responsible for the estrogenic activity of the isolated compounds were studied by investigating the activity of several synthetic compounds and chemically modifying the isolated compounds. The basic nucleus 1,3,8-trihyroxynaphthalene (T_3HN) was found to play a principal role in the binding affinity of these compounds to ER.

Key words *Cassia tora*; 6-hydroxymusizin; 1,3,8-trihyroxynaphthalene; estrogenic activity; rubrofusarin; toralactone

Estrogen is a key regulator of the cellular processes involved in the development and maintenance of the reproductive function.[1] Phytoestrogens are polyphenolic non-steroidal plant compounds with estrogen-like biological activity. Recently, an increasing number of epidemiological and experimental studies has suggested that the consumption of phytoestrogens may have a protective effect on estrogen-related conditions like menopause and estrogen related diseases such as prostate and breast cancers, osteoporosis and cardiovascular diseases.[2] In our search for new phytoestrogens, the ethanolic extract of *Cassia tora* exhibited a significant stimulation of estrogen dependent MCF-7 cells, suggesting its estrogenic activity. *Cassia tora* L. (Fabaceae) is widely distributed in tropical Asian countries and its seeds have been used as a traditional medicine for constipation, asthenia, eye disease, hepatitis, diuretic, hemoglobin disorders and as an antidysenteric.[3] Hypotensive activity of the seed extract has also been reported.[4,5] In India the plant is used for the treatment of snakebites and scorpion stings.[6] The seeds were found to contain a diversity of phenolic constituents, *i.e.* anthraquinones, naphthopyrones, naphthalenes, hydroanthracenes and their glucosides.[7—11] These phenolic constituents were reported to have various biological and pharmacological effects such as antibacterial,[11] antifungal,[12] antimutagenic,[13] hypolipidemic[14] and anti-allergic activities.[11] In addition, the aqueous extract of the seed[15] and naphthopyrones[9] and anthraquinones[10] isolated from the seeds exhibited hepatoprotective activity.

In this study, the isolation of two new phenolic triglucosides from a 70% ethanol extract of *Cassia tora* seeds is reported. Furthermore, a naringinase pretreatment, as a mimic of the metabolic activity of gastro-intestinal flora, was carried out for the ethanolic extract of *Cassia tora*, and the biologically active principles of this extract were also investigated.

Results

The 70% ethanolic extract of *Cassia tora* seeds significantly stimulated the proliferation of estrogen dependent MCF-7 cells indicating its possible estrogenic activity, which is significantly increased after being treated with naringinase (Table 1). The 70% ethanolic extract was fractionated on DIAON HP-20 to obtain 3 fractions (25% methanol, 50% methanol, 100% methanol). Estrogenic activity of these fractions was evaluated using MCF-7 cells before and after naringinase treatment (Table 1). The 50% methanol fraction and the naringinase pre-treated 70% ethanolic extract were

Table 1. Estrogenic Effects of Fractions of *Cassia tora* on the Proliferation of MCF-7 Cells

Fraction	MCF-7 cell proliferation (% of control)	
	1 μg/ml	10 μg/ml
Chloroform-soluble	147.8±12.8*	82.5±2.1
70% EtOH extract	126.8±4.1*	98.6±2.7
100% MeOH eluate	145.3±16.1*	115.2±12.7
50% MeOH eluate	152.5±17.4**	169.3±9.4**
25% MeOH eluate	153.8±1.7**	110.8±10.9
NT 70% EtOH extract	206.8±11.8**	157.6±18.9**
NT 100% MeOH eluate	142.4±7.8*	137.5±11.3*
NT 50% MeOH eluate	86.9±10.6	140.1±14.4*
NT 25% MeOH eluate	118.9±11.3	85.7±7.9

17β-Estradiol showed maximum activity (202.2±3.2%) at a concentration of 10^{-12} M. Each value represents the mean±S.E. (n=6). Asterisks indicate significant difference from the control at $p<0.05$ (*), $p<0.01$ (**). NT, naringinase treated.

* To whom correspondence should be addressed. e-mail: saibo421@inm.u-toyama.ac.jp

selected for further purification due to their relatively high estrogenic activity. The 50% methanol fraction was subjected to intensive chromatographic purifications using normal and reversed phase silica gel and Sephadex LH-20 to get two new compounds (**1**, **2**) along with seven known compounds [aurantio-obtusin 6-*O*-*β*-D-glucoside (**3**),[7] torachrysone 8-*O*-*β*-D-gentiobioside (**4**),[11] toralactone 9-*O*-*β*-D-gentiobioside (**5**),[8] 6-hydroxymusizin 8-*O*-*β*-D-glucoside (**6**),[16] torachrysone tetraglucoside (**7**), rubrofusarin triglucoside (**8**)[11] and chrysophanol triglucoside (**9**)].[9] From the naringinase pretreated total extract six known compounds were isolated and identified as [chrysophanol (**10**), physcion (**11**), 9-methoxychrysophanol (**12**),[17] aurantio-obtusin (**13**),[7] toralactone (**14**)[18] and rubrofusarin (**15**)].[11] In addition, torachrysone (**16**)[19,20] and 6-hydroxymusizin (**17**)[16] were obtained through acid hydrolysis of their corresponding isolated glycosides **6** and **7**. Finally, nor-rubrofusarin (**18**) and nor-toralactone (**19**) were obtained by the demethylation of compounds **14** and **15**. The known compounds were identified by comparison with reported data, and the structures of the new compounds were determined as follows.

