# Development of an Enzyme-Linked Immunosorbent Assay for the Herbicide Bentazon

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An ELISA method for the herbicide bentazon was developed. The approach to hapten synthesis addressed the problem of the presence of an ionizable NH group. Three immunogens were used to induce polyclonal antibodies toward bentazon and its derivatives in rabbits. One immunogen with a haptenic spacer at the sulfonamide NH of bentazon provided specific and sensitive antibodies to bentazon derivatives. The antibodies against succinylated KLH linked to bentazon through the NH showed very low affinity to bentazon and its derivatives. The third immunogen with a haptenic spacer at the aromatic ring of bentazon failed to induce bentazon-specific antibodies. The sensitivity and specificity of the resulting assays were investigated with different combinations of bentazon derivatives as immunogens and coating antigens. Solid-phase extraction and derivatization were employed to increase assay sensitivity. Detection limits for N-ethylated and N-methylated bentazon ranged from 0.01 to 0.1 μM (2–24 ppb of bentazon equivalent) in assay buffer. Gas chromatography (GC) was used as a comparison test to validate the ELISA procedure for *N*-methylbentazon. The correlation between data from GC and ELISA analyses was 0.95 with a slope of about 1.0.

## INTRODUCTION

Pesticide contamination of surface water and groundwater represents a major problem, frequently limiting water uses. Bentazon (3-isopropyl-1*H*-2,1,3-benzothiadiazin-4(3*H*)-one 2,2-dioxide) was one of the major herbicides used in rice fields in California until it was suspended in 1989 because of concerns over groundwater contamination (NCAMP, 1989). The major existing method for bentazon residue analysis uses gas chromatography (GC), of a bentazon derivative, and often produces cochromatographing interferences (BASF, 1978; Gaynor and MecTavish, 1981; Cessna, 1985; Ross et al., 1989). A rapid and economic analytical method for bentazon was urgently needed to monitor its presence in water. Immunochemical methods offer relatively quick and cost-effective approaches for environmental chemical analysis (Hammock and Mumma, 1980; Van Emon et al., 1989; Vanderlaan et al., 1988; Hammock et al., 1987; Jung et al., 1989; Newsome, 1986; Fukal and Kas, 1989).

Bentazon is a moderately water soluble and nonvolatile small molecule, possessing a unique cyclic sulfonamide structure. Because of these chemical and physical properties, it is a good target for ELISA-based residue analysis. However, many questions arose during hapten design, such as appropriate spacer position, type of spacer, and how to deal with the presence of an ionizable NH group. The key feature of bentazon is its acidity, such that the sulfonamide NH can be easily deprotonated, yielding multiple resonance forms. Therefore, haptens with a spacer attached at the NH and on the aromatic ring of bentazon allowed us to compare the production and characteristics of antibodies raised from neutral and anionic hapten conjugates. The present study addresses two spacer positions for haptenic synthesis: the NH of bentazon and the OH of 6-hydroxybentazon. Spacer coupling through the NH provided a hapten and resulting antibodies which yielded an assay with low detection limits for N-alkylated bentazon derivatives.

## MATERIALS AND METHODS

**Chemicals.** Bentazon, 6-hydroxybentazon [6-hydroxy-3-isopropyl-1*H*-2,1,3-benzothiadiazin-4(3*H*)-one 2,2-dioxide], and 8-hydroxybentazon (8-hydroxy-3-isopropyl-1*H*-2,1,3-benzothiadiazin-4(3*H*)-one 2,2-dioxide) were generously provided by BASF Corp. 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC) and proteins used in coupling were purchased from Sigma Chemical Co. Other chemical reagents were purchased from Aldrich Chemical Co. Thin-layer chromatography (TLC) was performed on 0.25-mm precoated silica gel 60 $F_{254}$ plastic sheets (E. Merck). Compounds were detected by viewing under UV light (254 nm) and then by exposure to iodine vapor. Column chromatography was carried out on silica gel (J. T. Baker, ca. 40 μm in diameter). The purity of all compounds was supported by TLC in several solvent systems (Table I), nuclear magnetic resonance spectrometry (NMR), and melting points which are uncorrected.

**Apparatus.** ELISAs were carried out in 96-well microplates (Nunc 442404) and read with a $V_{max}$ microplate reader (Molecular Devices, Menlo Park, CA). Infrared (IR) spectra were determined on an IBM IR/32 FTIR spectrometer. $^1$H NMR spectra were measured with a Varian EM-390 90-MHz spectrophotometer or QE-300 300-MHz spectrophotometer (General Electric) using tetramethylsilane as an internal standard. Mass spectra (MS) were obtained on a Trio-2 mass spectrometer (VG Masslab) using 70-eV EI. Ultraviolet–visible (UV) spectra were obtained on a HP 8452A diode array spectrophotometer (Hewlett-Packard). GC analysis was done on a Hewlett-Packard 5890 equipped with NP-TSD or flame photometric detectors.

**Extraction of Water Samples.** Tap water was fortified with bentazon at different levels and extracted according to the procedure of Higashi (1987) with slight modification. Fortified water samples (50–400 mL) were acidified with 10–80 mL (20%) of 1 N HCl. $C_8$ Bondelut cartridges (3 mL) were prewashed with 3 mL of methanol and then 3 mL of 0.1 N HCl. The acidified water samples were passed through the prewashed cartridge at a flow of about 15 mL/min under vacuum. The cartridges were next rinsed with ca. 3 mL of 0.1 N HCl and then placed in a centrifuge tube, where they were eluted with 3 mL of acetone while centrifuging (500*g*) for 1 min. After the removal of the acetone under nitrogen, diazomethane in ether was added until the solution maintained a permanent yellow color, and the resulting solution was allowed to sit at room temperature for 10 min with mixing. The excess of diazomethane was evaporated under a stream of nitrogen, and then the volume was adjusted with hexane for GC analysis. For ELISA analysis, acetonitrile was used.

**Gas Chromatography.** Quantitation was done with two different columns and two different detectors. In one case, a

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Table I. $R_f$ Values of Bentazon Derivatives[a]

| compd | $R_f$ I | $R_f$ II | $R_f$ III |
|---|---|---|---|
| 1 | 0.30 | 0.57 | 0.64 |
| 2 | 0.47 | 0.67 | 0.81 |
| 3 | 0.24 | 0.51 | 0.79 |
| | 0.30 | 0.56 | |
| 4 | 0.26 | 0.56 | 0.82 |
| 5 | 0.35 | 0.59 | 0.80 |
| 6 | 0.24 | 0.50 | 0.79 |
| 7 | 0.10 | 0.41 | 0.67 |
| 8 | 0.04 | 0.33 | 0.53 |
| 9 | 0.42 | | |
| 10 | | 0.66 | |
| 11 | | 0.66 | |

[a] The TLC solvent systems for I, II, and III are tetrahydrofuran (THF)/ethyl acetate/hexane (2:13:35), ethyl acetate/hexane/acetic acid (49.5:49.5:1), and chloroform/methanol/acetic acid (89:10:1), respectively. Two $R_f$ values of compound 3 indicate cis and trans isomers.

nitrogen–phosphorus (NP) detector and DB-5 column (30 m × 0.53 mm, 1.5 μM film thickness) were used with injector and detector temperatures at 235 and 250 °C, respectively, and a programmed oven temperature of 160 °C for 4 min and then 20 °C/min to 185 °C for 2 min and 25 °C/min to a final 220 °C for 2 min. The helium, hydrogen, and air flows were 12, 3.5, and 90 mL/min, respectively. In the second case, the GC was equipped with an flame photometric (FP) detector (sulfur mode) and DB-1701 column (15 m × 0.52 mm, 1.5 μM film thickness). The helium, hydrogen, and air flows were 12.4, 3.5, and 105.6 mL/min, respectively. The column, injector, and detector temperatures were 180, 250, and 275 °C, respectively. The quantitation was done relative to an external standard of *N*-methylbentazon by using peak area determined with a Hewlett-Packard Model 3393A integrator.

