Monoclonal-Based ELISA for the Identification of Herbicidal Cyclohexanedione Analogues That Inhibit Graminaceous Acetyl Coenzyme-A Carboxylase

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Cyclohexanediones are one of four known structural classes of herbicides that inhibit graminaceous acetyl coenzyme-A carboxylase (ACCase; EC 6.4.1.2). Five monoclonal antibodies were raised against cyclohexanediones conjugated to bovine serum albumin. Cross-reactivity studies using a homologous competitive indirect enzyme-linked immunosorbent assay (ciELISA) against 24 cyclohexanedione analogues revealed that two monoclonal antibodies (mAb A and mAb B) could segregate the analogues into active and inactive ACCase inhibitors on the basis of the analogue concentration required to inhibit 50% of antibody binding to the coating conjugate (IC_{50}). Both mAb A and mAb B were also found to cross-react with various members of the indolizidinedione structural class of ACCase inhibitors in ciELISA, suggesting that both cyclohexanediones and indolizidinediones possess features recognized by monoclonal antibodies important for the inhibition of ACCase activity. In conclusion, pharmacophore-specific antibodies may be potentially valuable screening tools for the identification of new lead chemistries in a pesticide discovery program.

Keywords: *Monoclonal; cyclohexanedione herbicides; ACCase*

INTRODUCTION

Pharmaceutical and agrochemical companies rely on three basic strategies to discover new lead chemistries: random screening, structural modification of an existing active lead, and rational or structure-based design (Schacter et al., 1992). The traditional discovery strategy is based on the random screening of large numbers or, more recently, a diverse series of compounds in search of desired biological activities (Moos et al., 1993). The screening assays used to identify new chemical leads from large random libraries of molecules differ between the two industrial sectors. In general, pharmaceutical companies must employ a series of in vitro or biological assays to screen compounds, whereas agrochemical companies can directly screen compounds against the actual in vivo target. Although the random screening strategy accounts for the discovery of the majority of pesticides available today, it remains an inherently inefficient process. Furthermore, the adoption of combinatorial synthesis has resulted in an exponential increase in the number of compounds available for screening, which may overwhelm the traditional in vivo target assays, thereby limiting the usefulness of the synthesis strategy (Gallop et al., 1994; Gordon et al., 1994).

To streamline the discovery process, new screening methods are being developed that allow for the rapid and efficient screening of large numbers of compounds (Kleinberg and Wanke, 1995). These methods, referred to as "high-throughput" screening, must be accurate, inexpensive, robust, and amenable to automation. Ideally, the in vitro pesticide target site would be used for the high-throughput screen assay, but in cases when the target site is unknown or not stable or the assay cannot be properly formatted, alternative screening methods must be developed. An alternative screening method that meets the assay requirements (i.e., rapid, inexpensive, robust, amenable to automation) is to use antibodies as screening tools. The rationale for this is based on the observation that antibodies when produced against small ligands may demonstrate binding properties similar to the natural receptor (Linthicum et al., 1988). It has been suggested that such antibodies are mimics of biological receptors; however, the precise nature of this mimicry has not yet been determined. Our interest in antibody mimics is their application as surrogates of pesticide target sites for use in high-throughput screens.

To assess the utility of an antibody-based approach to screening, a pilot study was initiated to develop antibody mimics to the graminaceous herbicide target site acetyl coenzyme-A carboxylase (ACCase). Initial research focused on the production of polyclonal antibodies to a cyclohexanedione−bovine serum albumin immunogen and the development of two indirect enzyme-linked immunosorbent assays (ELISAs). Cyclohexanedione analogues were tested in the two ELISAs for cross-reactivity to the polyclonal antiserum. These cross-reactivity studies revealed the polyclonal antiserum may be used to cluster analogues into active inhibitors of corn ACCase and inactive analogues on the basis of the analogue concentration required to inhibit 50% of the antiserum binding to the immobilized coating conjugate in the ELISA (unpublished results). However, there was no quantitative relationship between inhibition of antiserum binding and inhibition of ACCase activity. Fur-

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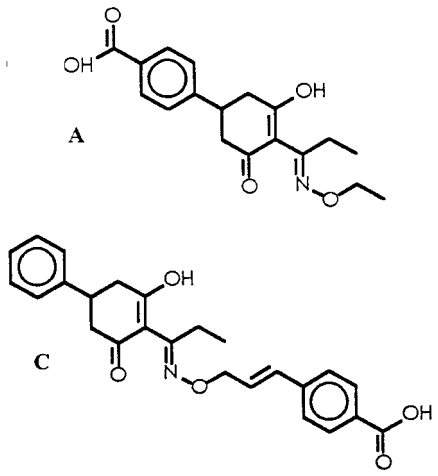

Figure 1. Structure of cyclohexanedione analogues used to produce the cyclohexanedione−protein conjugates. Immunogens IA and IC were produced by conjugating analogues **A** and **C** to BSA, respectively, as described under Materials and Methods. Coating conjugates CCA and CCC were prepared by conjugating analogues **A** and **C** to OVA, respectively, as described under Materials and Methods.

thermore, the polyclonal antiserum failed to cross-react with other known structural classes of ACCase inhibitors. To further evaluate the utility of an antibody approach to screening and to avoid potential problems inherent with the undefined nature of polyclonal antiserum, monoclonal antibodies to the cyclohexanedione inhibitor class of herbicides were produced. In this paper we report the production and characterization of cyclohexanedione pharmacophore-specific monoclonal antibodies and their cross-reactivity with other known structural classes of ACCase inhibitors.

MATERIALS AND METHODS

Chemicals and Reagents. All cyclohexanedione analogues and aryloxyphenoxypropionate, indolizidinedione, and triazinedione analogues were supplied by Dow AgroSciences (Indianapolis, IN). Bovine serum albumin (BSA), ovalbumin (OVA), Freund's complete and incomplete adjuvants, *N*-hydroxysuccinimide (NHS), *N*,*N*′-dicyclohexylcarbodiimide, urea, hydrogen peroxide, Sigma 104 phosphatase substrate, and 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) substrate tablets (ABST) were obtained from Sigma Chemical Co. (St. Louis, MO). Goat anti-mouse IgG conjugated to horseradish peroxidase, goat anti-mouse Ig−alkaline phosphatase conjugate, goat anti-mouse IgM conjugated to horseradish peroxidase, and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide were obtained from Pierce Immunochemcials (Rockford, IL). Pristane was obtained from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were of reagent or pesticide grade and obtained commercially.

