By-Products Produced by the Reaction of Estrogens with Hypochlorous Acid and their Estrogen Activities

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Estrogens that originate from humans and animals are thought of as a factor of endocrine disruptors in the environment. We investigated their halogenated derivatives, which could be produced by chlorine treatment at a sewage treatment plant. The chlorinated derivatives of estrone (E1), 17β-estradiol (E2), estriol (E3), and 17α-ethynylestradiol (EE2) were produced by the reaction with hypochlorous acid in organic solvents and also brominated derivatives when bromide ions were present. The structures of chlorinated and brominated estrogens isolated were determined by MS and NMR spectroscopy. The estrogenic activities of the halogenated derivatives were measured by yeast two-hybrid assays incorporating the human estrogen receptor α (hERα) or medaka fish (*Oryzias latipes*) estrogen receptor α (medERα). Although the activities of 4-chloroestrone (4-ClE1) and 10-chloro-1,4-estradiene-3,17-dione were similar to those of E1, the activities of 2-ClE1 and 2,4-dichloroestrone (2,4-diClE1) were approximately 4/5 and 1/50 that of E1, respectively, in an agonist assay for hERα. No activity was detected in 2,4,16,16-tetrachloroestrone (2,4,16,16-tetraClE1). The estrogenicities of chlorinated derivatives of E2, E3, and EE2 showed a similar tendency to that of E1. The brominated derivatives showed slightly weaker activity than the corresponding chlorinated derivatives. However, many estrogens halogenated at the 2 and 4 positions still had activity that was approximately 10^3–10^4 times stronger than that of bisphenol A.

Key words —— estrogen, halogenated estrogen, estrogenic activity, yeast two-hybrid assay, hypochlorous acid, bromide ion

INTRODUCTION

Estrogens produced from humans and animals are thought of as a factor of endocrine disruptors in the environment. Nakamuro *et al.* reported that the estrogen activities of steroidal sex hormones, such as estrone (E1), 17β-estradiol (E2), and estriol (E3), were approximately 10^2–10^4 times stronger than those of man-made compounds that are known as endocrine disruptors.[1] However, steroidal sex hormones that are collected at a sewage treatment plant are discharged into local sources of water through the disinfecting process. Organochlorine compounds, which are produced when hypochlor-ous acid is used as a disinfectant, are reported to have negative effects on the ecosystem.[2–6] Estrogens also can be chlorinated and oxidized with hypochlorous acid in sewage treatment plants. Therefore, the reactivity of hypochlorous acid for estrogens, the structure of the products, and the toxicities including estrogen activity need to be determined for estimation of the potential risk.

We therefore investigated in detail the reaction of E1, E2, E3, and 17α-ethynylestradiol (EE2) with hypochlorous acid in organic solvents.

MATERIALS AND METHODS

UV spectra were measured on a Tosoh PD-8020 photodiode array detector and Beckman DU 640 spectrophotometer. NMR spectra were taken on a JEOL JNM-GSX500 (^{1}H, 500 MHz: ^{13}C, 125 MHz)

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Fourier transform spectrometer with tetramethyl-silane (TMS) as an internal standard. Chemical shifts are expressed in δ values. FAB-MS measurements were taken on a JEOL JMS-SX103 mass spectrometer, and *m*-nitrobenzyl alcohol was used as a matrix. Analytical high performance liquid chromatography (HPLC) was performed on a Wakopack-navi-C30 column (4.6 × 250 mm) with 65% acetonitrile at a flow rate of 0.8 ml/min, and it was assessed with a UV-spectrophotometric detector at 254 nm. Preparative HPLC was carried out on a YMC-pack ODS-A (50 or 20 × 250 mm) at a flow rate of 8 ml/min.

Steroidal compounds were purchased from Aldrich Chem. Co., U.S.A. A sodium hypochlorite solution (> 5% available chlorine) was purchased from Wako Pure Chemicals, Industries Ltd., Osaka, Japan. The available chlorine concentration in the solution was measured using the iodometric titration method.

Reaction with Hypochlorous Acid ——— The reaction of estrogens with hypochlorous acid was carried out using the following general procedure.

