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Hapten Synthesis and Monoclonal Antibody-Based Immunoassay Development for the Detection of the Fungicide Kresoxim-methyl

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Strobilurin fungicides have been increasingly used for fungus pest control since they were introduced in 1996. For pesticide residue detection, immunoassays constitute nowadays a valuable approach. This paper describes the synthesis of functionalized haptens of kresoxim-methyl, the production of monoclonal antibodies, and the development of enzyme-linked immunosorbent assays. On the one hand, a two-step conjugate-coated immunoassay was optimized using extended or short incubation times, with limits of detection of 0.4 ng/mL for the extended assay and 0.3 ng/mL for the rapid assay. On the other hand, an immunoassay was optimized following a procedure consisting of just one incubation step. This one-step assay had a limit of detection of 0.4 ng/mL. All of these assays showed a similar performance, with sensitivities well below common maximum residue limits for this pesticide (50 μg/kg) and lower than the detection limits of the usual chromatographic detection methods.

KEYWORDS: Strobilurin; ELISA; dipeptide; linker; spacer arm; bridge; hapten heterology; pesticide; QoI fungicides

INTRODUCTION

The discovery of the strobilurin pesticides was inspired by a group of natural antibiotics produced by a range of Basidiomycete wood-rooting fungi. In 1977, Anke and co-workers (*1*) obtained two compounds from fermentations of *Strobilurus tenacellus*, strobilurins A and B (**Figure 1**), with a powerful antibiotic activity against a range of fungal species. It was observed that these molecules contained a common element essential to their mode of action, a β-methoxyacrylate subunit (*2*). Since those years, several synthetic derivatives of the strobilurin family have been prepared and commercialized, all of them incorporating the same, or slightly modified, toxophore moiety. Kresoxim-methyl (KM), with a β-methoxyiminoacetate group (**Figure 2**), was one of the first strobilurins to be patented (*3*) and, in 1999, it was included in Annex I of the EU Council Directive 91/414/EEC as an approved nonsystemic active substance for plant protection (*4*). Nowadays, strobilurins, classified as QoI fungicides, are extensively used for crop and culture protection and constitute a major class of agricultural fungicides; they represent approximately 10% of the fungicide market (*5, 6*). These chemicals have been approved for use in developed countries because they all are relatively readily degraded and represent low risk to human health as well as to birds, mammals, and bees, although they vary in their toxicity to aquatic organisms (*7*). Nevertheless, fungus resistance to strobilurins has already been reported (*8*), so the Fungicide Resistance Action Committee (FRAC) has recommended preventative application for optimum disease control (www.frac.info). Moreover, their extensive use and postharvest application makes it easier to reach human consumers. These facts have led the regulatory organisms to introduce controls for strobilurin residues in food products (*9*).

Current analytical methods for the detection of KM consist of extensive organic solvent extraction followed by gas chromatography analysis with nitrogen−phosphorus detection (*10*), although other chromatographic methods have been reported (*11, 12*). Biotechnology-based methods such as immunoassays (IAs) are an alternative or complementary tool for pesticide monitoring (*13*). Since they were introduced, many IAs have been described for pesticides of different families. Most IAs have been developed using polyclonal antisera raised in rabbits because the production of monoclonal antibodies (mAbs) has usually been considered to be an expensive and laborious technology. However, as IAs have been transferred to the industry, this initial cost becomes less important, considering the subsequent expense of assay validation and the potential of an unlimited supply of uniform immunoreagents.

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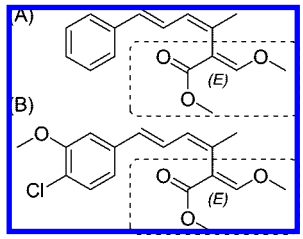

Figure 1. Structures of the natural strobilurin fungicides produced by *Basidiomycetes* fungi: (**A**) strobilurin A; (**B**) strobilurin B. The common (*E*)-methyl β-methoxyacrylate group acting as toxophore is circled.

In 2006, a research paper was published about the development of three highly sensitive IA formats using selective polyclonal antibodies for the detection of azoxystrobin (*14*), another member of this fungicide family. To our knowledge, no additional papers have appeared describing the development of IAs to any other member of the strobilurin family, and no mAbs have been obtained so far to any strobilurin. In this paper, we report a strategy for the synthesis of functionalized haptens for the methoxyiminoacetate strobilurins and the production of high-affinity monoclonal antibodies against KM. Three enzyme-linked immunosorbent assays (ELISAs) with different procedures and operation times for KM detection have been optimized and characterized.

MATERIALS AND METHODS

Chemicals and Instrumentation. Kresoxim-methyl [methyl (*E*)-2-methoxyimino-2-[2-(*o*-tolyloxymethyl)phenyl]acetate] (CAS Registry No. 143390-89-0), dimoxystrobin, and pyraclostrobin, Pestanal grade, were purchased from Riedel-de-Haën (Seelze, Germany). Technical grade KM was generously provided by BASF (Limburgerhof, Germany). Trifloxystrobin standard was kindly provided by Bayer CropScience (Frankfurt, Germany), and azoxystrobin and picoxystrobin were provided by Syngenta (Basel, Switzerland). Stock solutions of these compounds were prepared in dried *N*,*N*-dimethylformamide (DMF) and stored at −20 °C. *N*-Glycyl-glycine (gg) was obtained from Alfa Aesar (Karlsruhe, Germany), and *N*-β-alanyl-glycine (βag) and *N*-glycyl-γ-aminobutyric acid (gab) were from Bachem AG (Bubendorf, Switzerland). *N*,*N*-Dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (NHS), 4-aminobutyric acid, 6-aminohexanoic acid, and tributylamine were from Fluka (Madrid, Spain). Isobutyl chloroformate, ovalbumin (OVA), thimerosal, and *o*-phenylenediamine (OPD) were purchased 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). Bovine serum albumin (BSA) fraction V and Hybridoma Fusion and Cloning Supplement (HFCS) were purchased from Roche Applied Science (Mannheim, Germany). P3-X63-Ag 8.653 mouse plasmacytoma cell line was from the European Collection of Cell Cultures (Wiltshire, U.K.). HT and HAT supplements and gentamicine solution were obtained from Gibco BRL (Paisley, Scotland). Cell culture media (high-glucose Dulbecco's Modified Eagle's Medium), poly(ethylene glycol) (PEG1500), fetal bovine serum (FBS), 200 mM alanyl-glutamine solution, Red Blood Cell Lysing Buffer Hybri-Max, MEM nonessential amino acid solution, and Freund's adjuvants were from Sigma-Aldrich (Madrid, Spain). Culture plasticware 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, VE). ELISA plates were washed with an ELx405 microplate washer also from BioTek Instruments.

