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Hapten Syntheses and Antibody Generation for the Development of a Polybrominated Flame Retardant ELISA[||]

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Polybrominated diphenyl ethers (PBDEs) are a class of brominated flame retardants that are increasingly an environmental concern. Several antibodies were developed for the polybrominated diphenyl ether flame retardant BDE-47 (**1**), often found in the highest concentration in human milk, plasma, and adipose tissue. Four haptens with different bromine and linker substitution patterns were synthesized and utilized to generate five polyclonal antibodies from goats and two polyclonal antibodies from rabbits. Competition was assessed using four different coating antigens for all seven antibodies. The coating antigen showed marked effects on competition. When the same hapten was used for antibody and the coating antigen less competition was observed. The effect of BDE structure on competition was evaluated by using BDE-47 (**1**), BDE-99 (**2**), BDE-100 (**3**), BDE-153 (**4**), and BDE-183 (**5**). None of the compounds showed high competition with antibody I-KLH, presumably because steric hindrance prevented formation of an efficient binding site. As predicted from structural considerations, BDE-47 (**1**) competed well with the remaining antibodies, whereas BDE-100 (**3**) competed well with only II-KLH. The remaining congeners (BDE-99 (**2**), BDE-153 (**4**), and BDE-183 (**5**)) contain bromines that cannot be positioned in binding sites and thus cross-react poorly. The competition study demonstrated that a bromine substitution on the congener could occupy a position analogous to the linker's position.

KEYWORDS: ELISA; immunoassay; analysis; brominated flame retardants; PBDEs; polybrominated diphenyl ether

INTRODUCTION

Polybrominated diphenyl ethers (PBDEs) are brominated flame retardants consisting of a mixture of congeners. Extensive PBDE use has resulted in its increasingly widespread presence in the environment (*1*). Ingestion of PBDEs in foodstuffs and exposure to PBDEs in the environment has resulted in their bioaccumulation in fatty tissues and the milk of humans (*2*). In Sweden and Japan, decreased use of PBDEs, beginning in 1998, has apparently decreased PBDE burdens in human. While Swedish and Japanese women have similar levels of PBDEs in milk (*3*, *4*), averages in American women are nearly 10-fold higher (*1*), although there is considerable variability between individuals. The increase in PBDE levels in North America may be attributed to extensive usage of commercial pentaBDE formulations (*5*). Environmental contamination with PBDE and the observation of toxic responses to PBDEs (*6*, *7*) have resulted in proposals to ban their usage. The European Union banned use of pentaBDE and octaBDE in 2004 (*8*).

Commercial preparations of PBDEs contain a mixture of congeners; the structures of the ones used in this study are shown in **Figure 1**. In clinical samples of 47 nursing mothers (*9*) the percent of the congener in milk was 54% BDE-47, 16.8% BDE-99, 8.5% BDE-100, and 5.9% BDE-153 with other congeners making up the remainder. In plasma (*10*) the percent of the congener for the 2000−2002 sample group was 56% BDE-47, 18% BDE-99, 10% BDE-100, and 12% BDE-153. More alarming, these workers observed significant increases of PBDE levels over time. Other sample matrices have different congener distributions: for example BDE-99 is the most abundant in meat and poultry closely followed by BDE-47, (*11*, *12*); these are also the dominant congeners present in the commercial penta-

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[||] Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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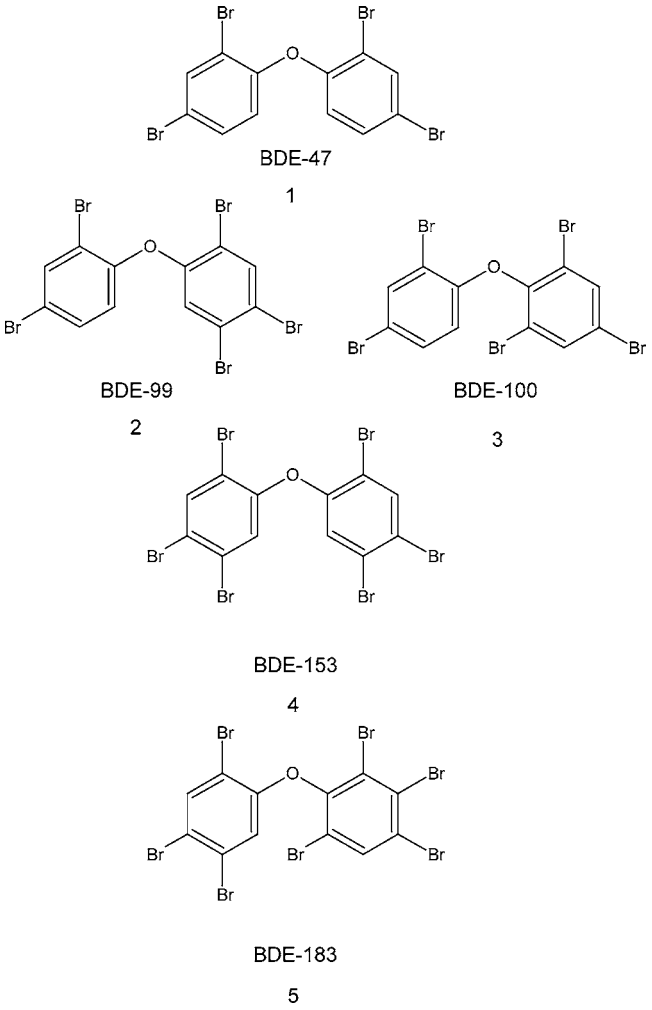

Figure 1. Structures of commonly found PBDEs used in this stdudy.

BDE (Bromkal-70-5DE), where they account for about 35% each in the mixture. Recent data indicate BDE-153 is becoming an increasingly more prevalent congener (*13*, *14*).