**Synthesis of Haptens.** *3-Isopropyl-1-(trifluoroacetyl)-2,1,3-benzothiadiazin-4(3H)-one 2,2-Dioxide* (1). Excess trifluoroacetic anhydride was added to a solution of bentazon in ether. After concentration on a rotary evaporator, the product was obtained as a white solid in 100% yield: mp 120–125 °C; IR (KBr) 1870 (s, C=O), 1710 (s, C=O), 1305, 1216 (s, $SO_2$) $cm^{-1}$; $^1H$ NMR ($CDCl_3$) δ 1.57 (d, $J$ = 7 Hz, 6 H, 2 $CH_3$), 5.1 (m, 1 H, CH), 7.2 (m, 1 H, aromatic), 7.6 (m, 2 H, aromatic), 8.2 (m, 1 H, aromatic); MS $m/z$ (relative intensity) 336 (0.4, $M^+$), 294 (26, $M^+ - C_3H_6$), 216 (100, $M^+ - C_3H_6NO_2S$).

*1-Carbisobutoxy-3-isopropyl-2,1,3-benzothiadiazin-4(3H)-one 2,2-Dioxide* (2). To a stirred solution of 480 mg (2 mmol) of bentazon and 272 mg (2 mmol) of isobutyl chloroformate in 10 mL of dry ether was added 202 mg (2 mmol) of triethylamine. The reaction mixture was heated under reflux for 30 min. After filtration, the filtrate was resolved on a silica gel column by eluting with hexane/ethyl acetate (2:1) to give 488 mg of colorless liquid oil (98%): IR (neat) 1752 (vs, C=O), 1262 (s, C—O), 1303, 1192 (s, $SO_2$) $cm^{-1}$; $^1H$ NMR ($CDCl_3$) δ 0.97 (d, $J$ = 6 Hz, 6 H, 2 $CH_3$), 1.60 (d, $J$ = 7 Hz, 6 H, 2 $CH_3$), 2.0 (m, 1 H, CH), 4.05 (d, $J$ = 6 Hz, 2 H, $CH_2$), 5.1 (m, 1 H, CH), 7.4 (m, 3 H, aromatic), 8.1 (m, 1 H, aromatic); MS $m/z$ (relative intensity) 342 (3, M + 1), 240 (23, $M^+ - C_5H_9O_2$), 198 (100, $M^+ - C_8H_{15}O_2$). Both compounds 1 and 2 were easily hydrolyzed back to bentazon in 7% $K_2CO_3$ in aqueous methanol at room temperature in 100% yield.

*3-Isopropyl-1-[(methoxycarbonyl)-2-propenyl]-2,1,3-benzothiadiazin-4(3H)-one 2,2-Dioxide* (3). Triethylamine (2 mmol) in chloroform was added dropwise to a stirred solution of bentazon (245 mg, 1.02 mmol) and methyl 4-bromocronate (270 mg, 1.5 mmol) in 40 mL of chloroform. After 1 h, the reaction mixture was filtered and the filtrate was washed successively with 0.5 N HCl, 0.5 N aqueous NaOH, and saturated $Na_2SO_4$. After drying ($Na_2SO_4$) and evaporation of solvent, the product was resolved by silica gel column chromatography and elution with hexane/ethyl acetate (2:1) to provide 241 mg (71%) of colorless oil: IR (KBr) 1717 (s, C=O), 1682 (s, C=C), 1606 (m, C=C), 1375 (s, C—N), 1307, 1196 (s, $SO_2$) $cm^{-1}$; $^1H$ NMR ($CDCl_3$) δ 1.58 (d, $J$ = 7 Hz, 6 H, 2 $CH_3$), 3.74 (s, 3 H, $CH_3$), 4.56 (d, $J$ = 6 Hz, 2 H, $CH_2CH$), 5.04 (septate, $J$ = 7 Hz, 1 H, CH), 6.08 (d, $J_{cis}$ = 15 Hz, $J_{trans}$ = 18 Hz, 1 H, $CHCO_2$), 7.0 (m, 1 H, $CH_2CH$=CH), 7.20 (d, $J$ = 9 Hz, 1 H, aromatic), 7.4 (m, 1 H, aromatic), 7.6 (m, 1 H, aromatic), 8.19 (d, $J$ = 9 Hz, 1 H, aromatic); MS $m/z$ (relative intensity) 338 (28, $M^+$), 237 (12, $M^+ - C_5H_9O_2$), 200 (9, $M^+ - CH_3SO_2CH_2CO_2$), 158 (16, $M^+ - C_5H_{10}NO_4S$), 99 (100, $M^+ - C_{10}H_{11}N_2O_4S$).

*3-Isopropyl-1-(4-nitrobenzyl)-2,1,3-benzothiadiazin-4(3H)-one 2,2-Dioxide* (4). A mixture of 2.40 g (10 mmol) of bentazon, 3.30 g (15 mmol) of 4-nitrobenzyl bromide, and 1.14 g (11 mmol) of triethylamine in 60 mL of dry ether was heated under reflux for 9 h in a $N_2$ atmosphere. The reaction mixture was washed successively with 0.5 M NaOH (2 × 50 mL), 0.5 N HCl (2 × 50 mL), and saturated $Na_2SO_4$ (50 mL). The ether extract was dried ($Na_2SO_4$) and concentrated. The final product was recrystallized from hot methanol to provide 0.75 g (31%) of 4 as light yellow crystal: mp 119–120 °C; IR (KBr) 1667 (s, C=O), 1527, 1348 (s, $NO_2$), 1304, 1199 (s, $SO_2$) $cm^{-1}$; $^1H$ NMR ($CDCl_3$) δ 1.50 (d, $J$ = 7 Hz, 6 H, 2 $CH_3$), 4.9 (m, 1 H, CH), 4.96 (s, 2 H, $CH_2$), 7.0 (m, 1 H, aromatic), 7.4 (m, 4 H, aromatic), 8.1 (m, 3 H, aromatic); MS $m/z$ (relative intensity) 375 (9, $M^+$), 360 (3.6, $M^+ - CH_3$), 333 (21.5, $M^+ - C_3H_6$), 136 (100, $M^+ - C_{10}H_{11}N_2O_4S$).

