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Haptens and Monoclonal Antibodies for Immunoassay of Imidazolinone Herbicides

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A set of haptens structurally resembling the herbicide imazethapyr (PURSUIT) was synthesized and used to derive monoclonal antibodies (MAbs) and direct and indirect competition enzyme immunoassays (EIAs) which could detect imazethapyr, imazaquin (SCEPTER), imazapic (CADRE), and imazamox (RAPTOR) in the 3−30 ng/mL (parts per billion) range, and imazapyr (ARSENAL) and imazamethabenz-methyl (ASSERT) in the 300−500 ppb range. Two MAbs, 3A2 and 3A5, had affinities of 10−75 nM for imazethapyr. MAbs 1A5, 1D2, and 3A5 were specific for the *S* isomers of the herbicides. Some MAbs were stable in solutions containing up to 15% methanol and 5% acetonitrile in indirect EIAs. Plates coated with hapten conjugates for indirect EIA could be stored frozen. Selectivity for the imidazolinones by some MAbs varied with different coating conjugates. These MAbs and haptens should prove useful in immunochemical analysis and residue recovery methods for imazethapyr and other imidazolinone herbicides.

KEYWORDS: Immunoassay; immunoaffinity; monoclonal antibody; imazethapyr; imidazolinone herbicide

INTRODUCTION

Imidazolinone compounds are potent and selective herbicides that exclusively inhibit some isozymes of acetohydroxyacid synthase (AHAS; also known as acetolactate synthase, abbreviated ALS), a key enzyme representing a control point in the pathway of leucine, valine, and isoleucine biosynthesis (*1*). The imidazolinone herbicides are relatively nontoxic to mammals, birds, and fish (*2*) and other species that do not have AHAS isoenzymes. Consequently, imidazolinones are increasingly used instead of more toxic herbicides such as atrazine. Some imidazolinones are broad-spectrum herbicides, whereas others act selectively on weeds in the presence of tolerant crops such as soybeans, corn, wheat, barley, rye, and peanuts (*3*). The selective herbicidal activity is due to differences in metabolism by different plant species. Plants tolerant to imazamethabenz-methyl convert it to an immobile metabolite. Species that tolerate imazapyr and imazaquin metabolize them to nontoxic forms, whereas tolerance to imazethapyr appears to be due to differential rates of metabolism (*3*).

Six imidazolinone herbicides are currently used in commercially important formulations: imazethapyr (PURSUIT), imazapyr (ARSENAL), imazamethabenz-methyl (ASSERT), imazaquin (SCEPTER), imazapic (CADRE), and imazamox (RAPTOR) (**Figure 1**). Their mode of action has been extensively investigated. They bind in an uncompetitive manner to the AHAS-pyruvate complex (*1*). The imidazolinone ring has a 4′ chiral carbon to which a methyl and isopropyl group are attached. Stidham et al. showed that the *R* isomer of imazethapyr and other pyridine imidazolinones are 10-fold more inhibitory to AHAS than the *S* isomer (*4*).

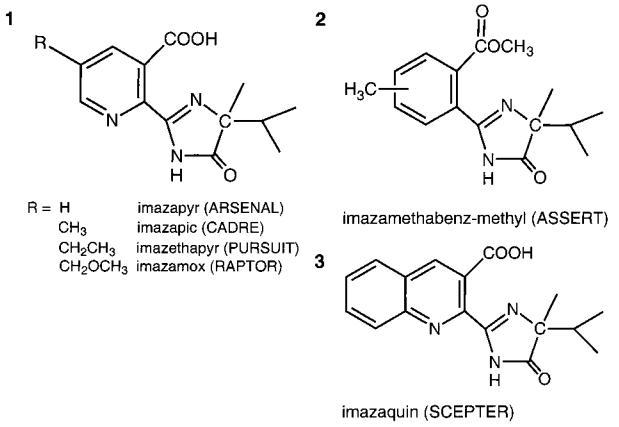

Figure 1. Structures of the major imidazolinone herbicides.

Despite the relative safety of the imidazolinones for organisms other than plants, their chemical properties differ and require special attention to how they are used in agriculture. Imazethapyr

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and others tend to persist in soils, with half-lives up to one year or more, depending on soil type, organic matter content, pH, temperature, and moisture (*5*−*8*). Periodic monitoring is thus important for effective weed control without crop damage.

Periodic monitoring may also reveal cross-resistance, in which plants resistant to one imidazolinone also have some degree of resistance to other imidazolinones or other herbicides such as sulfonylureas and triazolopyrimidines. Cross-resistance appears to be due to imidazolinones and other herbicides having the same site of action on AHAS (*9, 10*). New studies are also focusing on the secondary molecular biological effects of imidazolinone persistence, such as starvation for branched-chain amino acid and/or carbohydrate pools, and inhibition of the mitotic cycle (*11*). These types of studies all require inexpensive, efficient methods for recovery and analysis of imidazolinone residues from a variety of sources.

Sophisticated chemical residue isolation and instrumental analysis methods have been developed for imidazolinones in soil, water, forestry samples, sunflower seed, straw, leguminous vegetables, urine, kidney homogenates, and other substrates (*12*−*16*). Chemical extraction protocols for these diverse matrices are lengthy, and herbicide recovery efficiencies vary. Recently, Anisuzzaman et al. (*17*) prepared dimethyl derivatives to detect imidazolinone herbicides in environmental and biological matrices by gas chromatography. Several bioassays using tomato, canola, lentil, and other species have been developed for periodic monitoring, primarily of raw soil samples, with detection thresholds as low as 1 ppb (*8, 18*). These measure biological consequences such as root length or leaf area, but they do not identify the herbicide or other causal agents.

Immunochemical methods are potentially inexpensive, high-throughput solutions for analyte-specific periodic monitoring. Immunoaffinity chromatography with polyclonal antibodies and MAbs offers the possibility of efficient, reproducible, one-step cleanup and concentration of imidazolinone residues (*12*). Reliable immunoassays with whole (polyclonal) antisera have been reported for imidazolinone analysis. An EIA that used polyclonal rabbit sera to detect imazaquin in various matrices was developed by Wong and Ahmed (*19*). In addition, polyclonal EIAs for imazamethabenz residue in cereal grains (*20*), and for imazapyr in soil (*21*), have been published.

In this paper we describe the synthesis of several new haptens and their use to derive MAbs and format direct and indirect competition EIAs that detect imazethapyr and other imidazolinones in the low ppb to ppm range. The properties of the MAbs, including chiral isomer specificity of some MAbs, are described. These MAbs and haptens are starting materials for immunoassays and immunoaffinity methods for imazethapyr and other agriculturally important imidazolinone herbicides.

