JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Synthesis of Haptens and Protein Conjugates for the Development of Immunoassays for the Insect Growth Regulator Fenoxycarb

FERENC SZURDOKI,[†,‡] ANDRAS SZEKACS,[§] HONG M. LE,[§] AND BRUCE D. HAMMOCK*,[†]

Department of Entomology and Cancer Research Center, University of California, 1 Shields Avenue, Davis, California 95616, and Plant Protection Institute, Hungarian Academy of Sciences, H-1525 Budapest, POB 102, Hungary

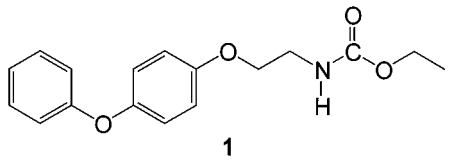
Sensitive and selective enzyme-linked immunosorbent assays (ELISAs) in the immobilized antigen format were developed for fenoxycarb (**1**), an insect growth regulator (IGR). The parent molecule [ethyl 2-(4-phenoxyphenoxy)ethylcarbamate] was derivatized at several positions to obtain haptens (**2**−**5**) that were used to produce protein conjugates and rabbit polyclonal antisera. Amino derivatives of fenoxycarb at the terminal and internal rings (**2** and **3**, respectively) were linked to carrier proteins by azo coupling. Carboxyalkyl-spacer groups were attached to the ethyl group and the nitrogen atom of the target compound (**1**) to obtain haptens **4** and **5**, respectively. Hapten-homologous ELISAs based on protein conjugates of compounds **2** and **4** determined fenoxycarb in the mid-ppb range (IC_{50}, 102 and 95 ppb, respectively). A more sensitive hapten-heterologous ELISA (IC_{50}, 17 ppb; detection limit 0.5 ppb) involved the antiserum raised against a conjugate of hapten **2** and the plate-coating antigen obtained from compound **3**. These assays displayed no significant interferences with photodegradation products of fenoxycarb, the IGRs methoprene and pyriproxyfen, and a variety of pesticides including the pyrethroids fenvalerate and cypermethryn, the phenoxyacetic acid herbicide 2,4-D, DDT, and the nitrodiphenyl ether herbicides acifluorfen and fluorodifen.

KEYWORDS: Fenoxycarb; hapten design; synthesis of haptens and protein conjugates; polyclonal antibodies; ELISA; immunoassay; heterology

INTRODUCTION

Environmental contamination from pesticides is an increasing concern for the public and regulatory agencies. Routine monitoring of these pollutants is required because of their human and environmental health hazards. Rapid, field-portable, and inexpensive methods of analysis also assist with product stewardship by facilitating timely and effective use of the pesticides. Immunoassays provide a simple and economical alternative to instrumental methods for environmental and agricultural trace analyses (*1*−*4*). As part of a collaborative research and development program (*1, 3, 5*), enzyme-linked immunosorbent assays (ELISAs) were developed for fenoxycarb, a novel insect growth regulator (IGR) insecticide (**1**, Figure 1).

Fenoxycarb (Ro 13-5223, Insegar, **1**) exhibits potent insect juvenile hormone-mimic activity, and thus causes serious disturbances in the development, reproduction, and behavior of a wide range of insects (*6*−*10*). Recent studies shed light on important details of the mode of action of fenoxycarb (*11*−*14*). This nonneurotoxic carbamate (**1**) is used for insect control in agriculture, forestry, and stored products, and is also employed as a public health insecticide (*6, 8, 15*−*17*). Fenoxycarb is registered for control of caterpillars, psyllids, and scales in Europe, and for fire ants in the U.S. (*18, 19*).



1

Figure 1. Structure of fenoxycarb (**1**).

Like most IGRs, fenoxycarb has remarkably low mammalian toxicity and is much more insect-selective than the conventional insecticides (*15*−*17*, *20*). Fenoxycarb is nontoxic to a number of beneficial insects and can be included in integrated pest management systems (*6*, *16*, *21*−*23*). However, this IGR (**1**) is harmful to some species of aquatic invertebrates, fish, and nontarget insects (*8*, *16*, *18*, *19*, *24*−*26*). The silkworm, *Bombyx mori*, is particularly sensitive to fenoxycarb (*8, 27*). Thus, this pesticide (**1**) has recently been suspected to cause the nonspin-

* To whom correspondence should be addressed. Phone: 530-752-7519. Fax: 530-752-1537. E-mail: bdhammock@ucdavis.edu.
† University of California.
‡ Current address: Polestar Technologies, Inc., 220 Reservoir Street, Suite 32, Needham Heights, MA 02494.
§ Hungarian Academy of Sciences.

10.1021/jf0107050 CCC: $22.00
Published on Web 11/22/2001

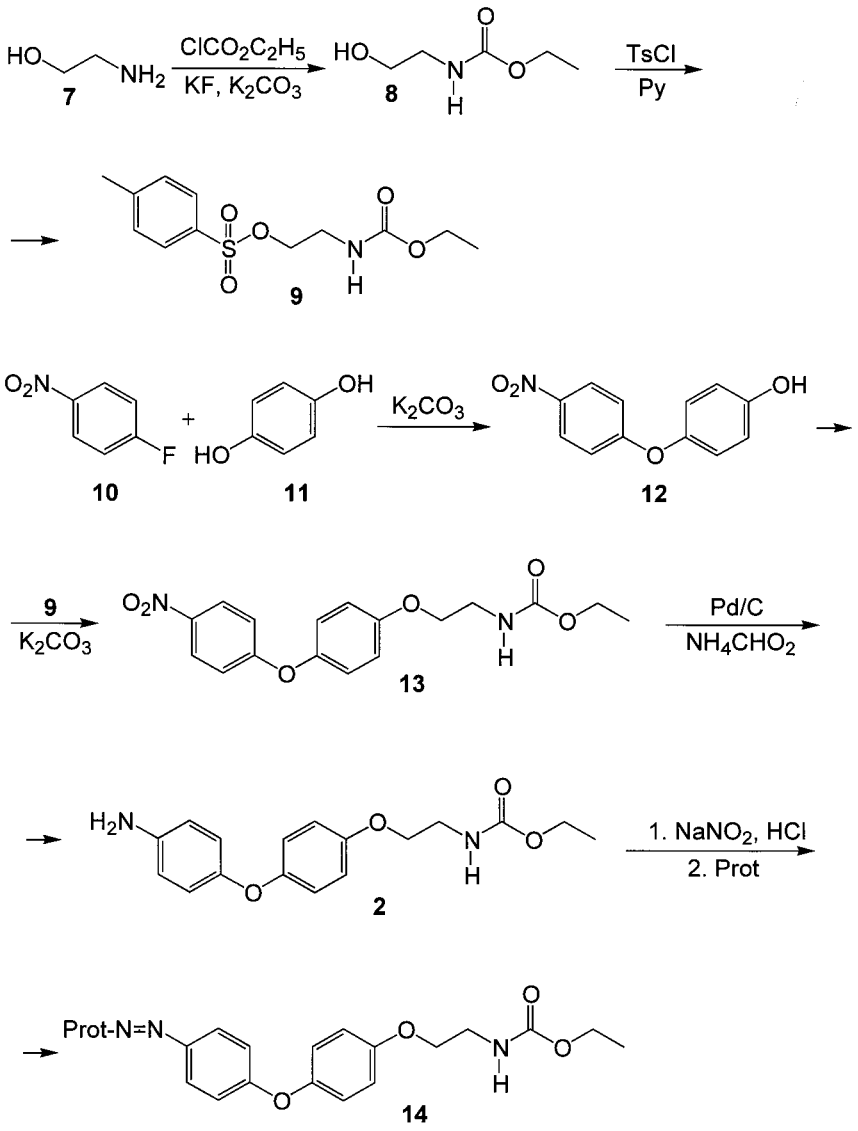


Figure 2. Syntheses leading to hapten **2** and conjugates **14** (Prot, carrier protein).

ning syndrome resulting in considerable economical losses in silkworm breeding (*8*, *14*, *28*).

Chromatographical techniques to detect residues of this pesticide involve laborious extraction and cleanup before analysis as well as costly instrumentation (*15*, *19*, *29*−*32*). Therefore, immunoassays with no or minimal sample pretreatment would facilitate the inexpensive and high-sample-throughput determination of fenoxycarb in environmental and agricultural samples. Recently, Giraudi et al. (*28*) published their results with a fenoxycarb ELISA based on a hapten comprising a part of the target structure (**1**). In our earlier studies, screening of several anti-fenoxycarb antisera led to preliminary ELISAs for this IGR (**1**) (*3*, *33*). This paper focuses on the synthesis of a panel of haptens (**2**−**5**, Figures 2−5) used to develop our antisera and immunoassays for fenoxycarb, and on the characterization of three resulting ELISAs.

MATERIALS AND METHODS

Reagents. Common reagents and organic solvents were purchased from Aldrich Chemical Co. (Milwaukee, WI), and immunochemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated. Aqueous solutions used in immunoassays and experiments with protein conjugates were prepared with Nanopure purified water. Fenoxycarb (**1**) and 2-(4-phenoxyphenoxy)ethylamine (**6**) were available from our related study (*10*) and synthesized according to known procedures (*34*). Protein concentrations of the conjugates were determined by the BCA protein assay (Pierce, Rockford, IL) following the manufacturer's instructions.

Instruments. Microtiter plates were read using an iEMS electronic microplate reader (Labsystems, Helsinki, Finland) controlled by the software Ascent provided by the vendor of the reader. Melting points (uncorrected) were taken with a Thomas−Hoover capillary apparatus. Ultraviolet−visible (UV−Vis) spectra were recorded on a Jasco 7850 spectrometer (Jasco Corp., Tokyo, Japan) and a Shimadzu UV-1601 spectrophotometer (Shimadzu Corp., Kyoto, Japan); wavelengths of characteristic peaks (λ_{max}, nm) are reported. Molar extinction coefficient (ϵ, L/mol/cm) values, given in parentheses, were also determined for certain compounds. Infrared (IR) spectra were recorded on a Perkin-Elmer 1600 series Fourier transform IR spectrometer; wavenumber values (λ_{max}, 1/cm) are given. NMR spectra were obtained with a General Electric QE-300 spectrometer (Bruker NMR, Billerica, MA) operating at 300 MHz for ^{1}H- and 75 MHz for ^{13}C-nuclei, if not otherwise stated. Some ^{1}H NMR spectra were obtained on a Bruker AW-80 (Bruker, Karlsruhe, Germany) instrument operating at 80 MHz. Chemical shifts (δ, ppm) are given relative to tetramethylsilane as internal reference. Electron impact mass spectra (MS) were determined on two instruments (A and B); data are reported as *m*/*z*, relative intensities (%) are given in parentheses. Instrument A consisted of the following: Trio-2 (VG Masslab, Altrincham, U.K.) apparatus using 70 eV electron ionization. Gas chromatography (GC) separations for

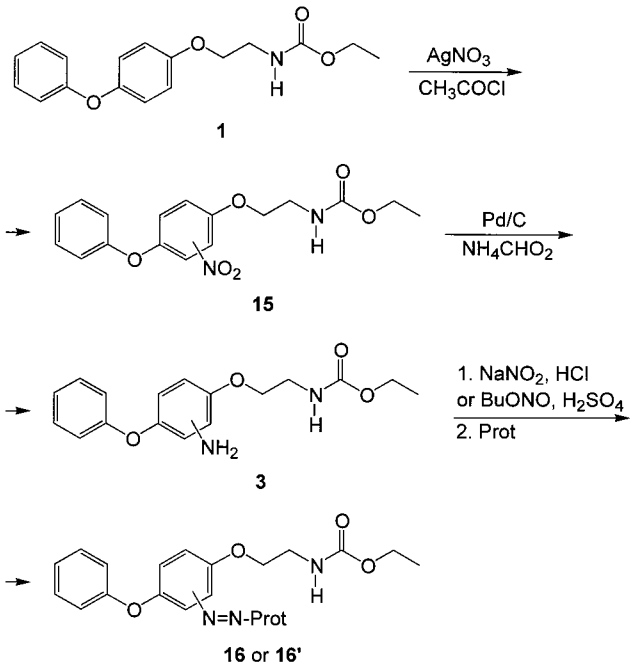


Figure 3. Syntheses leading to hapten **3** and conjugates **16** and **16′** (Prot, carrier protein).

