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Analytical Methods

Immunoassays for trifloxystrobin analysis. Part I. Rational design of regioisomeric haptens and production of monoclonal antibodies

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ABSTRACT

Trifloxystrobin is one of the main active principles belonging to the strobilurin family of crop protection compounds. In this article, the synthesis of a battery of regioisomeric functionalized derivatives of trifloxystrobin is described. The same aliphatic linear carboxylated chain was introduced as spacer arm in all of the synthesized haptens, but it was located at different positions of the parent molecule. *N*,*N*′-Disuccinimidyl carbonate was employed for hapten activation, so the resulting *N*-hydroxysuccinimyl ester could be readily purified and efficiently coupled to proteins. After immunization and hybridoma generation, a collection of 20 mouse monoclonal antibodies from different immunizing haptens was obtained. The analytical performance of these immunoreagents was evaluated in terms of affinity and selectivity with the aim to develop rapid and practical immunochemical procedures for trifloxystrobin determination.

1. Introduction

Strobilurins are a new growing class of synthetic fungicide compounds that are used for plant protection. During the last five years, the number of registered strobilurins has doubled, revealing the great commercial success of these bioactive substances. Nowadays, one of the best sold strobilurins is trifloxystrobin (Bayer, 2010). Structurally, trifloxystrobin is characterized by a methyl (*E*)-2-(methoxyimino)-2-phenylacetate toxophore group and a trifluoromethylphenyl moiety linked by a (*E*)-ethylideneaminooxymethyl bridge (Fig. 1). Out of the four possible geometric isomers of the trifloxystrobin molecule (*EE*, *EZ*, *ZE*, and *ZZ*), the *EE* isomer is by far the most effective to combat fungal diseases. Accordingly, just the *EE* isomer is used in the commercial fungicide formulations and therefore it is the only residue sought in common pesticide monitoring programs (Banerjee, Ligon, & Spiteller, 2005). Like the other members of this family of fungicides, it is classified as Q_oI inhibitor, blocking mitochondrial respiration in fungi of the four main groups of plant pathogens, *i.e.*, ascomycetes, basidiomycetes, deuteromycetes, and oomycetes (Balba, 2007; Bartlett et al., 2002). Trifloxystrobin shows low toxicity to mammals, birds, bees, terrestrial organisms, and some plants, but it is highly noxious to fishes, aquatic organisms, and algae. Microbial metabolism primarily consists in hydrolysis of the methyl ester group (Banerjee, Ligon, & Spiteller, 2006, 2007).

Immunochemical methods constitute alternative approaches for chemical residue analysis. The successful generation of specific antibodies and sensitive assays to a small organic molecule, such as a pesticide, is greatly dependent upon a proper design of immunizing and assay haptens. Conformation and electronic features of the target compound should be mimicked by the immunizing hapten, and a proper display of its main characteristic chemical moieties as part of a protein conjugate has to be maximized (Mercader, Agulló, Abad-Somovilla, & Abad-Fuentes, 2011; Shan, Lipton, Gee, & Hammock, 2003). Consequently, hapten and protein are commonly linked through a spacer arm, which must show low immunogenicity and must not interfere with antibody binding. Most commonly, a linear aliphatic chain of 3–6 carbon atoms is employed as linker (Abad, Moreno, & Montoya, 1998; Kim et al., 2003), though good results with shorter linkers have been reported (Kong, Zhang, Zhang, Gee, & Li, 2010; Suárez-Pantaleón, Mercader, Agulló, Abad-Somovilla, & Abad-Fuentes, 2008). In a previous article, antibodies to trifloxystrobin were produced from a single immunizing hapten without spacer arm (Mercader, Suárez-Pantaleón, Agulló, Abad-Somovilla, & Abad-Fuentes, 2008a). However, trifloxystrobin is a very flexible molecule that can adopt many different

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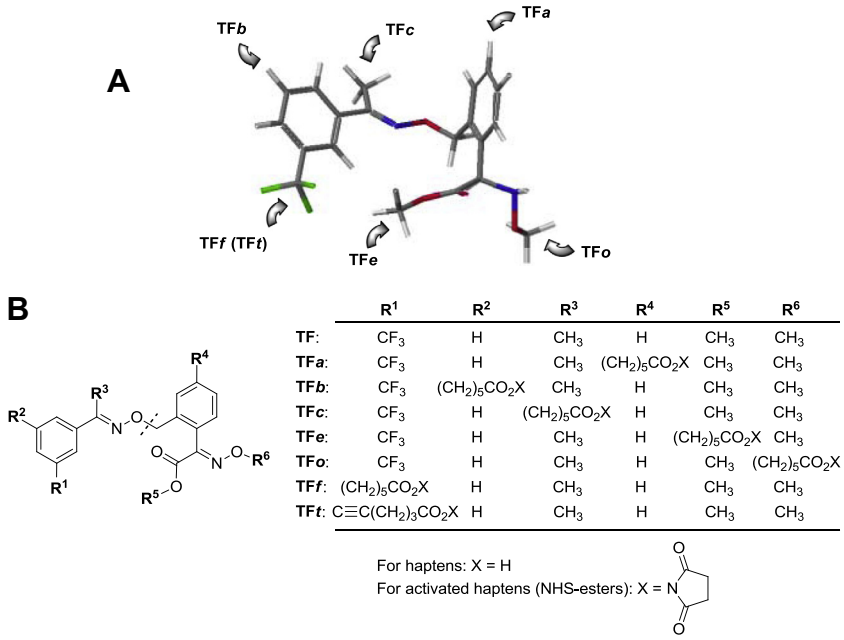


