

Synthesis of Haptens and Conjugates for an Enzyme Immunoassay for Analysis of the Herbicide Bromacil[†]

Ferenc Szurdoki,^{‡,§} Hassan K. M. Bekheit,^{‡,||} Maria-Pilar Marco,^{‡,⊥} Marvin H. Goodrow,[‡] and Bruce D. Hammock^{*,‡}

Departments of Entomology and Environmental Toxicology, University of California, Davis, California 95616, Plant Protection Institute, Hungarian Academy of Sciences, P.O. Box 102, H-1525 Budapest, Hungary, Plant Protection Research Institute, ARC, Dokki Giza, Nadi El-syd, Egypt, and Department of Biological Organic Chemistry, CID (CSIC), Jordi Girona 18-26, 08034 Barcelona, Spain

A competitive enzyme-linked immunosorbent assay (ELISA) was developed for quantitative detection of bromacil. Two haptens were synthesized, the handles having been attached at the N-1 and 6-methyl groups of the target molecule; both carboxylic acids were coupled to several proteins to obtain two sets of conjugates and then polyclonal antibodies. The standard curve based on the heterologous system with the antibody raised against immunizing antigen of the latter hapten conjugated to KLH and the coating antigen prepared from the former hapten and BSA was nearly linear at around the 0.25 ppb level, which is promising for the environmental monitoring of bromacil. There was only moderate handle recognition observed. Cross-reactivities for terbacil, a related herbicide, for metabolites of bromacil, and for the major metabolite of terbacil were less than 4%.

INTRODUCTION

Bromacil (E.I. du Pont de Nemours & Co., 1, Figure 1) is an economically important, persistent uracil herbicide used worldwide (Gardiner et al., 1969b; Moilanen and Crosby, 1974; Gardiner, 1975; Call et al., 1987; Worthing and Hance, 1991). There have been recent concerns about environmental and health hazards due to the contamination of groundwater and soil by bromacil (Valencia, 1981; Rao et al., 1985; Garrett et al., 1986; Call et al., 1987; Alva and Singh, 1990; Parent et al., 1990; California Code of Regulations 1991; Allender, 1991). The Lifetime Health Advisory Level in drinking water established by the U.S. Environmental Protection Agency for this pesticide is 90 µg/L (parts per billion, ppb) (U.S. Environmental Protection Agency, 1990). Conventional instrumental analyses for residues of this compound require laborious extraction and/or cleanup procedures prior to detection (Pease and Deye, 1967; Zweig and Sherma, 1972; Gardiner, 1975). Most novel chromatographic methods detecting traces of bromacil in environmental samples also employ complicated or expensive preconcentration (Putzien, 1987; Fröhlich and Meier, 1989; Lipschitz et al., 1989; Aakerblom et al., 1990; Di Corcia and Marchetti, 1991; Schlett, 1991; Allender, 1991; Tuinstra et al., 1991; Foster et al., 1991). Some recent procedures are based on still more sophisticated equipment (Wylie and Oguchi, 1990; Stan and Heil, 1991). Bioassays for bromacil appear to

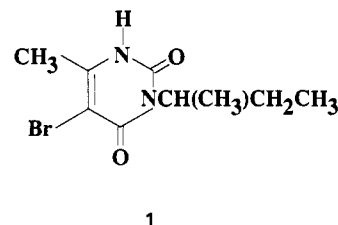


Figure 1. Structure of bromacil (1).

lack the necessary selectivity (Benett and de Beer, 1984; Yanase et al., 1990; Zimmermann et al., 1990). Immunoassays have been demonstrated to be simple and cost-effective alternatives to instrumental analyses when numerous samples need to be investigated for monitoring environmental pollutants or contaminants in agricultural products (Cheung et al., 1988; Vanderlaan et al., 1988; Hammock et al., 1990; Teshima et al., 1990; Hall, 1990; Newsome and Collins, 1991). In the course of our continuous interest in the development of sensitive and selective immunoassay systems for environmental contaminants (Hammock and Mumma, 1980; Goodrow et al., 1990; Harrison et al., 1991; Jung et al., 1991; Li et al., 1991) an enzyme-linked immunosorbent assay (ELISA) was devised to measure bromacil at around 0.25 ppb concentration. The minimal detection level of the routine HPLC method currently applied by the California Department of Pesticide Regulation (CDPR) is 0.1 ppb (K. S. Goh, CDPR, Sacramento, CA, 1991, personal communication). The minimum reporting limit of the U.S. EPA is 1.1 µg/L (ppb) of bromacil using GC with a selective N-P detector (U.S. Environmental Protection Agency, 1990).

Since small molecules are not recognized by the immune system, the target compound must be attached to a large carrier protein to effect the immune response of the host animal. Thus, the hapten, a target molecule to which an appropriate handle terminated by a reactive function is attached, must imitate the steric and electronic characteristics of the molecule to be detected.

The most obvious positions on the bromacil molecule for handle attachment are at the N-1 and the 6-methyl group (derivatives 2 and 3, Figures 2 and 3, respectively).

* Author to whom correspondence should be addressed.

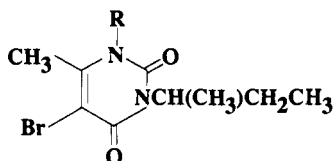
[†] This work was supported in part by the California Department of Food and Agriculture, NIEHS Superfund Grant ES04699-01, and the U.S. Environmental Protection Agency Cooperative Agreement CR-814709-01-0. H.K.M.B. is supported by the U.S. AID. M.-P.M.'s research is funded by the Doctores y Tecnologos Postdoctoral Fellowship (Ministerio de Educacion y Ciencia, Spain). B.D.H. is a Burroughs Wellcome Toxicology Scholar.

[‡] University of California.

[§] Hungarian Academy of Sciences.

^{||} ARC.

[⊥] CID (CSIC).



- 2a: R = (CH₂)₅CO₂C₂H₅, 2b: R = (CH₂)₅CO₂H,
 2c: R = (CH₂)₅CONH-PROT, 2cb: PROT = BSA,
 2cc: PROT = CONA, 2ck: PROT = KLH,
 2co: PROT = OVA, 2d: R = CH₃, 2e: R = (CH₂)₃CH₃

Figure 2. Structures of bromacil derivatives alkylated at N-1.

The 5-bromo and 3-(1-methylpropyl) substituents remained unchanged for maximum recognition and to minimize cross-reactivity with terbacil [5-chloro-3-(1,1-dimethylethyl)-6-methyl-2,4(1*H*,3*H*)-pyrimidinedione, a commonly used, related herbicide, 4] (Gardiner et al., 1969b; Worthing and Hance, 1991) and the photodegradation product of bromacil [5-bromo-6-methyl-2,4(1*H*,3*H*)-pyrimidinedione, 5] (Moilanen and Crosby, 1974). This approach is also expected to diminish the sensitivity of the assay for metabolites of the parent compound (1), 5-bromo-6-(hydroxymethyl)-3-(1-methylpropyl)-2,4(1*H*,3*H*)-pyrimidinedione (6), 5-bromo-3-(2- and 3-hydroxy-1-methylpropyl)-6-methyl-2,4(1*H*,3*H*)-pyrimidinedione (7 and 8, respectively), and 6-methyl-3-(1-methylpropyl)-2,4(1*H*,3*H*)-pyrimidinedione (9) (Gardiner et al., 1969a,b; Shriver and Bingham, 1973; Gardiner, 1975; Jordan and Clerx, 1981).

