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

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High-affinity and selective monoclonal antibodies have been produced against the strobilurin fungicide trifloxystrobin. A battery of functionalized haptens has been synthesized, and conjugate-coated enzyme-linked immunosorbent assays following different procedures have been developed. On the one hand, a two-step conjugate-coated immunoassay was optimized using extended or short incubation times, with limits of detection of 0.10 ng/mL for the extended assay and 0.17 ng/mL for the rapid assay. On the other hand, an immunoassay in the conjugate-coated format was optimized following a procedure consisting of just one incubation step. This one-step assay had a limit of detection of 0.21 ng/mL. All of these assays showed detection limits for trifloxystrobin in the low parts per billion range, well below the common maximum residue limits for this pesticide in foodstuffs (50 μg/kg).

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

INTRODUCTION

Strobilurins have been the most important lead compounds during more than a decade of intense competition between rival industrial fungicide research teams. In 1984/1985, ICI and BASF filed independently the first patent applications for synthetic derivatives of the natural fungicide strobilurin A (*1, 2*). Since then, numerous strobilurins have been described—it would be possible to encounter more than 900 patent applications—and nine strobilurin common names are ISO registered (http://www.alanwood.net/pesticides/index.htm). All of these molecules consist of three basic elements (**Figure 1**): the toxophore moiety, responsible for the fungicide activity (typically an enol ether, an oxime ether ester, or an oxime); a nucleus or bridge that helps to stabilize the molecule (in all cases a central arene subunit); and a side chain, which is necessary for optimal lipophilicity (it is a variable chemical group characteristic for each strobilurin that greatly determines the physicochemical properties of every molecule). In 2003, one of these strobilurins, trifloxystrobin (TF), was included in Annex I of the European Council Directive 91/414/EEC for its use as fungicide against foliar diseases in cereals, fruits, and vegetables. With global annual sales of about 180 million euros in 2006, trifloxystrobin ranks fifth in Bayer's best-selling active ingredients, and its use as an agrochemical has been registered in more than 80 countries worldwide. Like all strobilurins, TF inhibits mitochondrial respiration by influencing the function of the cytochrome bc_1 complex, which is located in the inner mitochondrial membrane of fungi and other eukaryotes. Although many inhibitors that bind at distinct sites on the respiration pathway are known, TF binds reversibly at a specific site on cytochrome *b*, a binding site to which no inhibitor had previously been identified. Trifloxystrobin has low toxicity to mammals, and it is unlikely to represent a serious hazard to nontarget organisms or to the environment provided that good agricultural practices are followed (*3*).

Immunochemical techniques have lately gained a position as alternative and/or complementary methods for the analysis of contaminants because of their ease to be adapted to specific analytical requirements for a certain application. Immunoassays may be developed in different formats and become portable and user-friendly (*4, 5*), and also they may be automated, integrated in biosensor devices, and used for multianalyte detection (*6*–*9*). Furthermore, the combination of antibodies and the accurate data treatment permit the development of immunoassays applied to the detection of a group of closely related antigens (*10*), couples of cross-reacting analytes (*11, 12*), or degradation products (*13*). However, to keep advancing the technique there is a need for antibodies and conjugates to novel analytes, because it is the availability of such immunoreagents that is the rate-limiting step. So far, we have obtained antibodies against two strobilurin fungicides. In a previous paper (*14*), we have

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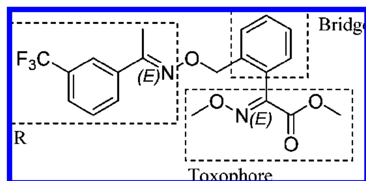

Figure 1. Basic chemical regions of synthetic strobilurin fungicides. R is the side chain characteristic of this strobilurin, trifloxystrobin. The central bridge subunit and the methyl methoxyimino acetate toxophore are also encircled.

described the development of monoclonal antibody-based immunoassays for the detection of kresoxim-methyl. Previously, Furzer et al. (*15*) had published the development and comparison of diagnostic immunoassays for the detection of azoxystrobin using polyclonal antibodies. Now, we describe the synthesis of functionalized haptens of TF, containing linker heterologies, and the production of high-affinity monoclonal antibodies (mAbs) against this fungicide. Three enzyme-linked immunosorbent assays (ELISAs) with different procedures and operation times for TF detection have been optimized and characterized.

MATERIALS AND METHODS

Chemicals and Instrumentation. Trifloxystrobin [methyl (*E*)-methoxyimino-{(*E*)-α-[1-α-(α,α,α-trifluoro-*m*-tolyl)ethylideneaminooxyl]-*o*-tolyl}acetate] (CAS Registry No. 141517-21-7; MW 408.13) standard was kindly provided by Bayer CropScience (Frankfurt, Germany). Kresoxim-methyl, dimoxystrobin, and pyraclostrobin, Pestanal grade, were purchased from Riedel-de-Haën (Seelze, Germany). Azoxystrobin and picoxystrobin standards were obtained from Syngenta (Basel, Switzerland). Stock solutions of these compounds were prepared in dried *N*,*N*-dimethylformamide (DMF) and stored at −20 °C. *N*-Glycylglycine (gg) was obtained from Alfa Aesar (Karlsruhe, Germany), and *N*-*β*-alanylglycine (*β*ag) and *N*-glycyl-*γ*-aminobutyric acid (gab) were from Bachem AG (Bubendorf, Switzerland). *N*,*N*-Dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (NHS), 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 anti-mouse immunoglobulin 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 alanylglutamine 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 (UV−vis) 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 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.