All current analytical methods for PBDEs (*15*) require extensive sample cleanup and require expensive instrumentation for separation, detection, and quantitation. Consequently these instrumental methods are less suitable for the high volume of samples required for environmental monitoring. Specific, high-throughput methods would aid the monitoring of this environmental pollutant. Enzyme-linked immunosorbant assays (ELISA) and other immunochemical methods have an established record of being sensitive, specific, and capable of high throughput. Previously, we developed immunoassays for other environmental contaminants such as polycyclic aromatic hydrocarbons (*16*) and polychlorobiphenyls (PCBs) (*17*) and believed this approach could be useful for the PBDEs. To our knowledge, there are no reported immunochemical methods available for the analysis of PBDEs.

The most critical step in producing useful antibodies for ELISA is the generation of suitable haptens for immunogens. Optimal hapten design will result in an antibody with the selectivity and specificity desired for the immunological technique. The incorporation of a "spacer arm" is often necessary to introduce an appropriate functional group that will enable conjugation with carrier proteins; however, the spacer may produce inappropriate haptens because of steric or electronic effects. The effect of hapten structure on antibody selectivity is difficult to precisely predict, although practice has been for haptens to resemble target ligands as closely as possible. To accomplish this aim a variety of approaches ranging from matching two-dimensional structures to sophisticated molecular modeling have been used. We have designed and synthesized a number of haptens mimicking the structure of BDE-47 with various approaches to the positioning of the linker, generated PBDE antibodies, utilized structures generated from semi-empirical quantum mechanics to assist in understanding the role of the linker, and carried out the initial evaluation of an ELISA from one of the antibodies. The work focused on utilizing the distal (from the linker) 2,4-dibromophenyl portion of BDE-47 and systematically varying the ring proximal to the linker. The development of four haptens allowed us to explore the effect of variation of the coating antigen on the assay's response to a fixed level of competitor. The completion of the study would allow a better understanding of the role of linkers in this type of assay and in addition move toward development of an ELISA useful in environmental monitoring of PBDEs.

MATERIALS AND METHODS

Materials. Bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH) were purchased from Sigma-Aldrich Chemical Co., (St. Louis, MO). Imject Alum and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) were purchased from Pierce Biotechnology, Inc. (Rockford, IL). SureBlue TMB microwell peroxidase substrate referred to as TMB was obtained from KPL (Gaithersburg, MD). [^{14}C]BDE-99 was synthesized following the method of Örn et al. (*18*). BDE-47, BDE-100, BDE−-153, and BDE-183 were synthesized according to Marsh et al. (*19*, *20*). Silica gel used for chromatography was 230−400 mesh (60 Å). Whatman 250 μm silica gel thin-layer chromatography (TLC) plates (UV254 fluorescence) were purchased from Fisher Scientific (Springfield, NJ). ELISA plates were purchased from Corning, Inc. (Corning, NY). All other chemicals and solvents were purchased from Sigma-Aldrich unless specifically stated otherwise.

Molecular Computations and Overlap Visualization. Spartan 02 version 1.0.5 running on a MacIntosh OSX was used for modeling (Wavefunction, Inc., Irvine, CA). The molecules were geometry optimized using the AM1 Semiempirical method. A methoxy group was substituted for the γ-propoxycarboxylate group in haptens I−III, and a formylamido group was substituted for the succinoylamido group in hapten IV. These substitutions preserved the steric and electronic nature of the linker juncture with the hapten but eliminated a large number of conformational isomers that would complicate the molecular orbital calculations while not providing additional information. The Molecular graphics were generated using the USCF Chimera package, and the molecules were superimposed manually (*21*). This simplified approach was utilized since we wished to view the overlap of the bromine of the ligands with the bromines or linker group of the hapten, or observe cases where the bromine of the ligand had to be positioned where no such overlap existed.

Hapten Synthesis. *General Method.* Unless otherwise indicated all glassware was flame-dried or was oven-dried for at least 12 h (110−120 °C) and cooled under nitrogen. All chemicals were used as purchased except dimethylformamide (DMF) and CH_2Cl_2, which were dried over CaH_2 and distilled under vacuum (DMF) or under nitrogen (CH_2Cl_2). Preparation of individual haptens required the synthesis of various polybromo-substituted diphenyl ethers carrying a phenolic or an amino group to which the linker was attached. Phenolic precursors were synthesized using the method of Marsh et al. (*22*) with only minor or no modifications. The identity of the products was confirmed by melting point (uncorrected), IR spectra (JASCO Fourier transform infrared spectrometer 460 Plus), high-resolution mass spectrometry (Agilent Technologies GC-MS 6890/5973N), and NMR (Bruker Avance 400 spectrometer). Purities were confirmed with TLC or HPLC, unless stated otherwise. Each of the target haptens contained a 2,4-dibromophenoxy group substituted on a second polybromophenyl ring containing the linking group. To our knowledge, the syntheses of the precursors of hapten IV have not been reported; therefore, the synthesis for hapten IV is described in detail.