Fig. 1. (A) Global minimum energy conformation of trifloxystrobin. Calculations were performed using Molecular Mechanics (MM3) as implemented in the CAChe program. A systematic conformational search was performed (all rotatable bonds were rotated by 15 degree steps) and the geometry of the generated conformers was refined by performing an optimize geometry calculation in MOPAC using PM3 parameters. This conformer represents the 2.6% of the equilibrium conformer population. Arrows denote the spacer-arm attachment site in each of the haptens. (B) Structure of trifloxystrobin (TF), the synthesized haptens, and the corresponding *N*-succinimidyl esters (activated haptens).

energetically similar conformations – even in the crystalline solid state, it adopts two different conformations which appear mixed in the obtained X-ray structure (Ziegler, Benet-Buchholtz, Etzel, & Gayer, 2003). Due to this mobility, it is not easy to predict, not even using computer-assisted molecular modeling, which position of the molecular skeleton would be more suitable for incorporation of the spacer arm in order to prepare an immunizing conjugate with prolonged and advantageous display of the hapten to the immune system, thereby increasing the chance of producing antibodies with enhanced binding properties against the target analyte. In the present study, we report the rational design and synthesis of seven haptens with the only difference of linker positioning in the trifloxystrobin framework, thus covering a great variety of possible spatial orientations of the molecule. The performance of the prepared regioisomeric molecules as immunogens was evaluated through the generation of mouse monoclonal antibodies (mAb). The novel collection of immunoreagents was characterized in the two main competitive enzyme-linked immunosorbent assay (cELISA) formats, *i.e.*, direct and indirect, and using homologous and heterologous conjugates. Moreover, trifloxystrobin structural analogues were synthesized and used as competitors in order to map epitope molecular interactions. In the following article of this series, the best combinations of antibody and conjugate were employed for immunoassay development, and subsequently for trifloxystrobin analysis in food samples.

2. Materials and methods

2.1. Chemicals and instrumentation

Analytical grade trifloxystrobin (methyl (*E*)-methoxyimino-[(*E*)-α-[1-(α,α,α-trifluoro-*m*-tolyl)ethylideneaminooxy]-*o*-tolyl]acetate) was kindly provided by Bayer CropScience (Frankfurt, Germany). Details about reagents, solvents, synthetic procedures, and techniques used in the structural characterization of synthesized compounds are described in Supplementary Data File. Horseradish peroxidase (HRP), ovalbumin (OVA), Freund's adjuvants, and *o*-phenylenediamine were purchased from Sigma/Aldrich (Madrid, Spain). Bovine serum albumin (BSA) fraction V was from Roche Applied Science (Mannheim, Germany). Sephadex G-25 HiTrap Desalting columns for protein–hapten conjugate purification and Sepharose HiTrap Protein G HP columns for antibody purification were obtained from GE Healthcare (Uppsala, Sweden). Rabbit anti-mouse immunoglobulin polyclonal antibody conjugated to peroxidase (RAM–HRP) was bought from Dako (Glostrup, Denmark). Immunoglobulin isotype was determined using the Mouse MonoAb-ID kit from Invitrogen (Carlsbad, CA, USA). Costar flat-bottom high-binding 96-well polystyrene ELISA plates were from Corning (Corning, NY, USA). UV–visible spectra and ELISA absorbances were read with a PowerWave HT from BioTek Instruments (Winooski, VT, USA). Microwells were washed with an ELx405 microplate washer also from BioTek Instruments.

2.2. Synthesis of haptens and active esters

Seven functionalized regioisomeric haptens of trifloxystrobin with equivalent spacer arms located at different positions of the trifloxystrobin skeleton were synthesized (Fig. 1). Full experimental details and physical and spectroscopic data of all of the haptens and synthetic intermediates are provided in Supplementary Data File (Figs. S1–S6). Carboxylated haptens were activated by formation of the corresponding *N*-hydroxysuccinimidyl ester (NHS-ester) using *N*,*N*′-disuccinimidyl carbonate (DSC) and Et_3N in dry acetonitrile as previously described (Esteve-Turrillas et al., 2010). Briefly, the hapten (0.074 mmol) and DSC (25 mg, 0.096 mmol) were dissolved in anhydrous acetonitrile (730 μL) under nitrogen in an ice-water bath. Et_3N (40 μL, 0.281 mmol) was then added and the resulting mixture was stirred at room temperature until complete consumption of starting material (as observed by thin-layer chromatography). The reaction mixture was diluted with $CHCl_3$, washed with a 10% aqueous solution of $NaHCO_3$ and brine, and dried over anhydrous Na_2SO_4. The residue obtained after

evaporation of the solvent was purified by column chromatography, using $CHCl_3$ as eluent, affording the NHS-esters in good yield (60–95%) and in highly pure form, as shown by ^1H NMR spectroscopy (copies of original spectra are included in Supplementary Data File).