An aqueous solution of sodium hypochlorite (0.6 ml, 6% available chlorine) was added to a solution of steroid (1 mmol) in methanol (100 ml) or acidic 80% methanol (including potassium bromide) and stirred. After 30 min of stirring at room temperature, an aqueous solution of sodium sulfite was added in an ice bath. The mixture was acidified with a 6% hydrochloric acid, and methanol was removed under reduced pressure. The aqueous residue was extracted with dichloromethane after adding sodium chloride. The dichloromethane solution was washed with brine and dried over sodium sulfate. After removal of the dichloromethane, the residue was dissolved in a solvent (20 ml) used for a mobile phase of preparative HPLC. The mixture of mono- and di-chlorinated derivatives separated into each component as a pure state by using methanol/water [70/30 to 80/20 (v/v)] as the mobile phase.

Estrogenic Activity of Halogenated Derivatives of Estrogens ——— The agonist activities of halogenated estrogens were measured with a yeast two-hybrid estrogenicity assay[7,8] using yeast cells (*Saccharomyces cerevisiae* Y190) into which the human (hERα) or medaka estrogen receptor α (medERα: *Oryzias latipes*) and coactivator TIF2 had been introduced. Expression plasmids for each hormone receptor ligand binding domain and pGAAD424-TIF2 were introduced into yeast cells that carried the β-galactosidase reporter gene. Both assays were adapted to a chemiluminescent (CLN) reporter gene (for β-galactosidase) in a method using a 96-well culture plate. Agonist activity was recorded as the EC$_{\times 10}$ that was defined as the concentration of the test solution producing a CLN intensity 10 times that of the blank control. The CLN intensity of each test solution was adapted to the mean of two measurements. Further details of the assay were described in a previous paper.[3]

RESULTS AND DISCUSSION

Reaction of Estrogens with Hypochlorous Acid

Many kinds of chlorinated derivatives produced by the reaction of E2 and hypochlorous acid were detected in the previous report[9] by LC-MS/MS analysis. However, those products were not isolated, and the structures of most of them were not obvious. The isolation of pure products and the determination of their structures are required for environmental problems caused by chlorination in sewage treatment plants to be elucidated. To isolate the pure products, we carried out the reactions of E1, E2, E3, and EE2 in organic solvents and used preparative HPLC to separate them. The chemical structures of the products and the experimental results are shown in Fig. 1 and Table 1, respectively.

Because E1 was reported to be more present in the environment than E2,[10] the reaction of E1 with sodium hypochlorite was first carried out in a methanol solution. Five chlorinated derivatives, 2-chloroestrone (2-ClE1), 4-chloroestrone (4-ClE1), 2,4-dichloroestrone (2,4-diClE1), 2,4,16,16-tetrachloroestrone (2,4,16,16-tetraClE1), and 10-chloro-1,4-estradiene-3,17-dione (10-Cl-1,4-estradiene-3,7-dione) were isolated.

The production of 10-Cl-1,4-estradiene-3,7-dione is considered to be produced by oxidation of the 3-hydroxy group with hypochlorous acid to a corresponding quinone. A further chlorination of active methylene at the 16 position leads to being formed. A small amount of 4-chloro-2-methoxy-estrone (4-Cl-2-MeOE1) seems to be formed by substituting chlorine with a methoxy group.