Preparation of Cyclohexanedione—Protein Conjugates. The cyclohexanedione haptens used in this study for the construction of immunogens and coating conjugates (Figure 1) contained a carboxylic acid group and were conjugated to proteins by the *N*-hydroxysuccinimide-active ester method as modified by Webb et al. (1997).

Immunogens. Two immunogens, IA and IC, were prepared by conjugating cyclohexanedione analogues **A** and **C** (Figure 1), respectively, to BSA as previously described (Webb et al., 1997).

Coating Conjugates. Coating conjugates CCA and CCC were prepared by conjugating analogues **A** and **C** (Figure 1), respectively, to OVA as previously described (Webb et al., 1997). Analogue **A** defined the minimum structural features of the cyclohexanedione pharmacophore required for the inhibition of ACCase.

Production of Monoclonal Antibodies. Monoclonal antibodies raised against immunogens IA and IC were prepared as previously described by Deschamps et al. (1990) with the following modifications.

Immunization. For each immunogen, 10 8-week-old Balb/c mice were injected intraperitoneally with a 1:1 (v/v) mixture of 140 μg of immunogen dissolved in PBS (pH 7.4) and Freund's complete adjuvant in a total volume of 250 μL. Secondary immunizations were given at two and five weeks following the primary immunization. The secondary immunizations consisted of a 1:1 (v/v) mixture of 70 μg of immunogen in PBS (pH 7.4) and Freund's incomplete adjuvant in a volume of 250 μL. Additional boosts (tertiary immunizations) were administered at 3 week intervals following the secondary immunization and consisted of 70 μg of immunogen in 250 μL of PBS.

Each week following the secondary and tertiary immunizations, the mice were bled from the retro-orbital plexus and the anti-cyclohexanedione serum titer was determined using the indirect ELISA procedure described below. The mice possessing the highest specific titer were given a final *intravenous* immunization consisting of 70 μg of immunogen in 150 μL of PBS (pH 7.4) and were sacrificed several days later by cervical dislocation.

Hybridization. The spleen of each mouse was freed of connective tissue and placed into a Petri dish containing serum-free RPMI medium. The spleen was cut into several small pieces and gently forced through a 400-mesh stainless steel screen. The cell suspension was transferred to a sterile centrifuge tube, and large tissue aggregates were removed by sedimentation as described by Shortman et al. (1972). The cell suspension was centrifuged at 200*g* for 10 min, and the resulting cell pellet was resuspended in fresh RPMI medium. The spleen cells were mixed with an equal number of log phase SP/2.0 myeloma cells in RPMI medium. The cell mixture was centrifuged as described above, and the pellet was resuspended in 1 mL of 50% (v/v) polyethylene glycol (3000−4000 molecular weight range) in RPMI medium at 37 °C. The cell suspension was mixed for 1 min, followed by the addition of 1 mL of RPMI medium and additional mixing for 1 min. An additional 9 mL of RPMI medium was slowly added to the suspension with mixing. The fusion products were centrifuged as described above, and the cell pellet was resuspended in RPMI medium supplemented with 10% (v/v) fetal bovine serum, 10% NCTC-109 medium, and 1% (v/v) hypoxanthine, aminopterin, and thymidine (HAT) to select for hybrid cells (Zola, 1987). The cell suspension was dispensed (100 μL/well) into microplates. The plates were incubated at 37 °C in an atmosphere of 5% (v/v) CO_2 in air. Four days following the fusion procedure, the cell cultures were resupplied with fresh medium daily, for 3 days.

Fusion Product Screening. Ten days following hybridization, the cell culture supernatants were screened for the presence of anti-cyclohexanedione antibodies. Immulon 2 U-shaped microplates (Fisher Scientific, Don Mills, ON) were coated with coating conjugate CCA or CCC diluted in coating buffer (10 mM bicarbonate, pH 9.5) and incubated at 37 °C for 2 h. The wells were washed three times with Tris/Tween [20 mM Tris-HCl, 0.15 M NaCl, 0.05% (v/v) Tween 20, pH 7.6]. Culture supernatant (100 μL/well) was transferred to the coated wells and incubated for 1 h at 37 °C. The wells were washed with Tris/Tween as described above, and 100 μL/well of goat anti-mouse Ig−alkaline phosphatase conjugate diluted 1:750 in PBS (7.4) was added and incubated as described above. After 1 h, the wells were washed as described above and 100 μL/well of substrate [1 mg of disodium *p*-nitrophenyl phosphate/mL of 1% (w/v) diethanolamine buffer, pH 9.8] was added. The color reaction was allowed to proceed for 30 min, followed by the addition of 50 μL/well of 2.0 M NaOH to terminate the reaction. Absorbance at 405 nm was measured using a Bio-Rad model 3550-UV microplate reader (Bio-Rad Laboratories, Hercules, CA). The presence of antibody-producing cultures capable of binding to the immobilized coating conjugate was determined by a positive color reaction ($A_{405nm} > 0.5$). Cultures

testing positive were transferred to 24-well culture plates for further proliferation.

After 10 days, the culture supernatants from the 24-well culture plates were assayed for the presence of cyclohexanedione-specific antibodies using the competitive indirect ELISA described below with analogue **A** (200 μM) being used to compete with either coating conjugate CCA or CCC for antibody binding. The presence of cyclohexanedione-specific antibodies was determined by a decrease of 50% in A_{405nm} in the presence of analogue **A** when compared to A_{405nm} without the competing analogue. Throughout this assessment process, the cultures were gradually depleted of HAT by resupplying the cultures with the same medium containing successively lower amounts HAT.

