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Production and Characterization of Monoclonal Antibodies Specific to the Strobilurin Pesticide Pyraclostrobin

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Strobilurin fungicides are nowadays among the most important fungicides in the market of active agrochemicals. Pyraclostrobin, which belongs to the last generation of this family of molecules, shows a broader antifungal activity spectrum and higher efficiency and security profiles than previous fungicides. This paper describes the synthesis of functionalized haptens, the production of monoclonal antibodies, and the development of enzyme-linked immunosorbent assays (ELISA) for the detection of pyraclostrobin. A conformational analysis of hapten structure was performed, which provided relevant data concerning the length of the spacer arm. A very useful strategy has been followed for the screening of hybridomas, leading to the selection of a panel of high-affinity monoclonal antibodies to pyraclostrobin. Moreover, different immunoassays have been characterized using the conjugate-coated indirect ELISA format, and limits of detection below 0.1 μg/L have been obtained. Also, a simplified one-step procedure has been carried out with two indirect assays. Finally, these results have been compared with the performance of the same antibodies in the antibody-coated direct ELISA format.

KEYWORDS: Strobilurin; fungicides; methoxycarbamates; enzyme-linked immunosorbent assay; antibody-coated direct competitive ELISA format; one-step conjugate-coated indirect immunoassay; simplified assay; conformational analysis; spacer arm length

INTRODUCTION

Since the discovery of the natural fungicide strobilurin A by Anke and co-workers in 1977 (*1*), a huge synthetic effort has been put forth to obtain industrially relevant derivatives of that molecule. Some good reviews have been published in this respect (*2*−*4*). The first molecules to be registered (azoxystrobin and kresoxim-methyl), and most strobilurin-active principles of the second generation, contain the same biologically active moiety as the natural compound or with minor changes, that is, an acrylate, an acetate, or an acetamide chemical group with the *E* configuration of the double bond in the toxophore moiety. Nevertheless, by application of quantitative structural−activity relationships, other compounds have been found that introduce more drastic chemical modifications without losing the antifungal activity. One of these exceptions is pyraclostrobin (PY), discovered by BASF scientists in 2000 and whose toxophore moiety is a methoxycarbamate group (**Figure 1**). Like all strobilurins, PY inhibits mitochondrial respiration by influencing the function of the cytochrome *b* (*5*). In 2004, the use of PY was accepted in the European Union, so it was included in Annex I of Directive 91/414/EEC, and by the end of 2005, it had been approved in more than 50 countries for over 100 crops in over 100 indications (www.agro.basf.com).

Immunochemical analysis is now a well-established technique that has provided solutions to many analytical requirements relevant to the agri-food sector, particularly for assessing food safety and quality. However, to advance the technique, there is

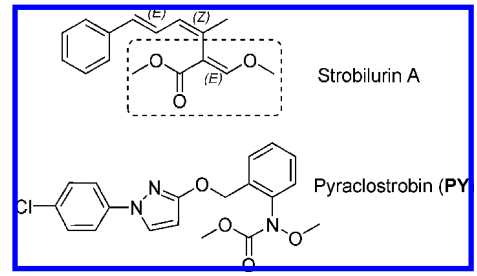

Figure 1. Chemical structure of strobilurin A, with the methyl methoxyacrylate toxophore group circled, and the synthetic fungicide PY.

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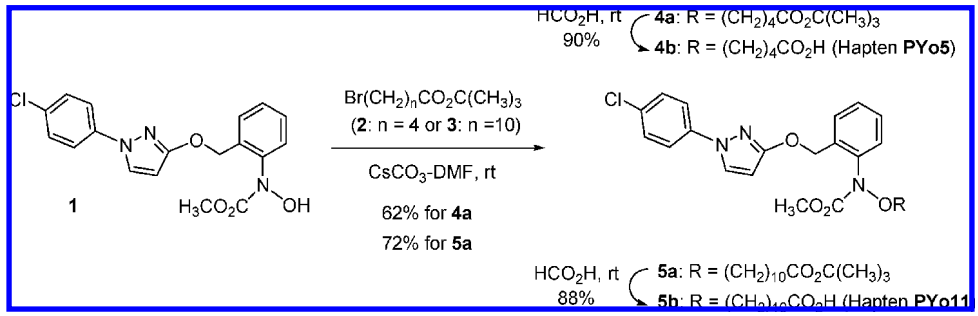

Figure 2. Scheme for the synthesis of haptens PYo5 and PYo11.

a need for antibodies against novel analytes, because it is the availability of such antibodies that is the rate-limiting step (*6*). Moreover, further research is required to provide innovative, fast, and cheap analytical devices with the proper analytical characteristics (*7, 8*). The competitive enzyme-linked immunosorbent assay (ELISA) is the most extended sort of immunoassay due to its simplicity, reliability, low cost, and high sample throughput. The immobilization and detection steps define the two main ELISA formats: the conjugate-coated indirect competitive ELISA (ic-ELISA) and the antibody-coated direct competitive ELISA (dc-ELISA). On the one hand, when the hapten conjugate is immobilized, the reacted antibody is usually indirectly detected using an enzymatically labeled secondary antibody. On the other hand, if the primary antibody is the immobilized species, a direct detection is preferentially carried out using an enzymatic hapten conjugate.

To our knowledge, three research articles have been published so far describing the production of antibodies and hapten conjugates for the immunochemical detection of strobilurin fungicides. Furzer et al. (*9*) produced polyclonal antibodies and developed three immunoassay formats (one assay using indirect enzymatic detection and two assays using fluorescence detection) for the analysis of azoxystrobin. In previous studies carried out in our laboratory, different ELISAs based on monoclonal antibodies (mAbs) had been developed against kresoxim-methyl (*10*) and trifloxystrobin (*11*), and a general strategy was established for the synthesis of functionalized derivatives and the introduction of spacer arms to strobilurins with a methyl methoxyiminoacetate toxophore. Now, we report a new strategy for the synthesis of derivatized haptens for a strobilurin with a different active chemical group and the production for the first time of mAbs against PY. The conjugate-coated ic-ELISA and the antibody-coated dc-ELISA formats have been employed, and different procedures have been optimized with the aim to characterize the prepared specific reagents.