The reactions of E2, E3, and EE2 with hypochlorous acid under similar conditions afforded the corresponding mono- and di-chlorinated derivatives, which were isolated in a pure state. E1 and its chlorinated derivatives were obtained in the reaction with E2 because a hydroxy group at the 17 position was oxidized with hypochlorous acid.
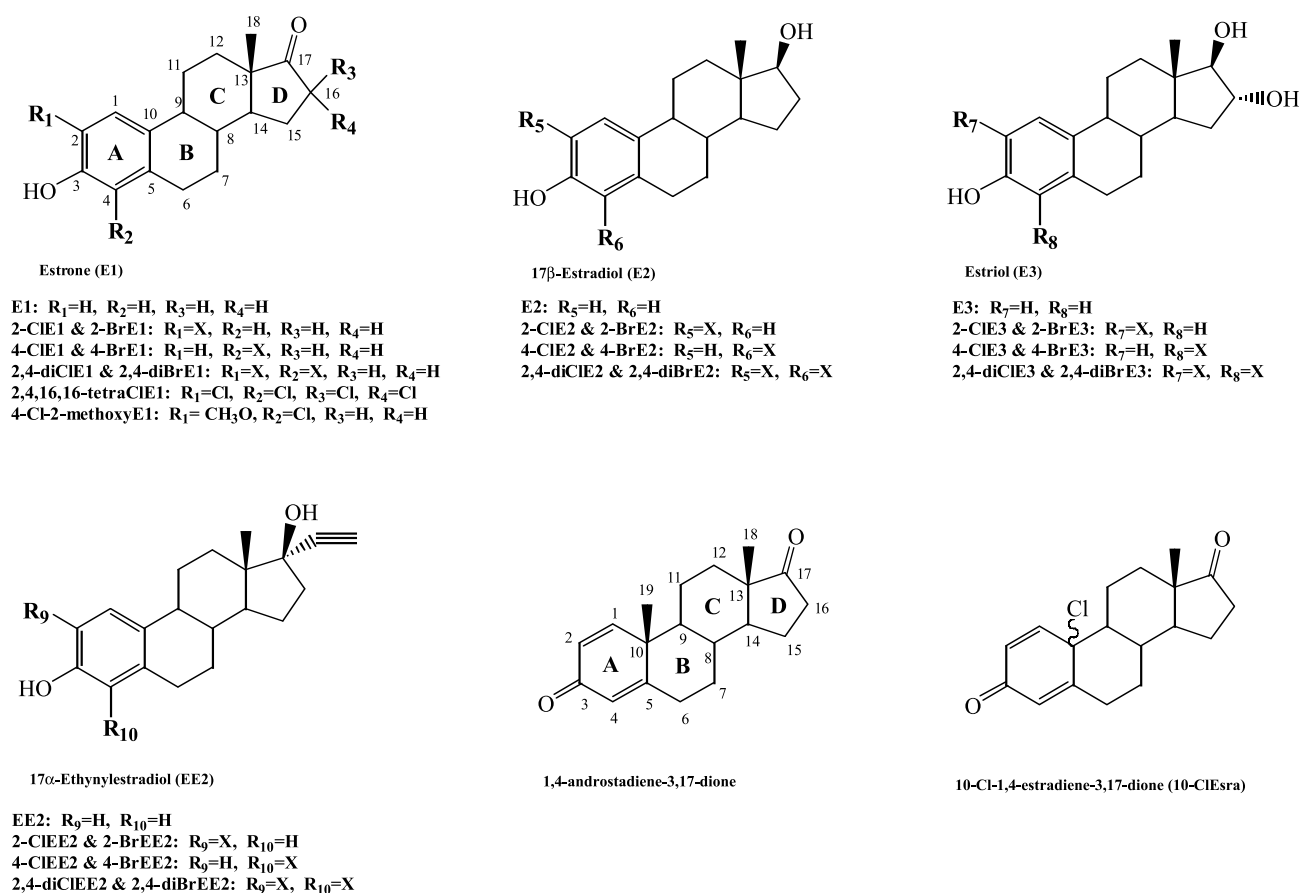
Estrone (E1)

E1: R$_1$=H, R$_2$=H, R$_3$=H, R$_4$=H
2-ClE1 & 2-BrE1: R$_1$=X, R$_2$=H, R$_3$=H, R$_4$=H
4-ClE1 & 4-BrE1: R$_1$=H, R$_2$=X, R$_3$=H, R$_4$=H
2,4-diClE1 & 2,4-diBrE1: R$_1$=X, R$_2$=X, R$_3$=H, R$_4$=H
2,4,16,16-tetraClE1: R$_1$=Cl, R$_2$=Cl, R$_3$=Cl, R$_4$=Cl
4-Cl-2-methoxyE1: R$_1$= CH$_3$O, R$_2$=Cl, R$_3$=H, R$_4$=H

17β-Estradiol (E2)

E2: R$_5$=H, R$_6$=H
2-ClE2 & 2-BrE2: R$_5$=X, R$_6$=H
4-ClE2 & 4-BrE2: R$_5$=H, R$_6$=X
2,4-diClE2 & 2,4-diBrE2: R$_5$=X, R$_6$=X

Estriol (E3)

E3: R$_7$=H, R$_8$=H
2-ClE3 & 2-BrE3: R$_7$=X, R$_8$=H
4-ClE3 & 4-BrE3: R$_7$=H, R$_8$=X
2,4-diClE3 & 2,4-diBrE3: R$_7$=X, R$_8$=X

17α-Ethynylestradiol (EE2)

EE2: R$_9$=H, R$_{10}$=H
2-ClEE2 & 2-BrEE2: R$_9$=X, R$_{10}$=H
4-ClEE2 & 4-BrEE2: R$_9$=H, R$_{10}$=X
2,4-diClEE2 & 2,4-diBrEE2: R$_9$=X, R$_{10}$=X

1,4-androstadiene-3,17-dione

10-Cl-1,4-estradiene-3,17-dione (10-ClEsra)

Fig. 1. Chemical Structures of E1, E2, E3, EE2, and their Halogenated Derivatives
X = Cl or Br.