Limiting Dilution. Cultures producing cyclohexanedione-specific antibodies were selected by limiting dilution to achieve clonality of the hybridoma cells. The cell concentration was estimated by trypan blue viability staining and diluted to give a final concentration of 1 cell/100 μL of medium. This dilution was dispensed (100 μL/well) into 96-well microplates and incubated as described above. The wells were checked daily for the presence of a single colony. Once a colony was observed, the culture medium was assayed for the presence of anti-cyclohexanedione antibodies as described above. Cells from colonies testing positive were transferred to 24-well culture plates, rescreened, and subjected to a second limiting dilution to ensure monoclonality. After a final assessment for the presence of anti-cyclohexanedione specific antibodies, hybridoma cells were collected for the production of ascitic fluid in mice.

Production of Ascites Fluid. Approximately 1×10^7 cells from selected hybridoma cell lines, suspended in 200 μL PBS, were injected intraperitoneally into Balb/c mice. These mice were injected intraperitoneally 10 days earlier with 500 μL of pristane. Three weeks following the injection of the mice with the hybridoma cell line, the peritoneal cavity was distended and the ascites fluid collected.

Monoclonal Antibody Isotyping. The monoclonal antibody heavy and light chain isotypes were determined using the Pierce Immunochemicals antibody ELISA isotyping kit with coating conjugate CCA as the antigen.

Indirect ELISA. Coating conjugate CCA or CCC in PBS (~1 μg/mL) were passively absorbed to 96-well polystyrene microplates (Immulon 2, U-shaped Fisher Scientific, Don Mills, ON) at 4 °C overnight. Unbound coating conjugate was removed by washing three times with 100 μL/well of a 0.05% (v/v) Tween 20 in PBS (PBST, pH 7.5) solution. Unoccupied sites were blocked with 200 μL/well of 0.1% (w/v) gelatin in PBS (pH 7.5) for 20 min at room temperature, followed by three washings with PBST as described above. Serial dilutions of culture supernatant or ascites fluid were prepared in PBS (pH 7.5), and 100 μL/well of this solution was incubated with the bound coating conjugate for 1 h as described above. Unbound antibody was washed as described above, and 100 μL/well of goat anti-mouse IgG or goat anti-mouse IgM conjugated to horseradish peroxidase (1:5000 dilution in PBS, pH 7.5) was incubated with the antibody−coating conjugate complex for 1 h as described above. All wells were washed with PBST as described above, and 100 μL/well of substrate, 0.01% (w/v) urea hydrogen peroxide, and 0.1% ABST in 24 mM citric acid monohydrate and 47 mM sodium monohydrogen phosphate buffer (pH 5.0) was added. Following a 20-min incubation in the dark at room temperature, the color reaction was terminated by the addition of 100 μL/well of 0.5 M citric acid. Color development was quantitatively determined by measuring the absorbance at 405 nm as previously described (Webb et al., 1997).

Competitive Indirect ELISA (ciELISA). Various analogues (Figures 2 and 3) were tested for their ability to compete with the immobilized coating conjugate CCA for binding to the monoclonal antibodies in ciELISA. Concentrated solutions of the test analogues were prepared by dissolving these compounds in acetone. When added to the monoclonal antibody, the concentration of acetone was 1% (v/v) and in these experiments antibody binding in the presence of 1% (v/v) acetone served as controls. The reaction mixture consisted of equal volumes of a known concentration of a test analogue and the appropriate dilution of antibody in PBS (Johnson and Hall, 1996). The reaction mixture was incubated for 30 min at room temperature. The reaction mixture was transferred to microplate wells previously coated with CCA (100 μL/well) and incubated for 1 h at room temperature. Once incubated with the appropriate goat-anti-mouse−horseradish peroxidase antibody conjugate and substrate, color development was inversely proportional to the concentration of the cross-reacting analogue.

Absorbance values of the reaction mixture containing known concentrations of test analogue (A) were normalized by dividing by the absorbance values of negative controls (wells containing only antibody in PBS; A_0). The A/A_0 values of the various analogues were plotted against the log concentration of the analogue to determine the concentration required to inhibit 50% of monoclonal antibody binding to the immobilized coating conjugate as described by Johnson and Hall (1996). All experiments were repeated two to five times, and the A/A_0 and apparent IC_{50} values were expressed as the mean ± standard error of the mean. In each instance, similar monoclonal antibody cross-reactivity patterns were observed.

RESULTS AND DISCUSSION

Cyclohexanedione—Protein Conjugates. The structures of the cyclohexanedione analogues used to produce both the immunogens and the ELISA coating conjugates are shown in Figure 1. Both cyclohexanedione analogue **A** [2-(1-ethoxyimino)propyl-5-carboxyphenyl-3-hydroxy-2-cyclohexen-1-one] and analogue **C** [2-[[[((4-carboxyphenyl-2-propenyl)oxy)]imino]propyl]-5-phenyl-3-hydroxy-2-cyclohexen-1-one] are in vitro inhibitors of maize ACCase. The concentrations of analogues **A** and **C** required to inhibit 50% of ACCase enzyme activity (IC_{50}) were 4.6 and 40 μM, respectively (C. T. Cseke, Dow AgroSciences, Indianapolis, IN, personal communication, 1993).

Analogues **A** and **C** (Figure 1) were selected as haptens for immunogen production in an attempt to present different aspects of the cyclohexanedione pharmacophore to the immune system. In previous studies it was shown that the hapten−protein conjugate that preserves the native structure and conformation of the ligand is most desirable for producing pharmacophore specific antibodies (Bolger et al., 1985; Flurkey et al., 1985; Linthicum et al., 1988; Sherman et al., 1986).