Reagents for hapten synthesis were obtained 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 melting points were determined using a Kofler hot-stage apparatus and are uncorrected. All NMR spectra were recorded in $CDCl_3$ or DMSO-d_6 at room temperature on a Bruker AC-300 spectrometer (300.13 MHz for ^{1}H and 75.47 MHz for ^{13}C). The spectra were referenced to residual solvent protons in the ^{1}H NMR spectra (7.26 and 2.50 ppm) and to solvent carbons in the ^{13}C NMR spectra (77.0 and 39.43 ppm). Carbon substitution degrees were established by DEPT pulse sequences. A combination of COSY and HSQC experiments was utilized for the assignment of ^{1}H and ^{13}C chemical shifts. IR spectra were measured as thin films for liquid compounds and as KBr pellets for solids using a Nicolet Avatar 320 spectrometer. High-resolution mass spectra were recorded with a VG AutoSpec spectrometer.

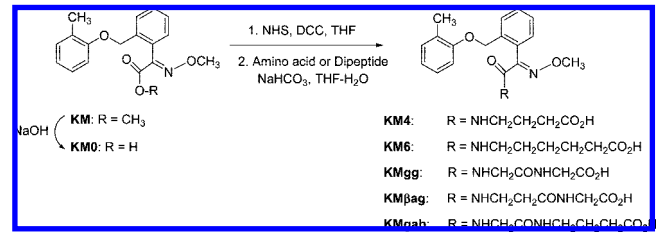

Figure 2. Scheme of hapten synthesis and structures of kresoxim-methyl (KM) and functionalized derivatives.

Hapten Synthesis. All KM derivatized haptens that were prepared in this study are summarized in **Figure 2**. Haptens were synthesized by alkaline hydrolysis of the KM ester, activation of the resulting carboxylic acid with DCC and NHS, and reaction with an amino acid (4-aminobutanoic acid and 6-aminohexanoic acid) or a dipeptide (*N*-glycyl-glycine, *N*-β-alanyl-glycine, and *N*-glycyl-γ-aminobutyric acid). Compounds used in this study present minor safety concerns. However, it is advisable to work in a well-ventilated fume hood during synthesis work.

(*E*)-2-(Methoxyimino)-2-(2-(*o*-tolyloxymethyl)phenyl)acetic Acid (KM0). Five grams of KM (15.96 mmol), methanol (70 mL), and 5 M aqueous sodium hydroxide (10.0 mL) were stirred at reflux. After the hydrolysis was complete, as monitored by TLC (around 5 h), most of the methanol was evaporated off under reduced pressure, water was added to the residue, and neutral impurities were removed by extraction with ethyl acetate. The aqueous layer was cooled to 0 °C and acidified to pH 3 with 2 M HCl aqueous solution, the product was extracted with ethyl acetate, and the extract was washed with brine and finally dried over anhydrous $MgSO_4$. Evaporation gave 4.09 g of a white solid (85.6%), which was used in the next steps without further purification. The preparation of this acid derivative has been described in the literature (*15, 16*), but no physical or spectroscopic data have been previously given for it. A detailed description of these data follows: mp 139–140 °C (from slow evaporation of a $CHCl_3$/hexane solution); ^{1}H NMR (DMSO-d_6), δ 7.43 (1H, dd, J = 7.5 and 1.5 Hz, H-3 Ph), 7.30 (1H, ddd, J = 7.5, 7.5, 1.5 Hz, H-4 Ph), 7.25 (1H, ddd, J = 7.5, 7.5, 1.5 Hz, H-5 Ph), 7.15–7.06 (3H, m, H-6 Ph, H-3 tolyl and H-5 tolyl), 6.87 (1H, br d, J = 7.5 Hz, H-6 tolyl), 6.81 (1H, dd, J = 7.5 and 7.5 Hz, H-4 tolyl), 4.98 (2H, s, CH_2O), 3.76 (3H, s, OCH_3), 2.22 (3H, s, CH_3-tolyl); ^{13}C NMR (DMSO-d_6), δ 164.70 (CO), 156.31 (C-2), 156.10 (C-1 tolyl), 134.44 (C-2 Ph), 133.01 (C-1 Ph), 130.32 (C-3 tolyl), 127.53 (C-6 Ph), 127.22 (C-5 tolyl), 126.81 (C-3 Ph), 126.56 (C-4 Ph), 125.79 (C-2 tolyl), 125.72 (C-5 Ph), 120.14 (C-4 tolyl), 111.34 (C-6 tolyl), 66.96 (CH_2O), 61.31 (CH_3O), 16.11 (CH_3); IR (KBr), 3472, 3070, 3025, 2977, 2943, 1710, 1616, 1494, 1377, 1251, 1008, 757, 744 cm^{-1}; MS (EI), *m*/*z* (%) 299 (M^+, 4), 268 (1), 223 (32), 192 (15), 116 (100), 89 (11); HRMS, calcd for $C_{17}H_{17}NO_4$ 299.11576, found 299.11652; UV (100 mM sodium phosphate buffer, pH 7.4), ϵ (280 nm) = 1.37 mM^{-1} cm^{-1}, ϵ (260 nm) = 2.86 mM^{-1} cm^{-1}.

General Procedure for Spacer Arm Introduction. The carboxylic acid (KM0) (1.0 mmol), NHS (1.1 mmol), and DCC (1.1 mmol) were dissolved in dry tetrahydrofuran (5.5 mL) at 0 °C under nitrogen

atmosphere. The mixture was stirred at 4 °C for 20 h and then at room temperature for 4 h. The precipitated *N*,*N*′-dicyclohexylurea was removed by vacuum filtration and a solution of 1.1 mmol of $NaHCO_3$, and 1.1 mmol of the corresponding amino acid or dipeptide in 1.8 mL of water was added. The reaction mixture was stirred at room temperature until TLC ($CHCl_3$/CH_3OH 5:1) showed complete consumption of the intermediate NHS ester (around 40 h); then the solvent was evaporated off, and the obtained residue was dissolved in 10% (w/v) aqueous citric acid and extracted with ethyl acetate. The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, and evaporated under reduced pressure to afford the crude coupling product, which was purified by column chromatography on silica gel using the eluent specified in each case.

