Detection of Thyroid Hormone Receptor-Binding Activities of Chemicals Using a Yeast Two-Hybrid Assay

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The adverse effects of chemicals exerted *via* estrogen receptors (ER) and androgen receptors (AR) have been studied extensively in recent years. However, those occurring *via* thyroid hormone receptors (TR) have not been studied enough. We examined the TR-binding activities of thyronine derivatives and alkylphenol derivatives (bisphenol A, parabens and antioxidants with *o*-*t*-butylphenol residue) using a yeast two-hybrid assay. In this assay system, the TR-binding activity of 3,5,3′-triiodo-L-thyronine (T_3) was detectable at concentrations as low as 3.0×10^{-8} M and reached a plateau at 1.0×10^{-6} M. The concentration of T_3 producing 10% of the activity stimulated at 1.0×10^{-6} M (10% relative effective concentration, REC_{10}) was 2.1×10^{-8} M. 3,5,3′,5′-tetraiodo-L-thyronine (T_4), 3,3′,5′-triiodo-L-thyronine (rT_3) and 3, 5-diiodo-L-thyronine (T_2) also exhibited TR-binding activities. The REC_{10} values of these chemicals were 4.2×10^{-8}, 5.0×10^{-7} and 1.0×10^{-5} M, respectively. *o*-Isopropylphenol and *o*-*t*-butylphenol exhibited TR-binding activities with REC_{10} values of 3.1×10^{-4} and 4.8×10^{-5} M, respectively, whereas *t*-butylbenzene, isopropylbenzene and the other chemicals tested had no detectable TR-bindng activity. These results suggest that a phenolic hydroxyl group and the *ortho*-substituents may play important roles in the TR-binding activities of these chemicals. This assay system will be a useful tool for screening the TR-binding activities of chemicals.

Key words —— thyroid hormone, yeast two-hybrid assay, phenolic hydroxyl group, *o*-isopropylphenol, *o*-*t*-butylphenol

INTRODUCTION

Since the publication of "Our Stolen Future"[1] in 1996, there has been considerable concern about the disruptive effects of chemicals on the endocrine systems of humans and wild life, especially *via* the hormone receptors.[2] Chemicals with such effects are referred to as endocrine disruptors (EDs). The effects of chemicals on the function of steroid hormones, especially estrogen and androgen, have been studied extensively.[3–6] Most of the activity of steroid hormones is mediated through their receptors, which reside in the cell nucleus and regulate the transcription of target genes in a ligand-dependent manner.[7] Nuclear hormone receptors constitute a large superfamily of ligand-inducible transcriptional factors.[8] TR is a member of this superfamily as are the receptors for steroid hormones. Thyroid hormone fulfils an important role in the development of neurons, multiplication of cells, cell death and energy metabolism of many organisms.[9] Thus, there may be chemicals which perturb the endocrine system by modulating TR function. For relevant examples, it is doubted that the dioxins and polychlorinated biphenyls (PCBs) produce their adverse effects by disrupting thyroid hormone function directly.[10,11] Compared to the numerous reports regarding estrogen receptors (ER)[3–5] and androgen receptors (AR),[2,6] there are few concerning the biological effects of chemicals that act through thyroid hormone receptors (TR).

As a method for evaluating the thyromimetic activities of chemicals, a frog metamorphosis assay[12] and a thyroid hormone receptor binding assay[10] have been established. We have developed a novel screening method for chemicals with hormonal activities

