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Synthesis of Polybrominated Diphenyl Ethers and Their Capacity to Induce CYP1A by the Ah Receptor Mediated Pathway

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Polybrominated diphenyl ethers (PBDEs) have become widely distributed as environmental contaminants due to their use as flame retardants. Their structural similarity to other halogenated aromatic pollutants has led to speculation that they might share toxicological properties such as hepatic enzyme induction. In this work we synthesized a number of PBDE congeners, studied their affinity for rat hepatic Ah receptor through competitive binding assays, and determined their ability to induce hepatic cytochrome P-450 enzymes by means of EROD (ethoxyresorufin-O-deethylase) assays in human, rat, chick, and rainbow trout cells. Both pure PBDE congeners and commercial PBDE mixtures had Ah receptor binding affinities 10^{-2}−10^{-5} times that of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. In contrast with polychlorinated biphenyls, Ah receptor binding affinities of PBDEs could not be related to the planarity of the molecule, possibly because the large size of the bromine atoms expands the Ah receptor's binding site. EROD activities of the PBDE congeners followed a similar rank order in all cells. Some congeners, notably PBDE 85, did not follow the usual trend in which strength of Ah receptor binding affinity paralleled P-450 induction potency. Use of the gel retardation assay with a synthetic oligonucleotide indicated that in these cases the liganded Ah receptor failed to bind to the DNA recognition sequence.

Introduction

Polybrominated diphenyl ethers are ubiquitously present in the environment, in a manner reminiscent of other highly halogenated and persistent aromatic compounds (HACs), such as the polychlorinated and polybrominated biphenyls (PCBs and PBBs), polychlorinated dibenzo-*p*-dioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs) (*1, 2*). Unlike these other HACs, which have either been phased out of production (PCBs and PBBs) or never synthesized commercially (PCDDs and PCDFs), tens of thousands of tonnes of PBDEs are presently manufactured annually as flame retardants for soft furnishings, electronic equipment, and automobiles (*3*). The "open" nature of these applications virtually guarantees that the PBDEs will end up dispersed into the environment. Levels of PBDEs in biota are currently rising rapidly and parallel the use pattern of PBDEs since their introduction in the late 1960s (*4*−*6*). Indeed, in one recent report, PBDE levels in biota surpassed those of PCBs (*2*).

The commercial PBDE mixtures presently in use are nominally deca-, octa-, and penta-brominated. Some 67 000 tonnes were manufactured in 1999 (*7*), an amount rivalling PCBs at the height of their production. Congeners 47 and 99 (2,2′,4,4′-tetraBDE and 2,2′,4,4′,5-pentaBDE) are the most frequently encountered PBDEs in the environment (*8*); these are major constituents of the "penta" commercial mixture, a minor commercial formulation (*7*). These congeners are found even in samples taken remotely from known sources (*5*), suggesting that, like other HACs having similar vapor pressures (*9*), they may undergo atmospheric transport (*10, 11*).

Very little is yet known about the toxicology of PBDEs. The structural similarities between PBDEs and other classes of HACs, notably the PCBs, suggest that PBDEs might activate the aryl hydrocarbon receptor (AhR) signal transduction pathway (*12*), which is a critical toxicological mechanism for many HACs. This response leads to the induction of the cytochrome P-450 isozyme CYP 1A1 (*12*−*14*), which can be assayed as 7-ethoxyresorufin-*O*-deethylase (EROD) activity. Induction of CYP 1A1 begins when the HAC binds to the intracellular AhR, causing the components of the AhR complex to dissociate and migrate to the nucleus, where the Ah receptor ligand binding subunit heterodimerizes with the nuclear protein ARNT. The dimer then associates with recognition sites on DNA called "dioxin response enhancers", triggering the transcription of mRNAs from which CYP 1A1 is translated. This sequence of events is relevant to environmental concerns because many HACs exhibit strong rank-order correlations between strength of Ah receptor binding, CYP 1A induction, and toxicity (*15*).

In the present study, 18 PBDE congeners were synthesized and evaluated, along with three commercial PBDE mixtures, for their capacity to bind and activate the AhR in rat hepatocytes and to induce EROD activity in cells from rainbow trout, chick, rat, and human.

Synthetic Methodology

Chemicals. 1,2-Dibromobenzene, 1,3-dibromobenzene, 1,4-dibromobenzene, 1,4-dibromo-2-fluorobenzene, 1,2,4-tribromobenzene, 2-bromophenol, 4-bromophenol, 2,4-dibromophenol, 2,4,6-tribromophenol, 2,5-bromoaniline, and 2,6-dibromoaniline were obtained from Aldrich. All solvents were HPLC Grade (Fisher Scientific, Toronto ON) and were used as received. Mass spectra were obtained using a Micromass VG70SE high-resolution mass spectrometer (HRMS), coupled with Hewlett-Packard 5890 Series II gas chromatograph, equipped with a DB-5 capillary column (J&W, 60 m × 0.25 mm × 0.25 μm). ^{1}H NMR analyses were performed using a Bruker AM400. PBDE congeners were purified by column chromatography on Kieselgel-60 using hexane as the eluent, followed by a cleanup on a small Celite-carbon column to remove possible traces of PBDD/PBDF and finally crystallized from methanol.

Synthesis of Intermediates. 2-Bromo-4-nitroaniline was obtained in 89% yield by bromination of 4-nitroaniline with

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1.01 equivalent of bromine (*16*), mp 104.5 °C; lit. (*17*) 104.5 °C; purity 99% by GC-MS. **2,6-Dibromo-4-nitroaniline** was prepared similarly by bromination of 4-nitroaniline with 2.2 equiv of bromine (89% yield; mp 205 °C; lit. (*18*) 206 °C; 99% purity).

3,4,5-Tribromonitrobenzene was prepared by the Sandmeyer reaction of diazotized 2,6-dibromo-4-nitroaniline with CuBr (*19*) (54% yield; mp 111 °C; lit. (*20*) 112 °C; purity 99%). **3,4-Dibromonitrobenzene** was prepared similarly from 2-bromo-4-nitroaniline (yield 62%, mp 59 °C; lit. (*21*) 58−59 °C; purity 98%), **1,2,3-tribromobenzene** from 2,6-dibromoaniline (92% yield, mp 88 °C; lit. (*22*) 87.8 °C; purity 99%), and **2,4,5-tribromoiodobenzene** by iodination of 2,4,5-tribromoaniline (72% yield, 99% purity), mp 108−109 °C.