TF*a*-NHS ester: 86% yield. ^1H NMR (300 MHz) δ (ppm) 7.87 (1H, br s, H-2″), 7.78 (1H, d, J = 7.8 Hz, H-6″), 7.59 (1H, br d, J = 7.8 HZ, H-4″), 7.46 (1H, br t, J = 7.8 Hz, H-5″), 7.29 (1H, br s, H-2′), 7.20 (1H, dd, J = 7.8, 1.6 Hz, H-6′), 7.11 (1H, d, J = 7.8 Hz, H-5′), 5.12 (2H, s, OCH_2), 4.03 (3H, s, $NOCH_3$), 3.81 (3H, s, CO_2CH_3), 2.83 (4H, br s, $COCH_2CH_2CO$), 2.67 (2H, t, J = 7.5 Hz, H-6), 2.60 (2H, t, J = 7.4 Hz, H-2), 2.22 (3H, s, CH_3), 1.83–1.65 (4H, m, H-3 and H-5), 1.49 (2H, m, H-4).

TF*b*-NHS ester: 80% yield. ^1H NMR (300 MHz) δ (ppm) 7.67 (1H, br s, H-4′), 7.59 (1H, br s, H-2′), 7.51 (1H, dd, J = 7.6, 1.5 Hz, H-3″), 7.46–7.36 (3H, m, H-5″, H-6′ and H-4″), 7.19 (1H, dd, J = 7.4, 1.5 Hz, H-6″), 5.14 (2H, s, OCH_2), 4.03 (3H, s, $NOCH_3$), 3.82 (3H, s, CO_2CH_3), 2.83 (4H, br s, $COCH_2CH_2CO$), 2.66 (2H, t, J = 7.3 Hz, H-2), 2.62 (2H, t, J = 7.4 Hz, H-6), 2.21 (3H, s, CH_3), 1.79 and 1.67 (2H each, each m, H-3 and H-5), 1.47 (2H, m, H-4).

TF*c*-NHS ester: 60% yield. ^1H NMR (300 MHz) δ (ppm) 7.85 (1H, br s, H-2″), 7.75 (1H, d, J = 7.8 Hz, H-6″), 7.59 (1H, d, J = 7.8 Hz, H-4″), 7.51–7.35 (4H, m, H-3′, H-4′, H-5′, and H-5″), 7.18 (1H, dd, J = 7.5, 1.3 Hz, H-6′), 5.11 (2H, s, OCH_2), 4.03 (3H, s, $NOCH_3$), 3.81 (3H, s, CO_2CH_3), 2.82 (4H, br s, $COCH_2CH_2CO$), 2.75 (2H, t, J = 7.9 Hz, H-6), 2.55 (2H, t, J = 7.4 Hz, H-2), 1.73 (2H, quint, J = 7.5 Hz, H-3), 1.56–1.42 (4H, m, H-4 and H-5).

TF*e*-NHS ester: 84% yield. ^1H NMR (300 MHz) δ (ppm) 7.86 (1H, br s, H-2″), 7.80 (1H, d, J = 7.8 Hz, H-6″), 7.59 (1H, d, J = 7.8 Hz, H-4″), 7.48 (1H, m, H-6′), 7.46 (1H, m, H-5″), 7.42 (1H, m, H-5′), 7.37 (1H, m, H-4′), 7.18 (1H, dd, J = 7.3, 1.5 Hz, H-3′), 5.14 (2H, s, OCH_2), 4.21 (2H, t, J = 6.7 Hz, H-6), 4.02 (3H, s, $NOCH_3$), 2.82 (4H, br s, $COCH_2CH_2CO$), 2.53 (2H, t, J = 7.3 Hz, H-2), 2.23 (3H, s, CH_3), 1.73–1.59 (4H, m, H-3 and H-5), 1.44–1.28 (2H, m, H-4).

TF*o*-NHS ester: 95% yield. ^1H NMR (300 MHz) δ (ppm) 7.85 (1H, br s, H-2″), 7.79 (1H, d, J = 7.9 Hz, H-6″), 7.59 (1H, d, J = 7.8 Hz, H-4″), 7.48 (1H, m, H-6′), 7.46 (1H, m, H-5″), 7.42 (1H, m, H-5′), 7.37 (1H, m, H-4′), 7.18 (1H, dd, J = 7.3, 1.5 Hz, H-3′), 5.14 (2H, s, OCH_2), 4.25 (2H, t, J = 6.7 Hz, H-6), 3.81 (3H, s, CO_2CH_3), 2.82 (4H, br s, $COCH_2CH_2CO$), 2.52 (2H, t, J = 7.4 Hz, H-2), 2.22 (3H, s, CH_3), 1.68 (4H, m, H-3 and H-5), 1.38 (2H, m, H-4).

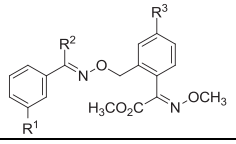
TF*f*-NHS ester: 88% yield. ^1H NMR (300 MHz) δ (ppm) 7.51 (1H, dd, J = 7.4, 1.3 Hz, H-3″), 7.45–7.34 (4H, m, H-2′, H-4′, H-4″ and H-5″), 7.25 (1H, t, J = 7.7 Hz, H-5′), 7.22–7.14 (2H, m, H-6″ and H-6′), 5.12 (2H, s, OCH_2), 4.03 (3H, s, $NOCH_3$), 3.81 (3H, s, CO_2CH_3), 2.82 (4H, br s, $COCH_2CH_2CO$), 2.65–2.58 (2H, two overlapped t, J = 7.5 Hz, H-2 and H-6), 2.20 (3H, s, CH_3), 1.83–1.61 (4H, m, H-3 and H-5), 1.50–1.41 (2H, m, H-4).