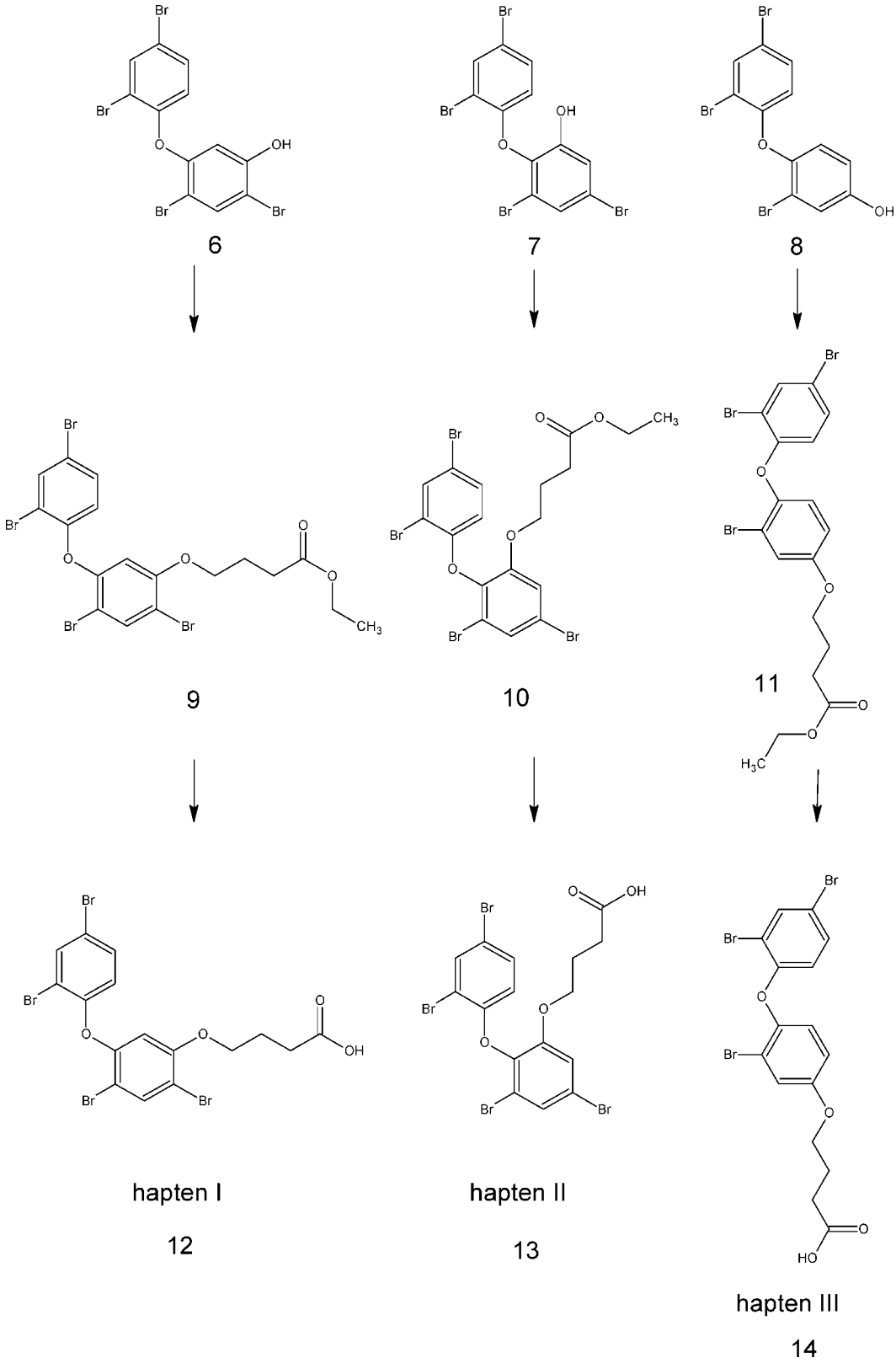

Figure 2. Synthesis scheme for haptens I–III.

Synthesis of Haptens I−III. *General Procedures for Synthesis of Butyrate Haptens.* **Figure 2** provides the general synthetic scheme for haptens I−III. Phenol precursors (2,4-dibromo-5-(2,4-dibromophenoxy)-phenol (**6**), 3,5-dibromo-2-(2,4-dibromophenoxy)phenol (**7**), and 3-bromo-4-(2,4-dibromophenoxy)phenol (**8**)) were synthesized using previously reported procedures (*22*). To a rapidly stirring solution of phenol precursor (2.2−12 mmol) in dried THF sodium hydride a 60% suspension was added (1.1 × molar concentration of phenolic precusor). After the mixture was stirred for 30 min, ethyl 4-bromobutyrate (1.1 × molar concentration of phenolic precursor) was added and the mixture was refluxed for 3−4 days. After THF was removed under vacuum, water (150 mL), concentrated HCl (2 mL), and CH_2Cl_2 (100 mL) were added. After phase separation, the CH_2Cl_2 layer was collected. The aqueous layer was further extracted with CH_2Cl_2 (100 mL), and the combined organic layers were dried over anhydrous sodium sulfate. After the removal of organic solvent, the residue was dissolved in CH_2Cl_2 and purified by the column chromatography. A glass column (2.9 cm i.d.) was packed with 120 g of silica gel, and the top was covered

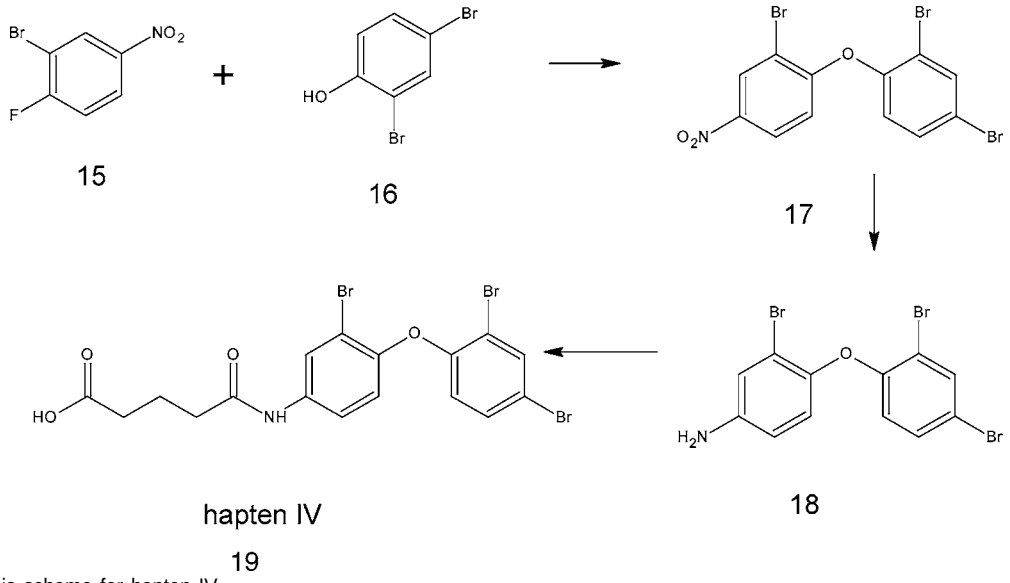

Figure 3. Synthesis scheme for hapten IV.

with anhydrous sodium sulfate (1 cm). The column was prewashed with a mixture of petroleum ether and CH_2Cl_2 (100 mL, 3/1 (v/v)). The sample was then loaded and eluted with a mixture of petroleum ether and CH_2Cl_2 (3/1 (v/v)). The yields ranged from 60 to 70%.

