Immunoassays for trifloxystrobin analysis.

Part I. Rational design of regioisomeric haptens and production of monoclonal antibodies

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22   **Abstract**

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

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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 (K. 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, Clough, Godwin, Hall, Hamer, & Parr-Dobrzanski, 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 (Kaushik 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, Agullo, 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 to 6 carbon atoms is employed as linker ([Abad, Moreno, & Montoya, 1998](#); [Y. J. Kim, Cho, Lee, Lee, Gee, & Hammock, 2003](#)), though good results with shorter linkers have been reported ([Kong, Zhang, Zhang, Gee, & Li, 2010](#); [Suarez-Pantaleon, Mercader, Agullo, Abad-Somovilla, & Abad-Fuentes, 2008](#)). In a previous article, antibodies to trifloxystrobin were produced from a single immunizing hapten without spacer arm ([Mercader, Suarez-Pantaleon, Agullo, Abad-Somovilla, & Abad-Fuentes, 2008a](#)). However, trifloxystrobin is a very flexible molecule that can adopt many different 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 -trifluoro-*m*-tolyl)ethylideneaminoxyl]-*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 the [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 the [Supplementary Data File \(Fig. S1 to S6\)](#). Carboxylated haptens were activated by formation of the corresponding *N*-hydroxysuccinimidyl ester (NHS-ester) using *N,N'*-disuccinimidyl carbonate (DSC) and Et₃N in dry acetonitrile as previously described ([Esteve-Turrillas, Parra, Abad-Fuentes, Agullo, Abad-Somovilla, & Mercader, 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. Et₃N (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 CHCl₃, washed with a 10% aqueous solution of NaHCO₃ and brine, and dried over anhydrous Na₂SO₄. 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 the Supplementary Data File).

TFa-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, $\text{COCH}_2\text{CH}_2\text{CO}$), 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).

TFb-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, $\text{COCH}_2\text{CH}_2\text{CO}$), 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).

TFc-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, $\text{COCH}_2\text{CH}_2\text{CO}$), 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).

TFe-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,

OCH₂), 4.21 (2H, t, *J* = 6.7 Hz, H-6), 4.02 (3H, s, NOCH₃), 2.82 (4H, br s, COCH₂CH₂CO), 2.53 (2H, t, *J* = 7.3 Hz, H-2), 2.23 (3H, s, CH₃), 1.73–1.59 (4H, m, H-3 and H-5), 1.44–1.28 (2H, m, H-4).

TFo-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, OCH₂), 4.25 (2H, t, *J* = 6.7 Hz, H-6), 3.81 (3H, s, CO₂CH₃), 2.82 (4H, br s, COCH₂CH₂CO), 2.52 (2H, t, *J* = 7.4 Hz, H-2), 2.22 (3H, s, CH₃), 1.68 (4H, m, H-3 and H-5), 1.38 (2H, m, H-4).

TFf-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, OCH₂), 4.03 (3H, s, NOCH₃), 3.81 (3H, s, CO₂CH₃), 2.82 (4H, br s, COCH₂CH₂CO), 2.65–2.58 (2H, two overlapped t, *J* = 7.5 Hz, H-2 and H-6), 2.20 (3H, s, CH₃), 1.83–1.61 (4H, m, H-3 and H-5), 1.50–1.41 (2H, m, H-4).

TFt-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, OCH₂), 4.03 (3H, s, NOCH₃), 3.82 (3H, s, CO₂CH₃), 2.84 (4H, br s, COCH₂CH₂CO), 2.82 (2H, t, *J* = 7.4 Hz, H-4), 2.58 (2H, t, *J* = 7.0 Hz, H-2), 2.18 (3H, s, CH₃), 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 TFa-I, an intermediate in the synthesis of hapten TFa, 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 the [Supplementary Data File \(Fig. S7\)](#).

2.4. Conjugate preparation

Conjugation was carried out with 1, 5, and 10 μ mol 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 *et al.* ([Mercader, Esteve-Turrillas, Agullo, Abad-Somovilla, & 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, Agullo, 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, Suarez-Pantaleon, Agullo, Abad-Somovilla, & Abad-Fuentes, 2008a). Selection of the best hybridomas was carried out by a double screening process (Mercader, Suarez-Pantaleon, Agullo, 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 100–150 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 μ L per well of trifloxystrobin standard solution in PBS and 50 μ L 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 μ L per well of analyte solution in PBS and 50 μ L per well of primary antibody dilution in PBST. For detection, 100 μ L 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*-phenyldiamine. 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 μ M 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 TFt, which was functionalized through the same attachment site that TFf 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 TFe 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 the 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 TFa and TFo implied the *O*-alkylation reaction of oxime **i** – prepared according to the procedure described in the literature by Neufeldt *et al.* (Neufeldt & Sanford, 2010) – and a benzyl bromide such as **ii**, whose preparation was carried out from readily available tolyl derivatives following previously reported methodology (Itoh, Nagata, Nomura, Takanaga, Sugita, & Ichikawa, 1984; Wenderoth, Sauter, Wingert, Hepp, Brand, Kuekenhoechner, 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 TFo, and a palladium catalyzed Sonogashira cross-coupling reaction followed by hydrogenation of a triple bond, in the case of hapten TFa.

The synthesis of haptens TFb and TFc 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; J.-K. Kim, Kim, Hwang, & Nam, 2009; Wenderoth, Sauter, Wingert, Hepp, Brand, Kuekenhoechner, et al., 1992)(Fig. 2B). For hapten TFb, 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 TFc 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 TFf and TFt were synthesized through the same synthetic sequence, similar to that described above for hapten TFb, 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 TFt. On the other hand, hydrogenation of the triple bond previously to hydrolysis of the *tert*-butyl ester group afforded hapten TFf.