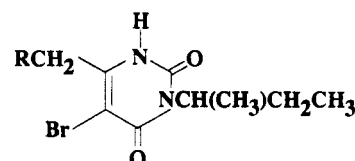
MATERIALS AND METHODS

Chemicals. Common solvents and chemicals of high quality were obtained from Aldrich Chemical Co. (Milwaukee, WI). Samples of bromacil, terbacil, and their metabolites were supplied by Du Pont (Wilmington, DE). Preparative TLC purifications were carried out on Whatman PLK5F 150A (F254, 20 cm × 20 cm × 1000 μm) silica gel plates (Fisher Scientific, Pittsburgh, PA). Immunochemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Instruments. Melting points uncorrected were taken on a Thomas-Hoover capillary apparatus. UV spectra were recorded on a DU-6 spectrometer (Beckman Instruments, Inc., Palo Alto, CA). Infrared spectra were determined on an IR/32 FTIR spectrophotometer (IBM Instruments, Inc.); wavenumber (cm⁻¹) values are given. ¹H and ¹³C NMR spectra were obtained with a QE-300 spectrometer (General Electric, 300 MHz for ¹H and 75 MHz for ¹³C nuclei). Chemical shifts (δ) are given in parts per million (ppm) relative to tetramethylsilane as internal reference. Electron impact mass spectra were determined on a Trio-2 (VG Masslab, Altrincham, U.K.) apparatus at 70 eV; data are reported as *m/z* (relative intensity). A Hewlett-Packard 5890 gas chromatograph equipped with a 15 m × 0.25 mm (i.d.) DB-5 column was interfaced to this mass spectrometer for GC-MS analyses. The FAB mass spectrum was obtained on a ZAB-HS-2F (VG Analytical, Wythenshawe, U.K.) instrument: matrix, 3-nitrobenzyl alcohol; xenon beam, 8 keV, 1-mA current. ELISA experiments were performed in 96-well microplates (Nunc, Roskilde, Denmark, no. 442404) and using V_{max} microplate reader (Molecular Devices, Menlo Park, CA).

Synthesis of Haptens. The structures of haptens 2b and 3d, the corresponding synthetic intermediates (2a, 3a-c) and conjugates (2c, 3e), and compounds 2d,e are shown in Figures 2 and 3. The substances were apparently pure on the basis of TLC and spectral investigations. The structures of the compounds were verified by spectral methods. Organic solutions were dried over Na₂SO₄.

5-Bromo-6-methyl-3-(1-methylpropyl)-2,4(1*H*,3*H*)-pyrimidinedione (1, Figure 1) of 99% purity (GC-MS) served as starting material: mp 158–159 °C [lit. mp 158–159 °C (Worthing and



- 3a: R = Br, 3b: R = (H₅C₂O₂C)₂CH,
 3c: R = (HO₂C)₂CH, 3d: R = HO₂CCH₂,
 3e: R = PROT-NHCOCH₂, 3eb: PROT = BSA,
 3ek: PROT = KLH, 3eo: PROT = OVA

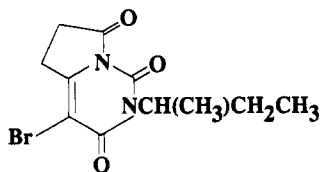
Figure 3. Structures of bromacil derivatives substituted at the 6-methyl position.

Hance, 1991)]; IR and MS characteristics were in accord with literature spectral data (Pease and Deye, 1967; Gardiner et al., 1969a); ¹H NMR (CDCl₃) δ 0.85 (t, *J* = 7.5 Hz, 3 H), 1.44 (d, *J* = 9 Hz, 3 H), 1.79 (m, 1 H), 2.06 (m, 1 H), 2.33 (s, 3 H), 4.94 (m, 1 H), 11.03 (bs, 1 H); ¹³C NMR (CDCl₃) δ 11.18, 17.36, 19.64, 25.84, 52.68, 97.37, 148.45, 152.57, 159.76.

*Ethyl 5-(5-Bromo-6-methyl-3-(1-methylpropyl)-2,4(1*H*,3*H*)-pyrimidinedione-1-yl)hexanoate* [2a, R = (CH₂)₅CO₂C₂H₅, Figure 2]. Synthesis was carried out under nitrogen with stirring. NaH (60% dispersion in mineral oil, 72 mg, 1.8 mmol) was added to a solution of bromacil (0.392 g, 1.50 mmol) in dry DMF (5 mL) at 4 °C. After 15 min at this temperature, ethyl 6-bromohexanoate (0.32 mL, 1.8 mmol) dissolved in 2 mL of dry DMF was added dropwise. The reaction mixture was ice-cooled for 1 h and allowed to stand at room temperature overnight. Water (20 mL) at 4 °C was added cautiously to the reaction mixture, the product was extracted with ether (3 × 15 mL), and the combined ethereal extracts were washed with water (5 × 5 mL), dried, and stripped of solvent. The crude product was purified by preparative TLC, using a hexane/ethyl acetate (Hex/EtOAc, 3:2) solvent system, to provide 0.435 g (72%) of oily ester: IR (NaCl) 1734, 1702, 1658, 1606, 1433, 766 cm⁻¹; ¹H NMR (CDCl₃) δ 0.83 (t, *J* = 7.5 Hz, 3 H), 1.25 (t, *J* = 7.5 Hz, 3 H), 1.43 (d, *J* = 7 Hz, 3 H), 1.55–2.15 (m, 8 H), 2.31 (t, *J* = 7.5 Hz, 2 H), 2.51 (s, 3 H), 3.89 (t, *J* = 8 Hz, 2 H), 4.13 (q, *J* = 7.5 Hz, 2 H), 4.98 (m, 1 H); ¹³C NMR (CDCl₃) δ 11.09, 14.04, 17.16, 19.95, 24.24, 25.71, 25.94, 28.41, 33.81, 46.28, 53.31, 60.10, 99.08, 149.17, 150.80, 159.05, 173.10; MS (EI) *m/z* (relative intensity) 301 (100), 303 (95), 347 (39), 349 (37), 373 (1), 375 (1), 387 (9), 389 (7), 402 (6, M⁺), 404 (6, M + 2).

*5-(5-Bromo-6-methyl-3-(1-methylpropyl)-2,4(1*H*,3*H*)-pyrimidinedione-1-yl)hexanoic Acid* [2b, R = (CH₂)₅CO₂H, Figure 2]. Aqueous NaOH (1.20 mL, 1 M) was added to the solution of compound 2a (0.323 g, 0.80 mmol) in THF (20 mL), and the mixture was stirred while heated under reflux overnight under nitrogen. The reaction mixture was concentrated in vacuo, diluted with 30 mL of 10% NaCl solution, washed with ether (2 × 10 mL), chilled, and acidified by 1 M HCl. The product was extracted with ether (10 × 5 mL). The collected extracts were dried and then evaporated in vacuo. The residue was then purified by preparative TLC (Hex/EtOAc/acetic acid, 3:2:0.25) to furnish 0.19 g (63%) of a crystalline product: mp 104–106 °C; IR (KBr) 3400–2500, 1711, 1658, 1437, 1338, 766 cm⁻¹; ¹H NMR (CDCl₃) δ 0.83 (t, *J* = 7.5 Hz, 3 H), 1.43 (d, *J* = 7 Hz, 3 H), 1.53–2.20 (m, 8 H), 2.38 (t, *J* = 7 Hz, 2 H), 2.52 (s, 3 H), 3.90 (t, *J* = 7.5 Hz, 2 H), 4.97 (m, 1 H), 9.66 (b, 1 H); ¹³C NMR (CDCl₃) δ 11.20, 17.26, 20.12, 24.12, 25.89, 25.97, 28.56, 33.67, 46.40, 53.60, 99.26, 149.23, 150.74, 159.03, 179.00; MS (EI) *m/z* (relative intensity) 204 (56), 206 (55), 259 (12), 261 (10), 273 (5), 275 (5), 301 (59), 303 (100), 305 (42), 319 (30), 321 (29), 357 (3), 359 (4), 361 (1), 374 (7, M⁺), 376 (7, M + 2).

