Synthesis of Haptens and Derivation of Monoclonal Antibodies for Immunoassay of the Phenylurea Herbicide Diuron†

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Diuron and related phenylurea herbicides and their metabolites are important candidates for sensitive and specific immunodetection. This paper describes a scheme for the synthesis of two different types of phenylurea haptens for immunization and use as detecting conjugates in enzyme immunoassays (EIAs). The haptens were used to develop indirect and direct EIAs and to derive a panel of monoclonal antibodies (MAbs) with different specificities for diuron and its analogs. One of six possible combinations of hapten–spacer arm conjugates tested as immunizing and screening antigens resulted in an indirect competition EIA that was 100–2000-fold more sensitive than the others. The eight most sensitive MAbs had I_{50} values of 2–20 ppb for diuron. These MAbs gave two different patterns of cross-reactivities with monuron and linuron and negligible recognition of other arylurea herbicides. These MAbs and EIAs are potentially suitable for identification as well as detection of diuron, monuron, and linuron.

INTRODUCTION

Diuron [3-(3,4-dichlorophenyl)-1,1-dimethylurea] is one of a family of arylurea herbicides. The first of these—monuron, fenuron, diuron, neburon—were developed in the early 1950s and have been in common use in agriculture since the 1960s. Subsequently, numerous related compounds have been tested for herbicidal activity. Their properties and uses are summarized in review articles by Maier-Bode and Hartel (1981) and Smith and Grover (1982).

Diuron and other arylurea herbicides are used extensively throughout the United States and in many other countries. Higher concentrations are applied as soil sterilants on noncrop lands, industrial sites, and rights-of-way, and smaller amounts are used for selective pre- or post-emergence control of grasses and broadleaf weeds among vegetables, potatoes, cotton, corn, beans, cereals, and ornamentals and in orchards and vineyards. They have also been used to control aquatic plants. Runoff and leaching from agricultural use may contaminate groundwater and surface water. In 1990, 40 of 782 wells sampled in California were contaminated with 0.06–3.95 ppb of diuron from nearby legal agricultural application (Miller et al., 1990).

The arylureas have low to moderate mammalian toxicity (Smith and Grover, 1982; Ware, 1983), but parts per million (ppm) amounts of phenylureas may affect embryonic and neonatal development of some fish and aquatic invertebrates (Call et al., 1987). Little is known about diuron toxicity in humans, but the Environmental Protection Agency's Lifetime Health Advisory Level (HAL) for diuron has been set at 10 ppb (Miller et al., 1990; Van Boven et al., 1990). This necessitates monitoring for residues in commodity and environmental matrices. The legal tolerance for diuron residues in meat and most commodities is 1–2 ppm but is as low as 100 ppb in commodities such as bananas, peaches, and nuts (U.S. Department of Agriculture, 1987; Duggan, 1988).

Standard analytical methods for detecting diuron include spectrophotometry, gas chromatography/mass spectrometry (GC/MS), and high-performance liquid chromatography (HPLC). The HPLC detection limit of 0.1–0.5 ppb of diuron in water (Smith and Grover, 1982; Goewie and Hogendoorn, 1987) is not sufficiently sensitive for the requirements of some regulatory agencies. Diuron and other substituted ureas are difficult to analyze by routine GC/MS because they are thermally unstable and decompose, although newer instrumental techniques may reduce or eliminate this problem (Mattern et al., 1989; Tamiri and Zitrin, 1987). Breakdown products (3,4-dichlorophenyl isocyanate) or thermostable derivatives are often measured rather than the parent compound itself (Van Boven et al., 1990; Goewie and Hogendoorn, 1985; Zahnow, 1987).

A rapid and reliable immunoassay for diuron and its analogs would improve the ability of regulatory agencies to monitor use and disposal. Quantitative enzyme immunoassays (EIAs) are an ideal primary screening method for three reasons: first, EIAs overcome technical difficulties encountered with decomposition of the analytes in

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GC/MS; second, many more controls, replicates, and dilutions can be run in each EIA at about $^1/_{10}$ the cost per sample compared with HPLC or GC/MS.

To develop a monoclonal immunoassay for diuron, we first undertook synthesis of haptens by a scheme that would be applicable to virtually all of the phenylurea herbicides, that would present the hapten in two different ways, and that would accommodate different lengths and structures of spacer arms. We tested the different presentations to identify the best combination of immunizing and screening antigens, and from these we derived a panel of specific monoclonal antibodies (MAbs) and indirect and direct competition EIAs. The EIAs are accurate, cost-effective, robust, and compatible with established procedures for recovery of triazines, bromacil, and other leachable herbicides from groundwater. The MAbs show differences in the recognition of monouron that may prove to be useful in identifying which phenylurea is present in a sample. They should also be useful in immunosensors, affinity columns, and other formats for environmental monitoring and dosimetry.

MATERIALS AND METHODS

Chemicals. All organic starting materials for the hapten syntheses were obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI). Thin-layer chromatography (TLC) utilized 0.2 mm precoated silica gel 60 F254 on plastic sheets from E. Merck (Darmstadt, Germany). Plates were eluted with solvent system A [tetrahydrofuran–ethyl acetate–hexane (2:13:35 v/v/v)] and/or other solvent systems indicated in each experiment. Compounds were detected first by viewing under UV light (254 nm) and then by exposure to iodine vapor. Flash chromatography was carried out on 40 mm average particle diameter Baker silica gel for flash chromatography (J. T. Baker, Inc., Phillipsburg, NJ).

Arylurea and carbamate herbicide standards >99% pure were purchased from AccuStandard, Inc. (New Haven, CT). Reference solutions of 1 mg/mL were prepared in methanol, standardized by UV spectrophotometry, and stored at 4 °C in Teflon vials (Pierce Chemical Co.). Dilutions were prepared in PBS–Tween containing 10% methanol (0.01 M KH_2PO_4–K_2HPO_4, pH 7.4–0.15 M NaCl–0.05% Tween 20–10% methanol). Unless otherwise specified, the EIAs were performed in Immulon 2 plates (Dynatech, Chantilly, VA). Enzyme–antibody conjugates were obtained from Sigma Chemical Co. (St. Louis, MO) or Boehringer-Mannheim (Indianapolis, IN). Solvents used in this work were spectrograde, and all other chemicals were analytical reagent grade or better. Cell culture media and additives were purchased from GIBCO Laboratories (New York), and fetal bovine serum was from Intergen, Inc. (Kankakee, IL). Swiss Webster mice were purchased from Simonsen Laboratories (Gilroy, CA), and Biozzi and B10.Q mice were from stock bred in the University of California (Berkeley) Hybridoma Facility mouse colony.

Apparatus for Hapten Synthesis. Melting points were determined with a Thomas Hoover apparatus and are uncorrected. Infrared spectra (IR) were determined on an IBM IR/32 FTIR spectrometer (IBM Corp., Danbury, CT). Most ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra, at 300.1 and 75.5 MHz, respectively, were measured on a General Electric QE-300 spectrometer (Bruker NMR, Billerica, MA); where indicated, a Varian EM-390 90-MHz spectrometer (Varian Associates, Palo Alto, CA) was used. Chemical shift values are given in ppm downfield from internal tetramethylsilane except where noted that 3-(trimethylsilyl)propanoic-2,2,3,3-d_4 acid, sodium salt (TSP), was the standard. Fast atom bombardment high-resolution (FAB-HRMS) and electron impact high-resolution (EI-HRMS) at 70 eV mass spectra were obtained on a ZAB-HS-2F mass spectrometer (VG Analytical, Wythenshawe, U.K.). FAB experiments utilized xenon (8 keV, 1 mA) for ionization and 3-nitrobenzyl alcohol as the matrix; polyethylene glycol 300 was added to the matrix as a mass calibrant. Low-resolution electron impact mass spectra (MS) were obtained on a Trio 2 mass spectrometer (VG Masslab, Altrincham, U.K.) or the ZAB-HS-2F instrument at 70 eV.