Production of Polyclonal Antibodies to Immunogens IA and IC. To evaluate the suitability of the two immunogens for producing antibodies specific for the cyclohexanedione pharmacophore, mice were immunized with immunogens IA and IC. After the tertiary immunization, all IA- and IC-immunized mice produced antiserum that recognized their respective homologous coating conjugate CCA and CCC, respectively. Antiserum from IA-immunized mice bound to both coating conjugate CCA and CCC, whereas IC-immunized mice recognized only the homologous coating conjugate. The antiserum was evaluated for binding to nonconjugated cyclohexanedione analogue **A** in the ciELISA. Antiserum from IA-immunized mice cross-reacted with analogue **A** in the ciELISA using both coating conjugates. The concentrations of analogue **A** required to inhibit 50% of IA antiserum binding to coating conjugate CCA and CCC were 12 ± 0.7 and 6.3 ± 0.3 nM, respectively. No inhibition of IC antiserum binding to coating conjugate CCC was observed at concentrations of analogue **A** up to 200 μM in the ciELISA.

Production of Anti-cyclohexanedione Monoclonal Antibodies. Results from the various cell fu-

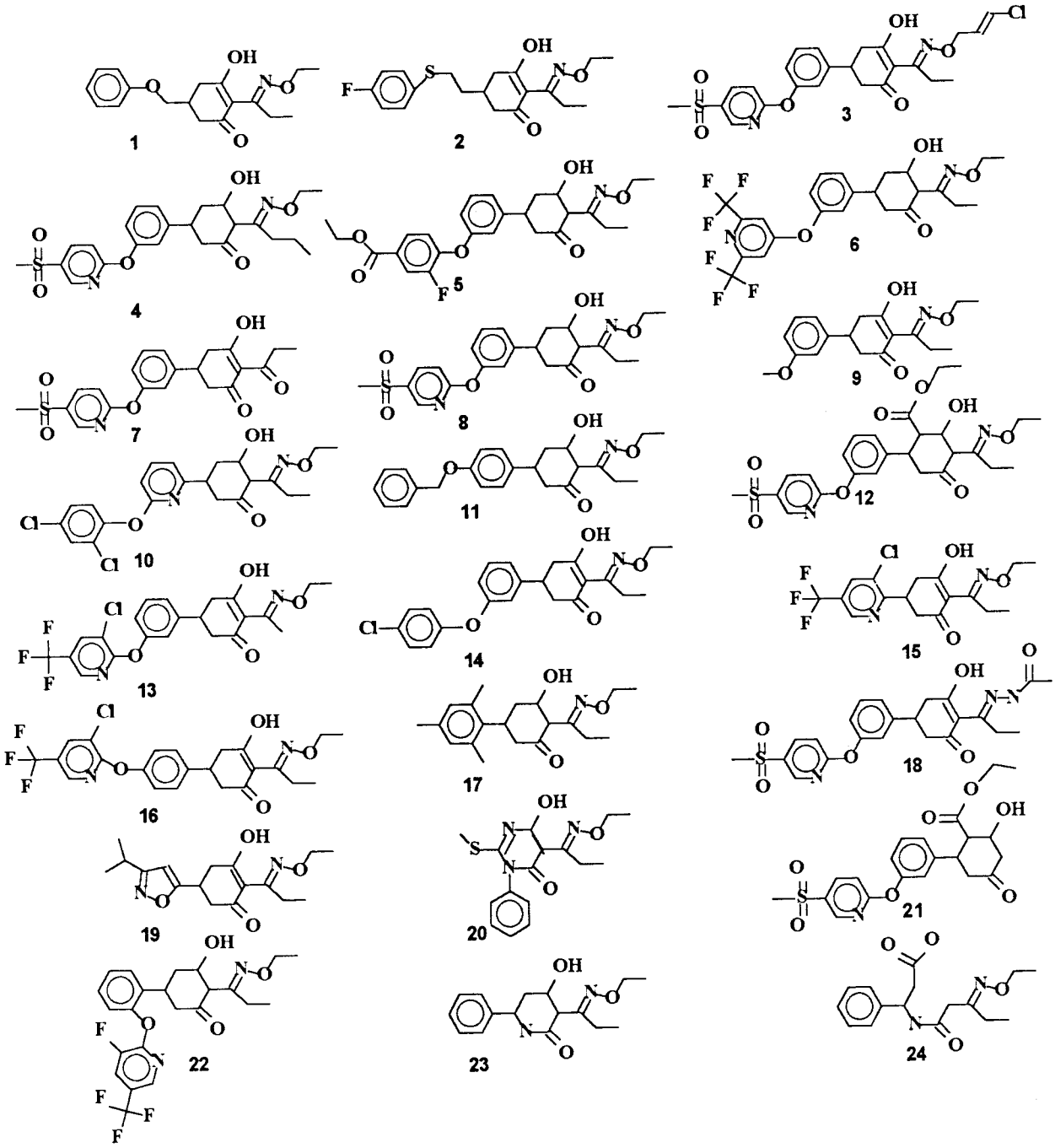

Figure 2. Structures of cyclohexanedione analogues used to evaluate the monoclonal antibody cross-reactivity profiles in the homologous ciELISA.

sions and the hybridoma selections for production of monoclonal antibodies that recognize the cyclohexanedione pharmacophore are summarized in Table 1. In general, all fusions resulted in antibodies that bound to the immobilized coating conjugates, but only a few of these antibodies were competitively inhibited by analogue **A** (Figure 1). The first fusion resulted in eight wells with antibodies that were competitively inhibited by analogue **A** in the ciELISA (Table 1). However, only one stable monoclonal antibody producing line, designated mAb A, was recovered. The second and third fusions (Table 1) using IA-immunized mice failed to produce any cyclohexanedione-specific antibody-producing cultures. The fourth fusion resulted in a single monoclonal antibody producing the cell line designated mAb B (Table 1). Both mAb A and mAb B bound only to coating conjugate CCA. The fifth fusion (fusion 5, Table 1) produced the largest number of positive antibody-producing wells to both coating conjugates and resulted in the recovery of three stable cell lines designated $mAbC_1$, $mAbC_2$, and $mAbC_3$. Like mAb A and mAb B, $mAbC_1$ bound only to coating conjugate CCA, whereas antibodies from $mAbC_2$, and $mAbC_3$ bound to both coating conjugates CCA and CCC.

