A Highly Sensitive and Rapid ELISA for the Arylurea Herbicides Diuron, Monuron, and Linuron

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A highly sensitive and rapid enzyme-linked immunosorbent assay (ELISA) for the detection of the arylurea herbicides monuron, diuron, and linuron is described. Diuron haptens with two different polymethylene handle locations were evaluated for use as immunizing and/or tracer antigens. One handle, consisting of three or five methylene groups, located at the terminal urea nitrogen distal to the aromatic ring provided the best antibodies and enzyme-labeled haptens with a variety of selectivities. The second methylene handle attachment at the internal urea nitrogen retained the basic structure of the target molecule, but was not recognized by the antibodies produced from the terminal substituted haptens, nor was it useful as an enzyme tracer. Rabbits were immunized with monuron hapten–bovine serum albumin conjugates that contained either a C3 or C5 methylene handle at the terminal nitrogen. The resulting selectivity of the antisera was not related to the immunizing antigen used. The analyses were class or compound selective for the individual herbicides depending upon the antisera used. Widely applied urea herbicides such as diuron, monuron, and linuron demonstrated 50% inhibition values at 0.4, 0.5, and 0.8 μg/L and detection limits of 0.04, 0.05, and 0.08 μg/L, respectively, in buffer. The test, applied to matrices such as water and orange juice, showed limited matrix effects which could be eliminated by moderate dilution of the sample. The assays were tested for tolerance to methanol, a commonly used solvent in the extraction of diuron from environmental matrices. Up to 50% methanol in the assay had no effect on assay parameters such as the 50% inhibition values. To develop a rational approach for the selection of a hapten for use as an enzyme tracer, a variety of monuron, diuron, and linuron haptens were tested for cross reactivity. Some of these same haptens were then used as hapten enzyme conjugates. In general, haptens recognized about 100 times less well than the "best" hapten were useful as enzyme tracers. In addition, we found that cross reactivity data for esters of carboxylic acid haptens may be better predictors of the binding of the carboxylic acids when conjugated as antigens or enzyme tracers.

INTRODUCTION

Diuron, monuron, and linuron are three members of the multitude of arylurea herbicides developed since the 1960s that have found broad use in agriculture. At a low rate of application, both pre- and postemergence, they display very selective control of germinating broadleaf weeds and grasses in crops such as cotton, alfalfa, some fruits, vegetables, cereals, and nuts. The largest quantity used, however, is at a high rate of application for total weed control on right-of-ways and for landscape maintenance. More than 455 000 kg of diuron was sold in California in 1991 (State of California, 1992).

As a result of non-point-source agriculture diuron has been found in ground water wells at concentrations of 0.1–3.0 ppb in California (State of California, 1991). Thus, the areas surrounding these wells have been designated as pesticide management zones which are subject to certain ground water protection restrictions and requirements, including continued monitoring. In an additional well water monitoring study covering the 20-year period of 1971–1991, the U.S. Environmental Protection Agency (EPA) found similar levels of diuron (U.S. EPA, 1992). With an EPA Lifetime Health Advisory Level for diuron of 10 ppb, there is a need for continuous monitoring of ground water. The high costs of conventional arylurea assays by classical residue analysis (high-pressure liquid chromatography (HPLC), HPLC/mass spectroscopy (MS), gas chromatography (GC), and GC/MS) makes the possibility of examining all well water samples required to protect public health and safety prohibitive. Immunoassays for arylurea herbicides may provide a rapid, cost-effective, accurate, and precise alternative.

Immunochemical detection methods for a variety of compounds of environmental or health concern have been developed during the past few years. For example, enzyme immunoassays for the determination of herbicides such as triazines (Goodrow et al., 1990; Lucas et al., 1992; Giersch and Hock, 1990), molinate (Gee et al., 1988), and paraquat (Van Emon et al., 1987) have been developed. These have proven to be a sensitive, fast, and cost-effective tool for environmental analysis. Immunoassays for arylurea herbicides have been developed by Newsome and Collins (1990) and Aherne (1991). Newsome and Collins developed an assay that was particularly sensitive for monuron in foods, whereas Aherne described assays for isoproturon and chlortoluron. In addition, we have collaborated with another researcher in the development of monoclonal antibody-based assays for diuron (Karu et al., 1994). In this report, the synthesis of a series of haptens used for the hapten–horseradish peroxidase (HRP) conjugates (enzyme tracers) resulted in highly sensitive assays for monuron, diuron, and linuron. The ELISA assays reported here were formatted for short analysis time (30 min); thus, they have potential for the rapid screening of a large number of samples.

Striking differences in antibody bonding due to hapten conjugation position, handle length, and/or alkyl substitution were found in our previous work on triazine herbicides. It was also observed that the relative binding of antibodies to these haptens was similar when testing the free hapten or the hapten conjugated to a protein

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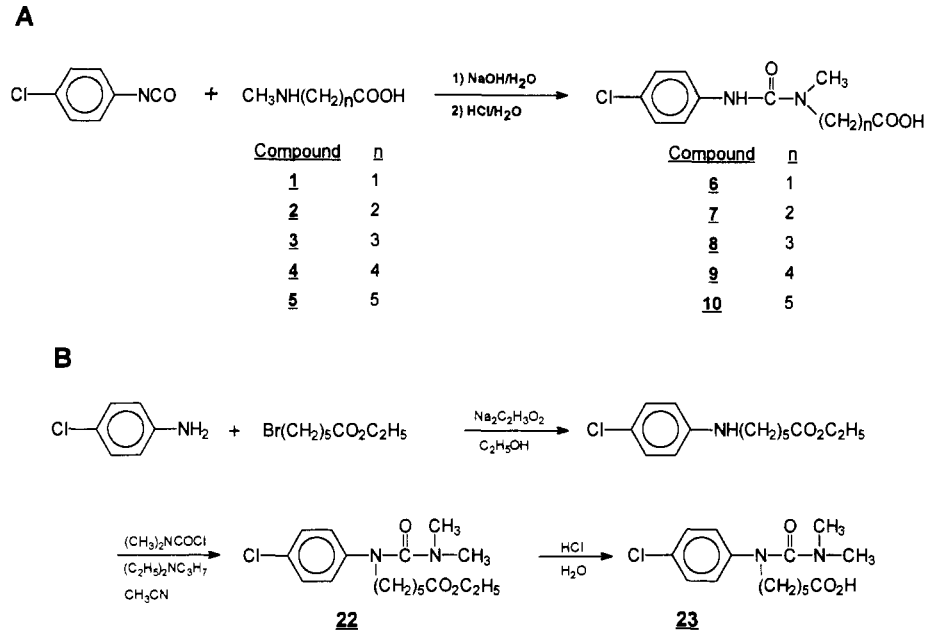


Figure 1. Hapten synthesis pathways. (A) Reaction of 4-chloro isocyanate and a corresponding amino acid yielding haptens with handle attachments at the terminal nitrogen of the arylurea compound. (B) Reaction sequence describing the formation of arylurea haptens with handle attachment at the internal nitrogen. Reaction conditions are given under Materials and Methods.

(Goodrow et al., 1990; Harrison et al., 1991a). Thus, a multihapten synthesis approach was undertaken to elucidate the most perfect mimic of the monuron molecule, in both geometry and electrostatic properties, which would ultimately provide maximum determinant exposure for antibody formation. Further haptens were designed to retain the basic features of the target molecule but with less affinity for the antibody, for example, by altered geometry and/or change in electronic nature of the hapten. These we predicted would be most useful as enzyme tracers or coating antigens. To test this hypothesis, haptens were screened for cross reactivity, and their utility as enzyme tracers was assessed.

MATERIALS AND METHODS

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). ^1H and ^{13}C NMR spectra, at 300.1 and 75.5 MHz, respectively, were measured on a General Electric QE-300 spectrometer (General Electric NMR Instruments, Fremont, CA). Analyses were performed in dimethyl sulfoxide-d_6 (DMSO-d_6); 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 low- (FAB-MS) and high-resolution (FAB-HRMS) mass spectra were obtained on a ZAB-HS-2F mass spectrometer (VG Analytical, Wythenshawe, U.K.) using 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 for FAB-HRMS. The absorbances of the ELISAs were read with a UV_{max} microplate reader (Molecular Devices, Menlo Park, CA) at 450 nm.

Chemicals. All organic starting materials and chemicals used for antigen preparation were obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI). Thin-layer chromatography (TLC) utilized 0.2-mm precoated silica gel 60 F 254 on plastic sheets from E. Merck (Darmstadt, Germany). Plates were eluted with solvent systems A (tetrahydrofuran (THF)–ethyl acetate–hexane (2:13:35 v/v/v)), B (ethyl acetate–hexane (1:1 v/v)), C (A plus 2% acetic acid, v/v), or D (B plus 2% acetic acid, v/v). Compounds were detected first by viewing under UV light (254 nm) and then by exposure to iodine vapor. Flash chromatography utilized silica gel of about 40 μm average particle diameter (J. T. Baker, Inc., Philipsburg, NJ). Bovine serum albumin (BSA), Tween 20, 3,3′,5,5′-tetramethylbenzidine (TMB), and Freund's complete and incomplete adjuvants were obtained from Sigma Chemical Co. (St. Louis, MO). Horseradish peroxidase (HRP) was obtained from Boehringer Mannheim (Indianapolis, IN). Ninety-six well microtiter plates were from Nunc (Roskilde, Denmark). Syntheses of haptens **16**, **18**, **24**, and **25** are reported in Karu et al. (1994).

Synthesis of Haptens. The first series of haptens with handle attachment at the terminal nitrogen was prepared from 4-chlorophenyl isocyanate and an appropriate ω-(methylamino) carboxylic acid (Figure 1A, compounds 1–5). The second series of haptens (Figure 1B), with handle attachment at the internal urea nitrogen, was obtained from the 4-(dimethylamino)pyridine (DMAP)-catalyzed dimethylaminocarbamoylation of ethyl 6-[(4-chlorophenyl)amino)]hexanoate followed by hydrolysis of the ester. A number of other monuron, diuron, and linuron derivatives were synthesized for cross-reactivity assessment. Carbon-13 chemical shift assignments were based on calculations from the additivity rule for monosubstituted benzenes (Pretsch et al., 1989), relative intensities of the substituted vs unsubstituted aryl carbon peaks, comparison with assignments by acknowledged experts, e.g., 1-(4-chlorophenyl)-3-methoxyurea (Sadtler, 1986) and 6-benzamidohexanoate (Sadtler, 1982), and performing distortionless enhancement by polarization transfer (DEPT) experiments (Sanders and Hunter, 1987) to confirm assignments.