Hapten Synthesis. Haptens of diuron with spacer arms of three or five carbons were prepared for conjugation to carriers, enzymes, or other ligands for use as immunizing and screening agents (Figure 1). Idealized gas-phase molecular models of diuron and the haptens were constructed in Chem3D Plus on a Macintosh IIci computer. The conformations were minimized for structural error and free energy using the Chem3D Plus implementation of Allinger's MM2 force field (Burkert and Allinger, 1982; Cambridge Scientific Computing, 1990).

Ethyl 4-[(3,4-Dichlorophenyl)amino]butanoate. Following the procedure of Wie et al. (1982), a solution of 3.24 g (20.0 mmol) of 3,4-dichloroaniline, 3.90 g (20.0 mmol) of ethyl 4-bromobutanoate, and 2.72 g (20.0 mmol) of sodium acetate trihydrate in 5 mL of ethanol was heated under reflux for 13 h. The cooled mixture was poured into 150 mL of ice/water and extracted with ether (3 × 50 mL). The combined ether extract was dried (Na_2SO_4) and concentrated to provide 5.35 g of a dark brown oil. This six-component (TLC) mixture was flash chromatographed on 125 g of silica gel by elution with 7:1 (v/v) hexane–ethyl acetate. The 1.24 g of crude product was recrystallized from 95% ethanol to provide 0.92 g (17%) of pure ester as white crystals: mp 71.0–72.0 °C; TLC R_f 0.62 (eluant solvent A); IR (KBr) 3394 (s, NH), 3364 (s, NH), 1713 (s, C=O, ester), 1191 (vs, CO), 1106 (m, CO) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 7.23 (d, J = 8.8 Hz, 1 H, Ar-H_5), 6.72 (d, J = 2.7 Hz, 1 H, Ar-H_2), 6.54 (dd, J = 2.7, 8.8 Hz, 1 H, Ar-H_6), 6.16 (t, J = 5.5 Hz, 1 H, NH), 4.06 (q, J = 7.1 Hz, 2 H, CH_2O), 3.01 (dt, J = 5.5, 7.0 Hz, 2 H, CH_2N), 2.39 (t, J = 7.4 Hz, 2 H CH_2CO_2), 1.76 (quin, J = 7.2 Hz, 2 H, CCH_2C), 1.18 (t, J = 7.1 Hz, 3 H, CH_3) (the 6.16 ppm peaks disappeared and the 3.01 ppm peaks became a single triplet with added D_2O); ^{13}C NMR (DMSO-d_6) δ 172.7 (ester C=O), 149.0 (Ar-C_1), 131.4 (Ar-C_3), 130.5 (Ar-C_5), 116.1 (Ar-C_4), 112.5 (Ar-C_2), 112.5 (Ar-C_2), 112.4 (Ar-C_6), 59.8 (CH_2O), 41.9 (CH_2N, C_4), 31.1 (CH_2CO_2, C_2), 23.9 (C_3), 14.2 (CH_3).

1-(3,4-Dichlorophenyl)-1-[3-(ethylcarboxy)propyl]-3,3-dimethylurea. A solution of 0.83 g (3.0 mmol) of the above ester, 3.0 mL of pyridine, and 4.0 mL (4.7 g, 43 mmol) of dimethylcarbamyl chloride in 10 mL of acetonitrile was stirred at room temperature in the dark for 10 days. Over this time the pale yellow solution became dark black-brown in color, and TLC suggested about 50% reaction had occurred. This mixture was poured into 100 mL of ice/water and extracted with chloroform (3 × 50 mL). The combined extract was washed sequentially with 1 M hydrochloric acid (2 × 25 mL), 0.1 M sodium hydroxide (2 × 25 mL), and saturated sodium chloride (25 mL) and dried (Na_2SO_4). Concentration of this solution provided 1.16 g of a brown oil showing two components by TLC, R_f 0.68 and 0.27, (eluant solvent A). Flash chromatography of this oil on 30 g of silica gel eluting with 2:1 (v/v) hexane–ethyl acetate provided 0.68 g (65%) of the urea ester as a viscous pale yellow oil: TLC R_f 0.24 (eluant solvent A); IR (neat) 1733 (s, C=O, ester), 1661 (vs, C=O, amide), 1168 (s, CO) cm^{-1}; ^{1}H NMR (DMSO-d_6) (Varian EM-390) δ 7.38 (d, J = 9 Hz, 1 H, Ar-H_5), 7.15 (d, J = 3 Hz, 1 H, Ar-H_2), 6.88 (dd, J = 3, 9 Hz, 1 H, Ar-H_6), 4.11 (q, J = 7 Hz, 2 H, CH_2O), 3.61 (t, J = 7 Hz, 2 H, CH_2N), 2.72 (s, 6 H, 2 CH_3), 2.34 (t, J = 7 Hz, 2 H, CH_2CO_2), 1.91 (quin, J = 7 Hz, 2 H, CCH_2C).

1-(3-Carboxypropyl)-1-(3,4-dichlorophenyl)-3,3-dimethylurea (Diuron I). The urea ester, 0.51 g (1.5 mmol), and 10 mL of 2 M hydrochloric acid were heated under reflux for 2 h. On slow cooling the crude acid crystallized as a white solid, 0.42 g (89%). Recrystallization from chloroform–hexane (1:2 v/v) provided pure diuron I (0.37 g, 77%): mp 115.0–116.0 °C; IR (KBr) 1728 (s, C=O, acid), 1616 (s, C=O, amide), 1203 (m, CO) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 12.1 (br, 1 H, OH), 7.55 (d, J = 8.8 Hz, 1 H, Ar-H_5), 7.33 (d, J = 2.6 Hz, 1 H, Ar-H_2), 7.00 (dd, J = 2.6, 8.8 Hz, 1 H, Ar-H_6), 3.57 (t, J = 7.3 Hz, 2 H, CH_2N), 2.66 (s, 6 H, 2 CH_3), 2.26 (t, J = 7.3 Hz, 2 H, CH_2CO_2), 1.71 (quin, J = 7.3 Hz, 2 H, CCH_2C), (the 12.1 ppm peak disappeared with added D_2O); ^{13}C NMR (DMSO-d_6) δ 174.3 (acid C=O), 159.6 (amide C=O), 145.4 (Ar-C_1), 131.8 (Ar-C_3), 131.1 (Ar-C_5), 125.1 (Ar-C_4), 123.8 (Ar-C_2), 122.6 (Ar-C_6), 49.8 (CH_2N, C_4), 37.5 (CH_3), 31.1 (CH_2CO_2, C_2), 23.5 (C_3); MS m/z (relative intensity) 320 (2, M^+ + 2), 318 (3, M^+), 176 (4, $Cl_2C_6H_3NHCH_2$ + 2), 174 (8, $Cl_2C_6H_3$-$NHCH_2$), 72 [100, $(CH_3)_2NCO$]; EI-HRMS m/z calcd for $C_{13}H_{16}$-$Cl_2N_2O_3$ 318.0538, obsd 318.0509.