MATERIALS AND METHODS

Chemicals and Instrumentation. PY (methyl{2-[1-(4-chlorophenyl)pyrazol-3-yloxymethyl]phenyl}methoxycarbamate) (CAS Registry no. 175013-18-0, MW 387.8 g/mol), kresoxim-methyl, and dimoxystrobin, Pestanal grade, were purchased from Riedel-de-Haën (Seelze, Germany). Trifloxystrobin standard was kindly provided by Bayer CropScience (Frankfurt, Germany), and azoxystrobin and picoxystrobin standards were obtained from Syngenta (Basel, Switzerland). Technical grade methyl 2-{[1-(4-chlorophenyl)-1*H*-pyrazol-3-yloxy]methyl}phenyl-(hydroxy)carbamate was generously provided by BASF. Reagents for protein conjugation and immunoassays were from Sigma-Aldrich (Madrid, Spain). Sephadex G-25 HiTrap Desalting columns and HiTrap Protein G HP columns from General Electric Healthcare (Uppsala, Sweden) were used for conjugate and antibody purification, respectively. Polyclonal rabbit antimouse immunoglobulins peroxidase conjugate (RAM-HRP) was from Dako (Glostrup, Denmark). Cell culture media and reagents were acquired from regular suppliers. Culture plastic ware and Costar flat-bottom high-binding polystyrene ELISA plates were from Corning (Corning, NY). Ultraviolet−visible spectra and ELISA absorbances were read (in dual wavelength mode, 492−650 nm) with a PowerWave HT from BioTek Instruments (Winooski, VT). ELISA plates were washed with an ELx405 microplate washer also from BioTek Instruments.

Reagents for hapten synthesis were acquired from commercial sources and used without purification. Tetrahydrofuran was distilled from sodium and benzophenone under an argon atmosphere. The reactions were monitored with the aid of thin-layer chromatography (TLC) using 0.25 mm precoated silica gel plates. Visualization was carried out with UV light and aqueous ceric ammonium molybdate solution or 50% (v/v) concentrated H_2SO_4 in water. Chromatography refers to flash column chromatography and was carried out with the indicated solvents on silica gel 60 (particle size, 0.040−0.063 mm). All NMR spectra were recorded in $CDCl_3$ at room temperature on a Bruker AC-300 spectrometer (300.13 MHz for ^1H and 75.47 MHz for ^{13}C). The spectra were referenced to residual solvent protons in the ^1H NMR spectra (7.26 ppm) and to solvent carbons in the ^{13}C NMR spectra (77.0 ppm). Carbon substitution degrees were established by distortionless enhancement by polarization transfer pulse sequences. A combination of correlation spectroscopy and heteronuclear single quantum coherence experiments was utilized for the assignment of ^1H and ^{13}C chemical shifts. Infrared (IR) spectra were measured as thin films between NaCl plates using a Nicolet Avatar 320 spectrometer. High-resolution mass spectra were recorded with a VG AutoSpec spectrometer.

Molecular Modeling: Determination of Minimum Energy Conformations. The two-dimensional structure of a molecule, obtained with the ChemDraw Ultra program, was converted into a three-dimensional molecular structure with Chem3D Ultra (CambridgeSoft). The generation of low energy conformations and geometrical optimizations was made using the conformational space search program CONFLEX6 running on Windows XP (*12*). The visualization and analysis interface BARISTA, a platform especially designed for conformational analyses, was used to create the initial parameters, and a conformation search was carried out with MMFF94S (2006-11-24HGTEMP) parameters using the default configuration resources, except for the upper limit value of conformational search that was set to cover an area of 10 kcal/mol within the most stable conformation (SEL = 10) (*13, 14*). The energy distribution of conformers was established under steric energy analysis.

Synthesis of Haptens. Haptens PYo5 and PYo11 (**Figure 2**) were synthesized from methyl 2-{[1-(4-chlorophenyl)-1*H*-pyrazol-3-yloxy]-methyl}phenyl(hydroxy)carbamate (**1**) (*15*) by *O*-alkylation with *tert*-butyl 5-bromopentanoate (**2**) and *tert*-butyl 11-bromoundecanoate (**3**), respectively, followed by hydrolysis of the *tert*-butyl ester moiety by treatment with formic acid. The required *tert*-butyl esters, **2** and **3**, were prepared from the corresponding ω-bromo alkanoic acids following a modification of the procedure described by Wright et al. for the preparation of *tert*-butyl esters from carboxylic acids (*16*).

General Procedure for the Preparation of the tert-Butyl Esters **2** *and* **3.** Concentrated H_2SO_4 (0.53 mL, 9.5 mmol) was added dropwise to a well-stirred suspension of anhydrous $MgSO_4$ (4.71 g, 39 mmol) in dry CH_2Cl_2 (23.8 mL), contained in a glass bottle mild pressure reactor. The mixture was stirred for 10 min, and the ω-bromo alkanoic acid (38 mmol) was added. The reactor was cooled down to −10 °C and

charged with isobutene (10.7 mL, 118 mmol) and then stirred at room temperature overnight (ca. 16 h). Upon completion of the reaction, the reactor was vented to remove the excess of isobutene, and the mixture was filtered to eliminate the $MgSO_4$, washing at the end with CH_2Cl_2. The filtrate and washing were combined and washed successively with 5% sodium bicarbonate solution and brine, then dried (Na_2SO_4), and concentrated under vacuum to give the *tert*-butyl ester as a colorless oil. *tert*-Butyl 5-bromopentanoate (**2**) (*17*) was obtained from 5-bromopentanoic acid in 90% yield and *tert*-butyl 11-bromoundecanoate (**3**) (*18*) was obtained from 11-bromoundecanoic acid in 89% yield. Both *tert*-butyl esters were very pure, as assessed by spectroscopic data, and were used directly in the alkylation reaction without further purification.

General Procedure for the O-Alkylation Reaction. The corresponding *tert*-butyl ω-bromo alkanoate (**2** or **3**, 1.0 mmol, 2 equiv) was added to a mixture of hydroxy carbamate **1** (187 mg, 0.5 mmol) and anhydrous $CsCO_3$ (262 mg, 0.8 mmol) in dry dimethylformamide (DMF) (4.9 mL) under nitrogen. The reaction mixture was stirred at room temperature for 3 h, then diluted with H_2O (70 mL), and extracted with EtOAc. The combined extracts were washed with brine, dried ($MgSO_4$), and concentrated. The residue was chromatographed on silica gel to give the pure *O*-alkylated carbamate.

***tert*-Butyl 5-[(2-{[1-(4-Chlorophenyl)-1*H*-pyrazol-3-yloxy]methyl}-phenyl)(methoxycarbonyl)aminooxy]pentanoate (4a).** Purification of the crude *O*-alkylated carbamate obtained from 183.5 mg of **1** and 233 mg of **2** by column chromatography, eluting with EtOAc−hexane (9:1), afforded 159.1 mg of pure **4a** (62% yield) as an oil. See the Supporting Information for characterization data.

***tert*-Butyl 11-[(2-{[1-(4-Chlorophenyl)-1*H*-pyrazol-3-yloxy]methyl}phenyl)(methoxycarbonyl)aminooxy]undecanoate (5a).** Purification of the crude *O*-alkylated carbamate obtained from 187.1 mg of **1** and 322 mg of **3** by column chromatography, eluting with EtOAc−hexane (9:1), afforded 222 mg of pure **5a** (72% yield) as an oil. See the Supporting Information for characterization data.