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 CO₂ 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 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 the Supplementary Data File](#). Immunogen BSA–TF α afforded the lowest number (1.2%) of positive clones – clones segregating antibodies that recognized the immobilized conjugate –, whereas conjugate BSA–TF ϵ 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 Tft. The employed double screening process using a differential and a checkerboard approach, as previously described (Mercader, Suarez-Pantaleon, Agullo, Abad-Somovilla, & Abad-Fuentes, 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 IgG₁ isotype, except antibodies TFt#35 and TFt#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). IC₅₀ 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 (TFa and TFc). Moreover, the mAbs recognized conjugates with the linker at equivalent positions, such as those of haptens TFe and TFo. Interestingly, no TFe- or TFo-type antibody bound to conjugates of TFf or TFt, whereas several TFf- or TFt-type antibodies recognized OVA–TFe or OVA–TFo, 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 TFe and TFo) or at the opposite side of the molecule (haptens TFb, TFf, and TFt).

Concerning direct assays, the two TFb-type mAbs and most of the TFa-, TFc-, and TFt-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, Manclus, Mojarrad, Mercader, Miranda, Primo, et al., 1997; Butler, 2000; Manclus, Abad, Lebedev, Mojarrad, Mickova, Mercader, 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 TFe- and TFo-type antibodies afforded enough signal with both TFe and TFo tracers (Table 3), and a similar behavior was observed between TFf- and TFt-type mAbs and tracers of TFt and TFf, respectively. Hapten heterology with this format afforded IC₅₀ values to trifloxystrobin lower than the homologous assays with monoclonal TFf#13 combined with tracer HRP-TFc, and with mAb TFt#316 together with HRP-TFe.

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 (TFa-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; Suarez-Pantaleon, Mercader, Agullo, 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 TFc-H and TFc-Et, showed varied CR values (between 10% and 100%) independently of the antibody origin, even though TFc-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 TFf- and TFt-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 (TFf-NO₂) was also well recognized by the other sorts of antibodies, particularly by TFb- and TFc-type mAbs – those generated from immunizing haptens with the spacer arm closer to the linker position of TFf. On the contrary, the presence of an electron donating group, such as -NH₂ (TFf-NH₂), drastically reduced the CR with TFa-, TFb-, TFc-, TFe-, and TFo-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 (TFf-H) brought about an intermediate outcome.

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.

Acknowledgements

This work was supported by the Spanish *Ministerio de Ciencia e Innovación* (MICINN) (AGL2006-12750-C02-01/02/ALI and AGL2009-12940-C02-01/02/ALI) and cofinanced by FEDER funds. R.L.-M. was hired by MICINN under a predoctoral FPI grant associated to the above project. J.V.M. was hired by CSIC with a postdoctoral contract under the *Ramón y Cajal* program, cofinanced by MICINN and by the European Social Fund

(ESF). We thank Ana Izquierdo-Gil and Laura López-Sánchez for excellent technical assistance.

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

Appendix A. Supplementary data

Supplementary material associated with this article can be found, in the online version, at doi:

References

- Abad, A., Manclús, J. J., Mojarrad, F., Mercader, J. V., Miranda, M. A., Primo, J., Guardiola, V., & Montoya, A. (1997). Hapten synthesis and production of monoclonal antibodies to DDT and related compounds. *Journal of Agricultural and Food Chemistry*, 45(9), 3694-3702.
- Abad, A., Moreno, M. J., & Montoya, A. (1998). Hapten synthesis and production of monoclonal antibodies to the N-methylcarbamate pesticide methiocarb. *Journal of Agricultural and Food Chemistry*, 46(6), 2417-2426.
- Balba, H. (2007). Review of strobilurin fungicide chemicals. *Journal of Environmental Science and Health Part B-Pesticides Food Contaminants and Agricultural Wastes*, 42(4), 441-451.
- Banerjee, K., Ligon, A. P., & Spiteller, M. (2005). Photoisomerization kinetics of trifloxystrobin. *Analytical and Bioanalytical Chemistry*, 382(7), 1527-1533.
- Banerjee, K., Ligon, A. P., & Spiteller, M. (2006). Environmental fate of trifloxystrobin in soils of different geographical origins and photolytic degradation in water. *Journal of Agricultural and Food Chemistry*, 54(25), 9479-9487.
- Banerjee, K., Ligon, A. P., & Spiteller, M. (2007). Spectral elucidation of the acid metabolites of the four geometric isomers of trifloxystrobin. *Analytical and Bioanalytical Chemistry*, 388(8), 1831-1838.
- Bartlett, D. W., Clough, J. M., Godwin, J. R., Hall, A. A., Hamer, M., & Parr-Dobrzanski, B. (2002). The strobilurin fungicides. *Pest Management Science*, 58(7), 649-662.
- Bayer. (2010). <http://www.annualreport2010.bayer.com/>. Accessed on 2 August 2013
- Butler, J. E. (2000). Solid supports in enzyme-linked immunosorbent assay and other solid-phase immunoassays. *Methods-a Companion to Methods in Enzymology*, 22(1), 4-23.
- Esteve-Turrillas, F. A., Parra, J., Abad-Fuentes, A., Agulló, C., Abad-Somovilla, A., & Mercader, J. V. (2010). Hapten synthesis, monoclonal antibody generation, and development of competitive immunoassays for the analysis of picoxystrobin in beer. *Analytica Chimica Acta*, 682(1-2), 93-103.
- Hwang, I. C., Kim, J. K., Kim, H. H., & Kyung, S. H. (2009). Synthesis and SAR of Methoxyiminoacetate and Methoxyiminoacetamide Derivatives as Strobilurin Analogues. *Bulletin of the Korean Chemical Society*, 30(7), 1475-1480.