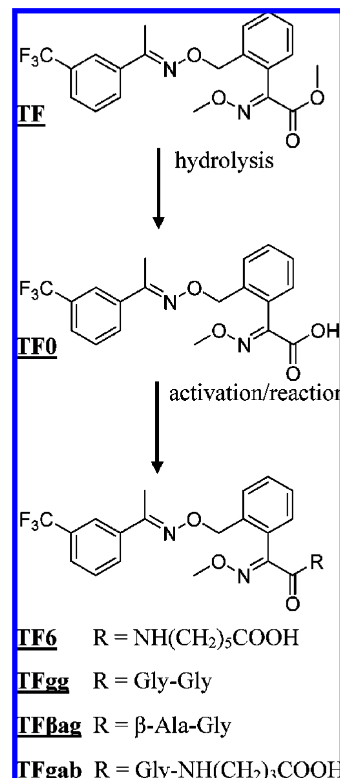

Figure 2. Scheme of the chemical synthesis of the hapten used in the immunizing conjugate (TF0) and the different derivatives obtained from that hapten by using several spacer arms for protein conjugation.

Hapten Synthesis. All TF derivatized haptens that were prepared in this study are summarized in **Figure 2**. Immunizing hapten TF0 was synthesized by alkaline hydrolysis of the TF methyl ester. All other haptens were synthesized from TF0 by activation of the carboxylic acid with DCC and NHS and reaction of the activated ester with either 6-aminohexanoic acid or a dipeptide (*N*-glycylglycine, *N*-*β*-alanylglycine, 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-(((*E*)-1-(3-(trifluoromethyl)phenyl)-ethylideneaminooxy)methyl)phenyl)acetic Acid (TF0). Five grams of TF (12.24 mmol), methanol (70 mL), and 5 M aqueous sodium hydroxide (12.5 mL) were stirred at reflux. After the hydrolysis was complete, as monitored by TLC (approximately 5 h), the reaction mixture was neutralized with 5 M HCl aqueous solution and the methanol evaporated off. 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 4.48 g of a clear liquid, which was further purified by recrystallization from hexane/acetone (95:5) to obtain 3.52 g of the acid derivative as white crystals (72.9%). The preparation of this acid derivative has been described in the literature (*16*), but no physical or

spectroscopic data have been previously given for it. A detailed description of these data follows: mp 102−103 °C (from slow evaporation of a $CHCl_3$/hexane solution); ^{1}H NMR (DMSO-d_6), δ13.29 (1H, br s, COOH), 7.91 (1H, br d J = 7.5 Hz, H-6 $PhCF_3$), 7.90 (1H, br s, H-2 $PhCF_3$), 7.73 (1H, br d, J = 7.5 Hz, H-4 $PhCF_3$), 7.63 (1H, br t, J = 7.5 Hz, H-5 $PhCF_3$), 7.47 (1H, br dd, J = 7, 2 Hz, H-6 Ph), 7.42 (1H, ddd, J = 7, 7, 1.5 Hz, H-5 Ph), 7.37 (1H, ddd, J = 7, 7, 2 Hz, H-4 Ph), 7.18 (1H, dd, J = 7, 1.5 Hz, H-3 Ph), 5.07 (2H, s, CH_2O), 3.89 (3H, s, OCH_3), 2.20 (3H, s, CH_3); ^{13}C NMR (DMSO-d_6), δ 163.73 (CO), 153.59 (C═N), 149.87 (C-2), 136.77 (C-1 $PhCF_3$), 135.39 (C-2 Ph), 130.76 (C-1 Ph), 129.89 (C-6 $PhCF_3$), 129.63 (C-5 $PhCF_3$), 129.21 (q, J = 32 Hz, C-3 $PhCF_3$), 128.76 (C-6 Ph), 128.55 and 128.52 (C-3 Ph and C-5 Ph), 127.61 (C-4 Ph), 125.70 (q, J = 3.5 Hz, C-4 $PhCF_3$), 123.98 (q, J = 272 Hz, CF_3), 122.12 (q, J = 3.5 Hz, C-2 $PhCF_3$), 74.38 (CH_2O), 62.92 (CH_3O), 12.22 (CH_3); IR (KBr), 3420, 3066, 2941, 2880, 1705, 1340, 1294, 1281, 1118, 1072, 1046, 1005, 728, 697 cm^{-1}; MS (EI), m/z 363 ($M^+ - OCH_3$, 0.5), 319 (1), 317 (7), 301 (4), 192 (3), 172 (3), 151 (2), 117 (11), 116 (100); HRMS, calcd for $C_{18}H_{14}F_3N_2O_3$ ($M^+ - OCH_3$) 363.09565, found 363.09614; UV (100 mM sodium phosphate buffer, pH 7.4), ϵ (280 nm) = 2.69 mM^{-1} cm^{-1}, ϵ (260 nm) = 10.20 mM^{-1} cm^{-1}.

General Procedure for Spacer Arm Introduction. The carboxylic acid (TF0) (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% 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.