1-(Carboxymethyl)-3-(4-chlorophenyl)-1-methylurea (6). The general procedure for the preparation of this compound was utilized for homologs of this series. The amino acid sarcosine (1) (0.891 g, 10.0 mmol) was dissolved in 1.0 M aqueous NaOH (10 mL), and 1.54 g (10.0 mmol) of powdered 4-chlorophenyl isocyanate was added. The heterogeneous mixture was shaken vigorously for 0.5 h and then allowed to stand overnight at room temperature. The mixture was filtered through Celite, and the filtrate was acidified with 6 M HCl to pH 1 and cooled in ice. The white solid precipitate was collected, washed thoroughly with ice–water, and dried in a vacuum desiccator to provide **6** (2.30 g, 94%): mp 125.0–126.0 °C dec with gas evolution; TLC R_f 0.05 (solvent C), 0.09 (solvent D); IR (KBr) 3401 (m, NH), 1719 (m, C=O, acid), 1638 (s, C=O, amide I), 1540 (vs, C=O, amide II), 1212 (s, CO) cm^{-1}; ^1H NMR (DMSO-d_6) δ 12.6 (br, 1 H, OH), 8.53 (s, 1 H, NH), 7.53 (d, J = 8.8 Hz, 2 H, Ar-$H_{3,5}$), 7.28 (d, J = 8.8 Hz, 2 H, Ar-$H_{2,6}$), 4.06 (s, 2 H, CH_2), 3.01 (s, 3 H, CH_3) (the 12.6 and 8.53 ppm peaks disappeared with added D_2O); ^{13}C NMR (DMSO-d_6) δ 171.7 (acid C=O) 155.9 (amide C=O), 139.7 (Ar-C_1), 128.4 (Ar-$C_{3,5}$), 125.8 (Ar-C_4), 121.5 (Ar-$C_{2,6}$), 50.4 (CH_2), 35.9 (CH_3); FAB-MS m/z (relative intensity) 245 (38, M + H^+ + 2), 243 (100, M + H^+); FAB-HRMS m/z calcd for $C_{10}H_{12}ClN_2O_3$ 243.0536, obsd 243.0540.

3-(Methylamino)propanoic Acid (2). A mixture of 44 mL

of 50% H_2SO_4/H_2O and 4.21 g (50.0 mmol) of 3-(methylamino)-propionitrile was heated under reflux for 3 h. To the cooled solution was added 150 mL of water followed by solid $Ba(OH)_2$ until the pH reached 10. The mixture was filtered and the precipitate washed thoroughly with water. Solid CO_2 was added to the total filtrate until the pH of the mixture was 7. This heterogeneous mixture was filtered through Celite and the filtrate concentrated. The residue was triturated with 10 mL of benzene to provide **2** as an off-white powder, 4.68 g (91%): samples failed to give a repetitive melting temperature range; IR (KBr) 1609 (vs, C=O), 1393 (vs, C=O); ^{1}H NMR (D_2O/TSP) δ 3.22 (t, J = 6.5 Hz, 2 H, CH_2N), 2.74 (s, 3 H, CH_3), 2.58 (t, J = 6.5 Hz, 2 H, CH_2CO_2); ^{13}C NMR (D_2O/DMSO-d_6 (2:1, v/v)) δ 178.7 (acid C=O, C_1), 47.5 (C_3), 34.1 (C_2), 34.0 (CH_3).

1-(2-Carboxyethyl)-3-(4-chlorophenyl)-1-methylurea (7). Compound **7** was obtained from 1.03 g (10.0 mmol) of crude **2**, 10.0 mL of 1.0 M NaOH, and 1.54 g (10.0 mmol) of 4-chlorophenyl isocyanate in 90% crude yield. Recrystallization from acetonitrile provided 2.19 g (85%) of **7**: mp 158.0–159.5 °C dec with gas evolution; TLC R_f 0.07 (solvent C), 0.20 (solvent D); IR (KBr) 3294 (m, NH), 3126 (m, NH), 1705 (vs, C=O, acid), 1645 (s, C=O, amide I), 1544 (vs, C=O, amide II), 1212 (s, CO) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 12.3 (br, 1 H, OH), 8.47 (s, 1 H, NH), 7.52 (dd, J = 8.2, 1.2 Hz, 2 H, Ar-$H_{3,5}$), 7.27 (dd, J = 8.2 Hz, 1.2 Hz, 2 H, Ar-$H_{2,6}$), 3.53 (t, J = 7.0 Hz, 2 H, CH_2N), 2.94 (s, 3 H, CH_3), 2.51 (t, J = 7.0 Hz, 2 H, CH_2CO_2) (the 12.3 and 8.47 ppm peaks disappeared with added D_2O); ^{13}C NMR (DMSO-d_6) δ 173.0 (acid C=O, C_1), 155.0 (amide C=O), 139.4 (Ar-C_1), 127.8 (Ar-$C_{3,5}$), 125.2 (Ar-C_4), 121.0 (Ar-$C_{2,6}$), 44.5 (C_3), 34.3 (CH_3), 32.5 (C_2); FAB-MS m/z (relative intensity) 259 (28, M + H$^+$ + 2), 257 (100, M + H$^+$); FAB-HRMS m/z calcd for $C_{11}H_{14}ClN_2O_3$ 257.0693, obsd 257.0713.

1-(3-Carboxypropyl)-3-(4-chlorophenyl)-1-methylurea (8). Treatment of 1.54 g (10.0 mmol) of 4-(methylamino)butyric acid hydrochloride (**3**) in 20.0 mL of 1.0 M NaOH with 1.54 g (10.0 mmol) of 4-chlorophenyl isocyanate produced 2.51 g (93%) of **8** which was recrystallized from dichloromethane (2.35 g, 87%): mp 111.0–113.0 °C dec with gas evolution [lit. (Newsome and Collins, 1990) mp 110–112 °C]; TLC R_f 0.10 (solvent C), 0.24 (solvent D); IR (KBr) 3355 (m, NH), 3310 (m, NH), 1701 (vs, C=O, acid), 1646 (s, C=O, amide I), 1539 (vs, C=O, amide II), 1235 (s, CO) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 12.1 (br, 1 H, OH), 8.35 (s, 1 H, NH), 7.54 (d, J = 8.9 Hz, 2 H, Ar-$H_{3,5}$), 7.27 (d, J = 8.9 Hz, 2 H, Ar-$H_{2,6}$), 3.32 (t, J = 7.2 Hz, 2 H, CH_2N), 2.94 (s, 3 H, CH_3), 2.26 (t, J = 7.3 Hz, 2 H, CH_2CO_2), 1.76 (quin, J = 7.2 Hz, 2 H, CCH_2C) (the 12.1 and 8.35 ppm peaks disappeared with added D_2O); ^{13}C NMR (DMSO-d_6) δ 174.8 (acid C=O, C_1), 155.6 (amide C=O), 140.1 (Ar-C_1), 128.5 (Ar-$C_{3,5}$), 125.7 (Ar-C_4), 121.6 (Ar-$C_{2,6}$), 47.8 (C_4), 34.7 (CH_3), 31.2 (C_2), 23.3 (C_3); FAB-MS m/z (relative intensity) 273 (35, M + H$^+$ + 2), 271 (100, M + H$^+$); FAB-HRMS m/z calcd for $C_{12}H_{16}ClN_2O_3$ 271.0850, obsd 271.0840.

5-(Methylamino)pentanoic Acid (4). The procedure of Benson and Cairns (1948) for the hydrolysis of *N*-methylcaprolactam was employed whereby 4.53 g (40.0 mmol) of 1-methyl-2-piperidone, 45 mL of water, and 5.1 g of concentrated H_2SO_4 were heated under reflux for 10 h. Solid $Ba(OH)_2$ was added to the cooled solution until the pH of the mixture reached 10. The $BaSO_4$ precipitate was separated by filtration. Excess solid CO_2 was then added to the filtrate until the pH reached 6. This mixture was filtered through Celite, and the filtrate was concentrated. Final drying to constant weight at 60 °C (0.2 Torr) provided 4.07 g (78%) of a white crystalline solid which was so hygroscopic that a melting point could not be determined: ^{1}H NMR (D_2O/TSP) δ 3.04 (t, J = 7.1 Hz, 2 H, CH_2N), 2.71 (s, 3 H, CH_3), 2.23 (t, J = 6.8 Hz, 2 H, CH_2CO_2), 1.6 (m, 4 H, CCH_2CH_2C); ^{13}C NMR (D_2O/DMSO-d_6 (1:1, v/v)) δ 181.6 (acid C=O, C_1), 49.6 (C_5), 38.2 (C_2), 33.7 (CH_3), 26.6 (C_4), 24.0 (C_3).

1-(4-Carboxybutyl)-3-(4-chlorophenyl)-1-methylurea (9). A mixture of crude **4**, 1.31 g (10.0 mmol), 1.54 g (10.0 mmol) of 4-chlorophenyl isocyanate, and 10 mL of 1.0 M NaOH produced 2.48 g (87%) of crude **9**, mp 107.0–110.0 °C. Recrystallization from acetonitrile gave pure **9**, 2.26 g (79%): mp 107.0–108.0 °C dec with gas evolution; TLC R_f 0.10 (solvent C), 0.28 (solvent D); IR (KBr) 3355 (m, N-H), 1708 (vs, C=O, acid) 1624 (s, C=O, amide I), 1525 (s, C=O, amide II), 1238 (s, CO) cm^{-1}, ^{1}H NMR (DMSO-d_6) δ 12.0 (br, 1 H, OH), 8.33 (s, 1 H, NH), 7.52 (d, J = 8.8 Hz, 2 H, Ar-$H_{3,5}$), 7.26 (d, J = 8.8 Hz, 2 H, Ar-$H_{2,6}$), 3.31 (m, 2 H, CH_2N), 2.92 (s, 3 H, CH_3), 2.25 (m, 2 H, CH_2CO_2), 1.5 (m, 4 H, CCH_2CH_2C) (the 12.0 and 8.33 ppm peaks disappeared with added D_2O); ^{13}C NMR (DMSO-d_6) δ 174.6 (acid C=O, C_1), 155.3 (amide C=O), 139.9 (Ar-C_1), 128.2 (Ar-$C_{3,5}$), 125.4 (Ar-C_4), 121.4 (Ar-$C_{2,6}$), 47.7 (C_5), 34.4 (CH_3), 33.6 (C_2), 27.0 (C_4), 21.9 (C_3); FAB-MS m/z (relative intensity) 287 (34, M + H$^+$ + 2), 285 (100, M + H$^+$); FAB-HRMS m/z calcd for $C_{13}H_{18}ClN_2O_3$ 285.1006, obsd 285.1034.