MATERIALS AND METHODS

All organic starting materials for hapten syntheses were obtained from Aldrich Chemical Co. Inc. (Milwaukee, WI). Solvents used were Spectrograde, water was deionized and then glass-distilled, and all chemicals were analytical reagent grade or better. Imidazolinone herbicide reference standards, prepared at 0.5 mg/mL in 50% acetone−PBS and stored at −20 °C in Tuf-Tainer Teflon vials (Pierce Chemical Co., Rockford, IL), were provided by Cyanamid Agricultural Research Center. Reference standard dilutions were prepared in PBS−Tween (0.01 M KH_2PO_4−K_2HPO_4, pH 7.4, 0.15 M NaCl, 0.05% Tween 20). Other herbicide standards (>99% pure) were purchased from AccuStandard Inc. (New Haven, CT), and 1 mg/mL stocks were prepared in PBS containing up to 50% methanol. Purified *R* and *S* isomers of the imidazolinones were generously provided by the Cyanamid Agricultural Research Center. Biotin-LC-hydrazide, NHS-LC-biotin, and cationized bovine serum albumin (cBSA) were from Pierce Chemical, BSA- and ovalbumin (OVA)-hydrazides were custom orders made by Molecular Probes (Eugene, OR). BIAcore CM5 chips were from Pharmacia (Piscataway, NJ). Keyhole limpet hemocyanin, Type VIII, (KLH) was purchased from Sigma.

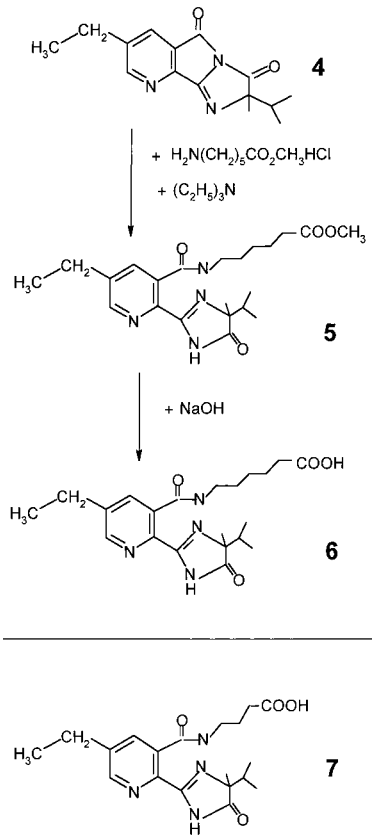

Figure 2. Synthesis of haptens with spacer arms on the pyridine ring

EIAs were performed using Immulon 2 plates (Dynatech, Chantilly, VA). Coating buffer for EIAs was 1.5 mM Na_2CO_3, 35 mM $NaHCO_3$, and 3 mM NaN_3, pH 9.6. PBS-Tween containing 0.05% gelatin, 0.45-μ filtered, was the blocking buffer and diluent for antibodies and antibody−analyte mixtures in EIAs. Washing buffer was PBS−Tween or TBS (0.05 M Tris−HCl, pH 7.5, and 0.15 M NaCl). The substrate solution for EIA color development was *p*-nitrophenyl phosphate (Sigma) 1 mg/mL, in 10% (w/v) diethanolamine−HCl, pH 9.8, 0.5 mM $MgCl_2$, and 3 mM NaN_3. Enzyme-conjugated secondary antibody, fluorescein isothiocyanate(FITC)-conjugated goat anti-mouse IgG (for KinExA affinity assays), and proteins and enzymes for hapten conjugate preparations were obtained from Sigma Chemical Co. or Boehringer-Mannheim.

Cell culture media and additives were purchased from GIBCO-BRL Laboratories (Grand Island, NY) and fetal bovine serum (FBS) was from Intergen Inc. (Kankakee, IL). Swiss Webster mice were purchased from Simonsen Laboratories (Gilroy, CA). Biozzi mice (*22*) were obtained from a colony maintained at U. C. Berkeley.

Hapten Synthesis. Haptens with a spacer attached at the carboxylic acid on the pyridine ring were synthesized in two steps from imazethapyr as shown in **Figure 2** (*23, 24*). To obtain **methyl 6-[5-ethyl-2-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl) nicotinamido] hexanoate (5)**, a stirred solution of 7-ethyl-2-isopropyl-2-methyl-5H-imidazo[1′,2′:1,2]pyrrolo[3,4-b]pyridine-3(2H) 5-dione **(4)** (7.25 g, 26.75 mmol) in anhydrous *N*,*N*-dimethylformamide (DMF) was treated successively with the addition of methyl 6-aminocaproate hydrochloride (5.10 g, 28.1 mmol) and triethylamine (3.00 g, 29.6 mmol). The resulting mixture was stirred at room temperature under nitrogen for 20.5 h then partitioned between water and ethyl acetate. The layers were separated, and the organic layer was washed with water, dried over magnesium sulfate, and vacuum-filtered through diatomaceous earth. The filtrate was concentrated in vacuo to yield a white

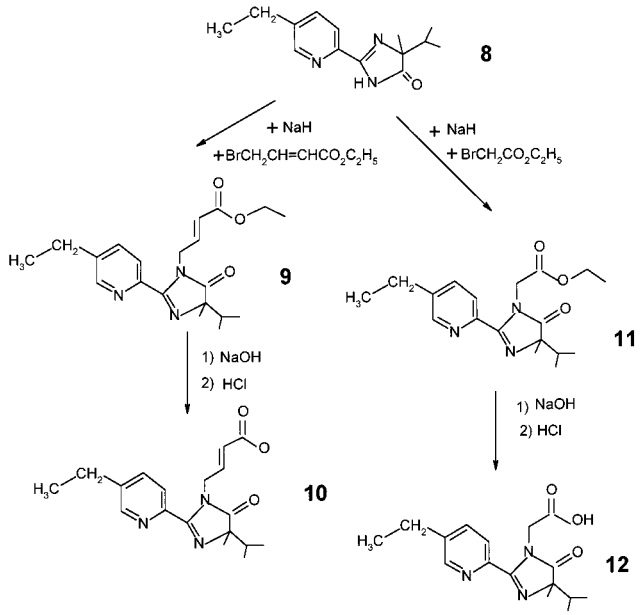

Figure 3. Preparation of haptens with spacer arms on the imidazole ring.

solid, which was triturated with 1:1 ether/petroleum ether to give compound **(5)** (mp 101−103 °C). ^{1}H NMR ($CDCl_3$, 300 MHz) 11.0 (bs, 1H); 9.1 (bs, 1H); 8.6 (s, 1H); 8.5 (s, 1H); 3.7 (s, 3H); 3.6−3.3 (m, 2H); 2.8 (q, J = 7.4 Hz, 2H); 2.3 (t, J = 7.4 Hz, 2H); 2.2−2.0 (m, 1H); 1.8−1.6 (m, 4H); 1.5−1.4 (m, 5H); 1.3 (t, J = 7.7 Hz, 3H); 1.1 (d, J = 6.9 Hz, 3H); 0.9 (d, J = 6.9 Hz, 3H).

6-[5-Ethyl-2-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl)nicotinamido] Hexanoic Acid (6). A stirred solution of methyl 6-[5-ethyl-2-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl)nicotinamido] hexanoate **(5)** (5.14 g, 12.35 mmol) in tetrahydrofuran (THF) was treated with the addition of 2 N NaOH (15 mL). The resulting mixture was stirred at room temperature for 24 h, and then concentrated in vacuo to obtain a residue. The residue was cooled in an ice bath and acidified with concentrated HCl. The resulting precipitate was isolated by vacuum filtration and washed with water to give a white solid product, **(6)** (mp 76−81 °C). ^{1}H NMR ($CDCl_3$, 300 MHz) 10.9 (bs, 1H); 8.5 (s, 1H); 3.6−3.4 (m, 2H); 2.8 (q, J = 7.7 Hz, 2H); 2.4 (t, J = 7.4 Hz, 2H); 2.2−2.0 (m, 1H); 1.7−1.6 (m, 4H); 1.5−1.4 (m, 1H); 1.4 (s, 3H); 1.3 (t, J 7.7 Hz, 3 H); 1.1 (d, J = 6.0 Hz, 3 H); 0.9 (d, J = 6.0 Hz, 3H).

