Development and Evaluation of an Immunological Approach for the Identification of Novel Acetyl Coenzyme-A Carboxylase Inhibitors: Assay Optimization and Pilot Screen Results

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Cyclohexanediones, aryloxyphenoxypropionates, indolizidinediones, and triazinediones are four known structural classes of herbicides that inhibit acetyl coenzyme-A carboxylase (ACCase; EC 6.4.1.2). An immunological study to determine the potential of ACCase inhibitor-specific monoclonal antibodies as screening tools to identify novel lead chemistry was undertaken. Using two cyclohexanedione-specific monoclonal antibodies (mAb A and mAb B; Webb, S. R.; Hall, J. C. *J. Agric. Food Chem*. **2000**, *48*, 1210−1218) and three different cyclohexanedione hapten coating conjugates, competitive indirect enzyme-linked immunosorbent assays (ciELISA) were developed. Cross-reactivity of the monoclonal antibodies with four structural classes of ACCase inhibitors revealed that the ciELISA using mAb A and a modified cyclohexanedione hapten coating conjugate detected analogues from all four known classes of ACCase inhibitors. A pilot screen using this ciELISA format identified two novel ACCase inhibitors, demonstrating the potential for antibodies as rapid and cost-effective screening tools for identifying novel lead chemistry in pesticide discovery programs.

Keywords: *ACCase; cyclohexanedione; monoclonal antibody; molecular mimicry; pesticide discovery*

INTRODUCTION

Herbicides belonging to the cyclohexanedione structural class are effective against a wide range of annual and perennial graminaceous species. Cyclohexanediones cause a rapid cessation of growth followed by destruction of shoot meristems in susceptible species. The biochemical target site of these herbicides is the enzyme acetyl coenzyme-A carboxylase (ACCase; EC 6.4.1.2; Burton et al., 1991; Rendina and Felts, 1988; Secor and Cseke, 1988), which catalyzes the first step in fatty acid biosynthesis. Other structural classes of herbicides, such as the aryloxyphenoxypropionates (Rendina et al., 1988; Secor and Cseke, 1988), indolizidine-2,4-diones (Babezinski and Fisher, 1991; Cressman, 1994), and triazinediones (Walker et al., 1990), are also potent inhibitors of ACCase activity.

Using partially purified enzyme preparations, it has been demonstrated that several cyclohexanediones and aryloxyphenoxypropionates are noncompetitive inhibitors of ACCase (Burton et al., 1991). On the basis of these results, Rendina et al. (1988) and Burton et al. (1991) suggested that the cyclohexanediones and aryloxyphenoxypropionates interact with ACCase at a location other than the active site. In addition, Yonetani−Theorell analysis of cyclohexanedione and aryloxyphenoxypropionate inhibition of ACCase activity indicates that these two structural classes are mutually exclusive inhibitors (D. Pernich, Dow AgroSciences, Indianapolis, IN, personal communication, 1993). Burton et al. (1991) suggested that both structural classes of inhibitors inactivate ACCase by binding to a common or shared inhibitor binding site. Subsequently, Rendina et al. (1995) used hybrid cyclohexanedione−aryloxyphenoxypropionate inhibitors to suggest that there is only a partial overlap between the cyclohexanediones and aryloxyphenoxypropionates at the inhibitor-binding site.

Our interest in ACCase is as a model system to evaluate the utility of antibodies as screening tools to identify new lead chemistry. This enzyme was selected for several reasons. First, ACCase is inhibited by four known structural classes of inhibitors (cyclohexanediones, aryloxyphenoxypropionates, indolizidinediones, and triazinediones), thereby providing a diverse set of structures to evaluate antibody recognition. Second, the cyclohexanedione class of inhibitors has a high degree of structural diversity, which provides flexibility in the design of haptens for synthesizing immunogens and coating conjugates. Third, a functional in vitro enzyme assay is available to verify results from the antibody screen. Finally, ACCase is a valid herbicide target, and any novel inhibitors identified during the development of the antibody-based screen may be commercially relevant.

The rationale for employing antibodies as screening tools is based on the observation that antibodies, when produced against small ligands, may have binding properties similar to those of 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 in their application as molecular probes for use in high-throughput pesticide screens.

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To evaluate the utility of antibodies as screening tools, monoclonal antibodies were produced against a cyclohexanedione−bovine serum albumin immunogen (Webb et al., 1997; Webb and Hall, 2000). Active cyclohexanediones were separated from inactive analogues using a monoclonal (mAb A or mAb B) antibody-based, homologous, competitive indirect enzyme-linked immunosorbent assay (ciELISA) (Webb and Hall, 2000). It was concluded from these studies that one of the two antibodies against the active cyclohexanedione analogues (mAb A) recognizes other classes of ACCase inhibitors, but the sensitivity of the homologous ciELISA was a major limiting factor for identifying new lead chemistry (Webb and Hall, 2000). In the current paper, we describe the development of two heterologous ciELISAs to which several active and nonactive ACCase inhibitors were evaluated for cross-reactivity with two monoclonal antibodies, mAb A and mAb B. We found that mAb A is more specific for the ACCase inhibitor pharmacophore than mAb B. Furthermore, in a pilot study, we identified two novel ACCase inhibitors using mAb A as a screening tool, thereby suggesting that an immunological approach may be viable for use in pesticide discovery programs.

MATERIALS AND METHODS

Chemicals and Reagents. The production and characterization of monoclonal antibodies mAb A and mAb B were previously described in Webb and Hall (2000). All cyclohexanediones, indolizidinediones, triazinediones, and the pilot screen analogues were supplied by Dow AgroSciences. Aryloxyphenoxypropionates **20** and **22** (Figure 4) were obtained from Hoechst-Roussel. All other aryloxyphenoxypropionates were provided by Dow AgroSciences. Ovalbumin (OVA), *N*-hydroxysuccinimide, 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 and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide were obtained from Pierce Immunochemicals (Rockford, IL). All other chemicals were of reagent grade and obtained commercially.

Cyclohexadione Analogues. The structures of the cyclohexanedione coating conjugate haptens **A**, **D**, and **E** are shown in Figure 1. The location and modification of functional groups introduced to haptens **D** and **E** were based on the cross-reactivity profile of the monoclonal antibodies, mAb A and mAb B, against a set of 24 cyclohexanediones using a ciELISA with **A** as the coating conjugate hapten (Webb and Hall, 2000). Position 2 oxime region of hapten **D** was modified by replacing the normal ethyl substituent that is associated with in vivo and in vitro ACCase inhibition with a sterically unfavorable *tert*-butyl substituent (Figure 1). The electronic profile of analogue **E** was altered by replacing the oxime functional group with an acyl hydrazone group (Figure 1). As a result of these structural and electronic modifications, both analogues **D** and **E** are nonactive ACCase inhibitors (C. Hamilton, Dow AgroSciences, Indianapolis, IN, personal communication, 1994).