General Procedure for the Hydrolysis of the tert-Butyl Esters. A solution of the *tert*-butyl ester (**4a** or **5a**, 0.5 mmol) in 95−97% formic acid (5 mL) was stirred at room temperature until TLC [developed with chloroform−EtOAc (9:1)] showed completion of the reaction (ca. 3−4 h). The reaction mixture was diluted with benzene (60 mL), washed with water and saturated brine, dried ($MgSO_4$), and filtered, followed by evaporation of the solvent under reduced pressure. The crude residue was purified via flash chromatography to give the pure carboxylic acid.

5-[(2-{[1-(4-Chlorophenyl)-1*H*-pyrazol-3-yloxy]methyl}phenyl)-(methoxycarbonyl)aminooxy]pentanoic Acid (Hapten PYo5, 4b). Purification of the crude carboxylic acid obtained from 136.5 mg of **4a** by column chromatography, eluting with chloroform, afforded 110.5 mg of pure **4b** (90% yield) as a viscous oil. ^{1}H NMR ($CDCl_3$): δ 7.69 (1H, d, J = 3 Hz, H-5 Pz), 7.64 (1H, m, H-6 Ph), 7.52 (2H, br d, J = 9 Hz, H-2 and H-6 Cl-Ph), 7.39−7.33 (3H, m, H-3, H-4 and H-5 Ph), 7.36 (2H, br d, J = 9 Hz, H-3 and H-5 Cl-Ph), 5.92 (1H, d, J = 3 Hz, H-4 Pz), 5.32 (2H, s, OCH_2), 3.95 (2H, t, J = 6 Hz, H_2-5), 3.78 (3H, s, OMe), 2.32 (2H, t, J = 7 Hz, H_2-2), 1.66 (4H, m, H_2-3 and H_2-4). ^{13}C NMR ($CDCl_3$): δ 178.69 (CO_2H), 164.27 (OCON), 156.07(C-3 Pz), 138.59 and 137.91 (C-1 and C-2 Ph), 134.38 (C-1 Cl-Ph), 130.64 (C-4 Cl-Ph), 129.34 (C-3 and C-5 Cl-Ph), 128.89, 128.63 and 128.56 (C-3, C-4 and C-5 Ph), 127.85 (C-5 Pz), 126.75 (C-6 Ph), 119.01 (C-2 and C-6 Cl-Ph), 94.41 (C-4 Pz), 74.03 (C-5), 66.89 (CH_2O), 53.55 (OMe), 33.31 (C-2), 27.15 (C-4), 21.18 (C-3). IR (NaCl): 3151, 3121, 2954, 2876, 1735, 1708, 1545, 1480, 1358, 1094, 936, 830, 751 cm^{-1}. MS (EI), *m*/*z* (%) 325 (14), 194 (7), 164 (15), 132 (100). HRMS (FAB) calcd for $C_{23}H_{25}{}^{35}ClN_3O_6$ (M^+ + H), 474.14319; found, 474.14342. UV (100 mM sodium phosphate buffer, pH 7.4): ε (280 nm) = 15.54 mM^{-1} cm^{-1}, ε (260 nm) = 15.90 mM^{-1} cm^{-1}.

11-[(2-{[1-(4-Chlorophenyl)-1*H*-pyrazol-3-yloxy]methyl}phenyl)-(methoxycarbonyl)aminooxy]undecanoic Acid (Hapten PYo11, 5b). Purification of the crude carboxylic acid obtained from 190.5 mg of **5a** by column chromatography, eluting with chloroform, afforded 157.5 mg of pure **5b** (88% yield) as a viscous oil. ^{1}H NMR ($CDCl_3$): δ 7.69 (1H, d, J = 3 Hz, H-5 Pz), 7.64 (1H, m, H-6 Ph), 7.53 (2H, br d, J = 9 Hz, H-2 and H-6 Cl-Ph), 7.41−7.33 (3H, m, H-3, H-4 and H-5 Ph), 7.36 (2H, br d, J = 9 Hz, H-3 and H-5 Cl-Ph), 5.92 (1H, d, J = 3 Hz, H-4 Pz), 5.34 (2H, s, OCH_2), 3.92 (2H, t, J = 7 Hz, H_2-11), 3.79 (3H, s, OMe), 2.33 (2H, t, J = 7.5 Hz, H_2-2), 1.65−1.53 (4H, m, H_2-3 and H_2-10), 1.29−1.19 (12H, m, H_2-4 to H_2-9). ^{13}C NMR ($CDCl_3$): δ 179.09 (CO_2H), 164.29 (OCON), 156.04 (C-3 Pz), 138.63 and 137.90 (C-1 and C-2 Ph), 134.45 (C-1 Cl-Ph), 130.57 (C-4 Cl-Ph), 129.33 (C-3 and C-5 Cl-Ph), 128.62, 128.48 and 128.41 (C-3, C-4 and C-5 Ph), 127.69 (C-5 Pz), 126.68 (C-6 Ph), 118.90 (C-2 and C-6 Cl-Ph), 94.45 (C-4 Pz), 74.80 (H_2-11), 66.87 (CH_2O), 53.51 (OMe), 33.86 (C-2), 29.31, 29.23, 29.18 and 29.11 (C-5, C-6, C-7 and C-8), 28.96 (C-4), 27.92 (C-10), 25.73 (C-9), 24.63 (C-3). IR (NaCl): 3151, 3121, 3062, 2954, 2876, 1735, 1708, 1545, 1480, 1358, 1094, 936, 830, 751 cm^{-1}. MS (EI): *m*/*z* (%) 364 (8), 357 (5), 327 (3), 325 (9), 194 (7), 165 (7), 132 (100). HRMS (FAB) calcd for $C_{29}H_{37}{}^{35}ClN_3O_6$ (M^+ + H), 558.23709; found, 558.23775. UV (100 mM sodium phosphate buffer, pH 7.4): ε (280 nm) = 11.57 mM^{-1} cm^{-1}, ε (260 nm) = 9.08 mM^{-1} cm^{-1}.

Preparation of Protein−Hapten Conjugates. All conjugates used in this study were prepared by activation of the free carboxylic group of the hapten and reaction with the amine groups of the carrier protein. Three carrier proteins were used as follows: bovine serum albumin (BSA) for the immunizing conjugate, ovalbumin (OVA) for coating conjugates, and horseradish peroxidase (HRP) for tracer conjugates. PYo5 and PYo11 were coupled to BSA using the carbodiimide method, while conjugates of these haptens to OVA and HRP were prepared with the mixed anhydride method. For more details, see the Supporting Information.

Production of mAbs. Animal manipulation was performed in compliance with the laws and guidelines of the Spanish Ministry of Agriculture, Fisheries, and Food. BALB/c female mice were immunized with BSA−PYo5 or BSA−PYo11 conjugate, and cell fusions were performed following standard procedures as described in the Supporting Information.