1,2,4-Tribromo-5-nitrobenzene (*1*) was prepared by a modification of the method of Oliver and Ruth (*23*). 1,2,4-Tribromobenzene (1.07 g, 3.39 mmol) was dissolved in a 2:2:1 mixture of methylene chloride, trifluoroacetic acid, and trifluoroacetic anhydride and cooled in an ice/water bath to 0° C. To the stirred solution was added ammonium nitrate (0.266 g, 4.15 mmol). The mixture was allowed to warm to room temperature and stirred overnight, then the solvent was removed on the rotary evaporator, and the residue, dissolved in methylene chloride, was eluted on a 40 × 3 cm silica column (Kieselgel-60) with 1:1 hexane:methylene chloride. The product crystallized from ethanol to give 1.13 g (92%) of 2,4,5-tribromonitrobenzene (purity >99% by GC-MS, mp 79−81 °C; lit. (*24*) 80−81 °C. The analogous method was used to prepare **2,3,4-tribromonitrobenzene** from 1,2,3-tribromobenzene (yield 54%, mp 85 °C, lit. (*25*) 85.4 °C, purity 98.5% by GC-MS) and **2,5-dibromo-4-fluoro-nitrobenzene (*6*)** from 2,5-dibromofluorobenzene (89%, brown oil, purity 98.5% by GC-MS).

The method of Krolski et al. (*26*) was used to reduce the following nitro compounds to anilines: **3,4-dibromoaniline** from 3,4-dibromonitrobenzene (yield 62%, mp 81 °C; lit. (*27*) 81 °C; purity 98% by GC-MS); **2,4,5-tribromoaniline** from 1,2,4-tribromo-5-nitrobenzene (yield 94.5%, mp 79−81 °C; lit. (*28*) 80−81 °C; purity >99% by GC-MS); **2,3,4-tribromoaniline** from 2,3,4-tribromonitrobenzene (yield 67%, mp 101 °C; lit. (*27*) 100.6 °C; 99% purity); and **3,4,5-tribromoaniline** from 3,4,5-tribromonitrobenzene (yield 88%, mp 123 °C; lit. (*29*) 123 °C; 99% purity).

2,4,5-Tribromophenol (2). Solid sodium nitrite (77 mg, 1.11 mmol) was slowly added to a stirred solution of 2,4,5-tribromoaniline (361 mg, 1.09 mmol) in 10 mL of TFA at 0 °C. The resulting light-yellow solution was transferred with a Pasteur pipet into a boiling solution of sodium sulfate in 50% sulfuric acid, then a little water was added, and the solution was heated to reflux for 50 min. The product was steam-distilled, and the distillate was extracted with 3 × 20 mL of methylene chloride. The combined extract was dried (Na_2SO_4), the solvent was evaporated, and the product was purified by flash chromatography (Kieselgel-60), eluting with 1:5 hexane:methylene chloride. Yield 278 mg (78%), mp 86−87 °C; lit. (*30*) 87 °C; purity >99% by GC-MS. Analogous methods were used to prepare **3,4-dibromophenol** (65% yield, mp 94 °C; lit. (*31*) 94 °C; purity 98%), **2,3,4-tribromophenol** from 2,3,4-tribromoaniline (45% yield, mp 95 °C; lit. (*32*) 95 °C; purity 98%), **2,5-dibromophenol** from 2,5-dibromoaniline (61% yield, mp 73 °C; lit. (*30*) 73−74 °C, purity 98%), and **3,4,5-tribromophenol** (68% yield, purity 98%).

2,3,4,6-Tetrabromophenol was prepared by reaction of 3-bromophenol with bromine (3.3 equiv) in the presence of iron filings, as described by Marsh et al. (*16*) (yield 82%, mp 112 °C; lit. (*33*) 112−113 °C; purity 98.5% by GC-MS).

Condensation of 2,5-Dibromo-4-fluoronitrobenzene with Bromophenols. In a typical procedure, 1.5 mmol of the bromophenol was added over 30 min to a boiling mixture of **6** (1.5 mmol) in 4.00 mL of dry acetone and 600 mg of anhydrous potassium carbonate. After 4 h at reflux, the brownish-red mixture was cooled to room temperature, the acetone was evaporated, and the residue was partitioned between methylene chloride and water. The organic layer was washed with 5% NaOH and brine, dried over anhydrous sodium, the solvent evaporated, and the residue was purified by flash chromatography over silica gel, eluting with 1:10 hexane:methylene chloride. Other polybromo-4-nitrodiphenyl ethers were prepared similarly except that the product was eluted with hexane:ethyl acetate (4:1).

Preparation of Iodonium Salts from Brominated Benzenes. The procedure of Jakobsson et al. (*34*) was followed at one-fifth scale, giving **2,2′,4,4′-tetrabromodiphenyliodonium chloride** from 1,3-dibromobenzene (5 g), yield 4.59 g, 68%, and **3,3′,4,4′-tetrabromodiphenyliodonium chloride** from 1,2-dibromobenzene (5 g), yield 1.54 g, 23%.

Synthesis of BPDEs from Iodonium Salts and Bromophenols. The method of Nilsson et al. (*35*) was used. The yields and spectral data of products are provided in Table 1.

Preparation of 4-Methoxyphenyl-2′,4′,5′-tribromophenyliodonium Bromide. To hydrogen peroxide (204 μL of 30%, 2 mmol) in a test tube equipped with a magnetic stirrer and maintained at 0 °C was added 1.56 mL (11.8 mmol) of trifluoroacetic anhydride during 5 min followed by slow addition of 2,4,5-tribromoiodobenzene (0.981 g, 2.21 mmol) in 5 mL of methylene chloride. The mixture was allowed to warm to room-temperature overnight, then the solvent was evaporated, and the residue was dissolved in 10 mL of methylene chloride/acetic anhydride (1:1 v/v), containing 170 μL of acetic acid. The solution was cooled to −20 °C and anisole (482 μL, 4.43 mmol) in 5 mL of acetic anhydride was slowly added with stirring. The reaction was kept at −20 °C for 45 min and then at room temperature for 48 h. After removal of solvents *in vacuo* below 70 °C, the oily residue was dissolved in a minimal amount of methanol, and a saturated solution of sodium bromide was added to precipitate off-white crystals of the product, 4-methoxyphenyl-2′,4′,5′-tribromophenyliodonium bromide (yield 0.276 g, 20%).

2,2′,3,4,4′,5′-Hexabromodiphenyl Ether (BDE-183). 2,3,4-Tribromophenol (102 mg, 0.31 mmol) was dissolved in a boiling solution of sodium hydroxide (12.5 mg, 0.31 mmol) in water (4 mL), and 4-methoxyphenyl-2′,4′,5′-tribromophenyliodonium bromide (196 mg, 0.31 mmol) was added in one portion. The mixture was held at reflux for 2 h, then the pH was brought to 11, and the water layer was extracted with 3 × 10 mL of methylene chloride. Workup by flash chromatography followed by a Celite/activated carbon column gave 80 mg (40% yield), purity 98%. Spectral data are provided in Table 1.