*5-Bromo-6-(bromomethyl)-3-(1-methylpropyl)-2,4(1*H*,3*H*)-pyrimidinedione* (3a, R = Br, Figure 3) was prepared by the bromination of bromacil similarly to the literature method (Gardiner et al., 1969a): mp 183–184 °C [lit. mp 183.5–184.5 °C (Gardiner et al., 1969a)]; IR (KBr) 3237, 3195, 1713, 1630, 1441, 1412, 768 cm⁻¹; ¹H NMR (CDCl₃/DMSO-*d*₆) δ 0.84 (t, *J* = 7.5 Hz, 3 H), 1.44 (d, *J* = 6 Hz, 3 H), 1.80 (m, 1 H), 2.05 (m, 1 H), 4.33 (s, 2



10

Figure 4. Structure of compound 10.

H), 4.91 (m, 1 H), 11.47 (b s, 1 H); ^{13}C NMR ($\text{CDCl}_3/\text{DMSO}-d_6$) δ 10.86, 16.80, 25.33, 25.76, 52.18, 104.57, 146.64, 150.24, 159.70; MS (EI) m/z (relative intensity) 259 (16), 261 (15), 283 (52), 285 (100), 287 (46), 309 (5), 311 (9), 313 (4), 338 (0.7, M^+), 340 (1.3, $\text{M} + 2$), 342 (0.6, $\text{M} + 4$).

Ethyl 3-[5-Bromo-3-(1-methylpropyl)-2,4(1H,3H)-pyrimidinedione-6-yl]-2-(ethoxycarbonyl)propanoate [3b, R = $\text{CH}(\text{CO}_2\text{C}_2\text{H}_5)_2$, Figure 3]. The dibromo compound (3a) (0.340 g, 1.00 mmol) was dissolved in dry DMF (15 mL). Diethyl malonate (0.77 mL, 5.0 mmol), potassium carbonate (0.69 g, 5.0 mmol), and Aliquat 336 (trioctylmethylammonium chloride, 0.020 g, 0.05 mmol) were added, and the mixture was stirred at ambient temperature under nitrogen for a day. The reaction mixture was ice-cooled and diluted with ether and water, and then 1 N HCl was added dropwise with stirring. The organic phase was separated, and the acidic aqueous phase was extracted with ether (3 \times 5 mL). The collected ethereal solutions were washed with water (4 \times 5 mL) and brine (5 mL) and dried; the solvent was removed in vacuo. Finally, the residue was subjected to preparative TLC (Hex/EtOAc, 3:2) to give 0.25 g (60%) of 3b as an oil which solidified on cooling: mp 101–104 $^\circ\text{C}$; IR (KBr) 3214, 3182, 1753, 1741, 1706, 1655, 1445, 769 cm^{-1} ; ^1H NMR (CDCl_3) δ 0.84 (t, $J = 7.5$ Hz, 3 H), 1.27 (t, $J = 7$ Hz, 6 H), 1.43 (d, $J = 7$ Hz, 3 H), 1.78 (m, 1 H), 2.04 (m, 1 H), 3.21 (d, $J = 7.5$ Hz, 2 H), 3.91 (t, $J = 7.5$ Hz, 1 H), 4.22 (m, 4 H), 4.90 (m, 1 H), 10.35 (b s, 1 H); ^{13}C NMR (CDCl_3) δ 11.20, 13.90, 17.28, 25.82, 31.95, 48.92, 52.91, 62.31, 98.81, 147.45, 151.48, 159.75, 167.77; MS (EI) m/z (relative intensity) 55 (100), 289 (24), 291 (26), 317 (44), 319 (42), 363 (64), 365 (61), 389 (1), 391 (1), 403 (2), 405 (1), 418 (3, M^+), 420 (3, $\text{M} + 2$).

This substance partly decomposed on standing at room temperature for a long period or upon heating. The intensity of the NH signal is decayed in the ^1H NMR spectrum of the aged sample. The GC-MS analysis indicated a component in this product, the structure of which was tentatively assigned as 10 (Figure 4): MS (EI) m/z (relative intensity) 245 (100), 247 (96), 271 (10), 273 (10), 285 (3), 287 (2), 300 (1, M^+), 302 (1, $\text{M} + 2$). This compound may have been formed by cyclization and loss of an ethoxycarbonyl group.

3-[5-Bromo-3-(1-methylpropyl)-2,4(1H,3H)-pyrimidinedione-6-yl]-2-carboxylpropanoic Acid [3c, R = $\text{CH}(\text{CO}_2\text{H})_2$, Figure 3]. Saponification of the freshly prepared malonester derivative (3b) (84 mg, 0.20 mmol) was carried out with 0.80 mL of aqueous 1 M NaOH in THF (10 mL) similarly to the hydrolysis of compound 2a. The crude product was recrystallized from ether; 47 mg (65%) of white crystals was obtained: mp 189–192 $^\circ\text{C}$ (dec); IR (KBr) 3450–2500, 1759, 1714, 1629, 1428, 1223, 770 cm^{-1} ; ^1H NMR ($\text{CDCl}_3/\text{DMSO}-d_6$) δ 0.83 (t, $J = 7.5$ Hz, 3 H), 1.42 (d, $J = 7$ Hz, 3 H), 1.77 (m, 1 H), 2.05 (m, 1 H), 3.17 (d, $J = 7.5$ Hz, 2 H), 3.87 (t, $J = 7.5$ Hz, 1 H), 4.87 (m, 1 H), 7.86 (b, 2 H), 10.64 (b s, 1 H); ^{13}C NMR ($\text{CDCl}_3/\text{DMSO}-d_6$) δ 10.84, 16.83, 25.33, 31.50, 48.80, 52.00, 97.19, 148.51, 150.25, 159.70, 169.63; MS (EI) m/z (relative intensity) 202 (79), 204 (68), 245 (100), 247 (90), 263 (11), 265 (11), 289 (1), 291 (1), 301 (1), 303 (1), 305 (0.3), 318 (0.3, $\text{M} - \text{CO}_2$), 320 (0.3, $\text{M} + 2 - \text{CO}_2$).