A scheme similar to that in **Figure 2** was used to prepare **4-[5-ethyl-2-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl)-*N*-methyl-nicotinamido]-butyric acid (7).** A stirred solution of 7-ethyl-2-isopropyl-2-methyl-5H-imidazo[1′,2′:1,2]pyrrolo[3,4-b]pyridine-3 (2H), 5-dione **(4)** (5.47 g, 20.19 mmol) in anhydrous DMF was treated successively with the addition of 4-(methylamino)butyric acid hydrochloride (3.29 g, 21.2 mmol) and triethylamine (4.29 g, 42.39 mmol). The resulting mixture was stirred at room temperature under nitrogen for 2.5 h, then diluted with water, acidified with concentrated hydrochloric acid, and extracted with ether. The organic extract was dried over magnesium sulfate, vacuum filtered through diatomaceous earth, and concentrated in vacuo to give **(7)** as a white solid (mp 42−49 °C). ^{1}H NMR ($CDCl_3$, 300 MHz) 8.5 (d, J = 2.2 Hz, 1H); 7.5 (d, J = 1.9 Hz, 1H); 3.6−3.2 (m, 2H); 3.1 (s, 1.5H); 2.8 (s, 1.5H); 2.8−2.7 (m, 2H); 2.6−1.8 (m,4H); 1.4−1.2 (m, 7H); 1.1 (d, J = 6.9 Hz, 3H); 0.9 (d, J = 6.9 Hz, 3H).

Haptens with a spacer arm on the imidazole ring were prepared as shown in **Figure 3. Ethyl 2-(5-ethyl-2-pyridyl)-4-isopropyl-4-methyl-5-oxo-2-imidazoline-1-crotonate (9).** A stirred suspension of sodium hydride (1.6 g, 53 mmol, 80% oil suspension) in dry THF was treated with the addition of solution of 2-(5-ethyl-2-pyridyl)-4-isopropyl-4-methyl-5-oxo-2-imidazoline **(8)** (10.24 g, 41.8 mmol) in dry THF by dropwise addition. After the addition was complete and the effervescence subsided, ethyl 4-bromocrotonate (technical grade; 12.9 g, 50.1 mmol, 75% pure) was added immediately. The resulting mixture was stirred for 19 h at room temperature under nitrogen. The reaction mixture was then partitioned between water and ether. The organic layer was recovered and dried over magnesium sulfate, decolorized with activated charcoal, vacuum filtered through diatomaceous earth, and concentrated in vacuo to a dark brown oil. The oil was purified by flash chromatography (2:1 hexanes/ethyl acetate eluent), to give compound **9** as a pale orange oil. ^{1}H NMR ($CDCl_3$, 300 MHz) 8.5 (d, J = 2.2 Hz, 1H); 8.2 (bs, 1H); 7.6 (m, 1H); 6.8 (m, 1H); 5.8 (d, J = 15.9 Hz; 1H); 4.9 (dd, J_1 = 5.2 Hz, J_2 = 1.4 Hz, 2H); 4.1 (q, J = 7.1 Hz, 2H); 2.7 (q, J = 7.4 Hz, 2H); 2.2 (m, 1H); 1.4 (s, 3H); 1.3−1.2 (m, 6H); 1.1 (d, J = 6.9 Hz, 3H); 0.9 (d, J = 6.9 Hz, 3H).

To compound **9** (1.16 g, 3.25 mmol) in 1:1 THF/H_2O, sodium hydroxide (0.45 g, 11.3 mmol) was added. The resulting mixture was stirred at reflux for 1.5 h, then at room temperature for 16.5 h. The mixture was subsequently diluted with water and washed with ether. The aqueous layer was acidified with 1 N hydrochloric acid (15 mL), and extracted with ether. The organic phase was dried over sodium sulfate, decolorized with charcoal, and vacuum filtered through diatomaceous earth. The filtrate was concentrated in vacuo to give **2-(5-ethyl-2-pyridyl)-4-isopropyl-4-methyl-5-oxo-2-imidazoline-1-crotonic acid (10)** as a yellow solid (mp 53−58 °C). ^{1}H NMR ($CDCl_3$, 300 MHz) 8.4 (d, J = 1.7 Hz, 1H); 8.0 (d, J = 7.9 Hz, 1H); 7.6 (dd, J_1 = 8.2 Hz, J_2 = 2.1 Hz; 1H); 6.7 (m, 1H); 5.6 (d, J = 15.7 Hz, 1H); 4.8 (dd, J_1 = 5.3 Hz, J_2 = 1.6 Hz, 2H); 2.6 (q, J = 7.4 Hz, 2H); 2.1 (m, 1H); 1.3 (s, 3H); 1.2 (m, 3H); 1.0 (d, J = 6.8 Hz, 3H); 0.8 (d, J = 6.8 Hz, 3H). Compound **10** slowly decomposed on standing.

Ethyl 2-(5-Ethyl-2-pyridyl)-4-isopropyl-4-methyl-5-oxo-2-imidazoline-1-acetate (11). A stirred mixture of sodium hydride (0.45 g, 15 mmol, 80% oil dispersion) in anhydrous THF was treated with the addition of solution of 2-(5-ethyl-2-pyridyl)-4-isopropyl-4-methyl-5-oxo-2-imidazoline **(8)** (3.3 g, 13.47 mmol) in anhydrous THF, dropwise over 40 min. After this addition was complete and the effervescence subsided, ethyl bromoacetate (2.30 g, 13.5 mmol) was added (**Figure 3**, right). The reaction was allowed to stir for 24 h at room temperature under nitrogen, and the mixture was then partitioned between water and ether. The organic layer was recovered, dried over magnesium sulfate, vacuum filtered through diatomaceous earth, and concentrated in vacuo. The residue was purified by flash chromatography (2:1 hexanes/ethyl acetate eluent) to give product **11** as a pale yellow oil. ^{1}H NMR ($CDCl_3$, 300 MHz) 8.4 (s, 1H); 8.1, d, J = 8.0 Hz, 1H); 7.7−7.6 (m, 1H); 4.8 (s, 2H); 4.0 (q, J = 7.1 Hz, 2H); 2.7 (q, J = 7.7 Hz, 2H); 2.2−2.1 (m, 1H); 1.4 (s, 3H); 1.3 (t, J = 7.7 Hz, 3H); 1.2 (t, J = 7.1 Hz, 3H); 1.1 (d, J = 6.9 Hz, 3H); 0.9 (d, J = 6.9 Hz, 3H).