Hybridoma Selection and Cloning. Twelve days after fusion, hybridoma culture supernatants were screened by simultaneous indirect noncompetitive and competitive ELISA with 1.0 μg/mL coating homologous conjugate as described by Abad et al. (*19*). The signal in noncompetitive conditions was compared with the competitive one when 0.5 μM PY was used as the competitor. The ratio of both absorbances was used as the criterion for selecting the best antibody-secreting clones. A rescreening of certain culture supernatants was done for better clone selection by bidimensional competitive ELISA. Those wells containing interesting clones received fresh culture medium, and they were assayed again the next day. This subsequent screening was done with serial dilutions of the culture supernatants by simultaneous indirect noncompetitive and competitive ELISA with 1.0 and 0.1 μg/mL homologous coating conjugate. In this case, the competing analyte was assayed at 10 and 100 nM. The selected hybridomas were cloned, and mAbs were produced, purified, and isotyped using standard procedures (*10, 11*).

ELISAs. Analytes were prepared as concentrated solutions in DMF and kept at −20 °C in amber glass vials. Sigmoidal curves were mathematically fitted to a four-parameter logistic equation using the SigmaPlot software package from SPSS Inc. (Chicago, IL). Assay sensitivity was estimated as the concentration of analyte that reduced 50% (IC_{50}) of the maximum signal (A_{max}) reached at the zero dose of analyte. The limit of detection (LOD) was estimated as the concentration of PY that provided a 10% reduction of A_{max}. Cross-reactivity (CR) values were calculated according to the formula:

$$CR = [IC_{50}(PY)/IC_{50}(compound)] \times 100$$

Competitive Two-Step Conjugate-Coated Indirect ELISAs. Ninety-six-well polystyrene ELISA plates were coated with 100 μL per well of OVA−conjugate solutions (at 1.0 or 0.1 μg/mL) in 50 mM carbonate−bicarbonate buffer, pH 9.6, by overnight incubation at room temperature. The optimum antibody concentration for competitive experiments was determined in each case, for a fixed conjugate concentration, to reach a maximum absorbance value around 1.0 in the absence of analyte. Coated plates were washed four times with washing solution [0.15 M NaCl containing 0.05% (v/v) Tween 20] and then received 50 μL per well of analyte in PBS plus 50 μL per well of mouse serum, hybridoma supernatant, or immunoglobulin solution in PBS containing 0.05% Tween 20 (PBST). Samples were usually run

in duplicate wells. The immunological reaction took place for 1 h at room temperature, and plates were washed again as described. Next, 100 μL per well of a 1/2000 dilution of RAM-HRP conjugate in PBST was added, and plates were incubated 1 h at room temperature. After the plates were washed, the retained peroxidase activity was determined by addition of 100 μL per well of freshly prepared 2 mg/mL OPD and 0.012% (v/v) H_2O_2 in 25 mM citrate and 62 mM sodium phosphate buffer, pH 5.4. The enzymatic reaction was stopped after 10 min at room temperature by the addition of 100 μL per well of 2.5 M sulfuric acid. The absorbance was immediately read at 492 nm with a reference wavelength at 650 nm.

Competitive One-Step Conjugate-Coated Indirect ELISAs. For this assay, only OVA−PYo5 was used as the coating conjugate. The previous procedure was followed except for the competitive reaction, for which 50 μL per well of standard solution in PBS and 50 μL per well of a 1:1 (v/v) mixture of mAb and RAM−HRP conjugate solutions in PBST was added. Single wells were assayed in these experiments. The reaction was allowed to happen for 1 h at room temperature, and after washing, the retained peroxidase activity was revealed as described above.

Competitive Antibody-Coated Direct ELISAs. This ELISA format employed the specific mAb immobilized onto the polystyrene plates. For coating, 100 μL of a 1 μg/mL mAb solution in 50 mM carbonate−bicarbonate buffer, pH 9.6, was added per well, and plates were incubated overnight at room temperature. Coated plates were washed four times with washing solution and received 50 μL per well of analyte in PBS plus 50 μL per well of HRP tracer solution in PBST. The optimum HRP conjugate concentration to reach a maximum absorbance around 1.0 was previously determined by noncompetitive assay. All samples were run in duplicate wells. The immunological reaction took place for 1 h at room temperature, and plates were washed again as described. Finally, the retained peroxidase activity was directly revealed as described above.

RESULTS AND DISCUSSION

Hapten Synthesis and Conjugate Preparation. It is known that the structure of the hapten and the appropriate length and composition of the spacer arm have a profound effect on the formation of specific and high-affinity antibodies (*20, 21*). Inasmuch as antibodies are directed toward parts of the molecule projecting from the protein, the correct molecular orientation of the hapten on the carrier is considered critical to obtain antibodies with the desired properties. Strobilurin pesticides hold two common structures: the toxophore, which displays the antifungal activity, and a bridging aryl ring, which separates the toxophore and the particular side chains of each compound (**Figure 1**). For these reasons and for the present project, the spacer arm was introduced at the toxophore group. In our previously developed immunoassays for two strobilurin fungicides, kresoxim-methyl and trifloxystrobin (*10, 11*), the strategy for the synthesis of haptens was based on the easy hydrolysis of the methyl methoxyiminoacetate group to the corresponding carboxylic acid, which allowed the ready attachment of different spacer chains at the toxophore moiety of the strobilurin molecule. For PY, the different nature of the toxophore moiety (a methyl methoxycarbamate group) precluded the use of this strategy because decarboxylation of the intermediate carbamic acid occurs almost instantaneously upon hydrolysis of the carbamate moiety. In the present work, the haptens used for immunization and as assay conjugates were prepared from methyl hydroxy(phenyl)carbamate **1** (**Figure 2**), using the hydroxyl group to covalently bind an aliphatic spacer arm, which holds a carboxylic group for subsequent linkage to carrier protein molecules. The haptens were prepared through a two-step procedure as follows (**Figure 2**). First, the spacer group was covalently bound to the hydroxy carbamate moiety of **1** as a *tert*-butyl ester, via *O*-alkylation reaction with the appropriate *tert*-butyl ω-bromo alkanoate (**2** or **3**) and $CsCO_3$ as a basic catalyst in DMF. This was followed by chemoselective hydrolysis of the *tert*-butyl ester moiety with formic acid at room temperature. Haptens PYo5 and PYo11 were thus obtained from compound **1** in 56 and 63% overall yields, respectively. In principle, the introduction of the spacer chain at this position of the molecule preserves the specific moieties of PY, introducing only a minimum modification at a distal point of the specific chemical groups of the fungicide. Furthermore, the prepared haptens contain either a five-carbon (hapten PYo5) or an 11-carbon (hapten PYo11) spacer attached to the oxygen moiety.