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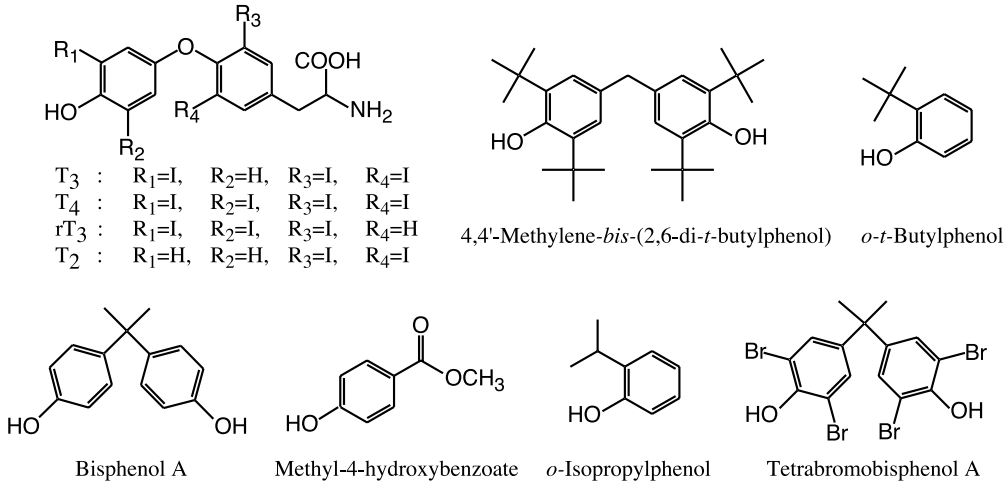

Fig. 1. Typical Structures of the Chemicals Tested in the Yeast-Two-Hybrid Assay

using a yeast two-hybrid system and demonstrated that this method detected the ER-, AR- and TR-binding activities of chemicals.[13] We tested the ER-binding activities of 517 chemicals using the yeast two-hybrid assay.[14] In that manuscript, we reported that a phenol with a hydrophobic moiety at the *para*-position is a key structural motif of ER-binding chemicals. These results were supported by the findings obtained from a crystallographic study of the ligand-binding ER. The study reported that the structural features of 17-β-estradiol (E_2), a hydrophobic steroid skeleton with a phenolic hydroxyl group, played a crucial role in the interaction between ER and E_2.[15] There is a reasonable relationship between the results obtained from the yeast two-hybrid assay and findings from the crystallographic study. Wagner *et al*. performed crystallographic analysis of TR and its ligands and reported that a phenol group in the ligands also played a key role in activation of the receptor.[16] For designing an iodo-free drug with thyromimetic activity, an *o*-isopropylphenol residue is useful for optimization of the activity.[17] Hydroxylated PCBs compete with 3,5,3′,5′-tetraiodo-L-thyronine (T_4) for the binding to transthyretin to perturb the thyronine hormone homeostasis.[18] Thus, there is relevance to studies of the TR-binding activities of chemicals with a phenol group. There are few reports concerning the thyromimetic activities of chemicals. Here, we examined the TR-binding activities of chemicals with a phenol group, such as alkylphenols, bisphenol A, tetrabromobisphenol A and antioxidants with a *t*-butylphenol residue, as well as thyronines, by our yeast two-hybrid assay.

MATERIALS AND METHODS

Chemicals —— 3,5,3′-Triiodo-L-thyronine (T_3; > 97%) was purchased from Fluca (Steinheim, German). T_4 was purchased from ICN Biomedicals Inc. (Costa Mesa, CA, U.S.A.). 3,3′,5′-Triiodo-L-thyronine (rT_3) and 3,5-diiodo-L-thyronine (T_2) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). L-Thyronine was purchased from Bachem AG (Bubendorf, Switzerland). All other chemicals were reagent grade and used without further purification. The chemical structures tested in the yeast two-hybrid assay are shown in Fig. 1.

The Yeast Two-Hybrid Assay —— The TR-binding activities of chemicals were examined with a yeast two-hybrid assay system with the thyroid hormone receptor, TRα, and the coactivator, transcriptional intermediary factor 2 (TIF2), as described previously.[13] Minor modifications of the duration of test chemical incubation were made in order to raise the assay sensitivity. The yeast expression plasmids, pGBT9 and pGAD424, were purchased from Clontech (Palo Alto, CA, U.S.A.). The LBD of TRα (codons 173-461) was amplified by reverse transcription (RT)-PCR using total RNA from rat liver. The *Eco*RI and *Bam*HI sites were introduced in 5′ and 3′ terminus of amplified fragments and subcloned into *Eco*RI-*Bam*HI sites of pGBT9 so that they were in the same translational reading frame as the vector's GAL4 DNA binding domain (GAL4DBD). The receptor interaction domain (RID) of coactivator, TIF2, was amplified by PCR from cDNAs[19] and subcloned into *Eco*RI-*Bam*HI-*digested* pGAD424 for the production of fusion pro-