Modified Suzuki Coupling to Prepare 3,4,4′,5-Tetrabromodiphenyl Ether (BDE-77). The procedure of Chan (*36*) was used to react the 4-bromophenyl monoester of boronic acid (218 mg, 1.09 mmol) and 3,4,5-tribromoiodobenzene (180 mg, 0.41 mmol) in the presence of copper(II) acetate (108 mg, 0.59 mmol) and triethylamine (0.245 mL, 1.76 mmol) in methylene chloride under nitrogen at room-temperature overnight with the following modifications: the amount of triethylamine was increased to 4.3 equiv; methylene chloride and triethylamine were dried over 4 Å molecular sieves before the reaction; and 0.5 g of finely ground 4 Å molecular sieves were present in the reaction slurry. Yield: 40%; purity 98%. 4,4′-Dibromodiphenyl ether (10%) was also formed (separated by flash chromatography).

Molecular Modeling. Molecular mechanics calculation and conformation searches were performed using "Spartan plus". The lowest energy and energy of coplanar conformation for each congener were calculated by the SYBYL method.

TABLE 1. Physical Properties of Synthesized PBDE Congeners

congener	Br pattern	method[a]	yield, %	M^+ (^{79}Br)	spectral details
28	2,4,4′-	I	63	404	7.80, d ($J = 2$ Hz); 1H, 7.44, m, 3H, 6.85, dd ($J = 2$ Hz, $J = 9$ Hz) 3H
47	2,2′,4,4′-	I	45	482	7.82, d ($J = 2$ Hz) 2H; 7.42, dd ($J = 2$ Hz, $J = 9$ Hz) 2H; 6.47, d ($J = 9$ Hz) 2H
49	2,2′4,5′-	I	67	482	7.83, d ($J = 2$ Hz) 1H; 7.53, d ($J = 8$ Hz) 1H; 7.45, dd ($J = 2$ Hz, $J = 8$ Hz) 1H; 7.19, dd ($J = 2$ Hz, $J = 8$ Hz) 1H; 6.93, d ($J = 2$ Hz) 1H; 6.81, d ($J = 8$ Hz) 1H
66	2,3′,4,4′-	I	32	482	7.81, d ($J = 2$ Hz) 1H; 7.58, d ($J = 8$ Hz) 1H; 7.46, dd ($J = 2$ Hz, $J = 8$ Hz) 1H; 7.19, d ($J = 2$ Hz,) 1H; 6.93, d ($J = 9$ Hz) 1H; 6.81, dd ($J = 2$ Hz, $J = 8$ Hz) 1H
71	2,3′,4′,6-	I	41	482	7.63, d ($J = 8$ Hz), 2H; 7.53, d ($J = 8$ Hz), 1H; 7.09, d ($J = 2$ Hz), 1H; 7.06, t ($J = 8$ Hz), 1H; 6.65, dd ($J = 2$ Hz, $J = 9$ Hz), 1H
77	3,3′,4,4′-	I	87	482	7.57, d ($J = 9$ Hz), 2H; 7.26, d ($J = 2$ Hz), 2H; 6.83, dd ($J = 2$ Hz, $J = 9$ Hz), 2H
81	3,4,4′,5-	III	40	482	7.80, s, 1H; 7.42, d ($J = 7$ Hz), 1H; 6.71, d ($J = 7$ Hz), 1H
85	2,2′,3,4,4′-	I	74	560	8.01, d ($J = 8$ Hz), 1H; 7.84, d,($J = 2$ Hz) 1H; 7.39, dd ($J = 2$ Hz, $J = 9$ Hz) 1H; 7.28, d($J = 8$ Hz), 1H; 6.51, d ($J = 9$ Hz) 1H
99	2,2′,4,4′,5-	I, II	69, 82	560	7.91, s, 1H; 7.84, d ($J = 2$ Hz), 1H; 7.47, dd ($J = 2$ Hz, $J = 9$ Hz), 1H; 7.02, s, 1H; 6.84, d ($J = 9$ Hz), 1H
100	2,2′,4,4′,6-	I	51	560	7.83, s, 2H; 7.82, d ($J = 2$ Hz), 1H; 7.30, dd, ($J = 2$ Hz, $J = 8$ Hz), 1H; 6.31, d ($J = 9$ Hz), 1H
119	2,3′,4,4′,6-	I	56	560	7.81, s, 2H; 7.55, d ($J = 8$ Hz), 1H; 7.09, d ($J = 2$ Hz), 1H. 6.69, dd ($J = 2$ Hz, $J = 8$ Hz), 1H
126	3,3′,4,4′,5-	II	84	560	7.637, d ($J = 9$ Hz), 1H; 7.32, d ($J = 2$ Hz), 1H; 7.27, s, 2H; 6.90, dd ($J = 2$ Hz, $J = 9$ Hz) 1H
153	2,2′,4,4′,5,5′-	II	81	638	7.94, s, 2H; 7.13, s, 2H
154	2,2′,4,4′,5,6′-	II	48	638	7.92, s, 1H; 7.83, s, 2H; 6.64, s, 1H
183	2,2′,3,4,4′,5,5′-	II, IV	12, 45	714	8.02, s, 1H; 7.93, s, 1H; 6.62, s, 1H

[a] I − iodonium salt coupling; II − nucleophilic condensation; III − modified Suzuki coupling; IV − unsymmetrical iodonium salt coupling.

The "planarization energy" means the energy difference between the coplanar and minimum energy conformations.

Bioassay Methodology. Supplies. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) was a generous gift from Wellington Laboratories (Guelph, Ontario, Canada). Glycerol, sodium ethylenediamine tetraacetate (EDTA), dimethyl sulfoxide (DMSO), and tris-(hydroxymethyl)-aminomethane (TRIS) were purchased from Fisher Scientific (Toronto, ON, Canada); resorufin was purchased from ICN Biomedicals Inc. (Aurora, OH); sodium pentobarbital (Somnitol) was obtained from MTC Pharmaceuticals (Cambridge, ON, Canada); Percoll, Hank's Balanced Salt Solution, Dulbelcco's phosphate buffered saline (PBS), fetal bovine serum, penicillin G, streptomycin, L-thyroxine (T4), sucrose, EGTA, N-2-hydroxylpiperazine-N-2-ethanesulfonic acid (HEPES), dithioerythritol (DTE), and 7-ER were purchased from Sigma (St. Louis, MO); hydroxylapatite was from Bio-Rad Canada (Missisauga, ON); hepatocyte qualified collagenase, minimal essential medium, and all components of attachment medium and serum free medium were purchased from GIBCO BRL (Burlington, ON, Canada). Immature Sprague−Dawley rats (100 g) were purchased from Charles River Breeding Laboratories (Canada); 19 day Barred Rock egg embryos were obtained from the Arkell Research Station, University of Guelph. Treatment of animals was in accordance with the University of Guelph Animal Care Policy C5.1.