The fusion using a mouse immunized with immunogen IC produced several wells that were positive for binding to both coating conjugates; however, analogue **A** did not compete with coating conjugates CCA or CCC for antibody binding. The difference between the two cyclohexanedione haptens (Figure 1) is the site of conjugation to the carrier protein. Analogue **C** (Figure 1) inhibited ACCase activity when conjugated to BSA. Incledon and Hall (1997) used this analogue as a ligand to affinity-purify corn ACCase, which suggests the

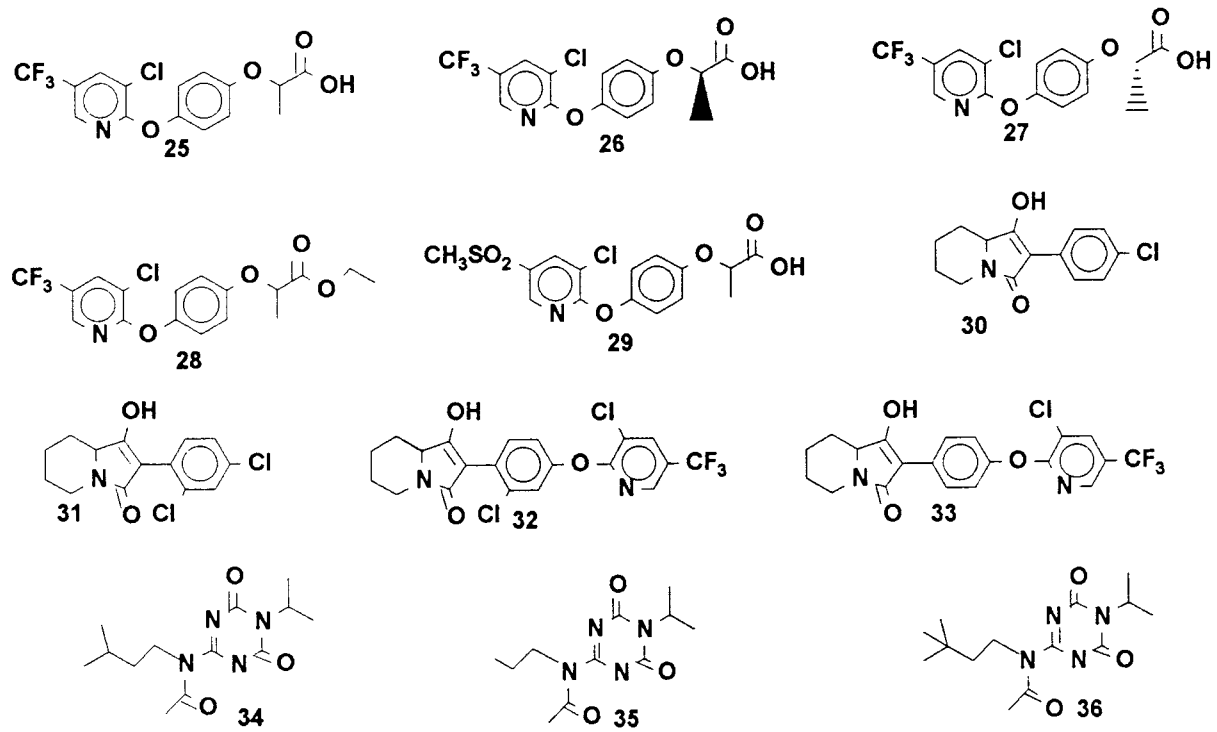

Figure 3. Representative analogues of other known structural classes of ACCase inhibitors. Analogues **25**−**29** are representative members of the aryloxyphenoxypropionate structural class. Analogues **30**−**33** are indolizidinedione structural class inhibitors. Analogues **34**−**36** are members of the triazinedione class of inhibitors.

Table 1. Summary of Cell Fusion and Hybridoma Production Results

immunogen[a]	fusion no.	no. of wells seeded	positive[b] CCA	positive[b] CCC	competitive[c] CCA	competitive[c] CCC	no. of cloned hybridomas
IA	1	576	127	NT[d]	8	NT	1
IC	2	576	86	256	0	0	0
IA	3	576	78	28	3	0	0
IA	4	576	151	75	4	0	1
IA	5	576	226	121	12	3	3

[a] Structures of the cyclohexanedione analogues used for construction of the immunogens and coating conjugates are shown in Figure 1. [b] Number of wells containing antibodies that bound to the coating conjugate in the indirect ELISA ($A_{405nm} > 0.5$). [c] The number of positive wells that when incubated with 200 μM analogue **A** (Figure 1) in the ciELISA resulted in 50% reduction in A_{405nm} compared to negative control. [d] NT, not tested.

cyclohexanedione pharmacophore is not altered during conjugation. In this study the lack of cyclohexanedione-specific antiserum or monoclonal antibodies suggests the pharmacophore is not a major antigenic determinant when analogue **C** is used as the hapten. These results are in agreement with a previous study in which immunogen IC failed to produce a cyclohexanedione-specific antiserum in rabbits (data not shown).

Characterization of the Monoclonal Antibodies. *Monoclonal Antibody Isotype.* The heavy and light chain isotype and coating conjugate binding profiles of the five monoclonal antibodies raised against immunogen IA are summarized in Table 2. Monoclonal antibody isotypes were identified to optimize the ciELISA (correct coating conjugate and secondary antibody; anti-IgG or anti-IgM). Furthermore, isotypes were determined to discover if heavy or light chain isotype influenced antibody utility in pesticide discovery applications. All monoclonal antibodies produced (IgG_1 and IgM classes) bound coating conjugate CCA, whereas only the IgM class

Table 2. Monoclonal Antibody Heavy and Light Chain Isotype and Coating Conjugate Binding Profile

monoclonal antibody	isotype: heavy chain	isotype: light chain	coating conjugate: CCA	coating conjugate: CCC
mAb A	IgG_1	kappa	+[a]	−[b]
mAb B	IgG_1	lambda	+	−
mAb C_1	IgG_1	kappa	+	−
mAb C_2	IgM	kappa	+	+
mAb C_3	IgM	kappa	+	+

[a] (+) positive, the antiserum recognized the cyclohexanedione−OVA conjugate. *b*(−) negative, the antiserum failed to recognize the cyclohexanedione−OVA conjugate.

monoclonal antibodies also bound to coating conjugate CCC (Table 2).