(*E*)-6-2-(Methoxyimino)-2-(2-(*o*-tolyloxymethyl)phenyl)acetamido)hexanoic Acid (KM6). Purification of the crude coupling product obtained from 2.52 g of KM0 by column chromatography, eluting with $CHCl_3$/EtOAc 8:2, afforded 1.75 g of pure KM6 (50% yield) as a viscous oil: ^{1}H NMR ($CDCl_3$), δ 7.54 (1H, dd, J = 7.4, 1.4 Hz, H-6 Ph), 7.42 (1H, ddd, J = 7.4, 7.4, 1.6 Hz, H-5 Ph), 7.37 (1H, ddd, J = 7.4, 7.4, 1.5 Hz, H-4 Ph), 7.23 (1H, dd, J = 7.4, 1.5 Hz, H-3 Ph), 7.15–7.08 (2H, m, overlapped H-3 tolyl and H-5 tolyl), 6.85 (1H, dd, J = 7.0 and 7.0 Hz, H-4 tolyl), 6.79 (1H, br d, J = 8.1 Hz, H-6 tolyl), 6.74 (1H, t, J = 5.9 Hz, NH), 4.95 (2H, s, CH_2O), 3.95 (3H, s, OCH_3), 3.30 (2H, q, J = 6.9 Hz, H-6), 2.32 (2H, t, J = 7.4 Hz, H-2), 2.23 (3H, s, CH_3-tolyl), 1.63 (2H, quint, J = 7.4 Hz, H-3), 1.51 (2H, quint, J = 7.3 Hz, H-5), 1.35 (2H, m, H-4); ^{13}C NMR ($CDCl_3$), δ 178.91 (C-1), 162.14 (CONH), 156.65 (C-1 tolyl), 151.05 (C═N), 135.78 (C-2 Ph), 130.60 (C-3 tolyl), 129.45 (C-5 Ph), 128.97 (C-3 Ph), 128.70 (C-1 Ph), 127.64 (C-6 Ph), 127.41 (C-4 Ph), 126.84 (C-2 tolyl), 126.72 (C-4 Ph), 120.48 (C-4 tolyl), 111.10 (C-6 tolyl), 68.25 (CH_2O), 63.26 (CH_3O), 39.29 (C-6), 33.69 (C-2), 29.14 (C-5), 26.25 (C-4), 24.22 (C-3), 16.24 (CH_3-Tol); IR (film), 3415, 3065, 2938, 2858, 2809, 1739, 1708, 1670, 1602, 1525, 1495, 1241, 1049, 752 cm^{-1}; MS (EI), *m*/*z* (%) 382 (5), 381 (18), 304 (17), 305 (89), 274 (20), 224 (13), 115 (19), 116 (100); HRMS (FAB), calcd for $C_{23}H_{29}N_2O_5$ (M^+ + 1) 413.20765, found 413.20673; UV (100 mM sodium phosphate buffer, pH 7.4), ϵ (280 nm) = 2.03 mM^{-1} cm^{-1}, ϵ (260 nm) = 4.62 mM^{-1} cm^{-1}.

(*E*)-4–2-(Methoxyimino)-2-(2-(*o*-tolyloxymethyl)phenyl)acetamido)butanoic Acid (KM4). The crude coupling product obtained from 2.0 g of KM0 was first purified by column chromatography, eluting with $CHCl_3$/EtOAc 8:2, and then recrystallized from EtOAc to afford 870 mg of pure KM4 (34% yield) as a white solid: mp 132–134 °C (from benzene/hexane); ^{1}H NMR ($CDCl_3$), δ 7.55 (1H, br d, J = 7.4 Hz, H-6 Ph), 7.43 (1H, ddd, J = 7.4, 7.4, 1.6 Hz, H-5 Ph), 7.38 (1H, ddd, J = 7.3, 7.3, 1.4 Hz, H-4 Ph), 7.22 (1H, dd, J = 7.3, 1.6 Hz, H-3 Ph), 7.15–7.08 (2H, m, overlapped H-3 tolyl and H-5 tolyl), 6.93 (1H, t, J = 6.0 Hz, NH), 6.85 (1H, dd, J = 7.3 and 7.3 Hz, H-4 tolyl), 6.79 (1H, br d, J = 8.1 Hz, H-6 tolyl), 4.96 (2H, s, CH_2O), 3.95 (3H, s, OCH_3), 3.37 (2H, q, J = 6.7 Hz, H-4), 2.36 (2H, t, J = 7.1 Hz, H-2), 2.24 (3H, s, CH_3-tolyl), 1.84 (2H, quint, J = 7.0 Hz, H-3); ^{13}C NMR ($CDCl_3$), δ 177.95 (C-1), 162.50 (CONH), 156.60 (C-1 tolyl), 150.81 (C═N), 135.81 (C-2 Ph), 130.61 (C-3 tolyl), 129.49 (C-5 Ph), 128.99 (C-3 Ph), 128.51 (C-1 Ph), 127.63 (C-6 Ph), 127.41 (C-4 Ph), 126.79 (C-2 tolyl), 126.73 (C-4 Ph), 120.51 (C-4 tolyl), 111.10 (C-6 tolyl), 68.23 (CH_2O), 63.32 (CH_3O), 38.74 (C-4), 31.29 (C-2), 24.49 (C-3), 16.23 (CH_3-tolyl); IR (KBr), 3386, 2979, 2939, 2900, 1731, 1629, 1603, 1540, 1496, 1247, 1184, 1013, 749 cm^{-1}; MS (EI), *m*/*z* (%) 354 (2), 353 (7), 277 (39), 259 (3), 246 (8), 224 (7), 223 (11), 192 (3), 144 (3), 117 (17), 116 (100); HRMS (FAB), calcd for $C_{21}H_{25}N_2O_5$ (M^+ + 1) 385.17635, found 385.17714; UV (100 mM sodium phosphate buffer, pH 7.4), ϵ (280 nm) = 2.19 mM^{-1} cm^{-1}, ϵ (260 nm) = 4.88 mM^{-1} cm^{-1}.

