Hapten Synthesis and Antibody Development for Polychlorinated Dibenzo-*p*-dioxin Immunoassays

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This paper reports the synthesis of haptens and the generation and preliminary evaluation of polyclonal antibodies for the detection of dioxins such as TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) by ELISA (enzyme-linked immunosorbent assay). These novel haptens contain unsaturation between the halogenated dibenzo-*p*-dioxin ring system and the protein to which it is conjugated, presenting a rigid handle structure. The substitution pattern is identical with or similar to that of TCDD (i.e., 2,3,7,8- or 1,2,3,7,8-). Finally, the haptens lack polar groups for hydrogen bonding. In direct binding assays using the new polyclonal antibodies there was excellent recognition of hapten−protein conjugates, including recognition of those hapten conjugates that were not used as immunogens (i.e., assay systems heterologous in hapten structure). These haptens do elicit selective immune responses in rabbits. Their evaluation in an ELISA format demonstrated the usefulness of these haptens for the detection of dioxins. An IC_{50} of 0.8 ng/well (16 ng/mL) was observed for an unoptimized system that used 2,3,7-trichloro-8-methyldibenzo-*p*-dioxin as an analytical surrogate standard.

Keywords: *TCDD; dioxin; immunoassay; polyclonal antibodies; hapten synthesis; polychlorinated hydrocarbons*

INTRODUCTION

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (Figure 1A) is the most toxic member of a group of halogenated aromatics including halogenated dibenzo-*p*-dioxins, dibenzofurans, and biphenyls. These compounds are known to exhibit a wide range of adverse effects on a variety of species (Whysner and Williams, 1996; Safe, 1995; Huff et al., 1994). Additionally, dioxins and related compounds are distributed ubiquitously in the environment (Safe, 1991). The toxicity of these chemicals to humans and the magnitude and relevance of the risk posed to humans by these compounds are topics of active debate and research (Whysner and Williams, 1996; Environ Dioxin Risk Characterization Expert Panel, 1995; Anonymous, 1995; Johnson, 1995; Clapp et al., 1995); however, the need for analytical methods to measure dioxins and related chemicals in environmental and biological samples is undisputed. Today, these compounds are heavily regulated on the basis of current knowledge about their toxicity to humans and other species (Federal Register, May 7, 1997). Effective enforcement of and compliance with these regulations necessitates analysis of vast numbers of samples. Furthermore, the debate over the significance of the impact that polychlorinated dibenzodioxins, dibenzofurans, and bi-

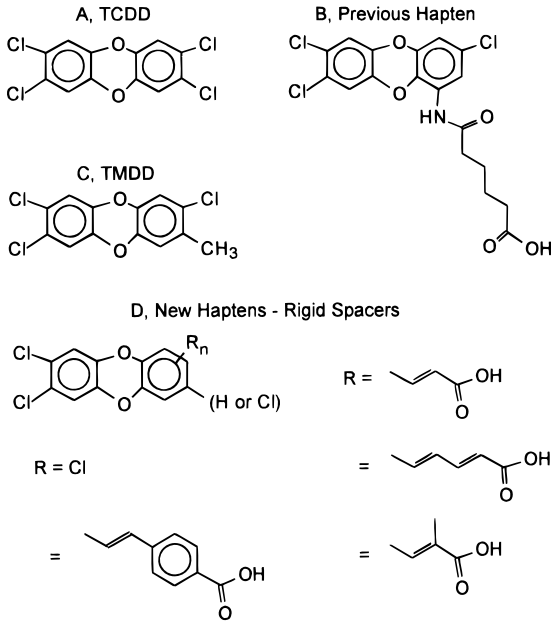

Figure 1. Structures of TCDD (A), the hapten used in previously published work (B), the surrogate standard (C), and new haptens with rigid spacers used in this paper for antibody development (D).

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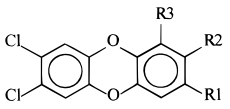
phenyls have on various species including humans can only be answered through further study of environmental levels of these compounds and related health effects. A fundamental element of this research is the ability to measure environmental and biological levels of these chemicals with high speed, accuracy, and precision.

Analysis of TCDD and related compounds is extremely challenging. To obtain a complete picture of environmental contamination by these chemicals, these lipophilic species need to be determined specifically at extremely low levels (typically parts per billion to parts per quadrillion) in various biological and environmental matrices. The current state-of-the-art methodology for analysis of these compounds is based on the use of gas chromatography with high-resolution mass spectrometric (HRMS) detection (de Jong and Liem, 1993; Ferrario et al., 1996; Tondeur et al., 1989; USEPA, 1990). The combined selectivity of a high-resolution capillary separation technique and a mass analyzer allows for specific detection of these molecules. Mass spectrometric detection is also very sensitive and provides confirmation of analyte identity based on molecular mass and fragmentation patterns. Extensive sample preparation involving organic extraction and sample concentration are also critical parts of these methods, improving the detection limits and selectivity of the analytical methods while reducing interference by environmental and biological matrixes. The time requirements and expense of these GC−MS-based methods are their major limitations. While these methods are well-suited to meet the analytical challenges posed by dioxins and related compounds, the time and money required to use these methods severely limits the numbers of samples that realistically can be analyzed. Effective use of these methods also requires highly trained analytical chemists.

Immunoassays (IA's) are analytical techniques that are well-suited to complement GC−MS-based methods for the analysis of polyhalogenated dibenzodioxins, dibenzofurans, and biphenyls. Many of the strengths of analysis by IA address the weaknesses of GC−MS-based analysis (Hammock and Gee, 1995). Typically, immunoassays are relatively inexpensive and rapid. Often sample preparation requirements are minimal in comparison to GC−MS-based methods, significantly reducing analysis time and expense. Reduced sample preparation has the added benefit of significantly decreasing the amount of organic solvent waste generated per sample analyzed. Immunoassays are generally less demanding instrumentally and more easily automated. Fully developed IA's also generally require less operator expertise to use.

A number of antibodies and IA's have been developed to polyhalogenated dibenzodioxins (Langley et al., 1992; Sherry et al., 1990; Stanker et al., 1987; Kennel et al., 1986; Watkins et al., 1989; Albro et al., 1979; Harrison and Carlson, 1997), polyhalogenated dibenzofurans (Luster et al., 1980), and polyhalogenated biphenyls (Waters et al., 1994; Donnelly et al., 1996; Franek et al., 1992; Mapes et al., 1993; Newsome and Shields, 1981; Johnson and Van Emon, 1996; Luster et al., 1979; Chiu et al., 1995). Hapten design, hapten synthesis, and antibody development are the initial and very critical aspects of immunoassay development. In the case of dioxins and related compounds, these two steps are particularly challenging because of the lipophilicity of these chemicals. Early reports of hapten synthesis and antibody development for radioimmunoassays (RIA's) designed to detect TCDD involved haptens with a 1,3,7,8 substitution. This earlier work with polyclonal antibodies resulted in immunoassays with low detection limits (25 pg) and fair selectivity for the target analyte, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (Albro et al., 1979). However, the hapten used in their work does not share the same substitution pattern as TCDD, and more selective assays with reduced detection limits might be developed through improved hapten design (Goodrow et al., 1995). Monoclonal antibodies were made to the same hapten with a 2,3,7,8 substitution, but IA's developed with these antibodies exhibited reduced sensitivity and selectivity (Kennel et al., 1986). Both of these haptens contained the polar carboxamido moiety on the dibenzo-*p*-dioxin ring system. Cross reactivity studies using an ELISA developed with the monoclonal antibody generated using this hapten (Figure 1B) indicated that this spacer was strongly recognized by the antibody (Stanker et al., 1987).