GC−MS analyses were done by a Hewlett-Packard 5890 instrument equipped with a DB-5 column (15 m × 0.25 mm i.d.; film thickness, 0.25 μm) interfaced to this mass spectrometer. Helium was employed as carrier gas at a velocity of 35 cm/sec. Split injections with a split ratio of 25:1 were used. The injector temperature was 250 °C, the ion source temperature was 200 °C, and the transfer line was held at 300 °C. Instrument B was the following: Varian Saturn 4D mass spectrometer (Varian Assoc., Walnut Creek, CA). A Varian 3400 CX gas chromatograph equipped with a DB-5 column (30 m × 0.25 mm i.d.) was interfaced to this mass spectrometer for GC−MS analyses, and was operated in splitless mode. Further parameters were similar to those reported for instrument A. GC separations were performed with column temperature programs as follows: Program A was 160 °C for 2 min, 160 to 300 °C at 10 °C/min, and 300 °C for 10 min. Program B was 50 °C for 1 min, 50 to 300 °C at 15 °C/min, and 300 °C for 10 min. Program C was 40 °C for 1 min, 40 to 300 °C at 20 °C/min, and 300 °C for 5 min. For GC−MS separations, retention time (min) and relative peak intensity (%) are given in parentheses. Electrospray mass spectra in positive mode (MS−ES$^+$) were recorded by a VG Quatro-BQ triple quadrupole mass spectrometer (VG Biotech, Altrincham, U.K.); samples were introduced in a mixture of acetonitrile/water (1:1). Data are reported as *m*/*z*. Elemental analysis data were determined by the combustion method.

Synthetic Procedures. Syntheses of haptens and protein conjugates are outlined in Figures 2−5. Yields of hapten synthesis steps were moderate to good. Spectral data consistent with the structures are provided.

Safety Notice. During preparation of compound **15**, the temperature of the nitration reaction mixture must be kept at or below room temperature because of the explosive nature of acetyl nitrate (*35*). A protective shield must be used, and special care must be exercised throughout this operation.

Ethyl 2-Hydroxyethylcarbamate (8). The experiment was carried out using anhydrous reagents and solvent with vigorous stirring under nitrogen. Ethanolamine (**7**, 99%, 305 μL, 5.0 mmol) was added to a mixture of tetrahydrofuran (THF, 15 mL), finely ground potassium fluoride (1.45 g, 25 mmol), and potassium carbonate (3.46 g, 25 mmol). Ethyl chloformate (97%, 517 μL, 5.25 mmol) was added dropwise with ice-cooling in 10 min; the reaction mixture was maintained at room temperature for 1 h, then at reflux temperature overnight. The mixture was cooled to room temperature and filtered. The solid material on the filter was washed with ether (3 × 10 mL). The combined filtrate and washings were concentrated under reduced pressure to give 0.64 g (92% based on 96% purity of the product) of oily ester **8**. IR (NaCl) 3340, 1696, 1265. ^{1}H NMR ($CDCl_3$) 1.24 (t, J = 7 Hz, 3 H), 3.15 (b, 1 H), 3.33 (m, 2 H), 3.69 (t, J = 5 Hz, 2 H), 4.12 (q, J = 7 Hz, 2 H), 5.38 (b, 1 H). ^{13}C NMR ($CDCl_3$) 14.24, 43.04, 60.73, 61.40, 157.32. GC−MS (instrument B, program C) main peak (5.5 min, 96%) 88 (33), 102 (100), 133 (4, M$^+$). The reported NMR spectra show the signals of compound **8**, the major component. This product was used without further purification in the following reaction.

Ethyl 2-(4-Methylphenylsulfonyloxy)ethylcarbamate (9). The experiment was carried out with stirring under nitrogen. *p*-Toluenesulfonyl chloride (98%, 0.856 g, 4.4 mmol) was added to a solution of the alcohol **8** (96%, 0.555 g, 4.0 mmol) and 4-(dimethylamino)pyridine (24.4 mg, 0.2 mmol) in anhydrous pyridine (5 mL) at 4 °C. The reaction mixture was stirred at 4 °C for 30 min, and at room-temperature overnight, then diluted with hexane/ethyl acetate 2:1 mixture (20 mL), then acidified by dropwise addition of 6 M HCl solution at 4 °C. The organic phase was separated, and the aqueous phase was extracted with hexane/ethyl acetate 2:1 mixture (4 × 5 mL). The combined organic extracts were washed with water (2 × 5 mL), saturated $NaHCO_3$ solution (2 × 5 mL), and water (3 × 5 mL) again. The solution was dried over Na_2SO_4, then concentrated under reduced pressure. The product was purified by recrystallyzation from hexane/ethyl acetate to yield 0.7 g (61%) of the solid tosilate **9**. mp 77−80 °C. IR (KBr) 3307, 1680, 1361, 1272, 1180, 814. ^{1}H NMR ($CDCl_3$) 1.21 (t, J = 7 Hz, 3 H), 2.45 (s, 3 H), 3.43 (m, 2 H), 4.0−4.2 (m, 4 H), 4.98 (b, 1 H), 7.36 (d, J = 8 Hz, 2 H), 7.79 (d, J = 8 Hz, 2 H). ^{13}C NMR ($CDCl_3$) 14.31, 21.36, 39.75, 60.72, 68.95, 127.98, 129.73, 132.28, 144.83, 156.30.

4-(4-Nitrophenoxy)phenol (12). The experiment was carried out using anhydrous reagents and solvent with vigorous stirring under nitrogen. 1-Fluoro-4-nitrobenzene (**10**, 99%, 2.14 mL, 20 mmol) was added dropwise to the mixture of hydroquinone (**11**, 99%, 2.45 g, 22 mmol), finely ground K_2CO_3 (13.8 g, 100 mmol), and *N*,*N*-dimethylformamide (DMF, 25 mL). The reaction mixture was heated to its boiling point in $1^1/_2$ h, kept at reflux temperature overnight, cooled to ambient temperature, then slowly poured into a stirred mixture of 6 M HCl (40 mL) and crushed ice (100 g). The resulting precipitate was collected by filtration, washed thoroughly with water, then dried in a vacuum-desiccator over KOH to yield 4.5 g of crude product. A portion (0.7 g) of this solid material was purified by preparative TLC using a solvent of hexane/ethyl acetate (3:2) to yield 0.4 g (corresponds to 56% overall yield) of **12** as a yellow crystalline product. mp 166−169 °C [lit. 171−172 °C (*36*)]. IR (KBr) 3436, 1589, 1505, 1342, 1240, 845. ^{1}H NMR ($CDCl_3$/d_6-DMSO) 6.8−7.15 (m, 6 H), 8.16 (d, J = 9 Hz, 2 H), 9.24 (b, 1 H). ^{13}C NMR ($CDCl_3$/d_6-DMSO) 116.00, 116.61, 121.60, 125.67, 141.63, 146.20, 154.69, 164.16. GC−MS (instrument B, program C) main peak (13.8 min) 173 (9), 185 (3), 201 (5), 215 (8), 231 (100, M$^+$).

Ethyl 2-[4-(4-Nitrophenoxy)phenoxy]ethylcarbamate (13). The experiment was performed using anhydrous reagents and solvent with stirring under nitrogen. A mixture of the phenol **12** (243 mg, 1.05 mmol), the tosilate **9** (287 mg, 1.0 mmol), finely ground K_2CO_3 (691 mg, 5.0 mmol), and DMF (10 mL) was kept at 50 °C overnight. The reaction mixture was poured into a stirred mixture of ether (20 mL) and ice−water (40 mL). The organic layer was separated, and the aqueous layer was extracted by ether (2 × 5 mL). The combined Etheral solutions were successively washed with saturated $NaHCO_3$ solution (10 mL) and water (4 × 5 mL), dried over Na_2SO_4, and evaporated under reduced pressure. The residue was purified by preparative TLC using a solvent of hexane/ethyl acetate (3:2) to yield 203 mg (59%) of **13** as an oily product. IR (NaCl) 3339, 1709, 1590, 1508, 1342, 1231, 845. ^{1}H NMR ($CDCl_3$) 1.26 (t, J = 7 Hz, 3 H), 3.4−3.7 (m, 2 H), 4.0−4.3 (m, 4 H), 5.16 (b, 1 H), 6.85−7.15 (m, 6 H), 8.18 (d, J = 9 Hz, 2 H). MS (instrument A) 88 (100), 116 (100), 139 (28), 231 (40), 300 (26), 346 (5, M$^+$).

Ethyl 2-[4-(4-Aminophenoxy)phenoxy]ethylcarbamate (2). Nitrogen atmosphere was maintained during the experiment. Palladium on activated carbon (palladium content, 10%; 61 mg) was suspended in water (2 mL) before use to avoid the fire hazard that would be caused by methanol being in contact with a dry catalyst in the presence of air (*37*). A solution of the nitro compound **13** (122 mg, 0.352 mmol) in

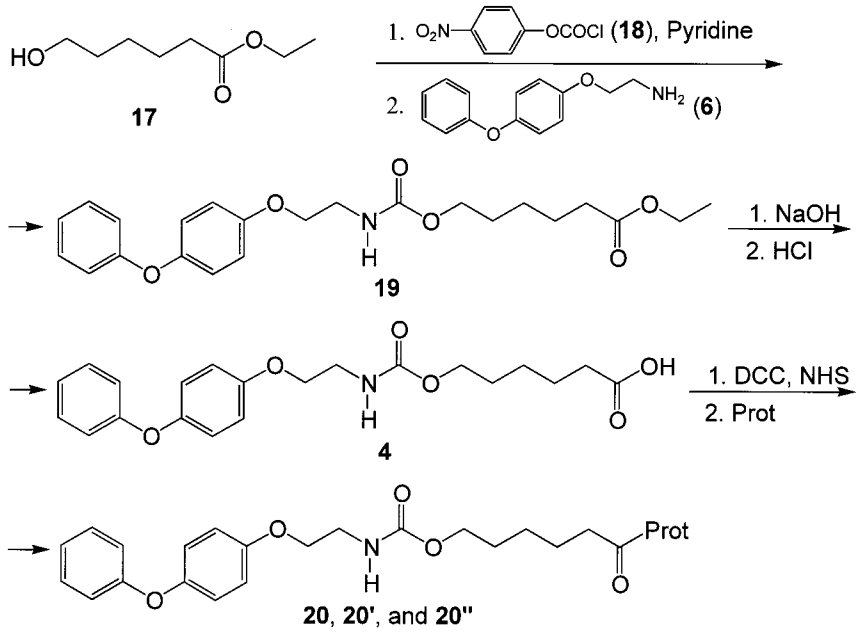


Figure 4. Syntheses leading to hapten **4** and conjugates **20**, **20′**, and **20′′** (Prot, carrier protein).