1-(5-Carboxypentyl)-3-(4-chlorophenyl)-1-methylurea (10). Compound **5** (Karu et al., 1993), 1.09 g (6.00 mmol), 6.0 mL of 1.0 M NaOH, and 0.932 g (6.07 mmol) of 4-chlorophenyl isocyanate gave 1.78 g (99%) of **10**: mp 148.5–149.5 °C dec with gas evolution; TLC R_f 0.13 (solvent C), 0.34 (solvent D); IR (KBr) 3358 (s, NH), 1711 (vs, C=O, acid), 1621 (vs, C=O, amide I), 1525 (vs, C=O, amide II), 1238 (m, CO) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 12.0 (br, 1 H, OH), 8.32 (s, 1 H, NH), 7.53 (d, J = 8.9 Hz, 2 H, Ar-$H_{3,5}$), 7.26 (d, J = 8.9 Hz, 2 H, Ar-$H_{2,6}$), 3.29 (t, J = 7.2 Hz, 2 H, CH_2N), 2.93 (s, 3 H, CH_3), 2.22 (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 8.32 ppm peaks disappeared with added D_2O); ^{13}C NMR (DMSO-d_6) δ 174.4 (acid C=O, C_1), 155.1 (amide C=O), 139.7 (Ar-C_1), 127.9 (Ar-$C_{3,5}$), 125.1 (Ar-C_4), 121.2 (Ar-$C_{2,6}$), 47.8 (C_6), 34.2 (CH_3), 33.6 (C_2), 27.0 (C_5), 25.7 (C_4), 24.3 (C_3); FAB-MS m/z (relative intensity) 301 (34, M + H$^+$ + 2), 299 (100, M + H$^+$); FAB-HRMS calcd for $C_{14}H_{20}ClN_2O_3$ 299.1162, obsd 299.1158.

3-(4-Chlorophenyl)-1-[5-(methoxycarbonyl)pentyl]-1-methylurea (11). The acid **10**, 1.69 g (5.66 mmol), was dissolved in 50 mL of methanol and saturated with gaseous HCl. After 24 h, the solvent was evaporated and the residue taken up in $CHCl_3$, washed with 5% $NaHCO_3$, and dried (Na_2SO_4). Evaporation of the solvent, followed by cooling and trituration of the residue, produced 1.56 g (88%) of an off-white solid, mp 75.0–77.0 °C. Recrystallization from methanol–water (2:3, v/v) with Norite treatment provided white crystals of **11** showing no impurities on TLC: mp 79.0–80.0 °C; TLC R_f 0.17 (solvent A), 0.51 (solvent B), 0.27 (solvent C), 0.58 (solvent D); IR (KBr) 3343 (m, NH), 1735 (vs, C=O, ester), 1648 (vs, C=O, amide I), 1536 (s, C=O, amide II), 1240 (s, CO) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 8.31 (s, 1 H, NH), 7.51 (d, J = 8.8 Hz, 2 H, Ar-$H_{3,5}$), 7.26 (d, J = 8.8 Hz, 2 H, Ar-$H_{2,6}$), 3.58 (s, 3 H, CH_3O), 3.28 (t, J = 7.2 Hz, 2 H, CH_2N), 2.92 (s, 3 H, CH_3N), 2.30 (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) (the 8.31 ppm peak disappeared with added D_2O); ^{13}C NMR (DMSO-d_6) δ 173.4 (ester C=O, C_1), 155.2 (amide C=O), 139.9 (Ar-C_1), 128.2 (Ar-$C_{3,5}$), 125.3 (Ar-C_4), 121.3 (Ar-$C_{2,6}$), 51.3 (CH_3O), 47.9 (C_6), 34.4 (CH_3N), 33.4 (C_2), 27.2 (C_5), 25.8 (C_4), 24.4 (C_3); FAB-MS m/z (relative intensity 315 (34, M + H$^+$ + 2), 313 (100, M + H$^+$).

1-(4-Carboxyphenyl)-3-(4-chlorophenyl)-1-methylurea (12). Equimolar quantities of 4-(methylamino)benzoic acid, 4-chlorophenyl isocyanate, and 1.0 M NaOH using a compound **6** synthetic procedure provided a 75% yield of **12** after one recrystallization of the crude product from acetonitrile: mp 205.0–206.0 °C dec with gas evolution; TLC R_f 0.32 (solvent C), 0.56 (solvent D); IR (KBr) 3319 (m, NH), 1700 (s, C=O, acid), 1648 (s, C=O, amide I), 1536 (s, C=O, amide II), 1239 (vs, C-O) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 12.9 (br, 1 H, OH), 8.70 (s, 1 H, NH), 7.95 (d, J = 8.6 Hz, 2 H, Ar'-$H_{3,5}$), 7.51 (d, J = 8.9 Hz, 2 H, Ar-$H_{3,5}$), 7.43 (d, J = 8.6 Hz, 2 H, Ar'-$H_{2,6}$), 7.29 (d, J = 8.9 Hz, 2 H, Ar-$H_{2,6}$), 3.33 (s, 3 H, CH_3) (the 12.9 and 8.70 ppm peaks disappeared with added D_2O); ^{13}C NMR (DMSO-d_6) δ 167.1 (acid C=O), 154.6 (amide C=O), 148.3 (Ar'-C_1), 139.1 (Ar-C_1), 130.5 (Ar'-$C_{3,5}$), 128.3 (Ar-$C_{3,5}$), 127.2 (Ar'-C_4) 126.2 (Ar-C_4), 125.1 (Ar'-$C_{2,6}$), 121.7 (Ar-$C_{2,6}$), 37.2 (CH_3); FAB-MS m/z (relative intensity) 307 (38, M + H$^+$ + 2), 305 (100, M + H$^+$); FAB-HRMS m/z calcd for $C_{15}H_{14}ClN_2O_3$ 305.0693, obsd 305.0703.

3-(4-Chlorophenyl)-1-(2-hydroxyethyl)-1-methylurea (13). Equimolar quantities of 4-chlorophenyl isocyanate and 2-(methylamino)ethanol in water produced crude product which was recrystallized from acetonitrile to provide **13** in 86% yield: mp 145.0–146.0 °C; TLC R_f 0.04 (solvent C), 0.12 (solvent D); IR (KBr) 3256 (m, br, OH), 3114 (m, NH), 1643 (vs, C=O, amide I), 1537 (s, C=O, amide II), 1043 (m, CO); ^{1}H NMR (DMSO-d_6) δ 8.46 (s, 1 H, NH), 7.47 (d, J = 7.9 Hz, 2 H, Ar-$H_{3,5}$), 7.26 (d, J = 7.9 Hz, 2 H, Ar-$H_{2,6}$), 4.96 (t, J = 4.7 Hz, 1 H, OH), 3.55 (dt, J = 4.7, 5.5 Hz, 2 H, CH_2O), 3.37 (t, J = 5.5 Hz, 2 H, CH_2N), 2.96 (s, 3 H, CH_3) (the 8.46 and 4.96 ppm peaks disappeared and the 3.55 dt became a single triplet with added D_2O); ^{13}C NMR (DMSO-

d_6) δ 155.6 (C=O), 139.9 (Ar-C_1), 128.3 (Ar-$C_{3,5}$), 125.3 (Ar-C_4), 121.1 (Ar-$C_{2,6}$), 59.5 (CH_2O), 51.1 (CH_2N), 35.4 (CH_3); FAB-MS *m/z* (relative intensity) 231 (35, M + H^+ + 2), 229 (100, M + H^+).

3-(4-Chlorophenyl)-1-(2-cyanoethyl)-1-methylurea (14). Compound **14** was obtained from 4-chlorophenyl isocyanate and **2** in benzene in 96% yield: mp 134.0–135.0 °C; TLC R_f 0.09 (solvent C), 0.28 (solvent D); IR (KBr) 3316 (m, NH), 2250 (w, CN), 1644 (vs, C=O, amide I), 1507 (m, C=O, amide II) cm^{-1}; ^1H NMR (DMSO-d_6) δ 8.54 (s, 1 H, NH), 7.52 (d, *J* = 8.9 Hz, 2 H, Ar-$H_{3,5}$), 7.30 (d, *J* = 8.9 Hz, 2 H, Ar-$H_{2,6}$), 3.61 (t, *J* = 6.7 Hz, 2 H, CH_2N), 3.03 (s, 3 H, CH_3), 2.75 (t, *J* = 6.7 Hz, 2 H, CH_2CN) (the 8.54 ppm peak disappeared with added D_2O); ^{13}C NMR δ 155.2 (C=O), 139.5 (Ar-C_1), 128.3 (Ar-$C_{3,5}$), 125.8 (Ar-C_4), 121.6 (Ar-$C_{2,6}$), 119.5 (CN, C_1), 44.4 (C_3), 34.9 (CH_3), 16.2 (C_2); FAB-MS *m/z* (relative intensity) 240 (34, M + H^+ + 2), 238 (100, M + H^+).

1-(2-Carboxyethyl)-3-(3,4-dichlorophenyl)-1-methylurea (15). Compound **15** was obtained from 0.619 g (6.00 mmol) of 3-(methylamino)propanoic acid, 6.0 mL (6.0 mmol) of 1.0 M aqueous sodium hydroxide, and 1.14 g (6.06 mmol) of 3,4-dichlorophenyl isocyanate in 94% crude yield and recrystallized from acetonitrile to provide refined **15** as white crystals, 1.36 g (78%): mp 167.0–168.0 °C dec with gas evolution; IR (KBr) 3340 (m, NH), 1710 (vs, C=O, acid), 1628 (s, C=O, amide I), 1512 (s, C=O, amide II), 1289 (vs, CO) cm^{-1}; ^1H NMR (DMSO-d_6) δ 12.3 (br, 1 H, OH), 8.63 (s, 1 H, NH), 7.8 (m, 1 H, Ar-H_2), 7.5 (m, 2 H, Ar-$H_{5,6}$), 3.53 (t, *J* = 7.1 Hz, 2 H, CH_2N), 2.94 (s, 3 H, CH_3), 2.50 (t, *J* = 7.1 Hz, 2 H, CH_2CO_2) (the 12.3 and 8.63 ppm peaks disappeared with added D_2O while the 7.8 multiplet resolved into a doublet at 7.81 ppm with *J* = 2.2 Hz and the 7.5 multiplet became a doublet centered at 7.48 ppm with *J* = 8.8 Hz plus a doublet of doublets centered at 7.43 ppm with *J* = 8.8 Hz, 2.2 Hz); ^{13}C NMR (DMSO-d_6) δ 173.4 (acid C=O), 154.9 (amide C=O), 141.0 (Ar-C_1), 130.7 (Ar-C_3), 130.1 (Ar-C_5), 123.0 (Ar-C_4), 120.7 (Ar-C_2), 119.5 (Ar-C_6), 44.7 (CH_2N), 34.7 (CH_3), 32.7 (CH_2); MS *m/z* (relative intensity) 292 (5, M^+ + 2), 290 (7, M^+), 274 (8, M^+ + 2 − H_2O), 272 (12, M^+ − H_2O), 189 (57, $Cl_2C_6H_3NCO$ + 2), 187 (100, $Cl_2C_6H_3NCO$); FAB-HRMS calcd for $C_{11}H_{13}Cl_2N_2O_3$ 291.0303, obsd 291.0296.