General Procedures for Converting Hapten Ester to Hapten Acid. Hapten ester [ethyl 4-(2,4-dibromo-5-(2,4-dibromophenoxy)phenoxy)-butyrate (**9**), ethyl 4-(3,5-dibromo-2-(2,4-dibromophenoxy)phenoxy)-butyrate (**10**), or ethyl 4-(3-bromo-4-(2,4-dibromophenoxyl)phenoxy)-butyrate (**11**)] (5 mmol) was dissolved in ethanol (100 mL), and aqueous sodium hydroxide (6 N, 3 mL) was added. The solution was stirred for 36 h at room temperature. After the removal of ethanol under vacuum, 10 mL of 2 N HCl and 150 mL of distilled water were added. The hapten acid was extracted with CH_2Cl_2 (150 mL × 2). After the removal of organic solvent, the residue was redissolved in CH_2Cl_2 (5 mL) and purified with silica gel TLC. The extracts were applied in a band (maximum 30−40 mg of mass/(TLC plate)) to a preparative TLC plate, and the plate was developed with a mixture of CH_2Cl_2 and hexane (4/1 or 3/1 (v/v)). The band near the origin was isolated by scrapping and extracted with CH_2Cl_2 (80 mL), and the extracts were filtered through a membrane filter (PTFE, 0.2 μm). The products were analyzed with a HP-1100 series HPLC, equipped with diode array detector and mass selective detector, using a VyDac C18 column (C18, 250 mm length × 4.6 mm i.d., 5 μm). The elution solvent was 80−20 acetonitrile−water at a flow rate of 0.7 mL/min. The eluant was monitored at 210 and 254 nm. Ionization was achieved in the APCI mode (fragmentor voltage, 220 V; chamber, 3500 V for positive and 3000 V for negative ion). The hapten purity after HPLC purification was >97%. The reaction yield was 60−80%, depending on the acid, after TLC purification. After the evaporation of solvent from TLC extracts, the residues were sticky oils, but after storing the residues in a vacuum chamber for 1−2 days, all the oils turned into white solids.

Synthesis of Hapten IV. Figure 3 shows the synthesis route for hapten IV.

2,2′,4′-Tribromo-4-nitrodiphenyl Ether (17). 2,4-Dibromophenol (**16**) (1.04 g, 4.13 mmol) was dissolved in dry DMF (8 mL) and cooled to 0 °C; sodium hydride (0.331 g, 60% in mineral oil, 8.28 mmol) was slowly added, and the solution stirred in an ice bath for 30 min and then at room temperature for 15 min. 3-Bromo-4-fluoronitrobenzene (**15**) (1.01 g, 4.13 mmol, 90% pure) was added, and the mixture was heated to 90 °C for 2 h. Water (10 mL) was added, and the mixture was extracted with CH_2Cl_2 (4 × 20 mL), the combined organic layers back-extracted with 25 mL of water, the organic layer dried using $MgSO_4$, and the volatiles removed under reduced pressure. The crude oil was purified by column chromatography on silica gel eluting with hexane and ether (50:50) and resulted in 1.19 g of the desired compound as a yellow-orange oil (63.8%). GC/MS analysis indicated greater than 86% purity. ^{1}H NMR (δ, $CDCl_3$) 6.69 (d, J = 9 Hz, 1H), 7.02 (d, J = 9 Hz, 1H), 7.53 (dd, J = 9 Hz, 3 Hz, 1H), 7.85 (d, J = 3 Hz, 1H), 8.10 (dd, J = 9 Hz, 3 Hz, 1H), 8.56 (d, J = 3 Hz, 1H); ^{13}C NMR (δ, $CDCl_3$) 113.2, 116.1, 117.1, 119.9, 123.9, 124.7, 130.2, 132.8, 137.1, 143.7, 151.1, 159.0; MS (GC-MS, EI, 70 eV) 217 ($M^+ - PhBr_2$), 219 ($M^+ + 2 - PhBr_2$), 324 ($M^+ - NO_2$, Br), 326 ($M^+ + 2 - NO_2$, Br), 328 ($M^+ + 4 - NO_2$, Br), 449 (M^+), 451 ($M^+ + 2$), 453 ($M^+ + 4$), 455 ($M^+ + 6$).

4-Amino-2,2′,4′-tribromodiphenyl Ether (18). The general reduction procedure described by Cortese and Heck (*23*) was followed. The 2,2′,4′-tribromo-4-nitrodiphenyl ether (**17**) from the previous reaction (0.30 g, 0.65 mmol) was dissolved in 3 mL of triethylamine. Platinum on carbon (0.0385 g, 5% on C) was added, followed by formic acid (0.2 mL, 5.20 mmol). The mixture was heated at reflux for 1.5 h and then CH_2Cl_2 (2 mL) was added; the solution was filtered, dried, and chromatographed on silca gel eluting with a hexanes−ethyl acetate mixture (50:50), giving a viscous, yellow oil (0.17 g, 62.1%). GC/MS analysis showed 91% purity. ^{1}H NMR (δ, $CDCl_3$) 3.70 (br s, 2H), 6.45 (d, J = 9 Hz, 1H), 6.60 (d, J = 8.5 Hz, 1H), 6.80 (d, J = 8.5 Hz, 1H), 6.90 (d, J = 2 Hz, 1H), 7.30 (s, 1H), 7.75 (s, 1H); ^{13}C NMR (δ, $CDCl_3$) 113.6, 115.2, 115.6, 116.6, 117.9, 120.0, 123.3, 131.6, 136.1, 136.3, 145.2, 155.0; MS (GC-MS, EI, 70 eV) 186 ($M^+ - PhBr_2$), 188 ($M^+ + 2 - PhBr_2$), 261 ($M^+ - 2Br$), 263 ($M^+ + 2 - 2Br$), 419 (M^+), 421-($M^+ + 2$), 423 ($M^+ + 4$), 425 ($M^+ + 6$).