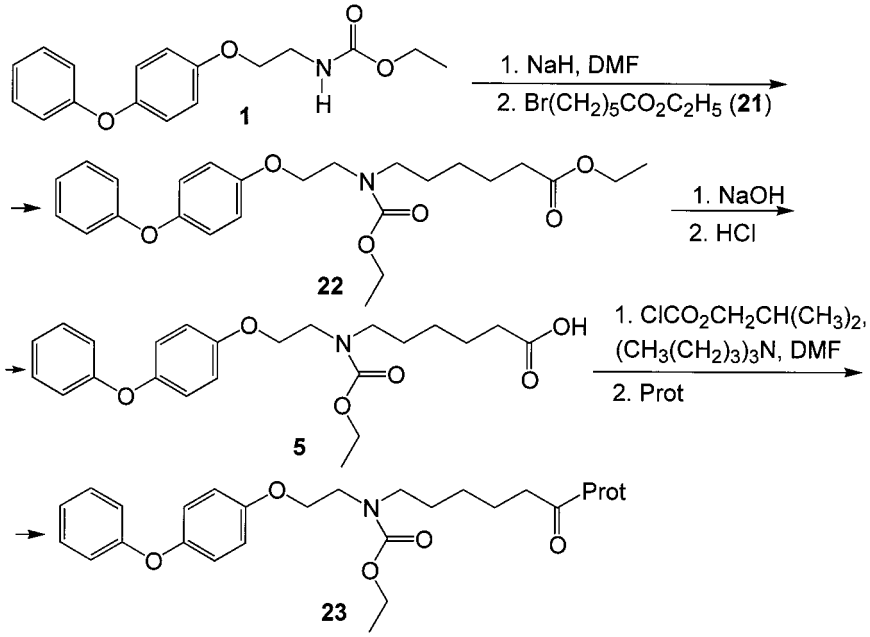


Figure 5. Syntheses leading to hapten **5** and conjugates **23** (Prot, carrier protein).

methanol (15 mL), and then ammonium formate (97%, 229 mg, 3.52 mmol), were added to the wetted catalyst with stirring at 4 °C. The reaction mixture was stirred at room temperature overnight, and the catalyst was filtered off and washed with methanol (2 × 7.5 mL) under nitrogen. The combined filtrate and washings were concentrated under diminished pressure. Ether (10 mL) and 5% $NaHCO_3$ solution (25 mL) were then added with stirring. The organic layer was separated, and the aqueous layer was extracted with ether (2 × 7.5 mL). The combined organic layers were washed with water (5 × 5 mL), dried over Na_2SO_4, and evaporated under reduced pressure. The residue was purified by preparative TLC using a solvent of hexane/ethyl acetate (1:2) to yield 80 mg (72%) of **2** as an oily product. An analytical sample was obtained by crystallyzation from carbon tetrachloride. mp 78−79 °C. IR (KBr) 3436, 3313, 1672, 1541, 1498, 1214, 829. ^{1}H NMR ($CDCl_3$) 1.24 (t, J = 7 Hz, 3 H), 3.56 (m, 2 H), 3.75 (b, 2 H), 3.98 (t, J = 5 Hz, 2 H), 4.12 (q, J = 7 Hz, 2 H), 5.13 (b, 1 H), 6.65 (d, J = 9 Hz, 2 H), 6.7−7.15 (m, 6 H). ^{13}C NMR ($CDCl_3$) 14.48, 40.35, 60.82, 67.30, 115.23, 116.06, 118.85, 119.90, 142.05, 149.63, 152.30, 153.70, 156.62. GC−MS (instrument A, program B) main peak (16.7 min) 88 (56), 92 (16), 116 (47), 201 (100), 270 (49), 316 (19, M^+).

Protein Conjugates of Hapten 2 (14). Hapten **2** (7.9 mg, 25 μmol) was dissolved in a mixture of DMF (2 mL) and aqueous HCl (0.5 M, 0.5 mL). The diazotation reaction was then carried out with stirring at 4 °C. An aqueous solution of sodium nitrite (0.1 M, 0.3 mL) was added dropwise to the hapten solution. After 30 min, urea (0.9 mg, 15 μmol) was added to decompose excess nitrous acid. After a further 5 min, the reaction mixture was divided into four equal portions. Meanwhile, each carrier protein was dissolved in 0.15 M $Na_2B_4O_7 \cdot 10\ H_2O$ (Borax) solution (5.5 mL) with stirring, and the solutions were then cooled to 4 °C. The proteins (15 mg each) used were bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), ovalbumin (OVA), and porcine thyroglobulin (TYG). Each aliquot of the diazonium salt solution was then added dropwise to a rapidly stirred protein solution at 4 °C. The reaction mixtures were stirred at 4 °C overnight, and at room temperature for 2 h, then the resulting yellow conjugates were purified by exhaustive dialysis in 0.01 M phosphate-buffered saline

(0.8% NaCl), pH 7.5 (PBS). The conjugates of proteins BSA, KLH, OVA, and TYG were designated as **14-BSA**, **14-KLH**, **14-OVA**, and **14-TYG**, respectively; UV−Vis (PBS) 355−378.

Ethyl 2-(4-Phenoxy-2/3-nitrophenoxy)ethylcarbamate (15). The experiment was carried out with intensive stirring under nitrogen. Acetyl chloride (98%, 120 μL, 1.65 mmol) was added dropwise to the solution of fenoxycarb (**1**, 0.452 g, 1.5 mmol) and silver nitrate (0.280 g, 1.65 mmol) in dry acetonitrile (5 mL) at 4 °C. The resulting suspension was kept at the same temperature for 30 min, then at room temperature for $1^1/_2$ h. Care has to be exercised that the temperature of the reaction mixture is kept at or below room temperature throughout the operation to avoid explosion (see Safety Notice above). The reaction mixture was poured into a stirred mixture of saturated NaCl solution (20 mL) and chloroform (10 mL) at 4 °C. The mixture was filtered, and the precipitate on the filter was washed with saturated NaCl solution (2 × 2.5 mL) and chloroform (2 × 5 mL). The chloroform-phase of the combined filtrates and washings was separated, and the aqueous phase was extracted with chloroform (3 × 3 mL). The combined chloroform solutions were successively washed with saturated $NaHCO_3$ (2 × 5 mL) and water (4 × 5 mL), dried over Na_2SO_4, and then concentrated under reduced pressure. The crude product was purified by preparative TLC using a solvent of hexane/ethyl acetate (1:1) to yield 351 mg (68%) of **15** as an oily product. IR (NaCl) 3336, 1712, 1589, 1528, 1487, 1350, 1268, 1216. ^{1}H NMR ($CDCl_3$) 1.24 (t, J = 7 Hz, 3 H), 3.61 (m, 2 H), 4.0−4.3 (m, 4 H), 5.35 (b, 1 H), 6.85−7.55 (m, 8 H). ^{13}C NMR ($CDCl_3$) 14.45, 40.11, 60.94, 69.43, 115.74, 116.28, 118.63, 123.98, 124.41, 124.76, 129.79, 129.98, 139.80, 147.81, 150.48, 156.46, 156.66. GC−MS (instrument A, program A) major isomer (9.5 min, ca. 94%) 77 (25), 88 (80), 116 (100), 231 (10), 300 (4), 346 (0.5, M^+); minor isomer (9.8 min, ca. 6%) 77 (18), 88 (100), 116 (82), 231 (3), 300 (2), 346 (0.5, M^+). The NMR spectra represent the signals of the major isomer. No efforts were made to identify or isolate the two isomers.

Ethyl 2-(4-Phenoxy-2/3-aminophenoxy)ethylcarbamate (3). The experiment was carried out with stirring under nitrogen. Palladium on activated carbon (palladium content, 10%; 200 mg) was suspended in water (4 mL); a solution of the nitro compound **15** (242 mg, 0.7 mmol) in methanol (30 mL), and then ammonium formate (97%, 455 mg, 7.0 mmol) were added to the suspension at 4 °C. The reaction mixture was stirred at room-temperature overnight, and then the catalyst was filtered off and washed with methanol (3 × 5 mL) under nitrogen. The combined filtrate and washings were concentrated under diminished pressure. Ethyl acetate (20 mL) and saturated $NaHCO_3$ solution (10 mL) were then added with stirring. The organic layer was separated, and the aqueous layer was extracted with ethyl acetate (3 × 3 mL). The combined organic layers were washed with saturated NaCl solution (4 × 5 mL), dried over Na_2SO_4, and evaporated under reduced pressure. The residue was purified by preparative TLC using a solvent of hexane/ethyl acetate (1:1) to yield 151 mg (68%) of **3** as a solid product. mp 83−86 °C. IR (KBr) 3409, 3313, 1708, 1594, 1511, 1268, 1222. ^{1}H NMR ($CDCl_3$) 1.23 (t, J = 7 Hz, 3 H), 3.57 (m, 2 H), 3.88 (b, 2 H), 4.02 (t, J = 5 Hz, 2 H), 4.12 (q, J = 7 Hz, 2 H), 5.28 (b, 1 H), 6.3−7.4 (m, 8 H). ^{13}C NMR ($CDCl_3$) 14.60, 40.48, 60.87, 68.06, 106.81, 108.57, 112.67, 117.93, 122.19, 122.50, 129.27, 129.56, 137.54, 142.27, 151.08, 156.73, 158.12. GC−MS (instrument A, program A) major isomer (9.4 min, ca. 98%) 77 (19), 88 (100), 116 (83), 200 (52), 201 (27), 270 (13), 316 (6, M^+); minor isomer (9.8 min, ca. 2%) 77 (22), 88 (100), 116 (64), 200 (43), 201 (29), 270 (14), 316 (4, M^+). The NMR spectra represent the signals of the major isomer. No efforts were made to identify or isolate the two isomers.

Protein Conjugates of Hapten 3 (16). Hapten **3** (31.6 mg, 100 μmol) was dissolved in aqueous HCl (0.5 M, 1.0 mL). The diazotation reaction was then carried out with stirring at 4 °C. An aqueous solution of sodium nitrite (0.2 M, 0.55 mL, 110 μmol) was added dropwise to the hapten solution. After 30 min, urea (1.8 mg, 30 μmol) was added. After a further 5 min, the reaction mixture was divided into five equal portions. Each aliquot was then added dropwise to a rapidly stirred solution of a carrier protein (20 mg) in Borax solution (0.2 M, 5 mL) at 4 °C. The proteins used were BSA, conalbumin (CONA), hemocyanin of *Limulus polyphemus* (LPH), OVA, and TYG. The reaction mixtures were stirred at 4 °C overnight and at room temperature for 2 h, and then the resulting yellow conjugates were purified by exhaustive dialysis in PBS. The conjugates of proteins BSA, CONA, LPH, OVA, and TYG were designated as **16-BSA**, **16-CONA**, **16-LPH**, **16-OVA**, and **16-TYG**, respectively. UV−Vis (PBS) 392−396.