3-(3,4-Dichlorophenyl)-1-[3-(methoxycarbonyl)propyl]-1-methylurea (17). To 0.61 g (2.0 mmol) of the urea acid **16** (Karu et al., 1993) in 10 mL of CH_2Cl_2 and 1.0 mL (25 mmol) of methanol was added 20 mg of DMAP and 0.45 g (2.2 mmol) of 1,3-dicyclohexylcarbodiimide (DCC), and the mixture was stirred rapidly. After 3 h, 15 mL of CH_2Cl_2 was added, and the organic layer was extracted with 5% $NaHCO_3$ (10 mL) followed by saturated NaCl (10 mL) and dried (Na_2SO_4). The solution was concentrated and the residue flash chromatographed using hexane/ethyl acetate (1:1, v/v) to provide 0.53 g (83%) of crude **17**. Recrystallation from methanol/water (3:1, v/v) gave 0.39 (61%) of **17** as transparent needles: mp 70.5–72.0 °C; TLC R_f 0.52 (solvent D); IR (KBr) 3304 (m, NH), 1744 (s, C=O, ester), 1646 (vs, C=O, amide I), 1515 (s, C=O, amide II), 1235 (s, CO) cm^{-1}; ^1H-NMR (DMSO-d_6) δ 8.50 (s, 1 H, NH), 7.9 (m, 1 H, Ar-H_2), 7.5 (m, 2 H, Ar-$H_{5,6}$), 3.58 (s, 3 H, CH_3O), 3.32 (t, *J* = 7.1 Hz, 2 H, CH_2N), 2.93 (s, 3 H, CH_3N), 2.32 (t, *J* = 7.3 Hz, 2 H, CH_2CO_2), 1.77 (quin, *J* = 7.2 Hz, 2 H, CCH_2C) (the 8.50 ppm peak disappeared with added D_2O); ^{13}C-NMR (DMSO-d_6) δ 173.2 (ester C=O, C_1), 155.0 (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), 51.4 (CH_3O), 47.4 (C_4), 34.4 (CH_3N), 30.6 (C_2), 22.8 (C_3).

1-(Carboxymethoxy)-3-(3,4-dichlorophenyl)-1-methylurea (19). Ester **20** (86 mg) was hydrolyzed by heating under reflux in 5 mL of 2 M HCl for 2 h. Cooling to room temperature provided **19** as a white solid in 77% yield: mp 183.0–184.0 C dec with gas evolution; TLC R_f 0.04 (solvent C), 0.16 (solvent D); IR (KBr) 3380 (m, NH), 1743 (vs, C=O, acid), 1672 (s, C=O, amide I), 1547 (vs, C=O, amide II), 1206 (s, CO) cm^{-1}; ^1H NMR (DMSO-d_6) δ 10.03 (s, 1 H, NH), 7.90 (s, 1 H, Ar-H_2), 7.53 (s, 2 H, Ar-$H_{5,6}$), 4.59 (s, 2 H, CH_2), 3.11 (s, 3 H, CH_3); ^{13}C NMR (DMSO-d_6) δ 172.1 (acid C=O, C_1), 157.3 (amide C=O), 139.0 (Ar-C_1), 130.9 (Ar-C_3), 130.4 (Ar-C_5), 124.1 (Ar-C_4), 120.0 (Ar-C_2), 118.8 (Ar-C_6), 71.3 (C_2), 36.1 (CH_3).

3-(3,4-Dichlorophenyl)-1-[(methoxycarbonyl)methoxy]-1-methylurea (20). A solution of 0.94 g (5.0 mmol) of 3,4-dichlorophenyl isocyanate, 0.46 g (5.5 mmol) of *N*-methylhydroxylamine hydrochloride, and 0.71 g (5.5 mmol) of *N,N*-diisopropylethylamine in 10 mL of dry benzene was stirred at room temperature for 2 days and then diluted with 50 mL of water. This mixture was extracted with ether (3 × 25 mL). The combined ether extract was dried (Na_2SO_4) and concentrated to provide an off-white solid. This was flash chromatographed using hexane/ethyl acetate (3:2, v/v) to provide 0.52 g (44%) of 3-(3,4-dichlorophenyl)-1-hydroxy-1-methylurea: mp 120.5–121.5 °C; TLC R_f 0.18 (solvent A); IR (KBr) 3384 (m, NH), 3357 (m, NH), 3170 (m, br, OH), 1650 (s, C=O, amide I), 1538 (vs, C=O, amide II) cm^{-1}; ^1H NMR (DMSO-d_6) δ 9.48 (s, 1 H, NH), 9.30 (s, 1 H, OH), 8.04 (d, *J* = 2.3 Hz, 1 H, Ar-H_2), 7.70 (dd, *J* = 8.7, 2.3 Hz, 1 H, Ar-H_6), 7.48 (d, *J* = 8.7 Hz, 1 H, Ar-H_5), 3.09 (s, 3 H, CH_3); ^{13}C NMR (DMSO-d_6) δ 157.5 (amide C=O), 140.1 (Ar-C_1) 130.8 (Ar-C_3), 130.3 (Ar-C_5), 123.6 (Ar-C_4), 120.4 (Ar-C_2), 119.3 (Ar-C_6), 37.9 (CH_3).

Compound **20** was prepared from 0.15 g (0.64 mmol) of the hydroxyurea, 0.26 g (0.65 mmol) of NaOH (10% in water), and 0.11 g (0.72 mmol) of methyl bromoacetate in 3 mL of methanol by stirring at room temperature and following the reaction progress by TLC. Additional quantities of NaOH and bromoester were added over 2 days until the TLC showed only a trace of starting material. The mixture was then diluted with 30 g of ice/water and triturated to provide a white solid. Recrystallization from methanol/water (2:1, v/v) gave 0.14 g (71%) of **20**: mp 84.0–85.0 °C; TLC R_f 0.32 (solvent A); IR (KBr) 3357 (m, NH), 1742 (vs, C=O, ester), 1689 (s, C=O, amide I), 1539 (vs, C=O, amide II), 1227 (s, CO) cm^{-1}; ^1H NMR (DMSO-d_6) δ 9.64 (s, 1 H, NH), 7.93 (s, 1 H, Ar-H_2), 7.6 (m, 2 H, Ar-$H_{5,6}$), 4.68 (s, 2 H, CH_2), 3.75 (s, 3 H, CH_3O), 3.12 (s, 3 H, CH_3N) (the 9.64 ppm peak disappeared with added D_2O); ^{13}C NMR (DMSO-d_6) δ 170.7 (ester C=O), 157.6 (amide C=O), 138.8 (Ar-C_1), 131.2 (Ar-C_3), 130.6 (Ar-C_5), 124.8 (Ar-C_4), 120.6 (Ar-C_2), 119.4 (Ar-C_6), 71.3 (CH_2), 52.2 (CH_3O), 36.4 (CH_3N).

1-(4-Carboxyphenyl)-3-(3,4-dichlorophenyl)-1-methylurea (21). A mixture of 0.907 g (6.00 mmol) of 4-(methylamino)-benzoic acid, 6.0 mL of 1.0 M NaOH, and 1.14 g (6.06 mmol) of 3,4-dichlorphenyl isocyanate after agitation, standing overnight, filtration, and acidification of the filtrate provided crude **21**. Recrystallization from acetonitrile gave 1.82 g (89%) of pure product: mp 198.5–199.5 °C dec with gas evolution; TLC R_f 0.34 (solvent C), 0.58 (solvent D); IR (KBr) 3317 (m, NH), 1711 (s, C=O, acid), 1646 (s, C=O, amide I), 1527 (vs, C=O, amide II), 1228 (s, CO) cm^{-1}; ^1H NMR (DMSO-d_6) δ 12.9 (br, 1 H, OH), 8.81 (s, 1 H, NH), 7.96 (d, *J* = 8.4 Hz, 2 H, Ar′-$H_{2,6}$), 7.8 (m, 1 H, Ar-H_2), 7.5 (m, 2 H, Ar-$H_{5,6}$), 7.44 (d, *J* = 8.4 Hz, 2 H, Ar′-$H_{3,5}$), 3.33 (s, 3 H, CH_3) (the 12.9 and 8.81 ppm peaks disappeared with added D_2O while the 7.8 multiplet resolved into a doublet centered at 7.80 ppm with *J* = 2.3 Hz); ^{13}C NMR (DMSO-d_6) δ 167.1 (acid C=O), 154.3 (amide C=O), 147.9 (Ar′-C_1), 140.4 (Ar-C_1), 130.8 (Ar-C_3), 130.5 (Ar′-$C_{3,5}$), 130.3 (Ar-C_5), 127.6 (Ar′-C_4), 125.5 (Ar′-$C_{2,6}$), 123.8 (Ar-C_4), 121.1 (Ar-C_2), 119.9 (Ar-C_6), 37.3 (CH_3); FAB-MS *m/z* (relative intensity) 341 (61, M + H^+ + 2), 339 (100, M + H^+); FAB-HRMS calcd for $C_{15}H_{13}Cl_2N_2O_3$ 339.0303, obsd 339.0325.