Table 1. Structures of Dioxin Haptens Used in This Study



compd no.	R1	R2	R3
IV	Cl	Cl	CH=CHCOOH
VII	H	$CH=C(CH_3)COOH$	H
X	Cl	$(CH=CH)_2COOH$	H
XI	Cl	CH=CHCOOH	H
XII	H	CH=CHCOOH	H
XIII	Cl	$CH=CHC_6H_4COOH$	H
XV	Cl	$O(CH_2)_5COOH$	H
XVI	Cl	NH_2	H

In this paper we report the syntheses of several novel haptens and their use for antibody and immunoassay development (Table 1). These haptens all feature three or four substituents in the same pattern as the chlorine atoms in 2,3,7,8-tetrachlorodibenzo-*p*-dioxin with the exception of hapten **IV** which has five substituents. One substituent on each hapten is an alkyl chain containing at least one double bond or aromatic ring in the chain, and these alkyl groups are all terminated with either a carboxylic acid or an amine group to facilitate covalent attachment of the hapten to protein molecules. A number of these haptens have been used to generate polyclonal antibodies in rabbits, and initial screening and characterization of these antibodies is reported.

MATERIALS AND METHODS

Safety Note. Although every effort was made to avoid the formation of TCDD during the synthetic work, caution is necessary because the toxicity of the compounds prepared in this study is unknown. The syntheses were carried out in fume hoods with an air flow of at least 100 linear ft/min. When handling these compounds, it is advisable to wear two pairs of protective gloves with some water between the two layers to avoid penetration of highly lipophilic compounds through the gloves. Activated carbon can be used to eliminate TCDD-like substances from waste solutions. UV light has been reported to degrade TCDD and some related compounds (Crosby and Wong, 1977; Qin, 1996) and thus may be useful for cleanup operations.

Reagents. All chemicals used were obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI), Lancaster Synthesis, Inc. (Windham, NH), or Fisher Scientific Co. (Pittsburgh, PA) and were of reagent quality or better. Bovine serum albumin

(BSA), keyhole limpet hemocyanin (KLH), hemocyanin from *Limulus polyphemus* hemolymph (LPH), Freund's Complete and Incomplete adjuvant, Tween 20, 3,3′,5,5′-tetramethylbenzidine, goat anti-rabbit IgG conjugated to alkaline phosphatase or horseradish peroxidase, and *p*-nitrophenyl phosphate were purchased from Sigma Chemical Co. (St. Louis, MO). 2-Amino-3,7,8-trichlorodibenzo-*p*-dioxin (**XVI**) and 2,3,7-trichloro-8-hydroxydibenzo-*p*-dioxin were gifts from Stephen Safe (Veterinary Physiology and Pharmacology, Texas A&M University, College Station, TX). TLC was performed on 0.2 mm silica gel 60-F254 plastic backed plates (Aldrich Chemical Co.). Preparative TLC was performed on Whatman PLK5F 150A (F254, 20 cm × 20 cm × 1 mm) silica gel plates (Whatman Inc., Clifton, NJ).

Instrumentation. Proton NMR data were obtained in either deuteriochloroform ($CDCl_3$) or deuteriomethylene chloride (CD_2Cl_2) with tetramethylsilane (TMS) as an internal standard on a 300 MHz NMR spectrometer (Bruker, Billerica, MA). Chemical shift values are reported downfield from TMS. The low-resolution GC−MS data were obtained using a Trio-2 GC−MS system (VG Masslab, Altrincham, UK) using 70 eV electron ionization (EI). A 30 m DB1 column (0.25 mm i.d., 0.25 μm film; J&W Scientific, Folsom, CA) was used with a helium flow rate of 30 cm/s. Samples were dissolved in tetrahydrofuran (THF), and splitless injections of 1 μL were made. The column was programmed from 80 °C (1 min hold) to 150 °C at 20 deg/min followed by an increase to 300 °C at 10 deg/min. Melting points were determined on a capillary melting point apparatus (Thomas-Hoover, Philadelphia, PA). Immunoassay microplates were read in a plate reader (UVmax, Molecular Devices, Sunnyvale, CA) interfaced to a computer for data collection. Data analysis for curve fitting was conducted using Softmax (v. 2.1, Molecular Devices, Sunnyvale, CA).

Synthesis of Haptens. The structures of all haptens used are shown in Table 1. The following is a detailed description of the synthesis of three of the novel haptens (**IV**, **VII**, **X**). The synthetic scheme for hapten **IV** is shown in Figure 2. This same general route was used to prepare haptens **VII**, **X**, **XI**, **XII**, and **XIII**. Mass spectral analysis, GC retention time, and TLC characteristics for haptens **XI**, **XII**, and **XIII** were consistent with the assigned structure. Hapten **XV** was synthesized using 2,3,7-trichloro-8-hydroxydibenzo-*p*-dioxin as the starting material.

The structures of all the intermediates used to prepare the haptens as well as the haptens themselves were characterized by NMR and GC−MS methods. Analysis of the compounds by GC gave one peak with the reported retention times. However, the structures of the carboxylic acid hapten derivatives were only characterized by low-resolution mass spectrometry using solid probe introduction. It was not possible to obtain NMR spectra of these haptens because they were insufficiently soluble in all deuterated organic solvents explored (chloroform, dichloromethane, methanol, acetone), alkaline (40%) deuterated water, and trifluoroacetic acid.

2,3,6-Trichloro-5-nitrobenzaldehyde (I). To 50 mL of sulfuric acid was added 4.0 g (0.019 mol) of 2,3,6-trichlorobenzaldehyde. This mixture was cooled to 8 °C, and 2.1 g (0.021 mol) potassium nitrate was added. The yellow solution was allowed to warm to room temperature and then was heated to 65 °C. After cooling, the reaction mixture was poured over ice. A tan solid, **I**, was collected (4.7 g, 96%): mp 77−80 °C; GC−MS (t_R = 10.01 min) calcd for $C_7H_2Cl_3NO_3$ 252.91; *m*/*z* (intensity) 252 ([M − H]$^+$, 42), 253 (M$^+$, 43), 254 (44), 255 (40), 143 (70), 108 (100); ^{1}H NMR ($CDCl_3$) δ 8.09 (Ar, s, 1 H), 10.38 (CH=O, s, 1 H).