6-((E)-2-(Methoxyimino)-2-(2-(((E)-1-(3-(trifluoromethyl)phenyl)-ethylideneaminooxy)methyl)phenyl)acetamido)hexanoic Acid (TF6). Purification of the crude coupling product by column chromatography, eluting with $CHCl_3$ and then $CHCl_3$/CH_3OH 99:1, afforded 1.2 g of pure TF6 from 2.0 g of TF0 (47% yield). A sample was further purified by semipreparative HPLC to obtain 400 mg of the product as a white solid: mp 149−150 °C (from $CHCl_3$/hexane); ^{1}H NMR (DMSO-d_6), δ 12.46 (1H, br s, COOH), 8.24 (2H, t, J = 5.8 Hz, 2 × NH), 7.91 (1H, br d J = 7.5 Hz, H-6 $PhCF_3$), 7.90 (1H, br s, H-2 $PhCF_3$), 7.74 (1H, br d, J = 7.5 Hz, H-4 $PhCF_3$), 7.63 (1H, br t, J = 7.5 Hz, H-5 $PhCF_3$), 7.46 (1H, br dd, J = 7.5, 1.5 Hz, H-6 Ph), 7.40 (1H, ddd, J = 7.5, 7.5, 1.5 Hz, H-5 Ph), 7.35 (1H, ddd, J = 7.5, 7.5, 1.5 Hz, H-4 Ph), 7.14 (1H, dd, J = 7.5, 1.5 Hz, H-3 Ph), 5.06 (2H, s, CH_2O), 3.88 (3H, s, OCH_3), 3.73 (2H, d J = 5.8 Hz, H-11), 3.37 (2H, overlapped with water signal from DMSO-d_6, H-7), 2.39 (2H, t, J = 7.2 Hz, H-8), 2.21 (3H, s, CH_3); ^{13}C NMR (DMSO-d_6), δ 171.20 (C-12), 170.73 (C-9), 161.79 (C-5), 153.49 (C═N), 151.18 (C-4), 136.73 (C-1 $PhCF_3$), 135.71 (C-2 Ph), 130.05 (C-1 Ph), 129.81 (C-6 $PhCF_3$), 129.54 (C-5 $PhCF_3$), 129.19 (q, J = 32 Hz, C-3 $PhCF_3$), 128.69 (C-6 Ph), 128.57 (C-3 Ph), 128.10 (C-5 Ph), 127.25 (C-4 Ph), 125.55 (q, J = 3.5 Hz, C-4 $PhCF_3$), 123.99 (q, J = 272 Hz, CF_3), 122.07 (q, J = 3.5 Hz, C-2 $PhCF_3$), 74.12 (CH_2O), 62.58 (CH_3O), 40.50 (C-11), 35.48 (C-7), 34.49 (C-8), 12.20 (CH_3); IR (KBr), 3358, 2945, 2850, 2820, 1749, 1675, 1605, 1560, 1529, 1345, 1224, 1122, 1034, 998 cm^{-1}; MS (EI), m/z (%) 508 (3), 507 (1), 323 (4), 322 (26), 321 (80), 306 (40), 305 (90), 186 (63), 116 (100); HRMS (FAB), calcd for $C_{25}H_{29}F_3N_3O_5$ ($M^+ + 1$) 508.20593, found 508.20725; UV (100 mM sodium phosphate buffer, pH 7.4), ϵ (280 nm) = 4.09 mM^{-1} cm^{-1}, ϵ (260 nm) = 13.90 mM^{-1} cm^{-1}.

(E)-5,9-Dioxo-4-(2-(((E)-1-(3-(trifluoromethyl)phenyl)ethylide-neaminooxy)methyl)phenyl)- 2-oxa-3,6,10-triazadodec-3-en-12-oic Acid (TF*β*ag). Purification of the crude coupling product by column chromatography, eluting with $CHCl_3$ and then $CHCl_3$/CH_3OH 99:1, afforded 66 mg of pure TF*β*ag from 104 mg of TF0 (48% yield) as a white solid: mp 149−150 °C (from $CHCl_3$/hexane); ^{1}H NMR (DMSO-d_6), δ 12.46 (1H, br s, COOH), 8.24 (2H, t, J = 5.8 Hz, 2 × NH), 7.91 (1H, br d J = 7.5 Hz, H-6 $PhCF_3$), 7.90 (1H, br s, H-2 $PhCF_3$), 7.74 (1H, br d, J = 7.5 Hz, H-4 $PhCF_3$), 7.63 (1H, br t, J = 7.5 Hz, H-5 $PhCF_3$), 7.46 (1H, br dd, J = 7.5, 1.5 Hz, H-6 Ph), 7.40 (1H, ddd, J = 7.5, 7.5, 1.5 Hz, H-5 Ph), 7.35 (1H, ddd, J = 7.5, 7.5, 1.5 Hz, H-4 Ph), 7.14 (1H, dd, J = 7.5, 1.5 Hz, H-3 Ph), 5.06 (2H, s, CH_2O), 3.88 (3H, s, OCH_3), 3.73 (2H, d J = 5.8 Hz, H-11), 3.37 (2H, overlapped with water signal from DMSO-d_6, H-7), 2.39 (2H, t, J = 7.2 Hz, H-8), 2.21 (3H, s, CH_3); ^{13}C NMR (DMSO-d_6), δ 171.20 (C-12), 170.73 (C-9), 161.79 (C-5), 153.49 (C═N), 151.18 (C-4), 136.73 (C-1 $PhCF_3$), 135.71 (C-2 Ph), 130.05 (C-1 Ph), 129.81 (C-6 $PhCF_3$), 129.54 (C-5 $PhCF_3$), 129.19 (q, J = 32 Hz, C-3 $PhCF_3$), 128.69 (C-6 Ph), 128.57 (C-3 Ph), 128.10 (C-5 Ph), 127.25 (C-4 Ph), 125.55 (q, J = 3.5 Hz, C-4 $PhCF_3$), 123.99 (q, J = 272 Hz, CF_3), 122.07 (q, J = 3.5 Hz, C-2 $PhCF_3$), 74.12 (CH_2O), 62.58 (CH_3O), 40.50 (C-11), 35.48 (C-7), 34.49 (C-8), 12.20 (CH_3); IR (KBr), 3358, 2945, 2850, 2820, 1749, 1675, 1605, 1560, 1529, 1345, 1224, 1122, 1034, 998 cm^{-1}; MS (EI), m/z (%) 290 (1), 288 (1), 274 (8), 261 (12), 234 (3), 203 (7), 187 (24), 172 (50), 145 (25), 116 (100); HRMS (FAB), calcd for $C_{24}H_{26}F_3N_4O_6$ 523.180445 ($M^+ + H$), found 523.17881; UV (100 mM sodium phosphate buffer, pH 7.4), ϵ (280 nm) = 2.85 mM^{-1} cm^{-1}, ϵ (260 nm) = 10.89 mM^{-1} cm^{-1}.