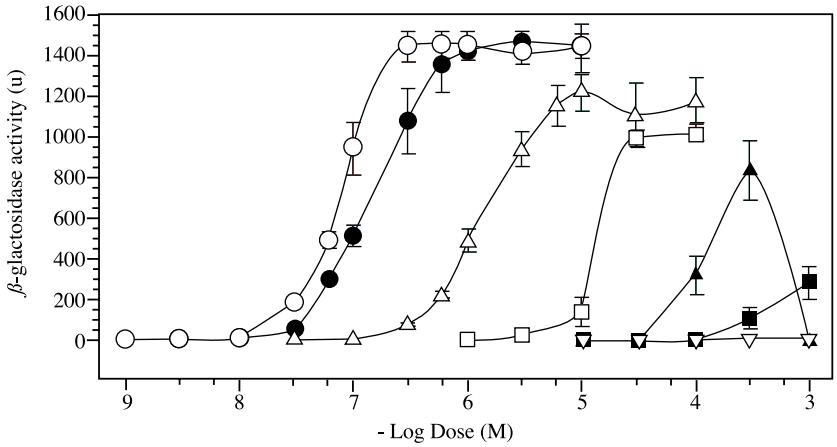

Fig. 2. Dose–Response Curves for the TR-Binding Activities of Thyronine Derivatives, *o*-Isopropylphenol and *o*-*t*-Butylphenol

The points represent averages β-galactosidase activities obtained from three independent experiments on separate days. Bars represent S.D. T_3 (○); T_4 (●); rT_3 (△); T_2 (□); *o*-*t*-butylphenol (▲); *o*-isopropylphenol (■) ; thyronine (▽).

teins with the GAL4 activation domain (GAL4AD). All sequences generated by PCR were confirmed by DNA sequencing. The yeast strain used in this study was Y190 (*MATa, ura3-52, his-D200, ade2-101, trp1-901, leu2-3, 112, gal4Dgal80D, URA3:::GAL-lacZ, cyhr2, LYS2:::GAL-HIS3*), obtaind from Clontech (Palo Alto). Yeast cells were transformed with the pGBT9-TRα and pGAD424-TIF2 using lithium acetate method and selected by growth on synthetic dropout (SD) medium (lacking tryptophan and leucine). Yeast transformants were preincubated overnight at 30°C in SD medium free from tryptophan and leucine. The culture (250 μl) was then mixed in a small test tube with a DMSO solution (2.5 μl) of test chemical and incubated for 24 hr at 30°C. After washing by centrifugation, the cells were digested enzymatically by incubation with 1 mg/ml Zymolyase 20T (100 μl) at 37°C for 15 min. The lysate was mixed with 4 mg/ml *o*-nitrophenyl-β-D-galactoside (40 μl) and reacted at 30°C until development of the yellow color before the reaction was stopped by the addition of 1 M Na_2CO_3 (100 μl). β-Galactosidase activity is calculated as described previously and represents the mean ± S.D. of 3 independent experiments performed on separate days. The results were evaluated by relative activity, expressed as 10% relative effective concentration (REC_{10}), that is the concentration of the test chemical showing 10% of the agonist activity of 1.0×10^{-6} M T_3, which is the optimum concentration for T_3. When the activity of test chemical was higher than REC_{10} within the concentration tested, we judged the chemical as positive. When it was judged to be negative, the highest dose tested is indicated.