For the immunogen, the use of spacers containing no strong immunodeterminant chemical groups is generally recommended. Moreover, aliphatic spacers seem to be the most appropriate linkers for immunizing and assay conjugates. Spacers of different lengths have been used during the last decades to produce high-affinity antibodies. Too long or too short linkers are not recommended, while medium-sized linkers (3−6 carbons) are considered as optimum. Nevertheless, Kim et al. (*22*) did not observe any influence of the linker length on the production of high-affinity polyclonal antibodies. It is possible to find many examples of high-affinity antibodies that have been produced using immunizing conjugates containing medium or short spacers. On the contrary, little work has been done regarding long spacers. As an example, Peterson et al. (*23*) have recently published a paper in which high-affinity mAbs were prepared using a 10-carbon long spacer in the immunogen. Such a conjugate seemed to produce antibodies with broader selectivity. For these reasons, we decided to investigate the influence of such a long spacer on the production of antibodies and on its convenience for assay conjugates.

Hapten PYo5 was coupled to BSA, OVA, and HRP with final estimated hapten-to-protein molar ratios of 19, 5, and 4, respectively. Likewise, PYo11 was bound to BSA, OVA, and HRP with calculated hapten-to-protein molar ratios of 21, 3, and 12, respectively. As preferred, immunizing conjugates BSA−PYo5 and BSA−PYo11 showed a high molar ratio, and they were stable in solution. The unusual hapten loading of the HRP−PYo11 tracer is probably an artifact caused by variations of the extinction coefficient at 280 nm of the protein, the hapten, or both, produced upon conjugation, which was observed as abnormally elevated absorbance values at that wavelength. On the contrary, the absorbance at 400 nm was similar to that of other tracer conjugates prepared in parallel. Enzymatic activity assays using the tracer in solution showed that the peroxidase activity of HRP−PYo11 was neither lost nor appreciably diminished (results not shown). All of these data suggest that PYo11 was normally coupled to HRP.

Mouse Immunization and Antiserum Analysis. Eight mice were immunized, four with the BSA−PYo5 conjugate and four with the BSA−PYo11 conjugate, following standard procedures. The immune response was followed by noncompetitive and competitive assays with antisera collected after the third injection. Sera were serially diluted with PBST, and competitive assays were performed with PY as competitor using 1.0, 0.3, and 0.1 μg/mL of the homologous coating conjugate and 1.0 μg/mL of the heterologous coating conjugate. Regarding antisera from mice immunized with BSA−PYo5, the titers were around $1/10^5$ at the highest OVA−PYo5 concentration. **Table 1** shows the IC_{50} values for PY found with the four antisera collected from mice immunized with BSA−PYo5. If the heterologous coating conjugate was used with these antisera, some binding and moderate inhibition were observed despite the low titers (results not shown). Although the four mice responded similarly

Table 1. Analysis of Antisera from Mice Immunized with BSA-PYo5

	antiserum #1			antiserum #2			antiserum #3			antiserum #4		
OVA-PYo5 (μg/mL)	A_{max}[a]	IC_{50}[a] (nM)	dil. ($\times 10^3$)	A_{max}[b]	IC_{50}[b] (nM)	dil. ($\times 10^3$)	A_{max}[b]	IC_{50}[b] (nM)	dil. ($\times 10^3$)	A_{max}[a]	IC_{50}[a] (nM)	dil. ($\times 10^3$)
1	0.93	91	240	1.17	124	120	1.26	272	240	1.17	198	240
0.3	0.91	28	120	1.17	27	60	1.17	154	120	1.07	81	120
0.1	1.25[b]	35[b]	8	1.36	28	10	1.14	62	8	1.15[b]	84[b]	20

[a] Mean value of three independent determinations. [b] Mean value of two independent determinations.

to the immunization process (comparable titers and affinity to PY), it could be observed that antiserum #1 and antiserum #2 showed the lowest IC_{50} values. Regarding the four mice immunized with BSA−PYo11, the collected antisera did not show any binding to the homologous coating conjugate, not even when it was employed at 1.0 μg/mL under noncompetitve conditions. Interestingly, two of the collected antisera did recognize the heterologous conjugate OVA−PYo5, although with low titers (1/5000 antisera dilutions afforded A_{max} values between 0.4 and 0.7). Nevertheless, no inhibition curves could be accurately fitted in competitive assays using PY as competitor. Therefore, it seems that the hapten with a long spacer arm (PYo11) was clearly less suitable than the hapten with medium-sized spacer (PYo5) for the generation of antibodies to PY.

Computational Studies. Given the poor results obtained with antisera from mice immunized with the conjugate of PYo11, computational studies were undertaken to gain some insights into the underlying molecular events associated with the observed behavior. We carried out an evaluation of the chemically significant conformations of PYo5 and PYo11 that could be relevant to the result of the immunizing process. Assuming that antibodies are generated against the most populated conformation of the haptens, this information should help to understand the influence of the linker length and flexibility in the outcome of the immune response. Energy-minimized structures and conformational energy calculations were performed for the corresponding amide derivatives of PYo5 and PYo11 (PYo5−NHMe and PYo11−NHMe, respectively) to mimic the conjugated haptens. These molecules are highly flexible, so the conformational search resulted in a very large number of conformations with little and no significant energy differences between many of them. For example, a conformational search of all conformational interspace within the search region of PYo5−NHMe resulted in a total of 9589 conformations. However, chemically significant conformations in a energy window not wider than 4 kcal/mol, which comprises about 94% of the total energy region, involved less than 100 conformations. In all of these conformers, the three rings of the PY molecule adopt an extended disposition, with the hydrocarbon arm chain positioned over the rings instead of being directed to the opposite direction, as seen in the superimposition of conformations shown in **Figure 3**.

The flexibility of PYo11−NHMe drastically increased as compared to PYo5−NHMe, resulting in 13418 possible conformations from which 508 enter within the 4 kcal/mol window (nearly 94% of the total energy region). The disposition of the rings in these more stable conformations is similar to those observed in PYo5−NHMe, but in this case, the hydrocarbon arm chain extended over the polycyclic aromatic core, showing a much higher degree of flexibility and orientation diversity than in the hapten with the shorter spacer arm (**Figure 3**). It seems that for this PY derivative there is a predisposition of the long and flexible linker to fold onto the rest of the molecule, probably due to attractive intramolecular van der Waals interactions. This observation shows the importance of the nature of the linker—an 11-carbon aliphatic arm is greatly hiding the characteristic

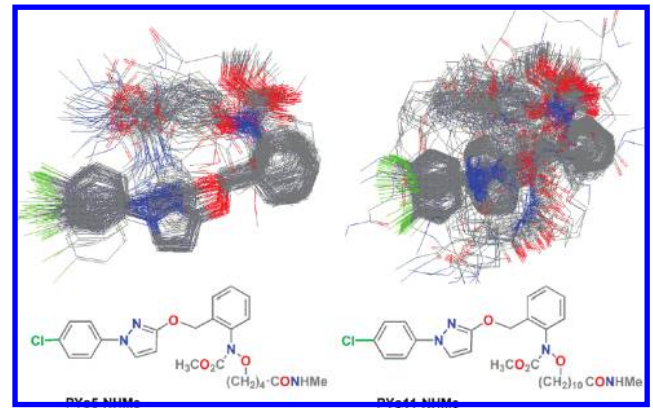

Figure 3. Superimposed lowest energy conformers of PY haptens. (**A**) One hundred conformers of PYo5-NHMe, recovered with an energy window of ca. 4.3 kcal/mol corresponding to 96.4% of the total energy region. (**B**) Two hundred conformers of PYo11-NHMe, recovered with an energy window of ca. 3.2 kcal/mol corresponding to 85.0% of the total energy region. The elements are represented in the following manner: carbon, gray; oxygen, red; nitrogen, blue; and chlorine, green. Hydrogens have been omitted for clarity.