Rodent Hepatic Cytosol. Immature male Sprague−Dawley rats (∼100 g) were euthanised by exposure to carbon dioxide followed by cervical dislocation. Livers were immediately perfused *in situ* with ice-cold HEGD buffer via the hepatic portal vein, then excised. Tissue was homogenized using three strokes of a Potter Elvhjem Teflon tissue homogenizer. The homogenate was spun at 9000 × g for 20 min in a Sorvall Superspeed RC2-B centrifuge at 4 °C and then spun at 100 000 × g for 68 min in a Beckman L7-65 ultracentrifuge at 4 °C. The cytosol was stored in 1 mL aliquots at −70 °C until required. The protein content of the cytosol was determined by the method of Bradford (*37*) using Bio-Rad Protein Assay Dye Reagent with bovine serum albumin (BSA) as a standard.

Hydroxylapatite (HAP) Ligand Binding Assay (*38*). One milliliter aliquots of tissue cytosol were diluted with HEGD buffer to give a final protein concentration of 2 mg/mL. Cytosol was incubated with a 1.0 nM [^{3}H]-TCDD and a range of concentrations of an unlabeled PBDE. The protein components of the cytosol were then adsorbed onto hydroxylapatite ($Ca_5(PO_4)_3OH$) and rinsed with a mild detergent (1.5% v/v Triton X-100) to remove free and loosely bound radioligand, and the bound radioactivity was quantitated by liquid scintillation counting. Nonspecific binding was determined in a parallel incubation of 1.0 nM [^{3}H]-TCDD plus 200 nM unlabeled TCDF and was assumed to be independent of the concentration of the unlabeled competitor (*39*). Data were elaborated by plotting "probit of percent specific binding" vs log[PBDE]; the EC_{50} was obtained from the log-[PBDE] corresponding to probit = 5.000; 100% specific binding was the value obtained when no PBDE was present.

Gel Retardation Assay. The following 32-base pair oligonucleotides containing the dioxin response enhancer (DRE) consensus binding sequence 5′-T-GCGTG-3′ were obtained from University Core DNA Services, University of Calgary.

5'-GATCTGAGCTCGGAGTTGCGTGAGAAGAGCCG-3'
3'-ACTCGAGCCTCAACGCACTCTTCTCGGCCTAG-5'

The oligonucleotides were [^{32}P]-end labeled by T4 polynucleotide kinase and annealed as follows. A single strand oligonucleotide was dissolved in TEN buffer (10 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 8.0) to give a concentration of 150 ng/μL. A mixture of 3 μL of each oligonucleotide, 3 μL of 10x T4 polynucleotide kinase reaction buffer (New England BioLabs), 18 μL of sterile water, 1.5 μL of T4 polynucleotide kinase (10 000 U/mL), and 1 μL of γ-[^{32}P]-ATP (7000 Ci/mmol, 160 mCi/mL) was incubated at 37 °C for 30 min. An additional 0.5 μL of T4 polynucleotide kinase was added, and the mixture was incubated for 30 min more. After adding 2.3 μL of 1.4

M KCl, the microfuge tube was sealed, heated to 85 °C, and allowed to cool slowly to room temperature to allow the two complementary oligonucleotides to anneal. The [^{32}P]-labeled oligonucleotide was then purified on a NICK Sephadex G-50 spin column according to the manufacturer's instruction and stored at −20 °C before use. One μL [^{32}P]-labeled oligonucleotide was taken for liquid scintillation counting.

Aliquots of tissue cytosol (16 mg protein /mL) were incubated with either 1 μL of DMSO (control), 10 nM of TCDD (reference), or various concentrations of PBDE or mixtures of TCDD and PBDE at 30 °C for 2 h. Aliquots of liganded cytosol were incubated at 23 °C with 500 ng of poly (dIdC) for 15 min; 1 μL of [^{32}P]-oligonucleotide (~500 000 cpm/μL) was then added, and the samples were mixed and incubated for a further 15 min at 23 °C. After mixing with bromophenol blue tracking dye, the samples were then electrophoresed in a 5% polyacrylamide gel in TBE buffer at 11 V/cm using a Bio-Rad Protean II x *i* cell with a Bio-Rad model 200/2.0 power supply. Gels were removed and placed on a piece of Whatman 3MM paper, sealed in Saran Wrap, and loaded into an autoradiography cassette with an 8" × 10" sheet of Kodak X-OMAT AR X-ray film. Following overnight exposure at −20 °C, the film was developed using Kodak Developer/Replenisher and Kodafix fixing solution.

Preparation of Primary Rat Hepatocytes. Cultures of primary rat hepatocytes were prepared by a modified protocol of Kreamer (*40*). An immature male Sprague−Dawley rat was anaesthetized with sodium pentobarbital, and the liver was perfused with collagenase, excised, rinsed, and desegregated in a sterile 150 mm Petri dish. The cells were filtered and resuspended in 25 mL of attachment media (William's E medium supplemented with 10 mM HEPES, 2 mM L-glutamine, and 10% fetal bovine serum) combined with 24 mL of Percoll in Hank's Balanced Salt Solution. The cells were then spun at 50 × g for 10 min, the pellet was washed with 50 mL of attachment media and spun for 3 min at 50 × g, and the pellet was resuspended in 30−40 mL of attachment media. The cells were then counted using a hemocytometer. Cells were plated in sterile 48-well collagen-coated culture plates at a density of 50 000 cells/well in 0.5 mL of attachment media. After 2 h, the medium was aspirated away, and 0.5 mL of serum-free media (William's E medium supplemented with 10 mM HEPES, 2 mM L-glutamine, 10 mM pyruvate, and 0.35 mM proline) was added to each well. The cells were then incubated for 24 h at 37 °C (95% air, 5% CO_2).

Preparation of Chicken Embryo Hepatocytes. Primary hepatocytes cultures were prepared from 19 day Barred Rock chicken embryos using the methods described by Kennedy (*41, 42*). Thirty eggs were used for one experiment. In brief, the embryos were decapitated, the livers were removed, rinsed with Krebs-Ringer buffer, and cut, and the combined livers were digested with collagenase with shaking for 45 min at 37 °C and then filtered through nylon gauze to remove undigested tissue. The filtrates were centrifuged at 300 g for 5 min, and the pellets were resuspended. The Percoll/sucrose isodensity centrifugation method was used to purify the hepatocytes. Hepatocytes were cultured in 48-well cell culture plates in Waymouth's MD serum free medium (0.5 mL/well) that was supplemented with insulin (1 μg/mL) and thyroxine (1 μg/mL). Because the chicken embryo hepatocytes are too small to be counted under the optical microscope, hepatocytes were plated in sterile 48-well plates at a concentration that resulted in total protein concentrations of approximately 60 μg/well. The cells were then incubated for 24 h at 37 °C (95% air, 5% CO_2), and then the medium was aspirated away and replaced with fresh Waymouth's MD serum free medium before the cells were treated with chemicals.