*3-Isopropyl-1-methyl-2,1,3-benzothiadiazin-4(3H)-one 2,2-Dioxide* (5). Diazomethane was prepared according to the method of De Boer and Backer (1963). Bentazon (0.28 g, 1.7 mmol) was dissolved in 10 mL of ether, and excess diazomethane in ether was added. After the mixture was stirred for 10 min at room temperature, the excess diazomethane was evaporated under a stream of nitrogen. The ether solution was then washed with 0.1 N HCl (2 × 20 mL) and followed by 0.1 N NaOH (2 × 20 mL). The extract was dried ($Na_2SO_4$) and concentrated to give 0.28 g (96%) of 5: mp 51–53 °C; IR (KBr) 1675 (vs, C=O), 1605 (m, C=C), 1375 (s, C—N), 1314, 1198 (s, $SO_2$) $cm^{-1}$; $^1H$ NMR ($CDCl_3$) δ 1.57 (d, $J$ = 7 Hz, 6 H, 2 $CH_3$), 3.30 (s, 3 H, $CH_3$), 5.01 (septate, $J$ = 7 Hz, 1 H, CH), 7.4 (m, 3 H, aromatic), 8.12 (d, $J$ = 8 Hz, 1 H, aromatic); MS $m/z$ (relative intensity) 254 (26, $M^+$), 212 (100, $M^+ - C_3H_6$).

*3-Isopropyl-1-(methylcarboxymethyl)-2,1,3-benzothiadiazin-4(3H)-one 2,2-Dioxide* (6). Compound 6 was prepared in the same fashion as 4 from bentazon and methyl bromoacetate with a yield of 63%: mp 89–90 °C; IR (KBr) 1746 (s, C=O), 1694 (s, C=O), 1606 (m, C=C), 1369 (s, C—N), 1321, 1205 (s, $SO_2$) $cm^{-1}$; $^1H$ NMR ($CDCl_3$) δ 1.58 (d, $J$ = 9 Hz, 6 H, 2 $CH_3$), 3.71 (s, 3 H, $CH_3$), 4.64 (s, 2 H, $CH_2$), 5.04 (septate, $J$ = 9 Hz, 1 H, CH), 7.21 (d, $J$ = 9 Hz, 1 H, aromatic), 7.4 (m, 1 H, aromatic), 7.6 (m, 1 H, aromatic), 8.19 (d, $J$ = 9 Hz, 1 H, aromatic); MS $m/z$ (relative intensity) 312 (1.7, $M^+$), 270 (9.4, $M^+ - C_3H_6$), 211 (58, $M^+ - C_5H_9O_2$), 132 (100, $M^+ - C_5H_{10}NO_4S$).

*1-[4-(Carboxymethyl)benzyl]-3-isopropyl-2,1,3-benzothiadiazin-4(3H)-one 2,2-Dioxide* (7). Bentazon (240 mg, 1 mmol) was treated with 230 mg (1 mmol) of 4-(bromomethyl)phenylacetic acid and 145 mg (1.4 mmol) of triethylamine in 3 mL of dry DMSO and stirred at room temperature overnight. The reaction mixture was washed with 0.5 N HCl (3 × 15 mL). After removal of the solvent, the liquid product was further purified by chromatography on a silica gel column eluted with hexane/ethyl acetate (1:1): IR (KBr) 3200 (br, OH), 1709 (s, C=O), 1682 (s, C=O), 1605 (m, C=C), 1373 (s, C—N), 1306, 1194 (s, $SO_2$) $cm^{-1}$; $^1H$ NMR ($CDCl_3$) δ 1.47 (d, $J$ = 7 Hz, 6 H, 2 $CH_3$), 3.53 (s, 2 H, $CH_2CO_2$), 4.83 (s, 2 H, $CH_2O$), 4.9 (m, 1 H, CH), 7.2 (m, 1 H, aromatic), 9.0 (m, 1 H, aromatic); MS $m/z$ (relative intensity) 388 (5, $M^+$), 344 (2.0, $M^+ - CO_2$), 149 (100, $M^+ - C_{10}H_{11}N_2O_3S$).

*6-[[4-(Carboxymethyl)benzyl]oxy]-3-isopropyl-1H-2,1,3-benzothiadiazin-4(3H)-one 2,2-Dioxide* (8). To 70 mg (0.27 mmol) of 6-hydroxybentazon in 1 mL of dry ether was added 57 mg (0.27 mmol) of trifluoroacetic anhydride while the flask was continuously shaken. After 1 h, the solvent was evaporated under a stream of $N_2$ to provide 96 mg of the *N*-trifluoroacetamide intermediate, mp 160–163 °C. The intermediate (80 mg, 0.23 mmol) and 60 mg (0.26 mmol) of 4-(bromomethyl)phenylacetic acid were dissolved in 15 mL of dry DMSO containing 2.2 equiv of triethylamine. After 1 h, the oily mixture was extracted with 30 mL of 5% $Na_2CO_3$ aqueous solution. The basic extract was washed with ether (4 × 30 mL) and then acidified to pH 1 with concentrated HCl. This solution was extracted with ethyl acetate (4 × 30 mL). After the solvent was removed from the combined extracts on a rotary evaporator, the residue was further purified on a silica gel column by elution with hexane/ethyl acetate (1:2).

Compound 8 was obtained as a brown solid: mp 174–176 °C; IR (KBr) 3260 (br, OH), 3200 (m, N—H), 1715 (s, C=O), 1655 (m, C=O), 1499 (m, C=C), 1367 (s, C—O), 1312, 1194 (s, $SO_2$) $cm^{-1}$; $^1H$ NMR ($CDCl_3$) $\delta$ 1.38 (d, $J$ = 7 Hz, 6 H, 2 $CH_3$), 3.50 (s, 2 H, $CH_2CO_2$), 4.7 (s, 2 H, $CH_2O$), 4.8 (m, 1 H, CH), 7.2 (m, 8 H, aromatic); $^1H$ NMR (DMSO-$d_6$) 1.30 (d, $J$ = 7 Hz, 6 H, 2 $CH_3$), 3.43 (s, 2 H, $CH_2CO_2$), 4.80 (s, 2 H, $CH_2O$), 4.8 (m, 1 H, CH), 7.2 (m, 8 H, aromatic), 10.0 (s, 1 H, $CO_2H$); MS $m/z$ (relative intensity), 404 (15, $M^+$), 256 (3, $M^+ - C_9H_8O_2$), 214 (13.4, $M^+ - C_9H_8O_2-C_3H_6$), 149 (100, $M^+ - C_{10}H_{11}N_2O_4S$).

*3-Isopropyl-1-(pentafluorobenzyl)-2,1,3-benzothiadiazin-4(3H)-one 2,2-Dioxide* (9). To 240 mg (1.0 mmol) of bentazon in 10 mL of methylene chloride was 10 mL of tetrapropylammonium hydroxide in 0.2 M NaOH with stirring, followed by the addition of 250 μL (1.2 mmol) of pentafluorobenzyl bromide over 1 h at room temperature. The reaction mixture was washed with 0.1 N NaOH (3 × 10 mL) and dried ($Na_2SO_4$). After removal of the solvent, the product was purified by chromatography on a silica gel using hexane/ethyl acetate (2:1) as eluant. Compound 9 was obtained in 97% yield: mp 102–103 °C; IR (KBr) 1684 (s, C=O), 1607 (m, C=C), 1385 (s, C—N), 1312 (s, $SO_2$) $cm^{-1}$; $^1H$ NMR ($CDCl_3$) $\delta$ 1.54 (d, $J$ = 7 Hz, 6 H, 2 $CH_3$), 4.92 (s, 2 H, $CH_2$), 5.0 (m, 1 H, CH) 7.22 (d, $J$ = 8 Hz, 1 H), 7.4 (m, 1 H, aromatic), 7.6 (m, 1 H, aromatic), 8.12 (d, $J$ = 8 Hz, 1 H, aromatic); MS $m/z$ (relative intensity) 420 (1, $M^+$), 378 (2, $M^+ - C_3H_6$), 181 (100, $M^+ - C_9H_{11}O_3N_2S$).