Table 1. Reaction of Steroids with Hypochlorous Acid

Substrate	Solvent	Isolated compounds (yield, %)
E1	MeOH	E1 (6.9), 2-ClE1 (1.6), 4-ClE1 (12.2), diClE1 (4.4), 10-ClEstra (7.7), tetraClE1 (2.5), 4-Cl-2-MeOE1 (1.2)
E2	MeOH	E2 (19.5), 2-ClE2 (0.9), 4-ClE2 (5.2), diClE2 (7.5), E1 (0.1), 2-ClE1 (1.7), 4-ClE1 (1.5), diClE1 (0.02)
E3	MeOH	E3 (20.7), 2-ClE3 (3.4), 4-ClE3 (9.9), diClE3 (9.9)
EE2	MeOH	EE2 (19.2), 2-ClEE2 (1.7), 4-ClEE2 (5.8), diClEE2 (4.8)
E1	80% MeOH[a]	E1 (1.5), 2-BrE1 (1.7), 4-BrE1 (2.6), diBrE1 (3.7)
E2	80% MeOH[a]	E2 (3.5), 2-BrE2 (16.1), 4-BrE2 (22.2), diBrE2 (16.7)
E3	80% MeOH[a]	E3 (1.2), 2-BrE3 (7.0), 4-BrE3 (6.4), diBrE3 (2.7)
EE2	80% MeOH[a]	EE2 (0.4), 2-BrEE2 (20.6), 4-BrEE2 (41.1), diBrEE2 (12.9)

a) In the presence of KBr.

Determination of the Structures of Halogenated Estrogens

The structures of the products, 2-ClE1, 4-ClE1, and 2,4-diClE1 were determined by FAB-MS and NMR spectroscopy. The ^{1}H-NMR spectrum of 4-ClE1, a typical halogenated estrogen, is depicted in Fig. 2. The FAB-MS and ^{1}H-NMR spectral data for all halogenated compounds are summarized in Table 2. The ^{13}C-NMR spectral data for chlorinated derivatives of E1 are shown in Table 3.

In the FAB-MS spectra, each compound shows a parent peak at a mass number corresponding to M$^+$, including the peaks of chlorine isotopes. In the ^{1}H-NMR spectra, the regions of aromatic protons
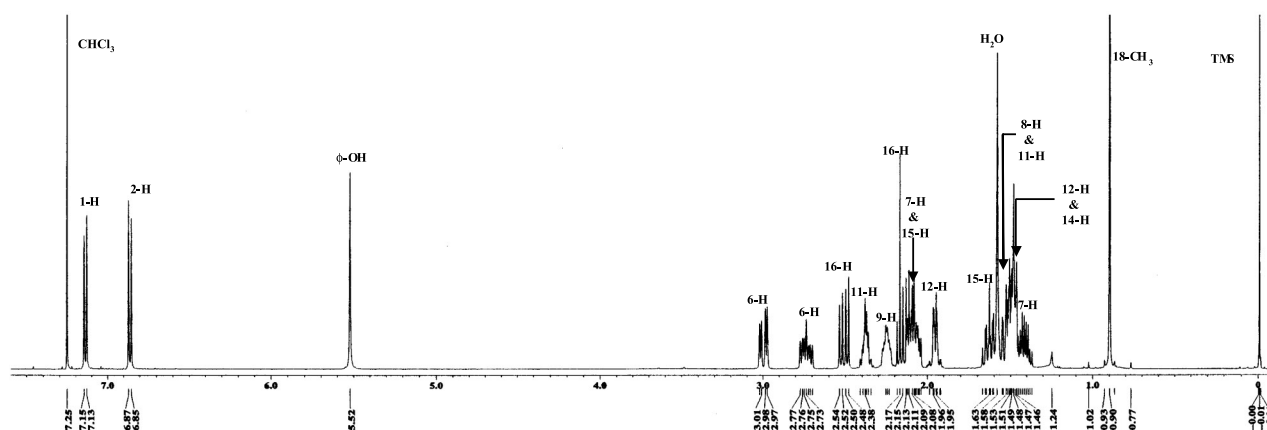
Fig. 2. ¹H-NMR Spectrum of 4-ClE1 as a Typical Halogenated Estrogen

show two singlet peaks (7.20 and 6.74 ppm) for 2-ClE1, two doublet peaks [7.14 ($J = 8.6$ Hz) and 6.86 ppm ($J = 8.6$ Hz)] for 4-ClE1, and a singlet peak (7.20 ppm) for 2,4-diClE1. The peaks for the other steroid skeleton exhibit a similar pattern to those of E1. The ¹³C-NMR spectra are also consistent with their structures.