(*E*)-5,8-Dioxo-4-(2-(*o*-tolyloxymethyl)phenyl)-2-oxa-3,6,9-triazaundec-3-en-11-oic Acid (KMgg). Purification of the crude coupling product obtained from 183 mg of KM0 by column chromatography, eluting with $CHCl_3$ and then $CHCl_3$/EtOAc 1:1, afforded 116 mg of pure KMgg (46% yield) as a white solid: mp 129–131 °C (from $CHCl_3$/hexane); ^{1}H NMR (DMSO-d_6), δ 12.60 (1H, br s, COOH), 8.39 (1H, t, J = 5.8 Hz, C_5-NH), 8.26 (1H, t, J = 5.8 Hz, C_8-NH), 7.54 (1H, br d, J = 7.5 Hz, H-6 Ph), 7.41 (1H, ddd, J = 7.5, 7.5, 1.5 Hz, H-5 Ph), 7.37 (1H, ddd, J = 7.5, 7.5, 1.5 Hz, H-4 Ph), 7.20 (1H, dd, J = 7.5, 1.5 Hz, H-3 Ph), 7.13 (1H, br d J = 7.5 Hz, H-3 tolyl), 7.09 (1H, br t, J = 8 Hz, H-5 tolyl), 6.89 (1H, br d, J = 8 Hz, H-6 tolyl), 6.82 (1H, dd, J = 7.5 and 7.5 Hz, H-4 tolyl), 4.92 (2H, s, CH_2O), 3.92 (3H, s, OCH_3), 3.83 (2H, d J = 5.8 Hz, H-7), 3.77 (2H, d J = 5.8 Hz, H-10), 2.16 (3H, s, CH_3-tolyl); ^{13}C NMR (DMSO-d_6), δ 171.07 (C-11), 168.62 (C-8), 162.17 (C-5), 156.02 (C-1 tolyl), 150.67 (C-4), 135.41 (C-2 Ph), 130.36 (C-3 tolyl), 129.36 (C-1 Ph), 129.01 (C-5 Ph), 128.65 (C-3 Ph), 127.15 (C-6 Ph), 127.05 (C-4 Ph), 126.79 (C-5 tolyl), 125.81 (C-2 tolyl), 120.29 (C-4 tolyl), 111.26 (C-6 tolyl), 67.18 (CH_2O), 62.77 (CH_3O), 41.99 (C-7), 40.54 (C-10), 15.86 (CH_3); IR (KBr), 3332, 3067, 2938, 1663, 1601, 1522, 1495, 1443, 1241, 1123, 1054, 1021, 983, 752 cm^{-1}; MS (EI), *m*/*z* (%) 414 (M^+ + 1, 0.5), 413 (M^+, 0.5), 383 (5), 382 (21), 307 (19), 306 97), 275 (11), 223 (10), 203 (11), 173 (8), 117 (17), 116 (100); HRMS, calcd for $C_{21}H_{23}N_3O_6$ 413.15869, found 413.15922; UV (100 mM sodium phosphate buffer, pH 7.4), ϵ (280 nm) = 1.55 mM^{-1} cm^{-1}, ϵ (260 nm) = 4.69 mM^{-1} cm^{-1}.

(*E*)-5,9-Dioxo-4-(2-(*o*-tolyloxymethyl)phenyl)-2-oxa-3,6,10-triazadodec-3-en-12-oic Acid (KMβag). Purification of the crude product obtained from 100 mg of KM0 by column chromatography, eluting with $CHCl_3$/methanol 99:1, afforded 69 mg of KMβag (48% yield). The compound was a white solid: mp 183–185 °C (from $CHCl_3$/hexane); ^{1}H NMR (DMSO-d_6), δ 12.48 (1H, br s, COOH), 8.26 (1H, t, J = 5.8 Hz, C_5-NH), 8.20 (1H, t, J = 5.7 Hz, C_9-NH), 7.52 (1H, br d, J = 7.5 Hz, H-6 Ph), 7.42 (1H, ddd, J = 7.5, 7.5, 1.5 Hz, H-5 Ph), 7.36 (1H, ddd, J = 7.5, 7.5, 1.5 Hz, H-4 Ph), 7.17 (1H, dd, J = 7.5, 1.4 Hz, H-3 Ph), 7.12 (1H, br d J = 7.5 Hz, H-3 tolyl), 7.09 (1H, br t, J = 8 Hz, H-5 tolyl), 6.86 (1H, br d, J = 8 Hz, H-6 tolyl), 6.81 (1H, dd, J = 7.5 and 7.5 Hz, H-4 tolyl), 4.91 (2H, s, CH_2O), 3.88 (3H, s, OCH_3), 3.73 (2H, d J = 5.7 Hz, H-11), 3.36 (2H, overlapped with water signal from DMSO-d_6, H-7), 2.35 (2H, t J = 7.2 Hz, H-8), 2.16 (3H, s, CH_3-tolyl); ^{13}C NMR (DMSO-d_6), δ 171.20 (C-12), 170.72 (C-9), 161.86 (C-5), 156.00 (C-1 tolyl), 151.06 (C-4), 135.30 (C-2 Ph), 130.32 (C-3 tolyl), 129.58 (C-1 Ph), 128.87 (C-5 Ph), 128.54 (C-3 Ph), 127.11 (C-4 Ph and C-6 Ph), 126.74 (C-5 tolyl), 125.82 (C-2 tolyl), 120.25 (C-4 tolyl), 111.11 (C-6 tolyl), 67.27 (CH_2O), 62.58 (CH_3O), 40.48 (C-11), 35.48 (C-7), 34.44 (C-8), 15.86 (CH_3); IR (KBr), 3365, 3290, 3081, 2934, 1755, 1651, 1623, 1568, 1495, 1231, 1050, 1002, 754 cm^{-1}; MS (EI), *m*/*z* (%) 396 (6), 320 (18), 223 (28), 191 (7), 144 (3), 116 (100); HRMS (FAB), calcd for $C_{22}H_{26}N_3O_6$ (M^+ + H) 428.18216, found 428.18244; UV (100 mM sodium phosphate buffer pH 7.4): ϵ (280 nm) = 1.70 mM^{-1} cm^{-1}, ϵ (260 nm) = 5.28 mM^{-1} cm^{-1}.