RESULTS

The β-galactosidase activities induced by T_3 were detectable from 3.0×10^{-8} M and reached a plateau at 1.0×10^{-6} M. The β-galactosidase activity obtained by incubation with 1.0×10^{-6} M T_3 was 1460 ± 60 u (Fig. 2). REC_{10} value of T_3 was 2.1×10^{-8} M. Except for thyronine, thyronine derivatives T_4, rT_3 and T_2, also exhibited TR-binding activities. Their REC_{10} values are listed in Table 1. The maximal activity induced by T_4 reached that of T_3 at 1.0×10^{-6} M and higher. The activity induced by rT_3 and T_2 reached a plateau from 10^{-5} M and 3.0×10^{-5} M at around 80 and 70% of 1.0×10^{-6} M T_3, respectively. Tyrosine and its iodinated derivatives did not exhibit TR-binding activities within their soluble concentration. However, 3.0×10^{-4} M 3,5-dibromotyrosine exhibited weak TR-binding activity (less than 10% activity of 1.0×10^{-6} M T_3).

Among alkylphenols, *p*-hydroxybenzoates and bisphenol A derivatives were examined, only *o*-isopropylphenol and *o*-*t*-butylphenol exhibited TR-binding activities (Fig. 2). The REC_{10} values of *o*-isopropylphenol and *o*-*t*-butylphenol were 3.1×10^{-4} and 4.8×10^{-5} M, respectively. Cytotoxic effects of these chemicals were observed at 1.0×10^{-3} M and higher. Other alkylphenols, *p*-hydroxybenzoates and bisphenol A derivatives did not exhibit TR-binding activities. Isopropylbenzene and *t*-butylbenzene also did not exhibit TR-binding activities. Chemicals with *t*-butylphenol residue, which are used as antioxidants commercially were examined and found to be negative in this assay system (Table 1).

Table 1. Detection of TR-Binding Activities of Chemicals Using a Yeast Two-Hybrid Assay

	Compounds	$REC_{10}(M)^{a)}$
Thyronine derivatives	3,5,3′-Triiodo-L-thyronine (T_3)	2.1×10^{-8}
	Thyroxine (T_4)	4.2×10^{-8}
	3,3′,5′-Triiodo-L-thyronine (rT_3)	5.0×10^{-7}
	3,5-Diiodo-L-thyronine (T_2)	1.0×10^{-5}
	L-Thyronine	$> 1.0 \times 10^{-4}$
Thyrosine derivatives	Thyrosine	$> 3.0 \times 10^{-4}$
	3-Iodotyrosine	$> 1.0 \times 10^{-5}$
	3,5-Diiodo-tyrosine	$> 1.0 \times 10^{-4}$
	3,5-Dibromo-tyrosine	$> 3.0 \times 10^{-4}$ *b)*
Phenols	*p*-Methylphenol	$> 3.0 \times 10^{-4}$
	p-Ethylphenol	$> 3.0 \times 10^{-4}$
	p-n-Propylphenol	$> 3.0 \times 10^{-4}$
	p-s-Butylphenol	$> 3.0 \times 10^{-4}$
	p-t-Butylphenol	$> 3.0 \times 10^{-4}$
	p-n-Butylphenol	$> 3.0 \times 10^{-4}$
	p-t-Pentylphenol	$> 3.0 \times 10^{-4}$
	p-n-Pentylphenol	$> 3.0 \times 10^{-4}$
	p-n-Hexylphenol	$> 3.0 \times 10^{-4}$
	p-n-Heptylphenol	$> 3.0 \times 10^{-4}$
	p-t-Octylphenol	$> 3.0 \times 10^{-4}$
	p-n-Octylphenol	$> 3.0 \times 10^{-4}$
	p-br-Nonylenol	$> 3.0 \times 10^{-4}$
	p-n-Dodecylphenol	$> 3.0 \times 10^{-4}$
	o-Methylphenol	$> 3.0 \times 10^{-4}$
	o-Ethylphenol	$> 3.0 \times 10^{-4}$
	o-Isopropylphenol	3.1×10^{-4}
	o-n-Propylphenol	$> 3.0 \times 10^{-4}$
	o-t-Butylphenol	4.8×10^{-5}
	o-n-Butylphenol	$> 3.0 \times 10^{-4}$
	o-Phenylphenol	$> 3.0 \times 10^{-4}$
	o-Iodophenol	$> 3.0 \times 10^{-4}$
	m-t-Butylphenol	$> 3.0 \times 10^{-4}$
Parabens	Methyl 4-hydroxybenzoate	$> 3.0 \times 10^{-4}$
	Ethyl 4-hydroxybenzoate	$> 3.0 \times 10^{-4}$
	Propyl 4-hydroxybenzoate	$> 3.0 \times 10^{-4}$
	Butyl 4-hydroxybenzoate	$> 3.0 \times 10^{-4}$
	Benzyl 4-hydroxybenzoate	$> 3.0 \times 10^{-4}$
Bisphenol A derivatives	Bisphenol A	$> 3.0 \times 10^{-4}$
	Tetrabromobisphenol A	$> 3.0 \times 10^{-4}$
Benzenes	Isopropylbenzene	$> 3.0 \times 10^{-4}$
	t-Butylbenzene	$> 3.0 \times 10^{-4}$