Cell Lines. The American Type Culture Collection (Manassas, VA) was the source of two human cell lines, hepatoma HepG2 (HB-8065) and intestinal Caco-2 (HTB-37), and of one rat cell line, hepatoma H4IIE (HB-8065). Caco-2 and HepG2 were grown at 37 °C in Minimal Essential Medium (MEM) with 10% fetal bovine serum (FBS). H4IIE and the rainbow trout liver cell line, RTL-W1, which was developed in Dr. Bols' laboratory, were maintained respectively in Dulbecco's MEM (DMEM) with 10% FBS at 37 °C and in Leibovitz's L-15 with 5% FBS at 22 °C as described previously (*43*).

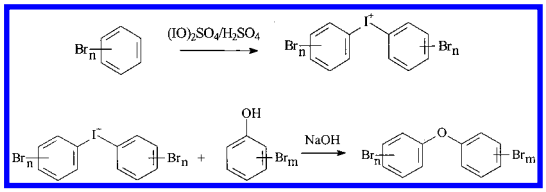
Exposure of Cells to PBDEs. All cells were exposed to TCDD and PBDEs in microwell cultures. The step-by-step protocol has been described in detail for TCDD and RTL-W1 (*44*). The same procedure was followed for the PBDEs in all cell lines. The mammalian cell lines were exposed at 37 °C; RTL-W1 was exposed at 22 °C. After 48 h, the medium was removed, the microwells were rinsed, and the cell monolayers in the microwells were assayed for EROD activity.

EROD Assays. The measurement of EROD activity on live cells in the microwell cultures has been described in step-by-step detail (*44*). The reaction mixture consisted of DMEM with 5.2 μM 7-ethoxyresorufin (ER) and 30 μM dicumarol for the mammalian cell lines and with 0.83 μM ER and no dicumarol for RTL-W1. Resorufin was measured with fluorometric plate readers, either a CytoFluor 4000 (Framingham, MA) or a BioRad Fluoromark (Richmond, CA). For each cell culture type, data are based on the means of at least two independent experiments, each of which involved 3−5 replicates. After measuring the fluorescence, the cells were washed and lysed in water at −70 °C overnight, and then the protein concentration was determined by the fluorescamine method (*45*) using bovin serum album (BSA) as standard.

Analysis of EROD Data and Calculation of REPs. PBDE congeners showed maximum EROD activities that were different from that of TCDD. The EC_{50} for each congener was obtained by assigning its own maximum EROD activity as 100% and plotting the probit of percent maximum activity against log[PBDE concentration]. Relative potencies (REPs) of individual PBDE congeners were expressed as EC_{50} of TCDD/EC_{50} of PBDE congener.

Results and Discussion

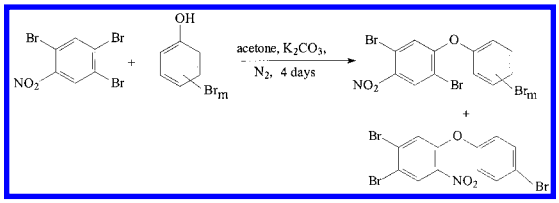
Synthesis of PBDE Congeners. Marsh et al. (*16*) recently described a general method for synthesizing symmetrical and unsymmetrical PBDE congeners from substituted bromobenzenes by conversion to an aryliodonium salt, which is then coupled to a bromophenol.



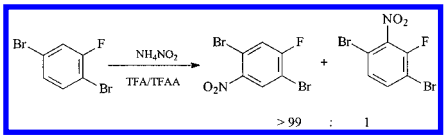
The authors synthesized PBDEs by condensing brominated phenols with aryliodonium salts having Br_n = unsubstituted, 4-bromo-, 2,4-dibromo-, and 3,4-dibromo-. We followed this method to synthesize PBDE congeners 28, 47, 49, 66, 71, 77, 85, 99, 100, 119, and 126, which were formed in yields 30−70% overall, and shown in each case to be >98% pure by GC-MS and ^{1}H NMR at 400 MHz (Table 1). Because they were to be used, inter alia, for studying Ah receptor binding affinities, their purification included chromatography over charcoal, to remove possible impurities of polybrominated dibenzodioxins or dibenzofurans.

We could not synthesize the 2,4,5-substituted iodonium salt precursor of PBDE congeners 99, 153, 154, and 183, which are prominent in commercial PBDE mixtures, and so we devised a new synthetic methodology based on S_N2Ar

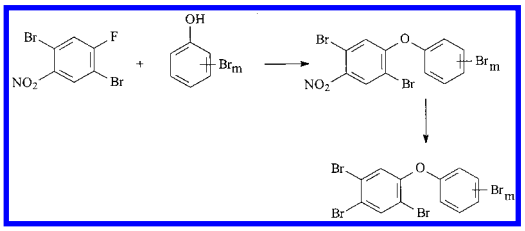
displacement of a halide ion activated by an *o*- or *p*-nitro group. Our initial condensation using 2,4,5-tribromophenol and 1,2,4-tribromo-5-nitrobenzene was unsatisfactory on account of excessive reaction times, poor yield (40%), and the formation of an inseparable mixture of two isomers, resulting from equal attack of the phenolate *o*- and *p*- to the nitro group.



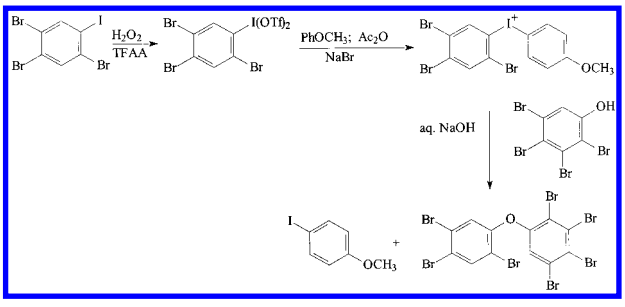
Instead, we synthesized the key precursor 2,5-dibromo-4-fluoronitrobenzene, by nitrating the commercially available 2,5-dibromofluorobenzene, which under our conditions occurred in >90% yield and with almost complete regiospecificity.



2,5-Dibromo-4-fluoronitrobenzene underwent nucleophilic displacement of the 4-fluoro substituent by a bromophenolate anion (acetone solvent, K_2CO_3 as base, 4 h). Introduction of the final Br substituent involved reduction of the NO_2 group (Sn/HCl/HOAc), diazotization, and Sandmeyer reaction with CuBr.



Congener 183 was prepared more successfully by condensing the iodosyl triflate with anisole, to give an unsymmetrical iodonium salt (20% yield) as shown below (*cf.* refs *46* and *47*). Subsequent reaction with 2,3,4,5-tetrabromophenol in alkaline solution led exclusively to the displacement of *p*-iodoanisole, affording the desired 2,2′,3,4,4′,5,5′-heptabromodiphenyl ether in 45% isolated yield. It is possible that this method may have more general application.