TF*t*-NHS ester: 75% yield. ^1H NMR (300 MHz) δ (ppm) 7.62 (1H, t, J = 1.5 Hz, H-2′), 7.57–7.47 (2H, m, H-6′ and H-3″), 7.46–7.33 (3H, m, H-5″, H-4″ and H-4′), 7.25 (1H, t, J = 7.8 Hz, H-5′), 7.18 (1H, dd, J = 7.4, 1.4 Hz, H-6″), 5.12 (2H, s, OCH_2), 4.03 (3H, s, $NOCH_3$), 3.82 (3H, s, CO_2CH_3), 2.84 (4H, br s, $COCH_2CH_2CO$), 2.82 (2H, t, J = 7.4 Hz, H-4), 2.58 (2H, t, J = 7.0 Hz, H-2), 2.18 (3H, s, CH_3), 2.06 (2H, quint, J = 7.2 Hz, H-3).

2.3. Synthesis of analogues

A small collection of trifloxystrobin analogues was synthesized, each one possessing a single structural modification of the trifloxystrobin molecule (Table 1). With the exception of TF*a*-I, an intermediate in the synthesis of hapten TF*a*, the rest of the analogues were prepared *ex novo* from readily available materials via the same type of strategy used for the preparation of the hapten framework, which was based on the *O*-alkylation reaction of a conveniently functionalized oxime with the appropriated bromo-benzyl derivative. A complete description of the preparation of these analogues is provided in Supplementary Data File (Fig. S7).

Table 1
Trifloxystrobin analogues.



Analogue	R^1	R^2	R^3
TF*a*-I	CF_3	CH_3	I
TF*c*-H	CF_3	H	H
TF*c*-Et	CF_3	C_2H_5	H
TF*f*-NO_2	NO_2	CH_3	H
TF*f*-H	H	CH_3	H
TF*f*-NH_2	NH_2	CH_3	H

2.4. Conjugate preparation

Conjugation was carried out with 1, 5, and 10 μmol of activated hapten (hapten–NHS ester) and HRP (2.2 mg), OVA (30 mg), and BSA (15 mg), respectively, in 50 mM carbonate buffer, pH 9.6. For details see Mercader, Esteve-Turrillas, Agulló, Abad-Somovilla, and Abad-Fuentes (2012). Protein–hapten conjugates were purified by gel filtration using 100 mM phosphate buffer, pH 7.4 as eluent, and stored frozen at −20 °C. Coupling degrees were calculated at 280 nm from absorbance values before and after conjugation.

2.5. Antibody production

Animal manipulation was carried out in compliance with Spanish laws and guidelines (RD1201/2005 and law 32/2007) and according to European Directive 2010/63EU concerning protection of animals used for scientific purposes. Each BSA conjugate was used to immunize a set of female BALB/c mice with 0.1 mg of conjugate per animal. Immunogen emulsions were prepared with BSA conjugates in sterile PBS (10 mM phosphate, pH 7.4 containing 140 mM NaCl) and Freund's adjuvant. Regular immunization schedules were followed as previously described (Parra, Mercader, Agulló, Abad-Somovilla, & Abad-Fuentes, 2012). Ten days after the third injection, a blood sample was taken by submandibular bleeding. For hybridoma generation, a standard cell fusion protocol was followed and cells were distributed in 96-well culture microplates (Mercader et al., 2008a). Selection of the best hybridomas was carried out by a double screening process (Mercader, Suárez-Pantaleón, Agulló, Abad-Somovilla, & Abad-Fuentes, 2008b). Briefly, a differential ELISA was performed with the supernatant of every microwell by parallel evaluation of a control well without competitor and a test well with 100 nM trifloxystrobin. Next, those supernatants exhibiting a clearly lower signal in the test well than in the control well were reevaluated by a checkerboard competitive screening test, in which diverse trifloxystrobin concentrations (0, 10, and 100 nM) were tested with serial dilutions of culture supernatant and different coating concentrations of homologous conjugate. Supernatants affording saturated signals in the control well were also included in this analysis. Hybridomas were cloned twice by limiting dilution. Antibodies were purified from 100 to 150 mL culture supernatants by affinity chromatography, and stored at 4 °C as ammonium sulfate precipitates.

