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ARTICLES

Hapten Syntheses and Antibody Generation for a New Herbicide, Metamifop

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To develop a competitive indirect enzyme-linked immunosorbent assay for metamifop, a new aryloxyphenoxypropionic acid herbicide, three structurally related haptens were synthesized. Hapten conjugates to keyhole limpet hemocyanin and bovine serum albumin were used as immunogens and plate-coating antigens, respectively. Various sets of polyclonal antibodies from rabbits and the coating antigens were screened for the assay in simple homologous and heterologous ELISA formats. A selected heterologous ELISA was optimized to show an average IC_{50} value as low as 20.1 ng/mL, detection ranges of 1.0−350 ng/mL, and a lowest detection limit of 0.1 ng/mL. The cross-reactivities of other aryloxyphenoxypropionic acid herbicides to the antibodies were less than 0.5% in the assays except fenoxaprop-P and fenoxaprop-P ethyl, having a diaryl ether group identical to that of metamifop. Molecular modeling studies revealed that the physicochemical properties of the diaryl ether group are the most important determinants of sensitivity and selectivity. The results strongly indicate that the selected set of ELISA is a highly sensitive and convenient tool for detecting metamifop.

KEYWORDS: Metamifop; aryloxyphenoxypropionic acid herbicide; ELISA; polyclonal antibodies

INTRODUCTION

Metamifop ((*R*)-2-[4-(6-chloro-1,3-benzoxazol-2-yloxy)phenoxy]-2′-fluoro-*N*-methylpropionanilide, **Figure 1**) is a new herbicide developed by Dongbu Hannong Chemical, Korea. It is used for post-emergence control of annual and perennial grass weeds in rice filed at a rate of 100−200 g/ha (*1*, *2*). Both acute oral and percutaneous LD_{50} (rat) values are over 2000 mg/kg, indicative of low mammalian toxicity (*1*).

The mode of action of metamifop and other aryloxyphenoxypropionic acid herbicides (AOPPs) such as fenoxaprop, haloxyfop, and quizalofop is the inhibition of acetyl-CoA carboxylase (ACCase), which catalyzes the first committed step in de novo fatty acid biosynthesis. The inhibition of ACCase leads to the interruption of lipid biosynthesis and results in death of the plant (*3*−*6*). In most cases, these herbicides effectively control weeds at a very low rate with limited or no toxicities to nontarget species. In addition, it is well known that these herbicides do not persist in the soil (*7*).

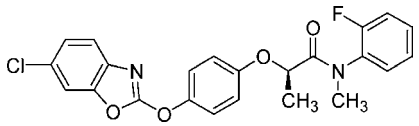

Figure 1. Structure of metamifop.

AOPPs are stereoactive compounds, among which the *R* (+) enantiomers usually have stronger herbicidal activities. It is well documented that stereoisomerism of haloxyfop bioactivity is originated from differential affinity of isomers on ACCase (*4*, *8*−*10*). Because of the different stereochemistry and activity relationships, most commercial AOPPs, including metamifop, are produced as *R* (+) isomer-enriched forms (e.g., fenoxaprop-P and quizalofop-P) (*1*).

In general, conventional pesticide residue analyses are comprised of laborious sample preparations including extensive sample extraction, cleanup, and utilization of high cost instruments. To ensure analytical sensitivity and reproducibility, it is also important that skilled analysts perform the conventional instrumental analysis.

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On the contrary, immunoassay is a fast, simple, and economic analytical method. Because of its strong selectivity and sensitivity, efforts for sample cleanup can be reduced to a minimum, which make the immunoassays highly convenient tools for high-throughput studies for a large number of samples in a short period of time (*11*). Analytical sensitivities of immunoassay are comparable to or better than those of the conventional methods such as high performance liquid chromatography (HPLC) and gas liquid chromatography (GLC). These characteristics of immunoassays make them the choice of on-site pesticide residue analysis (*12*, *13*).

The most popular form of immunoassay for pesticide residue analysis is ELISA (enzyme-linked immunosorbent assay), and many sensitive ELISAs for pesticides residues have been developed since the 1970s (*14*), especially for the compounds, of which the conventional analysis is very difficult (*15*−*19*). For metamifop, the analytical method involves HPLC with UV detection (*20*, *21*), and immunochemical detection methods have not been reported so far.

The most critical step in developing ELISA is the production of highly selective antibodies, and, therefore, the preparation of proper haptens for immunogen is very important. Reasonable hapten design will result in an antibody with the desired selectivity and specificity for immunoassay. To develop a competitive indirect enzyme-linked immunosorbent assay (ciELISA) for monitoring and efficient quantification of metamifop residue in agricultural and environmental samples, a number of haptens mimicking the structure of metamifop were prepared with the help of molecular modeling, and polyclonal antibodies were obtained from rabbit. Various combinations of coating antigens and polyclonal antibodies have been evaluated to improve the analytical sensitivity of ciELISA for metamifop.