The structure of 10-Cl-1,4-estradiene-3,7-dione was determined as follows. This compound was identified as a mono-chlorinated derivative by FAB-MS spectroscopy, where the relative isotopic abundance of chlorine in the parent peak is m/z 304 : 306 = 3 : 1. The ¹H-NMR spectrum shows an ABX spin coupled system in a low magnetic field: 7.10 ppm (¹H, d, $J = 10.3$ Hz), 6.18 ppm (¹H, dd, $J = 10.3$, 1.7 Hz), and 6.06 ppm (¹H, d, $J = 1.7$ Hz), which suggest that the A-ring is not an aromatic ring system. The UV spectrum also displays an absorption maximum at 238 nm — a shorter wavelength in comparison with that of an aromatic compound. A comparison of the ¹H-NMR spectrum with that of an authentic 1,4-androstadiene-3,17-dione revealed that both spectra are similar except for a signal caused by methyl at the 10 position of an authentic sample. In the ¹³C-NMR spectrum, a peak at 67.59 ppm assigned to C-10 shifts to the lower field by 24.10 ppm than the peak (43.49 ppm) assigned to C-10 of 1,4-androstene-3,17-dione, owing to chlorine substitution. These data support that this compound is 10-Cl-1,4-estradiene-3,17-dione formed by the oxidation with hypochlorous acid.

The FAB-MS spectrum of 2,4,16,16-tetraClE1 shows a parent peak at m/z 406 with a relative isotopic abundance of chlorine: m/z 406 : 408 : 410 : 412 : 414 = 18 : 23 : 12 : 3 : 1, indicating that it is tetra-

chlorinated estrone. The singlet peak at 7.19 ppm in the ¹H-NMR spectrum reveals that two chlorines were substituted at the 2 and 4 positions. In the ¹³C-NMR spectrum, a peak at 82.81 ppm appears as an obvious result of a low-field shift of a peak at 35.97 ppm due to methylene carbon at the 16 position of estrone, owing to the substitution of two chlorines. A peak due to the methyl group of estrone also shifts to the low field in the spectrum of this compound owing to the influence of two chlorines at C-16.

The data of the distortionless enhancement by polarization transfer (DEPT), ¹H-¹H correlation spectroscopy (¹H-¹H cosy), the heteronuclear multiple-quantum correlation (HMQC), and the heteronuclear multiple-bond correlation (HMBC) spectra were also consistent with the structure of each of the chlorinated compounds described above. The structures of chlorinated E2, E3, and EE2 were determined using a similar spectroscopic method to the E1 derivatives.

Reaction of Estrogens with Hypochlorous Acid in the Presence of Bromide Ions

Organobromine compounds were observed to form when the reaction with hypochlorous acid was carried out in the presence of bromide ions. We detected 2,4,6-tribromophenol, not 2,4,6-trichlorophenol, in an actual effluent from a sewage treatment plant near the sea.[11] Sea water including bromide ions was found to be introduced into the disinfection process of the plants. Therefore, formation of organobromine compounds and their toxicities are also not negligible for environmental pollution. On the basis of this point of view, we started the reac-

Table 2. ^{1}H-NMR (500 MHz) and FAB-MS Date for Estrogens and their Halogenated Derivatives