Compound **1** was obtained as yellow needles. The electron spray ionization mass (ESI-MS) spectrum showed an $[M+H]^+$ ion peak at *m/z* 733. On the basis of its ESI-MS and elemental analysis data, compound **1** was assigned the molecular formula $C_{32}H_{44}O_{19}$. The UV spectrum showed absorption maxima at 240, 265, 310, 324 and 340 nm, suggesting the torachrysone nucleus.[11,19,20] The ^{1}H-NMR spectrum showed an aromatic singlet at δ_H 7.09 (1H, s, H-4), two *meta*-coupled doublets at δ_H 7.06 (1H, d, J=2.1 Hz, H-7) and 6.89 (1H, d, J=2.1 Hz, H-5), one aromatic methyl at δ_H 2.21 (3H, s, CH_3-3), a methoxyl group at δ_H 3.82 (3H, s, OCH_3) and an acetyl group at δ_H 2.48 (3H, s, $COCH_3$), in addition to the chelated hydroxyl group signal at δ_H 9.46 (1H, s, OH-1). By comparison with the previously reported data, these ^{1}H-NMR data confirmed the torachrysone nucleus of **1**.[11,19,20] Moreover, the presence of three anomeric protons at δ_H 5.09 (1H, d, J=8.0 Hz, H-1′) , 4.32 (1H, d, J=8.0 Hz, H-1″) and 4.30 (1H, d, J=8.0 Hz, H-1‴) indicated the presence of three sugar moieties. The attachment of the sugar moieties to C-8 of the aglycone was confirmed by the heteronuclear multiple bond coherence (HMBC) correlation between the anomeric proton of glucose-I at δ_H 5.09 and C-8 of the aglycone at δ_C 155.2. All the glucose moieties were found to be in the β configuration as indicated by the J values of their anomeric protons (J=8.0 Hz). The ^{13}C-NMR chemical shifts of the sugar moieties, and the comparison of the sugar resulting from the acid hydrolysis of **1** with that of authentic samples, revealed that the three sugar moieties were D-glucose. In the ^{13}C-NMR spectrum, the downfield shifted C-6′ and C-3″ at δ_C 68.8 and at δ_C 88.1, respectively, revealed the presence of 1→6 and 1→3 linkages among the three glucose molecules.[3,11] In the HMBC spectrum, ^{1}H–^{13}C long-range correlations were observed between the anomeric proton at δ_H 4.32 (H-1″) and the carbon at δ_C 68.8 (C-6′), and between the anomeric proton at δ_H 4.30 (H-1‴) and the carbon at δ_C 88.1 (C-3″), which confirmed the 1→6 and 1→3 linkages. From these findings the structure of compound **1** was determined to be torachrysone 8-*O*-[*β*-D-glucopyranosyl(1→3)-*O*-*β*-D-glucopyranosyl(1→6)-*O*-*β*-D-glucopyranoside] (Fig. 1).

Compound **2** was obtained as yellowish amorphous powder. The ESI-MS spectrum showed an $[M+H]^+$ at *m/z* 759. On the basis of its ESI-MS datum and elemental analysis data, compound **2** was assigned the molecular formula $C_{33}H_{42}O_{20}$. The UV spectrum showed absorption maxima at 237, 247, 270, 277 and 383 nm, suggesting the naphtha-*α*-pyrone nucleus of this compound.[3,18] The ^{1}H-NMR spectrum showed two aromatic protons at δ_H 6.50 (1H, s, H-4) and 7.13 (1H, s, H-5), two doublet protons at δ_H 6.86 (1H, d, J=2.2 Hz, H-8) and 6.92 (1H, d, J=2.2 Hz, H-6), aromatic methyl protons at δ_H 2.22 (3H, s, CH_3-3) and a methoxyl proton at δ_H 3.88 (3H, s, OCH_3). These data confirmed the aglycone moiety to be toralactone.[3,18] The three anomeric protons at δ_H 5.09, 4.31 and 4.28 revealed the presence of three sugar moieties. The ^{13}C-NMR chemical shifts of the sugar moieties, and the comparison of the sugar resulting from the acid hydrolysis of **2** with authentic samples, revealed that the three sugar moieties were D-glucose.[3,11] The site of attachment of the three glucose units to the aglycone was found to be at OH-9 as indicated from the HMBC correlation between the proton at δ_H 5.09 (H-1′) and the carbon at δ_C 157.6 (C-9) of the aglycone. Similar to compound **1**, the downfield shifted carbons at δ_C 68.8 and 88.1 corresponding to C-6′ and C-3″, respectively, revealed the presence of 1→6 and 1→3 linkages among the three glucose units.[3] In the HMBC spectrum, long-range correlations were found between the anomeric proton at δ_H 4.31 (H-1″) and the carbon at δ_C 68.8 (C-6′), and between the anomeric proton at δ_H 4.28 (H-1‴) and the carbon at δ_C 88.1 (C-3″) which confirmed the 1→6 and 1→3 linkages. All the glucose moieties were found to be in the β configuration as indicated by the J values of the anomeric protons (J=8 Hz). Based on the formerly mentioned data and by comparison with reported data of rubrofusarin triglucoside and cassiacide C_2,[3,11] compound **2** was determined to be toralactone 9-*O*-[*β*-D-glucopyranosyl-(1→3)-*O*-*β*-D-glucopyranosyl-(1→6)-*O*-*β*-D-glucopyranoside].

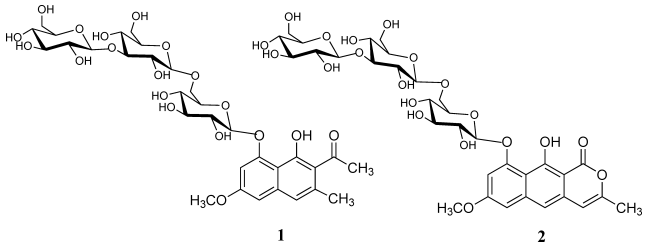

Fig. 1. New Compounds Isolated from *Cassia tora* Seeds

Estrogenic Activity of the Isolated Compounds Using estrogen-dependent MCF-7 cell-proliferation, yeast two hybrid assay expressing ERα and ERβ and the ligand binding screen, almost all the isolated compounds from the 50% methanol fraction and the naringinase-pretreated 70% ethanolic extract of *Cassia tora* were tested for their estrogenic activities at various concentrations. In addition, a group of synthetic compounds (**20**—**24**), torachrysone (**16**), 6-hydroxymusizin (**17**), nor-rubrofusarin (**18**) and nor-toralactone (**19**) (Fig. 2) were also investigated in order to study the structure activity requirements of these compounds.

Estrogen-Dependent MCF-7 Cells The positive control 17β-estradiol showed maximum cell proliferation (212.8% of the control) at a concentration of 10^{-12} M (Table 2). Aurantio-

obtusin 6-*O*-β-D-glucoside (**3**) showed a weak estrogenic activity at 10^{-5} M. The aglycones, aurantio-obtusin (**12**), 6-hydroxymusizin (**17**) and 1,3,8-trihydroxynaphtahlene (**24**) showed significant increase in their estrogenic activity upon decreasing their concentrations with a maximum activity at 10^{-7} M. Nor-rubrofusarin (**18**) and nor-toralactone (**19**) significantly stimulated the MCF-7 cell proliferation in a bell-shaped manner *versus* their concentrations, while 1,6-dihydroxynaphthalene (**23**) showed a significant stimulation of cell proliferation at 10^{-6} M.