3-[5-Bromo-3-(1-methylpropyl)-2,4(1H,3H)-pyrimidinedione-6-yl]propanoic Acid (3d, R = $\text{CH}_2\text{CO}_2\text{H}$, Figure 3). The substituted malonic acid 3c (29 mg, 0.08 mmol) was heated under reduced pressure (water aspirator) at 140–145 $^\circ\text{C}$ for 2 h while the flask was purged with a gentle stream of nitrogen. The reaction mixture was subjected to preparative TLC (Hex/EtOAc/acetic acid, 2:2:0.1) to yield 14 mg (55%) of solid product: mp 187–189 $^\circ\text{C}$ (dec); IR (KBr) 3400–2500, 1709, 1659, 1627, 1426,

766 cm^{-1} ; ^1H NMR (CDCl_3) δ 0.84 (t, $J = 7.5$ Hz, 3 H), 1.43 (d, $J = 7$ Hz, 3 H), 1.81 (m, 1 H), 2.02 (m, 1 H), 2.78 (m, 2 H), 2.99 (m, 2 H), 4.92 (m, 1 H), 9.8 (b, 1 H), 10.74 (b s, 1 H); ^{13}C NMR (CDCl_3) δ 11.24, 17.24, 25.82, 28.62, 30.94, 53.32, 98.81, 150.18, 152.96, 159.48, 176.83; MS (EI) m/z (relative intensity) 202 (100), 204 (92), 245 (89), 247 (82), 263 (62), 265 (58), 289 (3), 291 (3), 301 (2), 303 (3), 305 (1), 318 (2, M^+), 320 (2, $\text{M} + 2$); MS (FAB) m/z (relative intensity) 319 (100, $[\text{M} + \text{H}]^+$), 321 (98, $\text{M} + \text{H} + 2$).

Preparation of Conjugates. Conjugates of Acid 2b (2c). The mixed anhydride was formed under nitrogen with stirring. The solution of hapten 2b [R = $(\text{CH}_2)_5\text{CO}_2\text{H}$, Figure 2] (11.3 mg, 0.030 mmol) in 2.0 mL of dry THF and tributylamine (144 μL of 0.25 M solution in dry THF, 0.036 mmol) were mixed and chilled. Isobutyl chloroformate (144 μL of 0.25 M solution in dry THF, 0.036 mmol) was added dropwise at 4 $^\circ\text{C}$ to the mixture. The reaction mixture was maintained at this temperature for 10 min and then at ambient temperature for 30 min. The resulting mixed anhydride solution was divided into four equal portions, and these aliquots were added dropwise to the rapidly stirred protein solutions (15 mg each of BSA, CONA, KLH, and OVA in 2.5 mL of 0.2 M borate buffer, pH 8.7) for 20 min at 4–10 $^\circ\text{C}$. The reaction mixtures were stirred with ice-cooling for 1 h and finally at room temperature overnight. The immunogens (2c) with proteins BSA (2cb), CONA (2cc), KLH (2ck), and OVA (2co, Figure 2) were dialyzed extensively in PBS. The increased absorbance at 284 nm indicated the formation of the conjugates (UV spectroscopy in PBS). The number of hapten molecules bound to each molecule of carrier protein was estimated from the spectral data of the hapten, the protein, and the corresponding conjugate. Optical densities (ODs) at two different wavelengths (238 and 284 nm) were taken into account because the spectral maxima of the hapten and the proteins are approximately 280–285 nm (Wessendorf et al., 1990; Goodrow et al., 1990; Brinkley, 1992). The apparent substitution ratios of the conjugates 2cb, 2cc, 2co, and 2ck were 10, 12, 6.6, and 1.15×10^3 , respectively. Molecular weights of the proteins used in the calculations were as follows: BSA, 68 000; CONA, 76 000; OVA, 45 000; and KLH, 6 000 000 (Greirson et al., 1991).

Conjugates of Hapten 3d (3e). The procedure was analogous to that of compound 2c, described above. The mixed anhydride of acid 3d (R = $\text{CH}_2\text{CO}_2\text{H}$, Figure 3) (9.58 mg, 0.030 mmol) was generated in DMF. Proteins BSA, KLH, and OVA (25 mg of each) were used for obtaining conjugates 3eb, 3ek, and 3eo (Figure 2), respectively. There was no significant increase in the optical densities of the conjugates of 3d compared to those of the proteins. This may be due to the lower molar absorbance of hapten 3d compared to that of 2b and the lower epitope density in this set of conjugates. However, the successful assays employing these conjugates demonstrated eventually that the coupling reaction with the proteins did take place.

Preparation of Bromacil Derivatives for Cross-Reactivity Studies. 5-Bromo-1,6-dimethyl-3-(1-methylpropyl)-2,4(1H,3H)-pyrimidinedione (2d, R = CH_3 , Figure 2). A mixture of bromacil (0.261 g, 1.00 mmol), potassium carbonate (0.518 g, 3.75 mmol), acetone (5 mL), and iodomethane (0.163 g, 1.15 mmol) was stirred at room temperature under nitrogen overnight, diluted with ether, and then filtered. The filtrate was evaporated and purified by preparative TLC (Hex/EtOAc/triethylamine, 1:1:0.1) to give 0.24 g (87%) of white crystalline product: mp 97–99 $^\circ\text{C}$; IR (KBr) 1698, 1654, 1614, 1424, 764 cm^{-1} ; ^1H NMR (CDCl_3) δ 0.84 (t, $J = 7.5$ Hz, 3 H), 1.43 (d, $J = 7$ Hz, 3 H), 1.81 (m, 1 H), 2.09 (m, 1 H), 2.52 (s, 3 H), 3.47 (s, 3 H), 4.98 (m, 1 H); ^{13}C NMR (CDCl_3) δ 11.17, 17.24, 20.52, 25.96, 33.14, 53.64, 98.74, 149.64, 151.10, 158.89; MS (EI) m/z (relative intensity) 219 (100), 221 (97), 245 (6), 247 (6), 259 (4), 261 (2), 274 (5, M^+), 276 (5, $\text{M} + 2$).

5-Bromo-1-butyl-6-methyl-3-(1-methylpropyl)-2,4(1H,3H)-pyrimidinedione [2e, R = $(\text{CH}_2)_3\text{CH}_3$, Figure 2]. The oily compound (0.26 g, 82%) was obtained similarly to the preparation of the 1-methyl analogue (2d) above using 1-iodobutane (0.230 g, 1.25 mmol) instead of iodomethane: IR (NaCl) 1703, 1658, 1606, 1434, 766 cm^{-1} ; ^1H NMR (CDCl_3) δ 0.82 (t, $J = 7.5$ Hz, 3 H), 0.96 (t, $J = 7.5$ Hz, 3 H), 1.30–1.71 (m, 7 H), 1.78 (m, 1 H), 2.06 (m, 1 H), 2.51 (s, 3 H), 3.88 (t, $J = 8$ Hz, 3 H), 4.96 (m, 1 H); MS (EI) m/z (relative intensity) 204 (53), 205 (79), 206 (57), 207

(77), 245 (78), 247 (71), 261 (100), 263 (96), 287 (4), 289 (4), 301 (5), 303 (3), 316 (8, M⁺), 318 (8, M + 2).

Immunization. Three New Zealand white rabbits weighing 2–4 kg were used for each immunizing antigen for raising antibodies. Immunizing antigen (100 μ g) dissolved in physiological NaCl solution was emulsified with Freund's complete adjuvant (1:2 volume ratio) and injected intradermally at multiple sites on the back for 2 weeks (one injection a week). After 1 week, the animal was boosted with an additional 100 μ g of antigen in physiological NaCl solution and Freund's incomplete adjuvant (1:2 volume ratio) and bled 10 days later. If the resulting antibody displayed sufficiently high titer, boosting and bleeding continued as above for 10 days. The serum was isolated by centrifugation and stored at -20°C . All results of antibody characterization are for serum from terminal bleeds of six rabbits (three for each of **2ck** and **3ek**), which yielded 50–75 mL of serum per rabbit.

