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

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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 (Puttien, 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 Oghuchi, 1990; Stan and Heil, 1991). Bioassays for bromacil appear to 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 pg/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.

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Figure 1. Structure of bromacil (1).
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-dimethyleryl)-6-methyl-2,4(1H,3H)-pyrimidinedione, a commonly used, related herbicide, 4] (Gardiner et al., 1969a; Worthing and Hance, 1991) and the photodegradation product of bromacil [5-bromo-6-methyl-2,4(1H,3H)-pyrimidinedione, 5] (Mollanen 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(1H,3H)-pyrimidinedione (6), 5-bromo-3-(2- and 3-hydroxy-1-methylpropyl)-6-methyl-2,4(1H,3H)-pyrimidinedione (7 and 8, respectively), and 6-methyl-3-(1-methylpropyl)-2,4-(1H,3H)-pyrimidinedione (9) (Gardiner et al., 1969a; Shrifer 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 (Wilmingtom, DE). Preparative TLC purifications were carried out on Whatman PLK5F 150A (2F24, 20 cm x 20 cm x 1000 mm) silica gel plates (Fisher Scientific, Pittsburgh, PA). Immunoreagents 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 a model 259 FTIR spectrophotometer (IBM Instruments, Inc.); wavenumber (cm⁻¹) values are given. 1H and 13C NMR spectra were obtained with a JE-300 spectrometer (General Electric, 300 MHz for 1H and 75 MHz for 13C nuclei). Chemical shifts (6) are given in parts per million (ppm) relative to tetramethylsilane as internal reference. Electron impact mass spectra were determined on a Trio-2 (VG Maslab, 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 x 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, Wythenshaw, 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 Vmax microplate reader (Molecular Devices, Menlo Park, CA).

Synthesis of Hapten. The structures of haptenbs 2b and 3d, the corresponding synthetic intermediates (2a, 3a-e) 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(1H,3H)-pyrimidinedione (1, Figure 1) of 99% purity (GC-MS) served as starting material; mp 158-159 °C [lit. mp 158-159 °C (Worthing and Hance, 1991)] and MS characteristics were in accord with literature spectral data (Pease and Deye, 1967; Gardiner et al., 1969a); 1H NMR (CDCl3) 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) 25.54, 45.74, 79.75, 106.57, 157.82. Ethyl 5-I5-Bromo-6-methyl-3-(1-methylpropyl)-2,4(1H,3H)-pyrimidinedione-1-ylhexanoate (2a, R = (CH₂)₂CO₂H, 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 x 15 mL), and the combined ethereal extracts were washed with water (5 x 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), 2.51 (s, 3 H), 3.89 (t, J = 7 Hz, 2 H), 4.95 (m, 1 H), 11.18, 17.36, 19.64, 25.84, 52.68, 97.37, 148.45, 152.67, 159.78. Naltrexone (19) was prepared by the method of Worthing and Hance (1969a); IR (NaCl) 1739, 1702, 1658, 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), 2.51 (s, 3 H), 3.89 (t, J = 7 Hz, 2 H), 4.13 (q, J = 7 Hz, 2 H), 4.58 (m, 1 H), 11.18, 17.36, 19.64, 25.84, 52.68, 97.37, 148.45, 152.67, 159.78.
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 67-70°C; IR (NaCl) 3450, 1606, 1534, 1424, 764 cm⁻¹; 1H NMR (CDCl₃) δ 0.73-1.03 (m, 3 H), 1.77 (m, 3 H), 3.17 (t, J = 7.5 Hz, 2 H), 4.12 (m, 2 H); 13C NMR (CDCl₃) δ 17.71, 25.82, 30.99, 53.32, 98.81, 150.18, 152.96, 156.48, 178.83; MS (EI) m/z (relative intensity) 202 (100), 204 (92), 245 (89), 247 (82), 263 (62), 265 (58), 289 (39), 291 (3), 301 (2), 303 (3), 305 (1), 318 (2, M⁺), 320 (2, M + 2); and MS (M/2) m/z (relative intensity) 319 (100, [M + H⁺]), 321 (98, M + H₂⁺). 

**Preparation of Conjugates.** Conjugates of Acid 2b (2e). The mixed anhydride was formed under nitrogen with stirring. The solution of hapten 2b [R = (CH₂)₂CO₂H, Figure 2] (11.3 mg, 0.030 mmol) in 2.0 mL of a mixture of THF and triethylamine (144 μL of 0.25 M solution in dry THF, 0.036 mmol) was added dropwise to 4 °C to the reaction mixture. The solution was heated 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 °C. The reaction mixtures were stirred with ice-cooling for 1 h and finally at room temperature overnight.

**Preparation of Conjugates.** Conjugates of Acid 2d (2e). The mixed anhydride of acid 2d [R = CH₃CO₂H, 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 2eb, 2ek, and 2eo (Figure 2), respectively. There was no significant increase in the optical densities of the conjugates compared to those of the proteins. There may be due to the lower molar absorbance of hapten 2d compared to that of 2b, because the lower epitope density in this set of conjugates. However, the successful assays employing these conjugates demonstrated exactly that the coupling reaction with the proteins did take place.
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 \( R = \text{(CH}_2\text{)}_6\text{CO}_2\text{CH}_3 \), 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 2bc, 2cc, 2ck, and 2co, respectively (\( R = \text{(CH}_2\text{)}_6\text{CONH} - \text{PROT}; \text{PROT}, \text{protein, Figure 2} \)). 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 2bc, 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-
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 2h having been coupled to KLH (conjugate 2ck, Figure 2) and used for immunization resulted in only fair sensitivity (IC50 = 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 2ck, serum 2370, and coating antigen 3eb (dashed line). Bars indicate standard deviation for at least four independent replicates.

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### 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; IC50, analyte concentration required for 50% inhibition; IR, infrared spectroscopy; KLB, keyhole limpet hemocyanin; MS, mass spectrometry; NMR, nuclear magnetic resonance spectroscopy; OD, optical density; OVA, ovalbumin; PBS, phosphate-buffered saline (pH 7.5); PBST, 0.2 M phosphate buffer + 0.8% NaCl + 0.02% NaN3 + 0.05% Tween 20 (pH 7.5); PTC, phase-transfer catalysis; THF, tetrahydrofuran; TLC, thin-layer chromatography; UV, ultraviolet-visible spectroscopy.

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LITERATURE CITED


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