a) The concentration showing 10% activity of 10^{-6} M T_3 (relative activity).
b) The activity of 1.0×10^{-3} M L-Thyronine was less than 10% the activity of the 1.0×10^{-6} M T_3.

DISCUSSION

T_3 and T_4 have agonist activity at TR, whereas rT_3 and T_2 do not.[20] In this study, T_3, T_4, rT_3 and T_2 exhibited TR activating ability with the rank order of potency $T_3 > T_4 > rT_3 > T_2$. rT_3 and T_2 were partial-agonists at TR in this assay system. The substantial lengths of the TR and TIF2 were fused with

Table 1. Continued

	Compounds	$REC_{10}(M)^{a)}$
Antioxidants	BHT	$> 3.0 \times 10^{-4}$
	BHA	$> 3.0 \times 10^{-4}$
	4-Hydroxymethyl-2,6-di-*t*-butylphenol	$> 3.0 \times 10^{-4}$
	3-(3′,5′-Di-*t*-butyl-4′-hydroxyphenyl)propionic acid	$> 3.0 \times 10^{-4}$
	4-4′-Thiobis(3-methyl-6-*t*-butylphenol)	$> 3.0 \times 10^{-4}$
	4,4′-Methylenebis(2,6-di-*t*-butylphenol)	$> 3.0 \times 10^{-4}$
	2,2′-Methylenebis(4-methy-6-*t*-butylphenol)	$> 3.0 \times 10^{-4}$
	2,2′-Methylenbis(4-ethyl-6-*t*-butylphenol)	$> 3.0 \times 10^{-4}$
	1,1,3-Tris(2-methyl-4-hydroxy-5-*t*-butylphenyl)butane	$> 3.0 \times 10^{-4}$
	1,3,5-Trimethyl-2,4,6-tris(3,5-di-*t*-butyl-4-hydroxybenzyl)benzene	$> 3.0 \times 10^{-4}$
	Pentaerythritol tetrakis[3-(3′5′-di-*t*-butyl -4′-hydroxyphenyl)propionate]	$> 3.0 \times 10^{-4}$

the GAL4 DNA binding domain and activation domain, respectively. The intact TR and TIF2 were not produced in the yeast cells. This background in the assay system could be one reason for our observations.

Drug design studies on iodine-free TR agonists produced the following information: 1) a phenol group is essential for activity; 2) the amino acid residue of T_3 is not necessary; and, 3) 3′-iodine can be replaced by an isopropyl group.[17] By the crystallographic analysis of TR, it was also found that a phenol group in ligands plays an important role in activation of the receptor.[16] Thus, we conducted an examination of the TR-binding activities of alkylphenols, parabens and bisphenol A derivatives. *o*-Isopropylphenol and *o*-*t*-butylphenol exhibited activities. When β-galactosidase was induced by the interaction of these chemicals and the endogenous factor of the yeast, it was assumed that the activities did not occur *via* TR-TIF2. However, we have confirmed that these chemicals did not exhibit ER-binding activities in the yeast two-hybrid assay with ER-TIF2 (data not shown). Therefore these chemicals actually exert these actions *via* TR-TIF2. All other alkylphenols examined were negative. The size and position of alkyl group could be a restrictive factor. [4-(4-Hydroxy-3-isopropyl-benzyl)-3,5-dimethyl-phenoxy]-acetic acid,[17] 3,5-dimethyl-3′-isopropylthyronine[16] and 3,5-dibromo-3′-isopropylthyronine[16] were synthesized as iodine-free TR agonists. These chemicals adopted the isopropyl group as a substitute for iodine. In *o*-isopropylphenol and *o*-*t*-butylphenol, the isopropyl group and the *t*-butyl group seemed to mimic the iodine of the 3′ position of T_3, respectively. Interestingly, *o*-iodophenol did not exhibit activity. Iodine is an electron-accepting substituent, whereas the isopropyl and *t*-butyl groups are electron-donating substituents. The degree of dissociation of phenol may also affect the TR-binding activities of *o*-alkylphenols.