Ethyl 3-*trans*-(2,3,6-Trichloro-5-nitrophenyl)propenoate (II). To 20 mL of toluene were added 2.5 g (0.01 mol) of **I**, 2.52 mL (0.012 mol) of triethyl phosphonoacetate, 4.60 g (0.033 mol) of potassium carbonate, and 50 mg of dibenzo-18-crown-6. The reaction mixture was heated to reflux, cooled, and washed with water, and the toluene solution was dried over sodium sulfate. After filtration, 5 g of silica gel was added and the toluene was removed under vacuum. Column chromatography on silica gel with ethyl acetate/hexane in a gradient of 5:95 to 10:90 yielded 1.66 g (51%) of a yellow oil, **II**, that crystallized on standing: mp 41−45 °C; ^{1}H NMR ($CDCl_3$) δ 1.37 (CH_3, t, J = 7, 3 H), 4.31 (OCH_2, q, J = 7, 2 H), 6.46 (ArCH=C*H*, d, J = 16, 1 H), 7.65 (ArC*H*=CH, d, J = 16, 1 H), 7.91 (Ar, s, 1 H); GC−MS (t_R = 15.16 min) calcd for $C_{11}H_8Cl_3NO_4$ 322.95; *m*/*z* (intensity) M$^+$ 323 (13), 325 (17), 326 (18), 327 (17), 308 (46), 306 (45), 262 (58), 260 (100).

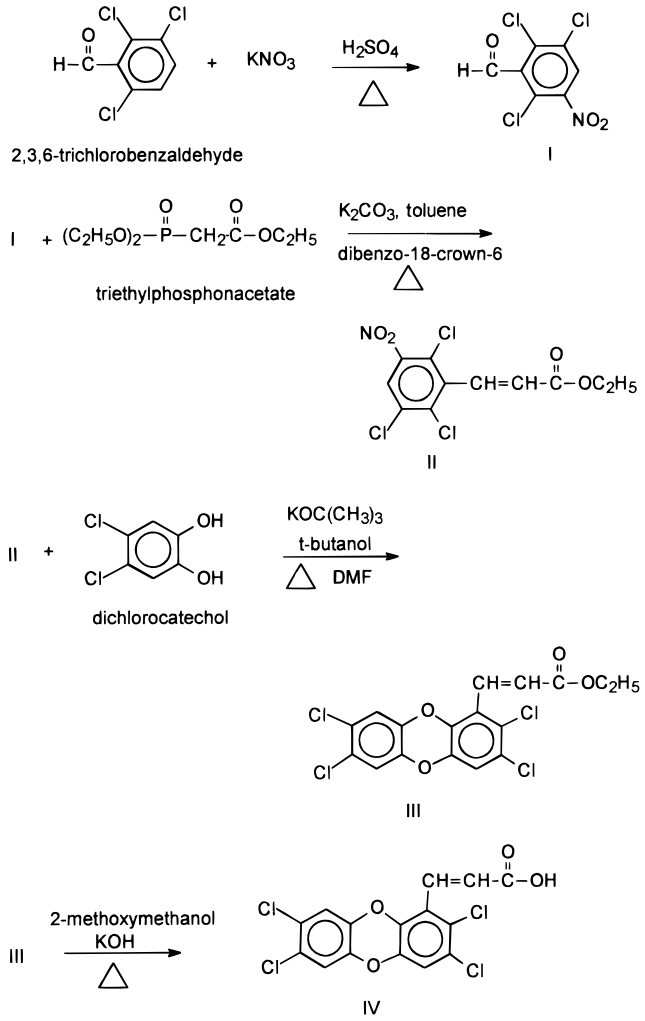


Figure 2. Scheme for the synthesis of hapten **IV**. A similar pathway was used for the synthesis of haptens **VII**, **X**, **XI**, **XII**, and **XIII**.

Ethyl *trans*-3-(2,3,7,8-Tetrachlorodibenzo-*p*-dioxin-1-yl)propenoate (III). In 2 mL of dry DMF under nitrogen were added 0.64 g (0.002 mol) of **II**, 0.40 g (0.0022 mol) of 4,5-dichlorocatechol (Gray et al., 1976), and 4 mL (0.004 mol) of 1 M potassium *tert*-butoxide in *tert*-butyl alcohol. This mixture was heated (140−150 °C) until the *tert*-butyl alcohol ceased to distill. The reaction mixture was allowed to cool, and 25 mL water was added to precipitate a brown solid. Column chromatography, of this solid over silica gel with hexane/ethyl acetate (95:5) yielded the product, **III** (0.083 g, 9.8%). A small amount was crystallized from hexane/toluene: mp 153−158 °C; ^{1}H NMR (CD_2Cl_2) δ 1.34 (CH_3, t, J = 7, 3 H), 4.31 (OCH_2 q, J = 7, 2 H), 6.73 (ArCH=C*H*, d, J = 17, 1 H), 7.76 (ArC*H*=CHC, d, J = 17, 1 H), 7.03 (Ar, s, 1 H), 7.04 (Ar, s, 1 H), 7.12 (Ar, s, 1 H); GC−MS (t_R = 22.16 min) calcd for $C_{17}H_{10}Cl_4O_4$ 417.93; *m*/*z* (intensity) M$^+$ 418 (30), 419 (4), 420 (34), 421 (19), 357 (92), 355 (87), 356 (27), 358 (16), 359 (30), 207 (100).

***trans*-3-(2,3,7,8-Tetrachlorodibenzo-*p*-dioxin-1-yl)propenoic Acid (IV).** To 0.5 mL of 2-methoxyethanol were added 0.065 g (0.00015 mol) of **III** and 0.040 g (0.0006 mol) of potassium hydroxide. This mixture was heated to reflux. After cooling, the reaction mixture was acidified with aqueous

hydrochloric acid, and the product, **IV**, was collected as a pale yellow solid (0.016 g 27%): mp 267−269 °C dec; solid probe MS calcd for $C_{15}H_6Cl_4O_4$ 389.90; *m/z* (intensity) M^+ 390 (34), 391 (9), 392 (42), 393 (8), 394 (20), 355 (100), 356 (23), 357 (92), 358 (13), 292 (45).

Ethyl *trans*-3-(4-Chloro-3-nitrophenyl)-2-methylpropenoate (V). To 10 mL of dry THF were added 2.5 g (0.011 mol) of triethyl 2-phosphonopropionate and 0.6 g (0.015 mol) of 60% sodium hydride in oil. This mixture was cooled with an ice bath and stirred for 1 h. To this reaction was added 1.83 g (0.01 mol) of 4-chloro-3-nitrobenzaldehyde. The reaction was stirred for 1 h and then poured into ice water. A yellow solid, **V**, was collected (2.4 g, 89%): mp 60−61.5 °C; ^{1}H NMR ($CDCl_3$) δ 1.36 (CH_3, t, J = 7, 3 H), 2.10 (CH_3, d, J = 3, 3 H), 4.29 (OCH_2, q, J = 7, 2 H), 7.51 (Ar, dd, J = 2, 1 H), 7.57 (Ar, s, 1 H), 7.59 (Ar, s, 1 H), 7.88 (ArC*H*, d, J = 1.5, 1 H); GC−MS (t_R = 7.40 min) calcd for $C_{12}H_{12}ClNO_4$ 269.05; *m/z* (intensity) M^+ 269 (32), 270 (5), 271 (11), 224 (62), 225 (12), 226 (19) 149 (42), 150 (19), 151 (16), 115 (100).