(*E*)-5,8-Dioxo-4-(2-(*o*-tolyloxymethyl)phenyl)-2-oxa-3,6,9-triazatridec-3-en-13-oic Acid (KMgab). Purification of the crude coupling product obtained from 132 mg of KM0 by column chromatography, eluting with $CHCl_3$/methanol 9:1, afforded 86 mg of pure KMgab (44% yield) as a white solid: mp 170–172 °C (from $CHCl_3$/hexane); ^{1}H NMR (DMSO-d_6), δ 12.04 (1H, br s, COOH), 8.31 (1H, t, J = 5.7 Hz, C_5-NH), 7.95 (1H, t, J = 5.5 Hz, C_9-NH), 7.55 (1H, dd, J = 7.5, 1.5 Hz, H-6 Ph), 7.43 (1H, ddd, J = 7.5, 7.5, 1.5 Hz, H-5 Ph), 7.38 (1H, ddd, J = 7.5, 7.5, 1.5 Hz, H-4 Ph), 7.20 (1H, dd, J = 7.5, 1.5 Hz, H-3 Ph), 7.11 (1H, br d J = 7.2 Hz, H-3 tolyl), 7.09 (1H, br t, J = 7.9 Hz, H-5 tolyl), 6.89 (1H, br d, J = 7.9 Hz, H-6 tolyl), 6.82 (1H, dd, J = 7.4 and 7.4 Hz, H-4 tolyl), 4.92 (2H, s, CH_2O), 3.92 (3H, s, OCH_3), 3.74 (2H, d J = 5.8 Hz, H-7), 3.07 (2H, q, J = 6.8 Hz, H-10), 2.21 (2H, t J = 7.4 Hz, H-12), 1.62 (2H, quint, J = 7.2 H-11); ^{13}C NMR (DMSO-d_6), δ 174.13 (C-13), 168.04 (C-8), 162.10 (C-5), 156.02 (C-1 tolyl), 150.71 (C-4), 135.40 (C-2 Ph), 130.34 (C-3 tolyl), 129.40 (C-1 Ph), 128.97 (C-5 Ph), 128.67 (C-3 Ph), 127.15 (C-6 Ph), 127.09 (C-4 Ph), 126.78 (C-5 tolyl), 125.79 (C-2 tolyl), 120.28 (C-4 tolyl), 111.24 (C-6 tolyl), 67.22 (CH_2O), 62.75 (CH_3O), 41.25 (C-7), 37.85 (C-10), 30.88 (C-12), 24.23 (C-11), 15.84 (CH_3); IR (KBr), 3408, 3323, 2937, 1730, 1652, 1568, 1523, 1417, 1240, 1168, 1044, 1014, 747 cm^{-1}; MS (EI), *m*/*z* (%) 441 (M+, 4), 410 (12), 335 (21), 334 (100), 216 (6), 303 (13), 224 (11), 203 (14), 173 (18); HRMS, calcd for $C_{23}H_{27}N_3O_6$ 441.19998, found 441.18935; UV (100 mM sodium phosphate buffer, pH 7.4), ϵ (280 nm) = 1.66 mM^{-1} cm^{-1}, ϵ (260 nm) = 4.86 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. Two carrier proteins were used: BSA for the immunogenic conjugate and OVA for coating conjugates.

Immunogenic Conjugate. Fifty micromoles of KM6 was dissolved in 500 μL of DMF and mixed with 50 μmol of NHS and 50 μmol of DCC also in DMF. Additional DMF was added to bring the final concentration of all reagents to 50 mM. The hapten was activated overnight at room temperature in amber vials. The day after, the reaction was centrifuged and the supernatant was collected. Next, 400 μL of the activated hapten solution was added dropwise to 2 mL of a 15 mg/mL BSA solution in 50 mM carbonate−bicarbonate buffer, pH 9.6. The coupling reaction was allowed during 4 h at room temperature with moderate stirring. The initial hapten-to-protein molar ratio in the mixture was aproximately 40:1. Finally, the conjugate was separated from the uncoupled hapten by gel filtration on Sephadex G-25, using 100 mM phosphate buffer, pH 7.4, as eluant. The degree of hapten conjugation to protein was measured spectrophotometrically. If conjugation occurred, the UV–vis spectrum of the conjugate was slightly different from that of the free protein. Therefore, the hapten-to-protein molar ratio was the average calculated from the absorbance values at 280 and 260 nm by assuming that the molar absorption of the haptens and the protein were the same for the free and the conjugated forms. The purified conjugate was stored at −20 °C.

Assay Conjugates. The mixed anhydride method was used to prepare conjugates of each hapten to OVA. Briefly, 18 μmol of hapten was dissolved in 180 μL of DMF and mixed with 18 μmol of tributylamine and 18 μmol of isobutyl chloroformate also in DMF. The same solvent was added to bring the final concentration of all reagents to 90 mM. Haptens were activated during 1 h at room temperature. Next, 100 μL of activated hapten solution was added dropwise to 2 mL of a 15 mg/mL OVA solution in 50 mM carbonate−bicarbonate buffer, pH 9.6. The coupling reaction was allowed during 2.5 h at room temperature with moderate stirring. The initial hapten-to-protein molar ratios in the mixture were approximately 15:1. Finally, conjugates were separated from uncoupled haptens by gel chromatography as described for the immunogenic conjugates.

Production of Monoclonal Antibodies. Animal manipulation has been performed in compliance with the laws and guidelines of the Spanish Ministry of Agriculture, Fisheries, and Food.

Immunization. BALB/c female mice (8–10 weeks old) were immunized with the BSA−KM6 conjugate by intraperitoneal injections. Doses consisted of an emulsion of 100 μL of PBS (10 mM phosphate buffer, pH 7.4, with 137 mM NaCl, 2.7 mM KCl) containing 100 μg of protein conjugate, estimated as protein concentration, and 100 μL of Freund's adjuvants. The first dose contained complete Freund's adjuvant, and subsequent doses were given at weeks 3 and 6 using incomplete Freund's adjuvant. After a resting period of at least 3 weeks from the last injection with adjuvant, mice received a booster intraperitoneal injection of 100 μg of protein conjugate in 200 μL of PBS, 4 days before cell fusion. Mice were tail-bled 9 or 10 days after the third injection.

Cell Fusion and Culture. P3-X63/Ag 8.653 murine myeloma cells were cultured in high-glucose DMEM supplemented with 2 mM alanylglutamine, 1 mM MEM nonessential amino acids, and 25 μg/mL gentamicin (referred to as s-DMEM) with 10% (v/v) FBS. Just before spleen extraction, mouse blood was collected by heart puncture and serum diluted 1/50 in PBS containing 0.01% (w/v) thimerosal and stored at 4 °C. Cell fusion procedures were carried out essentially as described by Nowinski et al. (*17*). Mouse spleen lymphocytes were fused with myeloma cells at a 4:1 ratio using PEG 1500 as the fusing agent. The fused cells were distributed in 96-well culture plates at an approximate density of 2.5×10^5 cells/well in 100 μL of s-DMEM with 20% FBS. Twenty-four hours after plating, 100 μL of HAT selection medium (s-DMEM supplemented with 100 μM hypoxanthine, 0.4 μM aminopterine, and 16 μM thymidine) with 15% FBS and 1% (v/v) HFCS was added to each well.