- Itoh, O., Nagata, T., Nomura, I., Takanaga, T., Sugita, T., & Ichikawa, K. (1984). Syntheses of Aryl Glyoxylate .1. The Reaction of Alkyl Dichloro(Alkoxy)Acetates with Aromatics in the Presence of Lewis Acid. *Bulletin of the Chemical Society of Japan*, 57(3), 810-814.
- Kim, J.-K., Kim, H.-H., Hwang, I.-C., & Nam, H.-T. (2009). Preparation of methoxyiminobenzene compounds as agrochemical fungicidal agents. In, (pp. 72pp.): Kyung Nong Corporation, S. Korea .
- Kim, Y. J., Cho, Y. A., Lee, H. S., Lee, Y. T., Gee, S. J., & Hammock, B. D. (2003). Synthesis of haptens for immunoassay of organophosphorus pesticides and effect of heterology in hapten spacer arm length on immunoassay sensitivity. *Analytica Chimica Acta*, 475(1-2), 85-96.
- Kong, Y., Zhang, Q., Zhang, W., Gee, S. J., & Li, P. W. (2010). Development of a Monoclonal Antibody-Based Enzyme Immunoassay for the Pyrethroid Insecticide Deltamethrin. *Journal of Agricultural and Food Chemistry*, 58(14), 8189-8195.
- Landsteiner, K. (1962). *The specificity of serological reactions* (Rev. ed.). New York,: Dover Publications.
- Manclús, J. J., Abad, A., Lebedev, M. Y., Mojarrad, F., Mickova, B., Mercader, J. V., Primo, J., Miranda, M. A., & Montoya, A. (2004). Development of a monoclonal immunoassay selective for chlorinated cyclodiene insecticides. *Journal of Agricultural and Food Chemistry*, 52(10), 2776-2784.
- Mercader, J. V., Agulló, C., Abad-Somovilla, A., & Abad-Fuentes, A. (2011). Synthesis of site-heterologous haptens for high-affinity anti-pyraclostrobin antibody generation. *Organic & Biomolecular Chemistry*, 9(5), 1443-1453.
- Mercader, J. V., Esteve-Turrillas, F. A., Agulló, C., Abad-Somovilla, A., & Abad-Fuentes, A. (2012). Antibody generation and immunoassay development in diverse formats for pyrimethanil specific and sensitive analysis. *Analyst*, 137(23), 5672-5679.
- Mercader, J. V., Suárez-Pantaleón, C., Agulló, C., Abad-Somovilla, A., & Abad-Fuentes, A. (2008a). Hapten synthesis and monoclonal antibody-based immunoassay development for detection of the fungicide trifloxystrobin. *Journal of Agricultural and Food Chemistry*, 56(8), 2581-2588.
- Mercader, J. V., Suárez-Pantaleón, C., Agulló, C., Abad-Somovilla, A., & Abad-Fuentes, A. (2008b). Production and characterization of monoclonal antibodies specific to the strobilurin pesticide pyraclostrobin. *Journal of Agricultural and Food Chemistry*, 56(17), 7682-7690.
- Neufeldt, S. R., & Sanford, M. S. (2010). O-Acetyl Oximes as Transformable Directing Groups for Pd-Catalyzed C-H Bond Functionalization. *Organic Letters*, 12(3), 532-535.
- Parra, J., Mercader, J. V., Agulló, C., Abad-Somovilla, A., & Abad-Fuentes, A. (2012). Generation of anti-azoxystrobin monoclonal antibodies from regioisomeric haptens functionalized at selected sites and development of indirect competitive immunoassays. *Analytica Chimica Acta*, 715, 105-112.
- Paulvannan, K., & Chen, T. (1999). Asymmetric synthesis of mercaptoalcohols - Matrix metalloproteinase inhibitors. *Synlett*(9), 1371-1374.
- Sanvicens, N., Varela, B., & Marco, M. P. (2003). Immunochemical determination of 2,4,6-trichloroanisole as the responsible agent for the musty odor in foods. 2. Immunoassay evaluation. *Journal of Agricultural and Food Chemistry*, 51(14), 3932-3939.
- Shan, G., Lipton, C., Gee, S. J., & Hammock, B. D. (2003). *Immunoassay, biosensors and other nonchromatographic methods* (Vol. 2): John Wiley & Sons Ltd.
- Snider, B. B., & Buckman, B. O. (1992). Total Synthesis of (+/-)-Velloziolone. *Journal of Organic Chemistry*, 57(18), 4883-4888.