MATERIALS AND METHODS

Chemicals and Reagents. Metamifop was donated by Dongbu Hannong Co., Korea. Reference standards of haloxyfop, fenoxaprop-P, fenoxaprop-P-ethyl, cyhalofop-butyl, propaquizafop, and fluazifop-butyl were purchased from Chem Service (West Chester, PA). 4-{2-[4-(6-Chloro-benzooxazol-2-yloxy)-phenoxy]-propionylamino}-3-fluoro-benzoic acid ethyl ester (K-13209), technical grade fenoxaprop-P-ethyl, and desmethyl-metamifop were kindly provided by the Korea Research Institute of Chemistry and Technology, Korea. 1,3-Dicyclohexylcarbodiimide (DCC), dimethyl aminopyridine (DMAP), ethyl 3-bromopropionate, 2-fluoroaniline, *N*-hydroxysuccinimide (NHS), oxalyl chloride, hydrochloric acid (HCl), lithium hydroxide monohydrate ($LiOH{\cdot}H_2O$), sodium acetate, sodium bicarbonate ($NaHCO_3$), sodium hydroxide (NaOH), dimethylformamide (DMF), 1,4-dioxane, tetrahydrofuran (THF), and pyridine for synthesis were purchased from Sigma (St. Louis, MO). Other reagents purchased from Sigma (St. Louis, MO) were goat anti-rabbit IgG-horseradish peroxidase (IgG-HRP, A-6154), keyhole limpet hemocyanin (KLH, H-7017), bovine serum albumin (BSA, A-7030), horse radish peroxidase (HRP, P-6782), phosphate−citrate buffer capsules with sodium perborate (P-4922), carbonate−bicarbonate buffer capsules (C-3401), *o*-phenylenediamine (OPD, P-9029), Freund's complete adjuvant (F-5881), and incomplete adjuvant (F-5506).

Silica gel (40−60 μm) for column chromatography was purchased from Merck.

All other reagents and solvents were analytical grade unless specified otherwise.

Instruments. LC/MS analysis was carried out with an Agilent 1100 series HPLC system (Agilent, Wilmington, CA) coupled with a Quattro LC triple quadrupole tandem mass spectrometer (Micromass, Manchester, UK) in eletrospray ionization (ESI^+) mode. The source temperature, desolvation temperature, con voltage, and capillary voltage were kept at 120 °C, 200 °C, 28 V, and 3.29 kV, respectively. The nebulizer and desolvation gas were set at 80 and 397 L/h, respectively.

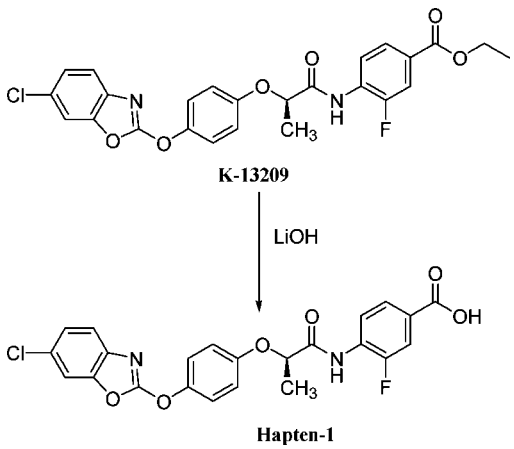

Figure 2. Synthetic scheme for hapten-**1**.

A Shimadzu 2010 gas chromatograph combined with a Shimadzu QP 2010 MS engine mass spectrometer was used for GC/MS confirmation. The ion source temperature was 200 °C, and mass spectra were obtained with electron impact (EI) mode at 70 eV.

^1H NMR spectra in acetone-d_6 or $CDCl_3$ were recorded on a JEOL LA400 (JEOL, Japan), and chemical shifts are expressed in ppm (δ) relative to tetramethylsilane (TMS) as internal standard.

ELISAs were carried out in a 96-well polystyrene microplate (MaxiSorp F96, Nunc, Denmark). A Taitec MBR-022UP plate shaking incubator (TAITEC, Japan) and Wellwash AC plate washer (Thermo Electronic Corp., Milford, MA) were used for incubation and washing the plate. The optical density of the 96-well plate was read at 492 nm with Multiskan plate reader (Thermolabsystems, Milford, MA).

Molecular Modeling. The conformational search was done with CaChe Worksystem Pro (Fujitsu Ltd., U.S.). Further refined structures were obtained from the selected low energy conformers using a PM3-water semiempirical force field. Electrostatic potential was mapped over an electron density isosurface using the same force field.

Synthesis of Haptens. *Hapten-**1**, 4-{2-[4-(6-Chloro-benzooxazol-2-yloxy)-phenoxy]-propionylamino}-3-fluoro-benzoic Acid* (**Figure 2**). To a cooled solution (0 °C) of K-13209 (200 mg) in 5 mL of 1,4-dioxane was added 20 mg of $LiOH{\cdot}H_2O$ in water (1 mL). The reaction mixture was stirred at room temperature for 6 h. After the pH was adjusted to 2, the reaction mixture was extracted with dichloromethane (2 × 50 mL). The organic layer was dried over anhydrous sodium sulfate and evaporated under reduced pressure. The residue in 0.5 mL of dichloromethane was purified with silica gel chromatography using an ethyl acetate/*n*-hexane mixture (1/3, v/v, containing 0.1% acetic acid) as an eluent. 32 mg of hapten-**1** was obtained as a white solid (yield 18%, purity 98.4%): ^1H NMR (acetone-d_6) δ 1.67 (d, 3H), 5.12 (q, 1H), 7.12−8.80 (m, 11H), 9.21 (s, 1H); LC−MS *m*/*e* 471 ($[M + H]^+$, 100), 473 (32).