(E)-5,8-Dioxo-4-(2-(((E)-1-(3-(trifluoromethyl)phenyl)ethylide-neaminooxy)methyl)phenyl)- 2-oxa-3,6,9-triazaundec-3-en-11-oic acid (TFgg). Purification of the crude coupling product by column chromatography, eluting with $CHCl_3$ and then $CHCl_3$/CH_3OH 99:1, afforded 103 mg of pure TFgg from 100 mg of TF0 (80%) as a white solid: mp 168−170 °C (from $CHCl_3$/hexane); ^{1}H NMR (DMSO-d_6), δ 8.32 (1H, t, J = 5.6 Hz, C_5−NH), 8.13 (1H, t, J = 5.5 Hz, C_8−NH), 7.91 (1H, br d, J = 7.5 Hz, H-6 $PhCF_3$), 7.90 (1H, br s, H-2 $PhCF_3$), 7.75 (1H, br d, J = 7.5 Hz, H-4 $PhCF_3$), 7.63 (1H, br t, J = 7.5 Hz, H-5 $PhCF_3$), 7.47 (1H, br dd, J = 7.5, 1.5 Hz, H-6 Ph), 7.41 (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, 1.5 Hz, H-3 Ph), 5.08 (2H, s, CH_2O), 3.91 (3H, s, OCH_3), 3.83 (2H, d, J = 5.6 Hz, H-7), 3.72 (2H, d, J = 5.5 Hz, H-10), 2.20 (3H, s, CH_3); ^{13}C NMR (DMSO-d_6), δ 171.09 (C-11), 168.32 (C-8), 162.01 (C-5), 153.57 (C═N), 150.84 (C-4), 136.78 (C-1 $PhCF_3$), 135.83 (C-2 Ph), 129.90, (C-1 Ph), 129.57 (C-6 $PhCF_3$), 129.15 (q, J = 32 Hz, C-3 $PhCF_3$), 128.78 (C-6 Ph), 128.64 (C-3 Ph), 128.12 (C-5 Ph), 127.28 (C-4 Ph), 125.62 (q, J = 3.5 Hz, C-4 $PhCF_3$), 123.96 (q, J = 272 Hz, CF_3), 122.09 (q, J = 3.5 Hz, C-2 $PhCF_3$), 74.10 (CH_2O), 62.72 (CH_3O), 42.01 (C-7), 41.11 (C-10), 12.21 (CH_3); IR (KBr), 3379, 3289, 3072, 2935, 2881, 2597, 2467, 1720, 1664, 1515, 1255, 1125, 751, 693 cm^{-1}; MS (EI), m/z (%) 378 (0.5), 332 (2), 318 (12), 317 (12), 305 (13), 304 (29), 286 (12), 260 (11), 304 (23), 247 (9), 172 (65), 145 (29), 116 (100); HRMS (FAB), calcd for $C_{23}H_{24}F_3N_4O_6$ ($M^+ + H$) 509.16479, found 509.16542; UV (100 mM sodium phosphate buffer, pH 7.4), ϵ (280 nm) = 2.71 mM^{-1} cm^{-1}, ϵ (260 nm) = 9.59 mM^{-1} cm^{-1}.

(E)-5,8-Dioxo-4-(2-(((E)-1-(3-(trifluoromethyl)phenyl)ethylide-neaminooxy)methyl)phenyl)- 2-oxa-3,6,9-triazatridec-3-en-13-oic Acid (TFgab). Purification of the crude coupling product by column chromatography, eluting with $CHCl_3$ and then $CHCl_3$/CH_3OH 99:1, afforded 62 mg of pure TFgab from 99 mg of TF0 (46%) as a white solid: mp 133−134 °C (from $CHCl_3$/hexane); ^{1}H NMR (DMSO-d_6), δ 12.04 (1H, br s, COOH), 8.22 (2H, t, J = 5.7 Hz, NH), 7.95 (2H, t, J = 5.6 Hz, NH), 7.90 (1H, br d, J = 7.5 Hz, H-6 $PhCF_3$), 7.89 (1H, br s, H-2 $PhCF_3$), 7.74 (1H, br d, J = 7.5 Hz, H-4 $PhCF_3$), 7.62 (1H, br t, J = 7.5 Hz, H-5 $PhCF_3$), 7.47 (1H, br dd, J = 7.5, 1.5 Hz, H-6 Ph), 7.41 (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, 1.5 Hz, H-3 Ph), 5.07 (2H, s, CH_2O), 3.92 (3H, s, OCH_3), 3.76 (2H, d, J = 5.7 Hz, H-7), 3.08 (2H, q, J = 6.7 Hz, H-10), 2.20 (2H, t, J = 7.5 Hz, H-12), 1.62 (2H, quint, J = 7.1 Hz, H-11), 2.20 (3H, s, CH_3); ^{13}C NMR (DMSO-d_6), δ 174.21 (C-13), 168.00 (C-8), 161.94 (C-5), 153.55 (C═N), 150.85 (C-4), 136.76 (C-1 $PhCF_3$), 135.79 (C-2 Ph), 129.91, (C-1 Ph), 129.87 (C-6 $PhCF_3$), 129.56 (C-5 $PhCF_3$), 129.15 (q, J = 32 Hz, C-3 $PhCF_3$), 128.77 (C-6 Ph), 128.66 (C-3 Ph), 128.13 (C-5 Ph), 127.28 (C-4 Ph), 125.63 (q, J = 3.5 Hz, C-4 $PhCF_3$), 123.50 (q, J = 272 Hz, CF_3), 122.08 (q, J = 3.5 Hz, C-2 $PhCF_3$), 74.11 (CH_2O), 62.71 (CH_3O), 42.08 (C-7), 37.92 (C-10), 31.14 (C-12), 24.52 (C-11), 12.20 (CH_3); IR (KBr),