Compound **11** (2.12 g, 6.41 mmol) in THF was treated with the addition of 2 N sodium hydroxide (8 mL), and the resulting mixture was stirred at room temperature for 16 h. It was then concentrated in vacuo to obtain a residue. The residue was acidified with concentrated hydrochloric acid and then extracted with ether. The organic extract was dried over magnesium sulfate, vacuum filtered through diatomaceous earth, and concentrated in vacuo to give **2-(5-ethyl-2-pyridyl)-4-isopropyl-4-methyl-5-oxo-2-imidazoline-1-acetic acid (12)** as a white solid (mp 134−136 °C). ^{1}H NMR ($CDCl_3$, 300 MHz) 8.4 (d, J = 2.2 Hz, 1H); 8.1 (d, J = 8.2 Hz, 1H); 7.7−7.6 (m, 1H); 4.8 (s, 2H); 2.7 (q, J = 7.4 Hz, 2H); 2.2−2.1 (m, 1H); 1.4 (s, 3H); 1.3 (t, J = 7.4 Hz, 3H); 1.0 (d, J = 6.9 Hz, 3H); 0.9 (d, J = 6.9 Hz, 3H).

Haptens **13** and **14** were synthesized as described by Wong and Ahmed (**Figure 4**) (*19*). Compounds **6**, **7**, **10**, **12**, **13**, and **14** were purified by thin-layer chromatography (TLC) on plastic sheets coated with 0.2 μm silica gel 60, F 254 (E. Merck, Darmstadt, Germany). Compounds were detected by viewing under UV light (254 nm). Flash chromatography was carried out on 40 μm average particle diameter silica gel (J. T. Baker Inc., Phillipsburg, NJ).

Apparatus for Hapten Analysis. Melting points were determined with a Thomas−Hoover apparatus. All ^{1}H nuclear magnetic resonance (NMR) spectra were measured at 300.1 MHz on Varian XL300 or Unity 300 spectrometers. Chemical shift values are given in ppm downfield from an internal tetramethylsilane standard.

Conjugation of Haptens to Carrier Proteins and Biotin. Hapten **6** (1.6 mg, 4 μmol) was dissolved in 100 μL of THF and diluted to 0.5 mL with PBS (pH 7.2−7.6). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (ECDI) (8.8 mg, 0.04 mmol) was added to activate carboxyl groups of the hapten for 2 h at room temperature. The mixture was slowly added to a solution of KLH (10 mg, 22 nmol)

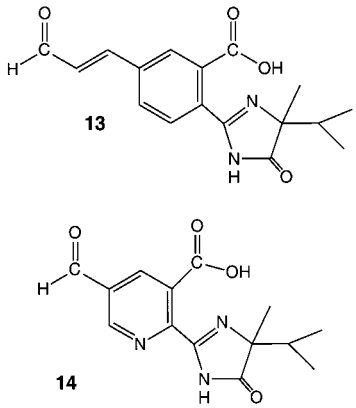

Figure 4. Additional haptens used for screening MAbs and in indirect EIAs.

in PBS (pH 7.2−7.6) so that the final molar ratio of hapten to total protein amino groups was greater than 100 to 1. The reaction mixture was stirred for 2 h at room temperature and then overnight at 4 °C. Excess reagents were removed by gel filtration. Hapten conjugate **6**-KLH eluted in the void fraction was collected, passed through a 0.22-μm filter and stored at 4 °C. This conjugate was used for immunization. Hapten density was not determined, but conjugation was verified by differences between UV absorption spectra of the modified and unmodified protein (data not shown). The ECDI activation method described above was also used to couple haptens **6** and **12** to cBSA (BSA treated with the addition of ethylenediamine to introduce aminoethyl amide groups) and hapten **12** to KLH for use as immunizing conjugates, and to link haptens **6**, **7**, **10**, and **12** to OVA for use as coating conjugates in EIAs. Additional coating conjugates were prepared by reacting the aldehyde groups on haptens **13** and **14** with hydrazide-derivatized BSA and OVA at pH 7.5 (*19*). These conjugates were purified by gel filtration as described above.

Haptens **13** and **14** were also coupled to biotin-LC-hydrazide at pH 7.5 to give conjugates **15** and **16**, respectively (**Figure 5**) (*19*). Imazethapyr was coupled to NHS-LC-biotin to give conjugate **17**, and imazaquin isothiocyanate was conjugated to NHS-LC-biotin to give conjugate **18** (**Figure 5**) by the method described by Wong and Ahmed (*19*).

Immunization of Mice. Pairs of female Swiss Webster and Biozzi mice were immunized with KLH and cBSA conjugates of haptens **6** or **12**, as previously described (*25*, *26*). Titers were determined by indirect EIA and sera were analyzed for ability to bind free imidazolinones in an indirect competition EIA. The responses of the mice to various immunizing conjugates, measured using different coating conjugates, are summarized in **Table 1**. The best-responding mouse (934-2) was given a final boost by tail vein injection of 50 μg of hapten **6**-KLH in 0.1 mL saline, 4 days prior to cell fusion and about 60 days after the first immunization, essentially as described (*26*).

Preparation of Hybridomas. All components of the cell culture media and details of the hybridoma techniques were published previously (*25*, *26*). Hybridomas were prepared by electrical fusion to P3X63AG8.653 myelomas (*26*, *27*). Of the hybridoma supernatant screened by indirect EIA, 83 gave a positive signal with coating hapten **13**-BSA, and only 1 gave a positive signal with coating hapten **10**-OVA. Cultures producing nonspecific binding antibodies as demonstrated by positive EIA response on plates coated with BSA alone were eliminated. A second indirect EIA established that 17 hybridomas maintained hapten specific binding, and 11 of these bound imidazolinones in competition EIAs. Hybridomas 1A5, 2C6, 3A2, 3A5, and 4A6 were chosen for MAb production in culture medium and ascites. The ascites fluid used in all subsequent assays was prepared in irradiated Swiss Webster mice as described previously (*26*). Immunoglobulin subclass was determined using a commercial EIA kit (Southern Biotechnology Associates, Birmingham, AL). Antibodies were purified by affinity chromatography on Hi-Trap Protein A or Protein G Sepharose (Pharmacia). Fab fragments were prepared by digestion of affinity-purified IgG with immobilized papain and purified using an ImmunoPure Fab Preparation Kit (Pierce Chemical Co.) according to the manufacturer's instructions.

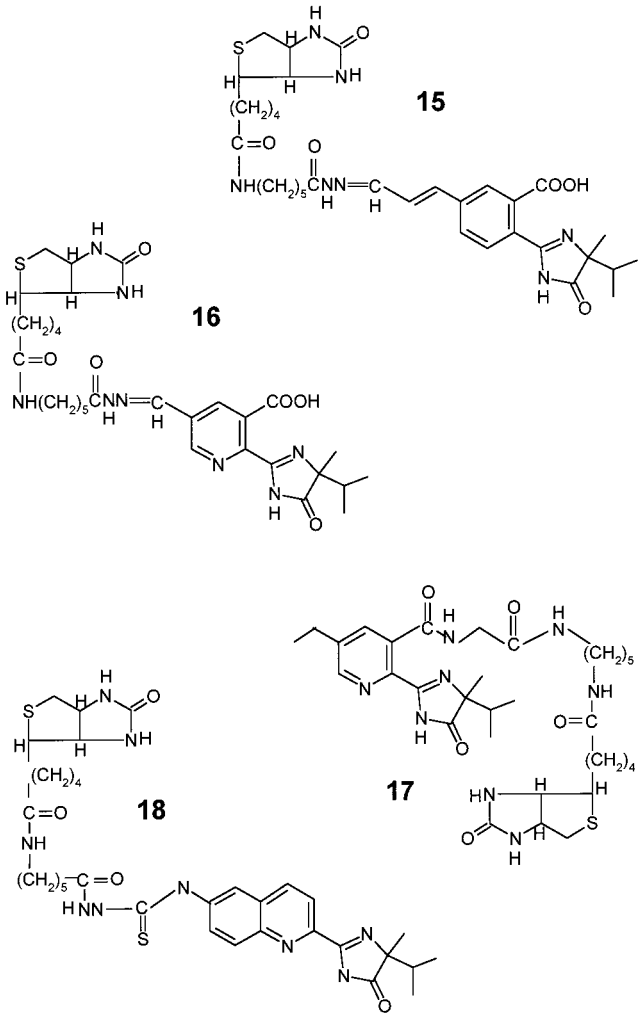

Figure 5. Haptens used as competitors in direct EIAs.