*Hapten-**2**, 2-[4-(6-Chloro-benzooxazol-2-yloxy)-phenoxy]-propionic Acid (**Figure 3**).* The reaction was performed as for hapten-**1** using fenoxaprop-P-ethyl (10.8 g), 50 mL of 1,4-dioxane, and 1.5 g of $LiOH{\cdot}H_2O$ in 10 mL of water. The residue was recrystallized in an ethyl ether/*n*-hexane (1:1) mixture. Hapten-**2** was obtained as a white solid (7.1 g, yield 71.7%, purity 98.9%): ^1H NMR ($CDCl_3$) δ 1.71 (d, 3H), 4.80 (q, 1H), 6.70−7.47 (m, 7H); LC−MS *m*/*e* 334 ($[M + H]^+$, 100), 336 (33).

*Synthesis of 3-(2-Fluoro-phenylamino)-propionic Acid Ethyl Ester (**1**, **Figure 4**).* Sodium acetate (8.2 g), 2-fluoroaniline (11.1 g), and ethyl 3-bromopropionate (18.1 g) were dissolved in 20 mL of ethanol and refluxed for 12 h. After the mixture was cooled to room temperature, water (50 mL) was added to the solution and the reaction mixture was extracted with ethyl acetate (2 × 100 mL). The organic layer was dried, evaporated, and purified to give 10.5 g of compound **1** as a dark brown oil (yield 49.8%): ^1H NMR ($CDCl_3$) δ 1.26 (t, 3H), 2.62 (t, 2H), 3.47 (t, 2H), 4.15 (q, 2H), 4.24 (br, 1H), 6.60−7.10 (m, 4H); GC−MS *m*/*e* 211 (M^+, 13), 124 (100).

*Synthesis of 3-[{2-[4-(6-Chloro-benzooxazol-2-yloxy)-phenoxy]-propionyl}-(2-fluoro-phenyl)-amino]-propionic Acid Ethyl Ester (**2**, **Figure***

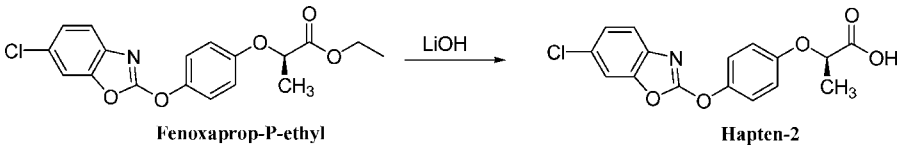

Figure 3. Synthetic scheme for hapten-**2**.

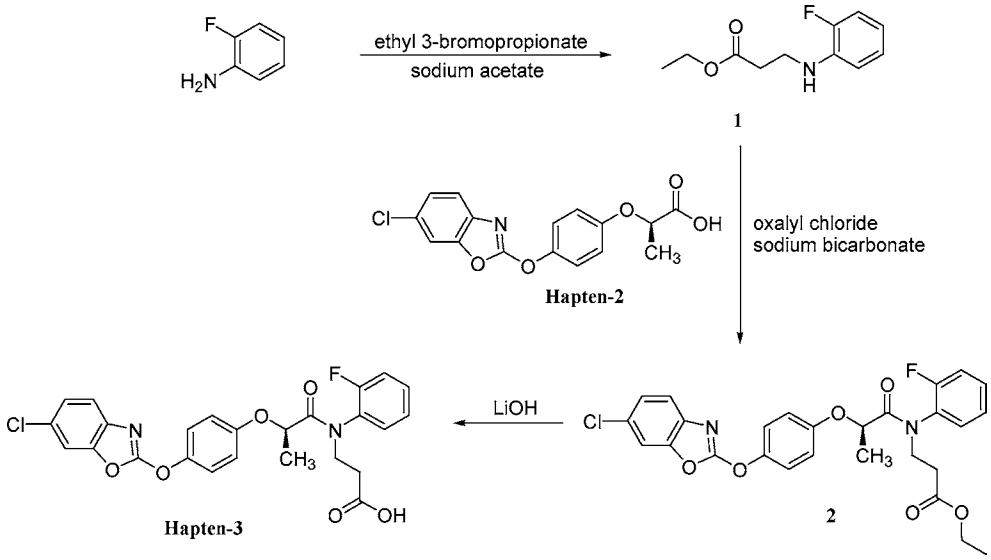

Figure 4. Synthetic scheme for hapten-**3**.