- Suárez-Pantaleón, C., Mercader, J. V., Agulló, C., Abad-Somovilla, A., & Abad-Fuentes, A. (2008). Production and Characterization of Monoclonal and Polyclonal Antibodies to Forchlorfenuron. *Journal of Agricultural and Food Chemistry*, 56(23), 11122-11131.
- Suárez-Pantaleón, C., Mercader, J. V., Agulló, C., Abad-Somovilla, A., & Abad-Fuentes, A. (2011). Forchlorfenuron-mimicking haptens: from immunogen design to antibody characterization by hierarchical clustering analysis. *Organic & Biomolecular Chemistry*, 9(13), 4863-4872.
- Wenderoth, B., Sauter, H., Wingert, H., Hepp, M., Brand, S., Kuekenhoechner, T., Roehl, F., Ammermann, E., & Lorenz, G. (1990). Preparation of new (phenoxyethyl)phenylglyoxylate oxime ethers as fungicides. In, (pp. 42 pp.): BASF A.-G., Germany .
- Wenderoth, B., Sauter, H., Wingert, H., Hepp, M., Brand, S., Kuekenhoechner, T., Roehl, F., Ammermann, E., & Lorenz, G. (1992). Oxime ethers, and fungicides containing same. In).
- Ziegler, H., Benet-Buchholtz, J., Etzel, W., & Gayer, H. (2003). Trifloxystrobin - A new strobilurin fungicide with an outstanding biological activity. *Pflanzenschutz-Nachrichten Bayer*, 56, 213-230.

Figure legends

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).

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

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

Table 1
Trifloxystrobin analogues.

Analogue	R ¹	R ²	R ³
TFa-I	CF ₃	CH ₃	I
TFc-H	CF ₃	H	H
TFc-Et	CF ₃	C ₂ H ₅	H
TFf-NO ₂	NO ₂	CH ₃	H
TFf-H	H	CH ₃	H
TFf-NH ₂	NH ₂	CH ₃	H

Table 2
IC₅₀ values (nM) from checkerboard assays using the indirect cELISA format.^a

mAb	OVA-hapten coating conjugate ^b						
	TFa	TFb	TFc	TFe	TFo	TFf	TFt
TFa#11	20.5	— ^c	—	—	—	—	—
TFa#24	11.7	—	—	12.2	—	—	—
TFa#26	29.9	—	—	—	25.1	—	—
TFb#14	—	3.2	—	3.0	2.1	2.3	3.4
TFb#32	—	16.7	20.7	—	—	15.4	—
TFc#146	—	—	8.2	—	—	—	—
TFc#151	—	—	17.4	—	—	—	—
TFc#163	—	—	10.2	10.1	3.1	—	—
TFe#23	—	—	—	6.1	6.7	—	—
TFe#25	—	—	—	4.2	4.1	—	—
TFe#38	—	—	—	1.8	1.8	—	—
TFe#310	—	—	—	22.6	23.1	—	—
TFo#14	3.8	—	—	8.5	5.5	—	—
TFo#110	12.1	—	—	9.3	11.6	—	—
TFo#112	—	—	—	—	8.0	—	—
TF#13	—	3.5	2.7	2.4	—	2.9	5.1
TF#21	—	—	—	—	4.0	5.1	4.4
TF#35	—	—	—	—	—	15.1	15.2
TF#211	1.4	1.8	—	2.4	—	3.8	5.3
TFt#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).

Table 3

IC₅₀ values (nM) from checkerboard competitive assay using the direct cELISA format.^a

mAb ^b	HRP-hapten tracer conjugate						
	TFa	TFb	TFc	TFe	TFo	TFf	TFt
TFa#24	45.1	– ^c	–	–	–	–	–
TFc#163	–	–	7.9	15.9	39.9	–	–
TFe#23	–	–	–	6.6	5.0	–	–
TFe#25	–	–	–	6.5	5.7	–	–
TFe#38	–	–	–	4.5	4.8	–	–
TFe#310	–	–	–	21.9	–	–	–
TFo#14	–	–	–	6.5	8.5	–	–
TFo#110	–	–	–	7.5	7.3	–	–
TFo#112	–	–	–	6.2	7.3	–	–
TF#13	–	–	4.6	–	–	8.1	5.6
TF#211	–	–	–	4.1	–	5.3	4.3
TF#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).

Figure(s)

Figure 1

Figure 2

Figure 3

SUPPLEMENTARY DATA

Immunoassays for trifloxystrobin analysis.

Part I. Rational design of regioisomeric haptens and production of monoclonal antibodies

Rosario López-Moreno, Josep V. Mercader, Consuelo Agulló, Antonio Abad-Somovilla, Antonio Abad-Fuentes

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1. Synthesis of haptens

1.1. General experimental details

Infrared (IR) spectra were measured as KBr pellets for solid compounds or as thin films between NaCl plates for liquids, using a Nicolet Avatar 320 spectrometer; peaks are reported using the following abbreviations: s (strong), m (medium), w (weak). Melting points were determined using a Kofler hot-stage apparatus or a Büchi melting point apparatus and are uncorrected. NMR spectra were recorded at 25 °C on a Bruker AC-300 spectrometer (300.13 MHz for ^1H and 75.47 MHz for ^{13}C), and data are relative to Me_4Si and referenced to residual solvent. Carbon substitution degrees were established by DEPT pulse sequences, and a combination of COSY and HSQC experiments was utilized for the assignment of ^1H and ^{13}C chemical shifts. Spectral splitting patterns are designated as s (singlet), d (doublet), t (triplet), q (quadruplet), quint (quintuplet), m (multiplet), and br (broad). Mass spectra (MS) and high-resolution mass spectra (HRMS) were recorded with a Micromass VG Autospec spectrometer using for ionization electron impact (70 eV) or fast atom bombardment (FAB) or by the electrospray ionization (ESI) mode, which was obtained with a Q-TOF premier mass spectrometer with an electrospray source.