Cross-Reactivity to Selected Cyclohexanedione Analogues. Monoclonal antibody specificity for the cyclohexanedione pharmacophore was evaluated by determining antibody cross-reactivity profiles against a set of cyclohexanedione analogues using the ciELISA. The structures of the 24 cyclohexanedione analogue set are shown in Figure 2. The concentrations of the cyclohexanedione analogues required to inhibit 50% of monoclonal antibody binding both to coating conjugate CCA (IC_{50}) in the ciELISA and to ACCase from corn (IC_{50}) are summarized in Table 3. Cyclohexanedione analogues within the training set may be grouped into two clusters, active ACCase inhibitors and inactive analogues, on the basis of their IC_{50} values. Active ACCase inhibitors are defined as analogues with an IC_{50} value of $\leq$100 μM against a partially purified corn ACCase preparation (Cseke, personal communication, 1993). Inactive analogues are defined as compounds with IC_{50} values >100 μM against a corn ACCase enzyme preparation (Cseke, personal communication, 1993).

In general, both mAb A and mAb B have higher affinities for active cyclohexanedione analogues compared to $mAbC_1$, $mAbC_2$, and $mAbC_3$ (Table 3). For

Table 3. Concentration of Selected Cyclohexanedione Analogues (Figure 2) Required To Inhibit 50% of the Monoclonal Antibody in the Homologous ciELISA

	monoclonal antibody IC_{50} ± SEM, nM					ACCase[b]
analogue[a]	mAb A	mAb B	mAb C_1	mAb C_2	mAb C_3	IC_{50}, nM
1	NT[c]	11.8 ± 0.6	>20000	>20000	>2000	4470
2	144 ± 2.5	37.2 ± 0.9	>20000	>20000	>2000	1780
3	214 ± 7.2	3.60 ± 0.8	1180 ± 28.9	>20000	>2000	20
4	224 ± 9.2	2.40 ± 0.2	693 ± 15.6	>20000	>2000	710
5	69.3 ± 3.1	21.3 ± 5.1	1260 ± 27.8	>20000	>2000	300
6	468 ± 8.5	25.4 ± 1.2	986 ± 19.2	>20000	>2000	40
7	5250 ± 130	>20000	874 ± 26.2	>20000	>2000	>100000
8	10.0 ± 0.8	6.50 ± 0.2	685 ± 48.2	>20000	>2000	260
9	15.3 ± 1.2	16.1 ± 1.7	136 ± 7.4	878 ± 24.1	>2000	2570
10	16.4 ± 0.9	NT	>20000	>20000	>2000	220
11	5.30 ± 0.2	4.10 ± 0.2	255 ± 6.8	>20000	>2000	290
12	933 ± 24.4	2.60 ± 0.3	6820 ± 25.6	864 ± 42.5	>2000	11750
13	77.8 ± 2.5	5.50 ± 0.9	1230 ± 3.8	6190 ± 34.8	>2000	1070
14	661 ± 12.8	13.4 ± 0.8	1870 ± 17.2	>20000	>2000	100
15	3.80 ± 0.2	12.4 ± 0.9	>20000	>20000	>2000	3890
16	12.3 ± 1.2	11.9 ± 1.2	1250 ± 32.3	>20000	>2000	1100
17	331 ± 6.1	248 ± 4.1	>20000	>20000	>2000	330
18	>20000	6280 ± 830	1440 ± 20.8	786 ± 34.2	>2000	>100000
19	31.3 ± 0.3	28.5 ± 0.5	>2000	>20000	>2000	710
20	>20000	5540 ± 896	8700 ± 83.1	>20000	>2000	>100000
21	>20000	>20000	>20000	>20000	>2000	>100000
22	324 ± 22.1	134 ± 3.1	>20000	>20000	>2000	>100000
23	34.8 ± 8.2	144 ± 7.5	>20000	>20000	>2000	36310
24	>20000	>20000	>20000	>20000	>2000	>100000

[a] The structures of the cyclohexanedione analogues are shown in Figure 2. [b] Cyclohexanedione analogues are characterized as active inhibitors of corn ACCase with IC_{50} values ≤100 μM. ACCase IC_{50} values were provided by Dow AgroSciences (C. T. Cseke, Dow AgroSciences, Indianapolis, IN, personal communication, 1993). [c] NT, not tested.

instance, all active cyclohexanedione analogues inhibited 50% of mAb A and mAb B binding to CCA at concentrations <1 μM (Table 3). In contrast, active cyclohexanedione analogues **1**, **2**, **10**, **15**, **17**, **19**, and **23** (Figure 2) failed to inhibit 50% of $mAbC_1$, $mAbC_2$, and $mAbC_3$ binding to CCA at concentrations as high as 100 μM (Table 3). None of the cyclohexanedione analogues tested in the ciELISA were observed to inhibit 50% of $mAbC_3$ binding to CCA at 100 μM (Table 3). Therefore, antibodies $mAbC_1$, $mAbC_2$, and $mAbC_3$ were not specific for the cyclohexanedione pharmacophore, on the basis of the limited cyclohexanedione cross-reactivity spectrum (Table 3), and were not subjected to further characterization.

The IgG_1 subclass monoclonal antibodies (mAb A, mAb B, $mAbC_1$) had lower IC_{50} values for more cyclohexanedione analogues in the ciELISA compared to the IgM class monoclonal antibodies ($mAbC_2$, $mAbC_3$; Table 3). The differences in cyclohexanedione analogue affinity between the IgG_1 and IgM antibodies may be attributed to their inherent structural and functional differences.