4). Hapten-**2** (330 mg) in 20 mL of ethyl acetate was cooled to 0 °C with ice. Oxalyl chloride (0.2 mL) was added dropwise, and a catalytic amount of DMF was added to the solution. After 3 h of stirring, the solvent was evaporated, and the residue was dissolved in 1 mL of ethyl acetate. It was added dropwise to the stirred mixture (200 mL) of ethyl acetate/0.1 N $NaHCO_3$ solution (1:1, v/v), which contained 220 mg of compound **1**. After 2 h, the organic phase was separated and dried as for compound **2**. Purification of residue dissolved in 0.5 mL of dichloromethane with silica gel column chromatography (eluent; ethylacetate/*n*-hexane = 1:8, v/v) gave 350 mg of compound **2** as a brown oil (yield 68.3%): ^{1}H NMR ($CDCl_3$) δ 1.23 (t, 3H), 1.82 (d, 3H), 2.65 (t, 2H), 3.49 (t, 2H), 4.12 (q, 2H), 4.83 (q, 1H), 6.60−8.12 (m, 11H); GC−MS *m/e* 526 (M^+, 40), 528 (15), 481 (7.3), 288 (38), 266 (28), 178 (76), 124 (100).

*Hapten-**3**, 3-[{2-[4-(6-Chloro-benzooxazol-2-yloxy)-phenoxy]-propionyl}-(2-fluoro-phenyl)- mino]-propionic Acid (**Figure 4**).* This reaction was performed in the same manner as for hapten-**1** using 5 mL of 1,4-dioxane and 200 mg of compound **2** to give 35 mg of hapten-**3** as a brown oil (yield 18.5%, purity 99.2%): ^{1}H NMR (acetone-d_6) δ 1.79 (d, 3H), 2.60 (t, 2H), 3.45 (t, 2H), 5.12 (q, 1H), 7.15−8.89 (m, 11H); LC−MS *m/e* 499 ([M + H]$^+$, 100), 501 (32).

Preparation of Protein−Hapten Conjugates. Haptens were conjugated to KLH and BSA by activated ester method (*22*). In brief, to the hapten was added a 0.5 mL DMF solution of 2.0 equiv of NHS and 1.5 equiv of DCC. The mixture was stirred at room temperature for 4 h and centrifuged to remove precipitated urea. The resulting activated ester was slowly added to KLH solution (KLH in 10 mL of 0.1 M borate buffer, pH 9.0) and BSA solution (BSA in 10 mL of 0.1 M borate buffer, pH 9.0). The reaction mixture was stirred overnight at 4 °C and then dialyzed against phosphate-buffered saline (PBS, 1 L) for 3 days at 4 °C with two buffer changes per day. Protein content was determined by the Bradford method (*23*). The conjugation ratio of hapten−protein conjugate was determined by the trinitrobenzenesulfonic acid (TNBSA) method (*24*). The amounts of hapten and protein for conjugation and conjugation ratio are shown in **Table 1**.

Immunization and Antibody Separation. Three New Zealand white rabbits (2−4 kg) were immunized with hapten−KLH conjugate (200 μg for each rabbit), emulsified in Freund's complete adjuvant. Five booster injections were given at 2 week intervals. Injection was made intradermally and subcutaneously at multiple sites on the animals' back.

Table 1. Amount of Haptens and Proteins for Conjugation, Final Concentration of Protein, and Conjugation Ratios

hapten	protein	amount of hapten (mg)	amount of protein (mg)	final concentration of protein (mg/mL)	conjugation ratio (%)
1	KLH	20	20	1.18	34
	BSA	20	50	6.15	45
2	KLH	25	20	2.05	24
	BSA	25	20	7.40	19
3	KLH	20	20	2.98	47
	BSA	20	50	6.33	60

Titers of antisera were monitored by noncompetitive ELISA using checkboard titration. Seven days after the last injection, the rabbits were bled, and antisera were collected and stored at −80 °C. The antiserum was purified with a HiTrap Protein G HP column (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's instructions. Purified IgG in PBS solution (phosphate-buffered saline solution, 10 mM phosphate, 137 mM NaCl, and 2.7 mM KCl, pH 7.5) was stored at −80 °C.

Checkboard Titration. A microplate was coated with hapten (**1**, **2**, **3**)−BSA conjugate solution (100 ng/well in 0.05 M carbonate−bicarbonate buffer, pH 9.6) by incubating at 4 °C overnight. The plate was washed five times with 0.05% Tween-PBS (PBST, 0.5 mL of Tween 20 in 1 L of PBS solution), dried, and 1% BSA/PBS (1 g of BSA in 100 mL of PBS, 200 μL/well) was added to block the uncoated sites. After preincubation of the plate at 37 °C for 1 h, the plate was washed, dried, and incubated at 37 °C for 1 h with antisera (50 μL/well, 1:5000-fold diluted) from a rabbit immunized with hapten (**1**, **2**, **3**)−KLH. The plate was washed and further incubated with the secondary antibody, goat anti-rabbit IgG-HRP (1:10 000 in PBST, 100 μL/well), at 37 °C for 1 h. After another washing, 100 μL/well of OPD solution (0.4 mg/mL in 0.05 M citrate−phosphate with 0.03% sodium perborate, pH 5.0) was added. The reaction was stopped with sulfuric acid solution (2 M, 50 μL/well) after 20 min incubation at room temperature. The absorbance was measured at 492 nm.