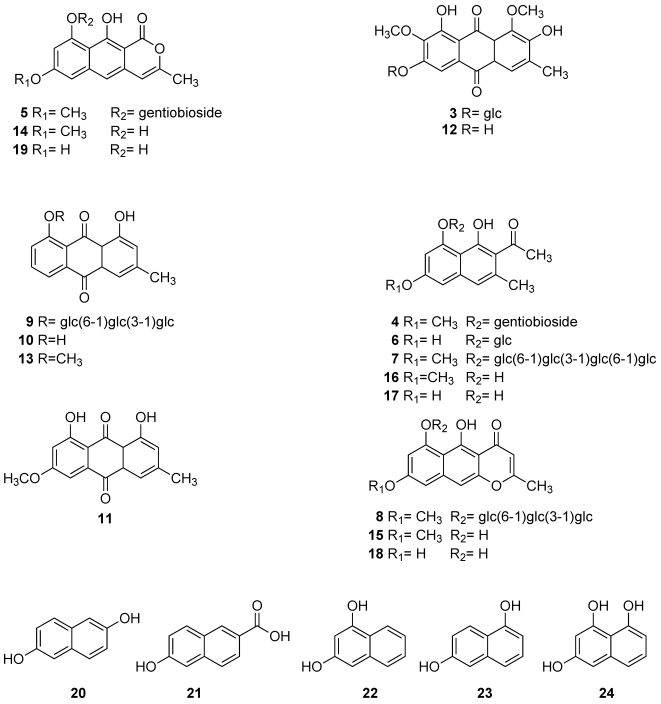

Fig. 2. Chemical Structures of Isolated Compounds (**3**—**15**) from the Seeds of *Cassia tora* and of Synthetic Compounds (**18**—**24**)

Yeast Two-Hybrid Assay In the yeast expressing ERα (Table 3), 6-hydroxymusizin 8-*O*-β-D-glucoside (**6**) showed estrogenic activity at 10^{-4} M. In addition, 6-hydroxymusizin (**17**), nor-rubrofusarin (**18**) and nor-toralactone (**19**) showed a significant estrogenic activity at 10^{-4} M. On the other hand, in the yeast expressing ERβ, 6-hydroxymusizin 8-*O*-β-D-glucoside (**6**) showed estrogenic activity at 10^{-4} M. An appreciable induction of β-galactosidase activity was observed for aurantio-obtusin (**12**), 6-hydroxymusizin (**17**), nor-rubrofusarin (**18**) and nor-toralactone (**19**) at a concentration of 10^{-4} M.

Ligand Binding Screen 6-Hydroxymusizin (**17**), nor-rubrofusarin (**18**), nor-toralactone (**19**) and T_3HN (**24**) showed concentration dependent increases in their binding to ERα with IC_{50} values of 2.5×10^{-4} M, 3.2×10^{-3} M, 3.9×10^{-4} M and 3.9×10^{-4} M, respectively, while physcion (**11**), aurantio-obtusin (**12**), toralactone (**14**), rubrofusarin (**15**), torachrysone (**16**), 2,6-dihydroxynaphthalene (**20**) and 1,6-dihydroxynaphthalene (**23**) showed IC_{50} values higher than 5×10^{-3} M (Table 4).

Anti-estrogenic Activity Using the estrogen-dependent MCF-7 cell proliferation and yeast two-hybrid assays, all the isolated compounds were tested for their anti-estrogenic activities at various concentrations. Inhibition of 17β-estradiol (E_2)-induced proliferation of MCF-7 cells and E_2-induced β-galactosidase activity by the isolated compounds were determined under the conditions at which these compounds did not inhibit the proliferation/growth of the MCF-7 and the yeast cells. Tamoxifen, an estrogen receptor antagonist, was used as the positive control.

Inhibition of 17β-Estradiol (E_2)-Induced Stimulation of

Table 2. Estrogenic and Anti-estrogenic Activities of Isolated and Synthetic Compounds on MCF-7 Cells

Comp.	Relative cell-proliferation (% of vehicle)			Relative cell-proliferation inhibition (% of 10^{-12} M E_2 proliferation value)		
	10^{-5} M	10^{-6} M	10^{-7} M	10^{-5} M	10^{-6} M	10^{-7} M
3	127.2±6.0*	110.2±4.6	110.3±5.1	104.1±2.9	94.1±1.9	98.3±4.4
5	110.4±4.3	116.2±4.4	84.1±12.7	96.7±13.2	93.0±5.3	104.1±3.2
6	94.7±2.3	103.3±6.5	96.9±1.2	122.8±2.9	104.8±3.8	106.9±5.8
7	116.3±6.4	125.3±9.6	106.4±3.9	106.1±2.3	98.1±6.0	93.8±1.2
8	104.9±2.8	103.8±9.8	106.8±2.6	104.0±2.4	104.6±2.5	103.9±2.5
9	85.1±9.7	106.7±4.6	99.8±4.6	105.1±5.3	103.1±2.1	106.0±3.3
10	93.1±12.2	99.8±8.6	95.9±8.8	96.2±2.8	93.6±2.3	91.4±4.3
11	101.1±8.8	94.5±6.3	94.4±2.8	89.7±2.1*	87.6±1.5**	90.4±2.7
12	96.6±17.8	112.8±9.4	147.5±7.5**	95.8±3.5	95.3±1.4	91.1±2.8
13	90.5±4.8	93.1±2.9	90.9±2.5	98.3±1.9	101.4±1.9	97.5±3.8
14	93.2±3.3	86.5±3.3	90.5±3.2	94.6±1.8	96.5±1.6	97.7±1.4
15	96.9±3.2	95.4±4.7	96.0±9.2	98.7±4.6	91.5±4.9	92.9±2.7
16	99.6±5.8	97.1±3.9	115.3±5.2	97.8±2.6	91.2±1.9	92.1±1.0
17	126.6±21.5	137.7±16.3*	157.7±8.5**	95.6±3.3	91.9±2.9	90.9±3.0
18	170.7±5.8**	179.2±24.7**	130.2±13.4	94.2±2.5	102.9±7.0	91.7±2.6
19	157.7±5.9**	157.7±3.5**	144.9±9.3**	92.7±3.4	92.1±2.5	91.9±5.0
20	97.6±8.3	95.1±6.0	96.8±3.4	93.3±2.0	102.2±4.4	106.7±4.0
21	93.5±9.2	106.4±9.7	105.2±8.9	107.7±2.9	111.2±3.2	113.2±1.9
22	90.0±4.9	106.7±4.4	100.7±8.6	99.7±4.0	92.7±2.9	88.9±4.4*
23	72.3±4.2	140.5±14.7**	110.5±6.1	94.3±3.2	96.5±4.0	91.3±2.5
24	125.3±16.5	140.8±8.5**	142.6±21.8**	95.0±2.7	98.3±2.5	97.1±3.7
Tam.				74.0±2.9**	104.1±1.9	101.2±4.4

Each value represents the mean±S.E. (n=6). Asterisks denote significant differences from the control at $p<0.05$ (*), $p<0.01$ (**). The estrogenic activity of 17β-estradiol was 212.8±19.6% at a concentration of 10^{-12} M (100%). The relative cell-proliferation inhibition was expressed as a percentage of the control (100%), observed in the presence of 10^{-12} M 17β-estradiol alone. Tam. denotes (tamoxifen).