Six inactive cyclohexanedione analogues (analogues **7**, **18**, **20**−**22**, **24**; Figure 2) were tested for their ability to inhibit monoclonal antibody binding in the ciELISA. These inactive analogues were included to determine antibody specificity for the active cyclohexanedione structure. In general, the IC_{50} values of inactive cyclohexanedione analogues were >1 μM for both mAb A and mAb B (Table 3). The only exception was analogue **22**, which had IC_{50} values of 0.32 ± 0.02 and 0.13 ± 0.003 μM for mAb A and mAb B, respectively. The concentration of analogue **22** required to inhibit 50% of mAb A and mAb B to coating conjugate CCA was similar to the IC_{50} values of several active cyclohexanedione analogues in the ciELISA (Table 3). The potency of this analogue against mAb A and mAb B binding is attributed to the structure of analogue **22** (Figure 2).

Monoclonal antibodies mAb A and mAb B were able to segregate most of the inactive cyclohexanedione analogues on the basis of their IC_{50} values because these analogues lack critical functional groups associated with the cyclohexanedione pharmacophore (Markley et al., 1995; Webb et al., 1997). For instance, the cyclohexane ring is absent in analogues **20** and **24** (Figure 2). Furthermore, analogue **20** has a phenyl substituent at position 6 of the six-member ring, which is not present on analogue **A** (Figure 1), the hapten used to construct the immunogen. Inactive analogues **7**, **18**, and **21** (Figure 2) are missing the critical oxime functional group at position 2 of cyclohexane ring (Markley et al., 1995; Webb et al., 1997). However, analogue **22** (Figure 2) possesses all of the critical functional groups associated with active cyclohexanedione inhibitors, namely, a cyclohexane ring with a carbonyl group at position 1, an oxyimino group at position 2, and a hydroxyl group at position 3 (Markley et al., 1995; Webb et al., 1997). The lack of ACCase inhibition by analogue **22** is attributed to the presence of the large ortho substituent on the phenyl group at position 5 of the cyclohexane ring (Figure 2). The large ortho substituent may prevent physical interaction between analogue **22** and the putative cyclohexanedione-inhibitor binding site on ACCase (Cseke, personal communication, 1993; Markley et al., 1995). Analogue **22** cross-reacts with both mAb A and mAb B because the critical pharmacophore functional groups on the cyclohexane ring and oxyimino substituents are identical to analogue **A** (Figure 1). Analogue **A** defined the minimum structural features of the cyclohexanedione pharmacophore required for the inhibition of ACCase. The structural feature of analogue **22** associated with the inactivity is outside the core structure defined by analogue **A**, which may explain why this analogue cross-reacts with both mAb A and mAb B. The cross-reactivity of analogue **22** with mAb A and mAb B in the ciELISA using coating conjugate CCA is in agreement with cross-reactivity results using a cyclohexanedione-specific polyclonal antiserum (unpublished results).

Table 4. Concentration of Other Known Structural Classes of ACCase Inhibitors Required To Inhibit 50% of mAb A or mAb B Binding to Coating Conjugate in the Homologous ciELISA

		$IC_{50} \pm$ SEM, μM		
analogue[a]	structural class	mAb A	mAb B	ACCase[b]
25	aryloxyphenoxypropionate	>138	>138	0.9
26	aryloxyphenoxypropionate	>138[c]	>138	0.5
27	aryloxyphenoxypropionate	>138	>138	>100
28	aryloxyphenoxypropionate	>128	>128	>100
29	aryloxyphenoxypropionate	>148	>148	>100
30	indolizidinedione	0.21 ± 0.006	11.3 ± 0.2	290
31	indolizidinedione	0.002 ± 0.0001	>168	10
32	indolizidinedione	>121	>121	0.3
33	indolizidinedione	>120	>120	2
34	triazinedione	>178	>178	>700
35	triazinedione	>197	>197	520
36	triazinedione	>169	>169	60

[a] Structures of the various analogues tested are shown in Figure 3. [b] ACCase IC_{50} values were provided by Dow AgroSciences (Cseke, personal communication, 1993). [c] Inhibition of mAb A binding observed at the highest concentration tested when compared to the negative control.

There was no apparent correlation between the concentration of cyclohexanedione analogue required to inhibit 50% of mAb A or mAb B binding in the ciELISA and the concentration required to inhibit 50% of corn ACCase activity (Table 3). This lack of a quantitative relationship between inhibition of antibody binding and inhibition of enzyme activity is similar to previous studies with cyclohexanedione polyclonal antiserum (unpublished results). Despite the lack of a quantitative relationship between antibody inhibition and enzyme inhibition, for the most part, both mAb A and mAb B differentiated active from inactive ACCase inhibitors on the basis of their IC_{50} values (Table 3). As a result, mAb A and mAb B were identified as two potential cyclohexanedione pharmacophore-specific antibodies that could be used in further studies to identify novel ACCase inhibitor structures from outside the cyclohexanedione structural class.

Cross-Reactivity to Other Known Classes of ACCase Inhibitors. To further characterize the pharmacophore specificity of mAb A and mAb B, their cross-reactivity with analogues representing other known structural classes of ACCase inhibitors was evaluated. The structures of the analogues representing the aryloxyphenoxypropionates (Burton et al., 1991; Rendina et al., 1988; Secor and Cseke, 1988), indolizidinedione (Babezinski and Fischer, 1991; Cressman, 1994), and triazinedione (Walker et al., 1990) structural classes of ACCase inhibitors are shown in Figure 3. The concentrations of the various analogues required to inhibit 50% of mAb A and mAb B binding to coating conjugate CCA (IC_{50}) in the ciELISA and 50% of corn ACCase activity are summarized in Table 4.

The cyclohexanediones and aryloxyphenoxypropionates are believed to interact with ACCase at a common or overlapping site (Rendina et al., 1988, 1990, 1995). However, none of the aryloxyphenoxypropionate analogues (**25**−**29**; Figure 3) inhibited 50% of mAb A or mAb B binding to CCA at the concentrations tested (Table 4). Similarly, none of the triazinedione analogues (**34**−**36**; Figure 3) inhibited 50% of mAb A or mAb B binding to CCA in the ciELISA (Table 4).