Ethyl 6-[(3,4-Dichlorophenyl)amino]hexanoate. A mixture of 3.24 g (20.0 mmol) of 3,4-dichloroaniline, 8.92 g (40.0 mol) of ethyl 6-bromohexanoate, 3.00 g (22.0 mmol) of sodium acetate trihydrate, and 5.0 mL of dimethyl sulfoxide was heated at 80 °C under a nitrogen atmosphere with vigorous stirring for 13 h. At this time a TLC indicated that most of the aniline had disappeared and at least five new products had formed. The mixture was poured into 100 mL of ice/water and extracted with chloroform (3 × 100 mL). The combined chloroform extract was washed sequentially with 5% sodium hydrogen carbonate solution (2 × 25 mL), 0.1 M hydrochloric acid (2 × 25 mL), and saturated sodium chloride (25 mL) and dried (Na_2SO_4). Evaporation of the solvent left 10.76 g of an amber oil consisting of six components as determined by TLC. Flash chromatography of 1.0 g of this oil on 30 g of silica gel with elution by hexane–ethyl acetate (7:1 v/v) provided 0.51 g of crude ester as a yellow semisolid. Recrystallization from 95% ethanol gave 0.19 g (33%) of pure ester as white crystals: mp 67.0–68.0 °C; IR (KBr) 3380 (vs, NH), 1714 (vs, C=O, ester), 1187 (s, CO) cm^{-1}; ^1H NMR (DMSO-d_6) δ 7.22 (d, J = 8.8 Hz, 1 H, Ar-H_5), 6.70 (d, J = 2.6 Hz, 1 H, Ar-H_2), 6.53 (dd, J = 2.6, 8.8 Hz, 1 H, Ar-H_6), 6.06 (t, J = 5.3 Hz, 1 H, NH), 4.05 (q, J = 7.1 Hz, 2 H, CH_2O), 2.96 (dt, J = 5.3, 6.8 Hz, 2 H, CH_2N), 2.29 (t, J = 7.2 Hz, 2 H, CH_2CO_2), 1.5 (m, 4 H, CH_2CCH_2), 1.3 (m, 2 H, CCH_2C), 1.17 (t, J = 7.1 Hz, 3 H, CH_3) (the 6.06 ppm peak disappeared and the 2.96 ppm peaks became a single triplet with added D_2O).

The bulk of the crude product was crystallized once from 95% ethanol to provide 1.94 g of slightly impure ester as pale yellow crystals. Although TLC showed traces of three other components, this material was found to be suitable for subsequent syntheses.

1-(3,4-Dichlorophenyl)-1-[5-(ethylcarboxy)pentyl]-3,3-dimethylurea. To 0.916 g (3.00 mmol) of the hexanoate ester and 3.0 mL of dry pyridine in 10 mL of dry acetonitrile under a nitrogen atmosphere and with vigorous stirring was added 4.0 mL (4.7 g, 43 mmol) of dimethylcarbamyl chloride over 5 min. An 8 °C exotherm resulted along with a darkening of the originally colorless solution. The mixture was placed aside in the dark for 7 months, after which time the urea ester and three other components were present as indicated by TLC. The dark brown mixture was poured into 150 mL of ice/water, and a viscous semisolid formed. The entire mixture was extracted with ether (4 × 50 mL); the combined extract was washed with 6 M hydrochloric acid (25 mL) followed by saturated sodium chloride solution (3 × 25 mL) and dried (Na_2SO_4). Concentration of the ether solution provided 1.20 g of a yellow-colored oil whose TLC displayed a major component at R_f 0.26 (eluant solvent A) and traces of four other components. Flash chromatography on 30 g of silica gel with elution by hexane–ethyl acetate (3:2 v/v) provided fractions showing only one component on TLC and totaling 1.06 g (94%) of the urea ester as a colorless oil: TLC R_f 0.28 (eluant solvent A); IR (neat) 1734 (s, C=O, ester), 1661 (vs, C=O, amide), 1182 (m, CO) cm^{-1}; ^1H NMR (DMSO-d_6) δ 7.55 (d, J = 8.8 Hz, 1 H, Ar-H_5), 7.29 (d, J = 2.6 Hz, 1 H, Ar-H_2), 6.99 (dd, J = 2.6, 8.8 Hz, 1 H, Ar-H_6), 4.03 (q, J = 7.1 Hz, 2 H, CH_2O), 3.52 (t, J = 7.4 Hz, 2 H, CH_2N), 2.64 (s, 6 H, 2 CH_3), 2.25 (t, J = 7.3 Hz, 2 H, CH_2CO_2), 1.5 (m, 4 H, CH_2CCH_2), 1.2 (m, 2 H, CCH_2C), 1.17 (t, J = 7.1 Hz, CH_3); ^{13}C NMR (DMSO-d_6) δ 172.7 (ester C=O), 159.6 (amide C=O), 145.4 (Ar-C_1), 131.7 (Ar-C_3), 131.0 (Ar-C_5), 125.0 (Ar-C_4), 123.7 (Ar-C_2), 122.5 (Ar-C_6), 59.6 (CH_2O), 50.2 (CH_2N, C_6), 37.4 (2 CH_3), 33.5 (CH_2CO_2, C_2), 27.6 (C_5), 25.8 (C_4), 24.2 (C_3), 14.1 (CH_3).

1-(5-Carboxypentyl)-1-(3,4-dichlorophenyl)-3,3-dimethylurea. A mixture of 0.93 g (2.5 mmol) of the urea ester and 30 mL of 2 M hydrochloric acid was heated under reflux for 2 h. On cooling and extensive trituration a white solid finally formed, 0.81 g (92%). Recrystallization of the solid from hexane–ethyl acetate (4:1 v/v) provided 0.61 g (71%) of glistening white needles of the urea acid: mp 53.0–55.0 °C; IR (KBr) 1730 (s, C=O, acid), 1659 (vs, C=O, amide), 1187 (m, CO) cm^{-1}; ^1H NMR (DMSO-d_6) δ 12.0 (br, 1 H, OH), 7.54 (d, J = 8.7 Hz, 1 H, Ar-H_5), 7.28 (d, J = 2.7 Hz, 1 H, Ar-H_2), 7.00 (dd, J = 2.7, 8.7 Hz, 1 H, Ar-H_6), 3.54 (t, J = 7.4 Hz, 2 H, CH_2N), 2.66 (s, 6 H, 2 CH_3), 2.20 (t, J = 7.2 Hz, 2H, CH_2CO_2), 1.5 (m, 4 H, CH_2CCH_2), 1.3 (m, 2 H, CCH_2C) (the 12.0 ppm peak disappeared with added D_2O); ^{13}C NMR (DMSO-d_6) δ 174.4 (acid C=O), 159.7 (amide C=O), 145.5 (Ar-C_1), 131.8 (Ar-C_3), 131.1 (Ar-C_5), 125.1 (Ar-C_4), 123.8 (Ar-C_2), 122.6 (Ar-C_6), 50.4 (CH_2N, C_6), 37.5 (2 CH_3), 33.7 (CH_2CO_2, C_2), 27.8 (C_5), 26.0 (C_4), 24.4 (C_3); MS m/z (relative intensity) 348 (M^+ + 2), 346 (21, M^+), 187 (5, $Cl_2C_6H_3$ NCO), 72 [100, $(CH_3)_2$-NCO]; FAB-HRMS m/z calcd for $C_{15}H_{21}Cl_2N_2O_3$ 347.0929, obsd 347.0929.