ELISA and Competitive Inhibition ELISA. Enzyme immunoassay studies were done according to the established solid-phase immunoassay principles (Voller et al., 1976; Wie and Hammock, 1982). The protocol was similar to that of Gee et al. (1988) with minor differences presented below. A volume of 100 μ L was maintained in the wells of 96-well microplates throughout the experiments. Coating antigens, dissolved in 0.1 M carbonate buffer (pH 9.6), were immobilized in the wells of the microplates. The wells were then treated with rabbit anti-hapten antibody, enzyme-labeled (alkaline phosphatase) goat anti-rabbit antibody, and enzyme substrate (*p*-nitrophenyl phosphate) following the standard protocol (Gee et al., 1988). The amount of rabbit anti-hapten antibody bound to the coating antigen was traced by the photometric quantification of the colored product generated by the enzyme. ELISA served for the measurement of the amount of specific antibody in a serum or for the determination of the affinity of an antibody to plate-coating antigens. In cases where weak binding of an antiserum to a coating antigen resulted in either a low signal (<0.200 absorbance for control) or the use of too great an amount of antiserum (dilution > 1/500), the system was no longer investigated. Antibodies raised against the KLH conjugates (**2ck** and **3ek**) were tested by ELISA for binding to plate-coating conjugates of BSA (**2cb** and **3eb**), CONA (**2cc**), and OVA (**2co** and **3eo**). Competitive inhibition ELISA (Gee et al., 1988) was then applied for the quantitative detection of bromacil. Bromacil (1 mg/mL in DMF) was diluted serially with PBSTA (phosphate-buffered saline plus Tween 20, azide) and incubated with antiserum overnight at room temperature to obtain standard curves with the ELISA method given above. (Cross-reactivity studies were performed similarly.)

RESULTS AND DISCUSSION

Synthetic Work. The amide anion, generated from bromacil by NaH in aprotic medium, was treated with ethyl 6-bromohexanoate to produce the ester **2a** [$\text{R} = (\text{CH}_2)_5\text{CO}_2\text{C}_2\text{H}_5$, Figure 2]. Saponification of this compound was best accomplished in THF solution. Hydrolysis in the usual way (1 M NaOH in aqueous ethanol) resulted in a product slightly contaminated with unidentified substances. These byproducts, detected by NMR, probably were formed due to the reaction of the uracil skeleton. The resulting hapten, **2b**, was coupled to proteins (BSA, CONA, KLH, and OVA) by the mixed anhydride method (Wie and Hammock, 1984) to yield conjugates **2cb**, **2cc**, **2ck**, and **2co**, respectively [$\text{R} = (\text{CH}_2)_5\text{CONH-PROT}$; PROT, protein, Figure 2]. The approximate numbers of the ligands covalently bound to 10 kDa of each protein, calculated from UV spectral data, were 1.5, 1.6, 1.9, and 1.5 for conjugates **2cb**, **2cc**, **2ck**, and **2co**, respectively.

In our preliminary studies, treatment of the dibromo compound (**3a**) (Gardiner et al., 1969a) with cyanide ion or 3-mercaptopropanoic acid failed to give acceptable yields of chain-elongated products of type **3** by substitution of bromine at the methylene group. Examination of the literature on similar uracil derivatives revealed the possibility of alternate pathways (e.g., dehalogenation, for-

Table I. Serum Titers of Anti-Bromacil Antibodies Determined by Enzyme-Linked Immunosorbent Assay^a

serum	coating antigen						
	2cb	2cc	2ck	2co	3eb	3ek	3eo
1960	+	+	++	+	-	+++	++
2005	+	+	++	+	-	+++	+
2007	+	+	++	+	+	+++	+
2365	++	++	++	++	++	++	++
2369	++	++	++	++	++	++	+
2370	++	++	+++	++	++	++	+

^a Sera 1960, 2005, and 2007 were raised against conjugate **3ek**; sera 2365, 2369, and 2370 were obtained by immunizing with antigen **2ck**. Titer, calculated as function of optical density at 405–650 nm with constant dilution, was ranked as follows: low titer, -, OD = 0.042–0.322; moderate titer, +, OD = 0.323–0.645; high titer, ++, OD = 0.646–0.910; extremely high titer, +++, OD = 0.911–1.353.

mation of cyclopropane compounds) for these reactions (Szabo et al., 1970; Sedor and Sander, 1973; Senda et al., 1975; Hirota et al., 1981). However, the bulkier diethyl malonate anion seemed to be a more promising reagent for our purposes. Malonester condensation of **3a** in a solid/liquid system with phase-transfer catalysis (PTC) (Dehmlow and Dehmlow, 1983) selectively gave the expected compound (**3b**). The transformation was carried out in DMF with excess diethyl malonate in the presence of solid potassium carbonate and a catalytic amount of Aliquat 336 at room temperature. Heating enhanced the formation of contaminants. Under these mild conditions the amount of side products (e.g., compounds derived from attack at C-5 and C-6 positions of the ring, substances alkylated or acylated at the NH group, double alkylation product of diethyl malonate) was not significant. Compound **3b**, however, is not stable. The structure of a component in an aged sample was tentatively designated **10** (see Figure 4). The malonester derivative **3b** was then hydrolyzed and decarboxylated (Spener and Mangold, 1973) to hapten **3d**. Conjugates **3eb**, **3ek**, and **3eo** ($\text{R} = \text{PROT-NHCOCH}_2$, Figure 3) with BSA, KLH, and OVA, respectively, were prepared via the mixed anhydride of carboxylic acid **3d**.

The mild solid/liquid two-phase process employing potassium carbonate as base was preferred to literature methods (Luckenbaugh and Soboczenski, 1966; Ogierman, 1986) to obtain the *N*-methyl and *N*-butyl derivatives of bromacil (**2d** and **2e**, respectively, Figure 2) in pure form for studying cross-reactivity. It is worthwhile to notice the different outcome of the smooth *N*-alkylations making use of the highly reactive iodoalkanes and the selective malonester reaction of bromo compound **3a**. Likewise, the formation of **2a** with ethyl 6-bromohexanoate under similar solid/liquid PTC conditions gave less satisfactory results than the method presented above.

ELISA Results. Competitive Inhibition Experiments. Immunization results demonstrated significant titers on homologous and heterologous ELISA antigens (Table I). In general, **2c** conjugates gave higher titer than the corresponding **3e** derivatives (Table I). There was no remarkable difference, however, in antibody titers among the sera raised against the same hapten. Thus, this effect may be only the consequence of the slightly higher loadings used in the case of **2c** conjugates.