*3-Isopropyl-1-methyl-6-methoxy-2,1,3-benzothiadiazin-4(3H)-one 2,2-Dioxide* (10). The synthesis and purification procedures were the same as for compound 5: starting material was 6-hydroxybentazon; yield, 100%; mp 62–63 °C; MS $m/z$ (relative intensity) 284 (32, $M^+$), 242 (100, $M^+ - C_3H_6$).

*3-Isopropyl-1-methyl-8-methoxy-2,1,3-benzothiadiazin-4(3H)-one 2,2-Dioxide* (11). The synthesis and purification procedures were the same as for compound 5: starting material was 8-hydroxybentazon; mp 93–95 °C; IR (neat) 1681 (s, C=O), 1587 (m, C=C), 1377 (s, C—O); MS $m/z$ (relative intensity), 284 (42, $M^+$), 227 (3, $M^+ - C_3H_6CH_3$).

*1-Ethyl-3-isopropyl-2,1,3-benzothiadiazin-4(3H)-one 2,2-Dioxide* (12). Diazoethane was prepared from *N'*-nitro-*N*-nitroso-*N*-ethylguanidine. The synthesis and purification procedures were the same as for compound 5. Compound 12 was obtained in 100% yield: mp 54–55 °C; IR (neat) 1680 (s, C=O), 1605 (m, C=C), 1373 (s, C—N), 1300, 1194 (s, $SO_2$) $cm^{-1}$; $^1H$ NMR ($CDCl_3$) $\delta$ 1.17 (t, $J$ = 7 Hz, 3 H, $CH_3$), 1.57 (d, $J$ = 7 Hz, 6 H, 2 $CH_3$), 3.82 (q, $J$ = 7 Hz, 2 H, $CH_2$), 4.97 (septate, $J$ = 7 Hz, 1 H, CH), 7.4 (m, 2 H, aromatic), 8.10 (d, $J$ = 7.8 Hz, 1 H, aromatic); MS $m/z$ (relative intensity) 268 (40, $M^+$), 226 (68, $M^+ - C_3H_6$), 211 (100, $M^+ - C_3H_6CH_3$).

**Preparation of Hapten–Protein Conjugates.** Bentazon was coupled to succinylated protein according to the method of Hung et al. (1980). Compound 7 was coupled covalently to proteins by the active ester method according to the method of Bauminger and Wilchek (1980). Compound 8 was coupled according to the mixed-anhydride method (Wie and Hammock, 1982, 1984) with slight modification. Specifically, compound 8 (42 mg) was dissolved in 1 mL of dry 1,4-dioxane with stirring, and 52.3 μL of tri-*n*-butylamine was added dropwise to this solution, followed by 28.4 μL of isobutyl chloroformate. Stirring was continued for 1 h at room temperature. Then 200 mg of KLH (or 300 mg of BSA) in 30 mL of 0.2 M borate buffer (pH 8.7) was added. The solution was then stirred for 6 h at room temperature. After complete dialysis of the reaction mixture against water, the conjugate solutions were lyophilized. Each conjugate (235 mg) was then treated with 100 mL of 7% $K_2CO_3$ in aqueous methanol (methanol/$H_2O$ 7:3) and stirred for 3 h at room temperature. The conjugate solution was again extensively dialyzed against water at 4 °C. All conjugates synthesized were confirmed by UV spectra.

**Immunization of Rabbits.** The immunization protocol was that of Gee et al. (1988) with slight modification. Female New Zealand white rabbits (2–4 kg) each initially received 100 μg (protein equivalent) of a hapten–protein conjugate in 0.1 M potassium phosphate buffer (pH 7.4) mixed 1:1 with Freund's complete adjuvant intradermally in multiple spots on the back, followed by three to five boosts with 200–300 μg of immunogen on a monthly basis. At the 10th day after each boost, about 10 mL of blood was collected to monitor the antibody production.

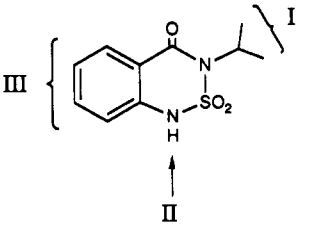



**Figure 1.** Available positions for spacer attachment on bentazon. These major sites include the isopropyl group (I), the reactive sulfonamide NH (II), and positions on the aromatic ring (III).

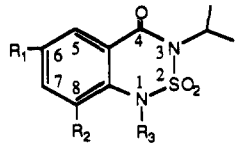
When the titer was sufficiently high, blood was collected by heart puncture. Serum was isolated by centrifugation and $NaN_3$ added to a final concentration of 0.02%. The serum was stored at −80 °C. Three rabbits were immunized with each of the immunogens (iAg1, iAg2, and iAg3).

**Enzyme Immunoassay and Competitive Enzyme Immunoassay Procedure.** Antisera were screened by solid-phase indirect EIA format using a 96-well plate coated with coating antigens (BSA conjugates) at 0.6–20 μg/mL (100 μL/well) in 0.1 M carbonate–bicarbonate coating buffer, pH 9.6. Plates were sealed with adhesive plate sealers to prevent evaporation and incubated overnight at 4 °C or 2 h at room temperature to adsorb the coating antigen to the wells. The plates were washed with PBS–Tween buffer (phosphate-buffered saline with 0.05% Tween 20 and 0.02% $NaN_3$). The samples, or standards mixed with appropriate dilution of antiserum (sample/antiserum solution 1:1), were added to the wells at 50 μL/well. For noncompetitive assay, no inhibitor was added. After 2 h or overnight, incubation at room temperature, the plates were washed again and goat anti-rabbit IgG conjugated to alkaline phosphatase diluted 1:2500 with PBS–Tween was added to each well (50 μL/well) and incubated for 1 h at room temperature. After another wash, 100 μL of a 1 mg/mL solution of 4-nitrophenyl phosphate in 10% diethanolamine buffer was added and incubated at room temperature for 20–60 min, and the absorbance was read at 405 nm with a $V_{max}$ microplate reader. Inhibition curves were analyzed by an IBM personal computer using a four-parameter logistic curve-fitting procedure which calculated $IC_{50}$ values (molar inhibitor concentration giving 50%). The coating antigen and serum concentrations were optimized by a checkerboard titration.