1-(4-Chlorophenyl)-1-[5-(ethoxycarbonyl)pentyl]-3,3-dimethylurea (22, Figure 1B). Ethyl 6-[(4-chlorophenyl)-amino]hexanoate was obtained following the procedure of Wie et al. (1982) whereby 5.10 g (40.0 mmol) of 4-chloroaniline, 6.00 g (44.0 mmmol) of sodium acetate trihydrate, 9.82 g (44.0 mmol) of ethyl 6-bromohexanoate, and 10 mL of ethanol were heated under reflux for 11 h. The hot solution was filtered, and the filtrate was cooled slowly to obtain 6.52 g (60%) of the crude product as white crystals, mp 67.0–69.0 °C. Recrystallization from ethanol–water (2:1, v/v) produced white platelets: mp 67.0–68.0 °C; TLC R_f 0.67 (solvent A); IR (KBr) 3391 (vs, NH), 1721 (vs, C=O, ester), 1185 (s, CO) cm^{-1}; ^1H NMR (DMSO-d_6) δ 7.06 (d, *J* = 8.8 Hz, 2 H, Ar-$H_{3,5}$), 6.53 (d, *J* = 8.8 Hz, 2 H, Ar-$H_{2,6}$), 5.75 (t, *J* = 5.3 Hz, 1 H, NH), 4.04 (q, *J* = 7.1 Hz, 2 H, CH_2O), 2.95 (dt, *J* = 6, 6.9 Hz, 2 H, CH_2N), 2.28 (t, *J* = 7.3 Hz, 2 H, CH_2CO_2), 1.5 (m, 4 H, 2 CH_2), 1.4 (m, 2 H, CH_2), 1.17 (t, *J* = 7.1 Hz, 3 H, CH_3) (the 5.75 ppm peak disappeared, and the 2.95 ppm dt became a single triplet with added D_2O); ^{13}C NMR (DMSO-d_6) δ 173.0 (ester C=O,C_1), 148.1 (Ar-C_1), 128.7 (Ar-$C_{3,5}$), 118.6 (Ar-C_4), 113.3 (Ar-$C_{2,6}$), 59.8 (CH_2O), 42.9 (C_6), 33.7 (C_2), 28.4 (C_5), 26.3 (C_4), 24.5 (C_3), 14.3 (CH_3).

To a solution of 2.16 g (8.00 mmol) of ethyl 6-[(4-chlorophenyl)-amino]hexanoate, 160 mg (1.3 mmol) of DMAP, 40 mL of *N,N*-

diisopropylethylamine, and 20 mL of dry acetonitrile under a nitrogen atmosphere was added 7.4 mL (8.6 g, 80 mmol) of dimethylcarbamyl chloride over 10 min. After the mixture was stirred at room temperature for 7 days, TLC still showed the presence of starting material. Thus, an additional 7.4 mL of carbamyl chloride was added and stirring continued for an additional 21 days. The mixture was poured cautiously into 200 g of ice and 200 mL of water with vigorous stirring. When effervescence subsided, 50 mL of concentrated HCl was added, and the solution was extracted with chloroform (5 × 75 mL). The combined extract was washed with water (50 mL) followed by saturated NaCl solution (50 mL) and dried (Na_2SO_4). Concentration provided 3.00 g of an amber-colored oil. Flash chromatography with elution by hexane/ethyl acetate (2:1, v/v) provided 2.04 g (75%) of pure **22** as a colorless oil which displayed only one component on TLC: TLC R_f 0.25 (solvent A); IR (neat) 1734 (s, C=O, ester), 1656 (vs, C=O, amide), 1181 (m, CO) cm^{-1}; ^1H NMR (DMSO-d_6) δ 7.37 (d, J = 8.9 Hz, 2 H, Ar-$H_{3,5}$), 7.04 (d, J = 8.9 Hz, 2 H, Ar-$H_{2,6}$), 4.03 (q, J = 7.1 Hz, 2 H, CH_2O), 3.49 (t, J = 7.4 Hz, 2 H, CH_2N), 2.60 (s, 2 H, 2 CH_3), 2.24 (t, J = 7.3 Hz, 2 H, CH_2CO_2), 1.5 (m, 4 H, 2 CH_2), 1.2 (m, 2 H, CH_2), 1.16 (t, J = 7.1 Hz, 3 H, CH_3); ^{13}C NMR (DMSO-d_6) δ 172.9 (ester C=O, C_1), 160.1 (amide C=O), 144.4 (Ar-C_1), 129.4 (Ar-$C_{3,5}$), 127.5 (Ar-C_4), 124.8 (Ar-$C_{2,6}$), 59.8 (CH_2O), 50.7 (C_6), 37.5 (2 CH_3), 33.5 (C_2), 27.8 (C_5), 26.0 (C_4), 24.4 (C_3), 14.2 (CH_3).

1-(5-Carboxypentyl)-1-(4-chlorophenyl)-3,3-dimethylurea (23). A mixture of 1.71 g (5.00 mmol) of **22** and 100 mL of 2 M HCl was heated under reflux for 5 h. On slow cooling and trituration, white crystals formed which were collected, washed with ice–water, and vacuum dried to obtain 1.45 g (93%) of **23**, mp 83.0–84.0 °C. Recrystallization from hexane–ethyl acetate (4:1 v/v) provided glistening white crystals of **23** (1.27 g, 81%): mp 83.5–84.5 °C, TLC R_f 0.20 (solvent C), 0.37 (solvent D); IR (KBr) 1728, 1716 (s, C=O, acid), 1618 (vs, C=O, amide I), 1196 (m, C-O) cm^{-1}; ^1H NMR (DMSO-d_6) δ 12.0 (s, 1 H, OH), 7.37 (d, J = 8.7 Hz, 2 H, Ar-$H_{3,5}$), 7.04 (d, J = 8.7 Hz, 2 H, Ar-$H_{2,6}$), 3.49 (t, J = 7.3 Hz, 2 H, CH_2N), 2.60 (s, 6 H, 2 CH_3), 2.17 (t, J = 7.2 Hz, 2 H, CH_2CO_2), 1.5 (m, 4 H, 2 CH_2), 1.4 (m, 2 H, CH_2) (the 12.0 ppm peak disappeared with added D_2O); ^{13}C NMR (DMSO-d_6) δ 174.3 (acid C=O, C_1), 160.1 (amide C=O), 144.4 (Ar-C_1), 129.3 (Ar-$C_{3,5}$), 127.5 (Ar-C_4), 124.6 (Ar-$C_{2,6}$), 50.7 (C_6), 37.4 (2 CH_3), 33.7 (C_2), 27.8 (C_5), 26.0 (C_4), 24.3 (C_3); FAB-MS m/z (relative intensity) 315 (33, M + H^+ + 2), 313 (100, M + H^+); FAB-HRMS m/z calcd for $C_{15}H_{22}ClN_2O_3$ 313.1319, obsd 313.1342.

1-(5-Carboxypentyl)-3-phenylurea (26). Compound **26** was prepared from phenyl isocyanate and 6-aminohexanoic acid using the same procedure as for compound **6**. Recrystallization of the crude product from acetonitrile provided an 84% yield of **26**: mp 129.0–130.0 °C; TLC R_f 0.09 (solvent C), 0.25 (solvent D); IR (KBr) 3328 (s, br, NH), 1695 (s, C=O, acid), 1629 (vs, C=O, amide I), 1565 (vs, C=O, amide II), 1240 (m, CO) cm^{-1}; ^1H NMR (DMSO-d_6) δ 12.0 (br, 1 H, OH), 8.38 (s, 1 H, ArNH), 7.38 (d, J = 7.9 Hz, 2 H, Ar-$H_{2,6}$), 7.20 (dd, J = 7.3, 7.9 Hz, 2 H, Ar-$H_{3,5}$), 6.87 (t, J = 7.3, 1 H, Ar-H_4), 6.11 (t, J = 5.6 Hz, 1 H, NH), 3.06 (dt, J = 6.8, 5.6 Hz, 2 H, CH_2N), 2.21 (t, J = 7.3 Hz, 2 H, CH_2CO_2), 1.5 (m, 2 H, CH_2-5), 1.4 (m, 2 H, CH_2-4), 1.3 (m, 2 H, CH_2-3) (the 12.0, 8.38, and 6.11 ppm peaks disappeared and the 3.06 ppm dt became a single triplet with added D_2O); ^{13}C NMR (DMSO-d_6) δ 174.7 (acid C=O, C_1), 155.4 (amide C=O), 140.8 (Ar-C_1), 128.8 (Ar-$C_{3,5}$), 121.1 (Ar-C_4), 117.8 (Ar-$C_{2,6}$), 39.2 (C_6), 33.9 (C_2), 29.8 (C_5), 26.2 (C_4), 24.5 (C_3).

1-(Carboxymethoxy)-3-(3,4-dichlorophenyl)urea (27). A mixture of carboxymethoxylamine hemihydrochloride, 0.55 g (5.0 mmol), 3,4-dichlorophenyl isocyanate, 1.10 g (5.3 mmol), 7.5 mL of 1.0 M NaOH, and 2 mL of DMSO was agitated vigorously for 1 h and then diluted with 50 mL of water. After filtration and acidification of the filtrate, 1.01 g (72%) of **27** was obtained as a white power: mp 189.0–190.0 °C dec with gas evolution; TLC R_f 0.04 (solvent C), 0.09 (solvent D); IR (KBr) 3231 (m, NH), 3131 (m, NH), 1700 (vs, C=O, acid), 1635 (s, C=O, amide I), 1551 (s, C=O, amide II), 1249 (s, CO) cm^{-1}; ^1H NMR (DMSO-d_6) δ 13.1 (br, 1 H, OH), 9.90 (s, 1 H, NH), 9.57 (s, 1 H, NH), 7.91 (s, 1 H, Ar-H_2), 7.53 (s, 2 H, Ar-$H_{5,6}$), 4.44 (s, 2 H, CH_2) (the 13.1, 9.90, and 9.57 peaks disappeared with added D_2O); ^{13}C NMR (DMSO-d_6) δ 171.8 (acid C=O, C_1), 157.2 (amide C=O), 139.3 (Ar-C_1), 131.1 (Ar-C_3), 130.7 (Ar-C_5), 124.2 (Ar-C_4), 120.2 (Ar-C_2), 119.1 (Ar-C_6), 73.2 (C_2).