3305, 3146, 2945, 2879, 1740, 1692, 1650, 1419, 1565, 1419, 1338, 1172, 1121, 1072, 1035, 934, 797, 695 cm^{-1}; MS (EI), m/z (%) 537 ($M^+ + 1$, 0.5), 536 (M^+, 1.5), 505 (0.5), 434 (0.5), 360 (1), 334 (47), 333 (19), 332 (100), 316 (8), 314 (7), 249 (3), 219 (4), 203 (9), 187 (13), 186 (35), 174 (8), 173 (11), 172 (20), 145 (23), 116 (70); HRMS, calcd for $C_{25}H_{27}F_3N_4O_6$ 536.18827, found 536.18960; UV (100 mM sodium phosphate buffer, pH 7.4), ϵ (280 nm) = 2.76 mM^{-1} cm^{-1}, ϵ (260 nm) = 10.58 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 immunizing conjugate and OVA for the coating conjugates.

Immunizing Conjugate. Typically, 25 μmol of TF0 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 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 approximately 40:1. Finally, the conjugate was separated from uncoupled hapten by gel filtration on Sephadex G-25, using 100 mM phosphate buffer, pH 7.4, as eluant. The degree of hapten to protein conjugation 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 absorptions of the hapten 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 immunizing conjugate. The purified conjugate was stored at −20 °C.

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−TF0 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 and 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) and containing 10% FBS. Just before spleen extraction, mouse blood was collected by heart puncture and serum diluted 1/50 with PBS containing 0.01% 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 per 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% 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 TF 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. Immunoglobulins 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 per well of OVA−conjugate solution (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. Coated plates were washed four times with washing solution (0.15 M NaCl containing 0.05% Tween 20) and then received 50 μL per well of analyte in PBS plus 50 μL per well of hybridoma supernatant or purified antibody solution in PBS containing 0.05% Tween 20 (PBST), unless otherwise stated. All samples were run in duplicate wells. The immunological reaction took place (typically 2 h) at room temperature, and plates were washed again as described. Next, 100 μL per 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 addition of 100 μL per well of freshly prepared 2 mg/mL OPD and 0.012% 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.

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

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 defined as the concentration of TF that provided a 10% reduction of A_{max}. Cross-reactivity (CR) values were calculated according to the following formula:

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

Competitive One-Step Conjugate-Coated ELISA. For this assay only OVA−TFgab was used as coating conjugate. The general 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 were added. The reaction was allowed to happen for 1 h at room temperature and, after washing, retained peroxidase activity was directly 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 the SigmaPlot software package from SPSS Inc. (Chicago, IL).

RESULTS AND DISCUSSION

Hapten Synthesis and Protein Conjugates. *Immunizing Conjugate.* Animal immunization and immunoassay development for detection of small organic molecules require the coupling of the target compound to a carrier protein. The most common coupling chemistries involve reaction of amine, thiol, or carboxylate groups with amino acid or carbohydrate residues of the protein. A step prior to protein conjugation of nonimmunogenic organic molecules, so-called haptens, is usually the synthesis of derivatives with chemically reactive groups. A suitable immunization derivative should preserve the structure of the target compound as much as possible with regard to its geometry, electronic distribution, and polarity. TF is a strobilurin fungicide containing a methyl methoxyiminoacetate group as toxophore and a distinctive side chain with a trifluorotolyl group (**Figure 1**). The most straightforward strategy to prepare a derivative of this pesticide was to hydrolyze the methyl acetate group to get the free carboxylic acid. Following this approach, the synthesis of hapten TF0 was undertaken (**Figure 2**), which was easily obtained with a 73% yield. This hapten preserves the specific moieties of TF, and a minimum modification is produced at a distal point of the specific chemical group of the pesticide. The resulting acid was directly coupled to BSA using the carbodiimide method, and it was used as immunogen. The calculated final hapten to protein molar ratio of the BSA−TF0 conjugate was 30, which indicated a strong modification of the protein. Although the use of a spacer arm is believed to favor the proper display of the hapten to the immune system, the described strategy is fast and easy to perform. Many authors have successfully obtained polyclonal and monoclonal antibodies also using hapten conjugates without a spacer arm as immunogen (*18*−*23*). Moreover, Furzer et al. (*15*) have recently published the development of high-affinity polyclonal antibodies against azoxystrobin, a strobilurin fungicide, using a conjugate with no linker. Strobilurins are rather large haptens, so the question arises whether the introduction of a bridge is really necessary and to what extent this requirement depends on the size or chemical complexity of the molecule.