5-[4-(2,4-Dibromophenoxy)-3-bromophenyl]carbamoylpentanoic acid (19). The procedure employed was similar to that described by Shelver and Smith (*24*). Briefly, 4-amino-2,2′,4′-tribromodiphenyl ether **(18)** (170.1 mg, 0.37 mmol) was dissolved in 3.0 mL of anhydrous pyridine, glutaric anhydride (89.4 mg, 0.78 mmol) was added, and the reaction mixture was stirred at room temperature for 45 h. The reaction was acidified with 2 M HCl and extracted 4 times with 2 mL of CH_2Cl_2. The combined organic layers were back-extracted with 2 M NaOH. The aqueous layer was separated, cooled, and acidified to pH 2, after which a white precipitate formed. The precipitate was collected by vacuum filtration, dissolved in ethyl acetate, and passed through a plug of silica gel. Evaporation of the solvent yielded a sticky white solid that was washed with several portions of cold CH_2Cl_2 to provide the target hapten 5-[4-(2,4-dibromophenoxy)-3-bromophenyl]carbamoyl-pentanoic (**19**) acid in acceptable yield and >99% purity as a colorless solid (73 mg, 37.6%): mp, 161−164 °C; ^{1}H NMR (δ, THF-d_8) 1.92 (m, 2H), 2.32 (t, J = 8 Hz, 2H), 2.37 (m, J = 8 Hz, 2H), 6.64 (dd, J = 9 Hz, 1 Hz, 1H), 6.96 (dd, J = 8 Hz, J = 1 Hz, 1H), 7.38 (ddd, J = 8 Hz, J = 2 Hz, J = 1 Hz, 1H), 7.56 (dd, J = 9 Hz, J = 2 Hz, 1H), 7.84 (dd, J = 2 Hz, J = 1 Hz, 1H), 8.11 (d, J = 2 Hz, 1H), 9.33 (s, 1H), 10.9 (br s, 1H); ^{13}C NMR (δ, $CDCl_3$) 21.3, 33.3, 36.4, 114.4, 115.3, 116.1, 119.5, 120.2, 122.0, 124.7, 132.4, 136.6, 138.7, 148.2, 154.6, 171.2, 174.3; IR (KBr) 3400 br, 3260, 1725, 1160; MS (GC-MS, EI, 70 eV) 419 ($M^+ - COCH_2CH_2CH_2COOH$), 421 ($M^+ + 2 - COCH_2CH_2CH_2COOH$), 423 ($M^+ + 4 - COCH_2CH_2CH_2COOH$), 425 ($M^+ + 6 - COCH_2CH_2CH_2COOH$), 515 ($M^+ - H_2O$), 517 ($M^+ + 2$

$- H_2O$), 519 ($M^+ + 4 - H_2O$), 521 ($M^+ + 6 - H_2O$); MS (HPLC-MS, ES, CE = 5) 533.8 (M^+), 535.8 ($M^+ + 2$), 537.8 ($M^+ + 4$), 539.8 ($M^+ + 6$).

Antigen Generation. Dry DMF (250 μL) was added to 0.02 mmol of each PBDE hapten and 0.05 mmol each of *N*-hydroxysuccinimide and EDC. The mixture was stirred at room temperature for 4 h and then centrifuged to remove precipitated urea. The clear supernatant was slowly added, approximately 10−20 μL/(20 min), to a KLH solution (15 mg of KLH in 5 mL of a 0.1 M borate buffer, pH 9.0) or BSA solution (75 mg of BSA in 5 mL of a 0.1 M borate buffer, pH 9.0). The reaction was stirred overnight at 4 °C and then dialyzed against PBS (1.5 L) for 3 days at 4 °C with two buffer changes per day. All the conjugated antigens were stored at −80 °C until used.

Antibody Generation. Goats were housed in the Biosciences Research Laboratory barn with ad libitum access to food and water. The individual PBDE-KLH conjugates (100 μg in 1.6 mL of PBS) were mixed with an equal volume of Alum Imject until an emulsion formed. The emulsion was initially injected subcutaneously at six sites. Goats received monthly injections containing 100 μg of immunogen. After the fourth (goats 133, 134, and 137) or fifth injection (goats 135 and 136), goats received an additional injection of 250 μg of immunogen for 4 months, since the titers particularly for goats 135, 136, and 137 were low. After the last injection the sera were used to evaluate the specificity and sensitivity toward different PBDE congeners. Because some of the titers from the goats were low, rabbits were immunized with 100 μg of the conjugate and given two more injections (100 μg) after which a satisfactory titer was obtained and the sera were utilized to evaluate the specificity and sensitivity toward different PBDE congeners.

Sera Evaluation. *(1) Titer Check.* In a 96-well ELISA plate, 100 μL/well of PBDE−hapten IV−BSA (2.5 μg/mL in bicarbonate coating buffer) was allowed to coat at room temperature for 2 h and at 4 °C overnight, after which the plate was washed 3 times with PBST. Control sera and sera from haptens I−IV were diluted with PBST + 1 mg/mL BSA, and 50 μL of corresponding diluted sera was pipetted into the ELISA plates. For goats 133 and 134 and rabbits 119 and 120 the dilutions ranged from 1:2000 to 1:1024480 using 1:2 serial dilutions. For goats 135, 136, and 137, because of low titer, the dilutions ranged from 1:50 to 1: 6400 using 1:2 serial dilutions. The plates were incubated at room temperature for 90 min and then washed three times with PBST (Wallac Columbus Washer, Turku, Finland). For the plates using goat sera 100 μL/well of a 1:6000 dilution (PBST + 1 mg/mL BSA) of rabbit anti-goat IgG-HRP was then added, and the plates were incubated at room temperature for 60 min. For plates using rabbit sera, goat anti-rabbit IgG-HRP diluted at 1:10000 was utilized. The plates were washed with PBST 3 times, and 100 μL/well of TMB substrate solution were added. The plates were incubated at room temperature for 30 min, after which the color development was stopped by adding 50 μL/well of 2 N H_2SO_4. The plates were read at 450 nm (Bio-Rad model 550 ELISA plate reader, Hercules, CA). The titers were defined as inverse of the highest dilution that produced absorbance greater than 0.2. The titers were determined monthly after each immunization.