Preparation of Enzyme-Linked Immunosorbent Assay Coating Conjugates. In this study, all cyclohexanedione haptens (Figure 1) used for the synthesis of the enzyme-linked immunosorbent assay (ELISA) coating conjugates contained a carboxylic acid group. Coating conjugates CCA, CCD, and CCE were prepared by conjugating haptens **A**, **D**, and **E** (Figure 1) to OVA, respectively, using the *N*-hydroxysuccinimide-active ester method as previously described (Webb et al., 1997).

Indirect ELISA. Monoclonal antibody binding to coating conjugate CCA, CCD, or CCE was determined using the ELISA procedure previously described by Webb et al. (1997). The appropriate dilutions of coating conjugate and monoclonal antibody for the ELISA were determined as described by Johnson and Hall (1996).

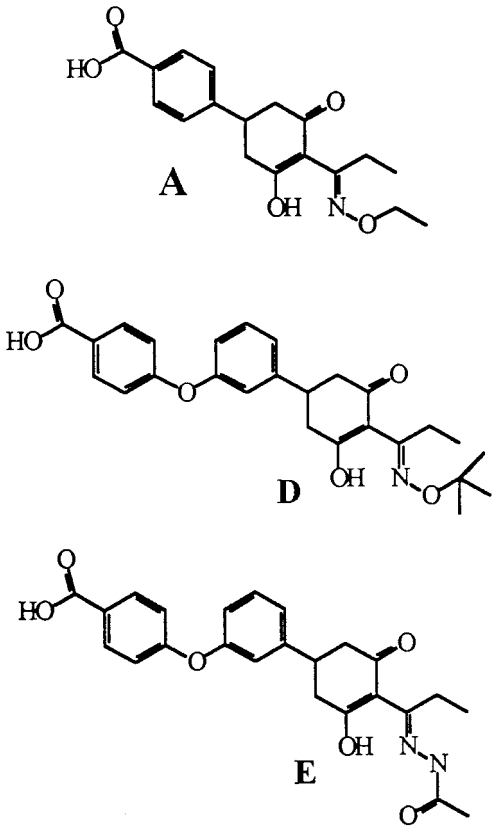

Figure 1. Structures of the cyclohexanedione haptens used to produce the various coating conjugates. Hapten **A** was conjugated to BSA and OVA for use as the immunogen (IA) and coating conjugate CCA, respectively, as previously described (Webb et al., 1997; Webb and Hall, 2000). Haptens **D** and **E** were conjugated to OVA for use as coating conjugates CCD and CCE, respectively. Haptens **D** and **E** were synthesized by C. Hamilton (Dow AgroSciences).

Competitive Indirect ELISA (ciELISA). To ensure that the antibody specificity for the ACCase inhibitor pharmacophore was not altered by modifying the coating conjugate structure, the cross-reactivity profiles of the monoclonal antibodies in the ciELISAs were determined as previously described (Webb and Hall, 2000) with the following modifications. Various known active inhibitors of corn ACCase (ACCase $IC_{50} \leq 100$ μM) and inactive analogues (ACCase $IC_{50} > 100$ μM) (Figures 2−5) were tested for their ability to compete with each immobilized coating conjugate CCA, CCD, or CCE for monoclonal antibody binding. Stock solutions of the test analogues were prepared by dissolving these compounds in dimethyl sulfoxide (DMSO). When added to the appropriate dilution of mAb A or mAb B in PBS, the concentration of DMSO was 1% (v/v). In these experiments monoclonal antibody binding in the presence of 1% (v/v) DMSO served as the control. Glyphosate (**33**; Figure 6) solutions were prepared by dissolving it in ultrapure water. When added to the appropriate dilution of mAb A or mAb B in PBS, the concentration of ultrapure water was 1% (v/v). In the experiments with glyphosate, monoclonal antibody binding in the presence of 1% (v/v) ultrapure water served as the control.

The effect of the various analogues on mAb A or mAb B binding to CCA, CCD, or CCE was determined by including specified concentrations of the test analogue in the reaction mixture. The reaction mixture consisted of equal volumes of a known concentration of a test analogue and the appropriate dilution of the monoclonal antibody in PBS. The reaction mixture was incubated at room temperature for 30 min and then transferred (100 μL/well) to microplate wells previously coated with CCA, CCD, or CCE, followed by an additional 1 h of incubation. Once incubated with the goat-anti-mouse-IgG−