Hybridoma Selection and Cloning. Twelve days after fusion, hybridoma culture supernatants were screened by simultaneous indirect noncompetitive and competitive ELISA with 1.0 and 0.1 μg/mL coating homologous conjugate. The signal in noncompetitive conditions was compared with the competitive one when 0.5 μM KM was used as competitor. The ratio of both absorbances was used as the criterion for selecting antibody-secreting clones. The selected hybridomas were cloned by limiting dilution in HT cloning medium (s-DMEM containing 20% FBS and supplemented with 100 μM hypoxanthine, 16 μM thymidine, and 1% HFCS). Stable antibody-producing clones were expanded and cryopreserved in liquid nitrogen.

Purification of Monoclonal Antibodies. MAbs were purified from late stationary phase culture supernatants by ammonium sulfate precipitation and protein G affinity chromatography following the manufacturer's instructions. The purified mAbs were stored at 4 °C as ammonium sulfate precipitates, and the immunoglobulin isotype was determined using the ImmunoPure Monoclonal Antibody Isotyping Kit I (HRP/ABTS) from Pierce (Rockford, IL).

ELISAs. *General Procedure for Immunoassays.* Ninety-six-well polystyrene ELISA plates were coated with 100 μL/well of OVA−conjugate solutions (at 1.0, 0.1, or 0.01 μg/mL) in 50 mM carbonate−bicarbonate buffer, pH 9.6, by overnight incubation at room temperature. The 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/well of analyte in PBS plus 50 μL/well of hybridoma supernatant or immunoglobulin solution in PBS containing 0.05% Tween 20 (PBST), unless otherwise stated. All samples were run in duplicate wells. Immunological reaction took place (typically 2 h) at room temperature, and plates were washed again as described. Next, 100 μL/well of a 1/2000 dilution (unless otherwise stated) of RAM−HRP conjugate in PBST was added, and plates were incubated (typically 1 h) at room temperature. After washing, retained peroxidase activity was determined by adding 100 μL/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 adding 100 μL/well of 2.5 M sulfuric acid. The absorbance was immediately read at 492 nm with a reference wavelength at 650 nm.

Noncompetitive Conjugate-Coated Assays. Purified mAbs were assessed by bidimensional checkerboard titration versus all coating conjugates. The stated general conditions were followed, but buffer containing no analyte was used. Immunoreagent titrations for final assay development were performed under the specific conditions of each assay. The optimum mAb concentration for competitive experiments was determined in each case, for a fixed conjugate concentration, to reach a maximum absorbance value of 1.0 in the absence of analyte.

Competitive Two-Step Conjugate-Coated ELISAs. These experiments were carried out according to the general assay conditions previously described. Assay sensitivity (IC_{50}) was estimated as the concentration of analyte that reduced 50% the maximum signal (A_{max}) reached at the zero dose of analyte. The limit of detection (LOD) was estimated as the concentration of KM that provided a 10% reduction of A_{max}. Cross-reactivity (CR) values were calculated according to the following formula: $CR = [IC_{50}(KM)/IC_{50}(compound)] \times 100$.

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

Stocks and Standard Curves. Analytes were prepared as concentrated solutions in DMF and kept at −20 °C in amber glass vials. For standard curves, a 1/1000 dilution was prepared in PBS from the stock in DMF, and then it was serially diluted in PBS, always using borosilicate glass tubes. Competitive curves were obtained by plotting mean absorbance values versus the logarithm of analyte concentration. Sigmoidal curves were mathematically fitted to a four-parameter logistic equation using SigmaPlot software package from SPSS Inc. (Chicago, IL).

RESULTS AND DISCUSSION

Hapten Synthesis and Protein Conjugates. Derivatization sites opposite to the characteristic chemical groups of a hapten are usually preferred to obtain highly specific mAbs. Also, to obtain high-affinity antibodies, it is advisable to introduce a spacer arm of four to six carbons and to preserve the main

Table 1. Results of Competitive Assays for KM Using mAb KM6#22 and Different Coating Conjugates

coating OVA conjugate	conjugate concn (μg/mL)	mAb concn[a] (ng/mL)	IC_{50} for KM[b] (nM)
KM0 (0 Å)[c]	1.00	60	669.0[d]
	0.10	80	27.0
	0.01	800	17.7
KM4 (7.5 Å)	1.00	150	—[e]
	0.10	70	136.7
	0.01	100	16.0
KM6 (10.6 Å)	1.00	40	—
	0.10	80	50.6
	0.01	400	23.9
KMgg (8.9 Å)	1.00	50	—
	0.10	50	40.7
	0.01	300	15.0
KMβag (10.4 Å)	1.00	100	—
	0.10	50	157.7
	0.01	150	17.0
KMgab (11.9 Å)	1.00	100	—
	0.10	60	92.7
	0.01	100	14.3

[a] Values determined by noncompetitive bidimensional checkerboard titration and affording a maximum absorbacne value around 1.0. [b] Mean of three independent determinations ($n = 3$). [c] Sum of the length of the C−C, CO−C, and N−C bonds in the spacer arm. [d] $n = 1$. [e] Not determined.

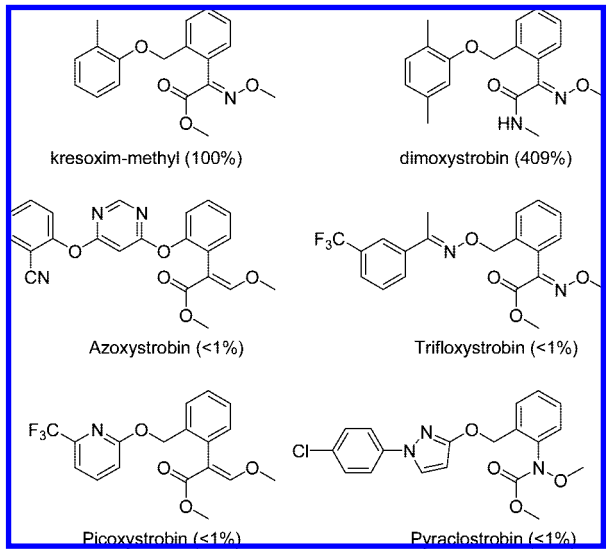

Figure 3. Chemical structures of some common strobilurin fungicides. The CR values are indicated in parentheses with respect to the IC_{50} value of kresoxim-methyl. They are the mean of three independent determinations.