Ethyl *trans*-3-(7,8-Dichlorodibenzo-*p*-dioxin-2-yl)-methylpropenoate (VI). To 2 mL of dry DMF under nitrogen were added 0.56 g (0.002 mol) of **V** and 0.40 g (0.0022 mol) of 4,5-dichlorocatechol. After the mixture was cooled in an ice bath, 4.0 mL (0.004 mol) of 1 M potassium *tert*-butoxide in *tert*-butyl alcohol was added. The reaction was heated (145−150 °C) until the *tert*-butyl alcohol ceased to distill. After the reaction cooled, it was poured into water and a solid was collected. Column chromatography of the solid on silica gel with hexane/ethyl acetate (9:1) yielded the product, **VI** (0.165 g, 23%): mp 121−123 °C; ^{1}H NMR ($CDCl_3$) δ 1.35 (CH_3, t, J = 7, 3 H), 2.11 (CH_3, d, s, 3 H), 4.26 (OCH_2, q, J = 7, 2 H), 6.85−7.0 (Ar, m, 5H), 7.53 (ArC*H*, d, J = 2, 1 H); GC−MS (t_R = 29.44 min) calcd for $C_{18}H_{14}Cl_2O_4$ 365.21; *m/z* (intensity) M^+ 365 (37), 366 (8), 367 (5), 290 (40), 291 (25), 292 (31), 115 (100).

***trans*-3-(7,8-Dichlorodibenzo-*p*-dioxin-2-yl)-2-methylpropenoic Acid (VII).** To 1 mL of 2-methoxyethanol were added 0.037 g (0.0001 mol) of **VI** and 0.016 g (0.000 26 mol) of potassium hydroxide dissolved in 0.5 mL of water. The reaction mixture was refluxed for 1 h. After cooling, aqueous hydrochloric acid was added. A pale white solid, **VII**, was collected by filtration (0.03 g, 89%); mp >280 °C; solid probe MS calcd for $C_{16}H_{10}Cl_2O_4$ 336.00; *m/z* (intensity) M^+ 336 (44), 337 (10), 338 (24), 290 (12), 291 (7), 292 (6), 163 (13), 115 (55), 45 (100).

Ethyl 5-(2,4-Dichloro-5-nitrophenyl)-*trans*,*trans*-2,4-pentadienoate (VIII). To 10 mL of dry THF were added 0.2 g (0.005 mol) of 60% sodium hydride in oil and 1.25 g (0.005 mol) of triethyl 3-phosphonocrotonate. The solution was cooled to 10−15 °C, and 0.96 g (0.0044 mol) of 2,4-dichloro-5-nitrobenzaldehyde (Aldous et al., 1974) was added in 5 mL of THF. The reaction mixture was allowed to warm to room temperature and then heated to reflux. After cooling, the THF was removed and the solid was dissolved in ethyl acetate. This solution was first washed with water, then dried over sodium sulfate, and filtered. The reaction mixture was adsorbed on silica gel. Following column chromatography with ethyl acetate/hexane (1:10), a solid, **VIII**, was obtained (0.40 g, 16%): mp 95−101 °C; ^{1}H NMR ($CDCl_3$) δ 1.25 (CH_3, t, J = 7, 3 H), 4.25 (OCH_2, q, J = 7, 2 H), 6.13 (CH=C*H*CO_2, d, J = 15, 1 H), 6.94 (C*H*=$CHCO_2$, dd, J = 12, 1 H), 7.23 (ArC*H*=CH, d, J = 15, 1 H), 7.43 (ArCH=C*H*, dd, J = 12, 1 H), 7.62 (Ar, s, 1 H), 8.17 (Ar, s, 1 H); GC−MS (t_R = 15.76 min) calcd for $C_{13}H_{11}Cl_2NO_4$ 315.00; *m/z* (intensity) M^+ 315 (80), 316 (24), 317 (54), 318 (17), 270 (76), 271 (18), 272 (48), 161 (50), 126 (100).

Ethyl 5-(3,7,8-Trichlorodibenzo-*p*-dioxin-2-yl)-*trans*,*trans*-penta-2,4-dienoate (IX). To 2 mL of dry DMF under nitrogen were added 0.20 g (0.00063 mol) of **VIII** and 0.19 g (0.0011 mol) of 4,5-dichlorocatechol. The mixture was cooled with an ice bath, and 2.0 mL (0.002 mol) of 1 M potassium *tert*-butoxide in *tert*-butyl alcohol was added. The mixture was heated (120−140 °C) until the *tert*-butyl alcohol ceased to distill. After cooling, water was added and a tan solid was collected. Column chromatography over silica gel with ethyl acetate/hexane (1:10) yielded a solid, **IX** (0.075 g, 27%): mp 136−141 °C; ^{1}H NMR (CD_2Cl_2) δ 1.30 (CH_3, t, J = 7, 3 H), 4.19 (OCH_2, q, J = 7, 2 H), 5.95 (CH=C*H*CO_2, d, J = 15, 1 H), 6.77 (C*H*=$CHCO_2$, dd, J = 17, 1 H), 6.86 (Ar, s, 1 H), 6.93 (Ar, s, 2 H), 7.08 (Ar, s, 1 H), 7.10 (ArC*H*=CH, d, J = 15, 1 H), 7.36 (ArCH=C*H*, dd, J = 17, 1 H); GC−MS (t_R = 22.27 min) calcd for $C_{19}H_{13}Cl_3O_4$ 409.99; *m/z* (intensity) M^+ 410 (30), 411 (4), 412 (6), 413 (26), 414 (4), 415 (10), 338 (36), 336 (34), 337 (11), 302 (100), 303 (28), 304 (70).

5-(3,7,8-Trichlorodibenzo-*p*-dioxin-2-yl)-*trans*,*trans*-penta-2,4-dienoic Acid (X). To 0.6 mL of methoxyethanol were added 0.1 mL of water, 0.02 g (0.0003 mol) of potassium hydroxide, and 0.065 g (0.00016 mol) of **IX**. The mixture was refluxed for 1 h and then cooled. After addition of aqueous hydrochloric acid, a solid, **X**, was collected (0.045 g, 74%): mp 266−267 °C; FAB-MS (negative mode) (in pyridine/glycerol) calcd for $C_{17}H_9Cl_3O_4$ 380.95; *m/z* (intensity) monoisotopic $[M - H]^-$ 381 (83), $^{37}Cl[M - H]^-$ 383 (100), $^{37}Cl_2[M - H]^-$ 385 (44).

Ethyl 6-(3,7,8-Trichlorodibenzo-*p*-dioxin-2-yloxy)hexanoate (XIV). 2,3,7-Trichloro-8-hydroxydibenzo-*p*-dioxin (3.1 mg, 10 μmol) (Mason and Safe, 1986; Singh and Kumar, 1993) was alkylated with ethyl 6-bromohexanoate (99%, 3.4 mg,15 μmol), with finely grounded potassium carbonate (0.02 g, 0.15 mmol) as base and Aliquat 336 (0.2 mg, 0.5 μmol) as a phase transfer catalyst (Dehmlow, 1983), in 1 mL of dry DMF. The mixture was vigorously stirred under nitrogen at 90−100 °C for a day. The reaction mixture was evaporated in vacuo and the residue was taken up in acetone. The mixture was filtered, and the filtrate was concentrated in vacuo. Purification by preparative TLC (hexane/ethyl acetate/triethylamine, 90:10:0.5) resulted in 3.8 mg (84%) of ester **XIV**: mp 92−94 °C; MS calcd for $C_{20}H_{19}Cl_3O_5$ 444.03; *m/z* (intensity) M^+ 444 (11), 446 (11), 448 (5), 302 (40), 304 (38), 306 (12), 143 (100).