1-(3-Carboxypropyl)-3-(3,4-dichlorophenyl)-1-methylurea (Diuron II). The following experimental procedure represents the general method for the preparation of this homologous series. 4-(Methylamino)butyric acid hydrochloride, 0.922 g (6.00 mmol), was dissolved in 12.0 mL of 1.0 M sodium hydroxide solution, whereafter 1.13 g (6.01 mmol) of powdered 3,4-dichlorophenyl isocyanate was added. The heterogeneous mixture was agitated vigorously on a vortex mixer for 0.5 h and then allowed to stand overnight at room temperature. A small amount of precipitate was removed by filtration through Celite, and the filtrate was acidified with 6 M hydrochloric acid to pH 1. The resultant white precipitate was collected, washed thoroughly with water, and vacuum dried to obtain 1.73 g (95%) of crude diuron II. Recrystallization from acetonitrile provided 1.66 g (91%) of glistening white crystals: mp 161.5–162.5 °C (dec with gas evolution) [lit. (Newsome and Collins, 1990) mp 123–125 °C]; TLC R_f 0.05 (eluant solvent A), 0.42 [eluant ethanol–chloroform (5:95 v/v)]; IR (KBr) 3376 (m, NH), 1719 (s, C=O, acid), 1635 (vs, C=O, amide I), 1517 (s, C=O, amide II), 1213 (s, CO) cm^{-1}; ^1H NMR (DMSO-d_6) δ 12.2 (br, 1 H, OH), 8.51 (s, 1 H, NH), 7.87 (d, J = 2.1 Hz, 1 H, Ar-H_2), 7.5 (m, 2 H, Ar-$H_{5,6}$), 3.31 (t, J = 7.3 Hz, 2 H, CH_2N), 2.93 (s, 3 H, CH_3), 2.23 (t, J = 7.3 Hz, 2 H, CH_2CO_2), 1.73 (quin, J = 7.3 Hz, 2 H, CCH_2C) (the 12.2 and 8.51 ppm peaks disappeared with added D_2O); ^{13}C NMR (DMSO-d_6) δ 174.4 (acid C=O), 154.9 (amide C=O), 141.1 (Ar-C_1), 130.6 (Ar-C_3), 130.1 (Ar-C_5), 123.0 (Ar-C_4), 120.7 (Ar-C_2), 119.5 (Ar-C_6), 47.5 (CH_2N, C_4), 34.4 (CH_3), 30.9 (CH_2CO_2, C_2), 22.9 (C_3); MS m/z (relative intensity) 306 (22, M^+ + 2), 304 (29, M^+), 189 (70, $Cl_2C_6H_3NCO$ + 2), 187 (100, $Cl_2C_6H_3NCO$), 161 (48, $Cl_2C_6H_3NCO$ + 2 − CO), 159 (22, $Cl_2C_6H_3NCO$ − CO); FAB-HRMS calcd for $C_{12}H_{15}Cl_2N_2O_3$ 305.0461, obsd 305.0463.

6-(Methylamino)hexanoic Acid Dihydrate. This amino acid was prepared according to the procedure of Benson and Cairns (1948) by the sulfuric acid hydrolysis of *N*-methylcaprolactam in 60% crude yield. Slow recrystallization of the crude white solid from ethanol–ether (1:1 v/v) provided a 51% yield of colorless crystals which gave nonreproducible melting ranges, usually commencing at 68 °C. Benson and Cairns (1948) reviewed the discrepant multiple melting points in the literature and recorded their own value as 66–67 °C. Theirs was accompanied by superior analytical data and chemical evidence that supported the identity of this product. Additionally, the following ^1H and ^{13}C NMR data confirmed the assigned structure and verified its identity as the dihydrate: IR (KBr) 1561 (s, CO_2^-), 1402 (vs, CO_2^-) cm^{-1}; ^1H NMR (D_2O/TSP) δ 4.75 (s, 6 H, 6 HOD), 3.04 (t, J = 7.5 Hz, 2 H, CH_2N), 2.70 (s, 3 H, CH_3), 2.20 (t, J = 7.2 Hz, 2 H, CH_2CO_2), 1.7 (m, 2 H, CH_2-5), 1.6 (m, 2 H, CH_2-3) 1.4 (m, 2 H, CH_2-4); ^{13}C NMR [D_2O/DMSO-d_6 (2:1 v/v)] δ 184.1 (C=O, C_1), 50.4 (C_6), 38.9 (C_2), 34.2 (CH_3), 27.1 (C_5), 26.8 (C_3 or C_4), 26.6 (C_3 or C_4).

1-(5-Carboxypentyl)-3-(3,4-dichlorophenyl)-1-methylurea (Diuron III). This hapten was obtained in 95% crude yield, mp 116.0–117.5 °C (dec with gas evolution), from 1.09 g (6.00 mmol) of 6-(methylamino)hexanoic acid dihydrate, 6.0 mL (6.0 mmol) of 1.0 M sodium hydroxide, and 1.13 g (6.00 mmol) of 3,4-dichlorophenyl isocyanate. Recrystallization from acetonitrile provided 1.78 g (89%) of pure diuron III: mp 116.5–117.5 °C (dec with gas evolution); IR (KBr) 3362 (m, NH), 1712 (vs, C=O, acid), 1634 (vs, C=O, amide I), 1522 (s, C=O, amide II), 1227 (s, CO) cm^{-1}; ^1H NMR (DMSO-d_6) δ 12.0 (br, 1 H, OH), 8.79 (s, 1 H, NH), 7.86 (d, J = 2.0 Hz, 1 H, Ar-H_2), 7.5 (m, 2 H, Ar-$H_{5,6}$), 3.29 (t, J = 7.3 Hz, 2 H, CH_2N), 2.93 (s, 3 H, CH_3), 2.21 (t, J = 7.3 Hz, 2 H, CH_2CO_2), 1.5 (m, 4 H, CH_2CCH_2), 1.3 (m, 2 H, CCH_2C), (the 12.0 and 8.79 ppm peaks disappeared with added D_2O); ^{13}C NMR (DMSO-d_6) δ 174.4 (acid C=O), 154.9 (amide C=O), 141.2 (Ar-C_1), 130.6 (Ar-C_3), 130.0 (Ar-C_5), 122.9 (Ar-C_4), 120.7 (Ar-C_2), 119.5 (Ar-C_6), 48.0 (CH_2N, C_6), 34.4 (CH_3), 33.8 (CH_2CO_2, C_2), 27.1 (C_5), 25.9 (C_4), 24.4 (C_3); MS m/z (relative intensity) 334 (2, M^+ + 2), 332 (4, M^+), 189 (73, $Cl_2C_6H_3NCO$ + 2), 187 (100, $Cl_2C_6H_3NCO$), 161 (25, $Cl_2C_6H_3NCO$ + 2 − CO), 159

(30, $Cl_2C_6H_3NCO - CO$); EI-HRMS m/z calcd for $C_{14}H_{18}Cl_2N_2O_3$ 332.0694, obsd 332.0685.

Conjugation of Haptens to Proteins. The diuron I–III haptens were attached to the protein carriers bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH) using an *N*-hydroxysuccinimide activated ester. These conjugates were for use as immunizing antigens and screening antigens in an indirect competition EIA. Using the same chemistry, the haptens were also attached to calf intestine alkaline phosphatase for use as a detecting probe in a direct competition EIA. The protein conjugation chemistry is schematized in Figure 2 and may be summarized as follows:

Diuron–BSA and Diuron–KLH Conjugates. The reactions were conducted in 1.5-mL microfuge tubes containing Teflon stir bars. To 0.200 mmol of the phenylurea–acid hapten was added 1.0 mL of dimethylformamide (DMF, dried over molecular sieves) containing 0.202 mmol of *N*-hydroxysuccinimide and 0.223 mmol of 1,3-dicyclohexylcarbodiimide (DCC). The mixture was stirred at room temperature for 3.5 h and centrifuged to remove precipitated urea. The precipitate was discarded; the solution is referred to as the "active ester supernate". Portions of the activated esters were stored at −78 °C.

Fifty milligrams of BSA (fraction V, Sigma A-6773) or KLH (type VIII, Sigma H-1757) was dissolved in 5.0 mL of borate buffer (0.05 M sodium borate·$10H_2O$–0.9% NaCl–0.02% NaN_3, pH 7.8) in a 10-mL glass vial with a Teflon "flea" stir bar. This solution was allowed to stir vigorously, and 1.05 mL of dry DMF was added very slowly to bring the protein solution to 20% in DMF. No visible precipitation occurred during this addition. The resulting solution was stirred at room temperature and 0.25 mL of the active ester supernate was added, a few microliters at a time, very slowly over a 20-min period. A small amount of precipitate developed during this addition. The solution was stirred gently overnight at 4 °C and then dialyzed *vs.* 0.2 M phosphate-buffered saline (PBS), pH 7.5, in a SpectraPor membrane with pores of 12 000–14 000 MW cutoff (six changes of 4 L each). The dialyzed solution was transferred into polypropylene vials. The other BSA and KLH conjugates of the hapten active esters were prepared in essentially the same manner.