chemical groups of PY, while a five-carbon aliphatic arm seems to better display the rings of the hapten—and it fits with the experimental results found during the production of antibodies, where PYo11 revealed as a less suitable hapten than PYo5 to induce the production of high-affinity antibodies to PY. Yet, the nature of the hapten backbone may also play a role in determining the optimum length and composition of the linker. Further molecular modeling studies with other compounds should help to predict and choose the best spacer arms for each analyte, both for immunizing conjugate and for assay conjugate preparation.

mAb Production. The results observed by computational studies and antiserum analysis led us to undertake the production of mAbs just from those mice immunized with the conjugate of PYo5. One of the cell fusions was unusually efficient since the screening assay revealed the presence of a high number of positive signals (99 wells with signals over 0.5 absorbance units). Moreover, 18 clones from this fusion gave absorbance signals over 3.0 (out of the measuring range of the microplate reader) under noncompetitive conditions, and most of them showed little or no inhibition. To differentiate those clones that produced antibodies with high affinity only to the coating conjugate from those that, in addition, strongly recognized PY in solution, an innovative rescreening analysis was introduced using bidimensional competitive assays. **Figure 4** illustrates the usefulness of this procedure. Two clones displaying the same signal in the first standard screening of postfusion supernatants showed a completely different behavior when evaluated under these new conditions. Thus, while clone 4A4 did not show a competitive response independently of the supernatant dilution, coating concentration, or analyte concentration, clone 4G3 gave a clear competitive response when the supertanant was diluted and the coating concentration was decreased. These results

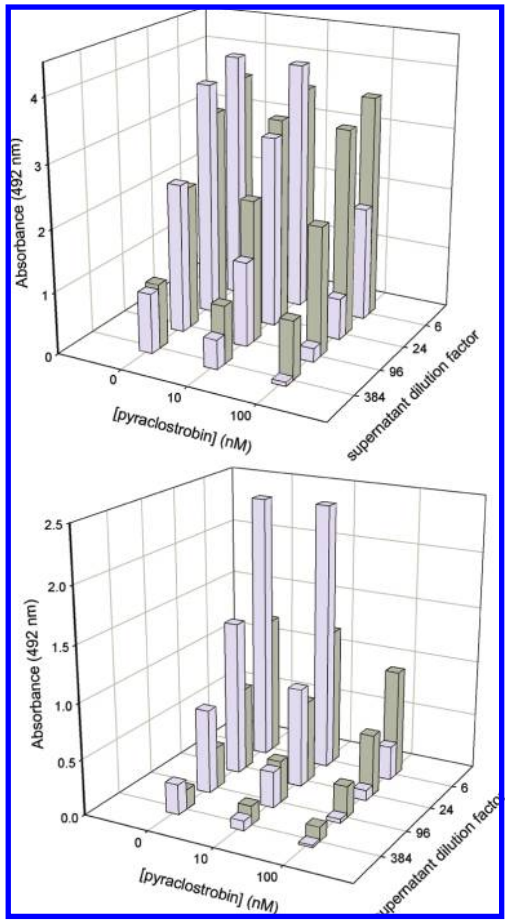

Figure 4. Bidimensional competitive rescreening assay of hybridomas 4G3 (light gray) and 4A4 (dark gray). Supernatants were serially diluted in PBST with a multichannel pipet and assayed against three PY concentrations (0, 10, and 100 nM) in plates coated with the conjugate OVA-PYo5 at 1.0 (upper graph) and 0.1 μg/mL (lower graph). With this scheme, up to four hybridomas can be included in a single ELISA plate, and just 50 μL of culture supernatant is required. This simple approach allows us to easily rank positive hybridomas according to their affinity for the analyte at a very early step of the whole process.

evidence the tremendous importance of the screening strategy to select the proper hybridomas. The clones showing the strongest inhibition were selected for cloning, and seven hybridomas were finally successfully stabilized. PYo5#31 was of the IgG_{2b} isotype, PYo5#32 was of the IgG_{2a} isotype, and all of the other mAbs were of the IgG_1 isotype. All of them contained κ-type light chains.

Two-Step Conjugate-Coated ic-ELISAs. Purified mAbs were analyzed in combination with the two OVA conjugates at 1.0 and 0.1 μg/mL using this ELISA format. The optimum mAb concentration was established in each case to reach a maximum absorbance of 1.0 by noncompetitve bidimensional titration. At 1.0 μg/mL OVA−PYo5 conjugate, the optimum concentration for most mAbs was between 10 and 15 ng/mL, except for antibody PYo5#38, which had to be used at a concentration of 80 ng/mL. If the coating conjugate was reduced to 0.1 μg/mL, the concentration of three mAbs (PYo5#33, PYo5#34, and PYo5#38) had to be increased 10 times and only two or three times for the other four mAbs (results not shown). Interestingly, OVA−PYo11 was recognized by most of the mAbs, although high concentrations of antibody were required (more than 500 ng/mL). Only mAb PYo5#21 was demanded at a regular concentration (40 ng/mL) with this conjugate, and it even provided a sensitive assay under these conditions (**Table 2**). It

Table 2. Assay Parameters Using the Two-Step ic-ELISA Format of Anti-PY mAbs

	OVA-PYo5[a] (1 μg/mL)			OVA-PYo5[a] (0.1 μg/mL)			OVA-PYo11[a] (1 μg/mL)		
mAb	A_{max}	IC_{50} (nM)	slope	A_{max}	IC_{50} (nM)	slope	A_{max}	IC_{50} (nM)	slope
PYo5#21	0.94	9.1	−1.07	**1.09**	**1.8**	**−1.03**	**0.96**	**1.5**	**−1.07**
PYo5#31	1.28	4.1	−0.96	**1.08**	**1.1**	**−1.38**	1.23	8.3	−3.07
PYo5#32	1.17	25.8	−1.10	1.29	5.9	−1.22	1.13	5.3	−1.25
PYo5#33	1.23	9.4	−0.97	1.23	7.9	−1.33	0.94	23.0	−1.46
PYo5#34	0.99	8.2	−1.18	1.07	7.1	−1.27	0.95	21.7	−1.67
PYo5#37	1.09	3.6	−0.99	**1.15**	**1.2**	**−1.37**	1.33	3.1	−1.65
PYo5#38	1.08	3.7	−1.15	1.03	6.7	−2.76	NR[b]	NR[b]	NR[b]

[a] Mean value of three independent determinations. [b] Not recognized.