6-((3,7,8-Trichlorodibenzo-*p*-dioxin-2-yl)oxy)hexanoic Acid (XV). To 3.3 mg (7.4 μmol) of ester **XIV** dissolved in 1 mL of THF was added 0.1 mL of 1 M NaOH. The mixture was stirred at reflux overnight. The reaction mixture was concentrated in vacuo, diluted with water, and washed with dichloromethane (2 × 5 mL). The alkaline aqueous solution was saturated with sodium chloride, cooled in ice, acidified by 6 M HCl, and extracted with dichloromethane/ether, 3:1, mixture (5 × 4 mL). The combined extracts were dried (Na_2SO_4) and then evaporated in vacuo to yield a semisolid acid, **XV** (2.5 mg, 81%): MS calcd for $C_{18}H_{15}Cl_3O_5$ 416.00; EI-MS *m/z* (relative intensity) M^+ 416 (12), 418 (12), 420 (4), 302 (100), 304 (99), 306 (35). This product was conjugated to proteins without further purification.

2,3,7-Trichloro-8-methyldibenzo-*p*-dioxin (TMDD) was synthesized according to the methods of Romkes et al. (Romkes et al., 1987a,b) and Denomme et al. (Denomme et al., 1985). The product had NMR and GC−MS data that were consistent with the assigned structure.

Preparation of Conjugates. Haptens containing carboxylic acids were activated by the mixed anhydride method. Haptens **IV**, **VII**, **X**, **XI**, **XII**, and **XIII** (0.03 mmol) were dissolved in dry *p*-dioxane. Isobutyl chloroformate and tri-*n*-butylamine were added in slight molar excess. The solution was stirred at room temperature for 30 min. Fifty milligrams of each protein (BSA or LPH) was dissolved in 30 mL of 0.2 M borate buffer, pH 8. The protein solution was ice-cooled. To improve the solubility of the activated hapten in the aqueous protein solution, 2 mL of *p*-dioxane was added to the protein solution. The addition of *p*-dioxane to the protein solution caused a slight cloudiness. The activated hapten solution was then added to the protein solution dropwise with stirring. Stirring was continued on ice for 30 min to an hour. To remove unreacted small molecules, the protein conjugates were precipitated with ice cold 100% ethanol. The precipitated protein was pelleted by centrifugation at 4 °C for 10 min, 4500*g*. The supernatant containing unreacted small molecules was decanted. The pellet was resuspended with cold ethanol three times, centrifuging between resuspensions. The supernatants were discarded, and the pellet was resuspended in distilled water to a concentration of approximately 5 mg of protein/mL. Conjugates of hapten **XV** were prepared similarly, except the hapten was dissolved in dry DMF and the reaction carried

out under nitrogen. After addition of the isobutyl chloroformate and tri-*n*-butylamine the mixture was stirred at 4 °C for 10 min, then at room temperature for an additional 20 min. Approximately 1 mL of the activated hapten solution (containing approximately 2.5 μmol) was added to each solution of protein (BSA, 14 mg/2 mL borate buffer or KLH, 40 mg/4 mL borate buffer) and stirred at 4−10 °C for 1 h. Stirring was continued at room temperature overnight. These conjugates were purified by extensive dialysis in phosphate-buffered saline (PBS). All conjugates were assayed for protein content, aliquoted, and stored at −20 or −80 °C until use.

Conjugates of hapten **XVI** were made by a diazotization reaction. The reaction was carried out with stirring under nitrogen in a flask cooled in a water bath (about 19−20 °C). Butyl nitrite (417 μL of 30 mM solution in dry DMSO, 12.5 μmol) was added dropwise to a solution of 2-amino-3,7,8-trichlorodibenzo-*p*-dioxin (**XVI**, 3.0 mg, 10 μmol) and sulfuric acid (625 μL of 20 mM solution in dry DMSO, 12.5 μmol) in dry DMSO (4.2 mL). Care was taken to avoid solidification of the reaction mixture because the freezing point of DMSO is 18 °C. After 30 min, the resulting diazonium salt solution was divided into aliquots. A small aliquot of the reaction mixture was added to excess 4-cresol dissolved in 0.2 M borate buffer, pH 8.7. This mixture was stirred at room temperature for 3 h, concentrated in vacuo, and then subjected to preparative TLC (hexane/acetone, 9:1). A yellow, amorphous azo compound was isolated: EI-MS *m*/*z* (relative intensity) M^+ 420 (8), 424 (3), 422 (8), 389 (1), 387 (4), 385 (7), 135 (16), 107 (100). The diazonium salt was split into two equal parts. Each half (ca. 2.5 mL) was added dropwise to a vigorously stirred solution of a carrier protein in 0.2 M borate buffer, pH 8.7, at 19−20 °C (BSA, 12 mg in 3 mL buffer or KLH, 50 mg in 5 mL buffer). After 2 h, the mixtures were allowed to stir at room temperature overnight. The yellow protein conjugates were purified by extensive dialysis in PBS.

Immunization of Rabbits. Female New Zealand white rabbits weighing 3−3.5 kg were purchased from Grimaud's Rabbitry (Stockton, CA). The immunogen was prepared by emulsifying the conjugate dissolved in sterile saline with Freund's complete adjuvant (1:1 v/v, Sigma Chemical Co., St. Louis, MO). Each rabbit was given an initial immunization of 100 μg of protein/rabbit subcutaneously in 5−10 droplets of about 20−50 μL each. After one month a boost of 50 μg/rabbit was given using Freund's incomplete adjuvant and a test bleed taken 10 days later. The boosting and bleeding continued at 1−2 month intervals for a total of 4−5 test bleeds. When serum titers no longer increased, a final bleed was taken by exsanguination 10 days after the final boost. Serum was separated from the clotted blood by centrifugation, aliquoted into small tubes, and stored at −80 °C.

Immunoassay Method. Enzyme-linked immunosorbent assays were conducted similar to Voller et al. (Voller et al., 1976).

Buffers. Coating buffer consisted of 0.795 g of Na_2CO_3 and 1.465 g of $NaHCO_3$ in 500 mL of distilled water, pH 9.6. Assay buffer was PBS containing 0.05% (v/v) Tween 20 (PBST, 8 g of NaCl, 0.2 g of KH_2PO_4, 1.15 g of Na_2HPO_4, 0.2 g of KCl, and 0.5 mL of Tween 20 in 1 L of distilled water, pH 7.5). Wash buffer (0.1XPBST) was prepared by diluting PBS 10-fold in distilled water and adding 0.05% Tween 20 (v/v). Substrate buffer for alkaline phosphatase (AP) was 10% (w/v) diethanolamine (97 mL of diethanolamine, 0.1 g of $MgCl_2$, in 1 L of distilled water, pH to 9.8 with concentrated HCl). Substrate solution for horseradish peroxidase (HRP) consisted of 0.4 mL of a 60 mg/mL DMSO solution of 3,3′,5,5′-tetramethylbenzidine, 0.1 mL of 1% H_2O_2 in water in a total of 25 mL of 0.1 M citrate acetate buffer (6.8 g sodium citrate in 500 mL of water, adjusted to pH 5.5 with acetic acid).