Compound	FAB-MS (m/z)	^{1}H-NMR (CDCl$_3$)[a]		
		Position		
		1	2	4
E1	270 (M$^+$)	7.15 d (8.6)	6.64 dd (2.9, 8.6)	6.58 d (2.9)
2-ClE1	304, 306 (M$^+$)	7.20 s		6.74 s
4-ClE1	304, 306 (M$^+$)	7.14 d (8.59)		6.86 d (8.59)
2,4-diClE1	338, 340, 342 (M$^+$)	7.20 s		
tetraClE1	406, 408, 410, 412, 414 (M$^+$)	7.19 s		
2-BrE1	348, 350 (M$^+$)	7.33 s		6.74 s
4-BrE1	348, 350 (M$^+$)	7.18 d (8.59)	6.86 d (8.59)	
2,4-diBrE1	426, 428, 430 (M$^+$)	7.40 s		
1,4-androstadiene	284 (M$^+$)	7.02 d (10.3)	6.20 dd (1.7, 10.3)	6.05 d (1.7)
10-Cl-1,4-estradiene	305, 307 (MH$^+$)	7.10 d (10.3)	6.18 dd (1.7, 10.3)	6.06 d (1.7)
4-Cl-2-methoxyE1	334, 336 (M$^+$)	6.88 s		
E2	272 (M$^+$)	7.15 d (8.6)	6.62 dd (2.9, 8.6)	6.55 d (2.3)
2-ClE2	306, 308 (M$^+$)	7.19 s		6.72 s
4-ClE2	306, 308 (M$^+$)	7.14 d (8.6)	6.85 d (8.6)	
2,4-diClE2	340, 342, 344 (M$^+$)	7.20 s		
2-BrE2[b]	350, 352 (M$^+$)	7.27 s		6.56 s
4-BrE2[b]	350, 352 (M$^+$)	7.09 dd (8.4)	6.68 dd (8.4)	
2,4-diBrE2[b]	428, 430, 432 (M$^+$)	7.35 s		
E3	288 (M$^+$)	7.05 d (8.6)	6.51 dd (2.3, 8.6)	6.45 d (2.3)
2-ClE3	322, 324 (M$^+$)	7.11 s		6.57 s
4-ClE3	322, 324 (M$^+$)	7.06 d (8.6)	6.70 d (8.6)	
2,4-diClE3	356, 358, 360 (M$^+$)	7.15 s		
2-BrE3[b]	366, 368 (M$^+$)	7.27 s		6.57 s
4-BrE3[b]	366, 368 (M$^+$)	7.09 dd (8.4)	6.69 dd (8.4)	
2,4-diBrE3[b]	444, 446, 448 (M$^+$)	7.38 s		
EE2	296 (M$^+$)	7.16 d (8.6)	6.63 dd (2.3, 8.6)	6.56 d (2.3)
2-ClEE2	330, 332 (M$^+$)	7.20 s		6.73 s
4-ClEE2	330, 332 (M$^+$)	7.15 d (8.6)	6.85 d (8.6)	
2,4-diClEE2	364, 366, 368 (M$^+$)	7.21 s		
2-BrEE2	374, 376 (M$^+$)	7.25 s		6.73 s
4-BrEE2	374, 376 (M$^+$)	7.19 dd (8.4)	6.85 dd (8.4)	
2,4-diBrEE2	452, 454, 456 (M$^+$)	7.40 s		

a) Chemical shifts are δ values and followed by multiplicit (s = singlet, d = doublet, dd = doublet of doublets) and J values (Hz). b) ^{1}H-NMR spectrum was measured in methanol-d_4.

tions of E1, E2, E3, and EE2 with hypochlorous acid in acidic 80% methanol in the presence of potassium bromide.

Brominations of the estrogens were revealed to take place rapidly under these conditions. Although HPLC did not detect many chlorinated derivatives, three kinds of corresponding brominated estrogens shown in Table 1 were isolated from every reaction mixture.

The structures of the brominated derivatives were determined by referring to MS and NMR spectra of the corresponding chlorinated derivatives (Table 2).

Estrogenic Activities of Halogenated E1, E2, E3, and EE2 in Yeast Two-Hybrid Assay

The agonist activities of chlorinated and brominated derivatives of E1, E2, E3, and EE2 were measured by means of a yeast two-hybrid estrogenicity assay using yeast cells (Y190) into which the hERα or medERα had been introduced.

The dose-response curves for the chlorinated derivatives of E1 are shown in Fig. 3. The estrogenic activities, EC$_{\times 10}$ (with hERα) of E1, 2-ClE1, 4-ClE1, 2,4-diClE1, and 2,4,16,16-tetraClE1 were 0.50, 0.74, 0.52, 24 nM, and not detected, respectively. Although the activity of 4-ClE1 was similar to that of E1, the