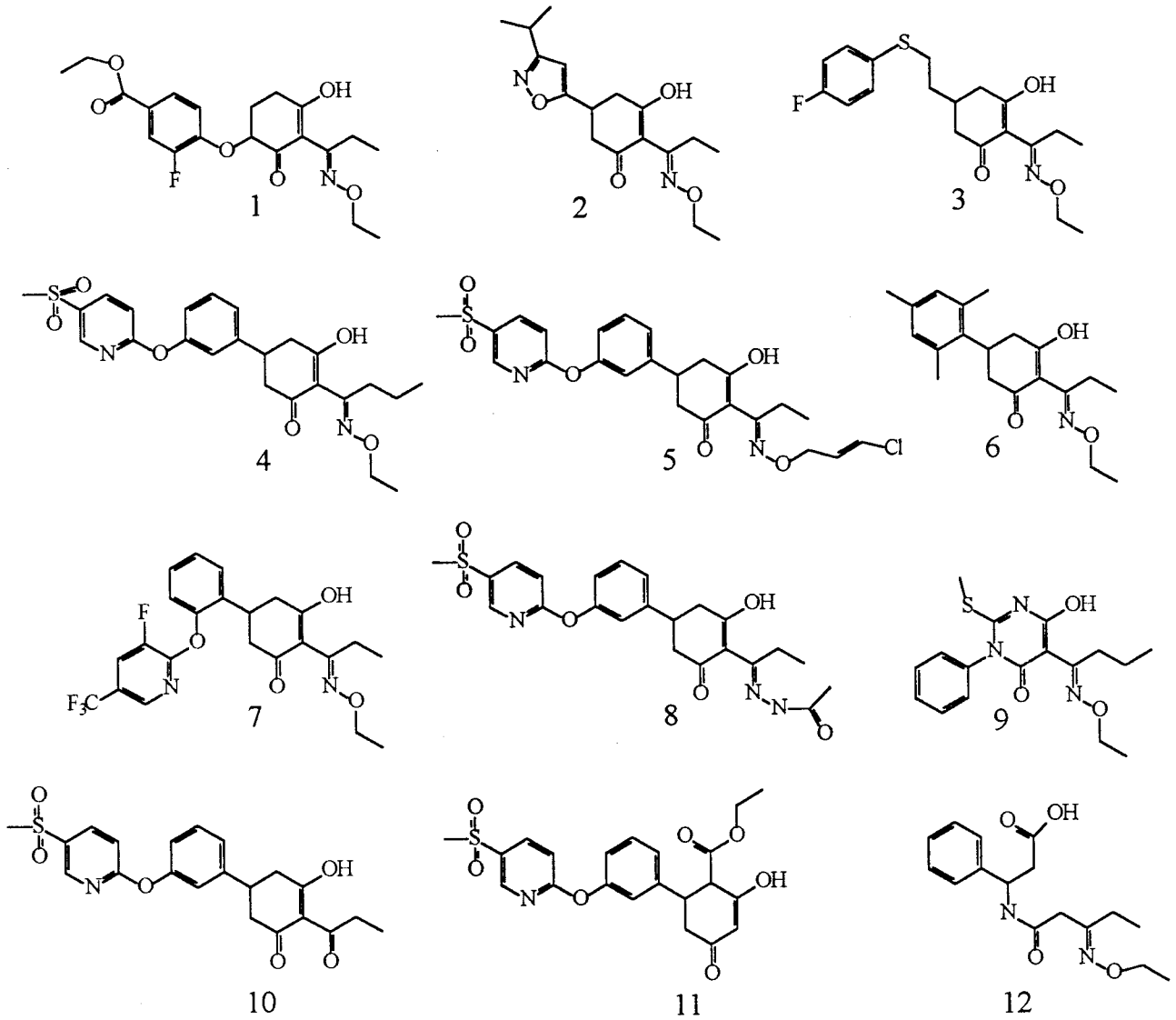

Figure 2. Structures of the various cyclohexanediones evaluated for cross-reactivity to mAb A and mAb B in ciELISA using coating conjugate CCA, CCD, or CCE as described under Materials and Methods. Analogues **1**−**6** are defined as active ACCase inhibitors (IC_{50} values ≤ 100 μM). Analogues **7**−**12** are defined as nonactive inhibitors (IC_{50} values > 100 μM).

horseradish peroxidase antibody conjugate and substrate, color development was inversely proportional to the concentration of the cross-reacting analogues.

Antibody-Based ACCase Inhibitor Screen. A high-throughput antibody screen was developed using the ciELISA with coating conjugate CCE and mAb A. Stock solutions of test analogues were prepared by dissolving an analogue (Figure 7) in DMSO. These stock solutions were diluted with PBS and added to the appropriate dilution of mAb A to give a final analogue concentration of 100 μM. In this reaction mixture the final concentration of DMSO was 1% (v/v). In these studies, mAb A binding to coating conjugate CCE in the presence of 1% (v/v) DMSO served as the control. The reaction mixture (100 μL/well) was added to wells coated with CCE (1:2400 dilution in PBS) as previously described (Webb et al., 1997). The ciELISA was performed as described above, and color development was determined by measuring absorbance at 405 nm with a Bio-Rad model 3550-UV microplate reader (Bio-Rad Laboratories, Hercules, CA) as previously described by Webb et al. (1997).

An analogue was considered a potential ACCase inhibitor if the A_{405nm} of wells containing the test analogue was reduced by 50% when compared to the control (A_0). All analogues from the pilot screen were tested at least two times. All analogues testing positive in the initial screen were subjected to serial dilution in PBS to confirm inhibition of mAb A binding to CCE and to determine their IC_{50} values. Controls and each dilution of analogue were run in triplicate (i.e., three wells), on duplicate plates, and absorbance values determined. Absorbance values (A) of samples containing a known concentration of test analogue were normalized by dividing by the mean absorbance (A_0) of the control (nine wells/plate). All IC_{50} value determinations from the pilot screen were repeated at least three times, and the IC_{50} values were expressed as the mean ± standard error of mean (SEM).

RESULTS AND DISCUSSION

Rationale for Modification of Cyclohexadione Hapten Structure. Modification of the structure of the coating conjugate hapten is routinely used to increase the sensitivity of immunoassays for residue analysis (Harrison et al., 1989; Marco et al., 1995; Schneider and Hammock, 1992; Wie and Hammock, 1984). For example, Johnson and Hall (1996) report an enhancement in sensitivity and reduction in cross-reactivity of a triclopyr ELISA from 5 to 0.1 ng/L by modifying the direct ELISA format from a homologous to a heterologous assay. In cross-reactivity studies, Webb and Hall (2000) also showed that higher concentrations of nonactive cyclohexanediones (IC_{50} > 100 μM; Markley et

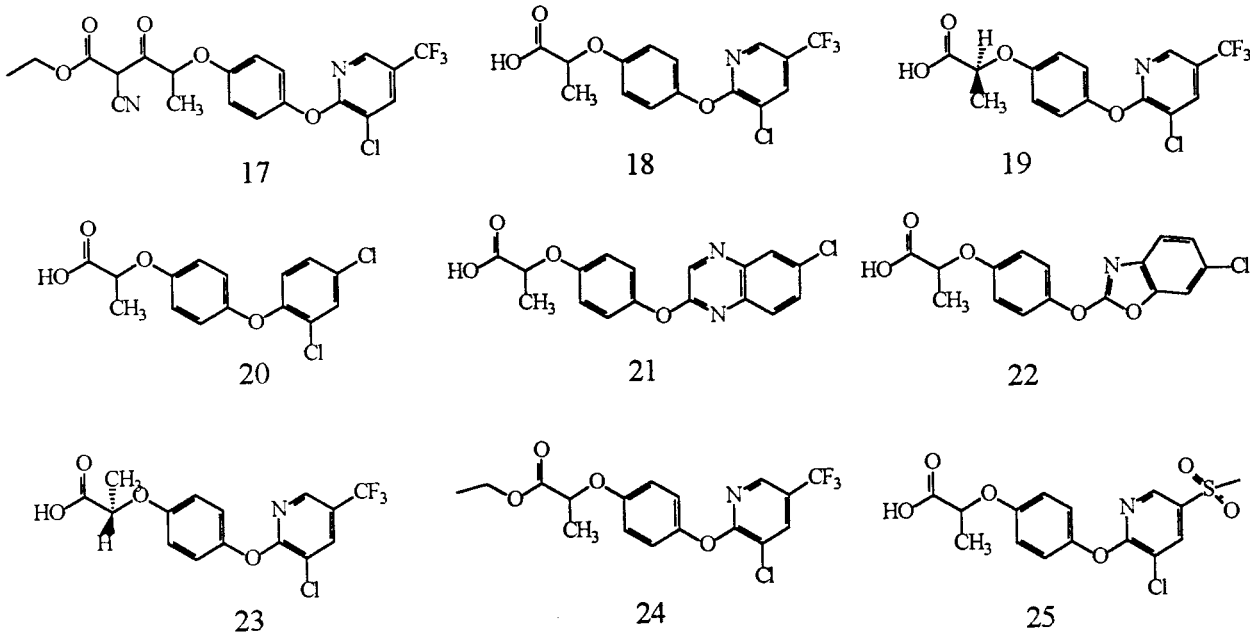

Figure 3. Structures of selected aryloxyphenoxypropionates. Analogues **17**−**22** are defined as active inhibitors because their IC_{50} values are ≤100 μM against corn ACCase. Analogues **23**−**25** are inactive analogues (ACCase IC_{50} values > 100 μM). The monoclonal antibody and enzyme IC_{50} values are shown in Table 2.