ELISA. Briefly, 96-well microtiter plates (Nunc Immunosorp, Roskilde, Denmark) were coated with 100 μL/well of the BSA−hapten conjugates in coating buffer. The plates were sealed with acetate plate sealers and incubated at 4 °C overnight. On the following day, the plates were washed five times with wash buffer. For titer experiments, 100 μL of rabbit antiserum diluted in assay buffer was added to each well, and the plate was covered and incubated for 1 h at room temperature. For competition experiments, 50 μL of antiserum and 50 μL of inhibitor were added to each well. Following the incubation, the plates were washed five times with wash buffer to remove unbound reactants and then 100 μL of goat anti-rabbit IgG conjugated to AP (1/5000 dilution in assay buffer) or HRP (1/10000 dilution in assay buffer) was added to each well. The plates were sealed and incubated at room temperature for 1 h. After five washings, 100 μL/well of substrate was added (1 mg/mL of *p*-nitrophenyl phosphate, in AP substrate buffer or HRP substrate in citrate−acetate buffer). The plates were incubated for 30−45 min at room temperature, and then the enzyme reaction was stopped by the addition of 50 μL of 4 N NaOH for AP or 4 N H_2SO_4 for HRP and the absorbance was read at 405 nm (AP) or 450 nm (HRP) in a microtiter plate reader.

Preparation of Standards. Because the dioxins are lipophilic molecules, care was taken to ensure solubilization during the immunoassay. Experiments by Stanker et al. (1987) using radiolabeled TCDD showed that DMSO, BSA, and detergent were important for keeping TCDD solubilized. Our stock solutions were 50 μg of analyte/mL of DMSO. Dilutions of analyte were made in a diluent consisting of one part DMSO to three parts PBST containing 0.2% BSA. Antiserum dilutions were prepared in PBST containing 0.2% BSA. Thus, the final DMSO concentration in the well was 12.5% (Stanker et al., 1987).

RESULTS AND DISCUSSION

Synthetic Chemistry. The spacers prepared in this paper are shown in Figure 1. Six novel haptens were synthesized. The method for preparation of hapten **IV** is illustrated in Figure 2. The other five haptens were prepared by analogous synthetic schemes, and their structures are shown in Table 1. Previous synthetic strategies for the development of haptens for TCDD detection by ELISA have employed a flexible adipic acid spacer attached to an amino chlorinated dibenzo-*p*-dioxin (Albro et al., 1979; Stanker et al., 1987; Langley et al., 1992) (Figure 1B). Examination of the literature suggested it might be possible to attach a more rigid spacer by direct reaction with an appropriately chlorinated dibenzo-*p*-dioxin ring system. For the preparation of the synthetic targets of this paper, this was not an effective route. For example, attempts to brominate 2,3-dichloro-7-methyldibenzo-*p*-dioxin under standard conditions of carbon tetrachloride/*N*-bromosuccinimide failed because this dioxin was not sufficiently soluble in this solvent for the reaction to proceed. It was hoped that this bromomethyl intermediate would serve as a useful synthetic intermediate for attachment of a rigid side chain using Wittig chemistry.

Since this literature route of spacer attachment after formation of the dioxin ring system was not successful, it was decided to append the spacer to one of the aryl rings before construction of the dibenzo-*p*-dioxin ring system. The route used to construct the dibenzo-*p*-dioxins with substituents utilized 4,5-dichlorocatechol and a chloro nitroaromatic ring with the appropriate side chain already in place. Use of chlorocatechols and chloro nitroaromatics has literature precedence for preparation of halogenated dibenzo-*p*-dioxins (Gray et al., 1976) as well as for the preparation of haptens for immunoassay (Chae et al., 1977). The aryl ring that served as the intermediate for attachment of the spacer was prepared from a chloronitrobenzaldehyde that was either commercially available or synthesized by nitrating a chlorobenzaldehyde under standard conditions in sulfuric acid. The chloronitrobenzaldehydes were treated

with Wittig or Wadsworth−Emmons reagents to add an unsaturated ester. The dibenzo-*p*-dioxin ring was formed using 4,5-dichlorocatechol and chloro nitroaromatics with unsaturated side chains in the presence of 2 equiv of potassium *tert*-butoxide in DMF at elevated temperatures. This synthetic route was specifically designed to avoid the formation of TCDD as it utilized intermediates that do not yield TCDD (Gray et al., 1976). While the yields for the dioxin ring formation were generally poor (<30%), sufficient product was obtained for spectral characterization, hydrolysis, and coupling after activation to various proteins for generation of antibodies in rabbits.

The application of ELISA to the analysis of lipophilic environmental contaminants, such as halogenated biphenyls, chlorinated hydrocarbon insecticides (Ibrahim et al., 1994a,b), halogenated dibenzo-*p*-dioxins, and dibenzofurans, has not been as frequent as for more water soluble species such as paraquat (Meulenberg et al., 1995; Vanderlaan et al., 1988) or the triazine herbicides (Wortberg et al., 1996; Kido et al., 1997; Wittmann and Hock, 1994). Among the reasons for fewer reports on the use of ELISA for the analysis of lipophilic species is the absence of sites on these molecules to directly append spacers (easily displaceable groups, carboxylic acids, amines, hydroxyl) for eventual conjugation to a protein for development of antibodies. Most often, specially designed chemistry must be undertaken to prepare haptens of lipophilic contaminants. Furthermore, for haptens prepared from more water soluble analytes that have polar functionalities, it is hypothesized that the type of spacer may be less important because there is a reduced tendency for the hapten to fold back onto the protein surface or into the hydrophobic protein core after conjugation. In contrast for water insoluble haptens, the role of the spacer may be more important because it is speculated that the hapten can fold back onto the protein surface or within the protein after conjugation. Such processes may result in suboptimal recognition of the lipophilic hapten during antibody production. A recent example from the literature used the flexible hexanoic acid spacer for the development of a monoclonal antibody (Mab) ELISA for chlorinated biphenyls. Reasonable success with respect to sensitivity was achieved with IC_{50} values ranging from 10 to 600 ng/mL (Johnson and Van Emon, 1996; Chiu et al., 1995).

Conjugation Chemistry. Haptens were coupled either by the mixed anhydride method (carboxylic acids) or diazotization (amines). Success of the coupling of hapten and protein were determined indirectly by adding the activated hapten to *n*-butylamine (a mimic of lysine groups in the protein) for the mixed anhydride method or to 4-cresol (a mimic of tyrosine groups in the protein) for the diazotization method. Successful reaction was indicated by the presence of a new spot on TLC with an R_f value different from that of the activated hapten or the *n*-butylamine or 4-cresol alone.