Table 1. Indirect EIA Responses of Mice to the First 3 Immunizations

immunizing hapten conjugates	mice		serum titers[a] on the indicated coating hapten-conjugates				
	strain	ID no.	unconjugated OVA	**6**-OVA	**7**-OVA	**10**-OVA	**12**-OVA
12–cBSA	S/W	931-1	high background on unconjugated OVA and all conjugates				
	S/W	931-2					
	Biozzi	912-1					
	Biozzi	912-2					
6–cBSA	S/W	932-1	NR	$>3\times10^4$	$>3\times10^4$	NR[c]	NR
	S/W	932-2	NR	$>3\times10^4$	$>3\times10^4$	NR	NR
	Biozzi	886	<500	NR	$>3\times10^4$	NR	NR
	Biozzi	died					
12–KLH	S/W	933-1	<200	$\geq5\times10^3$	$>10^4$	$>10^4$	$<10^3$
	S/W	933-2	<200	$\geq7\times10^2$	$\geq2\times10^3$	$\geq2\times10^3$	<200
	S/W	949-1	NR			$>5\times10^3$	
	S/W	949-2	NR[b]			NR[b]	
	S/W	950-1	NR			NR[b]	
	S/W	950-2	NR[b]			NR[b]	
6–KLH	S/W	934-1	NR	$>3\times10^4$	$>3\times10^4$	NR	NR
	S/W	934-2	NR	$>3\times10^4$	$>3\times10^4$	$>10^4$	NR
	Biozzi	887-1	NR	$>3\times10^4$	$>3\times10^4$	≤700	NR
	Biozzi	887-2	NR	$>3\times10^4$	$>3\times10^4$	NR	NR

[a] Titers are the greatest dilutions of antiserum giving an EIA rate greater than the mean plus two standard deviations above the background with unconjugated OVA as coating. [b] Response detectable, but too weak for use (EIA rate < 0.1 milli-absorbance unit per min. at serum dilution < 10^3). [c] NR = no response.

Enzyme Immunoassays. Indirect (immobilized hapten conjugate) and direct (immobilized antibody) EIAs were used for evaluating hapten

binding and competitive binding of imidazolinones and other analytes as previously described (*25*, *26*, *28*).

Direct competition EIAs were performed by capturing the MAb in microwells coated with affinity-purified goat anti-mouse IgG and allowing the analyte to compete with a hapten−biotin conjugate for binding (*26*). Bound hapten−biotin conjugate was detected by binding commercial avidin-alkaline phosphatase, washing, and then adding alkaline phosphatase substrate. The sensitivity of this assay was enhanced by use of a commercial enzymatic signal amplification kit (GIBCO-BRL).

EIA Data Analysis. Color development was monitored on a Multiskan EIA reader (Flow Laboratories) and rates of reaction (ΔA/min x 10^3) were calculated by linear regression. Competition EIA dose−response curves were fitted with a 4-parameter logistic equation (*26*).

Measurements of Binding Kinetics. Binding kinetics were determined by using a kinetic exclusion fluoroimmunoassay (KinExA) instrument (Sapidyne Instruments Inc., Boise, ID) and a BIAcore surface plasmon resonance system (BIAcore AB., Uppsala, Sweden). Measurements were made on 3A2 and 3A5 IgGs in PBS, affinity purified from mouse ascites on Protein A-Sepharose columns.

For BIAcore measurements, hapten **13**-OVA was immobilized onto the hydrogel layer of a CM5 chip by amide linkage after EDCI and NHS activation of the hydrogel's carboxyl groups according to BIAcore protocols. MAbs 3A2 and 3A5 were employed for binding kinetics determination of on- and off-rates (k_{on}, k_{off}). The procedures were set up according to manufacturer's instructions and controlled by BIALOGUE software. Binding affinities were calculated using the BIAEVALUATION program.

The Sapidyne KinExA instrument was used to measure antibody k_{on} and equilibrium dissociation constant (K_d) as recently described (*29*, *30*). Hapten **13**-BSA was adsorbed onto polystyrene-divinylbenzene (PS-DVB) beads (98 μm diameter, Sapidyne Inc., Boise, ID), or covalently coupled onto 1,1′-carbonyldiimidazole ReactiGel 6X beads (45−165 μm diameter, Pierce, Rockford IL). PS-DVB beads coated with hapten **13**-BSA gave optimal signal and lowest background for measurements with MAb 3A5, and ReactiGel beads coupled with hapten **13**-BSA gave the least aggregation and best reproducibility with MAb 3A2. The KinExA was controlled by software from Sapidyne Instruments Inc. Eight to 11 concentrations of analyte equilibrated with antibody were measured in duplicate or triplicate to obtain each K_d value. Binding constants were computed by iterative fitting using MATHCAD Plus 6.0 software (Mathsoft Inc., Cambridge MA) and worksheets developed by Sapidyne Instruments Inc.

RESULTS

Hapten Design. From the structures represented in **Figure 1**, it was evident that imidazolinones have significantly different molecular shapes and charge distributions. Accordingly, three different sets of haptens that differed in spacer arm position and length were prepared for this study (**Figures 2**−**4**). Haptens **6**, **7**, **10**, and **12** were designed to mimic the ethyl-substituted pyridine moiety that is the distinguishing feature of imazethapyr. The first set, haptens **6** and **7**, had spacers on the carboxylic acid group of the pyridine ring, whereas the second set, haptens **10** and **12**, had spacers on the imidazole ring. Conjugates of haptens **6** and **12** to KLH and cBSA were used for immunization because they would display the ethyl pyridine moiety most distal to the spacer arm. In the third set of haptens, **13** and **14,** the spacer arms replaced the ethyl moiety of imazethapyr. These haptens, designed primarily for screening hybridoma culture supernates, were expected to bind antibodies that recognized the imidazole ring characteristic of all imidazolinones.

Responses to Immunizing Hapten Conjugates. Mouse serum titers were determined by indirect EIA on OVA conjugates of haptens **6**, **7**, **10**, and **12** (**Table 1**). Sera from mice immunized with **6**-cBSA or **6**-KLH (**932**, **886**, **934**, and **887**) gave titers >30,000 when **6**-OVA or **7**-OVA was the coating hapten conjugate. The same sera showed 10% or less binding to **10**-OVA or **12**-OVA. Sera from two of six mice immunized with **12**-KLH (**933-1** and **933-2**) showed titers of 700 to 10,000 on **6**-OVA, **7**-OVA, or **10**-OVA coated wells and <1,000 on **12**-OVA coated wells. Although cBSA was shown to be an effective stimulator of anti-hapten immune responses in other studies (*31*−*34*), sera from all mice immunized with **12**-cBSA bound nonspecifically to unconjugated OVA and all four coating conjugates.