Modified Procedure to Obtain Protein Conjugates (16′) of Hapten 3 using the Anhydrous Diazotation Method. The diazotation reaction was carried out with stirring under nitrogen using dry DMSO in a vial cooled in a water bath kept at about 19−20 °C. Care was exercised to avoid solidification of the reaction mixture because the melting point of DMSO is 18 °C. Hapten **3** (28.5 mg, 90 μmol) was dissolved in a solution of sulfuric acid in DMSO (0.05 M, 2.25 mL, 112.5 μmol). A solution of butyl nitrite in DMSO (0.05 M, 2.25 mL, 112.5 μmol) was added dropwise to this solution. After 10 min, the resulting diazonium salt solution was divided into six equal portions. Each aliquot was added dropwise to a vigorously stirred solution of a carrier protein (15 mg of BSA, CONA, KLH, LPH, OVA, or TYG) in Borax solution (0.1 M, 7.5 mL) at 4 °C. The reaction mixtures were stirred at 4 °C for $1^1/_2$ h, and then stirred at room temperature overnight. The yellow conjugates were purified by exhaustive dialysis in PBS. The conjugates of proteins BSA, CONA, KLH, LPH, OVA, and TYG were designated as **16′-BSA**, **16′-CONA**, **16′-KLH**, **16′-LPH**, **16′-OVA**, and **16′-TYG**, respectively. UV−Vis (PBS) 395−399.

5-Ethoxycarbonylpentyl 2-(4-Phenoxyphenoxy)ethylcarbamate (19). The experiment was carried out with stirring under nitrogen. A solution of ethyl 6-hydroxyhexanoate (**17**, 97%, 1.16 g, 7.0 mmol) and 4-nitrophenyl chloroformate (**18**, 97%, 1.455 g, 7.0 mmol) in anhydrous pyridine (30 mL) was allowed to react at room temperature overnight. 2-(4-Phenoxyphenoxy)ethylamine (**6**, 1.605 g, 7.0 mmol) was then added, and the reaction mixture was kept at 55−60 °C for 24 h. The solution was diluted with water (160 mL) and extracted with dichloromethane (3 × 150 mL). The collected organic extracts were successively washed with 10% HCl (3 × 150 mL), saturated Na_2CO_3 (3 × 150 mL), and saturated NaCl solutions (150 mL), dried over Na_2SO_4, and evaporated under diminished pressure. The resulting crude product was purified by column chromatography using a solvent of chloroform/ethyl acetate (9:1) to provide 2.35 g (81%) of **19** as a solid product. mp 47 °C. Anal. found: C, 66.4, H, 7.2%. Calcd. for $C_{23}H_{29}NO_6$: C, 66.49; H, 7.03%. IR (KBr) 3316, 1731, 1687, 1506, 1490, 1466, 1222, 1196. ^{1}H NMR ($CDCl_3$, 80 MHz) 1.18 (t, J = 7.5 Hz, 3H), 1.3−1.85 (m, 6H), 2.23 (t, J = 7 Hz, 2H), 3.49 (m, 2H), 3.9−4.25 (m, 6H), 5.0 (b, 1H), 6.7−7.05 (m, 7H), 7.05−7.35 (m, 2H). MS−ES$^+$ 416 ($M + H^+$).

5-Carboxypentyl 2-(4-Phenoxyphenoxy)ethylcarbamate (4). A solution of ester **19** (1.04 g, 2.5 mmol) in 35 mL of ethanol was added to 1 M NaOH (3.0 mL), and the mixture was stirred at reflux temperature for 8 h. The reaction mixture was then concentrated under reduced pressure, diluted with water (100 mL), and cooled in an ice bath. After addition of ether (100 mL), the stirred mixture was acidified with 1 M HCl. The aqueous phase was separated, then extracted with ether (4 × 50 mL). The combined organic solutions were dried over Na_2SO_4 and concentrated under diminished pressure. The precipitated crystalline product was collected by filtration to afford 0.89 g (92%) of **4**. mp 114−115 °C. Anal. found: C, 65.2, H, 6.4%. Calcd. for $C_{21}H_{25}NO_6$: C, 65.10; H, 6.50%. IR (KBr) 3500−2500, 1696, 1508, 1490, 1468, 1241, 1210. ^{1}H NMR ($CDCl_3$, 80 MHz) 1.3−1.85 (m, 6H), 2.27 (t, J = 7 Hz, 2H), 3.50 (m, 2H), 3.85−4.2 (m, 4H), 5.08 (b, 1H), 6.7−7.05 (m, 7H), 7.1−7.35 (m, 2H), 9.24 (b, 1H). MS−ES$^+$ 388 ($M + H^+$).

Protein Conjugates of Hapten 4 (20, 20′, and 20″). Compound **4** (20 mg, 51.6 μmol) and *N*-hydroxysuccinimide (NHS, 6.9 mg, 60 μmol) were dissolved in dry THF (1 mL), and dicyclohexylcarbodiimide (DCC, 12.4 mg, 60 μmol) was added to this solution. The reaction mixture was stirred at room temperature for 2 h, and the precipitated solid (dicyclohexylurea) was removed by filtration. The resulting active ester solution was divided into two aliquots (300 μL and 700 μL). Each aliquot was added dropwise to a solution of BSA (100 mg) in water (10.5 mL) and THF (0.6 mL) with stirring at 4 °C (reagent ratios, 0.16 and 0.36 μmol hapten/mg protein, respectively). The reaction mixtures were stirred for 24 h at 4 °C. The conjugates were then purified by exhaustive dialysis in water. Employing a similar method, hapten **4** was also conjugated to BSA, CONA, and KLH using a 1 μmol hapten/mg protein reagent ratio. The conjugates of proteins BSA (reagent

ratios: 0.16, 0.36, and 1 μmol hapten/mg protein), CONA, and KLH were designated as **20-BSA**, **20′-BSA**, **20″-BSA**, **20″-CONA**, and **20″-KLH**, respectively; UV−Vis (PBS) 290−310. Lacking significant peaks above 310 nm, the UV−Vis spectra of conjugates **20**, **20′**, and **20″** were not useful for the determination of hapten/protein ratios because the hapten and protein bands overlapped.

Ethyl *N*-[2-(4-Phenoxyphenoxy)ethyl]-*N*-(5-ethoxycarbonylpentyl)-carbamate (22). The procedure was similar to that of reference *53*. The experiment was carried out using fenoxycarb (**1**, 0.452 g, 1.5 mmol), a suspension of NaH (60% dispersion in mineral oil, 72 mg, 1.8 mmol), and ethyl 6-bromohexanoate (99%, 324 μL, 1.8 mmol). The crude product was purified by preparative TLC using a solvent of hexane/ethyl acetate (3:2) to yield 0.56 g (84%) of **22** as an oily product. ^{1}H NMR: ($CDCl_3$) 1.25 (t, J = 7 Hz, 3 H), 1.26 (t, J = 7 Hz, 3 H), 1.3−2.0 (m, 6 H), 2.30 (t, J = 7.5 Hz, 2 H), 3.34 (m, 2 H), 3.58 (t, J = 5 Hz, 2 H), 3.95−4.25 (m, 6 H), 6.8−7.1 (m, 7 H), 7.2−7.35 (m, 2 H).

Ethyl *N*-[2-(4-phenoxyphenoxy)ethyl]-*N*-(5-carboxypentyl)-carbamate (5). The experimental method was similar to that employed to prepare compound **4** (above). A solution of ester **22** (490 mg, 1.105 mmol) in ethanol (20 mL) and 1 M NaOH (1.66 mL) was used. The crude product was purified by preparative TLC using a solvent of hexane/ethyl acetate/acetic acid (15:15:1) to give 0.24 g (52%) of **5** as an oily substance. UV−Vis (THF) 271, 279. IR (NaCl) 3500−2500, 1705, 1656, 1615, 1439, 1198. ^{1}H NMR ($CDCl_3$) 1.27 (t, J = 7 Hz, 3 H), 1.3−2.0 (m, 6 H), 2.35 (t, J = 7.5 Hz, 2 H), 3.34 (m, 2 H), 3.61 (m, 2 H), 3.95−4.25 (m, 4 H), 6.8−7.1 (m, 7 H), 7.2−7.35 (m, 2 H), 9.87 (b, 1 H). ^{13}C NMR ($CDCl_3$) 14.46, 24.22, 25.96, 28.04, 33.70, 47.11, 48.21, 61.27, 66.65, 115.23, 117.40, 120.59, 122.27, 129.40, 150.12, 154.65, 156.48, 158.19, 180.6. MS 77 (16), 184 (57), 186 (10), 199 (1.3), 230 (100), 342 (0.16), 370 (0.33), 398 (0.12) 415 (0.43, M^+).

Protein Conjugates of Hapten 5 (23). The mixed anhydride was formed using dry DMF with stirring under nitrogen. Hapten **5** (24.9 mg, 60 μmol) was dissolved in DMF (3.2 mL) and tributylamine (360 μL of 0.2 M solution in DMF, 72 μmol), then isobutyl chloroformate (360 μL of 0.2 M solution in DMF, 72 μmol) was added dropwise to the solution at 4 °C. The mixture was maintained at the same temperature for 10 min and at room temperature for 20 min, then divided into four equal portions. Each aliquot was added dropwise to a solution of a carrier protein (15 mg of BSA, CONA, KLH, or OVA) in 0.2 M borate buffer, pH 8.7 (5.0 mL) at 4 °C. The reaction mixtures were stirred at the same temperature for 2 h, and at room temperature overnight, and then the resulting conjugates were purified by exhaustive dialysis in PBS. The conjugates of proteins BSA, CONA, KLH, and OVA were designated as **23-BSA**, **23-CONA**, **23-KLH**, and **23-OVA**, respectively; UV−Vis (PBS) 275−278. In the UV−Vis spectra of hapten **5** and conjugates **23**, the peaks were very close to those of the carrier proteins; thus, it was not possible to estimate the hapten/protein ratios of these conjugates from the UV−Vis spectral data.

4-Methyl-2-(2-phenoxyphenyl)azo-phenol (26a). A suspension of 2-phenoxyaniline (**24a**, 185.2 mg, 1 mmol) in 0.1 M HCl (25 mL) was sonicated for 15 min. The diazotation and azo coupling reactions were then carried out with stirring. A solution of aqueous sodium nitrite (0.43 M, 2.4 mL, 1.03 mmol) was added to the amine at 4−5 °C in 15 min. The reaction mixture was kept at the same temperature for 30 min. Urea (12 mg, 0.2 mmol) was then added, and the mixture was stirred for 5 min. Meanwhile, 4-methylphenol (**25**, *p*-cresol, 108.1 mg, 1 mmol) was dissolved in Borax solution (0.2 M, 15 mL), the solution was cooled to 4 °C, and the diazonium salt solution was added to this solution dropwise at 4−5 °C. The mixture was kept at 5 °C for 30 min and at room temperature for 3 h. The precipitate was then collected by filtration, washed with water and hexane, and recrystallized from isopropyl ether to afford 248 mg (81%) of **26a** as a crystalline product. mp 67−68 °C. UV−Vis (methanol) 322 (ϵ = 21,200). IR (KBr) 1583, 1490, 1478, 1253, 1238, 810, 759. ^{1}H NMR ($CDCl_3$) 2.3 (s, 3 H), 6.88 (d, 1 H), 6.98−7.18 (m, 5 H), 7.3−7.5 (m, 4 H), 7.58 (d, 1 H), 7.68 (s, 1 H), 12.5 (s, OH).

4-Methyl-2-(3-phenoxyphenyl)azo-phenol (26b). This compound was prepared in a similar fashion from 3-phenoxyaniline (**24b**). 239 mg (78%) of **26b** as a crystalline product was obtained. mp 73−74 °C. UV−Vis (methanol) 324 (ϵ = 19,100). IR (KBr) 1585, 1489, 1477, 1254, 1208, 790, 691. ^{1}H NMR ($CDCl_3$,) 2.3 (s, 3 H), 6.92 (d, 1 H), 7.0−7.2 (m, 5 H), 7.3−7.5 (m, 4 H), 7.60 (d, 1 H), 7.68 (s, 1 H), 12.5 (s, OH).