geometric and electronic properties of the analyte. Thus, the immunizing hapten was synthesized with a simple, lineal hydrocarbonated spacer at the toxophore moiety of the strobilurin molecule distal to the characteristic phenoxy-methyl ring of KM, so minimum changes were introduced in the chemical structure of the pesticide. Besides, hapten heterology has been demonstrated as a proper strategy for the improvement of assay sensitivity (*18–20*). In the present work, heterologous haptens with linker-length and linker-composition differences have been prepared (**Figure 2**). The hydrolysis of KM afforded, with good yields (85.6%), the intermediate carboxylate product (KM0) used for the subsequent reaction with different spacer chains. Two sets of haptens resembling the molecule of KM were prepared with a spacer containing a free carboxyl moiety. One set (KM4 and KM6) was synthesized by reaction of the hydrolyzed ester of KM (KM0) with a linear amino acid. An amino acid of different length was used in each case (4-aminobutyric acid or 6-aminohexanoic acid) with the result of a hydrophobic spacer covalently linked to the molecule of KM through an amide group. The other set (KMgg, KMβag, and KMgab) was prepared similarly but using a dipeptide instead of a linear amino acid. In this way, a slight heterology (an amide group) was introduced inside the spacer arm and between the molecule of KM and the carrier protein, while maintaining very similar linker lengths (see **Table 1**). This straightforward synthesis strategy uses readily available spacers, and it has let us investigate the influence of a polar chemical group, such as an amide, on the performance of monoclonal antibodies in competitive immunoassays. Spacer arms prepared with dipeptides, such as β-Ala-β-Ala, or containing an amide group, such as L-Lys-ethylendiamine or 6-hydroxycaproyl-β-Ala, have been previously reported for the improvement of assay sensitivity or catalytic antibody production (*21–23*). Overall, the described synthetic procedure constitutes a general and new strategy for the direct and simple synthesis of haptens for methoxyiminoacetate strobilurins. Finally, two different chemistries were used for the coupling of haptens to proteins. The calculated hapten to protein molar ratios were 23 for the BSA conjugate and between 4 and 6 for all OVA conjugates.

Production of Monoclonal Antibodies. Five mice were immunized with BSA−KM6 conjugate, and the immunization was followed by noncompetitive and competitive assays with antisera collected after the third injection. Three cell fusions were performed with lymphocytes from those animals showing the strongest positive response. After cell fusions, seven different hybridomas were cloned and stored in liquid nitrogen. Six of the produced antibodies were of the IgG_1 isotype, and one of them was of the IgG_{2a} isotype; all immunoglobulins contained the κ light chain. For selection of one mAb for further assay development, competitive two-step conjugate-coated ELISAs were performed using purified antibodies, KM as analyte, and conjugate OVA−KM6 prepared at 1.0 and 0.1 μg/mL (results not shown). From the obtained inhibition curves, mAb KM6#22 was selected for further studies because it afforded the most sensitive assay (results not shown).

Two-Step Conjugate-Coated ELISAs. *Conjugate Selection.* Competitive assays were performed using all of the OVA conjugates as coating reagents at different concentrations. In general, it was observed that reducing any coating conjugate concentration to 0.01 μg/mL afforded more sensitive assays (**Table 1**). In fact, at a concentration of 1.00 μg/mL of any OVA conjugate, no competitive assays were obtained and, even at 0.1 μg/mL coating conjugate, the observed IC_{50} values remained generally high. Interestingly, the use of the lowest coating concentration (0.01 μg/mL) always resulted in sensitive assays, even though for some conjugates the required mAb concentration had to be significantly raised (800 ng/mL for OVA−KM0). In addition, at low coating concentrations, all heterologous conjugates afforded slightly lower IC_{50} values (around 16 nM) than the homologous conjugate (IC_{50} = 23.9 nM). Therefore, modest reductions of the IC_{50} values were observed in heterologous assays, which is in agreement with the commonly found variations when heterologies are used in monoclonal antibody-based immunoassays (*24, 25*). At 0.01 μg/mL coating conjugate, the lowest amount of mAb was required when OVA−KM4 and OVA−KMgab were used. Although the homologous and all

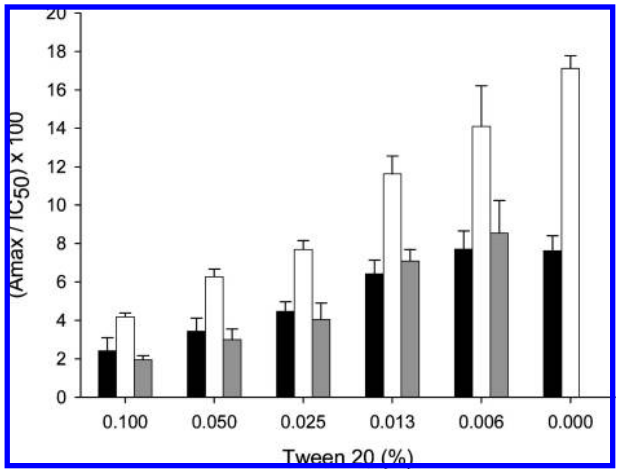

Figure 4. Results of the study on the influence of Tween 20 on the assay parameters for the two-step extended assay (black bars), the two-step rapid assay (white bars), and the one-step assay (gray bars). Results are the mean of three independent determinations.

heterologous conjugates resulted in similar and sensitive assays, OVA−KMgab was chosen for further assay optimization because it afforded a low IC_{50} value (14.3 nM) at low conjugate and antibody concentrations.

Antibody Selectivity. Competitive assays were performed to study antibody selectivity against five strobilurin fungicides: azoxystrobin, trifloxystrobin, picoxystrobin, pyraclostrobin, and dimoxystrobin. Assays were carried out with plates coated with 0.01 μg/mL OVA−KMgab, mAb KM6#22 at 200 ng/mL in PBST, and serial dilutions of each analyte in PBS. Only dimoxystrobin was also recognized by mAb KM6#22 with a CR value of 409% with respect to KM (**Figure 3**). This result is not surprising because kresoxim-methyl and dimoxystrobin have very similar chemical structures and the toxophore moiety, where the spacer arm had been introduced, contains an amide group in dimoxystrobin, which resembles the amide formed at the derivatization site upon reaction with the linker. Although the immunizing conjugate had been prepared with a derivative of KM distal to the characteristic ring of the fungicide, it seems that the toxophore moiety is part of the epitope recognized by the antibody.