Diuron–Alkaline Phosphatase (AP) Conjugates. An activated ester was prepared similar to diuron I, but with a five-carbon (hexanoic acid) spacer (Figure 1, scheme A) instead of the three-carbon (butanoic acid) spacer. A solution of 10^4 DEA units of alkaline phosphatase [Sigma catalog no. P00405; type VII-NT from bovine intestinal mucosa (the solution as purchased contained 8.4 mg of protein/mL by biuret assay and 2690 units/mg of protein); one DEA unit hydrolyzes 1.0 μmol of *p*-nitrophenyl phosphate/min at 37 °C in diethanolamine (DEA) buffer, pH 9.8] was prepared in 0.35 mL of 0.03 M triethanolamine-HCl, pH 7.6–3.0 M NaCl–10^{-3} M $MgCl_2$–10^{-4} M $ZnCl_2$, in a polypropylene tube with a Teflon "flea" stir bar at room temperature. The pH 7.8 borate buffer described above for dissolving BSA (0.35 mL) was added slowly. To this solution was added 10.0 μL of the activated ester. The solution was stirred at 4 °C and then dialyzed and stored as described above for the BSA conjugate.

Immunization and Monitoring of Mice. Pairs of mice of three strains (Swiss Webster, Biozzi, and B10.Q) were immunized with KLH conjugates of haptens I–III. The initial immunizing doses consisted of 50 μg of conjugate (as carrier protein) in 0.1 mL of physiological saline, and one mouse dose (approximately 50 μg) of Ribi adjuvant (MPL + TDM emulsion, Ribi Immunochem Research, Inc., Hamilton, MT). Booster doses given 7 and 22 days after the first dose consisted of 25 μg of conjugate protein in saline with Ribi adjuvant. Immunizations were subcutaneous, in three or four sites on the back of the mouse. Sera were taken on the 29th day after the first injection. Titers were determined by indirect EIA and sera further analyzed by competition EIA. Mice selected for hybridoma production were "hyperimmunized" by intravenous (tail vein) injection of 50 μg of conjugate in 0.05 mL of saline 3 days prior to cell fusion and about 45 days after the first immunizations. To lessen the risk of anaphylactic or delayed-type hypersensitivity responses, the mice were given a subcutaneous injection of antihistamine and antivasospasm drugs 1 h before the intravenous hyperimmunizing boost (Karu, 1993).

Media and Cell Culture. The "complete medium" for hybridoma culture was Iscove's modified Dulbecco's medium (IMDM), supplemented with 20% (v/v) fetal bovine serum (FBS), 10 μg/mL kanamycin sulfate, 5×10^{-5} M β-mercaptoethanol, and 1 μg/mL of 1/3 iron-saturated transferrin (human type III, Sigma) and Iscove's lipid emulsion (Iscove and Melchers, 1978; Iscove, 1984). Hybridomas were selected in complete medium containing 10% (v/v) J774A.1 macrophage-conditioned medium (Sugasawara et al., 1985), 10^{-4} M hypoxanthine, 8×10^{-7} M aminopterin, and 3×10^{-5} M thymidine (HAT). Myelomas were adapted to grow in medium with hypoxanthine and thymidine prior to fusion, and aminopterin was added to the hybridomas 24 h postfusion. The hybridomas were adapted to grow in complete IMDM without emulsion, macrophage medium, or HAT after they were expanded to 24-well culture plates. Cultures were grown at 37 °C in 5% CO_2 and saturating humidity. Hybridomas were frozen at 10^7 cells/mL in medium consisting of complete IMDM–FBS–dimethyl sulfoxide (6:3:1) using a Cryomed 910 programmable cell freezer and then stored in liquid nitrogen.

Hybridoma Production. The mice were euthanized, and splenocytes were recovered by standard aseptic techniques. Red blood cells were lysed by incubation of the spleen cells in 0.017 M Tris-HCl, pH 7.2–0.17 M NH_4Cl, and the lymphocytes were recovered by centrifugation (800*g*, 10 min) through 1 mL of sterile fetal bovine serum. These cells were counted and washed twice by suspension in electrofusion buffer (0.25 M glucose–0.1 mM calcium acetate–0.1 mM magnesium acetate–1.0 mM KH_2PO_4–K_2HPO_4, pH 7.2) and recovered by centrifugation. Hybridomas were prepared by electrical fusion, with P3X63AG8.653 myelomas as fusion partners. The myelomas were collected by centrifugation (800*g*, 10 min, room temperature), resuspended at 10^6 cells/mL in electrofusion buffer containing 0.6 unit/mL of freshly dissolved dispase (neutral protease from *Bacillus polymyxa*; Boehringer Mannheim), incubated for 15 min at room temperature, and then recovered by centrifugation. The treated cells were resuspended and washed twice by centrifugation in fusion buffer. All of the splenocytes were added to myelomas to give a ratio of five splenocytes per myeloma. Aliquots (0.8 mL) of the cell suspension were then fused in the FTC-03 chamber of an SSH-1 somatic hybridizer (Shimadzu Precision Instruments, Inc., Kyoto, Japan). The specific fusion conditions were as follows: temperature 35 °C; field strength, 2.30 kV/cm; first AC aligning pulse, 1 MHz, 40 V, 30 s; first rectangular DC fusing pulse, 460 V, width 20 μs; second AC aligning pulse, 40 V, 1 s; second rectangular DC fusing pulse, 460 V, width 20 μs; delay period after second DC pulse, 30 s. The decay rate for aligning and fusing pulses was 100% (instantaneous cut-off). The cultures were allowed to remain at 35 °C for 15 min after fusion to promote repair, and the cells were then resuspended in culture medium and seeded in 96-well plates at a density of approximately 3×10^4 cells per well; 10 656 cultures (111 96-well plates) were seeded. The aminopterin was added 24 h later.

Between 13 and 21 days postfusion, colonies were screened by automated sampling of 0.1 mL onto 96-well EIA plates coated with diuron I–BSA conjugate. A total of 2080 colonies were screened in three groups. Of these, 534 reacted specifically with diuron and were expanded to 24-well culture dishes. Two aliquots of each of these cultures were frozen and stored in liquid nitrogen. Of the 24-well cultures, 212 remained positive by indirect EIA, and 91 of these competitively bound diuron in competition EIAs. Cultures that produced nonspecific, adventitious binding antibodies were eliminated by EIA on plates coated with BSA. The I_{50} values for competitive diuron binding by the eight best antibodies ranged from 2 to 30 ppb. These cell lines were subcloned by limiting dilution, and 6–10 stable clones of each line were expanded and frozen. Selected clones were subsequently expanded to produce culture medium and ascites.

Production of Monoclonal Ascites. The mice used to produce the hybridomas were of the Biozzi and Swiss Webster strains, while the myeloma line was derived from Balb/c mice. To avoid rejection of these interstrain hybridomas, the host mice for ascites production were immunosuppressed. Female Swiss Webster retired breeders were given a "priming" injection of 0.5–0.8 mL of tetramethylpentadecane ("pristane oil") ip, depending on their size. Twelve days later they were immunosuppressed by exposure to 500 rads to whole-body γ radiation from a ^{60}Co

source. The next day, the mice received approximately 10^6 log-phase hybridoma cells ip in 0.5 mL of sterile IMDM salt solution. Ascites fluids were harvested by peritoneal tap with an 18-gauge needle from the seventh day after the cells were introduced.