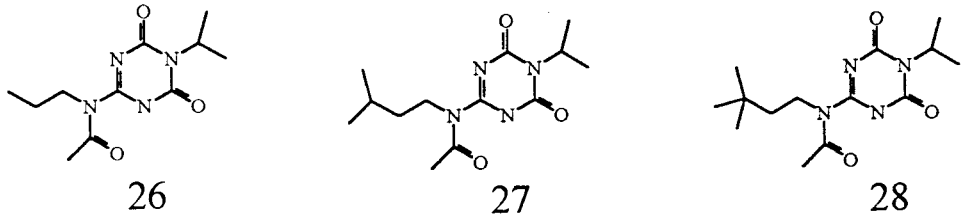

Figure 4. Structures of selected triazinedione structural class ACCase inhibitors. Analogues **26** and **27** are inactive (ACCase IC_{50} > 100 μM). Analogue **28** is an active inhibitor of corn ACCase (ACCase IC_{50} < 100 μM). The monoclonal antibody and enzyme IC_{50} values are shown in Table 3.

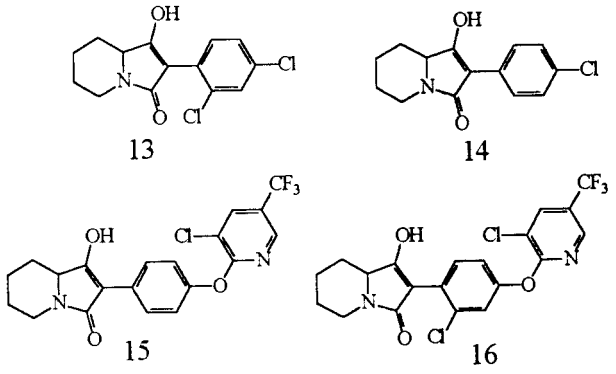

Figure 5. Structures of various indolizidinediones. Analogues **13**, **15**, and **16** are active (IC_{50} values ≤ 100 μM) inhibitors of ACCase. Analogue 1**4** is a nonactive inhibitor (IC_{50} > 100 μM) of ACCase. The monoclonal antibody and enzyme IC_{50} values are shown in Table 4.

al., 1995; Webb et al., 1997) were required to compete with CCA for antibody binding, as compared with active cyclohexanediones (IC_{50} < 100 μM). Conversely, structural deviations from the active pharmacophore on substituents at position 5 of the cyclohexane ring had little effect on antibody cross-reactivity (Webb and Hall, 2000). Furthermore, analysis of the effects of altering cyclohexanedione structure on mAb A and mAb B binding (Webb and Hall, 2000) indicates that structural and or electronic modifications to the position 2 oxime functional group specifically altered monoclonal antibody (mAb A and mAb B) binding. Therefore, the introduction of unfavorable (i.e., hapten has no ACCase inhibition activity) steric and/or electronic substitutions in the oxime region of the hapten should increase cross-reactivity of both by mAb A and mAb B to the cyclohexanediones. Furthermore, modification to the coating conjugate hapten was used to decrease antibody affinity for the cyclohexanediones and increase antibody binding to other structural classes of herbicides possessing the ACCase inhibitor pharmacophore. Selection of the oxime region for modification to increase ciELISA sensitivity for other structural classes of ACCase inhibitors is further supported by Rendina et al. (1995), who proposed that the cyclohexanedione and aryloxyphenoxypropionate structural classes of inhibitors overlap in the ACCase herbicide binding site through the oxime and phenoxypropanoic acid moieties, respectively.

Effect of Altering Coating Conjugate Structure on Monoclonal Antibody Specificity to the Cyclohexanediones. The effect of steric and electronic modifications to the coating conjugates on mAb A and mAb B specificity for ACCase inhibitors was evaluated by determining antibody cross-reactivity patterns against a set of cyclohexanedione analogues (Figure 2). The concentrations of various cyclohexanediones required to inhibit mAb A and mAb B binding to coating conjugate CCA, CCD, or CCE by 50% (IC_{50}), and to corn ACCase (IC_{50}) are summarized in Table 1. Although actual IC_{50} values (Table 1) of the cyclohexanediones vary depend-

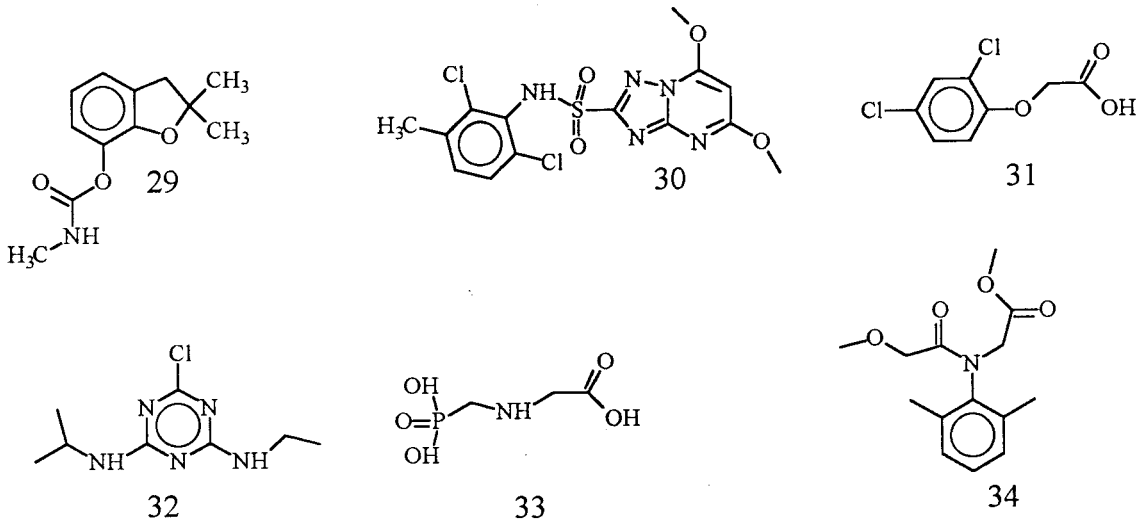

Figure 6. Structures of six known non-ACCase inhibitor pesticides. Compound **29** is the insecticide carbofuran. Compounds **30**, **31**, **32**, and **33** are the commercial herbicides metosulam, 2,4-dichlorophenoxyacetic acid, atrazine, and glyphosate, respectively. Compound **34** is the fungicide metalaxyl.