2.6. Competitive ELISAs

Immunoassays were carried out at room temperature using two different formats: the antibody-coated direct cELISA and the

conjugate-coated indirect cELISA. Briefly, plates were coated by overnight incubation at room temperature with immunoreagent solution in 50 mM carbonate buffer, pH 9.6. For direct assays, coating was done with 100 μL per well of antibody solution, and after washing the competitive step was performed during 1 h with 50 μL per well of trifloxystrobin standard solution in PBS and 50 μL per well of enzyme tracer dilution in PBST (PBS containing 0.05% (v/v) Tween 20). Indirect competitive assays were developed in OVA conjugate-coated plates using 50 μL per well of analyte solution in PBS and 50 μL per well of primary antibody dilution in PBST. For detection, 100 μL per well of RAM–HRP (diluted 1/2000 in PBST) was used. Both, the competitive and the secondary reaction were brought to equilibrium by incubation during 1 h. Plates were washed four times after each step and signal was generated using a freshly prepared 0.012% (v/v) H_2O_2 solution in 25 mM citrate and 62 mM phosphate buffer, pH 5.4 containing 2 mg mL^{-1} *o*-phenylendiamine. The enzymatic activity was stopped after 10 min with 2.5 M sulfuric acid. Absorbance was read at 492 nm with a reference wavelength at 650 nm.

2.7. Inhibition curves and data analysis

Starting from a 10 μM trifloxystrobin solution in PBS, standard curves were prepared by 10-fold serial dilution in the same buffer. A 10 mM trifloxystrobin stock solution in anhydrous *N*,*N*-dimethylformamide was used to prepare the first standard point. Experimental values were fitted to a four-parameter logistic equation using the SigmaPlot software package from SPSS Inc. (Chicago, IL, USA). Antibody affinity was estimated as the trifloxystrobin concentration at the midpoint of the sigmoidal curve, usually refers to as IC_{50}. Cross-reactivity (CR) was calculated as percentage value from the quotient between the IC_{50} for trifloxystrobin and the IC_{50} for the studied analogous compound, both in molar concentration units.

3. Results and discussion

3.1. Preparation of haptens and active esters

A battery of regioisomeric haptens of trifloxystrobin covering the entire molecular framework was prepared (Fig. 1B). All of the haptens maintained the structure and main functional groups of the parent molecule, introducing the minimum possible conformational and electronic changes, with the linker located at a variety of positions. In all cases, the spacer arm was a C-6 saturated hydrocarbon chain, with the exception of TF*t*, which was functionalized through the same attachment site that TF*f* and was synthesized to assess whether the introduction of a triple bond in the linker may contribute, through rigidity effects, to an improved hapten exposure.

Hapten TF*e* was prepared directly from trifloxystrobin in three steps via hydrolysis of the methyl ester moiety followed by *O*-alkylation of the carboxylate group with *tert*-butyl 6-bromohexanoate and acid hydrolysis of the *tert*-butyl ester group (see Fig. S4 and experimental details in Supplementary Data file). The rest of the haptens were obtained by total synthesis from commercially available starting materials. The synthetic strategy that was followed for their preparation was based on the Williamson-type *O*-alkylation reaction of a conveniently functionalized oxime, which provided the (1-oxyiminoalkyl)phenyl substructural moiety (left-hand side of the hapten planar structure in Fig. 1B), with the appropriate benzyl bromide, which supplied the methyl 2-(alkoxyimino)-2-(*o*-tolyl)acetate subunit (right-hand side of the structure in Fig. 1B). Incorporation of the alkyl C-6 carboxylated spacer arm was accomplished either through a palladium catalyzed Sonogashira cross-coupling reaction with *tert*-butyl hex-5-ynoate and hydrogenation of the triple bond, or by means of an alkylation reaction with the corresponding *tert*-butyl bromoester. In all cases, the synthesis was completed by acid hydrolysis of the *tert*-butyl ester moiety.

As illustrated in Fig. 2A, the synthesis of haptens TF*a* and TF*o* implied the *O*-alkylation reaction of oxime **i** – prepared according to the procedure described in the literature by Neufeldt and Sanford (2010) – and a benzyl bromide such as **ii**, whose preparation was carried out from readily available tolyl derivatives following previously reported methodology (Itoh et al., 1984; Wenderoth et al., 1990). The incorporation of the C-6 carboxylated spacer arm was realized by means of an *O*-alkylation reaction of a hydroxyimino group, in the case of hapten TF*o*, and a palladium catalyzed Sonogashira cross-coupling reaction followed by hydrogenation of a triple bond, in the case of hapten TF*a*.

The synthesis of haptens TF*b* and TF*c* involved, as the key step, the *O*-alkylation reaction of an oxime such as **iii** or **iv**, respectively, with benzyl bromide **v**, which was obtained following a modification of the literature procedures from commercial 1-(*o*-tolyl)ethanone (Hwang, Kim, Kim, & Kyung, 2009; Kim, Kim, Hwang, & Nam, 2009; Wenderoth et al., 1992) (Fig. 2B). For hapten TF*b*, the preparation of the intermediate oxime involved bromination of the phenyl ring of 3-(trifluoromethyl)benzoic acid and transformation of the carboxylic group into a methyl ketone group, followed by incorporation of the carboxylated alkyl chain through a Sonogashira cross-coupling reaction, hydrogenation of the triple bond, and condensation of the carbonyl ketone group with hydroxylamine. On the other hand, the oxime that was needed for the preparation of hapten TF*c* was synthesized from 1-(3-(trifluoromethyl)phenyl)ethanone through α-alkylation to the carbonyl group with *tert*-butyl 5-bromopentanoate followed by oxime formation. The first transformation did not take place by direct alkylation of the enolate derived from the methyl ketone group, so it was necessary to use an indirect alkylation process, based on the temporal transformation of the methyl ketone group into the corresponding allyl β-keto ester derivative (Paulvannan & Chen, 1999; Snider & Buckman, 1992).