Hapten load was estimated from UV absorption spectra of the hapten alone and the hapten−protein conjugate. For conjugate **X**−BSA and **XI**−BSA the hapten load was estimated to be 5 and 2.5 haptens/mol of protein, respectively. The absorbance maximum of the haptens was a 350 and 298 nm, respectively, and that of the protein at 280 nm. Hapten loads were not measured for the other conjugates, although their performance in subsequent ELISAs demonstrated that the hapten was attached to the protein.

Table 2. Serum Titer Response of Rabbits to Various Coating Antigens[a]

rabbit no.	coating antigen					
	IV−BSA	**VII**−BSA	**X**−BSA	**XI**−BSA	**XII**−BSA	**XIII**−BSA
5156	−[b]	−	−	−[c]	−	−
5163	−	−	−	−[c]	−	−
5164	+	−	+	−[c]	+	+
2492	+++	−	++	+	++	+++[c]
2493	++	−	+	−	+	++[c]
2494	+++	−	++	+	++	++[c]
2114	+++	−	+	+	+[c]	+++
2525	+	−	−	−	−[c]	+
7598	++	−	+[c]	−	+	+
7599	++	−	+[c]	+	+	++
7600	++	−	+[c]	−	+	+
69	++[c]	−	−	−	−	−
89	+++[c]	−	+++	+++	+++	+++
99	+++[c]	−	++	+	++	++

[a] The data shown are at a coating antigen concentration of 1 μg/mL and an antibody dilution of 1/2000. DMSO/PBST + BSA was used as the diluent for the antibody. [b] (−) absorbance <0.3; (+) absorbance 0.3−0.6; (++) absorbance 0.6−0.9; (+++) absorbance >0.9. [c] Homologous system where the coating hapten is the same as was used for immunization.

The hapten 2-amino-3,7,8-trichlorodibenzo-*p*-dioxin (**XVI**) (Poland et al., 1986; Hunter, 1988; Romkes et al., 1987b) is lipophilic, weakly basic, and poorly soluble in diluted aqueous mineral acids. Thus, the reaction conditions of the usual diazotization (Saunders and Allen, 1985) are not well suited for this compound. However, treatment of aromatic amines with alkyl nitrites in organic solvents such as ethanol and acetic acid has long been known as an alternative diazotization method (Saunders and Allen, 1985). The method reported here used DMSO as the solvent for the diazotization of amine **XVI** with butyl nitrite in the presence of sulfuric acid.

ELISA Screening. The sera from the 21 rabbits immunized with conjugates of haptens **IV**, **VII**, **X**, **XI**, **XII**, **XIII**, **XV**, and **XVI** were tested for binding to the various coating antigens, and the results are reported in Table 2. All rabbits showed high titers to coating antigens with the homologous hapten but a different protein (data not shown). The exceptions were for six antisera produced from **XV**−KLH and **XVI**−KLH. Sera from these rabbits showed high titers to their respective immunizing antigens, but not to the coating antigens with a different protein. Thus there did not appear to be any antibodies that recognize the hapten. In addition, none of the rabbit sera reported in Table 2 bound to haptens **XV** or **XVI** coupled to BSA. These results imply that the coupling of the hapten to the protein was not successful or that the hapten load was too low.

Because of their lipophilicity, dioxin standards were prepared in a diluent consisting of one part DMSO to three parts PBST containing 0.2% BSA. Since the antibodies would ultimately need to bind the dioxin under these conditions, the sera were screened in the presence of the DMSO/PBST + BSA diluent. The plates were coated with varying concentrations of coating antigen (0.1 to 10 μg/mL) and then probed with varying concentrations of antiserum (1/1000 to 1/16000 dilutions of sera). The data shown (Table 2) are at a coating antigen concentration of 1 μg/mL and an antiserum dilution of 1/2000. The signal (i.e., the maximum absorbance obtained) for all combinations of coating

Table 3. Percent Inhibition Using TMDD as an Inhibitor at Two Concentrations in Various Unoptimized ELISA Systems[a]

	coating antigen			
rabbit no.	**IV**−BSA	**X**−BSA	**XII**−BSA	**XIII**−BSA
2492 (**XIII**)[b]	NI[c]	4, 11	NI	NI
2493 (**XIII**)	5, 11	−[d]	−	8, 19
2494 (**XIII**)	0, 11	NI	NI	NI
2114 (**XII**)	7, 20	−	−	NI
7598 (**X**)	20, 35	−	−	−
7599 (**X**)	NI	−	−	5, 12
7600 (**X**)	0, 29	−	−	−
69 (**IV**)	12, 41	−	−	−
89 (**IV**)	6, 14	4, 24	6, 10	11, 15
99 (**IV**)	0, 12	NI	NI	1, 7

[a] Values reported are percent inhibition at 10 and 100 ppb TMDD, respectively. ELISA conditions were 1 μg/mL coating antigen, 1/1000 dilution antibody. For the competition step, 50 μL of antibody was added to the coated plate along with 50 μL of the inhibitor. Incubation times and other conditions were as described in the Methods. [b] The numeral in parentheses is the immunizing hapten. [c] No dose-dependent inhibition seen. [d] Not tested because the titer from the initial screen was <0.5 absorbance units.

antigens and antisera was lower (38−82%) using the DMSO/PBST + BSA diluent than when using assay buffer alone.

Data in Table 2 indicate that, under these conditions, the antibodies derived from haptens **IV** and **XIII** bind well to nearly all the coating antigens tested. Conversely, nearly all the antibodies generated in this study bind well to coating antigens derived from these same two haptens. More moderate binding is observed for coating antigens **X**−BSA, **XI**−BSA, and **XII**−BSA. The serum titers measured using **VII**−BSA ranged from 0.5 to 1.9 when measured in assay buffer (data not shown). However, titers were below 0.3 absorbance units for all rabbits when measured in the presence of the DMSO/PBST + BSA diluent, indicating that assays had poor tolerance to this diluent. All rabbit sera and coating antigen combinations that were positive in this initial titer screen were next screened for inhibition.

Screening for Inhibition by Analyte. Maximum absorbance values above 0.5 are desirable in order to develop an assay with a good dynamic range. Thus only coating antigen and antibody combinations that had titers in the presence of DMSO/PBST + BSA of >0.5 absorbance units were screened for inhibition by analyte. Our laboratory is also undertaking the development of surrogate standards for TCDD. That is, compounds that will behave like TCDD under analytical conditions but are less toxic. Such compounds would allow laboratories that are restricted in their ability to deal with TCDD waste to conduct these assays and provide useful internal standards for other analytical methods. 2,3,7-Trichloro-8-methyldibenzo-*p*-dioxin (TMDD, Figure 1C) is a close structural analogue to TCDD containing a methyl group in place of one chlorine in the same substitution pattern. Ongoing work in this laboratory with monoclonal antibodies to TCDD showed that TMDD responds similarly to TCDD in an ELISA (data not shown). In addition, other biological receptor studies have shown equivalency between TCDD and TMDD (Romkes et al., 1987a,b). Thus TMDD was used as the analyte for initial inhibition screening studies.