Coating Conjugates. Polyclonal and monoclonal antibody-based ELISAs derive great benefit from utilizing heterologous competitors to improve the assay sensitivity either by selecting coating haptens for which the antibody has a lower affinity than to the analyte or by using conjugates that better project the hapten in the aqueous phase (*24, 25*). With this idea in mind, a set of TF derivatives containing spacer arms of similar length but different composition (**Table 1**) was synthesized from TF0. These synthesis reactions were performed in a two-step mode as depicted in **Figure 2**. Such a strategy had also been used for the synthesis of derivatives of kresoxim-methyl, another methoxyiminoacetate strobilurin, as described in a previous paper (*14*). On the one side, a linear hydrocarbonated spacer was employed to obtain TF6 with a 47% yield. TF6 contains a 6-carbon-atom long hydrophobic linear linker typically used in immunoassays for small molecules. It was synthesized using 6-aminohexanoic acid, so an amide group is formed instead of the ester present in the toxophore moiety of TF. Moreover, TF6 brings in a linker-length heterology with respect to the hapten in the immunogen. On the other side, three different dipeptides were employed as linkers with yields of around 50% for TFβag and TFgab and 80% for TFgg. These three haptens also contain an amide at the toxophore as TF6 but now, a certain polarity is introduced by the internal amide group of the dipeptides. We supposed that the higher polarity of the dipeptides could facilitate the display of the hapten in the aqueous medium, leading to enhancement of the signal of the assay and consequently improving the sensitivity by allowing a decrease in the amount of required antibody. This sort of spacer has been shown to improve the sensitivity of polarization fluoroimmunoassays (*26*), and they have also been used to enhance the production of high-affinity antibodies or antibodies with certain catalytic properties (*27, 28*). All five synthesized haptens (TF0, TF6, TFgg, TFβag, and TFgab) were activated with a chloroformate reagent and conjugated to OVA. In this case, the obtained hapten-to-protein molar ratios were intentionally lower than those of the BSA conjugates, and they ranged between 3 and 8.

Table 1. Results of Competitive Assays for TF Using mAb TF0#17 and Different Coating Conjugates

coating OVA conjugate	conjugate concn (μg/mL)	mAb concn[a] (ng/mL)	IC_{50} for TF[b] (nM)
TF0 (0 Å)[c]	1.00	15	3.09
	0.10	100	2.67
	0.01	—[d]	—
TF6 (10.6 Å)	1.00	15	8.41
	0.10	100	7.40
	0.01	2500	34.39[e]
TFgg (8.9 Å)	1.00	15	4.06
	0.10	40	1.86
	0.01	400	5.75
TFβag (10.4 Å)	1.00	20	11.25[e]
	0.10	20	1.65
	0.01	300	5.56
TFgab (11.9 Å)	1.00	15	3.23
	0.10	30	1.45
	0.01	400	6.27

[a] Values determined by noncompetitive bidimensional checkerboard titration and affording a maximum absorbance 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] Not determined. [e] $n = 2$.

Production of Monoclonal Antibodies. Three mice were immunized with the BSA−TF0 conjugate following standard protocols. Mice immunization was verified by noncompetitive and competitive assays with mice sera collected after the third conjugate injection (results not shown). Two cell fusions were performed with the lymphocytes obtained from the spleen of immunized mice, and mAb-producing hybridomas were screened by simultaneous noncompetitive and competitive assays as described above. Although cell fusion efficiencies were normal (about 75% of the wells contained growing hybridomas) and the number of positive wells was not unusually low (50 wells showed an absorbance over 0.5), only a low number of them significantly recognized TF in solution. The question whether this limited number of high-affinity antibodies resulted from the absence of a bridge in the immunizing conjugate could be the subject of further studies. Finally, four hybridomas were cloned and expanded, and the mAbs were purified from culture supernatants and characterized. All four antibodies were of the IgG_1 isotype with κ light chains. Noncompetitive and competitive assays were carried out with the homologous conjugate at 1 μg/mL. The reaction between the antibody and the im-

mobilized conjugate was performed during 2 h, and the detection step with the enzyme-labeled conjugate took place during 1 h. From these results, mAb TF0#17 was selected for further studies because it afforded an IC_{50} value for TF in the low nanomolar range (results not shown).

Two-Step Conjugate-Coated ELISAs. *Conjugate Selection.* All five OVA conjugates were evaluated for assay performance using mAb TF0#17. Plates were coated with 1.00, 0.10, and 0.01 μg/mL conjugate in coating buffer. The appropriate mAb concentration in PBST (giving an A_{max} value of around 1.0) was determined for each coating conjugate and for each conjugate concentration by noncompetitive assays. Assay affinity for TF was calculated from competitive assays using serial dilutions of the analyte in PBS. Competitive reactions were run during 2 h at room temperature, and the secondary reaction with the enzyme-labeled conjugate was performed during 1 h also at room temperature. It could be observed that all conjugates, at any of the concentrations tested, rendered competitive assays with high sensitivities (**Table 1**); all of the calculated IC_{50} values were in the low nanomolar range. It was also found that the lowest IC_{50} values were obtained when the coating conjugate was used at 0.10 μg/mL, especially for haptens bearing dipeptide spacer arms. Assays with even lower coating conjugate concentrations (0.01 μg/mL) required too high mAb concentrations, which resulted in lower assay sensitivities. Moreover, the use of a long hydrocarbonated spacer (OVA−TF6 compared to OVA−TF0) reduced slightly the sensitivity of the assay. However, the use of a more hydrophilic spacer afforded around 4 times more sensitive assays if linkers of the same length are compared (OVA−TF6 compared to OVA−TFβag). In general, modest reductions of the IC_{50} values were observed with conjugates using dipeptides, which is in agreement with commonly found variations when heterologies are used in mAb-based assays (*29, 30*) and with the results obtained in a similar study for the development of immunoassays for kresoxim-methyl (*14*). When the conjugates with dipeptide linkers were used at 1.00 and 0.10 μg/mL, low and comparable concentrations of mAb were required to afford A_{max} values of around 1.0. Only when the concentration of these coating conjugates was reduced to 0.01 μg/mL did the amount of mAb have to be significantly raised (from 15−40 to 300−400 ng/mL). These results would fit with the hypothesis that a better display of the hapten occurs when more hydrophilic linkers are used, as observed by Nara et al. (*25*), thus improving the assay sensitivity. From this experiment, conjugate OVA−TFgab at 0.10 μg/mL was selected for further assay development. This assay showed an IC_{50} value of 1.45 nM before optimization.