(2) Specificity Determination. An indirect competition format was utilized for specificity determination. ELISA plates were coated with 100 μL/well of a solution of one of haptens I−IV−BSA at a concentration of 2.5 μg/mL and then washed. Competitors (BDE-47, BDE-99, BDE-100, BDE-153, and BDE-183) were added (100 μL/well at a concentration of 100 ng/mL), and co-incubated with 50 μL/well of sera. The dilution selected for the sera was the dilution that produced an OD_{450} nm of approximately 1.5 as determined by the titer check (except goat 135, at 1:50 OD_{450} nm = 0.7). The dilutions used were as follows: for goats 133 and 134, 1:2000; for goats 135, 136, and 137, 1:50; and for rabbits 119 and 120, 1:8000. Each animal's specificity pattern was determined in duplicate. The competitor and primary antibody were co-incubated at room temperature for 90 min. The secondary antibody and color development were the same as described above. After reading the plate, percent inhibition was determined from the OD_{450} nm of wells that contained PBDEs vs OD_{450} nm of wells that do not have PBDEs.

(3) Standard Curve Generation. PBDE−hapten I−BSA was used as a coating antigen, and indirect competitive ELISA was performed as described in Specificity Determination. Rabbit 120 sera at 1:8000 were utilized as primary antibody and co-incubated with BDE-47. The calibration curve used BDE-47 concentrations of 7.8, 15.6, 31.25, 62.5, 125, 250, and 500 ng/mL, performed in duplicate, and was repeated 3 times. The calibration curves were fitted with a logistic equation $Abs = (A - D)/[1 + (concn/C)^b] + D$, where A was the absorbance value when no BDE-47 is present; D was the absorbance value with the highest concentration of BDE; C was midpoint inhibition concentration (IC_{50}); and b was the slope near the midpoint inhibition concentration (Microplate Manager 4.0, Bio-Rad-Laboratories, Hercules, CA).

RESULTS AND DISCUSSION

Hapten Design and Syntheses. Because BDE-47 (**1**) is the congener present in the highest concentration in human milk, serum, and fatty tissue, we designed our haptens to produce antibodies to bind this molecule. When developing an immunoassay, careful hapten design must be utilized to achieve appropriate specificity. The hapten must be coupled with the protein using an appropriate spacer that allows the critical groups to play a role in forming the antibody binding pocket. For three of our haptens (**12**, **13**, **14**) we elected to use a three-carbon spacer between the ether linkage attached to the aromatic polybromodiphenyl ether and the carboxyl group used in the protein coupling, since this length spacer often worked well. This spacer should allow the polybromodiphenyl ether moiety to project from the carrier protein surface sufficiently to allow formation of an antibody binding pocket sensitive to the haptens structural features. Hapten IV (**19**) employed an amide rather than an ether linkage with three methylenes between the amide and the carboxylate group, to test the effect of altering the coupling functional group. The restricted rotation about the CO−NH bond could alter the geometry compared with the more freely rotating ether linkage. The amide can serve as a proton donor or acceptor, presenting a different chemistry from the ether group. Using a different linker in the immunogen and coating antigen could minimize the role of the linker in binding and produce a more sensitive ELISA. This is analogous to the studies in hetero ELISA and homo ELISA (*25*, *26*).

In addition to the linker, the bromine substitution pattern would be important in hapten design. All of our haptens contain the 2,4-dibromo substitution pattern found in BDE-47 (**1**) but also in BDE-99 (**2**) and BDE-100 (**3**) in the ring distal to the linker. Two of our haptens have a 2,4-dibromo substitution on the proximal ring attaching the linker to the 5 position in hapten I (**12**) or the 6 position in hapten II (**13**). Hapten III (**14**) and hapten IV (**19**) both have the linker positioned in place of the 4-bromo substituent on the proximal ring with hapten IV utilizing an amide rather than ether to connect the linker. The ether oxygen of the linker and bromine in PBDEs possess similar electronic structures with both atoms having nonbonded electron pairs. This similarity could allow the binding of a PBDE that places bromine in the same orientation as the linker of the hapten, an effect we will test experimentally. The antibody will form a binding site for the hapten with the linker pointing away from the antibody surface. For our haptens the group attaching the linker to the polybromodiphenyl ether would be expected to be part of the binding site with the flexible methylene chain oriented out in space terminating with the carrier protein. We explored haptens with different linker orientations in order to maximize our chances of finding an appropriate antibody and developing useful assays. To assist in the analysis of the results, we carried out molecular orbital calculations to generate three-dimensional chemical structures. These computations were carried out using the AM1 semiempirical molecular orbital