Heterologous ELISAs have been powerful tools for highly sensitive and selective detection of environmental pollutants (Wie and Hammock, 1984; Harrison et al., 1991). We selected a coating antigen with a hapten that the antibody binds less tightly than the analyte. The results supported the data and rationale presented by Harrison et al. (1991), which indicated that this is an effective way

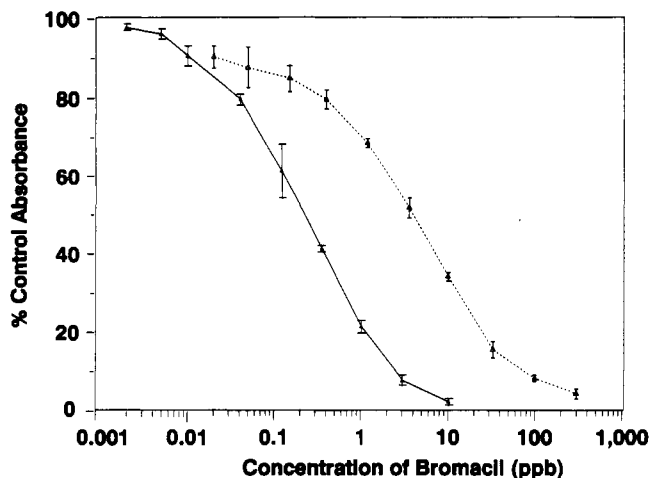


Figure 5. Standard curve constructed with system of antibody raised against immunizing antigen 3ek, serum 2005, and coating antigen 2cb (solid line) or against immunizing antigen 2ck, serum 2370, and coating antigen 3eb (dashed line). Bars indicate standard deviation for at least four independent replicates.

to increase the sensitivity of an immunoassay. Thus, heterologous combinations of antibodies and coating antigens displaying the highest titers were selected for competitive inhibition assays.

The systems of choice were optimized by two-dimensional titration according to the method of Gee et al. (1988), and then the sensitivities to bromacil were determined by competitive inhibition ELISA (Figure 5). Although the sera raised against conjugates of hapten 2b gave higher titer as mentioned above, antibodies derived from conjugates 3e were more sensitive for bromacil.

The position of hapten conjugation in the immunizing antigen strongly affects the binding strength of the target analyte to the antiserum. Modification of the 6-methyl group (hapten 3d, Figure 3) was hypothesized to give better results than derivatization at the N-1 position (hapten 2b, Figure 2) because the hydrogen bridge bonding with the participation of the amide hydrogen probably gives a significant contribution to the stability of the antibody-analyte complex. Literature analogies also supported this expectation (Wie and Hammock, 1982, 1984; Li et al., 1991). The N-alkylated uracil derivative 2b having been coupled to KLH (conjugate 2ck, Figure 2) and used for immunization resulted in only fair sensitivity ($IC_{50} = 4$ ppb, serum 2370, Figure 5), while when conjugate 3d-KLH (3ek, Figure 3) was the immunizing antigen, a higher affinity was observed (Figure 5). The standard curve obtained with an antibody raised against the immunizing antigen 3ek (serum 2005) and the coating antigen 2cd derived from hapten 2b and BSA had the IC_{50} value of 0.25 ppb and 0.01 ppb minimum detection concentration of bromacil (Figure 5). This system showed little cross-reactivity for metabolites of bromacil (6–9, Table II) as well as for the structurally most similar herbicide, terbacil (4). This is an important practical feature of the assay. The major metabolite of terbacil [5-chloro-3-(1,1-dimethylethyl)-6-(hydroxymethyl)-2,4(1H,3H)-pyrimidinedione, 11] (Rhodes et al., 1969; Gardiner, 1975) also produced only a weak affinity. Hapten recognition for hapten 2b, i.e., sensitivities for compounds 2a and 2b and also for the N-methyl and N-butyl derivatives of bromacil (2d, 2e, Figure 2), was marginal. Cross-reactivities against hapten 3d and its synthetic intermediates 3a and 3c were relatively slight as well. The low values displayed for substances 3d and 3c were possibly because of ionization of the carboxyl group. No inhibition was found with numerous related

Table II. Cross-Reactivity of the System Including Anti-Bromacil Antibody (Serum 2005, Raised against Conjugate 3ek) and Coating Antigen 2cb^a

compound	IC_{50} , ppb	cross-reactivity, %
1, bromacil	0.247	100
6, metabolite of bromacil	7.54	3.28
7, metabolite of bromacil	828	0.030
8, metabolite of bromacil	164	0.15
9, metabolite of bromacil	37.3	0.66
4, terbacil	7.58	3.26
11, metabolite of terbacil	63.0	0.39
2a, intermediate	952	0.026
2b, hapten used for coating	56.4	0.44
2d, N-methyl derivative of bromacil	414	0.060
2e, N-butyl derivative of bromacil	8020	0.003
3a, intermediate	3.38	7.31
3c, intermediate	56.4	0.44
3d, hapten employed for immunization	12.2	2.03

^a There was no cross-reactivity ($IC_{50} > 10\,000$) observed for numerous related compounds [e.g., 2,4(1H,3H)-pyrimidinedione, 5-bromo-2,4(1H,3H)-pyrimidinedione, 5-methyl-2,4(1H,3H)-pyrimidinedione, 5,6-diamino-1,3-dimethyl-2,4(1H,3H)-pyrimidinedione, thymidine, uridine, caffeine, and 1-methyluric acid].

compounds (e.g., various 5- and/or 6-alkyl-, amino-, halo-, and nitroaracils, caffeine, 1-methyluric acid) listed, in part, in Table II at concentration of 10 000 ppb. Further cross-reactivity studies are in progress.

The results obtained so far demonstrate the potential usefulness of these systems for selective quantitative detection of bromacil at the very low levels commonly found in environmental samples. These reagents are currently being examined in several immunoassay formats. Possibly assay sensitivity could be improved further by substituting enzyme tracers for coating antigens with homologous position attachment but heterologous handles. Further evaluation (e.g., application to environmental samples) of these assays will also be performed.

ABBREVIATIONS USED

BSA, bovine serum albumin; CONA, conalbumin; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; EI, electron impact; ELISA, enzyme-linked immunosorbent assay; EtOAc, ethyl acetate; FAB, fast atom bombardment; GC, gas chromatography; Hex, hexane; HPLC, high-performance liquid chromatography; IC_{50} , analyte concentration required for 50% inhibition; IR, infrared spectroscopy; KLH, keyhole limpet hemocyanin; MS, mass spectrometry; NMR, nuclear magnetic resonance spectroscopy; OD, optical density; OVA, ovalbumin; PBS, phosphate-buffered saline (pH 7.5); PBSTA, 0.2 M phosphate buffer + 0.8% NaCl + 0.02% NaN_3 + 0.05% Tween 20 (pH 7.5); PTC, phase-transfer catalysis; THF, tetrahydrofuran; TLC, thin-layer chromatography; UV, ultraviolet-visible spectroscopy.

ACKNOWLEDGMENT

The tremendous assistance of M. Dunlop and D. Jones (University of California, Davis, Facility for Advanced Instrumentation) in recording and interpretation of mass spectra is appreciated. S. J. Gee, M. Nasiri, and P. Schneider (University of California, Davis) and K. S. Goh (CDPR, Sacramento, CA) are thanked for helpful discussions. F. E. Henze and E. W. Zahnow (E. I. du Pont de Nemours & Co., Wilmington, DE) generously provided samples of bromacil, terbacil, and related compounds.