4-Methyl-2-(4-phenoxyphenyl)azo-phenol (26c). This compound was also synthesized in a similar way from 4-phenoxyaniline (**24c**). 251 mg (82%) of **26c** as a crystalline product was isolated. mp 62−63 °C. UV−Vis (methanol) 342 (ϵ = 20,900). IR (KBr) 1583, 1488, 1435, 1278, 1240, 849. ^{1}H NMR ($CDCl_3$) 2.3 (s, 3 H), 6.90 (d, 1 H), 6.98−7.18 (m, 6 H), 7.3−7.5 (m, 2 H), 7.7 (s, 1 H), 7.84 (d, 2 H), 12.6 (s, OH).

Immunization and Antiserum Preparation. A routine immunization protocol (*1*) was followed. Conjugates **14-KLH**, **14-TYG**, **16-LPH**, **16-TYG**, **20″-CONA**, **20″-KLH**, and **23-KLH** were used as immunogens. Rabbit polyclonal antisera were collected as described by Székács and Hammock (*1*).

ELISA. ELISA experiments in the immobilized antigen format were performed following a modified version of a protocol described earlier (*1*). 96-Well microtiter plates (Nunc, Roskilde, DK, #442404) were coated with appropriate fenoxycarb−protein conjugates which had been dissolved in carbonate buffer (0.1 M, pH 9.6), and then blocked with a 1% solution of gelatin (Reanal, Hungary) in PBS. Sample or standard solution and the antiserum solution, both in PBS containing 0.05% Tween 20 (PBST), were dispensed into the wells sequentially. After incubation, the wells were treated with goat anti-rabbit IgG (H + L)-horseradish peroxidase conjugate (Bio-Rad Laboratories, Richmond, CA, 1:12,000 dilution in PBST). Enzymatic activity was then measured using a chromogenic substrate solution containing *o*-phenylenediamine and hydrogen peroxide (*1*). The optical density (OD) values at 492 nm were recorded. To construct standard curves, stock solutions of fenoxycarb or related compounds in methanol or acetonitrile (10 mg/mL) were serially diluted with PBST. Sigmoidal standard curves were generated from the measured OD values using the common four-parameter curve fit method (*1*). The IC_{50} value is the analyte concentration required for 50% inhibition. The lower detection limit (LDL) is defined as the analyte concentration reducing the mean blank OD value by three standard deviations (SD) of the blank reading.

RESULTS AND DISCUSSION

Synthetic Work. Spacer groups were linked to the fenoxycarb molecule (**1**, Figure 1) at several positions to obtain haptens (**2**−**5**, Figures 2−5) that were then chemically activated to prepare their protein conjugates. Selected protein derivatives were used as synthetic immunogens (*2*) to elicit rabbit polyclonal antisera recognizing fenoxycarb. Some protein conjugates were used as microplate coating antigens in the ELISA procedure. The structure of fenoxycarb (**1**) offers various possibilities for spacer arm attachment (Figure 1). Amino derivatives at the terminal and internal rings (haptens **2** and **3**, respectively) were used to generate the corresponding diazonium salts (Figures 2 and 3). The diazonium compounds were linked to proteins by azo coupling (Figures 2 and 3). Elongation of the ethyl group of the parent structure led to hapten **4** with a carboxylic acid functional group (Figure 4). The nitrogen of the fenoxycarb molecule was alkylated to obtain another carboxylic acid hapten **5** (Figure 5). Protein conjugates of the latter two compounds (**4** and **5**, linker group for both: $(CH_2)_5CO_2H$) were obtained by activation of the carboxyl groups.

Our hapten design was guided by the principle of maximizing steric and electronic similarity with the parent molecule, as well as by the feasibility of the syntheses (*2*, *38*). Hapten synthesis often comprises the most time-consuming and difficult part of immunoreagent development (*38*). Thus, both the similarity criteria and the burden of preparative effort have to be carefully evaluated before undertaking the synthesis of a hapten. Synthesis of a near-perfect analogue of the target analyte is sometimes challenging. Such a high degree of structural similarity can,

Table 1. Characterization of Selected ELISA Systems

system	A (homologous)	B (homologous)	C (heterologous)
antiserum/immunogen	39189a/**20″-KLH**	4945/**14-KLH**	4945/**14-KLH**
dilution in competitive ELISA	1:1,000	1:3,000	1:1,500
coating antigen	**20″-BSA**	**14-BSA**	**16-BSA**
concentration (μg/mL)	5	1	2.5
titer	1:50,000	1:65,000	1:20,000
IC_{50} (ppb of fenoxycarb)	95	102	17
fenoxycarb detection range (ppb)	13–2,000	12–3,000	0.5–300

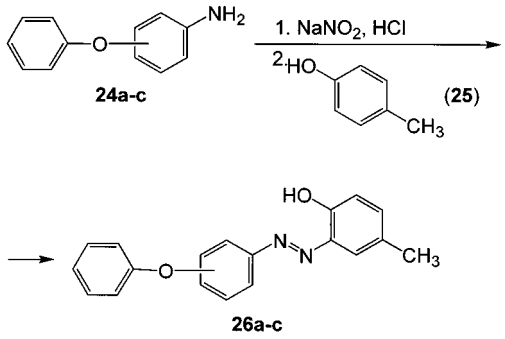


Figure 6. Syntheses leading to compounds **26a**–**c**.

however, be necessary to make a good immunizing hapten (*2*). In our synthetic work, preparation of hapten **2** (Figure 2) with structure closely related to fenoxycarb required the most effort. The ELISA (system C, Table 1) using the antiserum based on this immunizing hapten (**2**), however, resulted in the highest sensitivity in this study (see Competitive ELISAs below).

Synthetic precursor **13** of hapten **2** was assembled from the tosilate **9**, including the carbamate moiety of the fenoxycarb molecule, and 4-(4-nitrophenoxy)phenol (**12**, Figure 2). The high reactivity of fluorine in 1-fluoro-4-nitrobenzene (**10**) toward nucleophiles (*39*, *40*) enabled the development of a simple preparation of the phenol **12**, which was based on selective substitution reaction of compound **10** with hydroquinone (**11**). Such an approach can be a useful alternative to other methods (*36*, *41*) for the synthesis of diaryl ethers related to compound **12**, if the reactive fluoroaromatic starting material is readily available. Ethyl 2-hydroxyethylcarbamate (**8**) was obtained by selective derivatization of ethanolamine (**7**) in the presence of potassium fluoride. The role of fluoride ions was to enhance reactivity of the amino group by disrupting strong hydrogen bonds in the starting material (**7**) (*42*−*44*). This technique allowed a simple nonaqueous workup. The conventional variant of this reaction using aqueous base and extractive isolation gave less satisfactory results. The tosilate **9**, obtained from the alcohol **8**, was condensed with the phenol **12** in the presence of potassium carbonate. The nitro group of the resulting carbamate **13** was reduced by catalytic transfer hydrogenation (CTH) employing the ammonium formate/palladium-on-carbon system (*37*, *45*). This method produced the corresponding amine (**2**) in good yield under mild conditions without the difficulties involved using hydrogen gas. The amine hapten **2** was then diazotized in an aqueous solution following established procedures (*46*−*48*). The azo coupling reaction (*46*−*49*) of the resulting diazonium salt with proteins yielded the corresponding conjugates (**14**).

Adaptation of a general literature procedure (*35*, *50*) led to a mild and selective nitration method for fenoxycarb (Figure 3). Acetyl nitrate, formed from silver nitrate and acetyl chloride in situ (*50*), served as nitrating agent. The more activated internal ring of the fenoxycarb molecule was substituted selectively (Figure 3). It appears that a mixture of the two isomeric nitro compounds (**15**) was formed, but no efforts were made to isolate the individual isomers (Figure 3). This mixture (**15**) was reduced to the mixture of the corresponding amino compounds (**3**) by the CTH procedure (see above) with, again, no attempt to isolate the two individual isomers (Figure 3). The resulting hapten (**3**, isomer mixture) was diazotized by both the routine aqueous method (*46*−*48*) and a modified procedure using butyl nitrite in DMSO (Figure 3). We devised the latter diazotation method to conjugate a highly lipophilic dioxin derivative, weakly basic and poorly soluble in water, to proteins (*51*). It was also successfully applied to prepare conjugates of other lipophilic compounds such as pyrethroid haptens (*46*). The diazonium salt, formed from amine **3** by either method, was linked to proteins by azo coupling to furnish the corresponding conjugates **16** (obtained via the routine diazotation method) and **16′** (furnished by the modified procedure).

Following literature analogies (*52*), ethyl 6-hydroxyhexanoate (**17**) was successively treated with 4-nitrophenyl chloroformate (**18**) and 2-(4-phenoxyphenoxy)ethylamine (**6**) to produce the carbamate intermediate **19** (Figure 4). Carbamate groups such as that of compound **19** are usually fairly resistant to saponification. Thus, selective alkaline hydrolysis of the ester group of intermediate **19** to form hapten **4** could be performed in good yield (Figure 4). This carboxylic acid (**4**) was conjugated to proteins by the active ester method (*1*) to form conjugates (**20**, **20′**, and **20″**) with several hapten/protein ratios (Figure 4).

The amide anion, generated from fenoxycarb (**1**) by sodium hydride, was alkylated using ethyl 6-bromohexanoate (**21**) (*53*, *54*) to yield the ester **22** (Figure 5). This intermediate (**22**) was converted to hapten **5** by selective alkaline hydrolysis. The resulting carboxylic acid (**5**) was coupled to proteins by the mixed anhydride method (*53*) to furnish the corresponding conjugates (**23**, Figure 5).

Diazotation of the three phenoxyaniline-isomers (**24a**−**c**, Figure 6) and coupling of the resulting diazonium salts with 4-methylphenol (**25**) following standard protocols (*47*, *48*) gave **26a**−**c** model compounds for epitope density calculations (see below). The lack of λ_{OH} signals in the IR-spectra and the very high shifts of the O*H* signals in the ^{1}H NMR-spectra of azo derivatives **26a**−**c** (Figure 6) are due to strong intramolecular hydrogen bonds involving the hydroxyl and azo groups (*48*). These unusual spectral properties of compounds **26a**−**c** are similar to those of related azo compounds with hydrogen-bonded chelate structures (*48*).

Estimation of Hapten Densities (HDs) in Protein Conjugates. The colored protein derivatives **14**, **16**, and **16′**, obtained by azo coupling, display broad absorbance bands in the range of about 270−600 nm. We have utilized the strong UV−Vis absorbances due to azo moieties to calculate the approximate hapten/protein ratios of these conjugates (**14**, **16**, and **16′**). Under the conditions used in our azo coupling reactions (pH ∼9−10), diazonium salts readily form azo bridges with tyrosines and, to a lesser extent, a few other amino acids (e.g., histidine) in proteins (*49*, *55*, *56*). Thus, our estimation of HDs is based on molar extinction coefficient (ϵ) values of model azo compounds structurally related to the fenoxycarb-azo-tyrosine moieties in the protein conjugates **14**, **16**, and **16′**.

Because haptens **2** and **3** were available only in limited amounts, we choose commercially available aromatic amines (**24a**−**c**), structurally similar to these haptens, as starting materials for syntheses of model compounds **26a**−**c** (Figure 6).