Analogue **26**, the *R* enantiomer of haloxyfop, which is an active ACCase inhibitor, inhibited 37± 3.8% of mAb A binding to CCA at the highest concentration tested (138 μM). Conversely, analogue **27**, the inactive *S* enantiomer of haloxyfop at the same concentration, did not inhibit mAb A binding to CCA when compared to the negative control. Furthermore, analogue **25**, the racemic mixture of haloxyfop, at the same concentration (138 μM) did not inhibit mAb A binding in the ciELISA when compared to the negative control. These data suggest mAb A can specifically bind to the active enantiomer of haloxyfop but the homologous ciELISA format using coating conjugate CCA is not sensitive enough to generate IC_{50} values within the concentration ranges tested.

The aryloxyphenoxypropionate analogues, **28** and **29** (Figure 3), did not inhibit mAb A binding to CCA when compared to the negative control. Analogue **28**, the ethyl ester of haloxyfop, lacks a free carboxyl group and therefore is not an active in vitro inhibitor of ACCase. Analogue **29** is also an inactive analogue, although structurally similar to analogue **25** (Figure 3). The inactivity of analogue **29** is attributed to the presence of the methylsulfonyl group on the pyridine ring, which is hydrophilic and prevents binding to the proposed aryloxyphenoxypropionate inhibitor binding site on ACCase (Figure 3; D. Pernich, Dow AgroSciences, Indianapolis, IN, personal communication, 1993).

Only analogues **30** and **31** (Figure 3) from the indolizidinedione structural class were observed to inhibit 50% of monoclonal antibody binding in the ciELISA within the concentration range tested (Table 4). The concentrations of analogue **30** and **31** required to inhibit 50% of mAb A binding to coating conjugate CCA were 0.21 ± 0.006 and 0.002 ± 0.0001 μM, respectively. The only structural difference between these two indolizidinedione analogues is the presence of a chlorine at position 2 of the phenyl ring on analogue **31** (Figure 3). The presence of this chlorine accounts for the 100-fold increase in the potency of this analogue as an inhibitor of mAb A binding to CCA. Similarly, analogue **31** is a more potent inhibitor of ACCase activity than analogue **30** (Table 4). These differences in ACCase inhibition suggest that the chlorine in position 2 is important for fitting into a hypothetical indolizidinedione-inhibitor binding site on the enzyme. Analogue **31** is a more potent inhibitor of mAb A binding to CCA than any of the cyclohexanedione analogues tested (Table 3). The similarities among the mAb A IC_{50} values for the indolizidinedione analogues **30** and **31** and all active cyclohexanedione analogues (Tables 3 and 4) suggest these two indolizidinedione analogues have molecular features similar to the those of the cyclohexanedione analogue **A** (Figure 1) that are required for mAb A recognition. Although analogues **30** and **31** were potent inhibitors of mAb A binding, the other indolizidinedione

analogues tested (**32** and **33**; Figure 3), which are more potent inhibitors of ACCase, did not inhibit 50% of mAb A or mAb B binding (Table 4). The only structural difference between these two analogues and analogues **31** and **30** is the replacement of the chlorine at position 4 of the phenyl ring with a pyridinyloxy group (Figure 3). The failure of mAb B to bind analogue **31** may be due to the fact that mAb B and mAb A recognize different structural features of this analogue.

The pyridinyloxy group on analogues **32** and **33** may sterically interfere with fitting into the antigen binding site on mAb A and mAb B. Although mAb A did not preferentially bind the larger indolizidinedione analogues **32** and **33**, the antibody did cross-react with equivalently sized cyclohexanedione analogues in the ciELISA.On the basis of the mAb A IC_{50} values generated for active cyclohexanedione analogues and the indolizidinediones, we hypothesize that the cyclohexanedione structures defined by both analogue **A** (Figure 1) and indolizidinedione analogues **30** and **31** overlap with each other. Furthermore, the lack of recognition of analogues **32** and **33** by mAb A in the ciELISA suggests the orientation of the indolizidinediones in the antigen binding site of mAb A is different from that in the cyclohexanedione analogues. However, additional studies such as X-ray crystallographic structures of the mAb A antigen-binding site in the presence and absence of cyclohexanedione and indolizidinedione analogues would be required to determine the orientation of the ligands.

Although both monoclonal antibodies mAb A and mAb B failed to cross-react with representative analogues from all structural classes of ACCase inhibitors (Figure 3 and Table 4), recognition of a single analogue within a class is all that is required to identify a new lead chemistry. For example, the indolizidinedione analogues would have been identified as potential ACCase inhibitors on the basis of their IC_{50} values in the ciELISA. These results demonstrate monoclonal antibodies that recognize the pharmacophore of a small ligand can be used as molecular probes to identify other structures with the same pharmacophore. Both monoclonal antibodies, mAb A and mAb B, cross-reacted with at least one other structural class of ACCase inhibitor, which is in direct contrast to the polyclonal antiserum studies in which only cyclohexanedione structural class inhibitors were observed to cross-react with the polyclonal antiserum in the ciELISAs (unpublished results). Though none of the aryloxyphenoxypropionate analogues tested (Figure 3) were observed to inhibit 50% of monoclonal antibody binding in the ciELISA, the specificity of mAb A for the active enantiomer of haloxyfop (analogue **26**; Figure 3) suggests this antibody has low affinity but high specificity for the critical pharmacophore required for inhibition of corn ACCase. Furthermore, modification of the current homologous ciELISA format to a heterologous ciELISA by altering the structure of the cyclohexanedione hapten coating conjugate may improve the assay sensitivity for other structural classes of ACCase inhibitors. These optimizations and pilot screens to identify novel ACCase inhibitors are currently underway in our laboratory to evaluate the potential of antibody-based approaches to pesticide discovery programs.

ABBREVIATIONS USED

ABST, *N*-hydroxysuccinimide and 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) substrate tablets; ACCase, acetyl coenzyme-A carboxylase; CCA, coating conjugate A; CCC, coating conjugate C; ciELISA, competitive indirect enzyme-linked immunosorbent assay; HAT, hypoxanthin, aminopterin, and thymidine; NHS, *N*-hydroxysuccinimide; OVA, ovalbumin; PBS, phosphate-buffered saline.

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