Chemical reagents were purchased from regular sources and used without purification. All operations involving air-sensitive reagents were performed under an inert atmosphere of dry nitrogen or argon using syringe and cannula techniques, oven-dried glassware, and freshly distilled and dried solvents. Organic solvents were dried and distilled before use according to standard procedures.¹

Reactions were followed by thin-layer chromatography using 0.25 mm pre-coated silica gel plates and products were visualized by UV light (254 nm) and aqueous ceric ammonium molybdate solution or 50% (v/v) concentrated H_2SO_4 in water and then heating with a high-temperature air dryer. Unless otherwise stated, reaction mixtures were worked up by addition of water, and extraction with the appropriate solvent, the organic layer being washed with water and/or brine and dried using anhydrous magnesium or sodium sulfate. Evaporation of the solvent was performed under reduced pressure using a rotary evaporator. Synthesized compounds were purified by flash chromatography with a silica gel column (Merck 60, 230–400 mesh).

tert-Butyl hex-5-ynoate, *tert*-butyl 5-bromopentanoate and *tert*-butyl 6-bromohexanoate were prepared by esterification of commercial hex-5-ynoic acid, 5-bromopentanoic acid and 6-bromohexanoic acid, respectively, with isobutene following a procedure previously described for the preparation of related *tert*-butyl esters.²

1.2. Synthesis of hapten TFa

Figure S1. Synthetic sequence for the preparation of hapten TFa.

Methyl 2-(4-iodo-2-methylphenyl)-2-oxoacetate (2). Methyl 2-chloro-2-oxoacetate (323 μL , 3.510 mmol) was added to a solution of 3-iodotoluene (300 μL , 511.2 mg, 2.340 mmol) in anhydrous CH_2Cl_2 (5 mL). The mixture was stirred for 10 min and then cooled to -30°C followed by the addition of AlCl_3 (1.2 g, 9.015 mmol) in small portions (in about 10 min). The mixture was stirred at the same temperature for 90 min, during which the color changes to dark red. The reaction mixture was poured into a mixture of crushed ice and conc. HCl and extruded with CH_2Cl_2 . The combined organics layers were washed successively with a 10% aqueous solution of $\text{Na}_2\text{S}_2\text{O}_3$, a 0.1 M solution of NaOH and brine, dried over anhydrous Na_2SO_4 and concentrated to give a residue that was purified by chromatography, using hexane– Et_2O (9:1) as eluent, to afford, in order of elution, title compound **2** (348.6 mg, 49%), as a white solid, followed by methyl 2-oxo-2-(*m*-tolyl)acetate (92.4 mg, 13%), as a colorless oil, and regioisomeric methyl 2-(2-iodo-4-methylphenyl)-2-oxoacetate (248.5 mg, 35%) as a yellowish oil.

Physical and spectroscopic data of 2: Mp 86–88 °C (crystallized from hexane); IR (NaCl) $\nu_{\max}$ (cm⁻¹) 2954w, 2925w, 1734m, 1680s, 1585m, 1547m, 1429m, 1320m, 1203s, 922s, 912m, 735m; ¹H-NMR (300 MHz) δ (ppm) 7.22 (1H, br s, H-3), 7.68 (1H, dd, J = 8.2, 1.5 Hz, H-5), 7.38 (1H, d, J = 8.2 Hz, H-6), 3.95 (3H, s, CO₂CH₃), 2.53 (3H, s, CH₃); ¹³C-NMR (75 MHz) δ (ppm) 187.65 (CO), 164.22 (CO₂CH₃), 142.78 (C-2), 141.30 (C-3), 135.21 (C-5), 133.04 (C-6), 130.67 (C-1), 102.23 (C-4), 52.90 (CO₂CH₃), 20.95 (CH₃); MS (EI) m/z (%) 304 (M⁺, 7), 246 (9), 245 (100), 217 (12), 118 (5), 90 (17), 63 (5); HRMS m/z calcd. for C₁₀H₉IO₃ 303.9596, found 303.9595.

(E)-Methyl 2-(4-iodo-2-methylphenyl)-2-(methoxyimino)acetate (3). A suspension of keto-ester 2 (202.4 mg, 0.666 mmol) and *N*-methylhydroxylamine hydrochloride (240 mg, 2.87 mmol) in anhydrous pyridine (3.5 mL) was stirred at room temperature under nitrogen overnight. Work up as usual followed by purification by column chromatography with hexane–Et₂O (from 8:2 to 6:4) as eluent afforded methoxyimino derivative 3 (157.2 mg, 71%) as a white solid. Mp 50–52 °C (crystallized from hexane); IR (KBr) $\nu_{\max}$ (cm⁻¹) 2940w, 2886w, 1725s, 1597w, 1437m, 1323m, 1219s, 1066s, 1018s, 957m, 846m, 780m; ¹H-NMR (300 MHz) δ (ppm) 7.63 (1H, br s, H-3), 7.57 (1H, dd, J = 8.1, 1.4 Hz, H-5), 6.83 (1H, d, J = 8.1 Hz, H-6), 4.05 (3H, s, NOCH₃), 3.87 (3H, s, CO₂CH₃), 2.13 (3H, s, CH₃); ¹³C-NMR (75 MHz) δ (ppm) 163.07 (CO₂CH₃), 149.69 (C=N), 138.83 (C-3), 138.33 (C-2), 134.61 (C-5), 129.64 (C-1), 129.50 (C-6), 95.64 (C-4), 63.85 (NOCH₃), 53.02 (CO₂CH₃), 19.14 (CH₃); MS (EI) m/z (%) 333 (M⁺, 28), 302 (100), 270 (15), 259 (5), 243 (59), 242 (30), 175 (8), 143 (8), 116 (20), 89 (15), 63 (5), 59 (8); HRMS m/z calcd. for C₁₁H₁₂INO₃ 332.9862, found 332.9867.