Table 3. Induction of β-Galactosidase in the Yeast Two-Hybrid Assay Expressing ERα and ERβ by Isolated and Synthetic Compounds

Comp.	β-Galactosidase activity (*U*)					
	ERα			ERβ		
	10^{-4} M	10^{-5} M	10^{-6} M	10^{-4} M	10^{-5} M	10^{-6} M
3	39.9±12.4	47.6±2.4	36.5±0.6	70.1±12.4	54.4±2.2	48.1±0.6
5	50.7±1.5	41.9±2.7	41.2±2.6	66.0±9.4	48.4±2.1	53.4±1.9
6	81.0±5.9*	49.5±2.8	41.8±2.9	109.8±5.8**	60.0±10.4	48.9±2.2
7	32.8±7.2	39.8±6.3	31.2±9.7	52.0±7.5	39.9±11.6	42.4±9.1
8	59.5±4.8	51.4±2.4	38.1±4.5	66.4±13.2	53.5±4.9	50.2±5.6
9	32.2±3.7	35.8±3.2	39.9±2.2	41.0±1.6	44.1±4.4	42.0±1.8
10	34.6±3.7	40.7±3.2	39.1±2.2	54.4±1.6	46.2±4.4	55.1±10.8
11	56.4±11.1	48.8±5.1	48.5±4.5	56.6±1.4	65.9±4.3	72.6±2.2
12	47.0±12.4	38.7±2.2	46.5±0.6	321.4±12.4**	67.5±2.2	51.8±0.6
13	38.1±10.2	55.8±7.9	43.2±2.9	74.4±6.0	45.8±2.4	30.9±7.9
14	54.2±1.5	51.3±2.8	35.6±2.7	73.1±9.4	51.6±2.1	52.4±1.9
15	48.7±4.8	41.1±2.3	38.1±4.5	59.0±13.2	55.6±4.9	48.9±5.6
16	45.2±7.3	25.7±6.3	36.1±9.7	57.5±7.4	47.4±11.6	31.9±9.1
17	93.2±7.8*	31.2±10.9	44.2±4.1	348.4±23.6**	87.1±1.4**	55.5±2.2
18	128.4±12.1**	67.5±7.6	50.7±7.3	141.4±0.10**	54.8±5.8	61.3±4.1
19	190.3±19.1**	67.5±7.8	49.7±6.9	187.2±20.9*	66.0±1.5	66.7±7.6
20	52.2±8.5	42.3±3.1	78.6±5.4	52.2±8.5	42.3±3.1	78.6±5.4
21	36.3±2.8	36.9±6.2	53.0±5.4	36.3±2.8	36.9±6.2	53.0±4.4
22	42.7±4.6	44.5±0.4	55.6±1.4	42.7±4.6	44.5±0.4	55.6±1.4
23	41.6±0.5	40.4±1.8	42.6±1.9	41.6±0.5	40.4±1.8	42.6±1.9
24	41.5±6.3	35.2±6.8	24.0±1.3	55.0±10.6	54.7±1.5	53.9±9.5

Each value represents the mean±S.E. of three independent experiments (n=3). Asterisks denote significant differences from the control at $p<0.05$ (*), $p<0.01$ (**). DMSO (control) was 40.8±2.7 and 37.2±7.3 in the yeast expressing ERα and ERβ, respectively. 17β-Estradiol was 1553.7±13.5 and 2126.0±79.3 at a concentration of 10^{-7} M in the yeast expressing ERα and ERβ, respectively.

Table 4. ERα Binding Affinity of Isolated Compounds from *Cassia tora* and Synthetic Compounds

Compound	% Binding to ERα			IC_{50}
	5×10^{-3} M	10^{-4} M	10^{-5} M	
10	−2.5±1.7	−3.0±3.4	−4.1±0.2	$>5\times10^{-3}$ M
11	24.2±0.1**	−1.1±1.9	−12.0±0.5	$>5\times10^{-3}$ M
12	62.9±1.1**	10.9±4.3	−1.9±0.8	1.6×10^{-3} M
13	0.8±0.3	−8.5±0.2	−5.6±1.1	$>5\times10^{-3}$ M
14	20.1±0.2*	5.1±5.6	0.9±13.3	$>5\times10^{-3}$ M
15	27.1±5.0**	19.5±0.9	2.3±2.1	$>5\times10^{-3}$ M
16	35.6±2.3**	12.8±0.8	−0.9±0.5	$>5\times10^{-3}$ M
17	80.4±2.1**	37.8±0.5**	13.1±2.1	2.5×10^{-4} M
18	52.8±0.1**	24.7±0.9**	5.5±1.1	3.2×10^{-3} M
19	66.6±0.1**	42.1±9.1**	2.4±2.2	3.9×10^{-4} M
20	30.8±0.2**	−16.3±0.7	−7.4±0.6	$>5\times10^{-3}$ M
21	−10.7±3.1	−8.5±1.3	−4.2±0.3	$>5\times10^{-3}$ M
22	3.5±0.8	−32.2±28.5	−14.9±1.2	$>5\times10^{-3}$ M
23	32.8±1.5**	12.1±1.4	5.9±0.8	$>5\times10^{-3}$ M
24	86.6±1.2**	22.9±1.5**	−2.9±0.1	3.9×10^{-4} M

A percentage of 17β-estradiol (positive control) bound to ERα was 52.5±1.7% at a concentration of 10^{-7} M ($IC_{50}=8\times10^{-8}$ M). Asterisks indicate significant difference from the control at $p<0.05$ (*), $p<0.01$ (**), (n=4).

MCF-7 Cells Tamoxifen inhibited the 17β-estradiol-mediated proliferation of MCF-7 cells by 16% at 10^{-5} M (Table 2). Physcion (**11**) showed a significant anti-estrogenic activity at 10^{-5} and 10^{-6} M concentrations, while 1,3-dihydroxynaphthalene (**22**) showed significant anti-estrogenic activity at 10^{-7} M.