All sera that bound to one or more coating conjugates were tested in their ability to bind imazethapyr in indirect competition EIA. Only sera from Swiss Webster mice immunized with hapten **6** conjugates competitively bound free imazethapyr in these EIAs. The best competitive binding is generally obtained on coating haptens with a spacer arm at a position different from that of the immunizing hapten (*35*−*37*). That was not the case in this study. Sera from mice **934-1** and **934-2**, immunized with hapten **6**-KLH, gave the most sensitive competitive binding of imazethapyr (I_{50} values of 27−83 ppb) in assays with **7**-OVA and **10**-OVA as coating haptens. The **7**-OVA hapten is an imazethapyr mimic with a spacer arm similar to, but shorter than, the **6**-KLH immunizing hapten (**Figure 2**). The spacer arm of the **10**-OVA hapten is on the imidazole ring, a "heterologous" position likely to allow more conformational variations in the overall rotation and stacking of the imidazolinone ring against the pyridine ring, as compared with that which is possible in haptens **6** and **7** and imazethapyr. Mice **933-1** and **933-2**, immunized with **6**-cBSA, produced antisera that competitively bound imazethapyr in EIAs with **7**-OVA as coating conjugate. However, titers were low (**Table 1**) and I_{50} values were on the order of 2 ppm. EIA response with **10**-OVA as the coating conjugate could not be measured. These results indicate that factors other than spacer heterology are important. Mouse **934-2**, which was immunized with hapten **6**-KLH and had the best hapten binding and analyte competition responses to 3 of 4 coating hapten conjugates, was used for hybridoma production.

Properties of the MAbs. Hybridomas were initially screened by indirect EIA on hapten **10**-OVA and **13**-BSA conjugates. EIAs could not be done with cBSA because it tended to aggregate, raising background noise and lowering reproducibility. Of approximately four hundred hybridoma culture supernates tested, 83 recognized the class-specific conjugate **13** and only one (1A5) bound hapten **10**. Eleven MAbs competitively bound imidazolinone herbicides in solution. **Table 2** summarizes the selectivity of five MAbs and their immunoglobulin subclass. MAbs 1A5, 1D2, and 2C6 were most sensitive for imazethapyr, MAb 3A2 was most sensitive for imazaquin, and MAb 3A5 was most sensitive for imazamethabenz-methyl.

As with the antisera, competitive binding of imidazolinones by various MAbs differed with the coating conjugate used. MAbs 3A2 and 2C6 were equally sensitive for imidazolinone competitive binding with coating conjugates **13**-BSA and **14**-OVA whereas MAb 3A5 gave more sensitive detection limits when the **14**-OVA conjugate was used. MAb 1D2 gave competitive binding only on **14**-OVA.

MAb 1A5 was the only antibody that bound to the **10**-OVA coating conjugate. In indirect EIA it competitively bound free imazethapyr, imazapic, and to a lesser extent imazaquin, imazapyr, and imazamethabenz methyl. However, MAb 1A5 could not competitively bind imidazolinone compounds in EIAs with **13**-BSA or **14**-OVA as coating conjugates.

Table 2. Competitive Binding of Imidazolinones in Indirect EIA

		I_{50} (parts per billion)								
	MAb:	1A5	1D2		2C6		3A2		3A5	
	subclass:	$IgG_{2b}\kappa$	$IgG_{2a}\kappa$		$IgG_{2b}\kappa$		$IgG_{1}\kappa$		$IgG_{2a}\kappa$	
analyte	coating conjugate:	10-OVA	14-OVA	13-BSA	14-OVA	13-BSA	14-OVA	13-BSA	14-OVA	13-BSA
imazethapyr		60	20	–[a]	15	13	21	34	220	>1,000
imazapyr		500	>1,000	–	–	–	331	290	>5,000	–
imazaquin		>1,000	2,000	–	–	–	3	5	800	–
imazamethabenz-methyl		500	105	–	–	–	356	322	90	400
imazapic		100	250	–	204	171	27	31	340	>1,000
imazamox		–	240	–	15	13	26	34	280	>1,000

[a] – = no competition at 10 ppm.

Table 3. Responses of MAbs 1D2 and 3A2 in Direct Competition EIA Using Hapten–Biotin Conjugates

		I_{50} (ppb)				
	MAb:	1D2	3A2			
analyte	hapten–biotin conjugate:	15	16	15	16	18
imazethapyr		100	NC[a]	NC	27	33
imazamethabenz-methyl		2	NC	–[b]	NC	–
imazaquin		–	–	NC	34	14

[a] NC = no competition up to 10 ppm of analyte. [b] – = binding of conjugate was too weak to measure competition.

Table 4. Competitive Binding of Imidazolinones by MAb 3A2 IgG and Fab Fragments

		I_{50} (parts per billion)					
	assay:	indirect EIA		direct EIA		direct EIA	
	competitor:	14–OVA		16		18	
analyte		IgG	Fab	IgG	Fab	IgG	Fab
imazethapyr		7	17	52	47	31	31
imazaquin		2	2.9	22	21	15	13
imazapyr		132	189				
imazamethabenz-methyl		110	238				
imazapic		9	19				
imazamox		14	28				

The MAbs were also tested in direct EIAs with biotin conjugates of haptens **15**−**18**. MAbs 1A5, 2C6, and 3A5 bound haptens **15**−**17,** but did not competitively bind free imidazolinones in the presence of these conjugates (data not shown). Only MAbs 1D2 and 3A2 competitively bound imazethapyr and two other imidazolinones in direct EIAs with hapten−biotin conjugates as competitors (**Table 3**).

In an effort to improve resolution of the direct EIA with imidazolinone−biotin conjugates, the assay was done using a commercial EIA amplification system (GIBCO-BRL). In this procedure, NADPH was used as the alkaline phosphatase substrate. It was converted to the product NAD^+, which acted as cofactor for coupled redox reactions catalyzed by alcohol dehydrogenase and diaphorase. The diaphorase reaction reduced the dye iodonitotetrazolium violet to a colored formazan that was quantified by its absorbance at 495 nm. Use of this amplification system increased the rate of color development roughly 10-fold, but had no effect on the I_{50} values.

Because of steric hindrance and other effects, it is possible for MAbs and their proteolytic or recombinant Fab fragments to perform differently in assays with different hapten conjugates (*26*, *38*, *39*). Indirect and direct competition EIAs were done with MAb 3A2 and its proteolytic Fab to compare binding and competition on three hapten conjugates. MAb 3A2 and its Fab fragment gave nearly identical results as shown in **Table 4**.