Table 2. Results of Hapten Density Calculations[a,b]

conjugate	λ_{max} of the conjugate (nm)	carrier protein molecular weight (kDa)	ϵ used in the calculation (L/mol/cm)	hapten density[c]	number of hapten molecules/10 kDa carrier protein[d]
16-BSA	396	67	20,150[e]	28	4.2
16-CONA	394	76	20,150[e]	19	2.5
16-LPH	392	335	20,150[e]	28	0.84
16-OVA	393	43	20,150[e]	8.4	1.9
16′-BSA	399	67	20,150[e]	25	3.8
16′-CONA	396	76	20,150[e]	20	2.6
16′-KLH	397	6,000	20,150[e]	714	1.2
16′-LPH	398	335	20,150[e]	43	1.3
16′-OVA	395	43	20,150[e]	8.9	2.1
16′-TYG	397	660	20,150[e]	75	1.1
14-BSA	358	67	20,900[f]	23	3.5
14-KLH	378	6,000	20,900[f]	636	1.1
14-OVA	355	43	20,900[f]	9.2	2.2
14-TYG	360	660	20,900[f]	159	2.4

[a] Protein concentrations of the conjugates were determined by the BCA protein assay. [b] Conjugate **16-TYG** was not involved in this study. [c] Average number of hapten moleules linked to a carrier protein molecule; HD. [d] HD 10 kDa. [e] Average of ϵ values for **26a** and **26b** model compounds. [f] ϵ value for **26c** model compound.

In the structures of azo derivatives **26a**−**c**, the fragment according to 4-methylphenol (**25**) mimicks the phenolic group of tyrosine (Figure 6). Our approach is similar to that of Flanagan et al. (*57*), who determined epitope densities for protein conjugates prepared by azo coupling. The ϵ value for the azo linkage, used by these authors, was that of an azo derivative obtained by diazotation of an aromatic amine hapten and azo coupling with 4-methylphenol (**25**) (*57*, *58*). The ϵ values at the wavelengths of maximal absorbance (λ_{max}) of model compounds **26a**−**c** are taken into account in our calculations (see Materials and Methods and Table 2). The ring-substitution pattern of azoaromatic groups in the structure of protein conjugates **14** is analogous to that of compound **26c** synthesized from 1,4-phenoxyaniline (**24c**, Figures 2 and 6). Thus, the ϵ value of azo derivative **26c** is used to estimate HDs of this set of conjugates (**14**, Table 2). Conjugates **16** and **16′**, based on hapten **3**, are structurally related to azo compounds **26a** and **26b** (Figures 3 and 6). Azo derivatives **26a** and **26b** were synthesized from 1,2- and 1,3-phenoxyaniline (**24a** and **24b**, Figure 6), respectively. Because the two isomers, whose mixture comprises hapten **3**, are not identified, the average (20,150) of ϵ values for compounds **26a** and **26b** is employed in calculations for **16** and **16′** sets of conjugates (Figures 3 and 6, Table 2). Spectral characteristics (e.g., ϵ and λ_{max} values) of azo compounds **26a** and **26b** are very close (see Materials and Methods). The ϵ values used in our calculations (Table 2) and reported in the literature for azo derivatives structurally related to our model compounds are similar (*57*, *59*). The differences between the λ_{max} values of our model compounds **26a**−**c** and those of the corresponding protein conjugates (**14**, **16**, and **16′**) appear to be due to structural differences between azo compounds **26a**−**c** and conjugates (**14**, **16**, and **16′**) and/or to solvent effect and influence of the protein microenvironment. The molecular weights (kDa) of carrier proteins used in the calculations are as follows: BSA, 67 (*49*); CONA, 76 (*53*); KLH, 6,000 (*53*); LPH, 335 (*46*); OVA, 43 (*49*); TYG, 660 (*46*, *49*).

Although this HD estimation method is based on ϵ values of model compounds with only simple structures (**26a**−**c**), the obtained HDs (Table 2) are similar to those reported for other protein conjugates obtained by azo coupling (*56*, *57*). Our calculations give only approximate HDs; the real values may be somewhat higher due to azo coupling to a few amino acids (e.g., histidine) beside tyrosine in the carrier proteins. In our study, HDs for certain conjugate groups, each based on one of the carrier proteins BSA, CONA, KLH, and OVA, are markedly consistent between each group (Table 2). This trend is likely because of some structural properties of the individual native proteins. The hapten/10 kDa protein ratios (HD10kDa) allow comparison between conjugates of different carrier proteins (*53*). HD10kDa data range between 0.8 and 4.2, with the highest values for conjugates of BSA and CONA (Table 2).

Table 3. Selectivity of the ELISA Systems toward Compounds Related to Fenoxycarb

compound	cross-reactivity (CR (%))[a] system A[b,c]	system B[b,c]	system C[b,c]
fenoxycarb (**1**)	100	100	100
2-(4-phenoxyphenoxy)ethylamine (**6**)	9.2	–[d]	1.4
4-phenoxyphenol	–[d]	–[d]	0.2

[a] Results are expressed as percent cross-reactivity values. Cross-reactivity (CR) value (%) = (IC_{50} of fenoxycarb/IC_{50} of compound) × 100. [b] Assay systems were as described in Table 1. [c] Methoprene and a variety of compounds (e.g., pyriproxyfen, 4-phenylphenol, 2-hydroxydibenzofuran, fenvalerate, cypermethryn, 2,4-D, acifluorfen, fluorodifen, *p*,*p*′-DDT) structurally related to fenoxycarb did not produce significant inhibition (CR < 0.1%). [d] Significant inhibition was not observed in these cases.

Antisera Characterization, Titer. The results of antibody characterization were obtained from antisera of terminal bleeds. Raw antisera were used without purification. Titer is defined as the serum dilution giving three times the background absorbance (*60*). Titers were determined by the usual checkerboard titrations (*46*). Most antisera showed fairly high titers to their homologous antigens; typical reciprocal titers were on the order of 10^3 to 10^5. Selected examples (systems A and B) are shown in Table 1 and Figure 7. Some heterologous assays such as system C (Table 1, Figure 7) also displayed promising titers.

Competitive ELISAs. First, homologous assay systems were investigated in competition experiments. Selected assays (systems A and B, see Table 1 and Figure 8) detected fenoxycarb in the range of 12 to 3,000 ng/mL (ppb). Heterology provides the means of modifying equilibrium conditions in competitive immunoassays (*2*, *53*, *60*). Thus, heterologous assays are often used to enhance the sensitivity of ELISAs (*2*, *51*, *53*, *56*, *60*). In our work, site and carrier heterology in system C resulted in improved sensitivity (IC_{50}, 17 ppb; LDL, 0.5 ppb; Table 1, Figure 8) as compared to the homologous ELISA based on the same antiserum (system B, Table 1). The high sensitivity of system C using antiserum 4945, based on immunogen **14-KLH** and immunizing hapten **2** (Figure 2), may be also due to the relatively short and rigid azo group spacer in conjugate **14-KLH** of the lipophilic analyte (**1**). Such a linker group attached to the terminal ring of fenoxycarb may have helped to project the fenoxycarb moieties on the immunogen (**14-KLH**, Figure 2) for recognition by preventing the haptens from folding back on the adjacent lipophilic areas of the protein surface (*51*).

Cross-Reactivity. The assays showed no significant interferences (CR < 0.1%, Table 3) with 4-phenylphenol and 2-hydroxydibenzofuran, photodegradation products of fenoxycarb (*61*, *62*), which may be of advantage for environmental analysis of fenoxycarb. Likewise, the terpenoid methoprene and aromatic pyriproxyfen IGR insecticides did not cause significant inhibition with these ELISAs. The structures of the latter compound and fenoxycarb share the 4-phenoxyphenoxy moiety. A variety of further pesticides structurally related to fenoxycarb, including

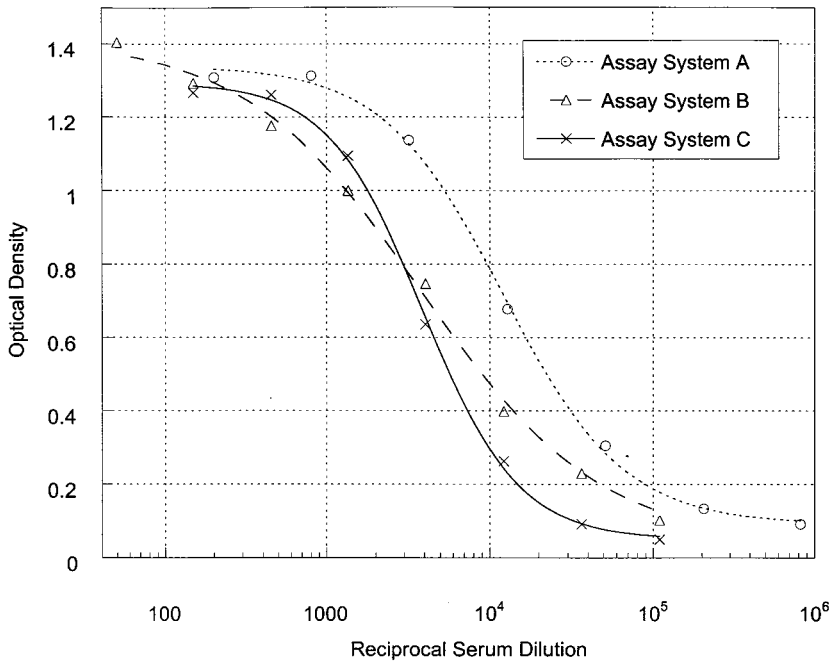


Figure 7. Titration curves obtained by ELISA systems used in this study. Assay systems were as described in Table 1. Microplate wells treated with a coating antigen were exposed to various dilutions of an antiserum. See Materials and Methods for further details. Each point represents the average of the OD values at 492 nm, which were from three replicate wells on the same microplates. Coefficients of variation for all points fell in the range of 0.3–13.3%.