Congener 81 (3,4,4′,5-tetrabromodiphenyl ether) was prepared by a modified Suzuki coupling, based on the chemistry of Evans et al. (*48*) in 40% yield. This method requires scrupulous care in drying the reagents, because the arylboronic ester is highly moisture sensitive; its hydrolysis gives *p*-bromophenol, which condenses with additional boronic ester to give 4,4′-dibromodiphenyl ether, which must be separated from the desired product.

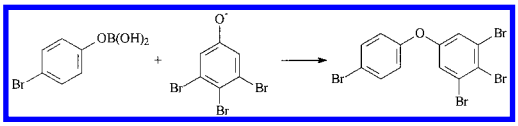


TABLE 2. Ah Receptor Relative Binding Affinities and "Planarization Energies" and DRE Binding Induced by PBDE Congeners

PBDE no.	Br substitution	AhR EC_{50}, μM (RBA)[a]	planarity energy kcal/mol	DRE binding activity[b]
3	4-	7.9 (1.3×10^{-4})	13.9	none
15	4,4′-	2.6 (3.8×10^{-4})	14.0	none
17	2,2′,4-	4.4 (2.3×10^{-4})	26.9	none
28	2,4,4′-	0.82 (1.2×10^{-3})	14.1	none
47	2,2′,4,4′-	1.8 (5.6×10^{-4})	26.3	none
49	2,2′,4,5′-	15 (6.7×10^{-5})	26.5	none
66	2,3′,4,4′-	0.49 (2.0×10^{-3})	14.2	none
71	2,3′,4′,6-	28 (13.6×10^{-5})	26.5	none
75	2,4,4′,6-	2.5 (4.0×10^{-4})	26.8	none
77	3,3′,4,4′-	0.46 (2.2×10^{-3})	14.1	medium
85	2,2′,3,4,4′-	0.053 (1.9×10^{-2})	26.3	none
99	2,2′,4,4′,5-	7.2 (1.4×10^{-4})	26.3	none
100	2,2′,4,4′,6-	13 (7.7×10^{-5})	27.5	weak
119	2,3′,4,4′-	0.88 (1.1×10^{-3})	26.8	medium
126	3,3′,4,4′,5-	0.37 (2.7×10^{-3})	14.0	medium
153	2,2′,4,4′,5,5′-	40 (2.5×10^{-5})	26.3	weak
154	2,2′,4,4′,5,6′-	43 (2.3×10^{-5})	27.6	none
183	2,2′,3,4,4′,5′,6-	4.0 (2.5×10^{-4})	27.8	weak
penta	mixture	2.4 (4.2×10^{-4})		none
octa	mixture	7.9 (1.3×10^{-4})		none
deca	mixture	no activity		none
TCDD		0.0010 (1.00)		strong

[a] Values in brackets are binding affinities relative to TCDD; concentration of [^{3}H]-TCDD was 1.0 nM. [b] Qualitative estimate based on visual examination of autoradiographs.

Ah Receptor Binding Assays. The EC_{50} values for Ah receptor binding of individual PBDE congeners were in the μM range, indicating weaker affinity than the reference toxicant TCDD (Table 2). Congener 85, which is not a major constituent in environmental samples, was the most active, but its relative binding affinity (RBA) was only 2% that of TCDD. RBAs of the 18 PBDEs studied spanned about 3 orders of magnitude, with some tendency for the congeners analogous to coplanar PCBs (PBDEs 77 and 126) to have larger RBAs than those with two or more *ortho*-bromines, such as 71, 100, 153, and 154.

The most abundant congeners in the commercial mixtures (PBDEs 47, 99, 153, 154, and 183) all had very small RBAs. The commercial mixtures also bound weakly to rat hepatic Ah receptor; their RBAs corresponded closely to those of their major components (PBDE 47 in the case of the "penta" mixture, and PBDE 183 in the case of the "octa"). We could not determine the RBA of the commercial decaBDE because of its very low solubility. The RBAs of the commercial mixtures were unaffected by prior passage through a carbon chromatography column, a treatment intended to remove possible traces of highly active minor components such as brominated dibenzodioxins or dibenzofurans, which would exaggerate the binding strength of the PBDEs, as is the case for PCBs (*49*).

For previously examined HAC families, the strength of binding to the AhR is determined by the planarity and lipophilicity of the ligand (*50*), indicating that the AhR has a nonpolar, planar binding site to which ligands must accommodate themselves. Recently, Kodavanti et al. (*51*) used molecular mechanics calculations to show, for the PCB family,

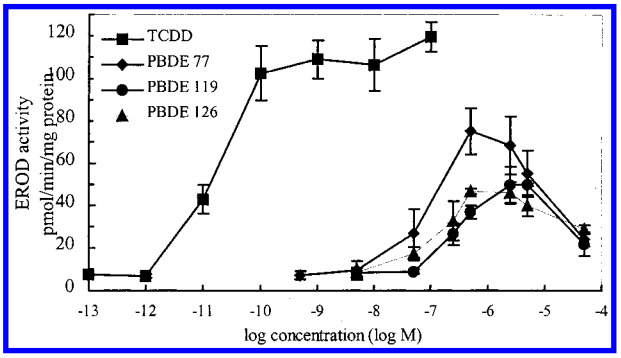


FIGURE 1. A typical dose-dependent EROD induction curve for TCDD and PBDE congeners 77, 119, and 126 in primary rat hepatocytes. Points represent mean level of triplicate doses and bars represent standard deviations. Similar dose response curves were obtained from other cell cultures.

that the "coplanar" congeners, which bind most strongly to the AhR, exhibited the smallest energy differences between their equilibrium and coplanar geometries. We used the SPARTAN software package to carry out similar calculations for the PBDEs, having first reproduced the "planarization energies" for PCB congeners reported by Kodavanti et al. (*51*). The calculated energies needed to force coplanarity of the PBDE molecules are larger than for PCBs and appear unrelated to the RBAs (Table 2): $\Delta E \sim 13-14$ kcal/mol for zero and one ortho Br and $\Delta E \sim 26-27$ kcal/mol for two or three ortho Br. One interpretation is that whereas PCBs (for example) must accommodate themselves to the geometry of the AhR binding site, for PBDEs the binding site becomes distorted by the large atomic volume of bromine and renders the issue of ligand planarity inconsequential.

EROD Induction. The ability of PBDEs to induce EROD activity was studied in chick and rat hepatocytes, in liver cell lines from rainbow trout (RTL-W1), rat (H4IIE) and human (HepG2), and in a human intestinal cell line (Caco-2). Figure 1 shows a typical set of induction curves, with the data summarized in Table 3. EROD induction was strongest in all cell types with PBDEs 77, 100, 119, and 126, although their maximal EROD activities were less than that of TCDD and their EC_{50}s were much larger. PBDEs 153 and 183 were weak inducers in all cells. PBDEs 66 and 85 were very weak inducers in rat hepatocytes and inactive in the other cells. The environmentally prominent congeners 47 and 99 were not inducers in any cell line; neither were PBDEs 28, 47, 99, and 154 and the commercial PBDE mixtures. Declines in EROD activity at high inducer concentrations have been previously ascribed to competitive inhibition by the inducer and are not due to cytotoxicity (*52*).