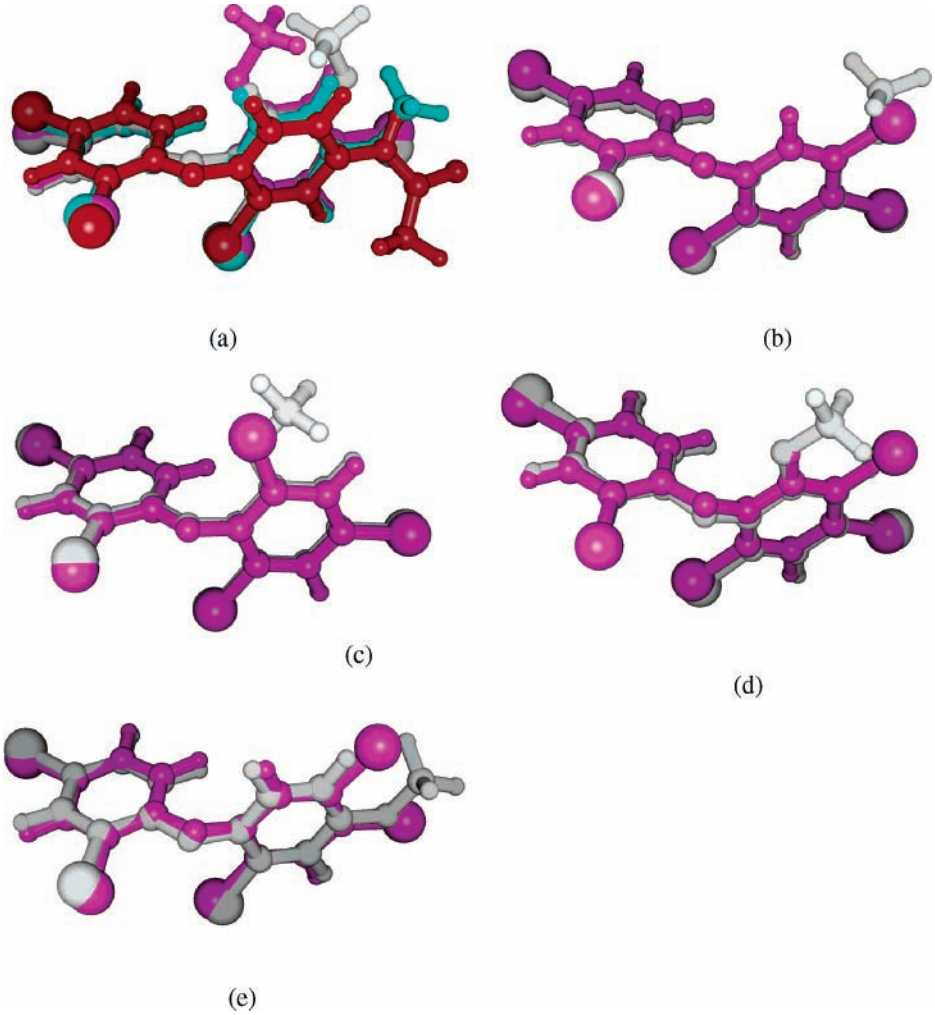

Figure 4. (a) Overlay of the four antigens with the distal ring commonly placed (hapten I, white; hapten II, magenta; hapten III, green; hapten IV, red). (b) Overlay of BDE-99 (magenta) and hapten I (white). (c) Overlay of BDE-100 (magenta) and hapten II (white). (d) Overlay of BDE-99 (magenta) and hapten II (white). (e) Overlay of BDE-99 (magenta) and hapten III (white).

method, and all molecules were geometry-optimized to obtain a stable conformation. The flexible chain of the haptens was eliminated since we intended to do a simple overlap comparison and the flexible portion of the linker would not affect these comparisons. Surrogate groups were used, a methoxy group for the γ-butoxycarboxy group and a formylamide for the glutaramide, as these groups preserve the electronic and steric properties of the haptens. The overlap analysis of the haptens is shown in **Figure 4**a, where the similarity and differences are clearly visible. The two bromines in the distal ring and the bromine 2 position of the ring containing the linker clearly overlap in all the haptens (**Figure 4b−e**).

Antibody Generation. All hapten syntheses and immunogen conjugation were uneventful. Initial evaluation showed immunogen of hapten I produced good titers after four injections. Conversely, immunogens from hapten III and IV did not result in an appreciable amount of antibody after the same dose regimen. Immunogen from hapten II produced intermediate results. Hence, the quantity of immunogen was increased to 250 μg/goat. However, after four more injections, immunogens of hapten III and IV still did not produce an appreciable increase in antibody titers; hence, rabbits were employed. After three immunizations at 100 μg/animal, rabbits showed titer greater than 8000 toward haptens II and III. Therefore, sera from hapten I−III with a high titer were available to test for specificity, whereas only low-titer sera from hapten IV was tested. Antibody titers tended to stabilize after four injections and did not increase with further injections. After eight injections goats 133 and 134 had titers of 16 000 and 137 had a titer of 800. After nine injections, goat 135 had a titer of 200 and goat 136 had a titer of 800. Thus, antibody production in goats was variable and sometimes problematic even after multiple boosts. Rabbits 119 and 120 had titers of 128 000 after three injections. Thus, rabbits were capable of producing good titer from hapten III, demonstrating a strong species difference.

Antibody Characterization. Table 1 shows the results of the inhibition of antibody binding by 10 ng/well of different BDE congeners with different coating antigens. Higher inhibition demonstrates a greater ability of the BDE congener to compete with the coating antigen for the antibody binding site. Clearly the degree of inhibition is dependent on both the competing PBDE congener and the coating antigen. As expected the antibodies from different haptens show different patterns of inhibition. Different inhibition patterns are also seen from antibodies produced from the same hapten but different animal species. BDE-47 showed relatively good inhibition with all antibodies, and all coating antigens indicated success of our hapten design. In general, the use of a coating antigen derived from the same hapten used to produce the antibody produced lower inhibition than the other coating antigens.

Rather surprising, none of the analytes competed to a great extent with the antibody produced from hapten I. Interestingly,

Table 1. Competition toward BDE-47, BDE-99, BDE-100, BDE-153, and BDE-183[a]

		% of inhibition			
immunogen, antisera	BDE congener	I-BSA[b]	II-BSA[b]	III-BSA[b]	IV-BSA[b]
I-KLH, goat-133	BDE-47	13	10	9	5
	BDE-99	10	18	9	9
	BDE-100	5	8	6	11
	BDE-153	7	6	8	15
	BDE-183	0	11	6	0
II-KLH, goat 134	BDE-47	40	23	49	24
	BDE-99	24	22	34	22
	BDE-100	49	39	52	29
	BDE-153	15	7	15	5
	BDE-183	8	11	20	9
II-KLH, rabbit 119	BDE-47	35	21	26	30
	BDE-99	19	17	13	11
	BDE-100	38	20	37	24
	BDE-153	2	12	0	3
	BDE-183	7	0	5	0
III-KLH, goat 137	BDE-47	26	19	8	4
	BDE-99	15	9	18	3
	BDE-100	21	20	14	6
	BDE-153	13	11	8	7
	BDE-183	10	10	8	0
III-KLH, rabbit 120	BDE-47	55	21	43	34
	BDE-99	33	19	21	6
	BDE-100	11	8	9	4
	BDE-153	7	7	3	2
	BDE-183	2	3	1	5
IV-KLH, goat 136	BDE-47	32	14	23	5
	BDE-99	14	13	6	1
	BDE-100	26	11	13	0
	BDE-153	27	18	9	0
	BDE-183	22	10	15	0
IV-KLH, goat 135	BDE-47	26	15	24	0
	BDE-99	11	8	6	1
	BDE-100	14	9	13	6
	BDE-153	9	7	8	2
	BDE-183	16	11	7	7