1-(5-Carboxypentyl)-3-(4-chlorophenyl)-1-methylthiourea (28). Equimolar quantities of 4-chlorophenyl isothiocyanate, **5**, and 1.0 M NaOH, using the conditions of compound **6** preparation, produced a 75% yield of crude product. Recrystallization from ethanol/water (1/1 v/v) provided pure **28** displaying only one spot on TLC: mp 90.0–91.5 °C dec with gas evolution; TLC R_f 0.13 (solvent C) 0.42 (solvent D); IR (KBr) 3194 (m, NH), 1700 (s, C=O, acid), 1523 (vs, amide II), 1334 (s, amide I), 1249 (m, CO) cm^{-1}; ^1H NMR (DMSO-d_6) δ 12.0 (br, 1 H, OH), 9.00 (s, 1 H, NH), 7.33 (s, 4 H, 4 Ar-H), 3.77 (t, J = 7.5 Hz, 2 H, CH_2N), 3.21 (s, 3 H, CH_3), 2.22 (t, J = 7.3 Hz, 2 H, CH_2CO_2), 1.6 (m, 4 H, CH_2CCH_2), 1.3 (m, 2 H, CCH_2C) (the 12.0 and 9.00 ppm peaks disappeared with added D_2O); ^{13}C NMR (DMSO-d_6) δ 180.8 (C=S), 174.6 (acid C=O, C_1), 140.3 (Ar-C_1), 128.6 (Ar-C_4), 127.9 (Ar-$C_{2,6}$ or Ar-$C_{3,5}$), 127.7 (Ar-$C_{2,6}$ or Ar-$C_{3,5}$), 53.0 (C_6), 38.7 (CH_3), 33.8 (C_2), 26.5 (C_5), 25.9 (C_4), 24.5 (C_3); FAB-MS m/z (relative intensity) 317 (37, M + H^+ + 2), 315 (100, M + H^+); FAB-HRMS m/z calcd for $C_{14}H_{20}ClN_2O_2S$ 315.0934, obsd 305.0944.

Preparation of Antigens and Enzyme Tracers. All conjugates were prepared according to the method of Langone and Van Vunakis (1975) as modified by Schneider and Hammock (1992). The resulting conjugates were diluted in 0.2 M phosphate buffer in 8% saline, mixed 1:1 with ethylene glycol, and stored at −20 °C. Coupling was confirmed by comparison of the UV spectra of BSA, haptens **8** and **10**, and the corresponding conjugates. The conjugates had an additional distinct strong maximum at 252 nm. The epitope density of both conjugates was approximately 10 haptens per BSA molecule, derived from the data of all spectra.

Immunization. Female New Zealand white rabbits were immunized with either the BSA conjugate of hapten **8** (rabbits 2164, 2180, 2249) or **10** (rabbits 2236, 2237, 2238, 2239). Injections of 100 μg of conjugate were given intradermally at weekly intervals for 3 weeks followed by intravenous boosts at 2–3-week intervals. Blood was collected from the marginal ear vein 7 days following the booster injections. Resulting antisera were stored at −20 °C. The immunogens were prepared by dissolving each conjugate in 0.5 mL of sterile saline and then emulsifying with 0.5 mL of Freund's complete adjuvant (first immunization) or 0.5 mL of Freund's incomplete adjuvant (second and third immunization). Subsequent intravenous boosts were without adjuvant.

ELISAs: Immobilization of Antibodies. Following determination of optimum concentrations by checkerboard titration, the antimonuron antisera dilutions were adsorbed onto the wells of a polystyrene microtiter plate overnight at 4 °C in 0.5 M carbonate buffer (pH 9.6). The dilutions providing highest sensitivity and sufficient color development (absorbance of 0.5 in less than 15 min) were found to be in a dilution range from 1:10 000 (antisera 2238, 2164, 2249) to 1:30 000 (antiserum 2180). After coating, the plates were washed three times with PBST (0.2 M phosphate buffer in 8% saline, supplemented with 0.1% (v/v) Tween 20, pH 7.8). The plates were used immediately or stored, sealed and frozen, at −20 °C for use up to several weeks later.

Preparation of Standard Solutions. The standards (monuron, diuron, and linuron) and the compounds tested for cross reactivities were each prepared at 100 μg/mL ethanol and serially diluted with PBST buffer for use.

ELISA Performance and Sample Determination. For the competition step of the ELISA, 50 μL of standard, or sample solution, and 50 μL of enzyme tracer (HRP-**10** conjugate) (1:20 000 for the antisera 2238, 2164, 2249) diluted in PBST were incubated on the antibody-coated plate for 15 min at room temperature. The plates were washed three times with PBST and dried. Substrate solution was added (100 μL). [The substrate solution was prepared by adding 200 μL of TMB (6 mg/mL DMSO) and 50 μL of H_2O_2 (1% in water) to 12.5 mL 0.1 M sodium acetate buffer, pH 5.5.] The reaction was stopped after 15 min at room temperature by adding 50 μL of 4 M H_2SO_4. The absorbances were measured at 450 nm on a 96 well microplate reader. Standards and samples were run in triplicate or quadruplicate well replicates.

Sample Preparation and Spiking. Water samples were obtained from a drinking water tap, an industrial water tap (drinking water supplied through taps which do not contain antisiphon devices), and a local oxbow lake (Putah Creek, Davis,

CA). Orange juice and milk were obtained from local vendors. All matrices were spiked and analyzed directly by ELISA.

RESULTS AND DISCUSSION

Hapten Synthesis. To maximize selective target molecule recognition, the immunizing hapten was designed to represent a near-perfect mimic of monuron in structure, electronic, and hydrophobic properties (Harrison et al., 1991b). From the three potential locations for handle attachment (the terminal urea nitrogen, the internal urea nitrogen, and directly on the aromatic ring system) the compounds with an extension of the terminal *N*-methyl group with innocuous methylene groups (Figure 1A, compounds **6-10**), provide the most perfect mimic of the monuron structure. The added methylene groups have little effect on the physical properties of the monuron since they have a negligible inductive effect on the adjacent urea functional group. A terminal carboxylic acid group provided a means of covalently bonding these haptens to various proteins via an activated ester (Langone and Van Vunakis, 1975). This particular handle location is also as distal as possible to the aryl ring for which selective antibody formation is most critical. Thus, a library of haptens varying in chain length (one to five methylene groups) and attached at the terminal urea nitrogen was prepared according to Figure 1A, from 4-chlorophenyl isocyanate and the respective ω-(methylamino) carboxylic acids (**1–5**, Figure 1A).

Haptens used for the preparation of the immunogen and the enzyme tracer used in the final assay can affect the assay sensitivity. Highly sensitive assays using heterologous hapten systems have been presented previously (Harrison et al., 1991a; Schneider and Hammock, 1992). The structure of the enzyme labeled hapten used for competition with the analyte ideally is less recognized by the antibodies than the hapten used for immunization. This moderately decreased tracer affinity relative to the analyte could lower the amounts of analyte required for an equilibrium under working conditions. In this way the analyte can compete even if it is present in lower concentrations, thus improving the assay sensitivity. For accomplishing such a heterologous system, hapten **23** (Figure 1B) with a C5 handle on the internal nitrogen of the urea was conjugated to HRP. Hapten **23** was bound poorly by all antisera as noted by its lack of cross reactivity and its poor performance as an enzyme label (Table 4). Whereas such a hapten structure may not alter the geometry of the arylurea molecule significantly, converting a secondary nitrogen to a tertiary nitrogen does effect the electronic and hydrogen bonding characteristics of the molecule. We have previously demonstrated antibody-binding variations due to the hapten conjugation position, handle length, and/or alkyl substitution (Goodrow et al., 1990; Harrison et al., 1991a). Attachment of haptens for immunization through the aromatic ring should maximize selectivity for substituents on the terminal nitrogen (such as monuron vs monolinuron) at the expense of variations in the aromatic substituents (such as monuron vs diuron). This approach was utilized by Newsome and Collins (1990) and was moderately successful. Their antibody clearly distinguished between monuron and monolinuron. However, it was also 10 times more selective for diuron than for monuron.

Immunization. High-affinity antibodies may be induced when initial immunization employs low amounts of antigen; high titer antibodies then follow with subsequent boosts using increased amounts of antigen. One rabbit receiving hapten **8** and two rabbits receiving hapten **10** were immunized with only 10 μg of antigen for the first four injections, with later injections of 100 μg. The rabbits immunized with larger amounts of antigen (rabbits 2164, 2180, 2249, 2238, 2239) produced antibodies with higher titers (1:10 000–1:30 000 optimum dilution of the crude antiserum after the fifth immunization) compared to those immunized with the lower amounts (rabbits 2236, 2237) (<1:2000). The antisera of the rabbits with the four highest titers [three rabbits immunized with hapten **8** (2164, 2180, 2249) and one rabbit (2238) immunized with hapten **10**] were characterized for sensitivity and selectivity. No significant difference between the two groups of antisera was seen with respect to relative optimum titer and test sensitivity for monuron (data not shown).

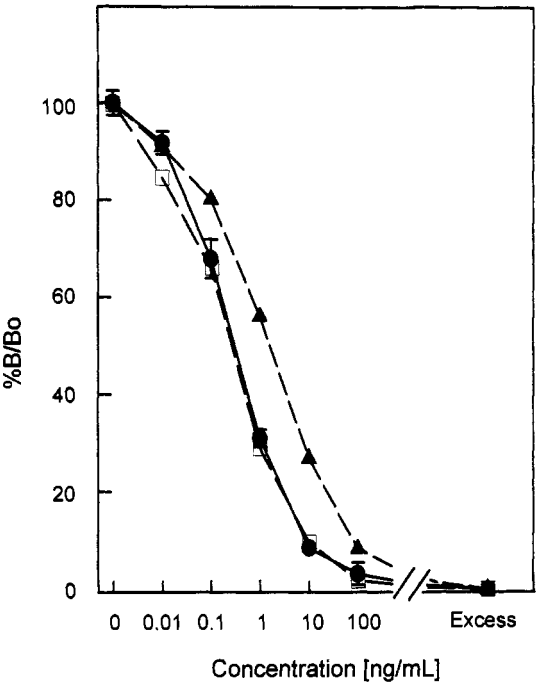
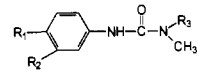
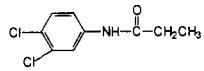
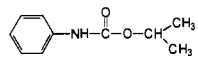
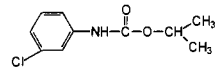
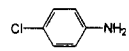
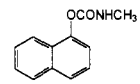


Figure 2. ELISA inhibition curves for monuron (●), diuron (□), and linuron (▲) using antiserum 2238 and the hapten **10**–HRP conjugate as enzyme tracer. The points represent the means and standard deviations for three to seven determinations. The absorbances (A) were converted to $\%B/B_0$ values for normalization according to the formula: $\%B/B_0 = [(A - A_{excess})/(A_{control} - A_{excess})]100$.