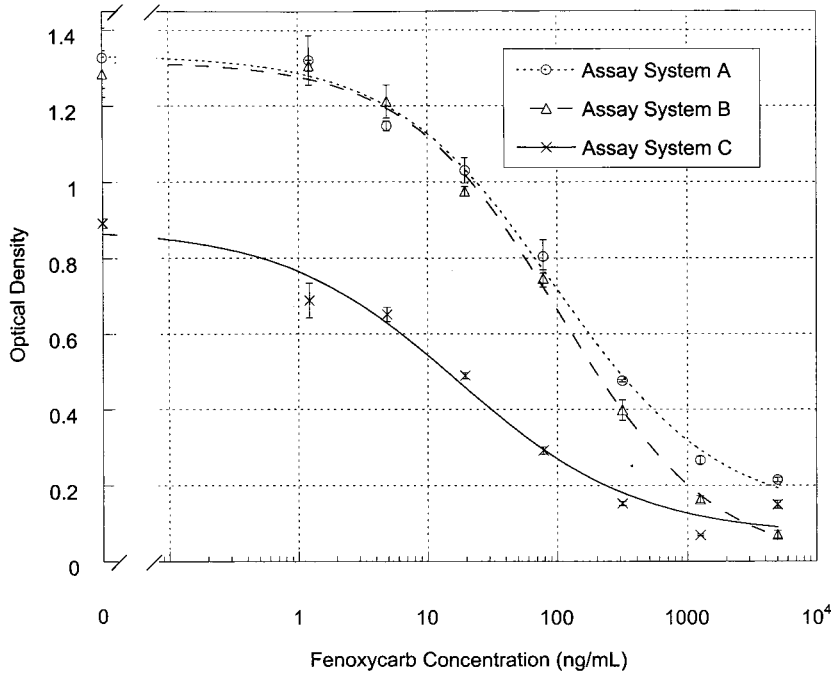


Figure 8. Fenoxycarb standard curves obtained by competitive ELISA systems used in this study. Assay systems were as described in Table 1. Microplate wells treated with a coating antigen were exposed to solutions containing an antiserum and various concentrations of fenoxycarb. See Materials and Methods for further details. Each point represents the average ± SD of the OD values at 492 nm, which were from three replicate wells on the same microplate.

the pyrethroids fenvalerate and cypermethryn, the phenoxyacetic acid herbicide 2,4-D, *p,p′*-DDT, and the nitrodiphenyl ether herbicides acifluorfen and fluorodifen, also produced no significant cross-reactivity (Table 3). 4-Phenoxyphenol (Table 3), a likely metabolite of fenoxycarb in *Heliothis virescens* (*63*), showed no or only weak interference with the assays. The higher cross-reactivity of ELISA system A than those of systems B and C for 2-(4-phenoxyphenoxy)ethylamine (**6**, Figure 4, Table 3) can be interpreted in terms of the structures of the immunizing haptens. The spacer arm in hapten molecule **4** is linked to the ethyl group of fenoxycarb (Figure 4). The 4-phenoxyphenoxy group is the immunodominant part of the corresponding immunizing antigen (**20′′-KLH**, Figure 4) because of the shielding effect of the carrier protein around the spacer group attachment (*2*, *54*). Thus, the resulting antiserum displays the highest affinity toward the aromatic moiety of fenoxycarb (system A, Table 3). The opposite tendency is expected for the antiserum based on hapten **2** considering that the linker azo

group is attached to the terminal phenyl ring of fenoxycarb in the immunogen structure (**14-KLH**, Figure 2, systems B and C in Table 3). Taken together, the cross-reactivity results (Table 3) demonstrate that our ELISAs, based on immunogen haptens possessing the entire target analyte structure (Figures 1, 2, and 4), are highly selective for fenoxycarb. It appears that an epitope encompassing at least the 2-(4-phenoxyphenoxy)ethylamine (**6**) molecule is required for potent inhibition of ELISAs A and C. ELISA B displays a high degree of specificity for fenoxycarb because the whole target analyte structure is necessary for strong inhibition.

Conclusions. The sensitivity and selectivity of our heterologous assay are similar to those of the ELISA reported by Giraudi et al. (*28*). The Italian group's hapten-homologous assay is based on the hapten 2-(4-phenoxyphenoxy)ethanol hemisuccinate, includes only a part of the fenoxycarb structure (**1**), and purified polyclonal antibodies (*28*). Their optimized ELISA is characterized with an IC_{50} of 9.5 ppb and an LDL of 0.42 ppb, and displays significant interference with pyriproxyfen (CR, 6.0%).

The assay system of Giraudi et al. (*28*) and our highly sensitive and selective ELISAs for fenoxycarb hold promise for rapid and inexpensive analysis of large numbers of samples for environmental and agricultural screening programs. These immunoassays may also provide a useful analytical tool for investigations on the biochemical mode of action and metabolism of this compound (**1**). The results presented here, our previous experience, and literature reports suggest that it is usually rewarding to explore several haptens with different spacer arm locations and sizes, various conjugation methods, and carrier proteins during ELISA development (*2*, *46*, *51*, *53*, *54*, *56*, *60*). Further studies with other assay systems and real samples are in progress.

ABBREVIATIONS USED

Borax, $Na_2B_4O_7 \cdot 10\ H_2O$; BSA, bovine serum albumin; CONA, conalbumin; CR, cross-reactivity; CTH, catalytic transfer hydrogenation; DCC, dicyclohexylcarbodiimide; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; EI, electron impact; ELISA, enzyme-linked immunosorbent assay; EPA, Environmental Protection Agency; GC, gas chromatography; HD, hapten density (hapten/protein ratio); HD10kDa, hapten/10 kDa protein ratio; IC_{50}, analyte concentration required for 50% inhibition; IR, infrared spectroscopy; KLH, keyhole limpet hemocyanin; LDL, lower detection limit; LPH, hemocyanin of *Limulus polyphemus*; MS, mass spectrometry; $MS-ES^+$, electrospray MS in positive mode; NHS, *N*-hydroxysuccinimide; NMR, nuclear magnetic resonance spectroscopy; OD, optical density; OVA, ovalbumin; PBS, phosphate-buffered saline (0.01 M phosphate buffer + 0.8% NaCl, pH 7.5); PBST, PBS + 0.05% Tween 20 (pH 7.5); ppb, parts per billion (ng/mL); SD, standard deviation; THF, tetrahydrofuran; TLC: thin-layer chromatography; TYG, porcine thyroglobulin; UV−Vis, ultraviolet−visible spectroscopy.

ACKNOWLEDGMENT

We are grateful to S. J. Gee (Department of Entomology, U. C. Davis) and L. Harshman (School of Biological Sciences, University of Nebraska, Lincoln, NE) for assistance with production of antisera, and to A. D. Jones (Department of Chemistry, Penn State University, University Park, PA), R. S. Mercer (Molecular Structure Facility, U. C. Davis), A. McClain (Department of Textile Engineering, Auburn University, Auburn, AL), and K. Bauer (CEQUA Kft., Budapest, Hungary) for assistance with spectral studies. We thank I. Morera and M. A. Miranda (Department of Chemistry, Polytechnic University of Valencia, Valencia, Spain) and D. Cerf (Novartis Crop Protection) for providing samples for cross-reactivity investigations. We also thank S. Dedos (Institute of Biology, N. C. S. R. "Demokritos", Athens, Greece), P. G. Schultz (Chemistry Department, Scripps Research Institute, La Jolla, CA), and I. Ujváry (Institute for Chemistry, Chemical Research Center, Budapest, Hungary) for helpful discussions.

LITERATURE CITED

(1) Székács, A.; Hammock B. D. Development of an enzyme-linked immunosorbent assay for the detection of the triazole fungicide myclobutanil. *J. Agric. Food Chem.* **1995**, *43*, 2083−2091.

(2) Szurdoki, F.; Bekheit, H. K. M.; Marco, M.-P.; Goodrow, M. H.; Hammock, B. D. Important factors in hapten design and enzyme-linked immunosorbent assay development. In *New Frontiers of Agrochemical Immunoassay*; Kurtz, D. A., Skerritt, J. H., Stanker, L., Eds.; AOAC International: Arlington, VA, 1995; pp 39−63.

(3) Szurdoki, F.; Jaeger, L.; Harris, A.; Kido, H.; Wengatz, I.; Goodrow, M. H.; Székács, A.; Wortberg, M.; Zheng, J.; Stoutamire, D. W.; Sanborn, J. R.; Gilman, S. D.; Jones, A. D.; Gee, S. J.; Choudary, P. V.; Hammock, B. D. Rapid assays for environmental and biological monitoring. *J. Environ. Sci. Health B* **1996**, *31*, 451−458.

(4) Sherry, J. Environmental immunoassays and other bioanalytical methods: overview and update. *Chemosphere* **1997**, *34*, 1011−1025.

(5) Le, H. M.; Székács, A.; Tőkés, G.; Ferguson, B. S. Detection of atrazine in Hungary by immunoanalytical (ELISA) method. *J. Environ. Sci. Health B* **1996**, *31*, 459−464.

(6) Dorn, S.; Frischknecht, M. L.; Martinez, V.; Zurflüh, R.; Fischer, U. A novel non-neurotoxic insecticide with a broad activity spectrum. *Z. Pflanzenkrankh. Pflanzenschutz* **1981**, *88*, 269−275.

(7) Okot-Kotber, B. M.; Ujváry, I.; Mollaaghababa, R.; Szurdoki, F.; Matolcsy, G.; Prestwich, G. D. Physiological influence of fenoxycarb pro-insecticides and soldier head extracts of various termite species on soldier differentiation in *Reticulitermes flavipes* (Isoptera). *Sociobiology* **1991**, *19*, 77−90.

(8) Grenier, S.; Grenier, A.-M. Fenoxycarb, a fairly new insect growth regulator: a review of its effects on insects. *Ann. Appl. Biol.* **1993**, *122*, 369−403.

(9) Schneider, M.; Wiesel, G.; Dorn, A. Effects of JH III and JH analogues on phase-related growth, egg maturation and lipid metabolism in *Schistocerca gregaria* females. *J. Insect Physiol.* **1995**, *41*, 23−31.

(10) Ujváry, I.; Matolcsy, G.; Bélai, I.; Szurdoki, F.; Bauer, K.; Varjas, L.; Kramer, K. J. Projuvenoids: synthesis and biological evaluation of sulfenylated, sulfinylated, and sulfonylated carbamates. *Arch. Insect Biochem. Physiol.* **1996**, *32*, 659−669.

(11) Dedos, S. G.; Fugo, H. Effects of fenoxycarb on the secretory activity of the prothoracic glands in the fifth instar of the silkworm, *Bombyx mori*. *Gen. Compar. Endocrinol.* **1996**, *104*, 213−224.

(12) Dhadialla, T. S.; Carlson, G. R.; Le, D. P. New insecticides with ecdysteroidal and juvenile hormone activity. *Annu. Rev. Entomol.* **1998**, *43*, 545−569.

(13) Dedos, S. G.; Fugo, H. Downregulation of the cAMP signal transduction cascade in the prothoracic glands is responsible for the fenoxycarb-mediated induction of permanent 5th instar larvae in *Bombyx mori*. *Insect Biochem. Molecular Biol.* **1999**, *29*, 723−729.

(14) Monconduit, H.; Mauchamp, B. Fenoxycarb, a potent contaminant of the silkworm, *Bombyx mori* L., does not influence its juvenile hormone titer. *Arch. Insect Biochem. Physiol.* **1999**, *40*, 141−149.

(15) Edwards, J. P.; Short, J. E.; Abraham, L. Large-scale evaluation of the insect juvenile hormone analogue fenoxycarb as a long-term protectant of stored wheat. *J. Stored Prod. Res.* **1991**, *27*, 31−39.

(16) Tomlin, C., Ed. *The Pesticide Manual*: *Incorporating The Agrochemicals Handbook*, 10th ed; British Crop Protection Council: Farnham, Surrey, UK, 1994; pp 442−443.

(17) Evans, R. G.; Sunley, A.; Bradford, C.; Patmore, R. I. Effects of fenoxycarb on development and reproduction of the Oriental cockroach, *Blatta orientalis*. *Med. Vet. Entomol.* **1995**, *9*, 235−240.

(18) U.S. EPA. *Pesticide Fact Handbook*; Noyes Data Corporation: Park Ridge, NJ, 1988; Vol. 1, pp 373−377.

(19) Key, P. B.; Scott, G. I. The chronic toxicity of fenoxycarb to larvae of the grass shrimp, *Palaemonetes pugio*. *J. Environ. Sci. Health B* **1994**, *29*, 873−894.

(20) Barr, A. C.; Abbitt, B.; Fiske, R. A.; Jaques, J. T.; Maynard, H. R.; Reagor, J. C. Reproductive effects of fenoxycarb on sheep. *J. Vet. Diagn. Invest.* **1997**, *9*, 401−406.

(21) Solomon, M. G.; Fitzgerald, J. D. Fenoxycarb, a selective insecticide for inclusion in integrated pest management systems for pear in the UK. *J. Hortic. Sci.* **1990**, *65*, 535−539.