Chiral Specificity. The *R* isomer of imazethapyr was shown to be about 10-fold more potent than the *S* isomer as an inhibitor of acetohydroxyacid synthase (*4*). The immunizing hapten was a racemic mixture of the *R* and *S* isomers at the 4′ carbon on the imidazole ring. Indirect competition EIAs were performed with pure *R* and *S* isomers and a technical mixture as competitor. The results, summarized in **Table 5**, show that MAbs 3A5, 1D2, and 1A5 preferentially recognized the *S* isomers of the imidazolinones tested. MAbs 3A2 and 2C6 bound either isomer about equally well, within the range of experimental variation. In most cases the sensitivity (I_{50} value) for the technical mixture of isomers commonly used as reference standards was between the I_{50} values for the pure isomers. The exception was imazapic, for which the most sensitive detection was obtained with the technical mixture. The reason for this was not determined.

Selectivity for Imidazolinones. Imidazolinone herbicides are used in combination with, or in place of, several other classes of herbicides. MAb 3A2 was tested for cross-reaction with sixteen other herbicide classes in an indirect competition EIA (wells coated with hapten **14**-OVA). No cross-reactivity was observed with up to 10 ppm of acifluorfen, alachlor, atrazine, EPTC, glyphosate, metolachlor, molinate, oxyfluorfen, paraquat, pendimethalin, phenmedipham, primisulfuron, sethoxydim, or thiobencarb. MAb 3A2 also did not cross-react with up to 5 ppm of chlorimuron-ethyl or chlorsulfuron, but 10−15% inhibition was observed with 5−10 ppm of these two herbicides. Thus, the indirect competition EIA is specific for imidazolinones in the presence of excess amounts of these other herbicides.

Stability of Conjugate-Coated EIA Plates. For immunoassay kit production, batchwise preparation of plates is economical and may improve reproducibility. The storage stability (shelf life) of EIA plates coated with hapten conjugates was tested as described by Schmidt et al. (*25*). Immulon 2 plates were coated in coating buffer with optimal amounts of **10**-OVA conjugate or **14**-OVA and stored at −80 °C in either coating buffer, blocking buffer, or dry. Plates were removed from storage after 8, 16, and 30 days and compared with freshly coated plates by indirect EIAs with MAbs 1A5, 3A2, and 3A5. Plates stored with wells filled with coating buffer at −80 °C gave good agreement between replicate samples, consistent response at the limits of detection, and very similar I_{50} values (data not shown). Coated plates frozen with wells containing blocking buffer showed the greatest variability. Lower I_{50} values were obtained with plates stored for 16 and 30 days compared with plates that were freshly coated or stored for 8 days (data not shown). These results indicated that it is feasible to store precoated plates for at least 30 days, with no loss, and even some improvement of competition EIA performance.

Table 5. Binding of Imidazolinone Chiral Isomers in Indirect EIA

			I_{50} (parts per billion)				
		MAb:	3A2	3A5	2C6	1D2	1A5
analyte	isomer	coating conjugate:	**13**-BSA	**14**-OVA	**13**-BSA	**14**-OVA	**10**-OVA
imazethapyr	*R*		46	>5000	76	>5000	>2000
	S		86	114	560	209	90
	tech. mix		41	186	44	248	247
imazaquin	*R*		8	>5000	–[c]	n.d.[b]	>10000
	S		7	277	–	n.d.	340
	tech. mix		15	~2000	–	n.d.	658
imazapyr	*R*		657	NC[a]	–	>10000	n.d.
	S		395	1050	–	2200	n.d.
	tech. mix		282	2670	–	11600	n.d.
imazapic	*R*		349	NC	n.d.	NC	NC
	S		1738	~1000	n.d.	323	2900
	tech. mix		59	317	n.d.	323	154
imazamox	*R*		42	>5000	85	n.d.	–
	S		66	136	67	n.d.	–
	tech. mix		54	689	35	n.d.	–

[a] NC = no competition by up to 10 ppm of analytes. [b] n.d. = not done. [c] – = no binding with this coating conjugate.

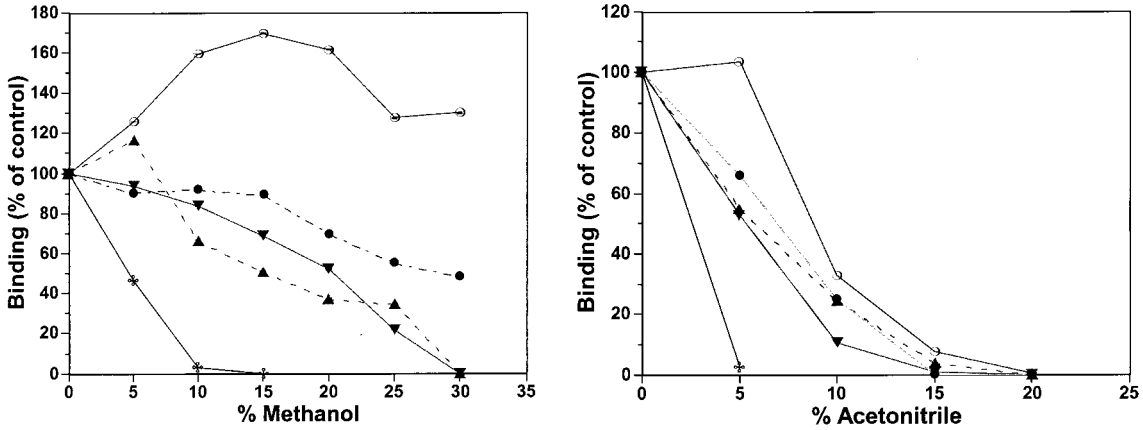

Figure 6. Binding of MAbs to coating conjugates after incubation with organic solvent. Indirect EIAs were performed as described in the text. MAb 3A2 (– ○ –); 3A5 (- -●- -); 1D2 (– ▼ –); 2C6 (- -▲- -); 1A5 (– + –).

Stability in Organic Solvents. Because standard imidazolinone residue recovery procedures often contain methanol or acetonitrile, we investigated the effect of these solvents on the indirect EIA. MAbs were incubated overnight in PBS−Tween containing no organic solvent, 5, 10, 15, 20, 25, or 30% methanol, or 5, 10, 15, or 20% acetonitrile. The coating conjugates were **13**-BSA for MAb 2C6, **14**-OVA for MAbs 1D2, 3A2, and 3A5, and **10**-OVA for MAb 1A5. Binding of MAb 3A2 was actually improved in PBS with 5−25% methanol (**Figure 6**, left). Binding of MAbs 1D2, 2C6, and 3A5 progressively decreased as methanol concentration increased (**Figure 6**, left). These antibodies retained roughly 40−60% binding activity in PBS containing 20% methanol. MAb 1A5 was much more sensitive to solvent concentration, as shown in **Figure 6**. All of the MAbs were less tolerant to acetonitrile than methanol (**Figure 6**, right). Although overnight exposure to solvents was harsher than necessary for indirect EIAs, it demonstrated that MAbs 3A2 and 3A5, and to a lesser extent 1D2 and 2C6, can potentially be used for assays and other applications in as much as 15% methanol or 5% acetonitrile.

Binding Kinetics. The K_d for MAbs 3A2 and 3A5 binding to imazethapyr were estimated by surface plasmon resonance in a BIAcore instrument (*40, 41*) and by KinExA (*30*). Because imazethapyr and hapten conjugates used in these studies were racemic mixtures, the dissociation constants were averages.