LITERATURE CITED

- Aakerblom, M.; Thoren, L.; Staffas, A. Determination of Pesticides in Drinking Water. *Vaar Foeda* 1990, 42, 236-243.
- Allender, W. J. Movement of Bromacil and Hexazinone in a Municipal Site. *Bull. Environ. Contam. Toxicol.* 1991, 46, 284-291.
- Alva, A. K.; Singh, M. Sorption of Bromacil, Diuron, Norflurazon, and Simazine at Various Horizons in Two Soils. *Bull. Environ. Contam. Toxicol.* 1990, 45, 365-374.
- Bennett, P. H.; de Beer, P. R. A Rapid Quantitative Bioassay for the Determination of Biologically Active Bromacil in Soils. *Pestic. Sci.* 1984, 15, 425-430.
- Brinkley, M. A Brief Survey of Methods for Preparing Protein Conjugates with Dyes, Haptens, and Cross-Linking Reagents. *Bioconjugate Chem.* 1992, 3, 2-13.
- California Code of Regulations. *Food and Agriculture. Title 3; Barclays Law Publishers: South San Francisco, CA, 1991; Section 6800.*
- Call, D. J.; Brooke, L. T.; Kent, R. J.; Knuth, M. L.; Poirier, S. H.; Hout, J. M.; Lima, A. R. Bromacil and Diuron Herbicides: Toxicity, Uptake, and Elimination in Freshwater Fish. *Arch. Environ. Contam. Toxicol.* 1987, 16, 607-613.
- Cheung, P. Y. K.; Gee, S. J.; Hammock, B. D. Pesticide Immunoassay as a Biotechnology. In *The Impact of Chemistry on Biotechnology: Multidisciplinary Discussions*; Phillips, M. P., Shoemaker, S. P., Middlekauff, R. D., Ottenbrite, R. M., Eds.; ACS Symposium Series 362; American Chemical Society: Washington, DC, 1988; pp 217-229.
- Dehmlow, E. V.; Dehmlow, S. S. *Phase Transfer Catalysis*; Ebel, H. F., Ed.; Verlag Chemie: Weinheim, FRG, 1983; pp 133-134.
- Di Corcia, A.; Marchetti, M. Multiresidue Method for Pesticides in Drinking Water Using a Graphitized Carbon Black Cartridge Extraction and Liquid Chromatographic Analysis. *Anal. Chem.* 1991, 63, 580-585.
- Foster, G. D.; Foreman, W. T.; Gates, P. M. Performance of the Goulden Large-Sample Extractor in Multiclass Pesticide Isolation and Preconcentration from Stream Water. *J. Agric. Food Chem.* 1991, 39, 1618-1622.
- Fröhlich, D.; Meier, W. HPLC Determination of Triazines in Water Samples in the ppt-Range by On-Column Trace Enrichment. *J. High Resolut. Chromatogr.* 1989, 12, 340-342.
- Gardiner, J. A. Substituted Uracil Herbicides. In *Herbicides. Chemistry, Degradation, and Mode of Action*; Kearney, P. C., Kaufman, D. D., Eds.; Dekker: New York, 1975; pp 293-322.
- Gardiner, J. A.; Reiser, R. W.; Sherman, H. Identification of the Metabolites of Bromacil in Rat Urine. *J. Agric. Food Chem.* 1969a, 17, 967-973.
- Gardiner, J. A.; Rhodes, R. C.; Adams, J. B.; Soboczenski, E. J. Synthesis and Studies with 2-¹⁴C-Labeled Bromacil and Terbacil. *J. Agric. Food Chem.* 1969b, 17, 980-986.
- Garrett, N. E.; Stack, H. F.; Waters, M. D. Evaluation of the Genetic Activity Profiles of 65 Pesticides. *Mutat. Res.* 1986, 168, 301-325.
- Gee, S. J.; Miyamoto, T.; Goodrow, M. H.; Buster, D.; Hammock, B. D. Development of an Enzyme-Linked Immunosorbent Assay for the Analysis of the Thiocarbamate Herbicide Molinate. *J. Agric. Food Chem.* 1988, 36, 863-870.
- Goodrow, M. H.; Harrison, R. O.; Hammock, B. D. Hapten Synthesis, Antibody Development, and Competitive Inhibition Enzyme Immunoassay for *s*-Triazine Herbicides. *J. Agric. Food Chem.* 1990, 38, 990-996.
- Greirson, B. N.; Allen, D. G.; Gare, N. F.; Watson, I. M. Development and Application of an Enzyme-Linked Immunosorbent Assay for Lupin Alkaloids. *J. Agric. Food Chem.* 1991, 39, 2327-2331.
- Hall, J. C.; Deschamps, R. J. A.; McDermott, M. R. Immunoassays to Detect and Quantitate Herbicides in the Environment. *Weed Technol.* 1990, 4, 226-234.
- Hammock, B. D.; Mumma, R. O. Potential of Immunochemical Technology for Pesticides Analysis. In *Recent Advances in Pesticide Analytical Methodology*; Harvey, J., Jr., Zweig, G., Eds.; ACS Symposium Series 136; American Chemical Society: Washington, DC, 1980; pp 321-352.
- Hammock, B. D.; Gee, S. J.; Harrison, R. O.; Jung, F.; Goodrow, M.; Li, Q. X.; Lucas, A.; Szekacs, A.; Sundaram, A. Immunochemical technology in environmental analysis: Addressing critical problems. In *Immunochemical methods for environmental analysis*; Van Emon, J., Mumma, R., Eds.; ACS Symposium Series 442; American Chemical Society: Washington, DC, 1990; pp 112-139.
- Harrison, R. O.; Goodrow, M. H.; Hammock, B. D. Competitive Inhibition ELISA for the *s*-Triazine Herbicides: Assay Optimization and Antibody Characterization. *J. Agric. Food Chem.* 1991, 39, 122-128.
- Hirota, K.; Yamada, Y.; Asao, T.; Senda, S. Pyrimidine Derivatives and Related Compounds. Part 36. Nucleophilic Addition Reaction of Cyanide Ion to 6-Substituted 1,3-Dimethyl-5-nitrouracils. Synthesis of 5,6-Dihydrouracil and 5,6-Dihydro-cyclothymine Derivatives. *J. Chem. Soc., Perkin Trans. 1* 1981, 1896-1899.
- Jordan, L. S.; Clerx, W. A. Accumulation and Metabolism of Bromacil in Pineapple Sweet Orange (*Citrus sinensis*) and Cleopatra Mandarin (*Citrus reticulata*). *Weed Sci.* 1981, 29, 1-5.
- Jung, F.; Székács, A.; Li, Q.; Hammock, B. D. Immunochemical Approach to the Detection of Aminotriazoles Using Selective Amino Group Protection by Chromophores. *J. Agric. Food Chem.* 1991, 39, 129-136.
- Li, Q. X.; Hammock, B. D.; Seiber, J. N. Development of an Enzyme-Linked Immunosorbent Assay for the Herbicide Bentazon. *J. Agric. Food Chem.* 1991, 39, 1537-1544.
- Lipschitz, C.; Irth, H.; De Jong, G. J.; Brinkman, U. A. T.; Frei, R. W. Trace Enrichment of Pyrimidine Nucleobases, 5-Fluorouracil and Bromacil on a Silver-Loaded Thiol Stationary Phase with On-Line Reversed Phase High-Performance Liquid Chromatography. *J. Chromatogr.* 1989, 471, 321-334.
- Luckenbaugh, R. W.; Soboczenski, E. J. Method for the Control of Undesirable Vegetation. U.S. Patent 3,235,363, 1966.
- Moilanen, K. W.; Crosby, D. G. The Photodecomposition of Bromacil. *Arch. Environ. Contam. Toxicol.* 1974, 2, 3-8.
- Newsome, W. H.; Collins, P. G. Determination of Imazamethabenz in Cereal Grain by Enzyme-Linked Immunosorbent Assay. *Bull. Environ. Contam. Toxicol.* 1991, 47, 211-216.
- Ogierman, L. Gas Chromatography of Uracil Herbicides by On-column Methylation with Trimethylanilinium Hydroxide. *J. Assoc. Off. Anal. Chem.* 1986, 69, 912-914.
- Parent, D.; Hourmant, N.; Guedes, Y.; Riche, C.; Thouvenot, D. Evaluation of the Hepatotoxicity and Hematotoxicity of Plant Protection Agents in Rat Cells in Culture. *Meded. Fac. Landbouwwet., Rijksuniv. Gent* 1990, 55, 1369-1375.
- Pease, H. L.; Deye, J. F. Bromacil. In *Analytical Methods for Pesticides, Plant Growth Regulators, and Food Additives. Additional Principles and Methods of Analysis*; Zweig, G., Ed.; Academic Press: New York, 1967; Vol. V, pp 335-346.
- Putzien, J. Enrichment and Determination of Pesticides in Drinking Water. *Vom Wasser* 1987, 68, 33-41.
- Rao, P. S. C.; Hornsby, A. G.; Jessup, R. E. Indices for Ranking the Potential for Pesticide Contamination of Groundwater. *Proc.—Soil Crop Sci. Soc. Fla.* 1985, 44, 1-8.
- Rhodes, R. C.; Reiser, R. W.; Gardiner, J. A.; Sherman, H. Identification of the Metabolites of Terbacil in Dog Urine. *J. Agric. Food Chem.* 1969, 17, 974-979.
- Schlett, C. Multi-Residue-Analysis of Pesticides by HPLC after Solid Phase Extraction. *Fresenius' J. Anal. Chem.* 1991, 339, 344-347.
- Sedor, F. A.; Sander, E. G. The Effect of Thiols on the Dehalogenation of 5-Iodo and 5-Bromouracil. *Biochem. Biophys. Res. Commun.* 1973, 50, 328-336.
- Senda, S.; Hirota, K.; Asao, T. Pyrimidine Derivatives and Related Compounds. XXV. The Synthesis of 6-Cyanouracil Derivatives and the Conversion of 6-Cyano-1,3-dimethyluracil to 5-Cyano Compound. *J. Org. Chem.* 1975, 40, 353-356.
- Shriver, J. W.; Bingham, S. W. Physiological Effects of Bromacil on Kentucky Bluegrass and Orchardgrass. *Weed Sci.* 1973, 21, 212-217.
- Spener, F.; Mangold, H. K. Reactions of Aliphatic Methanesulfonates VII. Chain Elongation by Two Methylene Groups. *Chem. Phys. Lipids* 1973, 11, 215-218.