Indirect Competitive ELISA. A microplate was coated with hapten-**3**−BSA conjugate solution (100 ng/well) by incubating at 4 °C overnight. The plate was washed five times with 0.05% Tween-PBS, dried, and 1% BSA/PBS was added to block the uncoated sites. After

the incubation at 37 °C for 1 h, the plate was washed, dried, and incubated at the same condition with a mixture of a constant concentration of rabbit antisera (50 μL/well) from hapten-**1**−KLH and various concentrations of analytes (50 μL/well). The plate was washed and further incubated with the secondary antibody, goat anti-rabbit IgG-HRP, at 37 °C for 1 h. After another washing, 100 μL/well of OPD solution was added. The reaction was stopped with sulfuric acid solution after 20 min incubation at room temperature. The absorbance at 492 nm was then read. Standard curve was fitted with a logistic 4-parameter equation Abs = $(A - D)/[1 + (\text{concentration}/C)^b] + D$, where A was the absorbance value with no chemical present; D was the absorbance value with the highest concentration of chemical; C was midpoint inhibition concentration (IC_{50}); and b was the slope near the midpoint inhibition concentration with SigmaPlot software (SPSS Inc., Chicago, IL). Linear detection range was established between IC_{20} and IC_{80} (*25*). Limit of detection (LOD) or lower limit of detection (LLD) was calculated as IC_{10} (*26*).

Cross-Reactivity. Cross-reactivity was calculated as the ratio of IC_{50} of the metamifop standard to the IC_{50} of the other compounds in PBST solution and expressed as a percentage. The sensitivity (IC_{50}) of the other compound was obtained by ciELISA described above.

RESULTS AND DISCUSSION

Preparation of Haptens and Their Protein Conjugates. Because small molecules, such as metamifop, usually cannot induce an immune response, it is necessary to prepare proper haptens that can be conjugated to a larger biomolecule (e.g., hapten−protein conjugates) and presented to the immune system (*27*). To develop a sensitive and specific immunoassay method, proper design of immunogen hapten is the most important part and requires a sophisticated understanding of its behavior on carrier proteins. For example, a highly lipophilic hapten, conjugated on the protein surface, may fold into the hydrophobic interior of the protein and, thus, result in a low affinity antibody (*28*). These findings suggest that a short or rigid handle (side chain) could be a better choice for lipophilic haptens or ligands with highly flexible bonds, such as metamifop and other AOPPs. In this study, synthetic procedures including hydrolysis and substitution have been applied to synthesize three haptens, which mimic metamifop. Those haptens contain a reactive carboxyl group for the production of protein conjugates, which were used as immunogens and plate-coating antigens. Therefore, three haptens containing the whole or partial structure of metamifop with a short handle on aromatic rings were designed with the help of molecular modeling to recognize the chlorobenzoxazolyloxyphenoxy moiety of metamifop. Haptens-**1** and -**2** were synthesized via alkaline hydrolysis of K-13209 and fenoxaprop-P-ethyl (**Figures 2** and **3**) (*29*). Hapten-**3** was synthesized by hydrolysis of compound **2** after condensation of hapten-**2** and compound **1** using oxalyl chloride (**Figure 5**). Because the diaryl ether bond of metamifop and the ester of haptens were labile in strong bases (e.g., sodium hydroxide and potassium hydroxide, data not shown), alkaline hydrolysis was carried out with weakly basic condition using lithium hydroxide in a mixture of dioxane and water.

Three haptens (haptens-**1**, -**2**, and -**3**) were conjugated to KLH to use as immunogens for the antibody production, and conjugated to BSA to use as coating antigens (**Table 1**). Conjugation of haptens and carrier proteins was performed by the activated ester method (*22*). The protein contents of the hapten−protein conjugates (0.7−9.9 mg/mL) were determined for the calculation of the amount of the hapten−protein conjugates to be used as immunogen or coating antigens. The coupling density was estimated by comparing the absorbance with the corresponding values of hapten-free proteins. From the available amino groups, 45% of hapten-**1**, 19% of hapten-**2**, and 60% of hapten-**3** were conjugated in the hapten−BSA conjugates, while 34% of hapten-**1**, 24% of hapten-**2**, and 47% of hapten-**3** were conjugated in the hapten−KLH conjugates.

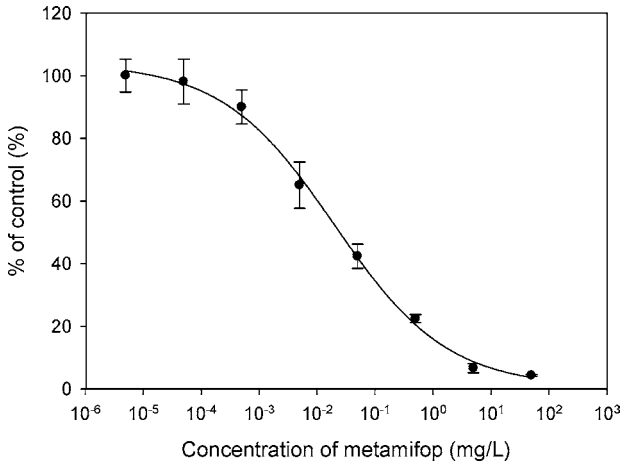

Figure 5. Potential standard curve obtained under optimized conditions in the ciELISAs for metamifop.

Table 2. Result of Cross Checkboard Titration[a]

	1-KLH	2-KLH	3-KLH
1-BSA	+++	+	+
2-BSA	+	++	+
3-BSA	+++	+	++

[a] +++, OD > 1.0; ++, 1.0 > OD > 0.5; +, 0.5 > O.D.; coating antigen, 100 ng/well; 1:5000-fold diluted antisera.