Assay Optimization. Optimum mAb TF0#17 concentrations were established for each assay by noncompetitive bidimensional checkerboard titration using a 0.1 μg/mL OVA−TFgab solution for plate coating and allowing the competitive reaction to proceed for 2.0 h (extended assay) or 0.5 h (rapid assay). Selected antibody concentrations were 30 and 100 ng/mL for the extended and rapid assays, respectively. The secondary reaction with the peroxidase conjugate was allowed during 1.0 h at a 1/2000 dilution for the extended assays and during 0.5 h at a 1/1000 dilution for the rapid ones. Other assay conditions were as described under Materials and Methods. It has been observed before that the detergent Tween 20 may have a detrimental influence on assay sensitivity (*31, 32*). For this reason, competitive assays with different amounts of this additive were carried out, and the A_{max}/IC_{50} ratios of the resulting curves were taken as a measure to set the maximum percentage of Tween 20 acceptable in the assay. As seen in **Figure 3**, the best A_{max}/IC_{50} ratio for the extended assay was obtained when no detergent at all was used. Moreover, Tween 20 could be eliminated without increasing the background signal, which was below 10% of the A_{max} value (result not shown). If no Tween 20 was employed, the assay IC_{50} value for TF was 1.00 nM with a calculated LOD of 0.10 ng/mL (mean of three independent experiments). No influence was observed on the slope of the curve when this assay was run without Tween 20 (−1.48 compared to −1.51 when 0.025% Tween 20 was used). **Figure 4** shows the inhibition curve of TF for the extended assay under these conditions.

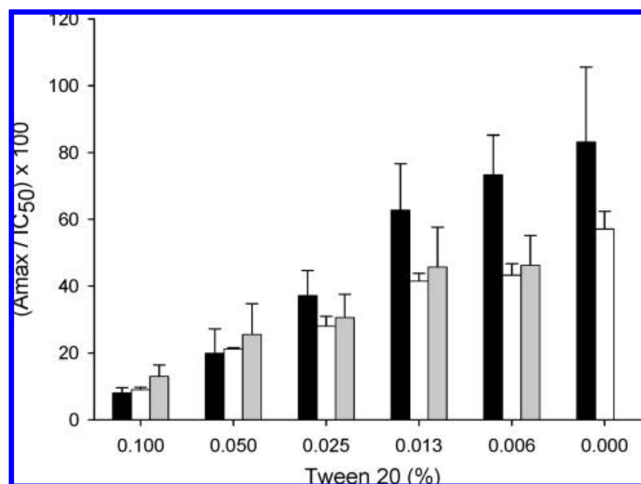

Figure 3. Results of the study on the influence of Tween 20 on the assay parameters for the two-step extended assay (black bars), the two-step rapid assay (white bars), and the one-step assay (gray bars). Results are the mean of three independent determinations.

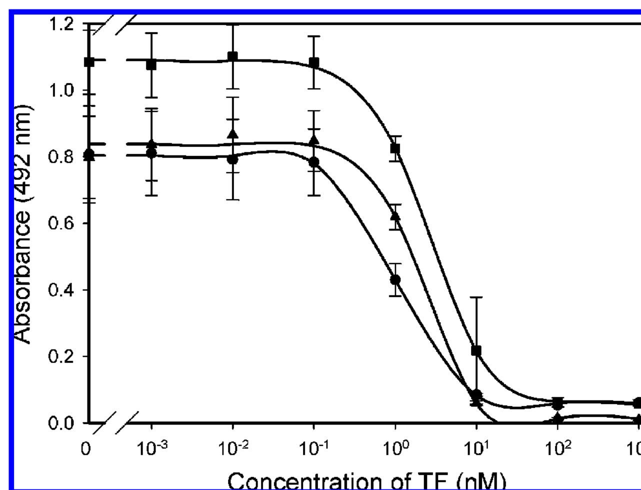

Figure 4. Inhibition curves of trifloxystrobin for the two-step extended assay (circles), the two-step rapid assay (squares), and the one-step assay (triangles). Each point is the mean of three independent determinations.

Concerning the rapid assay, the highest A_{max}/IC_{50} ratio was also found in the absence of Tween 20 (**Figure 3**). However, in this case the background signal was very high (around 40% of the A_{max} value), probably due to the higher mAb concentration required in this assay. A plateau was observed at the two lowest detergent concentrations that had been assayed (0.0125 and 0.0063%). Because higher A_{max} values were obtained with lower Tween 20 concentrations, a reduction of the antibody concentration was allowed. Therefore, further competitive assays for the rapid procedure were performed using 80 ng/mL mAb and 0.0125% Tween 20 with a first incubation period of 0.5 h and a secondary reaction of 0.5 h (**Figure 4**). Under these conditions,

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 TF[a] (ng/mL)	IC_{50} for TF[a] (nM)
two-step extended assay	2	1	30	0	1/2000	0.10	1.00
two-step rapid assay	0.5	0.5	80	0.0125	1/1000	0.17	2.45
one-step assay	1		100	0.0125	1/2000	0.21	1.84

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

an IC_{50} value of 2.45 nM and a LOD of 0.17 ng/mL were calculated from three independent experiments. The average curve presented a slope of −1.21, which was lower than that of the extended assay. The background signal was in any case lower than 10% of the A_{max} value. Therefore, sensitive two-step assays have been developed for TF following two different procedures. The two-step assay showed a higher sensitivity if extended incubation times were used. Nevertheless, it has been demonstrated that shorter incubation periods are possible while keeping a similar sensitivity. Immunoassay conditions and analytical parameters for these assays are summarized in **Table 2**.