(E)-Methyl 2-(2-(bromomethyl)-4-iodophenyl)-2-(methoxyimino)acetate (4). A mixture of 3 (300 mg, 0.900 mmol), *N*-bromosuccinimide (NBS, 160 mg, 0.900 mmol), and azo-*bis*-isobutyronitrile (AIBN, 8.8 mg, 0.054 mmol) in dry benzene (4 mL) was degassed by nitrogen bubbling and ultrasound for 15 min. The reaction mixture was refluxed under nitrogen for 14 h and the solvent was removed in the rotary evaporator to leave an oil that was purified by chromatography, using hexane–Et₂O (9:1) as eluent, to give benzyl bromide 4 (248.4 mg, 67%) as a yellowish oil. IR (NaCl) $\nu_{\max}$ (cm⁻¹) 3053w, 2941w, 1726s, 1595w, 1437m, 1388d, 1325m, 1302m, 1266m, 1223s, 1063s, 1017s, 957m, 785m, 736s; ¹H-NMR (300 MHz) δ (ppm) 7.84 (1H, d, J = 1.7 Hz, H-3), 7.70 (1H, dd, J = 8.1, 1.7 Hz, H-5), 6.88 (1H, d, J = 8.1 Hz, H-6), 4.23 (2H, s, CH₂Br), 4.07 (3H, s, NOCH₃), 3.88 (3H, s, CO₂CH₃); ¹³C-NMR (75 MHz) δ (ppm) 162.71 (CO₂CH₃), 148.09 (C=N), 138.97 (C-3), 137.71 (C-2), 137.45 (C-5), 130.21 (C-6), 129.79 (C-1), 95.64 (C-4), 64.02 (NOCH₃), 53.20 (CO₂CH₃), 29.37 (CH₂Br); MS (EI) m/z (%) 414 (M⁺+1 for ⁸¹Br, 12), 413 (M⁺ for ⁸¹Br, 97), 412 (M⁺ +1

for ^{79}Br , 13), 411 (M^+ for ^{79}Br , 99), 380 (21), 378 (5), 332 (29), 301 (100), 272 (30), 269 (22), 258 (6), 242 (36), 193 (2), 145 (13), 114 (10); HRMS m/z calcd. for $\text{C}_{11}\text{H}_{11}^{79}\text{BrINO}_3$ 410.8967, found 410.8966.

(E)-methyl 2-(4-iodo-2-(((*E*)-(1-(3-(trifluoromethyl)phenyl)ethylidene)amino)oxy)methyl) phenyl)-2-(methoxyimino)acetate (6). A mixture of benzyl bromide 4 (105 mg, 0.255 mmol), oxime 5 (synthesized in the laboratory following the procedure described by Neufeldt et al.,³ 55.3 mg, 0.255 mmol), and anhydrous Cs_2CO_3 (125 mg, 0.383 mmol) in dry DMF (1.2 mL) was stirred under nitrogen for 6 h, poured into water and worked up as usual using EtOAc to extract. Chromatography of the residue left after evaporation of the solvent, using hexane–Et₂O (8:2) as eluent, afforded the benzyl ether 6 (128 mg, 92%) as a white solid. Mp. 103–104 °C (crystallized from hexane); IR (KBr) ν_{max} (cm^{-1}) 2946w, 2878w, 1725s, 1595w, 1369m, 1340s, 1299m, 1277s, 1217m, 1169s, 1119s, 1068s, 1020s, 925m, 914m, 677m; ^1H -NMR (300 MHz) δ (ppm) 7.86 (2H, br s, H-3 and H-2'), 7.78 (1H, d, J = 7.8 Hz, H-6'), 7.71 (1H, dd, J = 8.1, 1.7 Hz, H-5), 7.60 (1H, d, J = 7.8 Hz, H-4'), 7.47 (1H, br t, J = 7.8 Hz, H-5'), 6.92 (1H, d, J = 8.1 Hz, H-6), 5.06 (2H, s, OCH_2), 4.03 (3H, s, NOCH_3), 3.82 (3H, s, CO_2CH_3), 2.23 (3H, s, CH_3); ^{13}C -NMR (75 MHz) δ (ppm) 162.90 (CO_2CH_3), 154.10 (CH_3CN), 148.70 ($\text{C}=\text{N}$), 138.23 (C-2), 137.67 (C-3), 137.00 (C-1'), 136.81 (C-5), 130.79 (C-3'', q, J = 32.5 Hz), 130.19 (C-6), 129.29 (C-6'), 129.20 (C-1), 128.85 (C-5'), 125.72 (C-4', q, J = 3.8 Hz), 123.94 (CF_3 , q, J = 272.9 Hz), 122.88 (C-2', q, J = 3.8 Hz), 95.62 (C-4), 73.99 (OCH_2), 63.90 (NOCH_3), 52.97 (CO_2CH_3), 12.61 (CH_3); MS (EI) m/z (%) 534 (M^+ , 0.1), 503 (12), 348 (100), 332 (50), 316 (33), 288 (8), 272 (11), 256 (53), 243 (21), 242 (90), 198 (5), 186 (18), 146 (5); HRMS m/z calcd. for $\text{C}_{20}\text{H}_{18}\text{F}_3\text{IN}_2\text{O}_4$ 534.0263, found 534.0282.