Table 1. Influence of ELISA Coating Conjugate Structure on the Concentration of Cyclohexanedione Required To Inhibit Antibody Binding by 50%

	$IC_{50} \pm$ SEM, μM ($n = 3$)					
	mAb A			mAb B		ACCase[b]
analogue[a]	CCA	CCD	CCE	CCA	CCE	IC_{50}, μM
1	0.07 ± 0.003	0.03 ± 0.001	0.06 ± 0.005	0.01 ± 0.001	0.008 ± 0.0003	0.30
2	0.03 ± 0.002	0.06 ± 0.003	0.02 ± 0.001	0.03 ± 0.005	0.009 ± 0.0005	7.70
3	0.14 ± 0.002	0.35 ± 0.004	0.17 ± 0.006	0.04 ± 0.005	0.01 ± 0.002	1.80
4	0.22 ± 0.009	0.23 ± 0.008	0.16 ± 0.003	0.002 ± 0.0001	>0.001	0.70
5	0.21 ± 0.007	>100	5.69 ± 0.011	0.004 ± 0.0006	7.54 ± 0.11	0.02
6	0.33 ± 0.006	0.74 ± 0.008	0.27 ± 0.004	0.25 ± 0.005	0.87 ± 0.006	1.00
7	0.32 ± 0.003	0.18 ± 0.002	0.34 ± 0.005	0.13 ± 0.003	0.74 ± 0.002	>100
8	>100	>100	54.4 ± 0.008	6.28 ± 0.83	9.75 ± 0.32	>500
9	>100	34.1 ± 0.08	24.4 ± 0.01	5.54 ± 0.90	1.38 ± 0.05	>200
10	5.25 ± 0.021	26.7 ± 0.02	5.47>0.030	>100	54.3 ± 0.87	>300
11	>100	>100	>100	>100	63.2 ± 0.45	NA[c]
12	>100	>100	>100	>100	25.2 ± 1.23	>400

[a] The structures of the cyclohexanediones are shown in Figure 2. [b] Cyclohexanediones are defined as active inhibitors of corn ACCase with IC_{50} values ≤100 μM (enzyme IC_{50} values provided by Dow AgroSciences; Cseke, personal communication, 1994). [c] Not available.

ing on the coating conjugate structure, the trend remains the same; active cyclohexanediones (analogues **1**−**6**; Figure 2) are more potent inhibitors of monoclonal antibody binding to all coating conjugates when compared to nonactive cyclohexanedione analogues (analogues **8**−**12**; Figure 2). Only analogue **7** (Figure 2), a nonactive ACCase inhibitor, was a potent inhibitor of both mAb A and mAb B binding to all coating conjugates (Table 1).

The objective of modifying the hapten structure of the coating conjugate from an active (**A**) to a nonactive cyclohexanedione structure (**D** and **E**) was to increase ciELISA cross-reactivity and sensitivity for the active ACCase inhibitor pharmacophore while decreasing cross-reactivity and sensitivity to inactive inhibitors. This proved to be true in many cases, but there were a few exceptions (i.e., active **5**, inactive **8** and **9**; Table 1). For example, active analogue **5** (Figure 2) was an exception because it inhibited antibody binding to CCA (IC_{50} = 0.21 μM) to a greater extent than it did with CCE (IC_{50} = 5.69 μM). Furthermore, even at the highest concentration tested (100 μM), analogue **5** did not inhibit binding of mAb A to CCD by 50% (Table 1). We hypothesize that the larger position 2 substituents on the cyclohexane ring of haptens **D** and **E** may increase antibody binding to the coating conjugate when compared to the smaller ethyl substituent on hapten **A**, thereby making inhibition more difficult (Figure 1).

Cross-Reactivity with Other Classes of ACCase Inhibitors. To determine if changing the coating conjugate hapten from an active (CCA) to nonactive structures (CCD and CCE) altered ciELISA sensitivity to other ACCase inhibitor structures, representative analogues from the aryloxyphenoxypropionate, triazinedione, and indolizidinedione structural classes were tested for cross-reactivity to mAb A and mAb B.

Aryloxyphenoxypropionates. The structures of the various aryloxyphenoxypropionates tested for cross-reactivity to mAb A and mAb B are shown in Figure 3, and their IC_{50} values are summarized in Table 2. As coating conjugates were changed (from CCA to CCD or CCE), our ability to predict active ACCase inhibitors using ELISA improved. For example, none of the active aryloxyphenoxypropionates tested inhibited mAb A binding to CCA by 50%. These results are consistent with earlier studies (Webb and Hall, 2000). However, active analogues **17**−**20** (i.e., 67% of all actives) (Figure 3) inhibited both mAb A and mAb B binding by 50% when coating conjugate CCE was used (Table 2). In contrast, only analogues **17** and **20** inhibited mAb A binding by 50% when coating conjugate CCD was used (Table 2). Therefore, the differences in mAb A and mAb