ELISA: Performance and Sensitivity. Monuron, diuron, and linuron standard curves were obtained with I_{50} values in the sub ppb (monuron, diuron) or low ppb level (linuron) (Figure 2 and Table 1). The detection limits based on the standard curve (80% B/Bo) were found to be 0.05 μg/L for monuron, 0.04 μg/L for diuron, and 0.08 μg/L for linuron (using antiserum 2238 and the hapten **10**-HRP). The I_{50} values and detection limits were calculated from the numbers derived from three to seven standard curves. Precoating procedures using anti-rabbit IgG or protein A (Schneider and Hammock, 1992) did not improve the assay with respect to color development or sensitivity. The entire assay procedure using the antibody coated plates was performed in 30 min.

Selectivity. The ELISA was selective for arylurea herbicides as none of the tested compounds outside this group displayed cross reactivity (Table 1). Selectivity of the assay for urea herbicides, particularly monuron, diuron, and linuron, was highly dependent on the antisera used. For example, serum 2164 (antihapten **8**) was more selective for monuron whereas serum 2238 (antihapten **10**) recognized monuron, diuron, and linuron with comparable sensitivity. There was 10 times less recognition by antibodies 2164 and 2249 when a methoxy group was substituted for a methyl group on the terminal nitrogen (monuron vs monolinuron and diuron vs linuron), even

Table 1. Cross Reactivities of Various Arylureas and Related Compounds

	compd			antisera[a] (immunizing antigen)					
				2238 (10–BSA)		2164 (8–BSA)		2180 (8–BSA)		2249 (8–BSA)	
	R_1	R_2	R_3	I_{50} (μg/L)	% CR[b]	I_{50} (μg/L)	% CR	I_{50} (μg/L)	% CR	I_{50} (μg/L)	% CR
											
monuron	Cl	H	CH_3	0.51 ± 0.10[c]	100	0.49 ± 0.15	100	1.5 ± 0.2	100	2.2 ± 0.3	100
diuron	Cl	Cl	CH_3	0.33 ± 0.16	180	2.0 ± 0.1	19	7.5 ± 2.4	24	43 ● 22	6.1
linuron	Cl	Cl	OCH_3	1.1 ± 0.66	58	37 ± 3.0	1.6	10.0 ± 1.0	19	340 ± 130	0.82
monolinuron	Cl	H	OCH_3	1.3	42	4.9	11	1.9	88	19	15
fenuron	H	H	CH_3	44	1.0	42	0.98	<200	<0.7	70	2.6
neburon	Cl	Cl	$(CH_2)_3CH_3$	0.05	1400	1.2	57	1.2	180	7.5	41
chlorbromuron	Br	Cl	CH_3	1.7	44	13	5.5	28	8.0	ni[d]	0
chlortoluron	CH_3	Cl	CH_3	15	4.2	9.1	6.6	48	3.9	ni	0
metabromuron	Br	H	CH_3	1.1	64	3.0	17	3.4	60	15	21
											
propanil				9.8	5.7	ni	0	57	2.91	ni	0
				11	4.0	ni	0	>200	<0.7	ni	0
propham											
											
chloropropham				36	1.7	ni	0	>200	<0.9	ni	0
											
4-chloroaniline				ni	0	ni	0	ni	0	ni	0
											
carbaryl[e]				ni	0	ni	0	ni	0	ni	0

[a] Antisera were used at concentrations of 1/10 000 for 2238, 2164, and 2249 and 1/30 000 for 2180. The hapten 8 conjugated to horseradish peroxidase was used as the enzyme label at a dilution of 1/20 000 for all antisera. [b] The percent cross reactivity was determined relative to monuron. [c] The I_{50} values for monuron, diuron, and linuron are the average ± standard deviation of three to five determinations. [d] ni = no inhibition. [e] Diverse other herbicides (atrazine, simazine, bromacil) did not cross react in any of the assays.

though these antibodies were made to the same immunogen. However, no such difference existed when using antibodies 2238 and 2180. Compounds in which chlorine was substituted with a bromine were less recognized (i.e., monuron vs metabromuron and diuron vs chlorbromuron). Compounds which contained no halogens, such as fenuron, were not recognized. Since monuron derivatives were used as immunogens, these antibodies characteristically recognized compounds containing one chlorine better than those containing two chlorines. Similar results were obtained by Newsome and Collins (1990). It has also been found that antibodies made to diuron derivatives containing two chlorines did not recognize monuron which contains only one chlorine (Karu et al., 1994).

The most sensitive assays utilized combinations of antibodies and hapten-HRP conjugates of hapten 8 or 10. With antibody 2180 (antihapten 8) increasing the chain length of the handle of the enzyme tracer from three to five (compounds 8–10) yielded slightly better assay sensitivity for monuron. No such pattern was observed for antibody 2238 (antihapten 10). Both antibodies showed no inhibition with haptens 6 and 7, whose handle chain lengths were one and two, respectively. The high recognition of neburon was likely due to the presence of the butyl side chain which mimics the handle of the immunogen (8). Aherne (1991) also reported highly selective assays for chlortoluron and isoproturon using a homologous assay system in which the immunogen was a hapten with a handle at the terminal nitrogen.

In contrast, Newsome and Collins (1990) report that no inhibition was obtained employing homologous assays using 1-(3-carboxypropyl)-3-phenyl-1-methylurea, 1-(3-carboxypropyl)-3-(chlorophenyl)-1-methylurea, or 1-dimethyl-3-(4-succinamidophenyl)urea haptens. They found that maximum sensitivity for monuron, diuron, and monolinuron was obtained from a heterologous assay system using 1-(3-carboxypropyl)-3-(4-chlorophenyl)-1-methylurea (hapten 8) for immunization and *N'*-(4-succinamidophenyl)-*N,N*-dimethylurea with a terminal nitrogen attachment for coating antigens.

Utilizing the same strategies described here, Karu et al. (1994) developed monoclonal antibodies to diuron haptens. These antibodies were selected on the heterologous hapten with the handle at the internal nitrogen, hapten 24 (Table 4). Three of the selected eight MAbs cross reacted significantly with linuron; otherwise, the resulting assays were highly selective for diuron.

The assays described here can be used for a qualitative as well as quantitative characterization of arylureas in environmental samples. For example, in states such as California, where monuron is not registered for use, serum 2238 (antihapten 10), selective for both monuron and diuron, could be used. For discrimination between diuron and linuron, antiserum 2164 (antihapten 8) could be used. Antiserum 2249 (antihapten 8) is very selective for monuron, and thus, the contribution of other registered arylurea compounds to immunoreactivity of this assay would be negligible. Alternatively, a more general class selective immunoassay could be used for monitoring immunoreactive material following selective HPLC separation of urea herbicides.

Sample Determinations. The applicability of the

Table 2. Recovery of Spiked Monuron and Diuron in Water Samples of Different Sources

	monuron (μg/L)			diuron (μg/L)		linuron (μg/L)	
spike (μg/L)	industrial (1/2)[a]	creek (1/5)	tap (1/1)	creek (1/5)	tap (1/1)	industrial (1/5)	creek (1/2)
0	<0.1	<0.3	<0.03	<0.1	<0.1	<0.5	<0.2
0.05			0.02 ± 0[b]	0.17 ± 0.05			
0.1	0.05 ± 0.01		0.07 ± 0.01				
0.5	0.33 ± 0.07	0.43 ± 0.12	0.52 ± 0.01	0.46 ± 0.15			
1	0.74 ± 0.06	1.1 ± 0.3	1.1 ± 0.1	1.0 ± 0.2	0.90 ± 0.04	1.0 ± 0.1	1.0 ± 0.10
10	7.9 ± 0.25	10.6 ± 1.2	10 ± 1.0	11 ± 1	8.2 ± 2.3	11 ± 1	8.5 ± 1.4

[a] Dilution of the sample tested. [b] The values given are the mean and standard deviation of three to four replicates.

Table 3. Recovery of Spiked Monuron and Diuron from Milk and Orange Juice

	monuron (μg/L)				diuron (μg/L)			
spike (μg/L)	1/1[a]	1/5	1/10	1/50	1/1	1/2	1/5	1/10
				milk				
0	<0.15	<0.75	<1	na	<0.04	<0.08	<0.2	na
0.1	na[b]	na	na	na	<0.04	0.12 ± 0.03	0.19 ± 0.04	na
1	1.5 ± 0.3[c]	1.2 ± 0.1	1.1 ± 0.1	na	0.54 ± 0.12	0.90 ± 0.11	1.6 ± 0.4	na
10	11 ± 2	9.0 ± 0.7	111 ± 1	na	4.9 ± 1.5	7.4 ± 1.6	11 ± 1	na
				orange juice				
0	5.0 ± 0.6	<0.75	<1.5	<7.5	na	na	0.26 ± 0.05	<0.5
0.1	na	na	na	na	na	na	0.42 ± 0.08	<0.5
1	11 ± 1	2.7 ± 0.1	1.7 ± 0.2	<7.5	na	na	2.3 ± 0.6	1.5 ± 0.3
10	43 ± 4	16 ± 1	13 ± 1	11 ± 1	na	na	21 ± 17	10 ± 1

[a] Sample dilution tested. [b] na: not analyzed. [c] Given are the mean values with standard deviations of the triplicate or quadruplicate determinations.

assay to a variety of matrices was studied by evaluating the recovery of analyte from spiked samples. Table 2 presents the recovery of monuron, diuron, or linuron from spiked samples of water from three different sources. The limit of detection varied, depending on the source of water; however, recoveries were acceptable when quantitating at the 0.1 ppb level, and minimal dilution was required to eliminate matrix effects (i.e., 1/1 or 1/2). Putah Creek water required the most dilution (1/5) in order to obtain adequate recovery of spiked compounds. This water was from an oxbow lake which is highly eutrophic. Interferences may be due to the high organic content of this water.

As examples of food matrices, recovery of the arylurea herbicides were measured from milk and orange juice. Milk is representative of a lipophillic matrix of high protein content. Diuron is used in citrus culture; thus, orange juice was tested. It is also representative of a highy acidic matrix. Monuron and diuron could be measured in milk with minimal dilution of sample (1/1 or 1/2) whereas in orange juice, diuron could be quantitated at a moderate dilution of 1/10. Monuron, however, required a greater than 1/50 dilution (Table 3). Nevertheless, these experiments demonstrate the ability to monitor diuron and monuron in a variety of matrices with minimal sample preparation.