tert-Butyl 6-(4-(((*E*)-2-methoxy-1-(methoxyimino)-2-oxoethyl)-3-(((*E*)-(1-(3-(trifluoromethyl)phenyl)ethylidene)amino)oxy)methyl)phenyl)hex-5-ynoate (7). A solution of *tert*-butyl hex-5-ynoate (156 mg, 0.952 mmol) in anhydrous degassed DMF (1.0 mL) was added to a mixture of aryl iodide 6 (127 mg, 0.238 mmol), CuI (1.3 mg, 0.007 mmol, 3% mol) and $(\text{Ph}_3\text{P})_2\text{PdCl}_2$ (5.0 mg, 0.007 mmol, 3% mol) under nitrogen atmosphere. After the addition anhydrous Et₃N (0.5 mL) the reaction mixture, initially yellow that turned deep-orange after a few minutes, was stirred at room temperature for 6 h, then poured into water and worked up in the usual manner. Chromatography of the crude product with hexane–Et₂O (7:3) as eluent afforded alkyne 7 (112.3 mg, 82%) as an oil. IR (NaCl) ν_{max} (cm^{-1}) 2977m, 2940s, 2231w, 1727s, 1609m, 1493m, 1437s, 1368s, 1301s, 1275s, 1214s, 1127s, 1070s, 1017s, 951m, 899m, 834m, 788m, 698m; ^1H -NMR (300 MHz) δ (ppm) 7.86 (1H, br s, H-2''), 7.78 (1H, d, J = 7.7 Hz, H-6''), 7.60 (1H, d, J = 7.8 Hz, H-4''), 7.51 (1H, d, J = 1.9 Hz, H-2'), 7.46 (1H, br t, J = 7.8 Hz, H-5''), 7.39 (1H, dd, J = 7.9, 1.9 Hz, H-6'), 7.11

(1H, d, J = 7.9 Hz, H-5'), 5.09 (2H, s, OCH₂), 4.02 (3H, s, NOCH₃), 3.81 (3H, s, CO₂CH₃), 2.47 (2H, t, J = 7.1 Hz, H₂-4), 2.42 (2H, t, J = 7.4 Hz, H₂-2), 2.22 (3H, s, CH₃), 1.88 (2H, quint, J = 7.3 Hz, H₂-3), 1.45 (9H, s C(CH₃)₃); ¹³C-NMR (75 MHz) δ (ppm) 172.41 (CO₂^tBu), 163.05 (CO₂CH₃), 153.74 (CH₃CN), 149.11 (C=N), 137.11 (C-3'), 136.17 (C-1''), 131.76 (C-2'), 130.72 (C-6'), 130.71 (C-3'', q, J = 32.5 Hz), 129.17 (C-6''), 128.93 (C-4'), 128.77 (C-5''), 128.57 (C-5'), 125.57 (C-4'', q, J = 3.8 Hz), 125.09 (C-1'), 123.94 (CF₃, q, J = 272.4 Hz), 122.83 (C-2'', q, J = 3.8 Hz), 90.44 (C-5), 80.69 (C-6), 80.24 (C(CH₃)₃), 74.54 (OCH₂), 63.73 (NOCH₃), 52.80 (CO₂CH₃), 34.32 (C-2), 28.05 (C(CH₃)₃), 24.00 (C-3), 18.80 (C-4), 12.49 (CH₃); MS (EI) m/z (%) 574 (M⁺, 14), 518 (72), 501 (33), 487 (4), 473 (4), 332 (12), 316 (12), 300 (9), 284 (10), 272 (23), 256 (10), 242 (10), 226 (49), 212 (8), 199 (6), 187 (20), 186 (100); HRMS m/z calcd. for C₃₀H₃₃F₃N₂O₆ 574.2291, found 574.2284.

tert-Butyl 6-(4-((*E*)-2-methoxy-1-(methoxyimino)-2-oxoethyl)-3-(((*E*)-(1-(3-(trifluoromethyl)phenyl)ethylidene)amino)oxy)methyl)phenyl)hexanoate (8). A mixture of alkyne 7 (117.8 mg, 0.205 mmol) and Rh(PPh₃)₃Cl (6 mg, 0.006 mmol) in anhydrous THF (1.9 mL) was stirred under an atmosphere of hydrogen at 4 atm overnight. The reaction mixture was concentrated to dryness and residue obtained purified by chromatography, using hexane–Et₂O (8:2) as eluent, to give the title compound 8 (102 mg, 90%) as an oil. IR (NaCl) $\nu_{\max}$ (cm⁻¹) 2975w, 2937m, 2858w, 1728s, 1613w, 1367m, 1341m, 1299m, 1275s, 1214m, 1167s, 1127s, 1070s, 1021m; ¹H-NMR (300 MHz) δ (ppm) 7.87 (1H, br s, H-2''), 7.79 (1H, d, J = 7.8 Hz, H-6''), 7.58 (1H, d, J = 7.8 Hz, H-4''), 7.46 (1H, br t, J = 7.8 Hz, H-5''), 7.29 (1H, d, J = 1.6 Hz, H-2'), 7.19 (1H, dd, J = 7.8, 1.6 Hz, H-6'), 7.11 (1H, d, J = 7.8 Hz, H-5'), 5.12 (2H, s, OCH₂), 4.03 (3H, s, NOCH₃), 3.81 (3H, s, CO₂CH₃), 2.65 (2H, t, J = 7.7, H₂-6), 2.21 (5H, m, H₂-2 and CH₃), 1.72–1.57 (4H, m, H₂-5 and H₂-3), 1.44–1.33 (11H, m, C(CH₃)₃ and H₂-3); ¹³C-NMR (75 MHz) δ (ppm) 173.12 (CO₂^tBu), 163.45 (CO₂CH₃), 153.52 (CH₃CN), 149.67 (C=N), 143.94 (C-1'), 137.26 (C-3'), 135.74 (C-1''), 130.74 (C-3'', q, J = 32.1 Hz), 129.23 (C-6''), 128.93 (C-2'), 128.78 (C-5''), 128.54 (C-5'), 127.78 (C-6'), 127.13 (C-4'), 125.54 (C-4'', q, J = 3.8 Hz), 124.00 (CF₃, q, J = 272.5 Hz), 122.85 (C-2'', q, J = 3.8 Hz), 79.95 (C(CH₃)₃), 75.14 (OCH₂), 63.68 (NOCH₃), 52.80 (CO₂CH₃), 35.58 (C-6), 35.43 (C-2), 30.67 (C-5), 28.79 (C-4), 28.08 (C(CH₃)₃), 24.90 (C-3), 12.50 (CH₃); MS (IE) m/z (%) 579 (M⁺+1, 3), 505 (12), 376 (10), 336 (19), 320 (6), 288 (5), 276 (27), 246 (6), 230 (31), 214 (10), 186 (40), 172 (11), 57 (100); HRMS m/z calcd. for C₃₀H₃₈F₃N₂O₆ [M+H]⁺ 579.2682, found 579.2690.