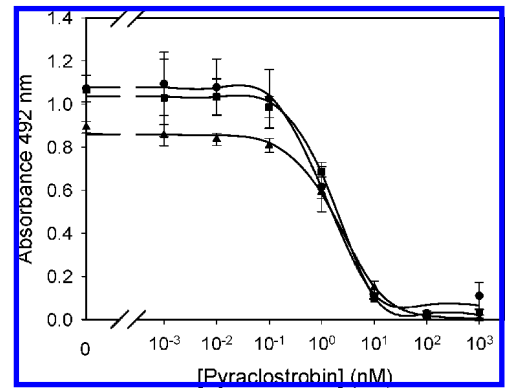

Figure 5. Inhibition curves of PY for the two-step ic-ELISA (circles), the one-step ic-ELISA (squares), and the dc-ELISA (triangles) using mAb PYo5#31 and conjugates of PYo5. Results are the means of three independent experiments.

should be noted that all mAbs came from mice immunized with a PYo5 conjugate. The positive binding of mAbs to OVA−PYo11 is probably the result of the interaction with the hapten when it adopts an unfolded conformation. The low probability of the unfolded state of the hapten could explain the higher concentrations of mAb that are required. The interaction of the antibody could stabilize a certain low-frequency hapten conformation, which better fits into the binding site. All antibodies showed lower IC_{50} values at 0.1 μg/mL than at 1.0 μg/mL coating conjugate concentration, except for antibody PYo5#38. If the homologous conjugate (OVA−PYo5) was used at 1.0 μg/mL, the resulting standard curves presented slopes around −1.0 in each case (**Table 2**). The heterologous conjugate (OVA−PYo11) at 1.0 μg/mL and the homologous conjugate (OVA−PYo5) at 0.1 μg/mL provided assays with standard curves showing high slope values. Again, mAb PYo5#21 was an exception; it rendered competitive assays with slopes around −1.0 in all cases (**Table 2**). No assays could be performed with 0.1 μg/mL OVA−PYo11 (results not shown). Under the assayed conditions, antibodies PYo5#21, PYo5#31, and PYo5#37 provided the best assays (highlighted in bold numbers in **Table 2**). **Figure 5** shows a typical standard curve obtained with mAb PYo5#31 and coating conjugate OVA−PYo5 at 0.1 μg/mL. The IC_{50} value for PY of this assay was 1.12 nM with a calculated LOD of 0.08 μg/L, well below the maximum residue limit (MRL) established by the European Commission (*24*), which is, at the lowest, 20 μg/kg for most food samples.

The CR of all seven mAbs was checked for other strobilurin fungicides as competitors using OVA−PYo5 at 0.1 μg/mL as a coating conjugate. None of these mAbs recognized dimoxystrobin, picoxystrobin, azoxystrobin, trifloxystrobin, or kresoximmethyl at 1 μM (results not shown). Haptens PYo5 and PYo11

were also evaluated as free competitors in solution. On the one hand, all of the mAbs bound PYo5 with a higher affinity than the fungicide PY, with CR values near or over 600% in most cases. On the other hand, hapten PYo11 was less recognized than PY (CR values between 20 and 50%). The long spacer arm of PYo11 seems to be hindering the binding of the mAb, not only to the conjugated form of the hapten, as observed before, but also to the free hapten, which would be in accordance with the folded molecular conformations suggested in **Figure 3**.

One-Step Conjugate-Coated ic-ELISAs. The aim of this experiment was to develop an assay in the ic-ELISA format with a procedure equivalent to an assay in the dc-ELISA format. These are the two most common ELISA formats, as previously mentioned, and both of them present different advantages and limitations. Good discussions in this respect have been published before (*25*−*27*). In our experience, the ic-ELISA format is more robust, it better allows the use of heterologous conjugates, the expenses of specific antibodies are lower, and sample matrix effects are more easily avoided. Conversely, the dc-ELISA format is simpler and shorter. The one-step assays developed in this study employ the same format as the previous two-step assays, but a washing step was omitted and just one incubation step was run, which implies two simultaneous reactions. A similar approach has been previously published (*10, 26, 27*). The specific mAb reacts with the analyte or the coating conjugate in a competitive mode, and simultaneously, the secondary antibody reacts with the primary and specific mAb. Therefore, this procedure allows the reduction of the total assay time to 1 h, while the amplification provided by the secondary antibody is maintained.

Because different immunological reactions take place simultaneously, we decided to examine the dependence of the assay performance on the concentration of the immunoreagents. We chose mAbs PYo5#31 and PYo5#38 because the optimum concentration for each antibody was very different in the two-step ic-ELISA (30 and 1000 ng/mL for PYo#31 and PYo5#38, respectively, at 0.1 μg/mL OVA−PYo5 coating conjugate). Competitive assays were run using the one-step procedure with 1.0 and 0.1 μg/mL OVA−PYo5 coating conjugate, and the results are shown in **Figure 6**. The mAb and the RAM−HRP conjugate were mixed as described, and they were immediately used. The concentration of the peroxidase conjugate was varied to reach a final dilution corresponding to 1/500, 1/1000, or 1/2000, and the range of mAb concentrations was adjusted for each mAb and for each coating−conjugate concentration. The best results (A_{max} around 1.0 and low IC_{50} value) for mAb PYo5#31 were achieved with 0.1 μg/mL OVA−PYo5 concentration, 100 ng/mL mAb, and a 1/1000 dilution of the secondary antibody (**Figure 6B**). On the contrary, antibody concentrations lower than 100 ng/mL afforded too low A_{max} values. The mean inhibition curve of three independent experiments using these immunoreagents and PY as competitor is shown in **Figure 5**. The average IC_{50} value obtained under these conditions was 1.59 nM for PY, and the calculated LOD was 0.36 ng/mL. If a 1.0 μg/mL coating−conjugate concentration was employed, an equivalent IC_{50} value was observed (1.5 nM PY), using in this case 20 ng/mL mAb and a 1/500 dilution of the RAM conjugate (**Figure 6A**). Nevertheless, under these conditions, the minimum asymptote of the fitted curve was around 10% of the A_{max} or higher. For these reasons, an assay with lower coating concentration seemed to be more adequate. Regarding mAb PYo5#38, it was only possible to reach A_{max} values of 1.0 at 1.0 μg/mL coating conjugate with an antibody concentration of 200−300 ng/mL. Interestingly, for this mAb, similar IC_{50} values (around 4.0 nM) for PY were observed, independently of the dilution of the secondary antibody (**Figure 6C**). Hence, it has been demonstrated that it was possible to adapt these immunoassays to the one-step ic-ELISA format even if high primary antibody concentrations are demanded.