The strength of EROD induction by HACs frequently parallels the strength of AhR binding (*53*), indicating that induction of EROD activity requires activation of the AhR-signal transduction pathway. PBDEs congeners were shown to follow this trend by comparing their EROD activities in primary cultures of rat hepatocytes with their ability to activate the AhR to its DNA binding form, as studied by the gel retardation assay, visualized by autoradiography (Tables 2). The gel retardation assay involves incubating the bound AhR-ligand complex with a synthetic oligonucleotide that contains the consensus binding sequence for liganded AhR and that is [^{32}P] end-labeled using [^{32}P]-ATP. Binding the massive AhR complex to the oligonucleotide increases its size and its mass:charge ratio, and it migrates more slowly in slab gel electrophoresis.

TCDD, the reference ligand, caused induction of a strong gel-shifted band (Table 2), as repeatedly observed previously (*54*, *55*). The stronger AhR agonists among the PBDEs, such as PBDEs 77 and 126, induced moderate intensities of the

TABLE 3. Maximum EROD Induction and EC_{50} for PBDE Congeners and Mixtures from Different Cell Cultures[b]

max EROD	RTL-W1 69 ± 7.3 pmol/min/mg protein		CEH 170 ± 34 pmol/min/mg protein		PRH 110 ± 17 pmol/min/mg protein		H4IIE 134 ± 11 pmol/min/mg protein		Caco 161 ± 20 pmol/min/mg protein		Hep-G2 495 ± 24 pmol/min/mg protein	
	EROD %	EC_{50} nM	EROD %	EC_{50} nM	EROD %	EC_{50} nM	EROD %	EC_{50} nM	EROD %	EC_{50} nM	EROD %	EC_{50} nM
TCDD	100.0	0.0092 ± 0.0021	100.0	0.026 ± 0.004	100.0	0.029 ± 0.002	100.0	0.014 ± 0.004	100.0	0.030 ± 0.005	100.0	0.053 ± 0.006
PBDE 28	< 2	nd[a]	4.8 ± 1.3	nd[a]	< 2	nd[a]	< 2	nd[a]	< 2	nd[a]	6.4 ± 1.2	nd[a]
PBDE 47	< 2	nd[a]	5.9 ± 2.1	nd[a]	< 2	nd[a]	< 2	nd[a]	2.4 ± 3.0	nd[a]	< 2	nd[a]
PBDE 66	< 2	nd[a]	6.8 ± 4.1	11.3 ± 0.7	23.4 ± 3.9	815 ± 39	11.4 ± 1.8	440 ± 57	2.2 ± 3.0	nd[a]	5.8 ± 2.5	9170 ± 474
PBDE 77	27.6 ± 7.3	27.3 ± 2.1	71.5 ± 14.6	8.3 ± 2.5	75.2 ± 8.7	38.7 ± 2.3	66.3 ± 11.5	61.5 ± 30	55.8 ± 11.3	98.8 ± 65	42.8 ± 7.3	615 ± 235
PBDE 85	< 2	nd[a]	14.5 ± 4.0	13.7 ± 2	26.4 ± 7	287 ± 336	16.7 ± 1.7	1420 ± 788	2.5 ± 2.9	nd[a]	2.3 ± 0.3	10500 ± 5940
PBDE 99	< 2	nd[a]	< 2	nd[a]	< 2	nd[a]	< 2	nd[a]	11.2 ± 6.2	nd[a]	< 2	nd[a]
PBDE 100	56 ± 20	752 ± 111	50.5 ± 10.7	111 ± 64	40.9 ± 4.9	1060 ± 88	53.4 ± 18	1040 ± 349	37.0 ± 5.6	2850 ± 658	32.3 ± 13.6	4670 ± 636
PBDE 119	46.6 ± 0.6	49.2 ± 7.3	62.5 ± 7.7	76 ± 60	61.0 ± 21.6	215 ± 130	79.7 ± 12	139 ± 66	46.8 ± 7.4	448 ± 378	73.5 ± 6.9	783 ± 44
PBDE 126	29.6 ± 9.0	25.4 ± 2.4	69.6 ± 9.2	11.2 ± 1.1	63.8 ± 29.5	69.4 ± 45	68.8 ± 6.6	34.1 ± 21.3	54.6 ± 11.8	196 ± 18	54.7 ± 1.0	417 ± 402
PBDE 153	9.3 ± 4.8	15800 ± 1270	13.8 ± 0.1	550 ± 245	19.1 ± 4.1	9080 ± 90	33.4 ± 1.1	4120 ± 233	27.3 ± 9.0	3500 ± 1710	9.4 ± 2.9	5680 ± 2990
PBDE 154	3.3 ± 1.5	nd[a]	< 2	nd[a]	< 2	nd[a]	< 2	nd[a]	< 2	nd[a]	< 2	nd[a]
PBDE 183	7.2 ± 0.1	1360 ± 7	9.5 ± 1.4	303 ± 106	39.4 ± 2.3	6050 ± 71	18.4 ± 3.3	3580 ± 948	23.8 ± 6.0	3400 ± 1100	34.6 ± 0.6	5180 ± 354
penta mixture	< 2	nd[a]	6.3 ± 3.7	nd[a]	< 2	nd[a]	< 2	nd[a]	< 2	nd[a]	7.3 ± 0.4	6020 ± 3480
octa mixture	< 2	nd[a]	< 2	nd[a]	< 2	nd[a]	< 2	nd[a]	< 2	nd[a]	< 2	nd[a]
deca mixture	< 2	nd[a]	< 2	nd[a]	< 2	nd[a]	< 2	nd[a]	< 2	nd[a]	< 2	nd[a]

[a] nd, not determined since there was no EROD activity. [b] Cell types: RTL-W1 = rainbow trout hepatoma; CEH = primary chick embryo hepatocytes; PRH = primary rat hepatocytes; H4IIE = rat hepatoma; Caco-2 = human adenocarcinoma; Hep G2 = human hepatoma. Max EROD represents EROD activities obtained from different cell cultures using 1 nM TCDD treatment as positive control; EROD% represents the highest EROD activities from individual congener compared with the activity of 1 nM TCDD.