Inhibition of 17β-Estradiol-Induced β-Galactosidase Activity in the Yeast Two-Hybrid Assay In the yeast expressing ERα (Table 5), rubrofusarin (**15**), nor-rubrofusarin (**18**), nor-toralactone (**19**) and T_3HN (**24**) showed significant anti-estrogenic activity in concentration dependent manner. Torachrysone (**16**), 6-hydroxymusizin (**17**), 1,3-dihydroxynaphthalene (**22**) and 1,6-trihydroxynaphthalene (**23**) showed activity at a 10^{-4} M concentration. On the other hand, in the yeast expressing ERβ, 9-methoxychrysophanol (**13**), toralactone (**14**), rubrofusarin (**15**), torachrysone (**16**), 6-hydroxymusizin (**17**), and T_3HN (**24**) showed activity at 10^{-4} M, while nor-rubrofusarin (**18**) and nor-toralactone (**19**) at 10^{-5} and 10^{-6} M.

According to the aforementioned data, among the isolated glycosides, aurantio-obtusin 6-*O*-β-D-glucoside (**3**) and 6-hydroxymusizin 8-*O*-β-D-glucoside (**6**), exhibited weak or no estrogenic activity. Regarding the aglycones and the synthetic compounds, aurantio-obtusin (**12**), 6-hydroxymusizin (**17**), nor-rubrofusarin (**18**), nor-toralactone (**19**) and 1,3,8-trihydroxynaphthalene (**24**) exhibited the most potent estrogenic and anti-estrogenic activities in the *in vitro* assays. On the other hand, toralactone (**14**), rubrofusarin (**15**) and torachrysone (**16**) showed the most significant anti-estrogenic activity, when evaluated by the yeast two-hybrid assay.

Discussion

Several phenolic compounds such as isoflavonoids, coumestans and lignans were reported to have estrogenic and/or anti-estrogenic activities due to their structural similarity to the mammalian estrogens. The main structural feature of these compounds was reported to be the presence of two phenolic groups separated by a planar core of about 12.8 Å.[21]

Despite lacking the formerly mentioned characteristic feature, the naphthopyrones, toralactone (**14**) and rubrofusarin (**15**), and the acetyl naphthalenes, torachrysone (**16**) and 6-hydroxymusizin (**17**), exhibited a significant estrogenic

Table 5. Inhibitory Effects of Isolated and Synthetic Compounds on the Induction of β-Galactosidase Activity by 17β-Estradiol in the Yeast Two-Hybrid Assay (ERα and ERβ)

Comp.	β-Galactosidase activity (% of control)					
	ERα			ERβ		
	10^{-4} M	10^{-5} M	10^{-6} M	10^{-4} M	10^{-5} M	10^{-6} M
3	92.8±9.0	93.4±1.9	107.1±6.2	97.1±2.7	102.4±3.6	95.9±3.4
5	97.3±7.4	104.5±1.1	105.8±2.0	90.4±2.9	91.9±4.6	100.1±1.1
6	94.0±6.5	98.8±18.6	120.1±5.6	99.6±2.8	110.5±3.1	110.3±2.5
7	103.1±2.3	116.5±4.9	104.2±4.8	121.8±1.8	102.9±2.7	105.8±1.7
8	118.7±8.3	110.1±5.9	91.8±1.9	95.8±5.0	101.5±12.8	111.1±3.3
9	95.7±1.4	104.0±1.4	106.9±3.2	90.7±0.7	97.3±6.5	103.7±4.2
10	95.7±3.7	110.4±5.9	110.1±3.9	90.9±6.7	103.3±9.2	110.1±7.8
11	82.4±6.7	89.4±3.1	97.7±2.2	82.6±1.2	89.0±3.2	98.1±6.7
12	101.7±7.3	90.3±6.4	103.4±5.3	129.1±11.7	117.2±3.3	123.3±3.9
13	82.9±0.9	102.5±2.8	111.8±7.5	78.8±1.3**	102.5±4.3	117.9±2.1
14	79.1±1.3*	78.9±3.2*	92.0±1.9	78.5±3.5**	88.2±4.8	124.2±4.3
15	63.6±0.8**	67.3±0.8**	80.4±5.6*	50.7±1.6**	82.2±3.2	99.8±9.6
16	66.4±2.4**	82.2±4.8	108.2±5.4	66.3±1.8**	88.2±8.9	99.7±11.0
17	52.4±3.9**	86.8±2.2	95.8±5.3	31.7±0.8**	99.8±4.7	117.7±7.9
18	—	57.4±3.9**	76.8±3.8*	—	63.6±2.1**	94.0±1.5
19	—	38.5±1.9**	73.7±2.2**	—	43.3±3.1**	75.8±4.3**
20	103.8±4.3	95.7±2.5	105.6±1.3	110.6±19.2	104.9±1.5	103.4±3.3
21	96.1±9.5	92.3±5.5	91.0±1.9	94.6±5.4	106.9±5.5	101.8±9.2
22	67.3±3.5**	90.6±4.7	105.7±5.9	81.7±5.8	125.4±8.01	117.9±6.6
23	76.1±3.2**	97.2±9.5	112.0±8.9	86.0±1.6	93.8±6.7	107.8±2.1
24	57.9±3.7**	76.4±3.4**	90.1±1.9	55.3±2.0**	85.7±1.4	94.1±1.9
Tam.	11.9±1.1**	114.2±4.2	117.9±1.5	22.4±2.5**	98.7±10.9	103.3±7.7

Each value represents the mean±S.E. of three independent experiments ($n=3$). Asterisks denote significant differences from the control at $p<0.05$ (*), $p<0.01$ (**). β-Galactosidase activity (U) of 17β-estradiol was 641.2±22.5 and 2333.4±112.5 in yeast expressing ERα and ERβ, respectively, at a concentration of 10^{-7} M (100%). β-Galactosidase activity (U) of the tested compounds was calculated as a percentage of the 17β-estradiol activity. Compounds **18** and **19** were toxic at a concentration of 10^{-4} M. Tam. denotes (tamoxifen).

and/or anti-estrogenic activities (Tables 4, 5). This finding has led us to investigate the main structure requirements responsible for the estrogenic activity of these compounds. Therefore, a group of synthetic compounds (**20**—**24**) possessing a partial similarity to the previously mentioned active compounds were investigated for their estrogenic activity. Moreover, demethylation of toralactone (**14**) and rubrofusarin (**15**) was carried out to study the effect of the methyl group at position 7 in these compounds on the activity.