## RESULTS AND DISCUSSION

**Hapten Synthesis.** The bentazon structure suggests at least three positions for the spacer attachment (Figure 1). There were two opposing arguments for spacer attachment at the isopropyl group (position I). Since the isopropylimido group is a unique and less stable part of the molecule (Moss et al., 1986), it is perhaps better left underivatized for eliciting bentazon-specific antibodies. On the other hand, if a spacer is an extension of the isopropyl group, it would minimally alter bentazon's basic chemical and physical properties because it is far removed from the resonating aromatic and heterocyclic rings. The second available position, the sulfonamide NH (position II), is a strong conjugate acid ($pK_a$ = 3.4), and it is the ideal spacer position to elicit antibodies for N-derivatized bentazon analogues. The third choice is on the aromatic ring (position III). This may yield a more specific and sensitive assay for parent bentazon because the hapten will be able to exist in its normal anionic form at physiological pH due to ionization at position II (NH). Therefore, the attachment of a spacer at the sulfonamide N (II) or the aromatic ring (III) would allow us to study the effect of spacer position and electron density and/or charge on the hapten in relation to antibody characteristics. In this study, haptens with a spacer on the aromatic ring or the sulfonamide N were synthesized. Four candidate haptens (Table II, compounds 3, 4, 6, and 7) with a spacer at the secondary nitrogen were synthesized. Attempted

**Table II. Structures of Bentazon and Its Derivatives[a]**



| compd | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| 1 | H | H | $C(O)CF_3$ |
| 2 | H | H | $O_2CCH_2CH(CH_3)_2$ |
| 3 | H | H | $CH_2CH{=}CHCO_2CH_3$ |
| 4 | H | H | $CH_2C_6H_4NO_2$ (4) |
| 5 | H | H | $CH_3$ |
| 6 | H | H | $CH_2CO_2CH_3$ |
| 7 | H | H | $CH_2C_6H_4CH_2CO_2H$ (4) |
| 8 | $OCH_2C_6H_4CH_2CO_2H$ (4) | H | H |
| 9 | H | H | $CH_2C_6F_5$ |
| 10 | $OCH_3$ | H | $CH_3$ |
| 11 | H | $OCH_3$ | $CH_3$ |
| 12 | H | H | $CH_2CH_3$ |
| 13 | H | H | H |
| 14 | OH | H | H |
| 15 | H | OH | H |

[a] 13, 14, and 15 are bentazon, 6-hydroxybentazon, and 8-hydroxybentazon, respectively. Compounds 1, 4, 9, and 12 are commonly used derivatives for analysis of bentazon by gas chromatographic procedure formed by reaction of bentazon (13) with trifluoroacetic anhydride, 4-nitrobenzyl bromide, diazomethane, pentafluorobenzyl bromide, and diazoethane, respectively.

hydrolysis of 3 and 6 to the free carboxylic acids under basic and acidic conditions was not successful, precluding further efforts to use them for protein conjugate preparation. Apparently, the heterocyclic ring of 3 and 6 was so unstable that it was cleaved with 7% $K_2CO_3$ in aqueous methanol at room temperature. The reduction of the nitro group on 4 to an amine was not successful using $Fe_3(CO)_{12}$/methanol (Landesberg et al., 1972), tin (Hartman et al., 1943), or ferrous ammonium sulfate (Smith and Opie, 1955), again precluding further efforts to prepare a hapten–protein conjugate. Compound 7 was synthesized by reacting bentazon with 4-(bromomethyl)phenylacetic acid in DMSO at room temperature in the presence of triethylamine. This derivative possessed the requisite free handle, a carboxylic acid, for further use in conjugate preparation.

Derivatization of 6- and 8-hydroxybentazon was investigated to put a spacer on the aromatic ring for subsequent conjugation. Protection/deprotection of the sulfonamide NH was necessary because derivatizing reagents which might react with a phenolic OH might also react with the NH. Trifluoroacetic anhydride and isobutyl chloroformate were suitable protecting reagents for the NH because both reagents reacted quantitatively with bentazon, and the protecting group could be removed quantitatively with 7% $K_2CO_3$ in aqueous methanol. Experiments with model compounds showed that both reagents preferentially protected the NH position, leaving the phenolic OH available for derivatization. Thus, 6-hydroxybentazon was likewise treated with 1 equiv of trifluoroacetic anhydride to form *N*-(trifluoroacetyl)-6-hydroxybentazon which then reacted with 4-(bromomethyl)phenylacetic acid to give 8 after the trifluoroacetyl group was removed during basic extraction.

**Conjugation.** Three pairs of hapten–carrier protein conjugates were synthesized by two approaches (Figure 2). One approach involved modification of the protein before coupling. Proteins (BSA or KLH) were treated with succinic anhydride, followed by activation and conjugation with bentazon. The conjugates (Figure 2[1]) were confirmed by UV–vis spectra in which the antigen showed maximum absorption at about 320 nm due to the bentazon ring system. This procedure offers the advantage

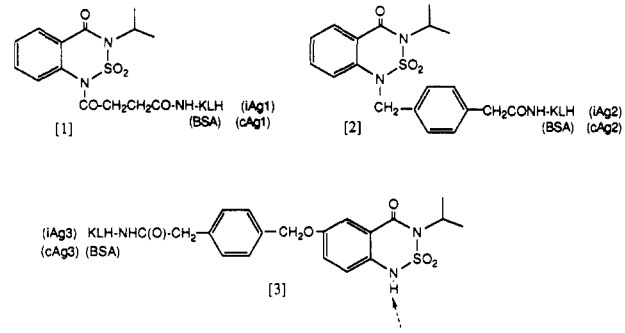



**Figure 2.** Hapten–protein conjugates prepared for bentazon immunoassay. Hapten–KLH conjugates and hapten–BSA conjugates were used as immunogen and coating antigen, respectively. With compound 8 the coupling procedure used may have led to dimerization at the NH shown with arrow.

that initial hapten synthesis is avoided. The second approach involved synthesis of a unique hapten followed by activation and coupling with carrier proteins. Two pairs of conjugates (Figure 2[2],[3]) were synthesized via the second approach. Compound 7 was treated with EDC and *N*-hydroxysuccinimide (NHS) to form the active ester which was coupled to proteins. Compound 8 was treated with isobutyl chloroformate to protect the NH, and the carboxylic acid was converted to a mixed anhydride. This "activated" hapten was then coupled by attachment at protein amine groups. A dimer might have occurred as a byproduct during the synthesis (Figure 3). These three pairs of conjugates were used to compare the effects of spacer position and type of spacer on polyclonal antibody characteristics.

**Antiserum Screening and Assay Optimization.** Titers of all antisera were tested on different coating antigens (Table III). The data show that homologous systems have higher titers than heterologous systems, indicating that the antibodies had high recognition of homologous haptens. The antisera against iAg2 gave very high titers with the three coating antigens. The antisera against iAg1 also gave very high titers with coating antigen 1 (cAg1) but much smaller values with cAg2. The antisera against iAg3 showed no recognition to cAg1. Ab1287 recognized cAg2 and cAg3 with high titers (Table III), and it did not recognize cAg1, *N*-isopropyl formate cAg3 (pcAg3 in Figure 3), and all controls. This indicated that the protecting group was indeed removed. The higher titer of Ab1287 to cAg2 is likely due to its higher hapten load (95 mol/mol of cAg2 vs 1 mol/mol of cAg3).

Primary inhibition studies for bentazon and methylbentazon were performed for each of the cAg and serum combinations giving good titer (i.e., good color development in a reasonable amount of time). To obtain a fully optimized assay, other parameters affecting the accuracy, sensitivity, and reproducibility were examined for those combinations showing good standard curves with a broad linear range, high slope, and high sensitivity. The assay combinations that met these criteria were Ab1283 (1:3000) with cAg1 or cAg3 and Ab1287 (1:800) with cAg3. The concentration of coating antigens was 5 μg/mL.