Table 3. ^{13}C-NMR (δ, CDCl$_3$) Data for Chlorinated Derivatives of Estrone

Position	Compound						
	2-ClE1	4-ClE1	2,4-diClE1	tetraClE1	1,4-androstadiene	10-Cl-1,4-estradiene	4-Cl-2-methoxyE1
1	125.83	124.75	124.84	124.61	155.35	147.53	111.18
2	117.29	113.05	117.96	118.07	127.78	126.96	141.5
3	149.15	149.42	145.75	145.89	186.24	184.97	147.27
4	116.11	120.29	121.2	121.18	124.2	124.17	126.96
5	137.17	134.8	134.2	133.77	168.35	160.52	137.71
6	29.08	27.96	27.77	27.44	32.61	32.14	27.17
7	26	26.15	26.1	26.1	32.37	31.51	26.06
8	38.11	37.62	37.44	36.2	35.18	35.61	37.51
9	43.87	44.14	43.93	43.61	52.37	53.34	44.44
10	133.41	133.48	133.98	133.07	43.49	67.59	127.14
11	26.42	26.33	26.18	25.49	22.17	22.55	26.45
12	31.53	31.58	31.46	32.2	31.27	30.75	31.59
13	48.03	47.97	47.89	47.17	47.75	47.7	47.92
14	50.42	50.35	50.31	45.55	50.5	49.8	50.49
15	21.65	21.64	21.61	45.74	21.99	21.97	21.63
16	35.93	35.95	35.91	82.81	35.7	35.68	35.95
17	220.82	220.82	220.56	205.27	219.95	219.86	220.74
18	13.91	13.89	13.84	15.68	13.89	13.83	13.86
19					18.8		
CH$_3$O							60.99

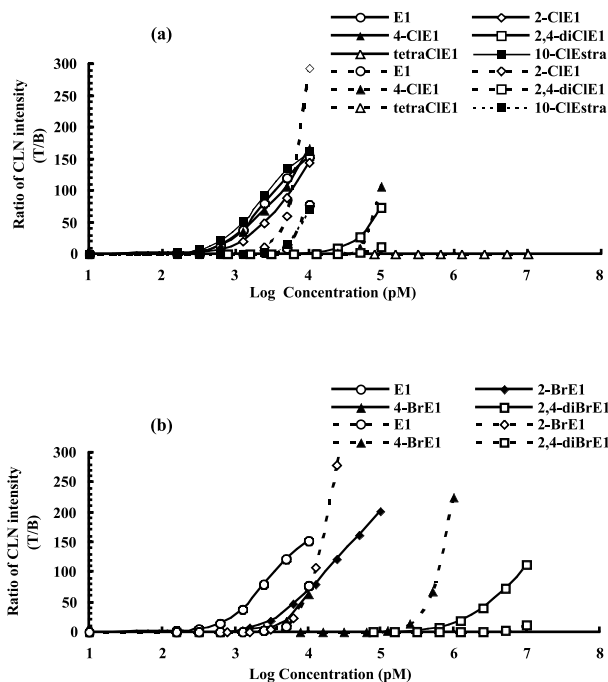**Fig. 3.** Dose–Response Curves for Chlorinated E1 (a) and Brominated E1 (b) Using the Agonist (–S9) Test for the Yeast Two-Hybrid Assay with hERα (Full Lines) and medERα (Dashed Lines)

The values are represented as the ratio of CLN intensity (T/B) of β-galactosidase.

activities of 2-ClE1 and 2,4-diClE1 were approximately 4/5 and 1/50 that of E1. Furthermore, no activity was detected in 2,4,16,16-tetraClE1 with two chlorines substituted at C-16. These results support the idea that the estrogenic activity of E1 is influenced by a chlorine substituted at the 2 and 4 positions on A-ring and by the number of chlorines substituted.

It is of interest that the activity of 10-Cl-1,4-estradiene-3,7-dione produced by oxidation of the A-ring with chlorination at C-10 was approximately 1.4 times stronger than that of E1. In order to clarify the effect of a steroidal skeleton on estrogenicity, we measured the activity of 1,4-androstadiene-3,17-dione under the same conditions. However, no estrogenicity was detected. A chlorine substituted at C-10 seems to play a important role in the estrogenic activity of 10-Cl-1,4-estradiene-3,7-dione. Dechlorination at the 10 position followed by protonation of the carbonyl group could aromatize the A-ring, resulting in E1 under the conditions.