Antibody Selectivity. Competitive assays were performed under optimized conditions with the rapid two-step assay described above and using different strobilurin fungicides as competitors. No inhibition was observed when kresoxim-methyl or pyraclostrobin was used up to 1000 nM. In the case of azoxystrobin, picoxystrobin, and dimoxystrobin, calculated CRs were 3.1, 2.3, and 1.3%, respectively (mean results of three independent experiments). No simple explanation for these results could be concluded by simple comparison of the chemical structures of these compounds. Competitive assays were also run with the uncoupled synthesized haptens (TF0, TF6, TFgg, TFβag, and TFgab) in solution. Interestingly, TF0 (the hapten that was used in the immunizing conjugate) was the least recognized molecule, with an IC_{50} value of 45.51 nM. A reasonable explanation could be that the free carboxyl moiety is interfering with the binding to the antibody, because the hapten did not contain a spacer arm. Concerning heterologous haptens, TF6 was bound with a better affinity (IC_{50} = 0.27 nM) than the fungicide TF, and haptens with dipeptide linkers (TFgg, TFβag, and TFgab) showed very low IC_{50} values, between 0.03 and 0.06 nM. The same cross-reactivity pattern was found independently of the coating conjugate that was used (results not shown). In our experience, when the immunogen is prepared with a hapten containing a spacer arm, that same hapten is frequently better recognized in solution than the target analyte, probably because the linker helps to accommodate the hapten in a cave-like binding site, a topography described in a recent paper by Lee et al. (*33*). In the present study, the immunizing hapten (TF0) contained no linker but, anyhow, the free haptens with a bridge, especially with a dipeptide, were better bound than TF0 and even better than the analyte. TF0 is a rather large hapten, and it was covalently coupled to the lysine residues of the carrier protein. Indeed, the ϵ-amine group of this amino acid could be acting as a spacer arm, or it could also happen that a different binding site topography occurs, comprising other parts of the immunogen than just the hapten.

One-Step Conjugate-Coated ELISA. The conjugate-coated ELISA format is usually performed in a two-step procedure. Often, this fact is considered a drawback compared to the antibody-coated format, which consists of just one incubation step. However, the conjugate-coated ELISA format is more robust and requires lower expenses of reagents. For these reasons, we have investigated the development of a conjugate-coated ELISA following a one-step procedure for the sensitive detection of TF. In this case, mAb TF0#17 and the enzyme-labeled secondary antibody were added simultaneously to the coated wells. Therefore, the amplification of the immunological reaction provided by the secondary antibody was maintained. The optimum mAb concentration was established at 100 ng/mL by noncompetitive bidimensional checkerboard tritration. A 1/2000 dilution of RAM−HRP was employed in this case. The mAb and the enzyme conjugate were mixed at a 1:1 ratio (v/v) just before use. Anyhow, no difference in A_{max} or IC_{50} parameters was observed when these reagents were incubated at room temperature for up to 30 min after mixing (results not shown). Again, the influence of detergent Tween 20 was assessed in competitive assays using TF as competitor. Interestingly, no competition was obtained in assays without Tween 20. The same result was found in a previous study for the development of immunoassays to kresoxim-methyl (*14*). Therefore, a certain amount of Tween 20 seems to be essential for the one-step conjugate-coated assays, most probably because if no additive is included in the reaction, unspecific adsorption of the enzyme-labeled polyclonal antibody to the well occurs. In this study, it could also be observed that the highest A_{max}/IC_{50} ratio was obtained when at least 0.0125% Tween 20 was used (**Figure 3**). The mean inhibition curve of three independent experiments performed under optimized conditions is shown in **Figure 4**. Assay conditions and analytical parameters are summarized in **Table 2**. Now, a 1 h incubation step afforded an assay with an IC_{50} value of 1.84 nM and a LOD of 0.21 ng/mL for TF, and the average curve showed a slope of −1.69. Therefore, a third immunoassay has been developed for the sensitive detection of TF with reduced incubation periods and just one washing step.

To the best of our knowledge, this is the first monoclonal antibody to trifloxystrobin that has been published so far. This high-affinity antibody was obtained using BSA−TF0 as immunogen. Conjugates with dipeptide bridges afforded assays with slightly lower IC_{50} values. A similar result was found in a previous study in which a mAb to kresoxim-methyl was obtained from a mouse immunized with a conjugate prepared with a long 6-carbon-atom aliphatic spacer (*14*). The obtained immunoreagents seem to be suitable for the development of sensitive analytical tests for this strobilurin fungicide. Three conjugate-coated ELISAs have been optimized with high sensitivities following diverse procedures. It has been shown that the concentration of Tween 20, a common additive reagent, has to be carefully optimized for proper assay performance. Future studies will investigate the performance of other linkers, derivatization sites, or structure heterologies for assay development, as well as the applicability of these assays to the analysis of trifloxystrobin in fortified or real samples.

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