The effect of pH on Ab1283 and its interactions with coating antigens (cAg1–3) and analytes were investigated according to the method of Harrison et al. (1989). The results indicated that pH effects vary from system to system and that the antibody binding is generally maximal near physiological pH. On all systems tested, very low (3.4–4.4) or high (>8.4) pH decreased the affinity of the antibodies to the analytes less than to the coating antigens, and thus the sensitivity was increased. However, when the pH was too far from neutrality, the color development

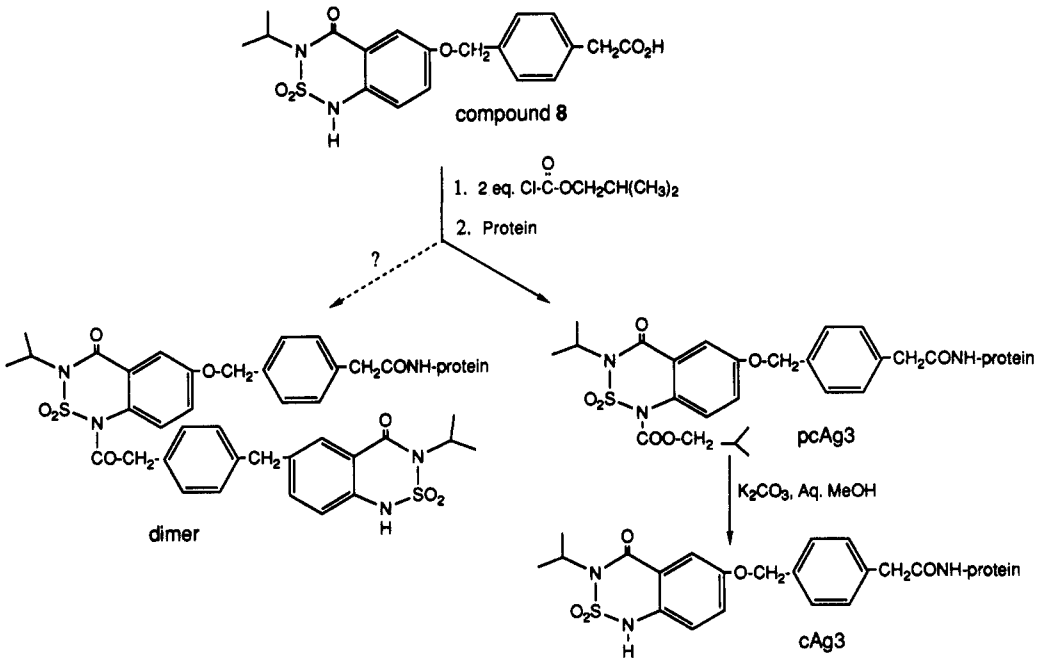



**Figure 3.** Conjugation of compound 8 with proteins to produce iAg3 and cAg3. A dimerization might occur during the reaction as indicated with dotted arrow.

**Table III. Summary of Titers of Antisera[a]**

| immunogen | rabbit no. | coating antigen[b] cAg1 | cAg2 | cAg3 |
|---|---|---|---|---|
| iAg1 | 1592[c] | >12 800 | 400 | <100 |
| | 2075[c] | 12 800 | 3 200 | 200 |
| | 2574[c] | >12 800 | 3 200 | <200 |
| iAg2 | 1282 | 12 800 | >25 600 | >25 600 |
| | 1283 | >25 600 | >25 600 | >25 600 |
| | 1284 | >25 600 | >25 600 | >25 600 |
| iAg3 | 1286 | <100 | 3 200 | 800 |
| | 1287 | 400 | 12 800 | 6 400 |
| | 1288 | <100 | <100 | 400 |

[a] The titer of antiserum is the antiserum dilution which gave twice optical density of the background. [b] BSA was used as control. All antisera did not significantly recognize BSA. [c] The antisera also gave high titers when succinylated BSA (sBSA) was used. There was no recognition of sBSA after the sera were mixed with 1 mg/mL of sBSA. This indicated that the antibodies had very high hapten–spacer recognition.

**Table IV. Optimization of Bentazon Immunoassay**

| parameter | range tested | antiserum 1283 opt range (cAg1) | opt range (cAg3) | antiserum 1287 opt range (cAg3) |
|---|---|---|---|---|
| pH | 3.4–9.4 | 6.4–7.4 | 6.4–7.4 | |
| solvent,[a] % | | | | |
| DMSO | 0–50 | <10 | <25 | <10 |
| MeOH | 0–50 | <1 | <1 | <10 |
| $CH_3CN$ | 0–50 | <10 | <50 | <10 |
| PBS concn, M | 0–4 | 0.1–0.2 | 0.1–0.2 | |
| incub time [Ag-Ab], min | 0–120 | >60 | >60 | >60 |
| incub time [Ag-well], min | 0–180 | >60 | >60 | >60 |

[a] The optimum range is defined as that range which has no effect on the standard curve. Higher solvent concentrations can be used if the same concentration is used for standards and samples. Higher solvent concentrations can reduce interference due to lipid micelles, and as explained in the text, methanol in some cases significantly improves assay sensitivity.

was very slow. It is also difficult, in practice, to provide a constant pH (even when buffer is used as the incubation medium), and thus experimental error may increase or reproducibility decrease.

The effect of ionic strength on the assays was also studied. The binding of Ab1287 to coating antigens decreased as the buffer concentration increased. The optimum buffer concentration to maximize binding was 0.1–0.2 M. The effect of calcium ion (up to 5 mM) was also tested. There was no significant change in bentazon inhibition curves using Ab1283 and Ab1287 as calcium concentration was varied.

The above results suggest that the affinities of antibodies for coating antigens differ from their affinities for analytes. Because *N*-methylbentazon is neutral and the $pK_a$ of bentazon is low (3.4), the population of the ionized form does not vary much as the buffer concentration and pH (except at pH 3.4) change. Therefore, the buffer concentration and pH appear largely to affect the antibodies and not the small hapten molecule in this case. The comparison of the effects of pH with those of the buffer concentration suggests that different mechanisms may be involved in the effect of $H^+/OH^-$ on antibody–analyte interaction and that of $Na^+/PO_4^{3-}$ on the same interaction. Although our understanding of these interactions was incomplete, this information was used effectively to improve assay performance. It is good practice to neutralize samples and to check for matrix effects due to ions.

The effect of different solvents on Ab1283 or Ab1287 and the Ab–cAg interaction was studied; the effect was very small if less than 10% acetonitrile or DMSO was added to the tested systems. Since acetonitrile, up to 50%, does not significantly affect the binding of Ab1283–cAg3 (Table IV), it can be used as the solvent to elute bentazon from solid-phase extraction cartridges employed to extract bentazon from water. Methanol has a very strong positive effect on the antibody–antigen binding. Similar effects were reported by Harrison et al. (1989). The use of a constant moderate amount of organic solvent in the assay can reduce assay variability by decreasing interference from lipid micelles with complex matrices as well as by reducing binding of analyte to surfaces.

The analysis time can be shortened by optimization of incubation time (Kemeny and Chantler, 1988; Niewola et al., 1983). The incubation time needed for coating antigen to adsorb on the well and the time required for antibody to bind coating antigen were about 30 min in both cases.