MAb Subtyping and Purification. Immunoglobin subclass was determined by EIA using a commercial kit (Southern Biotechnology Associates, Birmingham, AL). IgG antibodies were purified by affinity chromatography of protein A or protein G Sepharose (Pierce or Pharmacia).

Enzyme Immunoassays. The EIAs were done essentially as described by Voller et al. (1976). A standard indirect EIA (immobilized hapten conjugate) was used to evaluate the response of mice to the various immunizing conjugates and to screen the MABs. This format as well as a direct (immobilized antibody) format was used for competition EIAs.

For the indirect EIAs, conjugates were adsorbed to EIA plates in "coating buffer" (0.015 M Na_2CO_3–0.035 M $NaHCO_3$–0.003 M NaN_3, pH 9.6). The diluent for antibodies and antibody–analyte mixtures was PBS–Tween–BGG–methanol (0.01 M KH_2PO_4–K_2HPO_4, pH 7.4–0.15 M NaCl–0.02% NaN_3–0.05% Tween 20–10% methanol–0.1% bovine γ-globulin). The detecting antibody in indirect EIAs was alkaline phosphatase conjugated to goat anti-mouse IgG. The substrate solution for color development was *p*-nitrophenyl phosphate (Sigma 104 substrate tablets) 1 mg/mL, in 10% (w/v) diethanolamine-HCl, pH 9.8–0.4 mM $MgCl_2$–3 mM NaN_3. Individual EIA plates were tightly covered to prevent evaporation during incubation periods. Between steps, the plates were washed three times with PBS–Tween from a squeeze bottle and dried by rapping on lint-free towels. "Blocking solutions" to prevent adventitious binding were dissolved in PBS–Tween and filtered (0.45 μm) to remove aggregates before use.

An indirect competition EIA was used to quantify the phenylureas and to compare assay conditions and recognition by different antibodies. Subsaturating amounts of diuron–BSA conjugate and hybridoma culture supernate were determined by indirect EIA. Immulon 2 wells were coated overnight at 4 °C with a limiting amount of diuron I–BSA conjugate (25–250 ng of carrier protein) in 0.1 mL of coating buffer. Standards (0.05–200 ppb) or unknowns were mixed with the limiting dilution of antiserum or hybridoma culture fluid in the diluent and incubated overnight at room temperature in tightly sealed polypropylene tubes. The coated wells were washed, and aliquots (0.1 mL) of the antibody–analyte mixture were applied. After 2 h at room temperature, the wells were washed and the remainder of the assay performed as described above. When competitive binding occurred, color development was inversely proportional to the amount of phenylurea in the sample.

For the direct competition EIA format with hybridoma culture supernate or ascites fluids, wells were coated with 20–200 ng of affinity-purified goat antimouse IgG in the coating buffer at 4 °C overnight and then incubated for 2 h at room temperature with the fluid containing MAb. This EIA could also be done by coating wells directly with 20 ng of affinity-purified MAb. The plates were then washed with PBS–Tween, and 0.1 mL of the diluent containing the analyte and a limiting amount of diuron I–AP conjugate was added for 1 h at room temperature. After this step, the plates were washed again, alkaline phosphatase substrate was added, and the rate of color development was recorded.

Data Analysis. Color development was monitored on a Multiskan EIA reader (Flow Laboratories) interfaced with a Macintosh computer, and the rates of the reaction (ΔA_{405}/min $\times 10^3$) were calculated by linear regression. Competition EIA dose–response curves were fitted using the four parameter logistic model, and the data were analyzed as described previously (Schmidt et al., 1990).

RESULTS

Synthesis of Haptens and Conjugates. The structures and synthetic routes for the two synthesis schemes we used are shown in Figure 1. In scheme A, the diuron molecule was derivatized at the terminal nitrogen of the urea moiety with a methylene carbon chain to provide adequate separation from the carrier protein. In the second series of haptens (scheme B), the spacer arm wasattached at the internal nitrogen of the urea moiety. Haptens with methylene spacers of three and five carbons were made by following scheme A, and a hapten with a three-carbon methylene spacer was made by following scheme B. We refer to these as the diuron I, diuron II, and diuron III haptens, respectively (Figure 1, bottom row).

Table 1. Half-Maximal Inhibition (I_{50}, Parts per Billion of Diuron) in Competition EIAs with Sera from Mice Immunized with Diuron Conjugates

immunizing conjugate (KLH)	screening conjugate (BSA)		
	I	II	III
I		940–NC[a]	NC
II	27–247		850–1080
III	2790–NC	475–1660	

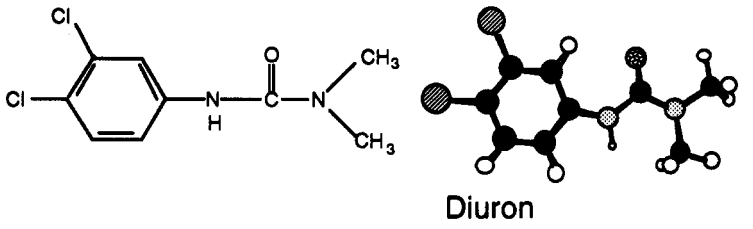
[a] NC, no competition with up to 2 ppm of diruon.

These strategies were designed to provide two different presentations of diuron, or any similar arylurea, with spacer arms of virtually any length and structure. Our scheme A resembles one originally used by Newsome and Collins (1990). Scheme B was designed to preserve both the dichlorophenyl and dimethylurea moieties as potential epitopes. Molecular modeling of the energy-minimized idealized structures indicated that derivatization which replaces the hydrogen on the internal nitrogen of diuron is likely to somewhat alter the electronic and steric properties. In the energy-minimized conformational models at the bottom of Figure 1, the carbonyl oxygen in the diuron I hapten lies at an angle to the dichlorophenyl ring. This oxygen is nearly coplanar with the ring in diuron II and diuron III.

The activated diuron esters shown in Figure 2 retained their reactivity after storage for several months at −70 °C. The active esters were also made efficiently in a modified coupling reaction using the water-soluble *N*-hydroxysulfosuccinimide and 1-ethyl-3-[3′-(dimethylamino)propyl]-carbodiimide, essentially as described by Staros et al. (1986) and Klibanov et al. (1989). The KLH and BSA conjugates could be stored at 4 °C for more than a year in phosphate-buffered saline containing 0.02% NaN_3, with no loss of activity.

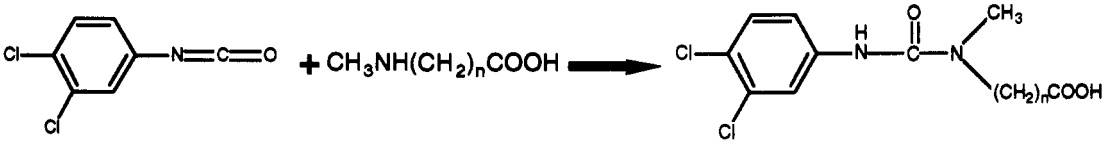
Responses of Mice to the Diuron Conjugates. To test the response of each hapten, pairs of Swiss Webster, Biozzi, and B10.Q mice were immunized with KLH conjugates of haptens diuron I, II, or III. Hapten-specific antibody titer was determined on EIA plates coated with diuron–BSA conjugates that had spacers different from those of the immunizing conjugate. In this assay, all three Diuron–KLH conjugates evoked high-titer anti-hapten responses in all of the mice. Serum dilutions of 1000–10000-fold contained sufficient antibody to give a strong response in an indirect competition EIA. The antisera differed dramatically in their ability to bind free diuron in the competition EIAs (Table 1). Sera from mice immunized with diuron II–KLH gave the most sensitive competition EIAs, with half-maximal inhibition (I_{50}) values of 27–247 ppb, on EIA plates coated with diuron I–BSA. No significant competitive binding of diuron occurred when diuron I was the immunizing hapten and diuron III was the screening hapten or *vice versa*. On the basis of these results, one Biozzi mouse and one Swiss Webster mouse immunized with diuron II–KLH were selected for hybridoma production.