Among the tested synthetic and chemically modified compounds, nor-rubrofusrain (**18**), nor-toralactone (**19**) and T_3HN (**24**) showed almost similar estrogenic and anti-estrogenic activities to those of 6-hydroxymusizin (**17**) (Tables 2—5). By comparing the structures of the formerly mentioned compounds it was found that trihydroxynaphthalene (T_3HN) is a common feature in these compounds, indicating its principal role in the estrogenic activity. It was also found that methylation of a free hydroxy group at position 3 of the T_3HN nucleus as in toralactone (**14**), rubrofusarin (**15**) and torachrysone (**16**) resulted in reduction of estrogenic activity and binding affinity to the estrogenic receptor ERα, but retained anti-estrogenic activity. On the other hand, 1,3- (**22**) and 1,6-dihydroxylated naphthalene (**23**) showed weaker estrogenic activities, which confirmed the significance of the trihydroxylated system for effective binding to estrogen receptors.

The estrogenic activity of aurantio-obtusin (**13**) is in accordance with the previously reported data about the importance of the phenolic group at 2 and 6 position in the antharquinone nucleus for their estrogenic activity.[22] Moreover, physcion (**11**), which has a methyl group at the 6 position, showed only anti-estrogenic activity.

To the best of our knowledge, this is the first report about the estrogenic and anti-estrogenic activities of these naturally occurring phenolic compounds based on naphthalene and naphthopyrone nucleuses. Finally, the increase in estrogenic activity of the naringinase pretreated extract of *Cassia tora* suggests the activation of this extract by intestinal flora.

In conclusion, the current study introduced the 1,3,8-trihydroxynaphthalene (T_3HN) as a new basic nucleus responsible for estrogenic activity, which might be of interest in the discovery of new estrogenic and anti-estrogenic compounds based on this nucleus.

Experimental

Chemicals and Reagents Naringinase was purchased from Sigma Co. (St. Louis, MO, U.S.A.). Dulbecco's modified Eagle's medium (DMEM) was obtained from Gibco BRL (Grand Island, NY, U.S.A.). Fetal bovine serum (FBS) was purchased from ICN Biomedicals, Inc. (Aurora, OH, U.S.A.). Streptomycin, 0.25% trypsin, and *O*-nitrophenyl β-D-galactoside (ONPG) were purchased from Nacalai Tesque Co. (Kyoto, Japan). 17β-Estradiol was purchased from Calbiochem Co. (Darmstadt, Germany). Human serum was obtained from Bio-Whittakar (Walkersville, MD, U.S.A.). 3-(4,5-Dimethyl-2-thiazolyl)-2,3-diphenyl-2*H*-tetrazolium bromide (MTT), penicillin, norit SX-II charcoal, 2,6-dihydroxynaphthalene, 6-hydroxy-2-naphthoic acid, 1,3-dihydroxynaphthalene, 1,6-dihydroxnaphthalene and tamoxifen were purchased from Wako Chem. Co. (Osaka, Japan). 20T-Zymolyase from Seikagaku Kogyo Co. (Tokyo, Japan). Dextran 70T was obtained from Amersham Pharmacia Biotech AB. (Uppsala, Sweden). 1,3,8-Trihydroxynaphthalene (T_3HN) was kindly provided by Professor Yutaka Ebizuka (Graduate School of Pharmaceutical Sciences, The University of Tokyo).

General Experimental Procedures The melting point was measured on a Yanagimoto microhot stage melting point apparatus. Optical rotations were measured with a DIP-360 automatic polarimeter (Jasco Co., Tokyo).

UV spectra were measured with a UV-2200 UV–VIS recording spectrophotometer (Shimadzu Co., Kyoto). IR spectra were measured with a Jasco FT/IR-230 infrared spectrometer. ^{1}H- and ^{13}C-NMR were measured with a JHA-LAA 400 WB-FT (^{1}H, 400 MHz; ^{13}C, 100 MHz; Jeol, Tokyo) spectrometer, the chemical shifts being represented as ppm with tetramethylsilane as an internal standard. ESI-MS was carried out on an Esquire 3000 mass spectrometer (Bruker Daltanik GmbH, Bremen, Germany) system with ESI ionization source. Elemental analysis was carried out on a Perkin Elmer analyzer 2400 II. TLC was carried out on precoated silica gel 60 F_{254} (0.25 mm, Merck) and RP-18 F_{254}S (0.25 mm, Merck Co.). Column chromatography (CC) was carried out on (BW-820MH silica gel), ODS DM 1020T (ODS, Fuji Silysia, Nagoya, Japan), Diaion HP-20 (Mitsubishi Kasei, Tokyo) and Sephadex LH-20 (Pharmacia Co.). Medium pressure liquid chromatography (MPLC) was performed on LiChroprep Rp-18 (size A, Merck Co.). Preparative HPLC was performed on a Tosoh CCPM-CCPM-II system (Tosoh Co., Tokyo) equipped with a UV 8020 detector and TSK gel ODS-80Ts column (21.5×300 mm, Tosoh Co.).

Plant Material *Cassia tora* seeds were purchased at the Japanese market and identified by a specialist in botanical identification of Suntory Company, Japan (Lot No. T-260).

Extraction and Isolation The seeds of *Cassia tora* were extracted with 70% ethanol and the extract was concentrated in vacuum. The ethanolic extract (100 g) was suspended in water and fractionated with $CHCl_3$ (3×1 l) to obtain a chloroform fraction (7 g). The remaining water solution was applied to a Diaion HP-20 column (60 cm×6 cm) and stepwisely eluted with H_2O, 25% methanol, 50% methanol and 100% methanol. The 50% methanol fraction (60 g) was subjected to a silica gel column eluted with $CHCl_3$–MeOH–H_2O (9 : 1 : 0.1→5 : 5 : 0.5 v/v/v) to yield 8 fractions. Compound **3** (13 mg) was obtained from fraction 2 after purification on Sephadex LH-20 using methanol as eluent followed by a medium pressure liquid chromatography (MPLC) Rp-18 column using MeOH–H_2O (1 : 1 v/v). Fraction 4 was subjected to an ODS column to obtain compounds **5** (25 mg) and **6** (14 mg). Compound **4** (5 mg) was obtained from fraction 5 by purification on a MPLC Rp-18 column eluted with MeOH–H_2O (4 : 6 v/v). Fraction 8 was further purified using a MPLC Rp-18 column gradiently eluted with MeOH–H_2O to obtain compounds **8** (70 mg) and **9** (14 mg) in addition to two other fractions 8-1 and 8-2. Fraction 8-1 was purified on Sephadex LH-20 using MeOH–H_2O (1 : 1 v/v) to get compounds **1** (20 mg) and **7** (75 mg). Finally, compound **2** (6 mg) was obtained from fraction 8-2 by prep HPLC (acetonitrile: 0.1% TFA/H_2O (30% v/v) 5 ml/min, monitored at 285 nm).