Finally, haptens TF*f* and TF*t* were synthesized through the same synthetic sequence, similar to that described above for hapten TF*b*, starting from 1-(3-bromophenyl)ethanone (Fig. 2C). After preparation of the required key oxime **vi,** *O*-alkylation with benzyl bromide **iv** and acid hydrolysis of the *tert*-butyl ester led to hapten TF*t*. On the other hand, hydrogenation of the triple bond previously to hydrolysis of the *tert*-butyl ester group afforded hapten TF*f*.

In order to allow coupling to carrier proteins, the carboxylic acid group of the haptens was activated through formation of the corresponding *N*-succinimidyl ester (see Fig. 1 for structure of the corresponding NHS-esters). This was readily performed using DSC as activating reagent. This activation procedure is highly efficient giving exclusively CO_2 and *N*-hydroxysuccinimide as the only by-products, which allowed an easy purification of the intended active esters.

3.2. Preparation of hapten–protein conjugates

The availability of the activated haptens in the purified form allowed us to readily prepare both the immunizing and the assay conjugates using the same coupling procedure and with a good control of the hapten-to-protein molar ratio (MR). A set of BSA, OVA, and HRP conjugates for every hapten was obtained with high yields. Calculated hapten-to-protein MRs were between 8 and 22, 3 and 5, and 3 and 7 for BSA, OVA, and HRP conjugates, respectively. MRs of BSA and OVA conjugates were in the usual range, whereas the unlikely estimated values that were observed with some of the

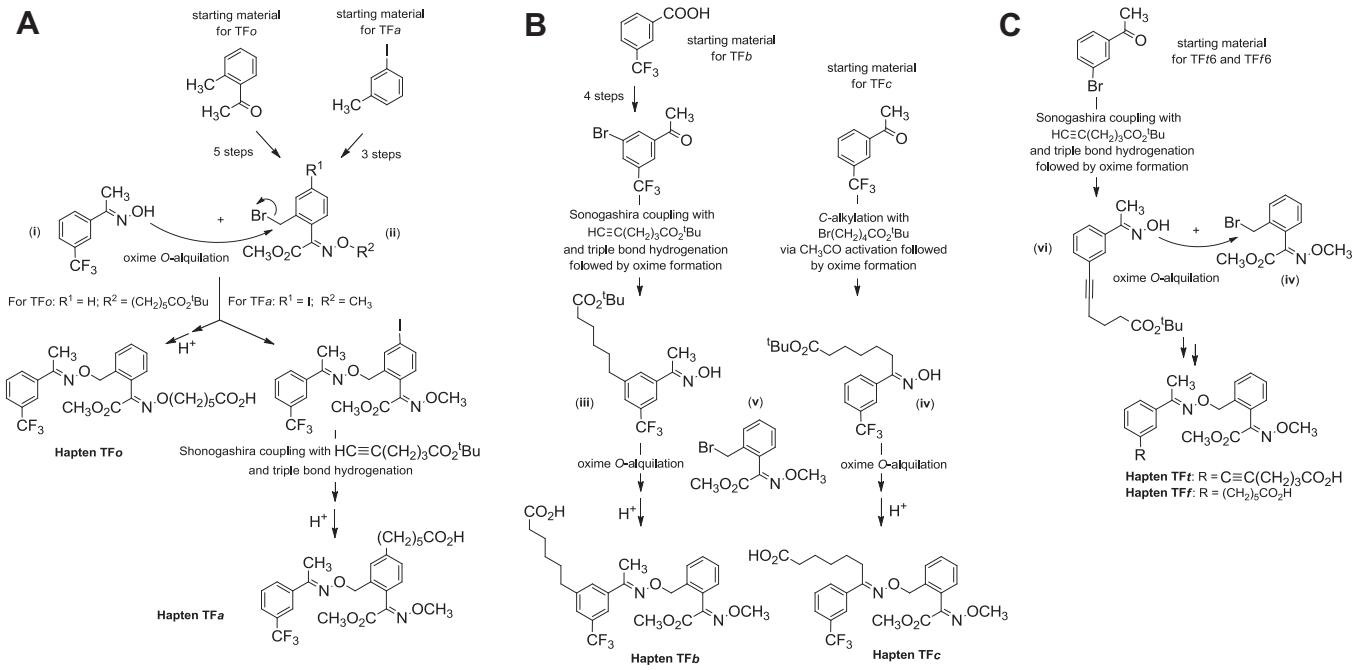


Fig. 2. Schematic diagram showing the rational design and general strategy followed for hapten synthesis.

HRP conjugates were probably due to modified molar extinction coefficients of the protein and/or the hapten after conjugation.