- Stan, H. J.; Heil, S. Two-Dimensional Capillary Gas Chromatography with three Selective Detectors as a Valuable Tool in Residue Analysis—State-of-the Art. *Fresenius' J. Anal. Chem.* 1991, 339, 34–39.
- Szabo, L.; Kalman, T. I.; Bardos, T. J. The Reaction of 5-Bromouracil Derivatives with Sulfur Nucleophiles, and a Novel Synthetic Route to 5-Sulfur-Substituted Uracils and Nucleotides. *J. Org. Chem.* 1970, 35, 1434–1437.
- Teshima, R.; Kawase, M.; Tanaka, T.; Hirai, K.; Sato, M.; Sawada, J.; Ikebuchi, H.; Ichinoe, M.; Terao, T. Production and Characterization of a Specific Monoclonal Antibody Against Mycotoxin Zearalenone. *J. Agric. Food Chem.* 1990, 38, 1618–1622.
- Tuinstra, L. G. M. T.; Roos, A. H.; Matser, A. M.; Traag, W. A.; Van Rhijn, J. A. Development of a Multi-Residue/Multi-Matrix Method for Pesticide Analysis in Agricultural Products. *Fresenius' J. Anal. Chem.* 1991, 339, 384–386.
- U.S. Environmental Protection Agency. *National Pesticide Survey, Survey Analytes. Fall 1990*; U.S. EPA Office of Water, Office of Pesticides and Toxic Substances: Washington, DC, 1990.
- Valencia, R. Mutagenesis Screening of Pesticides *Drosophila*. *Gov. Rep. Announce. Index (U.S.)* 1981, 81, 2555; *Chem. Abstr.* 1981, 95, 92276g.
- Vanderlaan, M.; Watkins, B. E.; Stanker, L. Environmental Monitoring by Immunoassay. *Environ. Sci. Technol.* 1988, 22, 247–254.
- Voller, A.; Bidwell, D. E.; Bartlett, A. Enzyme Immunoassay in Diagnostic Medicine: Theory and Practice. *Bull. W.H.O.* 1976, 53, 55–65.
- Wessendorf, M. W.; Tallaksen-Greene, S. J.; Wohlhueter, R. M. A Spectrophotometric Method for Determination of Fluorophore-to-Protein Ratios in Conjugates of the Blue Fluorophore 7-Amino-4-methylcoumarin-3-acetic Acid (AMCA). *J. Histochem. Cytochem.* 1990, 38, 87–94.
- Wie, S. I.; Hammock, B. D. Development of Enzyme-Linked Immunosorbent Assays for Residue Analysis of Diflubenzuron and BAY SIR 8514. *J. Agric. Food Chem.* 1982, 30, 949–957.
- Wie, S. I.; Hammock, B. D. Comparison of Coating and Immunizing Antigen Structure on the Sensitivity and Specificity of Immunoassays for Benzoylphenylurea Insecticides. *J. Agric. Food Chem.* 1984, 32, 1294–1301.
- Worthing, C. R., Hance, R. J., Eds. *The Pesticide Manual. A World Compendium*, 9th ed.; The British Crop Protection Council: Farnham, Surrey, U.K., 1991.
- Wylie, P. L.; Oguchi, R. Pesticide Analysis by Gas Chromatography with Novel Atomic Emission Detector. *J. Chromatogr.* 1990, 517, 131–142.
- Yanase, D.; Andoh, A.; Yasudomi, N. A New Simple Bioassay to Evaluate Photosynthetic Electron-Transport Inhibition Utilizing Paraquat Phytotoxicity. *Pestic. Biochem. Physiol.* 1990, 38, 92–98.
- Zimmermann, G. M.; Weil, L.; Quentin, K.-E. Bioassay for the Determination of Herbicides in Water. Sensitivity and Determination. *Vom Wasser* 1990, 74, 195–206.
- Zweig, G.; Sherma, J. Gas Chromatographic Analysis. In *Analytical Methods for Pesticides and Plant Growth Regulators*; Zweig, G., Ed.; Academic Press: New York, 1972; Vol. VI, pp 65, 603–604.

Received for review November 12, 1991. Accepted May 6, 1992.

Registry No. 1, 314-40-9; 2a, 142103-13-7; 2b, 142103-14-8; 2d, 7692-45-7; 2e, 142103-15-9; 3a, 142103-16-0; 3b, 142103-17-1; 3c, 142103-18-2; 3d, 142103-19-3.