Naringinase Treatment of the Extract and Isolation of the Aglycones[23] The 70% ethanolic extract (25 g) was incubated with naringinase enzyme (12.5 g) in 1000 ml of 0.2 M acetate buffer (pH=4.7) at 37 °C for 24 h. The solution was extracted with EtOAc three times (500 ml×3). The pooled EtOAc extract was evaporated under vacuum to obtain the naringinase treated extract (15 g). This extract was applied to a silica gel column (30 cm×5 cm) eluted with hexane–ethyl acetate (9.5 : 0.5—5 : 5 v/v). Five fractions were obtained and screened for their estrogenic activity using MCF-7 cells. Because of their relatively high estrogenic activity fractions 1 and 2 were selected for further purification. Fraction 1 was applied to silica gel column (15 cm×2 cm) using silica gel 60 and gradiently eluted with hexane–ethyl acetate (9.5 : 0.5—9 : 1 v/v) to obtain compounds **10** (300 mg), **11** (25 mg) and **12** (10 mg). Fraction 2 was injected to an MPLC Rp-18 column eluted with MeOH–H_2O (8 : 2 v/v) affording compound **13** (30 mg) and a mixture of compounds **14** and **15**. The isolation of compounds **14** and **15** was achieved by repeated purification using prep. HPLC (acetonitrile: 0.1% TFA/H_2O (75% v/v) 5 ml/min, monitored at 285 nm) to afford compounds **14** (30 mg) and **15** (130 mg).

Torachrysone 8-*O*-[*β*-Glucopyranosyl(1→3)-*O*-*β*-D-glucopyranosyl(1→6)-*O*-*β*-D-glucopyranoside] (1) Yellow needles (MeOH). mp 249—251 °C. $[\alpha]_D^{25}$ −38.5° [*c*=0.32, MeOH]. UV (MeOH) nm λ_{max} (log ε): 240 (4.95), 265 (4.61), 310 (4.12), 324 (4.11), 340 (4.12). IR (KBr) cm^{-1}: 3390, 1629, 1398, 1260, 1165. ^{1}H-NMR (DMSO-d_6, 400 MHz): δ 2.21 (3H, s, C$\underline{H_3}$-3), 2.48 (3H, s, CO$\underline{CH_3}$), 3.82 (3H, s, O$\underline{CH_3}$), 4.00 (1H, d, *J*=12.0 Hz, H-6′), 4.30 (1H, d, *J*=8.0 Hz, H-1‴), 4.32 (1H, d, *J*=8.0 Hz, H-1″), 5.09 (1H, d, *J*=8.0 Hz, H-1′), 6.89 (1H, d, *J*=2.1 Hz, H-5), 7.06 (1H, d, *J*=2.1 Hz, H-7), 7.09 (1H, s, H-4), 9.46 (1H, s, OH-1). ^{13}C-NMR (DMSO-d_6, 100 MHz): δ 19.5 (C$\underline{H_3}$-3), 32.2 (CO$\underline{CH_3}$), 55.4 (O$\underline{CH_3}$), 60.7 (C-6″), 61.0 (C-6‴), 68.3 (C-4″), 68.8 (C-6′), 70.0 (C-4′), 70.1 (C-4‴), 72.2 (C-2″), 73.3 (C-2′), 73.8 (C-2‴), 76.0 (C-5‴), 76.1 (C-5′), 76.4 (C-5″), 76.9 (C-3′, C-3‴), 88.1 (C-3″), 101.1 (C-5), 102.4 (C-1′), 102.8 (C-1″), 103.3 (C-7), 104.1 (C-1‴), 108.6 (C-4a), 118.8 (C-4), 123.2 (C-2), 133.6 (C-3), 136.8 (C-8a), 151.0 (C-1), 155.2 (C-8), 158.4 (C-6), 204.5 (CO). ESI-MS *m/z* (rel. int.): 733 $[M+H]^+$ (100), 571 $[M-Glc.+H]^+$ (69), 409 $[M-2\times Glc.+H]^+$ (50), 247 $[agl.+H]^+$ (77). *Anal.* Found: C, 52.4; H, 6.2. $C_{32}H_{44}O_{19}$ requires: C, 52.4; H, 6.0%.

Toralactone 9-*O*-[*β*-D-Glucopyranosyl-(1→3)-*O*-*β*-D-glucopyranosyl-(1→6)-*O*-*β*-D-glucopyranoside] (2) Yellowish amorphous powder. $[\alpha]_D^{25}$ −38.7° [*c*=0.15, MeOH]. UV (MeOH) nm λ_{max} (log ε): 237 (4.26), 247 (4.14), 270 (4.68), 277 (4.7), 383 (3.78). IR (KBr) cm^{-1}: 3360, 1681, 1650, 1624, 1579, 1407, 1370, 1231, 1165, 1040, 868, 812. ^{1}H-NMR (DMSO-d_6, 400 MHz): δ 2.22 (3H, s, C$\underline{H_3}$-3), 3.88 (3H, s, O$\underline{CH_3}$), 4.00 (1H, d, *J*=12.0 Hz, H-6′), 4.28 (1H, d, *J*=8.0 Hz, H-1‴), 4.31 (1H, d, *J*=8.0 Hz, H-1″), 5.09 (1H, d, *J*=8.0 Hz, H-1′), 6.50 (1H, s, H-4), 6.86 (1H, d, *J*=2.2 Hz, H-8), 6.92 (1H, d, *J*=2.2 Hz, H-6), 7.13 (1H, s, H-5), 12.5 (1H, s, OH-9). ^{13}C-NMR (DMSO-d_6, 100 MHz): δ 18.8 (C$\underline{H_3}$-3), 55.6 (O$\underline{CH_3}$), 60.5 (C-6″), 61.0 (C-6‴), 68.6 (C-4″), 68.8 (C-6′), 70.1 (C-4′), 70.1 (C-4‴), 72.2 (C-2″), 73.2 (C-2′), 73.8 (C-2‴), 75.7 (C-5‴), 75.7 (C-5′), 76.1 (C-5″), 76.3 (C-3′), 76.9 (C-3‴), 88.1 (C-3″), 98.5 (C-10a), 100.5 (C-6), 100.9 (C-1′), 101.9 (C-8), 102.7 (C-1″), 104.1 (C-1‴), 104.1 (C-4), 109.2 (C-9a), 111.6 (C-5), 132.4 (C-4a), 141.6 (C-5a), 152.7 (C-3), 157.6 (C-9), 161.4 (C-7), 162.8 (C-10), 166.8 (C-1). ESI-MS *m/z* (rel. int.): 781 $[M+Na]^+$ (50), 759 $[M+H]^+$ (100), 597 $[M-Glc.+H]^+$ (24), 273 $[agl+H]^+$ (43). *Anal.* Found: C, 52.03; H, 5.77. $C_{33}H_{42}O_{20}$ requires: C, 52.28; H, 5.59%.