Each BIAcore measurement of K_d required seven serial 2-fold dilutions of MAb. K_d estimates in the KinExA used duplicates or triplicates of 8−11 concentrations of imazethapyr equilibrated with MAb. In BIAcore experiments, MAbs 3A2 and 3A5 had K_d values of 24.7 and 9.6 nM, respectively, for hapten **13**. The KinExA study estimated a K_d of 11.4 nM for 3A2, and 73 nM for 3A5 binding imazethapyr. The BIAcore and KinExA experiments could not be directly compared because BIAcore measures association and dissociation of antibody from an immobilized hapten conjugate, whereas KinExA measures unoccupied antibody from solutions at equilibrium. Nevertheless, the data indicate that MAbs 3A2 and 3A5 have affinities for imazethapyr or an imidazolinone class compound in the 10−75 nM range.

DISCUSSION

The primary aim of this work was to develop MAbs that preferentially recognized imazethapyr using a combination of rationally designed compound- and class-specific haptens. The immunizing conjugates were designed to emphasize the presentation of the ethyl group characteristic of imazethapyr on the pyridine ring. The haptens with spacer arms on the carboxylic acid group of the pyridine ring proved to be better immunogens than haptens with spacer arms on the imidazole ring. Use of mouse strains that differed in immunoglobulin

variable-region gene repertoires and immunoregulatory genes may have contributed to the characteristics of the MAbs that were produced. The competitive binding results of **Table 2** show that four of the five MAbs selected for further study (1A5, 1D2, 2C6, and 3A2) were highly sensitive for imazethapyr, and all five differed in cross-reactivity toward the other imidazolinones. Three results were particularly noteworthy. First, all the MAbs bound poorly or not at all to imazapyr. This was consistent with the rationale for our hapten design because the lack of an ethyl group on the pyridine ring is the only difference between imazapyr and imazethapyr. Second, MAb 3A2, which bound best to imazaquin, cross-reacted strongly with imazapic, imazamox, and imazethapyr. This MAb might be recognizing part of the molecule away from the substituent site (R on the pyridine ring in **Figure 1**) and toward the imidazolinone ring common to all of these herbicides. Third, the hybridoma panel included one MAb, 1A5, that bound hapten **10**-OVA with a spacer arm on the imidazolinone ring and also competitively bound with imazethapyr. No other MAb possessed the same property.

The equilibrium constants measured by KinExA and BIAcore confirmed results from the competition EIAs indicating that MAbs 3A2 and 3A5 were of moderately high affinity for imazethapyr. Differences between the values determined with the two instruments were due in part to the differences in procedures. We consider KinExA results more valid, primarily because they were direct measurements of free antibody from solutions at analyte−antibody equilibrium. K_d values from the BIAcore were computed from the relative rates of antibody binding and dissociation rates with a hapten conjugate immobilized on the sensor chip. These values might be affected by diffusion rates of antibody in the hydrogel and the likelihood that some fraction of dissociated antibody might re-bind (*41, 42*).

In previous projects in our lab, we observed differences in the hapten binding preference of intact IgG and proteolytic or recombinant Fab fragments of the same MAb (*38, 39*). This was probably due to steric effects. As shown in **Table 4**, approximately equal amounts of MAb 3A2 IgG and proteolytic Fab fragments gave nearly identical results in indirect and direct EIAs.

The MAbs described here have already been used by others in practical applications. MAb 3A2 proved useful for imidazolinone residue recovery by immunoaffinity chromatography (*12*), and Lee et al. developed a capillary column flow injection liposome immunoassay to quantify imazethapyr (*43*). MAb 3A2 also showed excellent performance characteristics in a reusable fiber optic sensor for imazethapyr that was originally developed using a sheep antiserum (*44*; and R. B. Wong and M E. Eldefrawi, unpublished).

These MAbs were originally developed to acquire data for pesticide registration and research into imidazolinone toxicology and mechanisms of herbicidal action. Potential applications include studies of absorption and transport from roots, persistence, bioavailability, mobility, microbial metabolism in various soils, and immunohistochemical localization. The immunochemical methods can be used in studies that previously could be done only with radiolabeled imidazolinones. The chirally selective antibodies may be useful for quality assurance monitoring during herbicide manufacturing. MAbs that differ in specificity for some of the parent compounds and haptens may be usable in multiplexed or array-based assays to identify the imidazolinones in a sample. We hope that the MAbs described here will prove usable and cost-effective in some of the roles described above, and in future applications.

IUPAC Names and CAS Registry Numbers. Imazethapyr (PURSUIT), 5-ethyl-2-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl) nicotinic acid, 81335-77-5; Imazapyr (ARSENAL), 2-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl) nicotinic acid, 81510-83-0; Imazamethabenz-methyl (ASSERT), a mixture of methyl 6-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl) *m*-toluate and methyl 2-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl) *p*-toluate, 81405-85-8; Imazaquin (SCEPTER), 2-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl)-3-quinolinecarboxylic acid, 81335-47-9; imazapic (CADRE), 81334-60-3; imazamox (RAPTOR), 114311-32-9; Acifluorfen (BLAZER, TACKLE), 5-[2-chloro-4-(trifluoromethyl)phenoxy]2-nitrobenzoate, 50594-66-6; Alachlor, (LASSO), 2-chloro-2′-6′-diethyl-*N*-(methoxymethyl)-acetanilide, 15972-60-8; Atrazine, 1912-24-9; Primisulfuron (BEACON), 3-[4,6-bis(difluoromethoxy)-pyrimidin-2-yl]-1-(2-methoxycarbonyl-phenylsulfonyl)-urea, 86209-51-0; EPTC (EPTAM), S-ethyl dipropylthiocarbamate, 759-94-4; Glyphosate (ROUNDUP), *N*-(phosphono-methyl) glycine isopropylamine salt, 1071-83-6; Metolachlor, 2-chloro-*N*-(2-ethyl-6-methylphenyl)-*N*-(2-methoxy-1-methylethyl) acetamide, 51218-45-2; Molinate (ORDRAM), S-ethyl-hexahydro-1*H*-azepine-1-carbothioate, 2212-67-1; Oxyfluorfen (GOAL), 2-chloro-1-(3-ethoxy-4-nitrophenoxy)-4-(trifluoromethyl) benzene, 42874-03-3; Paraquat, 1,1′-dimethyl-4,4′-bipyridinium dichloride, 1910-42-5; Pendimethalin (PROWL), *N*-(1-ethylpropyl)-3,4-dimethyl-2,6-dinitro-benzenamine, 040487-42-1; Phenmedipham, 3-methoxycarbonylaminophenyl-*N*(3'-methylphenyl)carbamate, 13684-63-4; Sethoxydim (POAST), 2[1-ethoxyimino)butyl]-5-[2-ethylthio)propyl-3-hydroxy-2-cyclohexen-1-one, 74051-80-2; Thiobencarb (BOLERO), S-[(4-chlorophenyl)methyl]diethylcarbamothioate; Chlorimuron ethyl (CLASSIC), ethyl 2-[[[[(4-chloro-6-methoxypyrimidin 2-yl)amino]carbonyl]amino]sulfonyl]-benzoate, 90982-32-4; Chlorsulfuron (GLEAN), 2-chloro-*N*[(4-methoxy-6-methyl-1,3,5 triazin-2-yl)aminocarbonyl]-benzenesulfonamide, 64902-72-3.

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