The phenolic system antioxidants, listed in Table 1, have an *o*-*t*-butylphenol residue in the molecule. These antioxidants are important industrial materials, which are widely used in foods and resins. We also evaluated the TR-binding activities of these antioxidants. No antioxidants exhibited TR-binding activities. Except for BHA and BHT, the structures of antioxidants were larger than those of thyroid hormones and *o*-*t*-butylphenol. Steric hindrance may prevent the antioxidants from binding to TR and/or activating it. These antioxidants are decomposed to the smaller molecules with *o*-*t*-butylphenol *in vivo*.[21,22] Their TR-binding activities could be examined subsequent to this report. Both butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), derivatives of 2,6-di-*t*-butylphenol, were negative. The additional substitution at the *ortho*-position of *o*-*t*-butylphenol may reduce the TR-binding activity as 5′-iodine of T_4 reduced the activity of T_3.

3,5-Dibromo-tyrosine exhibited weak TR-binding activity. This result suggests that chemicals with *o*-bromophenol residue may affect the activation of TR. Hydroxylated polybrominated biphenyls (PBBs) and tetrabromobisphenol A (TBBPA) compete with T_4 for binding to transthyretin.[23] The study of TR-binding and anti-TR-binding activities of PBBs and polybrominated dipheyl ethers (PBDEs) and their metabolites are ongoing in our laboratory.