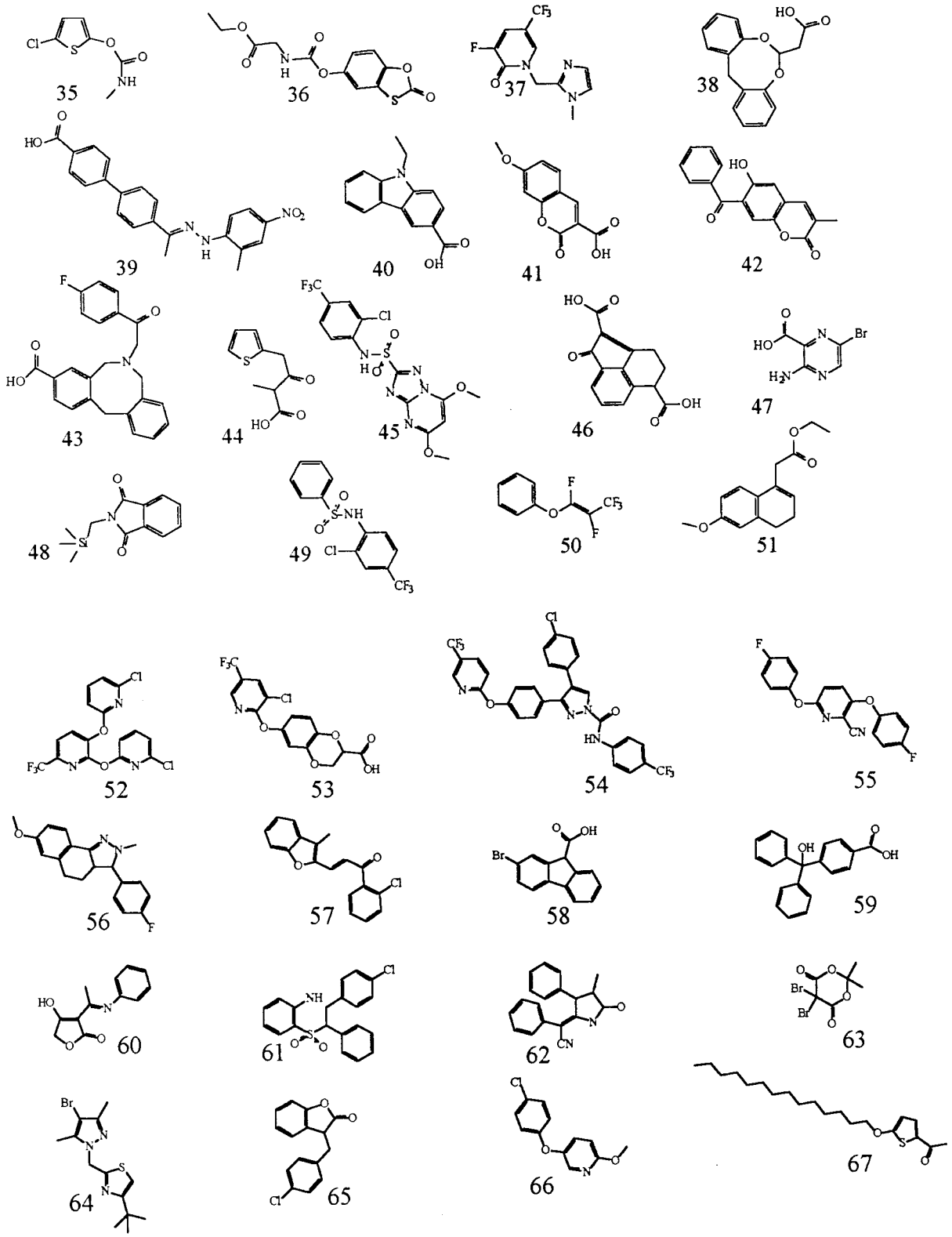

Figure 7. Structures of analogues used in the pilot screen using ciELISA.

B cross-reactivity with the aryloxyphenoxypropionates indicate that changing coating conjugate structures influence assay sensitivity. Specifically, changing the coating conjugate to CCE with mAb A optimized the correlation between inhibition of antibody and ACCase binding.

Even with this ELISA optimization, not all of the active aryloxyphenoxypropionates ACCase inhibitors tested (**17**−**22**) inhibited mAb A binding to coating conjugate CCE within the concentration range tested. For example, active analogues **21** and **22** did not inhibit mAb A binding to coating conjugate CCE (Table 2). The lack of mAb A recognition of analogues **21** and **22** may be attributed to the presence of the larger benzoheterocyclic rings when compared to the phenyl and pyridyl rings of **17**−**20** (Figure 3).

None of the inactive aryloxyphenoxypropionates (**23**−**25**) competed with CCE for mAb A binding within the

Table 2. Influence of ELISA Coating Conjugate Structure on the Concentration of Aryloxyphenoxypropionates Required To Inhibit Antibody Binding by 50%

	$IC_{50} \pm SEM$, μM ($n = 5$)					
	mAb A			mAb B		ACCase[b]
analogue[a]	CCA	CCD	CCE	CCA	CCE	IC_{50}, μM
17	NT[c]	0.83 ± 0.04	0.61 ± 0.01	NT	8.53 ± 0.6	0.03
18	>138	>138	73.8 ± 1.2	>138	14.7 ± 0.6	0.90
19	>138[d]	>138	45.9 ± 3.5	>138	5.76 ± 0.4	0.50
20	>141	72.8 ± 3.6	64.0 ± 2.4	>141	21.3 ± 1.9	1.50
21	NT	>145	>145	NT	>145	0.03
22	>149	>149	>149	>149	>149	1.00
23	>138	>138	>138	>138	19.5 ± 1.6	>3000
24	>128	>128	>128	>128	>128	>3000
25	>148	>148	>148	>148	>148	>100

[a] Structures of the aryloxyphenoxypropionates are shown in Figure 3. [b] Corn ACCase IC_{50} values were provided by Dow AgroSciences (Cseke, personal communication, 1994). [c] Not tested. [d] At 138 μM of *R* enantiomer of haloxyfop the A/A_0 was reduced to $58.8 \pm 2.3\%$ when compared to the positive control.

concentration range tested (Table 2). Analogue **24** is the ethyl ester of analogue **18**; without the free propanoic acid group this analogue has no in vitro ACCase inhibiting activity (Table 2). Although the propanoate group is important for activity, it does not appear to be essential for enzyme inhibition. For example, analogue **17**, which is a β-ketocyano ester, is an active in vitro enzyme inhibitor as well as the most potent inhibitor of mAb A binding (Table 2). Therefore, like the propanoic acid moiety of analogue **18**, the β-ketocyano ester moiety of **17** functions as an acidic group. These results suggest that the presence of an acidic group, such as a propanoic acid (**18**−**20**; Figure 3) or β-ketocyano ester (**17**; Figure 3), is important for inhibition of ACCase activity and for recognition by mAb A.

The importance of an acid group can be offset by other structural modifications to the aryloxyphenoxypropionates. For example, analogue **25** (Figure 3), which has a propanoic acid moiety, does not inhibit ACCase or mAb A binding to CCE (Table 2) due to the presence of a methylsulfonyl group on the pyridine ring, which is thought to be too hydrophilic to allow binding to the proposed hydrophobic pocket of the aryloxyphenoxypropionate binding site within ACCase (D. Pernich, personal communication, 1993). In contrast, several ACCase active cyclohexanediones contain a methylsulfonyl group at position 5 of the cyclohexane ring (**4**, **5**; Figure 2; Markley et al., 1995). These analogues are also potent inhibitors of mAb A binding to both CCA and CCE (Table 1). Differences in mAb A recognition between the methylsulfonyl substituent on a cyclohexanedione (**4**, **5**; Figure 2) versus an aryloxyphenoxypropionate (**25**; Figure 3) suggest these two classes of herbicides may only partially overlap at the ligand binding site. This hypothesis is further supported by Rendina et al. (1995), who demonstrated that the cyclohexanediones and aryloxyphenoxypropionates only partially overlap through the oxime and propanoic acid regions. Several authors (Gronwald et al., 1992; Marshall et al., 1992; Parker et al., 1990) also hypothesize that the partial overlap between these enzyme inhibitors may account for the occurrence of grasses with mutant ACCase that are resistant to both inhibitor classes and grasses with resistance to only the aryloxyphenoxypropionates.