Effect of Methanol on Test Sensitivity. The addition of methanol to the standards affected the sensitivity and, in some cases (lower dilutions of enzyme tracers) enhanced the color development. Antiserum 2238 could tolerate up to 50% methanol (Figure 3) with almost no loss of assay sensitivity. Antiserum 2180, however, could only tolerate up to 10% methanol. Using 75% methanol or greater, little or no color development was observed. The high tolerance of antiserum 2238 and acceptable loss of sensitivity in up to 40% methanol for antiserum 2180 offers broad application possibilities for samples such as methanolic extracts or HPLC eluates.

Screening of Potential Haptens for Conjugation as Enzyme Tracers. To make a rational choice of a hapten for use as an enzyme tracer, the recognition of several haptens by antiserum 2238 (antihapten 8) and 2180 (antihapten **10**) was tested by measuring their respective

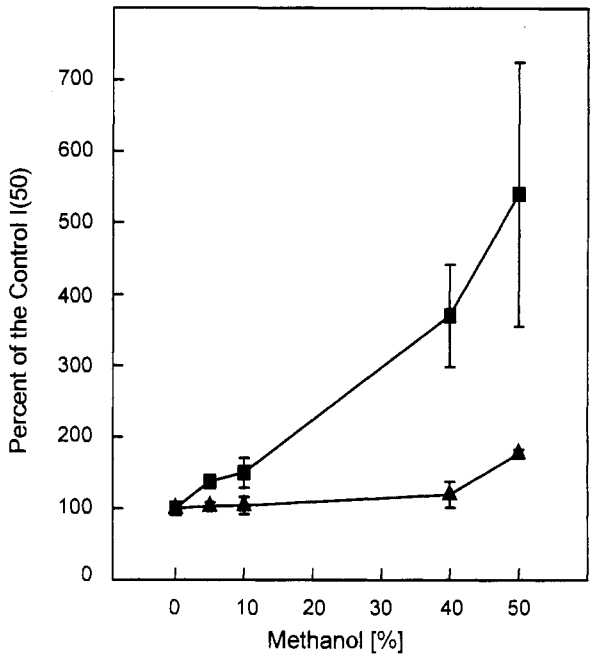
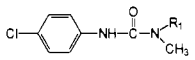
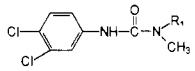
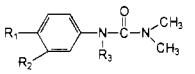
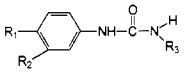
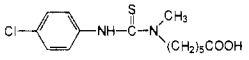


Figure 3. Differential effects of methanol on the assay sensitivity. The fortified standards were performed simultaneously with the control standards on the same plate. All experiments were performed using the hapten 10–HRP conjugate as enzyme tracer. Antibodies used were 2238 (▲) and 2180 (■). The points represent the mean and standard deviation from three determinations.

cross reactivity. Some of these haptens were then used as enzyme tracers in an ELISA. The tracer efficiency, expressed as maximum absorbances and as the 50% inhibition values in the ELISA for monuron are shown in Table 4. Haptens which were usable as enzyme tracers had about a 100-fold higher I_{50} than the "best" hapten. For example, hapten **10** had an I_{50} of 0.025 μg/L. All haptens with I_{50} values of ≤2.5 μg/L (i.e., haptens **8**, **9**, **12**, **18**, **26**, and **28**), when used as enzyme tracers, resulted in assays which could be used to detect monuron sensitively. From this information, we would now consider haptens **15**, **16**, and **21** as potential candidates as enzyme tracers. Compound **7** seems to be an exception to this generali-

Table 4. Relationship between the I_{50} for a Hapten in a Given Assay System and the Relative Sensitivity of an Assay for Monuron Using That Hapten as an Enzyme Label

compd				antisera (immunizing antigen)					
				2238 (10–BSA)			2180 (8–BSA)		
	R_1	R_2	R_3	% of A[a]	I_{50} for monuron[b] (μg/L)	I_{50} for hapten[c] (μg/L)	% of A	I_{50} for monuron (μg/L)	I_{50} for hapten (μg/L)
									
monuron series									
hapten **6**	CH_2COOH			<5	ni[d]	5.3	<5	ni	6.9
hapten **7**	$(CH_2)_2COOH$			7.7	ni	2.0	30	ni	0.79
hapten **8**	$(CH_2)_3COOH$			55	0.46 ± 0.12	0.12	96	2.3 ± 0.5	0.091
hapten **9**	$(CH_2)_4COOH$			97	1.0 ± 0.6	0.028	110	2.1 ± 0.7	0.058
hapten **10**	$(CH_2)_5COOH$			100	0.71 ± 0.06	0.025	100	1.0 ± 0.2	0.032
hapten **11**	$(CH_2)_5COOCH_3$					0.051			0.14
hapten **12**	$(C_6H_4)COOH$			10	1.8 ± 0.6	1.9	17	10 ± 5	0.14
hapten **13**	$(CH_2)_2OH$					ni			0.32
hapten **14**	$(CH_2)_2CN$					0.11			0.18
									
diuron series									
hapten **15**	$(CH_2)_2COOH$					0.7			4.0
hapten **16**	$(CH_2)_3COOH$					0.28			1.3
hapten **17**	$(CH_2)_3COOCH_3$					0.071			0.91
hapten **18**	$(CH_2)_5COOH$			62	3.0 ± 1.2	0.016	63	1.8 ± 0.6	0.27
hapten **19**	OCH_2COOH			<5	ni	69	<5	ni	ni
hapten **20**	OCH_2COOCH_3					1.9			9.3
hapten **21**	$(C_6H_4)COOH$					1.1			1.9
									
internal nitrogen series									
hapten **22**	Cl	H	$(CH_2)_5COOC_2H_5$			85			130
hapten **23**	Cl	H	$(CH_2)_5COOH$	<5	ni	ni	<5	ni	ni
hapten **24**	Cl	Cl	$(CH_2)_3COOH$			ni			ni
hapten **25**	Cl	Cl	$(CH_2)_5COOH$			ni			ni
									
misc compds									
hapten **26**	H	H	$(CH_2)_5COOH$	51	0.41 ± 0.18	2.5	15	ni	130
hapten **27**	Cl	Cl	OCH_2COOH			3.1			ni
									
hapten **28**				60	0.46 ± 0.14	0.52	53	0.93 ± 0.40	2.4

[a] The maximum absorption obtained using the various compounds as enzyme tracers are given as a percent of the maximum absorption obtained using hapten **10**–HRP as the enzyme tracer. [b] The I_{50} for monuron was obtained by utilizing an enzyme tracer made from the compound shown in the first column and the respective antiserum. [c] The I_{50} for the hapten was obtained by utilizing hapten **10**–HRP as the enzyme tracer and the respective antiserum. [d] ni = no inhibition.

zation, which may be due to poor hapten loading during conjugation.

Haptens with longer chain length handles were recognized better than those with shorter chain length handles (see monuron haptens **6**–**10** and diuron haptens **15**, **16**, and **18**). This effect may arise from the highly polar carbonyl group and its close proximity to the antibody binding pocket in the case of the shorter handled haptens. Thus, haptens with short handles (**6**, **7**) were less efficient as tracers.

The relatively low cross reactivity characteristic of linuron (Table 1) was also seen with the corresponding HRP conjugate (hapten **19**, Table 4). Compound **26**, with no Cl on the aryl ring system, was recognized by antiserum 2238 (showing broad recognition for a wide range of ureas) and worked efficiently as a tracer. In contrast, using antiserum 2180 (antihapten **8**) there was almost no recognition and it was not useful as a tracer. Hapten **28**, a thiourea with a sulfur group in place of the carbonyl oxygen, had lower cross reactivity (compared to **10**) but was still well recognized and could be used as an enzyme conjugate. The aromatic handle of hapten **12** was valuable when used as a tracer with inhibition values which ranged between shorter (C_2–C_3) and longer handles, since the length was similar to the size of an aromatic ring system. The recognition of this hapten type **12** and **21**, however, by both antibodies used was relatively low. Compounds, modified at their handle (two methylene groups), **13** and **14**, were recognized very differently by each antisera. Compound **13** was recognized by antibody 2180, but not 2238, whereas compound **14** was recognized by both antibodies. It is possible that the cyano group of hapten **14** mimicked a longer handle.

Esters of carboxylic acid haptens may better mimic the characteristics of the hapten bound to a protein than the corresponding free acids normally examined for antibody recognition. To evaluate this hypothesis initially and to examine haptens more closely analogous to their being bound to a protein (e.g., as hapten–HRP conjugate) the cross reactivities of several methyl and one ethyl ester

were examined and compared to the corresponding free acids (10 vs 11; 16 vs 17, 23 vs 22; 19 vs 20). In general, haptens with high cross reactivity values (10, 16) were also recognized strongly when presented as an ester. Thus, presentation as an ester was not critical. However, the recognition of haptens with low CR values (19, 17) was enhanced by their presentation as an ester. In addition, although we would predict that haptens 23 and 24 would be useless as enzyme tracers due to their lack of recognition, since compound 12 (the ester) is recognized better than 25 (the acid), then perhaps the promise as tracers of these compounds may have been revealed if these had been tested as esters. As further evidence of this possibility, Karu et al. (1994) made antibodies from diuron immunogens (similar to the ones used here for monuron) and were able to bind antigens made from the haptens with a handle at the internal nitrogen in a direct binding test.

The positive correlation between hapten recognition and tracer efficiency supports our previously presented hypothesis, and thus for the prediction of the sensitivity of heterologous assay systems (Goodrow et al., 1990; Harrison et al., 1991b) supplies a simple and fast screening method for determining the optimum hapten to be used for conjugation. On the basis of the data presented here, haptens to be used as potential tracers should be tested as their corresponding esters since the esters better represent the actual chemical properties of the hapten–protein conjugate. Furthermore, since the ester is often a precursor to the formation of the acid derivative, it is readily available for testing.

Use of heterologous assay systems has been shown to produce more sensitive assays than homologous assays (Harrison et al., 1991a,b). The position of the handle and the composition of the handle offer a variety of possibilities for generating heterologous assays. Another approach to a heterologous hapten would be a slight modification in the target molecule. In hapten 28, a sulfur was substituted for an oxygen (hapten 10), but the handle position and composition remain the same. It was predicted that this hapten should have a somewhat lower affinity for the antibody as a result of reduced hydrogen bonding capabilities, and in fact, it does as the CR is about 50%. This compound, when used as a tracer, resulted in an assay among the most sensitive reported here. Thus, this strategy may be useful in design of tracers for other pesticides.

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