Titers of the Antisera. Three hapten−KLH conjugates were injected seven times into each of three rabbits as immunogens, respectively. The antisera collected after each boost was subjected to titration by the homologous indirect ELISA through checkboard titration. All antisera after the fifth or sixth boosting showed higher titers than those of the others. These results indicate that specific antibodies in the rabbit antiserum were produced against each immunogen. The final antisera were used for the subsequent screening in search of specific antibodies to the target compound metamifop. All antisera did not show any significant affinity for BSA itself as a coating antigen (**Table 2**).

Screening and Selection of Antisera by ciELISA. The lowest detection level is observed when the affinity of the antibody for the analyte is greater than that for the plate-coating hapten−protein conjugate. This can be achieved by selecting a different hapten−protein conjugate as coating antigen with an immunizing hapten−protein conjugate, and the equilibrium shifts to the antibody−analyte complex providing a low level of detection (*30*). So, heterology is commonly used to eliminate problems coming from no or poor inhibition by the target compound, associated with the strong affinity of the antibodies for the bridging groups. It usually results in somewhat weaker recognition of plate-coating antigens than recognition of the target analyte. Thus, lower analyte concentrations can compete with these reagents under equilibrium conditions, which results in better assay sensitivity than homologous system (*31*, *32*). For the heterologous metamifop ELISA in this study, heterology included hapten heterology using different hapten structures, site heterology, and linker heterology using spacer arms with different lengths (*33*).

To establish a sensitive ELISA, all combinations between coating antigens and antisera were screened via the inhibition

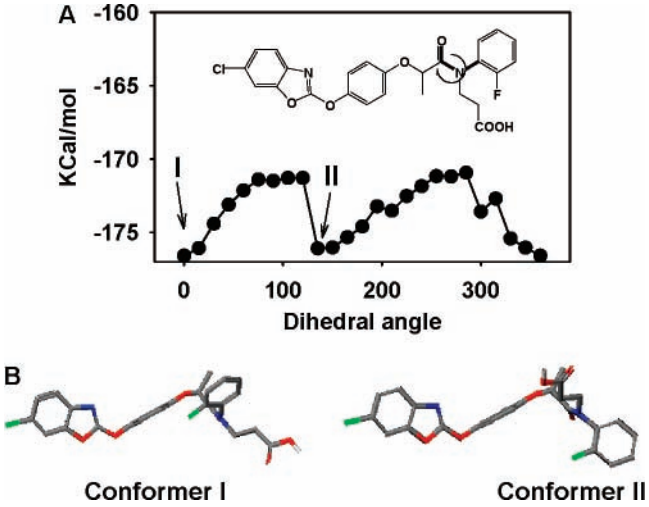

Figure 6. Potential energy map of various conformers of hapten-**3** at specified dihedral angle (**A**) and the structures of the lowest energy conformer I and local energy minimum conformer II (**B**).

by different concentrations of the analyte dissolved in the assay buffer, using the homologous or heterologous competitive indirect ELISA system. The inhibition ratio was expressed as a percentage of the difference between the absorbance of the analyte-free buffer and that of the analyte containing buffer, divided by the former. Therefore, the antisera of terminal bleeds from three rabbits were screened against three different coating antigens using two-dimensional titration with the coated antigen format. Mostly, the homologous assay, in which the same hapten was used in coating antigen and immunogen, had a higher titer than the heterologous assay. The results are consistent with a previous study on the deltamethrin (*34*) and bensulfuron-methyl (*25*), anti hapten-**1**−KLH antiserum had the highest affinity for coating antigen hapten-**1**−BSA, followed by hapten-**3**−BSA. Combinations of coating antigen and antiserum that resulted in high optical densities (OD > 1.0) were selected for further development (**Table 2**).

There was almost no or very low inhibition by the analyte in homologous ELISAs using the same hapten for a coating antigen and an immunogen, whereas there were very high inhibitions in heterologous ELISAs using the combinations of hapten-**3**−BSA as coating antigens with the antisera raised against the hapten-**1**−KLH.

The IC_{50} values of the rabbit antiserum **1**−KLH immunogen were 5000 and 20.1 ng/mL, on a plate coated with hapten-**1**−BSA, or with hapten-**3**−BSA, respectively.

The linear detection range was 1.0−350 ng/mL, which corresponds to IC_{20}−IC_{80}, and the LOD (IC_{10}) was 0.1 ng/mL (**Figure 5**).

Affinity and Cross-Reactivity. The recognition of specific ligand by antibody can be assessed by (a) steric properties and (b) chemical properties of atoms/molecules (e.g., electrostatic potentials and hydrophobicities). To prepare proper haptens for metamifop or its related pesticides, both steric and electrostatic properties of metamifop and other possible ligands were evaluated by computational modeling.

AOPPs, including metamifop, have many freely rotatable bonds, which make a low-energy conformer search difficult. For example, potential energy barriers of the rotation of amide bond were approximately 2−6 kcal/mol for hapten-**3** (**Figure 6**). From the conformational searches, many local minima were found. In this study, only the lowest or second lowest energy conformers were considered for further studies.