The estrogenicities of chlorinated derivatives of E2, E3, and EE2 showed a similar tendency to those of E1. Although brominated derivatives showed slightly weaker activity than the corresponding chlorinated derivatives, the effect of bromine substitution at the 2 and 4 positions on the estrogenic activi-

Table 4. Estrogen-Agonist Activities of Halogenated Derivatives Using the Yeast Two-Hybrid System (hERα and medERα)

Compound	hERα Assay ($-$S9)			medERα Assay ($-$S9)	
	Concentration range (pM)[a]	EC$_{\times 10}$[b] nM	Relative activity BPA = 1	Concentration range (pM)[a]	EC$_{\times 10}$[b] nM
E1	0–10	0.5	8.4×10^3	0–10	4.1
2-ClE1	0–10	0.74	5.7×10^3	0–10	2.1
4-ClE1	0–10	0.52	8.1×10^3	0–10^2	40
2,4-diClE1	0–100	24	1.8×10^2	0–10^2	90
2,4,16,16-tetraClE1	0–10^4	$< 10^4$	ND[c]	0–10^4	$< 10^4$
1,4-androstadiene	0–10^4	$< 10^4$	ND[c]	0–10^4	$< 10^4$
10-Cl-1,4-estradiene	0–10	0.36	1.2×10^4	0–10	3.7
2-BrE1	0–10^2	2.0	2.1×10^3	0–10^2	3.7
4-BrE1	0–10	3.0	1.4×10^3	0–10^3	2.0×10^2
2,4-diBrE1	0–10^4	8×10^2	5.0	0–10^4	9.2×10^3
E2	0–5	0.17		0–5	0.56
2-ClE2	0–10	0.34		0–10	0.62
4-ClE2	0–10	0.16		0–10	1.2
2,4-diClE2	0–10^2	15		0–10^2	24
2-BrE2	0–10^2	1.2		0–10^2	1.9
4-BrE2	0–10^2	1.1		0–10^2	5.8
2,4-diBrE2	0–10^3	5×10^2		0–10^3	5.8×10^2
E3	0–10^3	40		0–10^3	2.2×10^2
2-ClE3	0–10^3	1.4×10^2		0–10^3	4.2×10^2
4-ClE3	0–10^3	29		0–10^3	3.7×10^2
2,4-diClE3	0–10^4	1.6×10^3		0–10^4	4.9×10^3
2-BrE3	0–10^4	3.4×10^2		0–10^4	7.0×10^2
4-BrE3	0–10^3	72		0–10^3	1.3×10^2
2,4-diBrE3	0–10^4	$< 10^4$		0–10^4	ND[c]
EE2	0–10	0.14		0–10	0.42
2-ClEE2	0–10	0.38		0–10	0.68
4-ClEE2	0–10	0.43		0–10	3.3
2,4-diClEE2	0–10^2	36		0–10^2	47
2-BrEE2	0–10	0.73		0–10	1.3
4-BrEE2	0–10^2	7.1		0–10^2	69
2,4-diBrEE2	0–10^4	6.3×10^3		0–10^4	5.4×10^3

a) Concentration ranges prepared for evaluation of EC$_{\times 10}$. b) EC$_{\times 10}$ was defined as the concentration at which the ratio of the chemiluminescent signal of the sample to that of a blank control was 10. c) ND: not detected.

ties also had a similar tendency to those of chlorinated derivatives.

Serious differences in the sensitivity were not observed between human and medaka estrogen receptors. However, it is of interest that in the yeast with medERα, estrogens halogenated at the 2 position showed stronger activities than those at the 4 position except halogenated E3. When with hERα the derivatives halogenated at the 4 position showed stronger activities.

Fukazawa *et al*. reported that bisphenol A (BPA) and its chlorinated derivatives were detected in the effluent from several pulping processes for waste paper containing thermal paper and/or other printed paper.[3] Also, chlorination enhanced the activity using the same assay system as the one in this study. A summary of the comparison of the estrogenic activities (with hERα) of BPA is in Table 4. The estrogenic activities of chlorinated estrogens are entirely stronger than those of BPA. The mono-chlorinated derivatives of E1, E2, and EE2 show approximately 10^3–10^4 times stronger activity. Even di-chlorinated derivatives are approximately 150–250 times stronger. The brominated estrogen also shows stronger estrogenic activity than BPA.

Thus, the hypochlorous acid was revealed to react easily with estrogens to create the chlorinated derivatives and brominated derivatives when in the

presence of bromide ions. The halogenation of estrogens at the 2 and 4 positions tends to decrease the estrogenic activity. In particular, the di-chlorinated derivatives result in weak activity. It is assumed to be due to disruption in the interaction of the 3-hydroxyl group with the receptor. Because we have already detected chlorinated derivatives of E1 in the effluent from one sewage treatment plant (data not shown), we have to investigate the features of halogenated derivatives for biodegradation and accumulation in the body. We also need to assess not only the estrogenic activity but also the other bioactivities in them.

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