Properties of the Hybridomas. On the basis of the results of the serum tests, the hybridoma supernates were



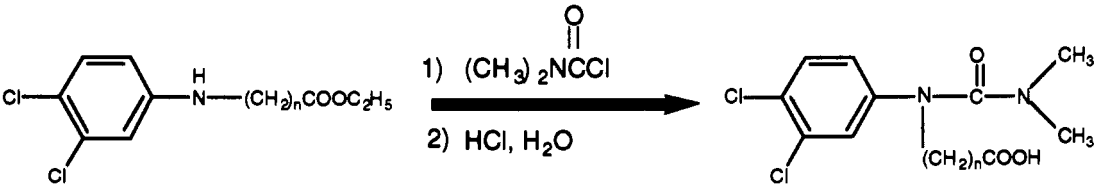
Diuron

SCHEME A



(n = 3 or 5)

SCHEME B



(n = 3 or 5)

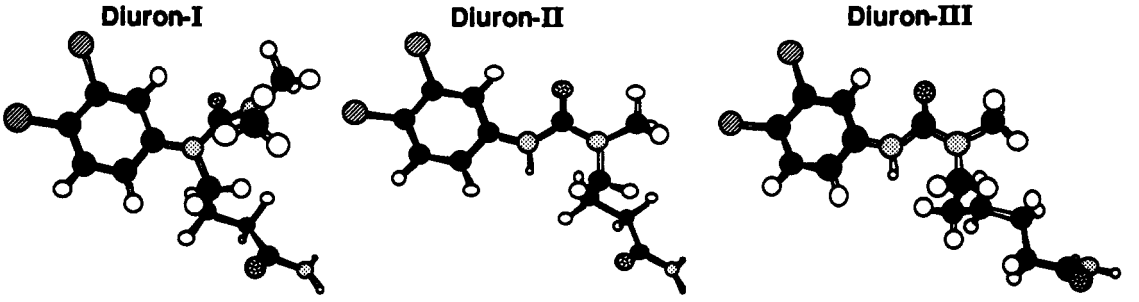
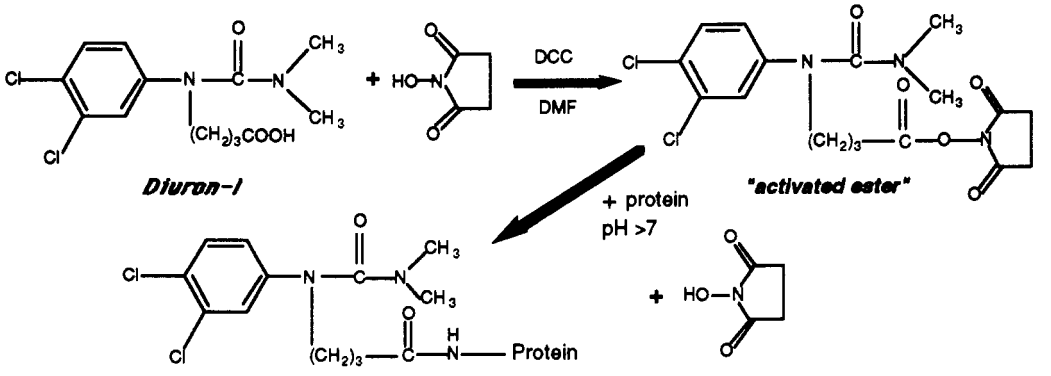


Figure 1. (Top) Structure and conformation of diuron. (Center) The two hapten synthesis schemes detailed under Materials and Methods. (Bottom) Energy-minimized conformations of the three haptens used for immunization and screening.



Hapten-protein conjugate

Figure 2. Formation of the *N*-hydroxysuccinimide active esters of the haptens, and the subsequent conjugation to protein carriers, as described under Materials and Methods.

initially screened for antibodies that bound to diuronI–BSA. Table 2 summarizes some properties of the eight MAbs that most sensitively recognized diuron. Two MAbs were derived from the Swiss Webster mouse, and the remaining six MAbs came from the Biozzi mouse. All of the MAbs were of the IgG subclasses, which can be easily purified to near homogeneity with commercially available affinity media. The most sensitive competitive binding of diuron by all of the MAbs occurred when the coating antigen was diuron I–BSA, as with the original mouse sera.

Table 2. Origin, Ig subclass, and I_{50} Values of the Eight Most Sensitive MAbs for Binding Diuron in Competition EIA with the Indicated Coating Conjugates[a]

MAb	mouse	antibody subclass	I_{50} (ppb) for diuron		
			diuron I–BSA	diuron II–BSA	diuron III–BSA
21	S/W	$IgG_{2b\kappa}$	8.0 ± 3.9	NC[b]	NC
60	S/W	$IgG_{2b\kappa}$	21.9 ± 8.7	NC	NC
195	Biozzi	$IgG_{3\kappa}$	4.6 ± 1.2	NC	2030
202	Biozzi	$IgG_{1\kappa}$	9.9 ± 1.5	924 ± 155	454 ± 43
275	Biozzi	$IgG_{2b\kappa}$	2.5 ± 0.6	NC	575 ± 40
481	Biozzi	$IgG_{1\kappa}$	2.3 ± 0.5	234 ± 95	188 ± 41
488	Biozzi	$IgG_{2b\kappa}$	3.1 ± 1.1	NC	1016 ± 13
520	Biozzi	$IgG_{3\kappa}$	7.9 ± 0.7	NC	NC

[a] Data are mean ± SE for triplicates. [b] NC, no competition by diuron up to 10 ppm.

Table 3. Relative Cross-Reactivity of MAbs with Other Phenylureas and Similar Aryl Carbamates

analyte	% cross-reactivity (I_{50} for diuron/I_{50} for compound) × 100							
	MAb 21	MAb 60	MAb 195	MAb 202	MAb 275	MAb 481	MAb 488	MAb 520
diuron	100	100	100	100	100	100	100	100
linuron	40.0	61.1	3.5	7.4	31.3	2.7	8.7	3.0
monuron	2.4	1.4	3.4	3.5	1.7	3.2	5.2	2.8
fenuron	NC[a]	0.2	0.2	0.2	0.1	0.03	0.9	0.4
siduron	NC	NC	NC	0.1	NC	NC	0.3	NC
tebuthiuron	NC	NC	NC	NC	NC	NC	NC	NC
propham	NC	NC	NC	NC	NC	NC	NC	NC
barban	NC	NC	NC	0.1	NC	0.0	0.1	1.1

[a] NC, no competition up to 5 ppm.

The cross-reactivity of each MAb with other phenylurea herbicides and the structurally similar carbamates was also determined by indirect competition EIA on wells coated with diuron I–BSA (Table 3). Three of the eight MAbs were 30–60% cross-reactive with linuron, while the other five showed 9% or less cross-reactivity with linuron. The maximum recognition of monuron was 5.2%, relative to diuron, with MAb 488. None of the MAbs competitively bound the nonchlorinated phenylureas fenuron or siduron, the related herbicide tebuthiuron (Spike), or the aryl carbamates propham or barban in this assay.

The results of Table 3 indicate that the dichlorophenyl moiety is the primary recognition feature. However, the urea moiety also influences binding; linuron differs from diuron only by having one *N*-methoxy and one *N*-methyl group. None of the eight MAbs showed any significant binding of 1,2-dichlorobenzene or 1,3-dichlorobenzene (data not shown). The metabolites 3,4-dichloroaniline, 3,4-dichlorophenylurea, and 1-(3,4-dichlorophenyl)-3-methylurea in amounts up to 1 ppm did not compete with diuron I–AP for binding to MAb 481 in the direct EIA (data not shown). None of the MAbs reacted with the triazine herbicides atrazine and simazine or with bromacil (data not shown). Thus, the MAbs appear to be suitable for quantifying diuron in the presence of other leachable herbicides that may be found in environmental samples.