3.3. Antibody affinity

A collection of mAbs to trifloxystrobin was generated using BSA conjugates of all of the synthesized regioisomeric haptens. A set of 4 mice was immunized with each conjugate, and positive immune response was confirmed with a small serum sample that was obtained 10 days after the third injection. Cell fusions were carried out with all of the mice with mostly high efficiencies for hybrid cell formation. Spleens were processed individually or several spleens from the same set of animals were pooled. A summary of cell fusion results can be found in Table S1 of Supplementary Data File. Immunogen BSA-TF*a* afforded the lowest number (1.2%) of positive clones – clones segregating antibodies that recognized the immobilized conjugate, whereas conjugate BSA-TF*t* gave the highest amount of positive clones (60.7%). Further work with other analytes and haptens is required to conclusively ascribe this high ratio of positive clones to the presence of a triple bond in the spacer arm of hapten Tf*t*. The employed double screening process using a differential and a checkerboard approach, as previously described (Mercader et al., 2008b), allowed identification of those clones producing antibodies that bound free trifloxystrobin with high affinity. Following that process, a total of 20 hybridomas were generated and stabilized – at least two hybridomas were cloned from each set of immunized animals. All antibodies were constituted by κ light chains and were of the IgG_1 isotype, except antibodies TF*t*#35 and TF*t*#316 which were of the IgG_{2b} isotype.

Checkerboard competitive assays were performed by the indirect and direct cELISA formats. Regarding indirect assays, all antibodies recognized the homologous OVA conjugate, and most of them also afforded enough signal with one or more heterologous coating antigens (Table 2). IC_{50} values below 5 nM were obtained with most types of antibodies, except with those from haptens with the linker at a more central position of the molecule (TF*a* and TF*c*). Moreover, the mAbs recognized conjugates with the linker at equivalent positions, such as those of haptens TF*e* and TF*o*.

Table 2
IC_{50} values (nM) from checkerboard assays using the indirect cELISA format.[a]

mAb	OVA–hapten coating conjugate[b]						
	TF*a*	TF*b*	TF*c*	TF*e*	TF*o*	TF*f*	TF*t*
TF*a*#11	20.5	–[c]	–	–	–	–	–
TF*a*#24	11.7	–	–	12.2	–	–	–
TF*a*#26	29.9	–	–	–	25.1	–	–
TF*b*#14	–	3.2	–	3.0	2.1	2.3	3.4
TF*b*#32	–	16.7	20.7	–	–	15.4	–
TF*c*#146	–	–	8.2	–	–	–	–
TF*c*#151	–	–	17.4	–	–	–	–
TF*c*#163	–	–	10.2	10.1	3.1	–	–
TF*e*#23	–	–	–	6.1	6.7	–	–
TF*e*#25	–	–	–	4.2	4.1	–	–
TF*e*#38	–	–	–	1.8	1.8	–	–
TF*e*#310	–	–	–	22.6	23.1	–	–
TF*o*#14	3.8	–	–	8.5	5.5	–	–
TF*o*#110	12.1	–	–	9.3	11.6	–	–
TF*o*#112	–	–	–	–	8.0	–	–
TF*f*#13	–	3.5	2.7	2.4	–	2.9	5.1
TF*t*#21	–	–	–	–	4.0	5.1	4.4
TF*t*#35	–	–	–	–	–	15.1	15.2
TF*t*#211	1.4	1.8	–	2.4	–	3.8	5.3
TF*t*#316	–	–	–	4.5	1.6	3.4	3.3

[a] Average of three independent experiments.
[b] Conjugate concentration was 1.0 μg mL^{-1}.
[c] No or low signal at the highest assayed antibody concentration (1.0 μg mL^{-1}).

Interestingly, no TF*e*- or TF*o*-type antibody bound to conjugates of TF*f* or TF*t*, whereas several TF*f*- or TF*t*-type antibodies recognized OVA–TF*e* or OVA–TF*o*, or both of them. As a result, it could be stated that antibodies with the highest affinity were mainly obtained from haptens containing the spacer arm either at the methoxyacetate moiety (haptens TF*e* and TF*o*) or at the opposite side of the molecule (haptens TF*b*, TF*f*, and TF*t*).

Concerning direct assays, the two TF*b*-type mAbs and most of the TF*a*-, TF*c*-, and TF*t*-type antibodies did not properly bind any enzyme tracer. Poor recognition of enzyme tracers by mAbs directly immobilized to ELISA plates has been previously observed with other immunoreagents (Abad et al., 1997; Butler, 2000;

Manclús et al., 2004). Although the tracer recognition capacity of several anti-trifloxystrobin antibodies clearly improved when they were assayed in capture-antibody pre-coated plates (data not shown), this alternative assay format was not further explored with those mAbs. All of the TF*e*- and TF*o*-type antibodies afforded enough signal with both TF*e* and TF*o* tracers (Table 3), and a similar behaviour was observed between TF*f*- and TF*t*-type mAbs and tracers of TF*t* and TF*f*, respectively. Hapten heterology with this format afforded IC_{50} values to trifloxystrobin lower than the homologous assays with monoclonal TF*f*#13 combined with tracer HRP–TF*c*, and with mAb TF*t*#316 together with HRP–TF*e*.

3.4. Antibody specificity to structural analogues

The main structural motifs of the target analyte involved in antibody binding as a function of the immunizing hapten was studied by homologous indirect cELISA using as competitors a small collection of trifloxystrobin analogues carrying single structural differences that were designed to introduce steric and electronic modifications in the molecule (Table 1). Interestingly, high CR values were found (between 100% and 250%) with the iodinated analogue (TF*a*-I), independently of the antibody type. Equivalent or even superior recognition of iodinated analogues has been observed in our group with other strobilurins (unpublished results) and by other authors with brominated compounds (Sanvicens, Varela, & Marco, 2003; Suárez-Pantaleón, Mercader, Agulló, Abad-Somovilla, & Abad-Fuentes, 2011). Most probably, the increased hydrophobicity would compensate the larger size of the molecule. On the other hand, analogues with slight modifications at a central position of the molecule, such as TF*c*-H and TF*c*-Et, showed varied CR values (between 10% and 100%) independently of the antibody origin, even though TF*c*-type antibodies, derived from the hapten with the linker at that position, coherently displayed the highest recognition of these two analogues (results not shown).