In the ciELISA using CCE, the only difference in the aryloxyphenoxypropionate cross-reactivity profiles between mAb A and mAb B occurred with inactive analogue **23** (Table 2). Analogue **23** (Figure 3) inhibited mAb B binding to coating conjugate CCE but did not inhibit the binding of mAb A by 50% within the concentration range tested (Table 2). Analogue **23** (inactive), the stereoisomer of analogue **19** (active), does not inhibit ACCase activity (Figure 3; Table 2). These data indicate that mAb A is stereoselective and is highly specific for the active aryloxyphenoxypropionate conformation. In contrast, mAb B lacks stereospecificity and, as a result, may not be as specific for the ACCase inhibitor pharmacophore as mAb A.

Table 3. Influence of ELISA Coating Conjugate Structure on the Concentration of Triazinedione Required To Inhibit Antibody Binding by 50%

	$IC_{50} \pm SEM$, μM ($n = 3$)					
	mAb A			mAb B		ACCase[b]
analogue[a]	CCA	CCD	CCE	CCA	CCE	IC_{50}, μM
26	>178	>178	>178	>178	54.3 ± 15.3	inactive
27	>197	>197	>197	>197	76.1 ± 21.6	inactive
28	>169	>169	76.3 ± 11.7	>169	48.8 ± 8.1	active

[a] Structures of the various analogues tested are shown in Figure 4. [b] Analogues are defined as active if they inhibit at least 50% of corn enzyme activity at concentrations ≤ 100 μM.

Triazinediones. The concentrations of triazinediones (Figure 4) required to inhibit binding of mAb A and mAb B by 50% to various coating conjugates are summarized in Table 3. None of the triazinedione analogues (i.e., one active and two inactives) tested using CCA inhibited binding of mAb A or mAb B by 50% (Table 3). Similar to the aryloxyphenoxypropionate analogues, differences in analogue cross-reactivity between the monoclonal antibodies were observed. For example, only active analogue **28** (Figure 4) inhibited binding of mAb A to coating conjugate CCE by 50%. In contrast, all triazinediones tested using coating conjugate CCE inhibited mAb B binding by 50% (Table 3). Differences in the cross-reactivity profiles of mAb A and mAb B suggest the antibodies "recognize" different features of the triazinediones. Therefore, changing the coating conjugate structure from CCA to CCE improves the accuracy of determining active versus inactive ACCase inhibitors, which agrees with our results using cyclohexanediones and aryloxyphenoxypropionates (Tables 2 and 3).

Indolizidinediones. The concentrations of various indolizidinediones (Figure 5) required to inhibit binding of mAb A and mAb B to the coating conjugates by 50% are summarized in Table 4. Similar to the trends observed with the previous three classes of chemistry (i.e., cyclohexadiones, triazinediones, and aryloxyphenoxypropionates), as the coating conjugates were changed (from CCA to CCD or CCE) the cross-reactivity profiles of both mAb A and mAb B to indolizidinedione ana-

Table 4. Influence of ELISA Coating Conjugate Structure on the Concentration of Indolizidinediones Required To Inhibit Antibody Binding by 50%

	$IC_{50} \pm$ SEM, μM ($n = 3$)					
	mAb A			mAb B		ACCase[b]
analogue[a]	CCA	CCD	CCE	CCA	CCE	IC_{50}, μM
13	0.001 ± 0.0001	0.07 ± 0.003	0.009 ± 0.0002	>168	>168	10.00
14	0.21 ± 0.008	8.7 ± 0.02	4.9 ± 0.06	11.3 ± 0.21	7.3 ± 0.51	290.00
15	>120	14.6 ± 0.13	24.7 ± 0.12	>120	8.4 ± 0.37	2.00
16	>121	>121.0	>121.0	>121	3.70 ± 0.16	0.30

[a] Structures of the indolizidinediones are shown in Figure 5. [b] Corn ACCase IC_{50} values provided by Dow AgroSciences (Cseke, personal communication, 1994).

logues changed. For example, both analogues **13** (ACCase active) and **14** (inactive) (Figure 5) were potent inhibitors of mAb A binding to CCA, whereas only analogue **14** inhibited mAb B binding to CCA by 50%. When CCE was used, analogue **14** (inactive) inhibited binding to both mAb A and mAb B by 50%, whereas analogue **13** (active) inhibited binding to mAb A but not to mAb B (Table 4). Analogue **15** (active) did not inhibit mAb A binding to coating conjugate CCA by 50% but it did when CCD and CCE were used as coating conjugates (Table 4). Furthermore, analogue **16** (active) was a potent inhibitor of only mAb B binding to CCE but failed to inhibit binding of mAb A by 50% (Table 4). These differences in the cross-reactivity profiles of mAb A and mAb B with the indolizidinediones (Table 4) suggest that the important molecular features involved in the antibody−antigen interaction can be modified by changing the coating conjugate structure. However, changing the coating conjugates did not improve our prediction of ACCase inhibition to the same extent as it did with the other three classes of chemistry.

Cross-Reactivity with Other Pesticide Structures. The results of cross-reactivity studies against analogues representing the aryloxyphenoxypropionate and triazinedione structural classes of inhibitors indicate that changing the coating conjugate structure from CCA to CCD or preferably CCE increases our ability to discriminate active versus inactive ACCase inhibitors from chemistry outside the cyclohexanedione structural class. The ELISA using CCD or CCE was altered to the point that it may detect herbicides with diverse chemistry that have target sites other than ACCase. Therefore, a cross-reactivity study using both antibodies (mAb A and mAb B), the three coating conjugates (CCA, CCD, and CCE) and six known non-ACCase inhibiting pesticides was undertaken.

The structures of the six pesticides tested are shown in Figure 6. These pesticides include the carbamate insecticide carbofuran (compound **29**), the fungicide metalaxyl (compound **34**), and four commercial herbicides, metosulam (compound **30**), 2,4-dichlorophenoxyacetic acid (2,4-D; compound **31**), atrazine (compound **32**), and glyphosate (compound **33**). None of these compounds had any activity against ACCase within the concentration range tested (IC_{50} > 100 μM; C. T. Cseke, Dow AgroSciences, Indianapolis, IN, personal communication, 1994). The cross-reactivity profiles and calculated IC_{50} values against mAb A and mAb B are summarized in Table 5. All six pesticides tested, except glyphosate (compound **33**; Figure 6), inhibited mAb B binding to CCE by 50%, within the concentration range tested (Table 5). The most potent inhibitor of mAb B binding to CCE was the fungicide metalaxyl (**34**; Figure 6; Table 5). These results indicate that mAb B lacks the specificity required for use as a screening tool for novel

Table 5. Influence of Coating Conjugate Structure on mAb A and mAb B Cross-Reactivity with Six Known Non-ACCase Inhibitor Pesticides

	$IC_{50} \pm$ SEM, μM ($n = 3$)			
	mAb A		mAb B	common name
compound[a]	CCD	CCE	CCE	of pesticide
29	>226	>226	117.3 ± 9.9	carbofuran
30	>120	>120	5.32 ± 0.21	metosulam
31	22.3 ± 0.64	>226[b]	193.2 ± 8.6	2,4-D
32	>232	>232	70.6 ± 1.3	atrazine
33	>295	>295	>295	glyphosate
34	>179	>179	<0.02	metalaxyl

[a] Structures of the non-ACCase inhibitor pesticides are shown in Figure 6. [b] At 226 μM of 2,4-D the A/A_0 was reduced by 33.2 ± 2.3% compared to the positive control.