Preparation of the Naringinase-Treated Extracts and Fractions[23] The 70% alcoholic extract and 25%, 50% and 100% MeOH fractions of *C. tora* (40 mg, each) were incubated with naringinase enzyme (20 mg) in 2 ml of 0.2 M acetate buffer (pH=4.7) at 37 °C for 4 h. The resulting solution was then extracted with BuOH (10 ml×3) and the combined BuOH extracts were evaporated under vacuum to get the naringinase-treated extract and fractions. These, in turn, were dissolved in DMSO (10 mg/ml) as stock solutions to test their estrogenic activity.

Acid Hydrolysis of Torachrysone Tetraglucoside (4) and 6-Hydroxymusizin 8-*O*-*β*-D-Glucoside (6)[11] A solution of **4** and **6** (10 mg of each) in 1% H_2SO_4 (0.5 ml) in a sealed tube was heated separately on a boiling water bath for 1 h. The solution was then extracted with EtOAC, and concentrated under vacuum to obtain torachrysone (**17**) (2 mg) and 6-hydroxymusizin (**16**) (5 mg). Both compounds were identified by ^{1}H-NMR and EI-MS.

Acid Hydrolysis of Compounds 1 and 2[11] A solution of **1** and **2** (4 mg of each) in a 1% H_2SO_4 (0.2 ml) in a sealed tube was heated on a boiling water bath for 1 h. The solution was then extracted with EtOAc, and concentrated under vacuum to get torachrysone and toralactone, respectively, and the identity of the aglycones was verified by ^{1}H-NMR, EI-MS, TLC and by comparison with the reported data.[11,13,18—20] The aqueous layer was neutralized with sodium carbonate and freeze dried. The sugar component of the residue was detected by TLC through comparison with authentic samples to be glucose. The absolute configuration of the sugar was determined as D-glucose according to the method described by Hara *et al.*[25]

Demethylation of Toralactone (14) and Rubrofusrin (15)[24] Toralactone and rubrofusarin (5 mg each) were separately dissolved in acetic anhydride (5 ml), treated gradually with HI (*d*=1.7) and refluxed gently for 2 h with occasional shaking. At the end of the reaction, a saturated solution of sodium thiosulfate (5 ml) was added. The resulting mixture was extracted by EtOAc and evaporated under vacuum. The resulting demethylated compounds were purified using prep HPLC (acetonitrile: 0.1% TFA/H_2O (80% v/v), 5 ml/min, monitored at 285 nm) to afford nor rubrofusrin (**18**) (2 mg) and nor toralactone (**19**) (1.5 mg).

MCF-7 Cells Proliferation Assay Estrogen-sensitive human MCF-7 breast cells were grown in DMEM supplemented with 5% FBS, penicillin, and streptomycin. The cells were harvested by trypsinization (0.25% trypsin) and plated at a concentration of 5×10^3 cells/well in DMEM supplemented with 5% FBS in 96-well tissue culture plates (Iwaki Co., Chiba, Japan) and allowed to attach for 24 h. Then the culture medium was replaced with phenol red-free DMEM containing 10% heat-inactivated dextran/charcoal-stripped (DC) human serum prior to the addition of compounds and 17β-estradiol. Stock solutions of test compounds in DMSO were diluted with DC medium. The final DMSO concentration in culture medium did not exceed 0.1%, and this concentration did not affect cell viability. After 4 d in a humidified incubator with 5% CO_2 at 37 °C, the proliferation of the cells was measured using the MTT method.[26]

Yeast Two-Hybrid Assay The yeast two-hybrid assay was carried out according to the method of Nishikawa and Kanayama.[27,28] Briefly, yeast cells expressing ERα and ERβ were separately grown overnight at 30 °C with shaking in a synthetic defined medium (SD) lacking tryptophan and leucine. Yeast cells were treated with 17β-estradiol and the isolated compounds for 4 h at 30 °C, and β-galactosidase activity was determined as fol-

lows. The growth of the yeast cells was monitored by measuring the turbidity at 600 nm. The treated yeast cells were collected by centrifugation (8000×***g***, 5 min) and re-suspended in 200 μl of *Z*-buffer (0.1 M sodium phosphate, pH 7.0, 10 mM KCl, and 1 mM $MgSO_4$) containing 1 mg/ml of zymolyase at 37 °C for 15 min. The reaction was started by the addition of 40 μl of 4 mg/ml *O*-nitrophenol β-D-galactopyranoside (ONPG) as a substrate. When a yellow color developed (incubation time: *t*), 100 μl of 1 M Na_2CO_3 was added to stop the reaction. The absorbance of the solution (150 μl) was measured at 420 and 550 nm. The β-galactosidase activity was determined using the following formula:

$$U=1000\times(A_{420}-1.75\times A_{550})/(t\times 0.05\times A_{600})$$

Ligand Binding Screen An estrogen-R (α) competitor screening kit was purchased from Wako Chemicals Japan Inc. The assay depends on the competition between the samples applied in different concentrations and the labeled estrogen mixture. The amount of the ligand that binds to the ERα coated on the microplate well is determined by the dynamic equilibrium among all the ligand concentrations in the mixture, the difference of their binding affinities to the receptor and the incubation time. Therefore, the reduction in fluorescence intensities from the labeled estrogen retained is an indication of the affinity of the added compounds to the estrogen receptor. The isolated compounds were tested in 10^{-5}, 10^{-4} and 5×10^{-3} M concentrations. Estradiol was used as a positive control and a labeled estrogen mixture was used as a negative control. The results were calculated as percentages of the negative control.

Anti-estrogenic Assay To examine the antagonistic activity of the test compounds, the inhibition of β-galactosidase activity and the proliferation of MCF-7 cells, which had been induced by 10^{-7} M and 10^{-12} M 17β-estradiol, respectively, were measured at various concentrations of test compounds.

Statistical Analysis Each set of experiments was repeated at least three times. Values are expressed as mean±S.E.M. One-way analysis of variance followed by Dunnett's test was used for statistical analysis.

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