[a] The values are the mean of two determinations with an average difference between replicates of 8% for BDEs-47 and -99, 5% for BDEs-100 and -153, and 6% for BDE-183. [b] Coating antigen.

when **12** was used to form the coating antigen, generally high inhibition was observed throughout the study. This could be interpreted as hapten 1, showing weak binding of the ligand making competition with the antibody easier. The structure of **12** has the linking group adjacent to the 4 bromine, and the formation of an antibody with this congested site could result in less specific binding of ligands with bromine in this position. The lowered affinity would negatively affect the function of the antibody but would make displacement from the coating antigen easier. When the additional bromine of **2** is positioned so it overlaps the linker of hapten I (**Figure 4b**) for three of the four coating antigens used to study this antibody, the competition was higher than the similar experiments with BDE-47. For all the other antibodies **2** shows distinctly poorer inhibition than **1**. This provides strong evidence that the bromine of a ligand can fit into the binding pocket produced by the linker, and overlap studies shown in **Figure 4** can be useful in understanding the experimental results.

The antibody produced by **13** in either goats or rabbits produced rather good results. High inhibition was produced by **1** and **3** as predicted from examination of the overlap of the ligand and hapten. The efficient inhibition of **3** is expected because the additional bromine relative to **1** occupies the position of the ether linker (**Figure 4c**). In contrast, **2** places the additional bromine in the space occupied by hydrogen in the hapten but adjacent to the linking group (**Figure 4d**). Consequently **2** shows lower inhibition than **1** or **3** but much more than the other competitors. As expected, BDE-153 (**4**) and BDE-183 (**5**) show reduced inhibition due to steric conflicts since they have additional bromines that do not match the hapten binding pockets.

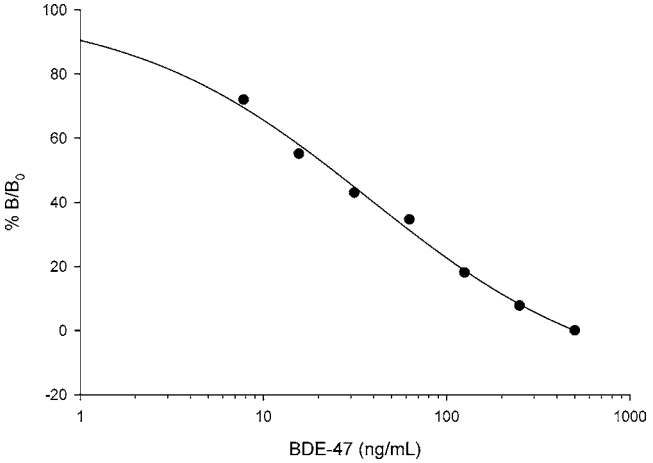

Figure 5. Standard curve from the antibody produced from hapten III–KLH immunized rabbit with hapten I–BSA as the coating antigen. The mean IC_{50} was 27.7 ng/mL ($n = 3$).

Hapten III produced antibodies in goat 137 and rabbit 120, but the antibodies were distinctly different from each other. The low-titer goat antibody produced only very modest inhibition, but the rabbit antibody produced high inhibition, particularly for **1**. As overlap considerations predict (**Figure 4e**), inhibition from **3** is less than **1** but more than the other ligands since the bromine that is not in a bromine site is adjacent to the linker as in the binding between **3** and **13**.

For **19** the antibodies produced from two different goats showed competition for **1** but poor differentiation from the other ligands. Although the chemistry of the linkers differs between **14** and **19**, the low-titer antibodies produced from these two haptens in goats are at least qualitatively similar. Interestingly, the use of the **19** derived coating with the antibodies from the same hapten produced very poor competition. Again, this indicates at least for this system using the same hapten for both coating antigen and forming the antibody may not produce the optimum analytical system.

The most promising antibody for ELISA development was that produced against hapten III in the rabbit and employing hapten I−BSA as the coating antigen. This combination produced the highest inhibition using **1** as the competing ligand. Other PBDEs showed less inhibition, and thus this system would show considerable specificity. The standard calibration curve using BDE-47 as a competitor is shown in **Figure 5**. The data were well-fit by the standard four-parameter equation and had an IC_{50} of 27.7 ng/mL ($n = 3$).

Conclusion. Four different PBDE−haptens were synthesized, and three of the immunogens produced antibodies, which showed good competition with BDE-47. The immunogen from hapten I with the poorest competition had the linker adjacent to a critical binding site, indicating this design should be avoided. However, the use of this hapten coupled with BSA as a coating antigen generally produced greater competition relative to the other coating antigens. The results of the competition study and examination of overlap between ligands and haptens confirmed that the linker position was suitable as a binding site for the aromatic bromine on the ligand. As overlap analysis predicted, only one of the immunogens produced an antibody (II-KLH) that showed good competition with BDE-100. The

analysis also explained why BDE-99 showed slightly improved binding for antibody I-KLH. These results demonstrated the importance of the linker position. The decreased competition when BDE-153 and BDE-183 were used as competing compounds was compatible with the model. The results also indicated the coating antigen played an important role in determining competition with the interacting ligand.

ABBREVIATIONS USED

BSA, bovine serum albumin; BDE, polybrominated diphenyl ethers; BFR, brominated flame retardant; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; ELISA, enzyme-linked immunosorbent assay; KLH, keyhole limpet hemocyanin; MES, 2-[*N*-(morpholino)ethanesulfonic acid]; OD, optical density; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline plus 0.05% Tween 20; TMB, 3,3′,5,5′-tetramethylbenzidine.

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