ACCase inhibitors; therefore, additional characterization of this antibody was not performed. In contrast, none of these pesticides inhibited the binding of mAb A to CCE, whereas only 2, 4-D inhibited binding of mAb A to CCD. We hypothesize that this cross-reactivity of 2,4-D with CCD is due to the structural similarity between the phenoxyacetic acid moiety of 2,4-D and phenoxypropanoic acid moiety of an aryloxyphenoxypropionate such as haloxyfop (analogue **18**; Figure 3). On the basis of these results and the cross-reactivity profiles of the known ACCase inhibitors, mAb A and coating conjugate CCE were selected for use in the ciELISA pilot screen.

Pilot Screen. A pilot screen was initiated to determine the specificity of the antibody for other classes of ACCase inhibitors and to develop a format for screening large numbers of analogues. A total of 33 analogues representing the major clusters of chemistry other than the cyclohexanediones, triazinediones, aryloxyphenoxypropionates (except for analogues **53**−**55** and **60**, which were known nonactive aryloxyphenoxypropionates), and indolizidinediones within the Dow AgroSciences chemical library (Figure 7) were screened. The fact that these four analogues (**53**−**55** and **60)** were non-ACCase inhibitors was not revealed to us by Dow AgroSciences prior to the ELISA screen. None of the remaining analogues, representing acids, non-acids, diones, and lipophilic molecules, were believed to have any appreciable corn ACCase inhibiting activity (i.e., corn ACCase IC_{50} values > 100 μM).

All analogues were initially screened at 100 μM for their ability to compete with CCE for binding to mAb A. A total of six analogues (analogues **35**, **39**, **42**, **45**, **62**, and **64**; Figure 7) were observed to inhibit binding of mAb A by 50% (Table 6). The calculated hit rate was 18% (6 of 33). Of the six hits, analogues **35** and **42**, not previously thought to be ACCase inhibitors, were found to inhibit corn ACCase with IC_{50} values of 19 and 90 μM, respectively. The remaining four analogues identi-

Table 6. Monoclonal Antibody A Recognition of Chemistry Outside Known ACCase Inhibitor Structural Classes Using the CiELISA Screen; Concentrations of Analogue Required To Inhibit mAb A Binding and Corn ACCase Activity by 50%

	antibody screen	ACCase[b]	
analogue[a]	$IC_{50} \pm$ SEM, μM ($n = 3$)	IC_{50}, μM	screen outcome
35	38.8 ± 2.3	19.0	+ ve
36	>100	18.0	false −ve[c]
37	>100	22.0	false −ve
39	44.9 ± 1.5	140	false +ve[d]
42	0.22 ± 0.03	90.0	+ve
45	10.4 ± 0.05	170	false +ve
62	79.5 ± 1.3	366	false +ve
64	83.4 ± 4.5	303	false +ve

[a] Structures of the pilot screen analogues are shown in Figure 7. [b] ACCase IC_{50} values supplied by Dow AgroSciences (Cseke, personal communication, 1994). [c] False −ve, false negative; the antibody screen failed to identify the analogue as a potential ACCase inhibitor; antibody $IC_{50} > 100$ μM when in fact enzyme $IC_{50} < 100$ μM. [d] False +ve, false positive; the antibody screen identified the analogue as a potential ACCase inhibitor when the ACCase $IC_{50} > 100$ μM.

fied as active by ELISA were considered to be false positives. Conversely, analogues **36** and **37**, previously known to be active inhibitors of corn ACCase, were not identified as actives by the ciELISA screen (false negatives). All other analogues (Figure 7) were not inhibitors of ACCase and did not compete with coating conjugate CCE for mAb A binding (IC_{50} values > 100 μM).

The monoclonal antibody screen identified two novel ACCase inhibitors (analogues **35** and **42**; Figure 7). Although the antibody-based screen did not identify all ACCase inhibitors in the pilot study, the number of actual inhibitors in the set of analogues identified as hits by the antibody screen was enriched 3-fold when compared to the initial sample set (i.e., of 33 starting molecules, 6 and 2 were positive by Ab and ACCase assays, respectively, and the two were a subset of the 6 identified by Ab assay; 6/2 = 3). It is important to note that none of the nonactive aryloxyphenoxypropionate analogues (**53**−**55** and **60**; Figure 7) were identified as potentially novel ACCase inhibitor pharmacophore. The presence of false positives and negatives in a screening procedure involving comparisons between antibody binding and enzyme inhibition reveals the intricate differences in the mechanisms of small-molecule binding to antibodies versus an enzyme. Additional studies with a larger set of selections from a chemical library may provide a more conclusive answer regarding hit rates and accuracy of the antibody screen.

In summary, an antibody raised against one structural class of inhibitors can be used as a molecular probe to identify other structural classes of inhibitors with similar biological activity. For instance, the modification of the coating conjugate hapten increased ciELISA cross-reactivity, thereby allowing identification of all four structural classes of ACCase inhibitors. Furthermore, in the pilot screen two novel ACCase inhibitor structures were identified using the antibody-based screen. However, this ELISA-based screen for identifying new lead chemistry does not predict in vitro ACCase activity in every case. Furthermore, like in vitro ACCase activity, the ELISA-based screen does not necessarily predict in vivo herbicidal activity. Nonetheless, our results demonstrate that the antibody-based ELISA does have some utility as a rapid, inexpensive, high-throughput screening tool to identify new lead chemistries.

ABBREVIATIONS USED

ABST, *N*-hydroxysuccinimide and 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) substrate tablets; ACCase, acetyl coenzyme-A carboxylase; BSA, bovine serum albumin; CCA, coating conjugate A; CCD, coating conjugate D; CCE, coating conjugate E; ciELISA, competitive indirect enzyme-linked immunosorbent assay; DMSO, dimethyl sulfoxide; OVA, ovalbumin; PBS, phosphate-buffered saline.

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