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REFERENCES

1) Colborn, T., Dumanoski, D. and Myers, J. P. (1996) *Our Stolen Future*, A Dutton Book Press, New York.
2) Kelce, W. R., Stone, C. R., Laws, S. C., Earl Gray, L., Kemppainen, J. A. and Wilson, E. M. (1995) Persistant DDT metabolite p,p′-DDE is a potent androgen receptor antagonist. *Nature* (London), **375**, 581–585.
3) Connor, K., Ramamoorthy, K., Moore, M., Mustain, M., Chen, I., Safe, S., Zacharewski, T., Gillesby, B., Joyeux, A. and Balaguer, P. (1997) Hydroxylated PCBs as estrogens and antiestrogens: Structure-activity relationships. *Toxicol. Appl. Pharmacol.*, **145**, 111–123.
4) Welch, R. M., Levin, W. and Conney, A. H. (1969) Estrogenic action of DDT and its analogs. *Toxicol. Appl. Pharamacol.*, **14**, 358–367.
5) Zacharewski, T. R., Meek, M. D., Clemons, J. H., Wu, Z. F., Fielden, M. R. and Matthews, J. B. (1998) Examination of the in vitro and in vivo estrogenic activities of eight commercial phthalate esters. *Toxicol. Sci.*, **46**, 282–293.
6) Kelce, W. R., Lambright, C. R., Earl Gray, L. and Roberts, K. P., Jr. (1997) Vinclozolin and p,p′-DDE alter androgen-dependent gene expression: in vivo confirmation of an androgen receptor-mediated mechanism. *Toxicol. Appl. Pharamacol.*, **142**, 192–200.
7) Oro, A. E., Umesono, K. and Evans, R. M. (1989) Steroid hormone receptor homologs in development. *Development*, **107**, 133–140.
8) Weatherman, R. V., Fletterick, R. J. and Scanlan, T. S. (1999) Nuclear-receptor ligands and ligand-binding domains. *Annu. Rev. Biochem.*, **68**, 559–581.
9) Kirsten, D. (2000) The thyroid gland: physiology and pathophysiology. *Neonatal Netw.*, **19**, 11–26.
10) McKinney, J., Fannin, R., Jordan, S., Chae, K., Rickjenbacher, U. and Pedersen, L. (1987) Polychlorinated biphenyls and related compound interactions with specific binding sites for thyroxine in rat liver nucler extracts. *J. Med. Chem.*, **30**, 79–86.
11) Porterfield, S. P. and Hendry, L. B. (1998) Impact of PCBs on thyroid hormone derected brain development. *Toxicol. Ind. Health*, **14**, 103–120.
12) Veldhoen, N. and Helbing, C. C. (2001) Disruptor effects on gene expression in live Rana catesbeiana tadpoles using a tail fin biopsy technique. *Environ. Toxicol. Chem.*, **20**, 2704–2708.
13) Nishikawa, J., Saito, K., Goto, K., Dakeyama, F., Matsuo, M. and Nishihara, T. (1999) New screening methods for chemicals with hormonal activities using interaction of nuclear hormone receptor with coactivator. *Toxicol. Appl. Pharmacol.*, **154**, 76–83.
14) Nishihara, T., Nishikawa, J., Kanayama, T., Dakeyama, F., Saito, K., Imagawa, M., Takatori, S., Kitagawa, Y., Hori, S. and Utsumi, U. (2000) Estrogenic activities of 517 chemicals by yeast Two-Hybrid assay. *J. Health Sci.*, **46**, 282–298.
15) Brzozowski, A. M., Pike, A. C. W., Dauter, Z., Hubbard, R. E., Bonn, T., Engström, O., Öhman, L., Greene, G. L., Gustafsson, J. and Carlquist, M. (1997) Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature* (London), **389**, 753–758.
16) Wagner, R. L., Apriletti, J. W., McGrath, M. E., West, B. L., Baxter, J. D. and Fletterick, R. J. (1995) A structural role for hormone in the thyroid hormone receptor. *Nature* (London), **378**, 690–697.
17) Chiellini, G., Apriletti, J. W., Yoshihara, H. A., Baxter, J. D., Ribeiro, R. C. J. and Scanlan, T. S. (1998) A high-affinity subtype-selective agonist ligand for the thyroid hormone receptor. *Chem. Biol.*, **5**, 299–306.
18) Rickenbacher, U., Mckinney, J. D., Oatley, S. J. and Blake, C. C. F. (1986) Structurally specific binding of halogenated biphenyls to thyroxine transport protein. *J. Med. Chem.*, **29**, 641–648.
19) Voegel, J. J., Heine, M. J. S., Zechel, C., Chambon, P. and Gronemeyer, H. (1996) TIF2, a 160kDa transcriptional mediator for the ligand-dependent activation function AF-2 of nuclear receptors. *EMBO J.*, **15**, 3667–3675.
20) Visser, T. J. and Schoenmakers, C. H. (1992) Charactaristic of type III iodothyronine deiodinase. *Acta Med. Austriaca.*, **19**, 18–21.
21) Wright, A. S., Crowne, R. S. and Hathway, D. E. (1996) The fate of di-(3,5-*tert*-butyl-4-hydroxyphenyl)methane. *J. Biochem.* (Tokyo), **99**, 146–154.
22) Dacre, J. C. (1970) Toxicologic studies with 2,6-di-*tert*-butyl-4-hydroxymethylphenol. *Toxicol. Appli. Pharmacol.*, **17**, 669–678.
23) Meerts, I. A. T. M., Zanden, J. J., Luijks, E. A. C., Leeuwen-Bol, I., Marsh, G., Jakobsson, E., Bergman, Å. and Brouwer, A. (2000) Potent competitive interaction of some brominated flame retardants and related compounds with himan transthyretin in vitro. *Toxicol. Sci.*, **56**, 95–104.