Table V. Cross-Reactivity of Antiserum 1283

| | cAg1 | | | cAg3 | | |
|---|---|---|---|---|---|---|
| compd | $IC_{50}$, μM | CR % | inh % at 400 μM | $IC_{50}$ μM | CR % | inh % at 400 μM |
| 1 | >1600 | <0.1 | 36 | >1600 | <0.1 | 36 |
| 2 | 4.6 | 41 | 98 | 3.9 | 74.7 | 97 |
| 3 | <0.01 | >19000 | 97 | 0.06 | >4800 | 98 |
| 4 | <0.01 | >19000 | 97 | <0.01 | >29000 | 97 |
| 5 | 1.9 | 100 | 78.7[a] | 2.9 | 100 | 88.4[a] |
| 6 | 1.75 | 107 | 97 | 0.7 | 414 | 97 |
| 8 | 62.9 | 3 | 77 | 400 | 0.7 | 54 |
| 9 | 0.8 | 237 | 72 | 3.2 | 91 | 84 |
| 10 | 15.4 | 12 | 67 | >2000 | <0.1 | 27 |
| 11 | 244 | 1 | 55 | >2000 | <0.1 | 37 |
| 12 | 1.78 | 107 | 97 | 1.0 | 291 | 97 |
| 13 | 110 | 58.4 | 71 | 1590 | <0.1 | 40 |
| 14 | >2000 | <0.1 | 2.3 | >2000 | <0.1 | 6 |
| 15 | >2000 | <0.1 | 10 | >2000 | <0.1 | 15 |

[a] The analyte concentration was 6.4 μM. CR % = (($IC_{50}$ of compound 5/compound $IC_{50}$) × 100). The extrapolated $IC_{50}$ values of compounds 3 and 4 are about 0.2 (67 ppt, equivalent to 48 ppt of bentazon) and 0.1 nM (37 ppt, equivalent to 24 ppt of bentazon), respectively. The data showed that the assay sensitivity was dramatically improved by derivatization.

Table VI. Cross-Reactivity of Antiserum 1287[a]

| compd | $IC_{50}$, μM | CR % | inh % at 400 μM |
|---|---|---|---|
| 1 | >2000 | <0.2 | -2 |
| 2 | 0.11 | 3518 | 95 |
| 3 | 1.13 | 342 | 95 |
| 4 | 0.02 | 19350 | 91 |
| 5 | 3.87 | 100 | 94 |
| 6 | 2.01 | 193 | 94 |
| 8 | 20.6 | 19 | 80 |
| 9 | 3.4 | 114 | 65 |
| 10 | 49.7 | 8 | 64 |
| 11 | >400 | <1 | 31 |
| 12 | 0.62 | 619 | 91 |
| 13 | >2000 | <0.2 | 1.8 |
| 14 | >2000 | <0.2 | 9.7 |
| 15 | >2000 | <0.2 | 5.3 |

[a] The coating antigen is cAg3. The calculation of CR % is the same as that shown in Table V.

The plates were routinely coated overnight at 4 °C. An incubation time of overnight at room temperature of the Ab–cAg was usually used to minimize variation. However, the times could be shortened dramatically even in an equilibrium format if fast assays are needed.

**Assay Sensitivity and Cross-Reactivity.** Inhibition data for bentazon and its derivatives using Ab1283 are summarized in Table V. Derivatization improved the assay sensitivity by increasing the slope of the standard curve and decreased the detection limit by several orders of magnitude. The same was true for Ab1287 (Table VI). Thus, both sensitivity and precision of the assays were improved by derivatization.

Very little inhibition (8–44%) was obtained with Ab2574 (1:1600, mixed with 1 mg/mL of succinylated BSA, cAg1 was used as coating antigen) at 2000 μM of bentazon and its derivatives 1–13. Due to this lack of sensitivity, this antiserum could not be used for the analysis of bentazon at residue levels using currently available coating antigens.

The inhibition studies and optimization results above demonstrate that the combinations of Ab1283 with cAg1 or cAg3 and Ab1287 with cAg3 can be used for bentazon residue analysis. The $IC_{50}$ values of *N*-methylbentazon in the three assays are 1.9, 2.9, and 3.9 μM, respectively. To characterize the specificity of the assay, several pesticides, bentazon derivatives, and bentazon metabolites were examined in the three ELISA systems (Tables V and VI). In general, derivatization at NH of bentazon increased

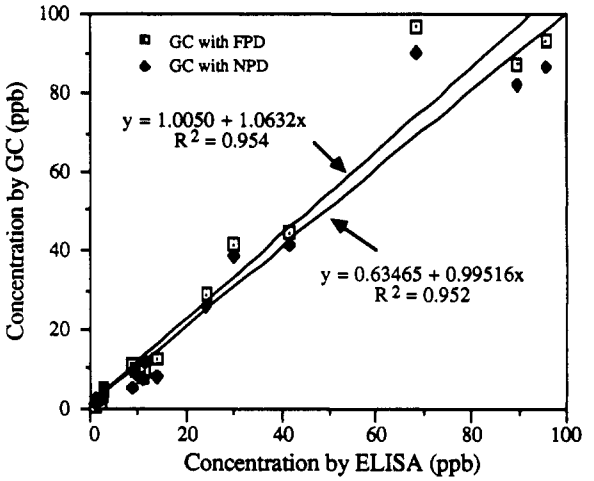



**Figure 4.** Correlation of ELISA and GC. The GC results were determined with NP and FP detectors. The data are the average of three replicates with CV % from 1 to 9 for ELISA and from 3 to 10 for GC. When the data from the two different GC detectors were correlated, an $R^2$ of 1.00 was obtained.

the sensitivity, as demonstrated by compounds 2–6, 9, and 12. The exception was compound 1, which may hydrolyze back to bentazon under assay conditions. Since these derivatives do not occur in environmental samples, they would not interfere in residue analysis of bentazon. There was no cross-reactivity found for high concentrations of other pesticides (carbaryl, 2,4-D, molinate, parathion, methyl parathion, atrazine, diazinon, carbofuran, MCPA, and thiobencarb) commonly used in rice fields or for the major bentazon metabolites (anthranilic acid, *N*-isopropylanthranilic amide, 6-hydroxybentazon, and 8-hydroxybentazon) in any of the three assay combinations (Ab1283-cAg1/cAg3 and Ab1287-cAg3).

**Assay Validation.** The *N*-(*p*-nitrobenzyl) derivative gave the highest sensitivity when either Ab1283 or Ab1287 was used. Unfortunately, we were unable to form this derivative at the bentazon NH at residue levels. The *N*-ethyl derivative of bentazon provided somewhat more sensitive assays than the *N*-methyl derivative using either Ab1283 or Ab1287. The combination of Ab1287 with cAg3 was the most sensitive combination for *N*-ethylbentazon. This was discovered after the method validation had been conducted by using *N*-methylbentazon.