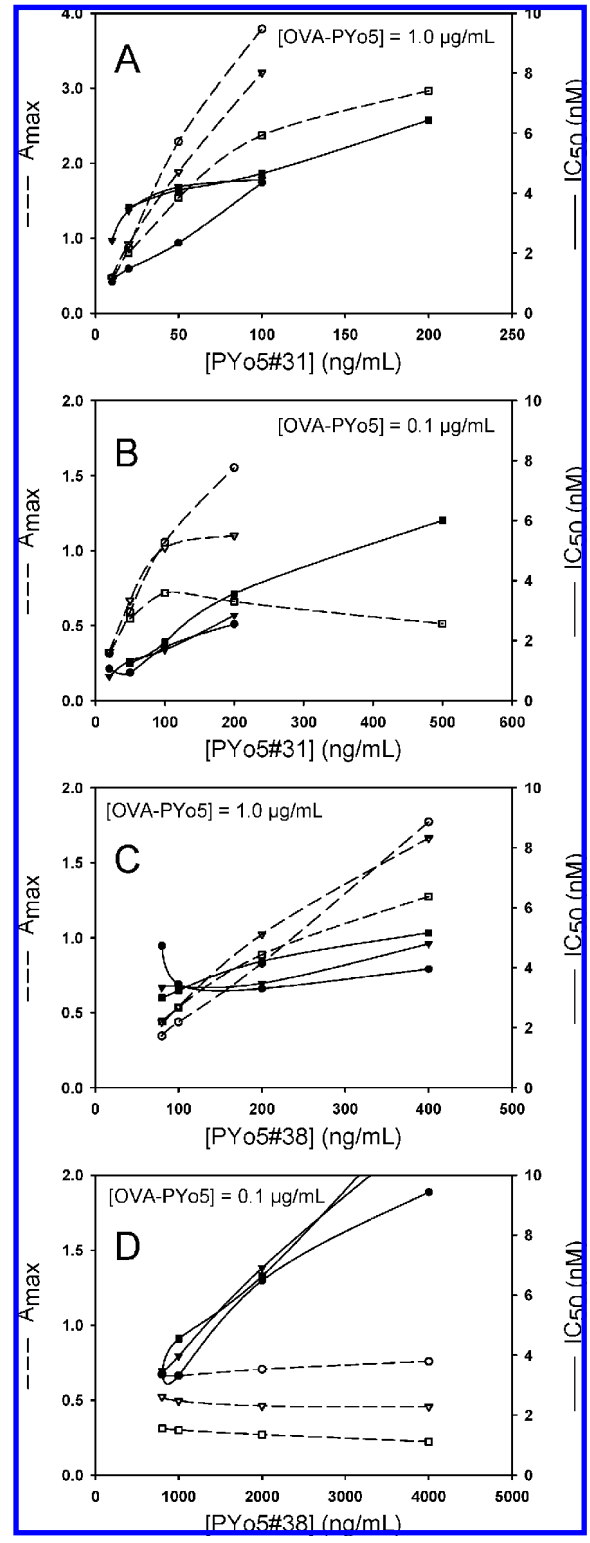

Figure 6. Optimization of the different immunoreagents used in the one-step ic-ELISA. The variation of the IC_{50} (—) and A_{max} (- - -) values is represented for two mAbs (PYo5#31 and PYo5#38) at two different coating conjugate concentrations (1.0 and 0.1 μg/mL) and three different dilutions of the RAM-HRP conjugate: 1/500 (circles), 1/1000 (triangles), and 1/2000 (squares).

Table 3. Assay Parameters Using the dc-ELISA Format

mAb	A_{max}[a]	IC_{50}[a] (nM)	slope[a]
PYo5#21	0.75	7.4	−3.08
PYo5#31	**0.83**	**2.3**	**−1.05**
PYo5#32	0.99	11.5	−1.08
PYo5#33	1.10	13.0	−1.15
PYo5#34	1.06	11.3	−1.31
PYo5#37	0.73	6.9	−2.68
PYo5#38	**1.14**	**3.2**	**−1.25**

[a] Mean values of three independent assays.

Antibody-Coated dc-ELISAs. This is probably the most widespread ELISA format in the in vitro diagnostics market, maybe because of its simplicity and rapidity. It is known that tracer conjugates with short spacer arms (two or less carbon atoms) usually do not afford a sufficient signal (*28, 29*) in noncompetitive direct assays. In the present research, noncompetitive assays with the dc-ELISA format revealed that the tracer HRP−PYo11 was not recognized by any of the mAbs, which indicated that such long spacers are not convenient for enzyme conjugates applied to direct ELISAs. This assay format is revealed again as a more constrained format with regard to linker length. Competitive assays were performed using all seven mAbs and the homologous enzyme tracer HRP−PYo5. The optimum concentrations of the enzyme tracer were established by noncompetitive assays, and they were around 10 ng/mL. The parameters of the achieved competitive assays are summarized in **Table 3**. The lowest IC_{50} value was obtained with mAb PYo5#31, which was also among those antibodies that afforded the best assays in the ic-ELISA format. With the dc-ELISA format, mAb PYo5#31 rendered an assay with a lower slope (−1.05) but a double IC_{50} value (2.3 nM) than with the ic-ELISA format. **Figure 5** shows the inhibition curve for PY attained under the optimized conditions. The calculated LOD of the direct assay using this mAb and the homologous enzyme tracer was 0.09 ng/mL. Interestingly, mAb PYo5#38 provided competitive assays with equivalent IC_{50} values (between 3.0 and 4.0 nM) independently of the format and the procedure that was used. Finally, the binding of all mAbs to the different strobilurin fungicides and to haptens PYo5 and PYo11 in solution was also evaluated in the dc-ELISA format. As with the indirect format, none of the assayed strobilurins (azoxystrobin, trifloxystrobin, kresoxim-methyl, dimoxystrobin, and picoxystrobin) was recognized by any of these mAbs. The CR values found using the two haptens in solution as competitors were comparable to those observed with the ic-ELISA format.

Two derivatives of PY with very different spacer lengths have been synthesized. Three-dimensional studies showed that aliphatic linkers have the tendency to bend over the molecule of PY, which would explain that medium-sized arms rendered the best results as both immunogens and assay conjugates. A too long spacer seems to favor intramolecular and/or intermolecular interactions, which probably hinders the binding to the antibody. The double-screening strategy applied in this study emerged as a very useful approach to rate a high number of strongly positive hybridomas at early steps of the process, when supernatant availability is limited. A collection of mAbs has been produced from mice immunized with a conjugate using a five-carbon linker, whereas a too long linker was not productive. These mAbs bind specifically the fungicide PY with high affinity. The produced immunoreagents allowed the development of different competitive immunoassays, both in the conjugate-coated indirect ELISA and in the antibody-coated direct ELISA. Finally, an assay using the indirect ELISA format has been developed using a simplified one-step procedure. Future work will study the performance of these ELISAs for the analysis of PY in relevant food matrices.

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Supporting Information Available: Characterization data for compounds **4a** and **5a** and the procedures for conjugate synthesis and mAb production. This material is available free of charge via the Internet at http://pubs.acs.org.

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