Assay Optimization. Detergents are often added to the assay buffer to improve the solubility of the analyte or to decrease nonspecific binding. Tween 20 is one of the additives commonly used. Nevertheless, it has been published before that this detergent may also reduce the sensitivity of the assay (*26, 27*). Therefore, the influence of detergent concentration on the A_{max} and IC_{50} values was studied. Assays were performed using a solution of 0.01 μg/mL OVA−KMgab for plate coating. Previous to competitive assays, mAb KM6#22 concentrations were established by noncompetitive bidimensional checkerboard titration, wherein the immunoreaction was carried out during 2.0 (extended assay) or 0.5 h (rapid assay) in buffer containing 0.025% Tween 20. For the extended assay, the mAb concentration was established at 100 ng/mL and the secondary reaction with the enzyme-labeled conjugate, at a 1/2000 dilution, was allowed during 1 h. For the rapid assay, the mAb concentration was fixed at 200 ng/mL and detection with the RAM−HRP conjugate, at a 1/1000 dilution, was performed during 0.5 h. For competitive assays, mAb solutions were prepared in PBS containing decreasing concentrations of Tween 20. The A_{max}/IC_{50} ratios of the resulting curves were calculated, and these values were taken as a measure to set the maximum percentage of Tween 20 acceptable in the assay. **Figure 4** shows the dependence of the A_{max}/IC_{50} ratio for various concentrations of this detergent. From this study, it was found that Tween 20 had an important and negative influence on assay parameters. On the one hand, for the extended assay, a plateau was reached for the A_{max}/IC_{50} ratio at Tween 20 concentrations below 0.00625%. On the other hand, even the lowest Tween 20 concentration had a detrimental effect to assay parameters when the rapid assay was used, the highest A_{max}/IC_{50} ratio for the rapid assay being obtained with no detergent in the assay buffer. Low background signals were obtained for the extended and rapid assays (below 0.1 and 0.2 absorbance unit, respectively). Inhibition curves obtained under optimized immunoreagent and Tween 20 concentrations are shown in **Figure 5**, and assay conditions and parameters are summarized in **Table 2**. Lower IC_{50} values were observed with the rapid assay (5.6 nM compared to 9.7 nM for the extended assay), but an increase of mAb and RAM−HRP concentrations was required and a higher background was obtained. Under these conditions, the calculated LODs for KM were 0.4 ng/mL for the extended assay and 0.3 ng/mL for the rapid assay. Therefore, two-step indirect ELISAs with different incubation times have been developed that could be valid for different application, the most suitable assay depending on time, cost, and sensitivity requirements.

One-Step Conjugate-Coated ELISA. Conjugate-coated ELISAs are usually robust and easy to perform. In addition, compared to the antibody-immobilized format, conjugate-coated ELISAs are more tolerant to hapten heterologies for competitive assays. Nevertheless, it usually takes longer to carry out because it includes two incubation steps and an additional washing step. This drawback could be overcome by incubation of mAb, analyte solution, and labeled antimouse IgG antibody in just one step. In this respect, the optimum concentrations of mAb KM6#22 and RAM−HRP were ascertained by noncompetitive bidimensional checkerboard titration. Solutions of mAb KM6#22 and RAM−HRP in PBST were mixed in a 1:1 ratio (v/v) and immediately used. Anyhow, no difference in A_{max} or IC_{50} values was observed when the mixture was incubated for up to 30 min at room temperature before use (results not shown). Next, the influence of Tween 20 on the assay parameters of this one-step assay was also studied using 300 ng/mL final mAb concentration and 1/2000 RAM−HRP final dilution. From results depicted in **Figure 4**, it could be concluded that the lower the concentration of Tween 20, the higher the A_{max}/IC_{50} ratio. Nevertheless, at 0% Tween 20, high unspecific binding was observed and the experimental results could not be fitted to an inhibition curve

Table 2. Summary of Assay Conditions and Sensitivity

assay	time, first reaction (h)	time, second reaction (h)	mAb concn (ng/mL)	Tween 20 (%)	enzyme-labeled conjugate dilution	LOD for KM[a] (ng/mL)	IC_{50} for KM[a] (nM)
two-step extended assay	2	1	80	0.00625	1/2000	0.4	9.7
two-step rapid assay	0.5	0.5	150	0	1/1000	0.3	5.6
one-step assay	1		300	0.00625	1/2000	0.4	9.2

[a] Values are the mean of three independent determinations.

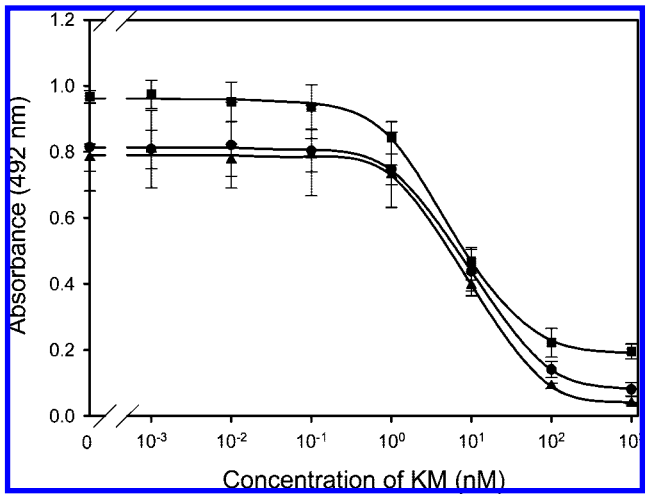

Figure 5. Inhibition curves of kresoxim-methyl for the two-step extended assay (circles), the two-step rapid assay (squares), and the one-step assay (triangles). Each point is the mean of three independent determinations.

and no A_{max}/IC_{50} ratio could be calculated. Therefore, the lowest assayed Tween 20 concentration providing a reproducible inhibition curve was chosen for further assay development. Optimized mAb, RAM−HRP, and Tween 20 concentrations are summarized in **Table 2**. The mean inhibition curve of three independent experiments is shown in **Figure 5**. An IC_{50} value of 9.2 nM was obtained when the experimental values were fitted to a sigmoidal curve from which the LOD for KM was established at 0.4 ng/mL. Thus, a third ELISA has been developed with reduced washing and incubation steps for the detection of KM in the low nanomolar range, although a higher concentration of mAb was required. Similar results were found by Moreno et al. (*28*) using the same approach.

To the best of our knowledge, this is the first monoclonal antibody to KM so far reported in the literature. The obtained immunoreagents have proved to be suitable for the development of sensitive strobilurin analytical tests. No large differences in assay sensitivity were found by the use of linker heterologies. Three conjugate-coated ELISAs have been optimized using one or two incubation steps during different incubation periods. Tween 20 is usually included as additive reagent in buffer, but our results confirm that a careful optimization of this component is advisable for proper assay performance. Pending studies include the performance of dipeptide linkers in immunizing conjugates and the evaluation of other linker, site, or structure heterologies for assay development.

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