Antibody selection was based upon providing the standard curve with lowest $IC_{50}$, greatest slope, largest range of inhibition, smallest cross-reactivity and interferences, and best reproducibility. The combination of Ab1283 with cAg1 best fulfilled these criteria and was thus used in the method for bentazon in water samples after methylation. Tap water spiked with bentazon (1–100 ppb) was analyzed by GC and ELISA to determine the correlation between the two methods. The acidified water samples (pH <2) were first extracted with $C_8$ Bondelut, and then acetone was used to elute bentazon from the cartridges. The eluate was methylated with diazomethane and split for GC and ELISA analysis after the solvent was exchanged to hexane and acetonitrile, respectively. The recoveries ranged from 99.3 to 118.7% with standard deviations of 1.8–13.4. There was good correlation between GC and ELISA results ($R^2$ = 0.95) with a slope of about 1.0 ($R^2$ of two GC detectors was 1.00) (Figure 4). This demonstrated that the ELISA procedure can be used for bentazon residue analysis if the bentazon is first extracted and then derivatized.

**Discussion of Antibody Specificity.** It has been previously observed that antibodies raised from a tertiary amine hapten do not recognize its secondary and primary

amine analogues and vice versa (Kabakoff, 1980; Schlaeppi et al., 1989; Harrison et al., 1989; Wie and Hammock, 1984). Thus, the finding that the antibodies against iAg1 and iAg2 would not bind to bentazon was predictable because the position of spacer attachment, at the bentazon NH, changed the molecule from an acidic secondary sulfonamide to a neutral tertiary sulfonamide. Ab1283 recognized N-derivatized bentazon analogues better than bentazon. The explanation may be that the Ab binding site cannot accept the charged bentazon, which is ionized in the incubation medium, either because of the steric effect of the water layer around the ionized nitrogen or because of the repulsion which might exist between possible hydrophobic binding sites of the antibody and ionized bentazon.

Ab1287 failed to recognize bentazon and 6-/8-hydroxybentazon (14 and 15). It had very high affinity for N-derivatized bentazon analogues but low affinity with compounds 10 and 11 (Table VI). There are four possible reasons: (1) The introduction of an electron-donating group (Hammett constant $\sigma_{para}$ of $OCH_2C_6H_5$ is −0.42) at the 6-position can alter the electron distribution in the molecule so that it is significantly different from that in bentazon. (2) A possible masking (Vallejo et al., 1982) of the sulfonamide NH as a determinant group may occur from a complex involving ionized bentazon and macromolecules. (3) The possibility exists that some dimer was formed as a byproduct during conjugate synthesis (Figure 1), leading to antibodies which recognize the dimer rather than hapten monomer. (4) The hapten may inherently have poor immunogenicity because of the resonance properties when the NH is deprotonated. A strong influence of an electron-donating group, methoxy group with a Hammett constant $\sigma_{para}$ of −0.268, was demonstrated by the increase in $IC_{50}$ from 3.9 μM for compound 5 to 49.7 and >400 μM for compounds 10 and 11, respectively. The inhibition differential of compounds 10 and 11 shows that the position of methoxy substitution exerts an effect, and in either case $IC_{50}$ was increased substantially when compared with the N-derivatives (Table VI). It is possible with compound 8 that dimer formation occurred, resulting in antibodies directed to the more readily recognized tertiary nitrogen. A hapten [with a spacer of $OCH_2CO_2H$ at the 8-position of bentazon] similar to compound 8 was synthesized, and this hapten was coupled to carrier proteins (BSA and KLH) via a water-soluble carbodiimide procedure (Goodfriend et al., 1964). However, none of the antibodies raised against the conjugates recognize bentazon even at the highest concentrations.

Inhibition studies demonstrated that the sensitivity is increased as the chain length of $R_3$ (Table II) increases; the slopes of compounds 3 (with exception of 3 to Ab1287), 4, and 9 are smaller than those of 5, 6, and 12, and parent bentazon gave very low inhibitions in all of the systems tested.

It is known that antibody–antigen binding is dependent on noncovalent interactions which include hydrogen bonds, van der Waals forces, hydrophobic bonds, etc. (Bennett and Glaudemans, 1979; Goetze and Richards, 1978; Marquart et al., 1980; Satow et al., 1986; Saul et al.; 1978; Segal et al., 1974). Bennett and Glaudemans (1979) concluded that the antibody combining site can possess much flexibility in accommodating ligands of different sizes. Logically, the more space within the pocket of the antibody binding site that the inhibitor molecule occupies, the greater the total binding energy between antibody and inhibitor. If this is the case, the binding site of Ab1283/Ab1287 may involve uncharged amino acid residues and be quite flexible, which was suggested by the data in Table V and VI.

To our knowledge, the immunogenicity of sulfonamide compounds such as bentazon has not been studied, and the effects of the type, position, and length of spacer to the induction of specific antibody for small molecules in general have not been studied systematically. Further work in these areas could offer the opportunity to generate hapten-specific antibodies for small molecules, such as bentazon, with minimum assay development work.

## CONCLUSION

Three groups of polyclonal antibodies against three different conjugates for bentazon and its derivatives were raised in rabbits. The N-derivatized bentazon conjugate (iAg2) successfully induced the antibodies (Ab1283) which are specific and sensitive for N-derivatized bentazon analogues, such as *N*-ethyl- and *N*-methylbentazon. These antibodies were designed to compare the properties of antibodies raised from neutral and anionic hapten conjugates and to detect derivatized bentazon from environmental samples. In contrast, iAg3 (the spacer is at the 6-position of bentazon) failed to raise bentazon-specific antibodies (Ab1287). Instead, Ab1287 is more like Ab1283 and even more sensitive to some of the N-derivatives such as *N*-ethylbentazon ($IC_{50}$ of 160 ppb). The conjugate formed from succinylated KLH (iAg1) also failed to produce useful antibodies for bentazon and its N-derivatives, likely due to the excess of unreacted succinic acid residues on the protein and the large difference in chemical and physical properties between the hapten and the analytes. However, this approach provided a very useful coating antigen (cAg1). Although free bentazon is not detected by the antibodies reported here, both antisera, Ab1287 and Ab1283, have high affinity for N-derivatives of bentazon such as *N*-ethyl- and *N*-methylbentazon. Thus, the antibodies can be applied to the residue analysis of bentazon provided that bentazon is first extracted and derivatized. Solid-phase extraction cartridges were used in the extraction step. The assays were validated for analysis of water spiked with bentazon by comparison with GC data.

There are several approaches which might yield a workable ELISA for parent bentazon. First, it is possible that the ether linkage in compound 8 reduced recognition of free bentazon. Although ether handles generally can be successful, using $(CH_2)_nCO_2H$ as a spacer on the aromatic ring, that is, a $CH_2$–Ar linkage, may offer advantages (Goodrow et al., 1990). Second, the isopropyl functionality also offers an alternative location for spacer placement. Derivatives in which the isopropyl group is replaced with an alkyl group terminating with an $NH_2$ or COOH could potentially be used to prepare the requisite protein conjugates. Finally, preparation of monoclonal or recominant antibodies may offer more opportunity to obtain a bentazon-specific antibody.

## ABBREVIATIONS USED

Ab, antiserum/antibody; BSA, bovine serum albumin; cAg, coating antigen; CR %, percentage of cross-reactivity; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; GC, gas chromatography; $IC_{50}$, concentration giving 50% inhibition; iAg, immunizing antigen; IR, infrared spectroscopy; KLH, keyhole limpet hemocyanin; MeOH, methanol; NMR, nuclear magnetic resonance spectroscopy; PBS, phosphate-buffered saline; pcAg, protected coating antigen; sBSA, succinylated BSA; TLC, thin-layer chromatography; UV, ultraviolet–visible spectroscopy.

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