(22) Darvas, B.; Abd El-Kareim, I. A.; Camporese, P.; Farag, A. I.; Matolcsy, G.; Ujváry, I. Effects of some new proinsecticide type fenoxycarb derivatives and related compounds on some scale insects and their hymenopterous parasitoids. *J. Appl. Entomol.* **1994**, *118*, 51−58.

(23) Hoddle, M. S.; Van Driesche, R. G.; Lyon, S. M.; Sanderson, J. P. Compatibility of insect growth regulators with *Eretmocerus eremicus* (Hymenoptera: Aphelinidae) for whitefly (Homoptera: Aleyrodidae) control on poinsettias: I. Laboratory assays. *Biol. Control* **2001**, *20*, 122−131.

(24) Lee, B. M.; Scott, G. I. Acute toxicity of temephos, fenoxycarb, diflubenzuron, and methoprene and *Bacillus thuringiensis* var. *israelensis* to the mummichog (*Fundulus heteroclitus*). *Bull. Environ. Contam. Toxicol.* **1989**, *43*, 827−832.

(25) Hosmer, A. J.; Warren, L. W.; Ward, T. J. Chronic toxicity of pulse-dosed fenoxycarb to *Daphnia magna* exposed to environmentally realistic concentrations. *Environ. Toxicol. Chem.* **1998**, *17*, 1860−1866.

(26) Bortolotti, L.; Porrini, C.; Sbrenna, A. M.; Sbrenna, G. Ovicidal action of fenoxycarb on a predator, *Chrysoperla carnea* (Neuroptera: Chrysopidae). *Appl. Entomol. Zool.* **2000**, *35*, 265−270.

(27) Kamimura, M.; Kiuchi, M. Effects of a juvenile hormone analogue, fenoxycarb, on 5th stadium larvae of the silkworm. *Bombyx mori* (Lepidoptera: Bombycidae). *Appl. Entomol. Zool.* **1998**, *33*, 333−338.

(28) Giraudi, G.; Giovannoli, C.; Baggiani, C.; Rosso, I.; Coletto, P.; Dolci, M.; Grassi, G.; Vanni, A. Enzyme immunoassay for the determination of the insecticide fenoxycarb. *Anal. Commun.* **1998**, *35*, 183−185.

(29) Haenni, R. P.; Mueller, P. A. Fenoxycarb. In *Analytical Methods for Pesticides and Plant Growth Regulators*; Sherma, J., Ed.; Academic Press: San Diego, CA, 1988; Vol. XVI, pp 21−29.

(30) Bicchi, C.; D'Amato, A.; Tonutti, I.; Cantamessa, L. Simultaneous determination of clofentezine, fenoxycarb and hexythiazox by HPLC on apples, pears and their pulps. *Pestic. Sci.* **1990**, *30*, 13−19.

(31) Natangelo, M.; Tavazzi, S.; Fanelli, R.; Benfenati, E. Analysis of some pesticides in water samples using solid-phase microextraction-gas chromatography with different mass spectrometric techniques. *J. Chromatogr. A* **1999**, *859*, 193−201.

(32) Wang, Z.; Hennion, B.; Urruty, L.; Montury, M. Solid-phase microextraction coupled with high performance liquid chromatography: a complementary technique to solid-phase microextraction-gas chromatography for the analysis of pesticide residues in strawberries. *Food Addit. Contam.* **2000**, *17*, 913−923.

(33) Székács, A.; Szurdoki, F.; Hammock, B. D. Immunoassay development for pesticides and toxins. *PACIFICHEM '95. International Chemical Congress of Pacific Basin Societies* (Honolulu, HI, December 17−22, 1995), *Book of Abstracts*; American Chemical Society: Washington, DC, 1995; AGRO 7.

(34) Fischer, U.; Schneider, F.; Zurflueh, R. Carbamic acid derivatives. U.S. Patent 4,215,139, 1980.

(35) Klamann, D.; Behnisch, R. Organische Stickstoff-Verbindungen IV, Teil I. Aromatische Nitro-Verbindungen. In *Methoden der organischen Chemie (Houben-Weyl), Vol. E16d*; Büchel, K. H. et al., Eds.; G. Thieme: Stuttgart/ New York, 1992; pp 327, 328.

(36) Ikawa, K. Syntheses of acid hydrazide derivatives containing a diphenyl ether nucleus. XIV. Cleavage of diphenyl ether derivatives by hydrazine hydrate. *Yakugaku Zasshi* **1959**, *79*, 1498−1502; *Chem. Abstr.* **1960**, *54*, 10923e.

(37) Anwer, M. K.; Sherman, D. B.; Roney, J. G.; Spatola, A. F. Applications of ammonium formate catalytic transfer hydrogenation. 6. Analysis of catalyst, donor quantity, and solvent effects upon the efficacy of dechlorination. *J. Org. Chem.* **1989**, *54*, 1284−1289.

(38) Galve, R.; Camps, F.; Sanchez-Baeza, F.; Marco, M.-P. Development of an immunochemical technique for the analysis of trichlorophenols using theoretical models. *Anal. Chem.* **2000**, *72*, 2237−2246.

(39) Rarick, M. J.; Brewster, R. Q.; Dains, F. B. The formation of aromatic ethers from *p*-nitrofluorobenzene. *J. Am. Chem. Soc.* **1933**, *55*, 1289−1290.

(40) March, J. *Advanced Organic Chemistry: Reactions, Mechanism, and Structure*, 4th ed.; Wiley: New York, 1992; pp 641−676.

(41) Wang, L.; Xi, H.; Sun, X.; Shen, Y.; Yang, Y.; Pan, Y.; Hu, H. Synthesis of functionalized *p*-phenylene oxide oligomers. *Synth. Commun.* **2000**, *30*, 227−234.

(42) Clark, J. H.; Holland, H. L.; Miller, J. M. Hydrogen bonding in organic synthesis, 4: A simple, high-yield method for the methylenation of catechols. *Tetrahedron Lett.* **1976**, *17*, 3361−3364.

(43) Huber, I.; Fülöp, F.; Kóbor, J.; Bernáth, G. Cyclic amino alcohols and related compounds, 36: Simple methods for the preparation of *N*-alkyltetrahydroisoquinoline-1,3-amino alcohols. *Acta Chim. Hung.* **1991**, *128*, 375−379.

(44) Kozikowski, A. P.; Ding, Q.; Spiegel, S. Synthesis of *erythro*-ω-aminosphingosine and preparation of an affinity column for sphingosine kinase purification. *Tetrahedron Lett.* **1996**, *37*, 3279−3282.

(45) Ram, S.; Buchsbaum, D. J. Synthesis of a new class of isothiocyanatopeptide bifunctional chelating agents for coupling to monoclonal antibodies. *Int. J. Peptide Protein Res.* **1996**, *48*, 79−86.

(46) Wengatz, I.; Stoutamire, D. W.; Gee, S. J.; Hammock, B. D. Development of an enzyme-linked immunosorbent assay for the detection of the pyrethroid insecticide fenpropathrin. *J. Agric. Food Chem.* **1998**, *46*, 2211−2221.

(47) Székács, A.; Le, H. M.; Knopp, D.; Niessner, R. A modified enzyme-linked immunosorbent assay (ELISA) for polyaromatic hydrocarbons. *Anal. Chim. Acta* **1999**, *399*, 127−134.

(48) Szurdoki, F.; Ren, D.; Walt, D. R. A combinatorial approach to discover new chelators for optical metal ion sensing. *Anal. Chem.* **2000**, *72*, 5250−5257.

(49) Hermanson, G. T. *Bioconjugate Techniques*; Academic Press: San Diego, CA, 1996.

(50) Olah, G. A.; Lin, H. C.; Olah, J. A.; Narang, S. C. Electrophilic and free radical nitration of benzene and toluene with various nitrating agents. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 1045−1049.

(51) Sanborn, J. R.; Gee, S. J.; Gilman, S. D.; Sugawara, Y.; Jones, A. D.; Rogers, J.; Szurdoki, F.; Stanker, L. H.; Stoutamire, D. W.; Hammock, B. D. Hapten synthesis and antibody development for polychlorinated dibenzo-*p*-dioxin immunoassays. *J. Agric. Food Chem.* **1998**, *46*, 2407−2416.

(52) De Marre, A.; Soyez, H.; Permentier, D.; Schacht, E. 4-Nitrophenyl chloroformate activation of poly-α,*β*-[*N*-(2-hydroxyethyl)-D,L-aspartamide] and poly-α,*β*-[*N*-(2,3-dihydroxypropyl)-D,L-aspartamide]. *Polymer* **1995**, *36*, 4031−4037.
(53) Szurdoki, F.; Bekheit, H. K. M.; Marco, M.-P.; Goodrow, M. H.; Hammock, B. D. Synthesis of haptens and conjugates for an enzyme immunoassay for analysis of the herbicide bromacil. *J. Agric. Food Chem.* **1992**, *40*, 1459−1465.
(54) Li, K.; Li, Q. X. Development of an enzyme-linked immunosorbent assay for the insecticide imidacloprid. *J. Agric. Food Chem.* **2000**, *48*, 3378−3382.
(55) Wong, S. S. *Chemistry of Protein Conjugation and Cross-Linking*; CRC Press: Boca Raton, FL, 1991.
(56) Danilova, N. P. ELISA screening of monoclonal antibodies to haptens: influence of the chemical structure of hapten-protein conjugates. *J. Immunol. Methods* **1994**, *173*, 111−117.
(57) Flanagan, M. E.; Jacobsen, J. R.; Sweet, E.; Schultz, P. G. Antibody-catalyzed retro-aldol reaction. *J. Am. Chem. Soc.* **1996**, *118*, 6078−6079.
(58) Schultz, P. G. Personal communication, 2001.
(59) Traylor, P. S.; Singer, S. J. The preparation and properties of some tritiated diazonium salts and related compounds. *Biochemistry* **1967**, *6*, 881−887.
(60) Manclús, J. J.; Primo, J.; Montoya, A. Development of enzyme-linked immunosorbent assays for the insecticide chlorpyrifos. 1. Monoclonal antibody production and immunoassay design. *J. Agric. Food Chem.* **1996**, *44*, 4052−4062.
(61) Climent, M. J.; Miranda, M. A. Gas chromatographic-mass spectrometric study of photodegradation of carbamate pesticides. *J. Chromatogr. A* **1996**, *738*, 225−231.
(62) Climent, M. J.; Miranda, M. A. Erratum to "Gas chromatographic-mass spectrometric study of photodegradation of carbamate pesticides" [J. Chromatogr. A, 738 (1996) 225−231]. *J. Chromatogr. A* **1997**, *761*, 341.
(63) Mauchamp, B.; Malosse, C.; Saroglia, P. Biological effects and metabolism of fenoxycarb after treatment of the fourth and the fifth instars of the tobacco budworm, *Heliothis virescens* F. *Pestic. Sci.* **1989**, *26*, 283−301.

Received for review May 31, 2001. Revised manuscript received October 10, 2001. Accepted October 11, 2001. Partial financial support was provided by the NIEHS Superfund Basic Research Program P42 ES04699 and the NIEHS Center P30 ES05707. This work was also supported by a collaborative grant of the European Union (EC INCO-COPERNICUS ERBIC15CT960802), the US−Hungarian Joint Fund (J.F. No. 051/90), and Hungarian research funds (OTKA Grant T03232, OM 02193/1999). A.S. is a Bolyai Scholar (1998-2001, Hungarian Academy of Sciences).

JF0107050