Comparison of Direct and Indirect Competition EIAs. All eight MAbs competitively bound diuron in the direct competition EIA using diuron I–AP conjugate but with less sensitivity than in the indirect EIA. Cross-reactivities of the other phenylureas were not significantly different in the two assays.

The precompetition and competition steps of the indirect EIA could be as short as 2 h, and the competition step in the direct EIA could be as short as 1 h without affecting the I_{50}. Bovine γ-globulin was superior to bovine serum albumin, gelatin, and nonfat dry milk as a blocking agent in these assays. Coefficients of variation of 10% or less were routinely obtained using hybridoma culture fluid, ascites fluid, or purified IgG.

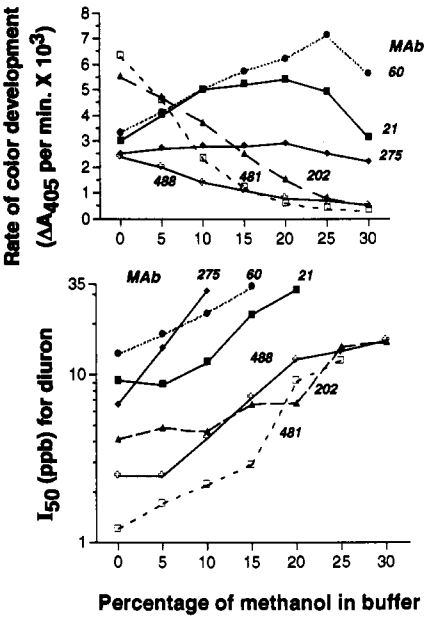


Figure 3. Effect of methanol on the indirect competition EIA. Limiting dilutions of the indicated MAb and various amounts of diuron were incubated at room temperature overnight in PBS–Tween containing the indicated amounts of methanol. These mixtures were applied to wells coated with 25 ng of diuron I–BSA, and the indirect competition EIA was conducted as described under Materials and Methods.

The effect of methanol was tested on the indirect competition EIA, because methanol is an efficient eluant for phenylureas and triazines bound to C_{18} solid-phase extraction resin. Figure 3 illustrates how the I_{50} value and the rate of color development for six of the MAbs were affected by the presence of methanol in the competitive binding step of the indirect competition EIA. MAb 488 appeared to be the best choice for routine assays. It had the second lowest I_{50} in the absence of methanol and an acceptable decrease of sensitivity (increase of I_{50}) and assay color development rate when the competition step contained up to 15% (v/v) methanol. MAb 21 functioned

very well in EIAs containing as much as 15% methanol, but it was less sensitive than MAb 488.

DISCUSSION

To our knowledge, these are the first MAbs developed for the arylurea herbicides. The hapten structures were selected for evaluation as immunizing and/or detecting conjugates on the basis of our previously reported criteria (Harrison et al., 1991). In fact, the results of this study strongly support the hapten design criteria specified by Harrison et al. Of the six possible combinations of hapten–spacer arm conjugates that we tested as immunizing and screening antigens, one combination resulted in a competition EIA that was 100–2000-fold more sensitive than the others. This combination employed the diuron II hapten as immunizing antigen. This hapten consists of an electronically innocuous three-methylene spacer group on the terminal nitrogen of the urea moiety, distal to the major determinant groups. This exposes for antibody manufacture a near-perfect mimic of the target molecule with respect to electronic, steric, and hydrophobic properties. A structurally different mimic, diuron I, used the same spacer arm, but it was attached to the internal nitrogen. Diuron I hapten was recognized less well by the MAbs and thus proved to be the best screening hapten.

Newsome and Collins (1990) raised polyclonal sera in rabbits to two phenylurea haptens and were able to format a competition EIA of similar sensitivity for diuron and related arylureas. However, they were unable to evoke a response in rabbits to their *N'*-(3,4-dichlorophenyl)-*N*-methyl-*N*-butylurea hapten, which was presumably the same as our diuron II hapten, that gave us the most sensitive competition EIA. This difference led us to more closely examine the synthesis, purification, and analysis of our diuron II. We repeated the synthesis five times with slight variations, and we recrystallized the product from different solvents. The melting points of our materials, 161.5–162.5 °C, remained substantially above Newsome and Collins's reported value of 123–125 °C. In contrast to their result, we found diuron II to be virtually insoluble in dichloromethane, which therefore was unsuitable for recrystallization. To more thoroughly characterize the structure and confirm the identity of our material, we used IR, ^{13}C NMR, and HRMS. Our ^{1}H NMR was in fair agreement with the results reported by Newsome and Collins, except that we observed a downfield carboxylic acid OH peak that they did not report. Because Newsome and Collins (1990) did not report an IR spectrum, there is no evidence that their hapten had a carboxylic acid group. We speculate that their material may have cyclized, thus preventing its conjugation to the carrier protein.

Like Newsome and Collins, we observed the most sensitive competition with free herbicide using a heterologous coating hapten. During development of our MAbs, additional, polyclonal rabbit antisera that react with diuron, monuron, and linuron were prepared using haptens similar to those used to derive our MAbs (Schneider et al., 1992). The schemes we describe here allow synthesis of these and other arylurea haptens with spacers of virtually any degree of rigidity and chain length. These should prove useful for deriving polyclonal or monoclonal antibodies and formating assays with more specificity for other arylureas.

The direct competition EIA is faster and simpler than the indirect EIA, and it had I_{50} values similar to those of the indirect EIA for four of the eight MAbs. The effect of methanol on the rate of color development and the increase in I_{50} for diuron was different for every MAb. Tolerance of up to 15% methanol facilitates the assay of samples recovered from solid-phase extraction columns. Since we can concentrate residues from a groundwater sample 400-fold by solid-phase extraction, the extract taken into the EIA may be as much as 60-fold more concentrated than the original groundwater. If the assay detection limit is defined as the concentration required for 10% inhibition (approximately 0.6 ppb for diuron in the indirect EIA), then the practical detection limit for samples is on the order of 0.01 ppb.

In California, the Department of Pesticide Regulation is required to monitor five major leachable herbicides found in the state's groundwater: simazine, atrazine, prometon, bromacil, and diuron. Since all of these compounds can be determined by conventional GC or HPLC, EIA would only be cost-effective if it could be used as a screening method for all five compounds. We recently demonstrated that we could use multivariate statistical methods to identify the three individual triazines (simazine, atrazine, and prometon) from their patterns of cross-reaction with triazine MAbs we derived previously (Kauvar et al., 1992; Cheung et al., 1993). Differences in the cross-reactivity for different phenylurea analogs may similarly make it possible to use three or more of the diuron MAbs to distinguish individual urea herbicides. An EIA recently developed for bromacil may complete the suite of tests that could be run concurrently, eliminating the need for instrumental analysis as a primary screen for these leachable herbicides in groundwater (Szurdoki et al., 1992). A validation of the extraction method and EIA for diuron in groundwater is presented in an accompanying paper.

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Registry No. Supplied by Author: Diuron, 330-54-1; linuron, 330-55-2; monuron, 150-68-5; siduron, 1982-49-6; fenuron, 101-42-8; tebuthiuron (Spike), 34014-18-1; propham (IPC), 122-42-9; barban (carbyne), 101-27-9; atrazine, 1912-24-9; simazine, 122-34-9; prometon, 1610-18-0; bromacil, 314-40-9. The hapten we call diuron II was assigned CAS 66320-26-1 in the work of Newsome and Collins (1990).

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