Table 3 shows those antibody and coating antigen combinations that gave a dose dependent inhibition using the surrogate standard at 10 and 100 ppb. The majority of the coating antigen/antibody combinations that showed inhibition by the surrogate standard utilized coating antigen **IV**−BSA (a pentasubstituted hapten). In addition, assays utilizing antibodies derived from hapten **IV** (rabbits 69, 89, and 99) showed inhibition with the homologous hapten as the coating antigen. Under these screening conditions, system **X**−BSA/89 demonstrated dose dependent inhibition and had the highest maximum absorbance (about 1.3 absorbance units), and thus this system was selected for further study.

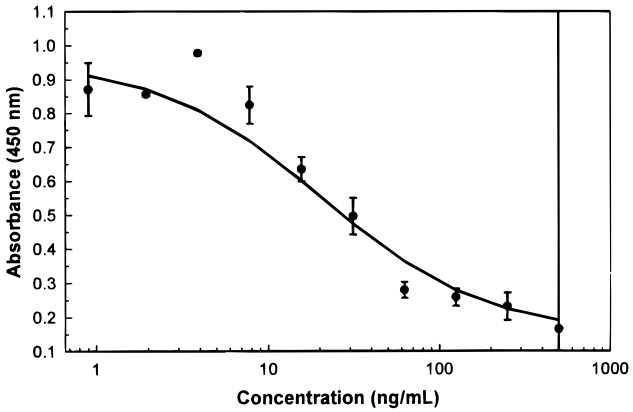


Figure 3. Calibration curve for surrogate standard, TMDD. The coating antigen was **X**−BSA at a concentration of 0.05 μg/mL and the antibody was rabbit 89 at a dilution of 1/100000. Each point is the mean of four well replicates. Standards were prepared and the assay run as described in Materials and Methods. The IC_{50} is approximately 16 ng/mL (0.8 ng/well).

Although the inhibition by TMDD shown in Table 3 for system **X**−BSA/89 was not among the highest seen, the response of the competitive ELISA to an analyte can be improved by decreasing the coating antigen and antibody concentrations, thus lowering the IC_{50}. The drawback to decreasing reagent concentrations is that there is a concomitant reduction in the dynamic range of the signal. Consequently when the coating antigen concentration was reduced to 0.25 μg/mL and the antibody concentration to 1/20000, the inhibition by the surrogate standard at 100 ppb was increased from 24 to 52%, but the signal decreased from about 1.3 to 0.12 absorbance units for the uninhibited control.

The choice of the reporting enzyme is a factor in the determination of the sensitivity of an immunoassay (Ishikawa, 1987). In this study, using HRP in place of AP increased the absorbance from 0.12 to 0.8 absorbance units using only one-fourth of the substrate reaction time and the same antibody and antigen concentrations. This allowed further reductions to the reagent concentrations, i.e., coating antigen concentration was decreased to 0.05 μg/mL and antibody dilution increased to 1/200000. The corresponding inhibition by 100 ppb of TMDD was then increased from 52 to 73%. These results indicate that further screening of the antigen/antibody combinations reported in Table 3, using HRP, may result in even more sensitive ELISAs than the example reported here.

Figure 3 shows the standard curve obtained in system **X**−BSA/89 utilizing HRP as a reporting label. The IC_{50} is approximately 16 ng/mL (0.8 ng/well). It is difficult to compare these results directly to the work of others since TMDD was used as the standard and often IC_{50} is not reported or the method for the determination of the limit of detection is not stated. However, an

examination of the respective calibration curves published indicates that the IC_{50} value reported here for TMDD is on par with the results reported by Langley et al. (Langley et al., 1992), Watkins et al. (Watkins et al., 1989), and Stanker et al. (Stanker et al., 1995) for TCDD. In Watkins' work a lower detection limit of 0.1 ng/well was reported although the assay was amplified by an avidin−biotin system. Our results indicate that the haptens reported here were successfully used to generate antibodies that can recognize the chlorodibenzodioxins and can be formatted into a sensitive ELISA. Further screening and optimization has yielded an assay with detection limits (calculated as 80% of the maximum signal in assay buffer) below 10 pg/well for TMDD. This assay shows 100% cross reactivity with TCDD (Sugawara et al., 1998).

The incorporation of rigid hydrocarbon spacers might aid in the recognition of TCDD haptens during antibody formation, thus possibly influencing the sensitivity of the assays. However, from a synthesis standpoint it was not possible to prepare the corresponding flexible haptens. Furthermore, it is recognized that the heterologous ELISA formats used so successfully in this laboratory may contribute equally to the success of the assays (Goodrow et al., 1995). In previous assays for TCDD, homologous ELISA formats have been employed, i.e., the hapten used for the immunization was also used for the coating of the wells (Albro et al., 1979; Langley et al., 1992; Stanker et al., 1987). Since, in this paper, a direct comparison of a hapten with a rigid spacer to its analogous hapten with a flexible spacer has not been made either for immunization or in heterologous and homologous assay formats, it is not possible to state unequivocally that the incorporation of rigid spacers in these haptens has been the primary reason for the levels of sensitivities observed in the ELISA evaluations reported in this paper.

Another reason for incorporating spacers with only hydrocarbon constituents adjacent to the hapten is to reduce handle recognition. In the earlier efforts to develop haptens for TCDD, there appeared to be significant handle recognition which is likely related to the presence of the polar carboxamido moiety on the dibenzo-*p*-dioxin ring system (Stanker et al., 1987). It was hoped that the hydrocarbon spacer haptens used in this work would result in less handle recognition and increased recognition of the chlorinated dibenzo-*p*-dioxin ring. Cross reactivity studies in progress for some of the TCDD assays found in later screens support this conclusion (Sugawara et al., 1998).

CONCLUSIONS

The focus of this paper was the preparation of novel haptens with rigid hydrocarbon spacers for the generation of new polyclonal antibodies for use in the analysis of dioxins, such as TCDD, by ELISA. This has been accomplished, and the haptens and antibodies have been found to be useful in heterologous assay formats with IC_{50} values of 16 ng/mL (~0.8 ng/well) for recognition of TMDD (a TCDD surrogate) that are comparable to or better than those reported in the literature for TCDD. The rationale for attachment of these spacers onto the chlorinated dibenzo-*p*-dioxin ring system was 2-fold: To promote optimal recognition of this ring system during antibody formation through decreased handle recognition and to promote more effective presentation of the hapten during antibody synthesis through decreased flexibility of the unsaturated handles. These same haptens may be useful for improving existing assays for TCDD based on monoclonal antibodies since they provide heterologous structures for assay development.

ABBREVIATIONS USED

AP, alkaline phosphatase; BSA, bovine serum albumin; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; GC−MS, gas chromatography with mass spectral detection; HRP, horseradish peroxidase; IA, immunoassay; IC_{50}, concentration of analyte that gives a 50% reduction in signal; KLH, keyhole limpet hemocyanin; LPH, hemocyanin from *Limulus polyphemus* hemolymph; Mab, monoclonal antibody; PBS, phosphate-buffered saline; PBST, PBS plus 0.05% Tween 20; RIA, radioimmunoassay; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; THF, tetrahydrofuran; TMDD, 2,3,7-trichloro-8-methyldibenzo-*p*-dioxin; TMS, tetramethylsilane; UV, ultraviolet.

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