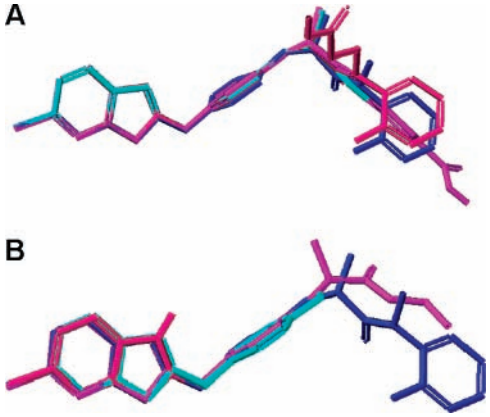

Figure 7. Superimposed structures of (**A**) hapten-**1** (purple), -**2** (fenoxaprop-P, pale blue), and conformer II of hapten-**3** (magenta) on metamifop (blue), (**B**) fenoxaprop-P-ethyl (purple), 4-(6-chlorobenzoxazol-2-yl)-phenol (pale blue), and 6-chloro-3H-benzoxazol-2-one (magenta) over metamifop (blue).

The linker groups of hapten-**1** and -**2** were positioned at the peripheral side of the molecules, where these haptens were prepared for antibodies, interacting with diaryl ether groups. Superimposing the energy minimized structures showed strong similarities between metamifop and its haptens, fenoxaprop-P and fenoxaprop-P-ethyl (**Figure 7**). Although *N*-phenylpropionamide groups were not superimposed well between structures, complete overlaps were found in the diaryl ether groups, which may be important for the antibody recognition. As one of the possible determinant of antibody−antigen recognition, electrostatic potentials of various haptens and related compounds were also calculated (**Figure 8**). Electrostatic potential maps of hapten-**1** and fenoxaprop-P (hapten-**2**) were slightly different from that of metamifop (**Figure 8B**, **C**, and **A**, respectively). In summary, antibodies from haptens-**1** and -**2** may recognize a common functional group in ligands, the diaryl ether group.

The linker group was placed on the nitrogen of propionamide in hapten-**3**. Because of the presence of many feasible conformers (e.g., **Figure 6**), the electrostatic potential maps were evaluated with two representative conformers (**Figure 8D** and **E**). Conformer I has its *N*-phenyl group, located perpendicular to the long axis of the molecule (**Figure 8D**). From the molecular modeling, antibodies from this conformation were expected to interact with the diaryl ether group, which was shared by many ligands (metamifop, fenoxaprop-P, and fenoxaprop-P-ethyl). The overall shape of conformer II (**Figure 8E**), in which the linker was positioned perpendicular to the long axis of the molecule, was more similar to metamifop than any other ligands, and the antibodies from this conformer were expected to be specific for metamifop. Both conformers, however in general, showed electrostatic potential maps very similar to that of metamifop. In addition, the low-energy barrier between two conformers suggested that both conformers may exist in relevant conditions and contribute equally during the immunization procedures.

Cross-reactivities of various ligands against the antibody, obtained by hapten-**1**−KLH conjugate, are described in **Table 3**. Among the various parts of molecules, 4-(6-chlorobenzoxazol-2-yloxy)-phenoxy group was the common functional group in metamifop, fenoxaprop-P, and fenoxaprop-P-ethyl. Although the electrostatic potential maps of fenoxaprop-P and fenoxaprop-P-ethyl were slightly different from those of metamifop and hapten-**3**, the overall shapes and distributions of charges were quite similar (**Figure 8**). Appreciably high cross-reactivities of these ligands suggested that the 4-(6-chlorobenzoxazol-2-yloxy)-