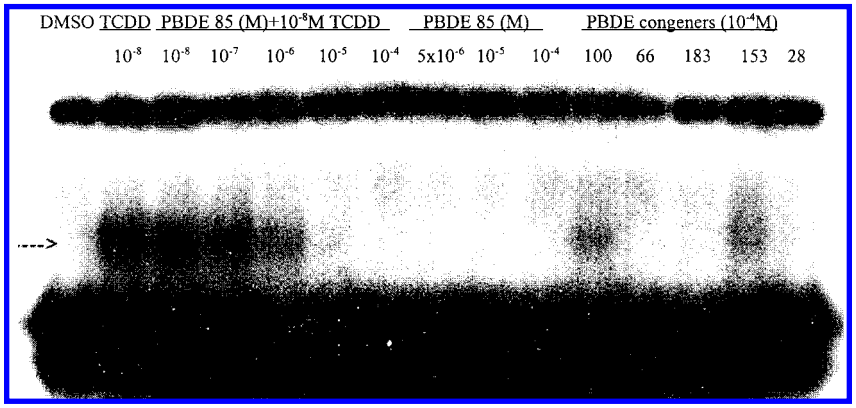


FIGURE 2. Gel retardation assay for PBDE congener 85 as agonist and antagonist in Ah receptor/Arnt/DRE complex formation. An arrow indicates the AhR/Arnt/DRE complex. 10^{-8} M TCDD as positive control. Congeners 100, 66, 183, 153, and 28 were also tested for synthetic DRE activation.

TABLE 4. Relative Induction Potencies (REPs) for PBDE Congeners in Different Cell Cultures

	REP = EC_{50} of TCDD/EC_{50} of PBDE					
PBDE no.	RTL-W1[a]	CEH[b]	PRH[c]	H4IIE[d]	Caco-2[e]	Hep G2[f]
TCDD	1.0	1.0	1.0	1.0	1.0	1.0
PBDE 126	0.00036	0.0024	0.00042	0.00041	0.00016	0.00013
PBDE 77	0.00034	0.0032	0.00076	0.00023	0.00031	0.000086
PBDE 119	0.00019	0.00035	0.00014	0.00010	0.000068	0.000068
PBDE 100	0.000012	0.00024	0.000028	0.000013	0.000011	0.000011
PBDE 183	0.0000068	inactive	0.0000048	0.0000039	0.0000088	0.000010
PBDE 153	0.0000006	0.000048	0.0000032	0.0000034	0.0000087	0.0000093
PBDE 85	inactive	inactive	0.00010	0.0000099	inactive	inactive
PBDE 66	inactive	inactive	0.000036	0.000032	inactive	inactive
PBDE 154	inactive	inactive	inactive	inactive	inactive	inactive
PBDE 99	inactive	inactive	inactive	inactive	inactive	inactive
PBDE 47	inactive	inactive	inactive	inactive	inactive	inactive
PBDE 28	inactive	inactive	inactive	inactive	inactive	inactive

[a] RTL-W1= rainbow trout liver cell line. [b] CEH = primary chick embryo hepatocytes. [c] PRH= primary rat hepatocytes. [d] H4IIE = rat hepatoma cell line. [e] Caco-2= human intestinal cell line. [f] Hep G2 = human hepatoma cell line.

shifted band, i.e., they activated the AhR to a form that could bind DNA and also induced EROD greater activity. Conversely, weak AhR agonists such as PBDEs 100 and 153 gave weak activation of the AhR to its DNA binding form and weak EROD induction. The commercial mixtures and their major constituents, PBDEs 47 and 99, were almost completely inactive. PBDE 85 was exceptional: despite its relatively strong AhR affinity, it showed no evidence of activating the AhR to its DRE-binding form and was only a very weak EROD inducer. This behavior is reminiscent of that of PCB 153, which also binds the AhR but fails to induce EROD activity due to failure to cause DRE binding (*55*). To pursue the parallel with PCB 153 further, the gel shift assay was carried out using AhR preparations activated with a fixed aliquot of TCDD and varying concentrations of PBDE 85. Increasing concentrations of PBDE 85 decreased the intensity of the gel shifted band and at the highest concentrations completely out-competed TCDD for AhR binding sites (Figure 2); hence PBDE 85 appeared to act as an AhR antagonist. Similar experiments carried out with the stronger AhR agonists PBDEs 77 and 126 only partly inhibited DRE activity because these congeners themselves activate the AhR to its DRE binding form.

The relative potencies (REPs) of individual PBDEs for EROD induction were expressed as the EC_{50} for TCDD/EC_{50} for PBDE congener, where the EC_{50} is the concentration needed to induce 50% of the maximal EROD response (Table 4). The REP values are empirical only because of the variation of the maximal EROD induction among congeners and cell lines. Most REPs were similar in cells from different species. Exceptions were PBDEs 66 and 85, which were weakly active

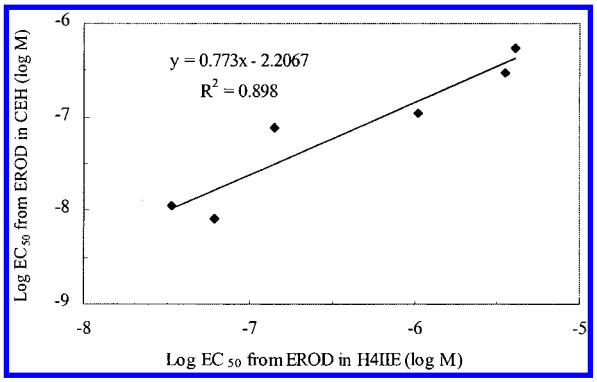


FIGURE 3. Correlation of EC_{50}s (M) for PBDE congeners 77, 119, 126, 100, 153, and 183 from primary chicken embryo hepatocytes (CEH) and rat hepatoma cell line (H4IIE). Points represent the mean level of log EC_{50} (log M). If TCDD is included in the correlation analysis, the R^2 is 0.9782.

in rat liver cells but not in the other cells, and PBDEs 77 and 126, which were approximately 10-fold more potent in chick hepatocytes (which have been noted to be particularly sensitive HACs (*59*)). The concordance of REP values for a given congener across species is illustrated in Figure 3, which shows the correlation between log(EC_{50}) in H4IIE cells and chick embryo hepatocytes for the six PBDE congeners most active as EROD inducers. Correlations ranged from a high of $r^2 = 0.96$ between H4IIE and Hep G2 cells to a low of 0.71 between primary rat hepatocytes and chick embryo hepatocytes.

In conclusion, PBDEs contribute to the total "dioxin-like" activity of environmental samples, although their activity is much less than that of potent HACs such as PCDDs, PCDFs, and coplanar PCBs. Only a subset of the PBDEs induces EROD activity, as is true also for PCDDs (*56*), PCDFs (*57*), PCBs (*43, 58, 59*), PBBs (*60*), polychlorinated naphthalenes (PCNs) (*61*), and polycyclic aromatic hydrocarbons (PAHs) (*62, 63*). The most active PBDE congeners are more potent than mono ortho PCBs (*43, 58, 59*), with REPs similar to those observed for PCNs (*60*) and PAHs (*61, 62*). Despite their limited ability to induce hepatic monooxygenase enzymes, pure PBDE congeners and their commercial mixtures are persistent, bioaccumulative, and increasing in abundance in environmental biota (*64*), implying that exposed organisms may experience their biochemical or toxic effects for protracted periods.

Acknowledgments

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