Finally, when only the electron withdrawing trifluoromethyl group of trifloxystrobin was changed, remarkable results were found. As shown in Fig. 3, average CR values were near 100% for TF*f*- and TF*t*-type antibodies, independently of the modification that was introduced, as could be predicted by Landsteiner's principle, *i.e.* antibody specificity is directed primarily to the portion of the hapten located furthest from or opposite to the functional group linking it to the carrier protein (Landsteiner, 1962). Besides, the analogue better mimicking the electron withdrawing properties of the trifluoromethyl group (TF*f*-NO_2) was also well recognized by the other sorts of antibodies, particularly by TF*b*- and TF*c*-type mAbs – those generated from immunizing haptens with the spacer arm closer to the linker position of TF*f*. On the contrary, the presence of an electron donating group, such as $-NH_2$ (TF*f*-NH_2), drastically reduced the CR with TF*a*-, TF*b*-, TF*c*-, TF*e*-, and TF*o*-type antibodies, which evidenced the relevance, for antibody binding, of the electronic modifications introduced by those moieties located opposite to the spacer arm of the immunizing hapten. Binding to an analogue with a neutral substituent (TF*f*-H) brought about an intermediate outcome.

Table 3
IC_{50} values (nM) from checkerboard competitive assay using the direct cELISA format.[a]

mAb[b]	HRP–hapten tracer conjugate						
	TF*a*	TF*b*	TF*c*	TF*e*	TF*o*	TF*f*	TF*t*
TF*a*#24	45.1	–[c]	–	–	–	–	–
TF*c*#163	–	–	7.9	15.9	39.9	–	–
TF*e*#23	–	–	–	6.6	5.0	–	–
TF*e*#25	–	–	–	6.5	5.7	–	–
TF*e*#38	–	–	–	4.5	4.8	–	–
TF*e*#310	–	–	–	21.9	–	–	–
TF*o*#14	–	–	–	6.5	8.5	–	–
TF*o*#110	–	–	–	7.5	7.3	–	–
TF*o*#112	–	–	–	6.2	7.3	–	–
TF*f*#13	–	–	4.6	–	–	8.1	5.6
TF*t*#211	–	–	–	4.1	–	5.3	4.3
TF*t*#316	–	–	–	5.2	–	10.5	9.7

[a] Only those mAbs that recognized at least one enzyme tracer are shown. Values are the mean of three independent experiments.
[b] Coating antibody concentration was 1.0 μg mL^{-1}.
[c] No or low signal at the highest assayed tracer concentration (300 ng mL^{-1}).

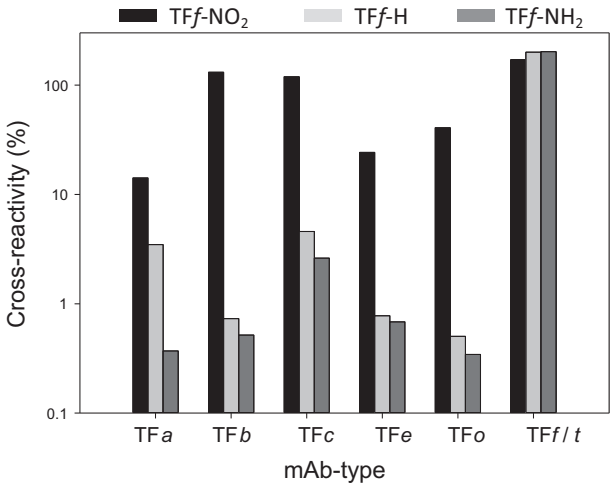


Fig. 3. Average CR values for three trifloxystrobin analogues obtained with the different types of antibodies.

4. Conclusions

A battery of regioisomeric haptens was prepared with the same spacer arm – a C-6 hydrocarbon chain ending in a carboxylate group – located at a variety of positions in order to cover the entire trifloxystrobin skeleton. In most cases, haptens were synthesized through formation of a C–C bond using Sonogashira cross-coupling methodology or *C*-alkylation reactions, whereas in other cases formation of a C–O bond using an *O*-alkylation reaction was followed. Carboxylate activation with DSC and purification of the active ester was shown to be an attractive strategy for immunogen and assay conjugate preparation by a single efficient procedure. A key influence of linker positioning over antibody affinity and specificity was revealed. For this agrochemical, derivatization over the methoxyacetate group – an aliphatic peripheral moiety with high-conformational freedom and displaying the aromatic and more rigid parts of the molecule – was shown to be the most adequate approach in terms of both synthetic convenience and antibody production. Several mAb/conjugate combinations were shown to be good candidates for immunoassay development with suitable analytical properties.

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Limited amounts of the immunoreagents described in this work are available upon request.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2013.11.150.

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