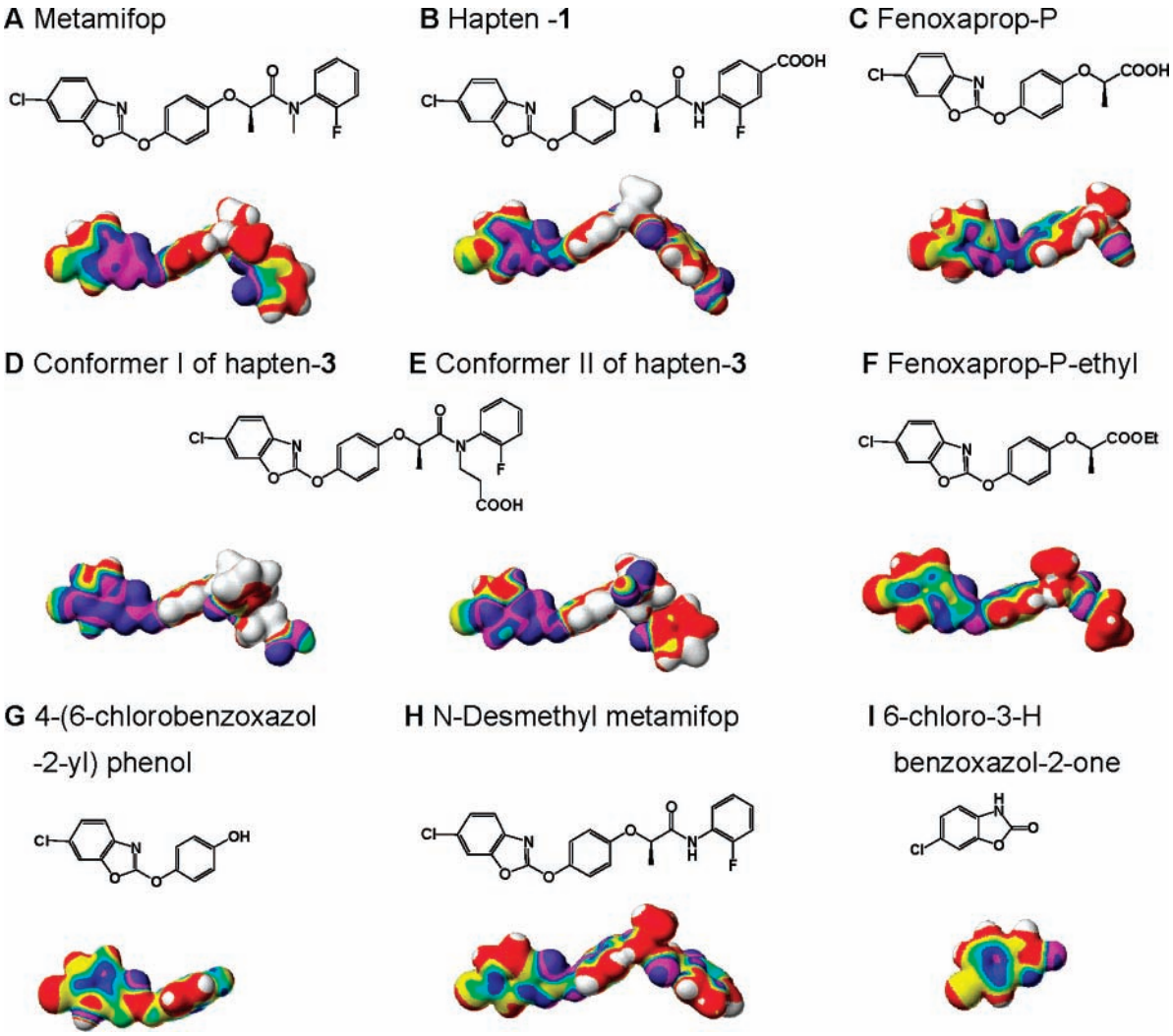

Figure 8. Structures and electrostatic potential maps on electron density isosurface of metamifop (**A**), haptens (**B**–**E**), and other ligands (**F**–**I**). Color code for electrostatic potential maps: white > 0.090 > red > 0.030 > yellow > 0.010 > green > 0.000 > pale blue > –0.010 > blue > –0.030 > pink > –0.06 > violet.

Table 3. Cross-Reactivity of the ELISA System[a] for Metamifop and Its Structurally Related Compound

compound	IC_{50} (ng/mL)	CR[b] (%)
metamifop	20.1	100
fenoxaprop-P	35.6	56.4
fenoxaprop-P-ethyl	19.2	104.2
4-(6-chlorobenzoxazol-2-yl)phenol	79.4	25.3
N-desmethyl metamifop	107.5	18.7
6-chloro-3H-benzoxazol-2-one	NI[c]	0

[a] The plate was coated with 5.0 ng of 3-BSA, and 1:10 000-fold diluted anti-1-KLH antiserum was used. [b] % cross-reactivity (CR) = (IC_{50} of metamifop/IC_{50} of other compound) × 100. [c] NI, no inhibition.

phenoxy group plays an important role in antigen−antibody recognition (**Table 3**). In addition, no cross-reactivity of 6-chloro-3H-benzoxazol-2-one supported that the 6-(4-chlorobenzoxazol-2-yl)-phenyl group is the most important part in antibody binding. To determine the effects of other parts of molecules on antibody binding, the cross-reactivities of several synthetic precursors were measured. Both 4-(6-chlorobenzoxazol-2-yloxy)-phenol and *N*-desmethyl metamifop showed appreciable cross-reactivity (**Table 3**). Practically similar cross-reactivities of both compounds suggested that the *N*-phenyl group has a negligible effect on antibody binding affinity. In comparison with the above two synthetic intermediates, the presence of a propionate group greatly enhanced the affinity [e.g., cross-reactivity of fenoxaprop-P vs 4-(6-chlorobenzoxazol-2-yl)-phenol] (**Table 3**). Esterification of carboxylic groups (fenoxaprop-P-ethyl) further increased the cross-reactivity. However, the electrostatic potential maps of 4-(6-chlorobenzoxazol-2-yl)-phenol groups of these ligands were not significantly different (e.g., **Figure 8A**, **C**, and **F**). These finding suggested that higher hydrophobicity of linker groups (ester of propionate vs propionic acid and *N*-methylamide vs amide) is also important for proper binding. Through the interpretation of cross-reactivity, molecular modeling proved to be useful for antibody recognition (*35*) as well as hapten design (*36*).

Fenoxaprop-P-ethyl showed the highest cross-reactivity, but it is expected that the probability of coexistence in analytical sample is low because the application spectrum of that herbicide is similar to metamifop; therefore, only one of those would be used in field. The ELISA, when it is fully developed, will help primarily to detect the concentration of this herbicide in paddy water to prevent ground or underground water contamination in addition to the detection of residues in crop.

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