6-(4-((*E*)-2-Methoxy-1-(methoxyimino)-2-oxoethyl)-3-(((*E*)-(1-(3-(trifluoromethyl)phenyl)ethylidene)amino)oxy)methyl)phenyl)hexanoic acid (hapten TFa). A solution of *tert*-butyl ester 8 (86 mg, 0.149 mmol)

in formic acid (3.3 mL) was stirred at room temperature under nitrogen for 3 h. Then the reaction mixture was diluted with benzene and washed with cold water until pH 6–7 and dried over anhydrous Na₂SO₄. Chromatographic purification of the residue left after evaporation of the solvent, using CHCl₃–MeOH (99:1) as eluent, afforded hapten TFa (63.4 mg, 82%) as a colorless oil. IR (NaCl) $\nu_{\max}$ (cm⁻¹) 3371m (broad), 2984s, 2859m, 1738s, 1709s, 1614w, 1437m, 1341s, 1299s, 1275s, 1214s, 1167s, 1127s, 1070s, 1020s, 737m, 699m; 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'), 7.19 (1H, dd, *J* = 7.8, 1.6 Hz, H-6'), 7.11 (1H, d, *J* = 7.8 Hz, H-5'), 5.12 (2H, s, OCH₂), 4.03 (3H, s, NOCH₃), 3.82 (3H, s, CO₂CH₃), 2.66 (2H, t, *J* = 7.5 Hz, H-6), 2.35 (2H, t, *J* = 7.4 Hz, H-2), 2.21 (3H, s, CH₃), 1.71–1.56 (4H, m, H-3 and H-5), 1.42 (2H, m, H-4); ¹³C-NMR (75 MHz) δ (ppm) 178.40 (CO₂H), 163.5 (CO₂CH₃), 153.6 (CH₃C=N), 149.6 (C=N), 143.8 (C-1'), 137.2 (C-3'), 135.8 (C-1''), 130.8 (C-3'', q, *J* = 32.5 Hz), 129.2 (C-6''), 129 (C-2'), 128.8 (C-5''), 128.6 (C-5'), 127.8 (C-6'), 127.2 (C-4'), 125.6 (C-4'', q, *J* = 3.8 Hz), 124.0 (CF₃, q, *J* = 272 Hz), 122.86 (C-2'', q, *J* = 3.8 Hz), 75.1 (OCH₂), 63.7 (NOCH₃), 52.8 (CO₂CH₃), 35.5 (C-6), 33.8 (C-2), 30.6 (C-5), 28.7 (C-4), 24.5 (C-3), 12.5 (CH₃); MS (EI) *m/z* (%) 522 (M⁺, 0.6), 491 (11), 337 (21), 336 (100), 320 (96), 304 (30), 289 (5), 276 (45), 260 (12), 246 (37), 230 (45); HRMS *m/z* calcd for C₂₆H₂₉F₃N₂O₆ 522.1978, found 522.1985; UV (100 mM sodium phosphate buffer, pH 7.4) ϵ (280 nm) 4.08 mM⁻¹ cm⁻¹.

1.3. Synthesis of hapten TFb

Figure S2. Synthetic sequence for the preparation of hapten TFb.

3-Bromo-5-(trifluoromethyl)benzoic acid (10). NBS (842 mg, 4.734 mmol) was added in small portions to a solution of 3-trifluoromethylbenzoic acid (600 mg, 3.156 mmol) in trifluoroacetic acid (1.6 mL) and conc. H₂SO₄ (480 μL). The resulting suspension was vigorously stirred at room temperature overnight, poured into crushed ice-water and the solid which separated out was filtered and dried to give title compound 10 (765 mg, 90%). The physical and spectroscopic properties of this compound were identical to those reported earlier.⁴

3-Bromo-N-methoxy-N-methyl-5-(trifluoromethyl)benzamide (11). A solution of acid 10 (433.1 mg, 1.609 mmol) in SOCl₂ (1.7 mL, 2.785 g, 23 mmol) was refluxed under nitrogen for 4 h. The excess of SOCl₂ was removed under vacuum and the residue dissolved in dry CH₂Cl₂ (5 mL). The obtained solution was added to a suspension of MeO(Me)NH·HCl (187 mg, 1.920 mmol) and Et₃N (535 µL (3.840 mmol) in anhydrous CH₂Cl₂ (10 mL) and the mixture stirred at room temperature under nitrogen for 18 h. After this time, the reaction mixture was poured into water and worked up in the usual way. Chromatography of the residue left after evaporation of the solvent, using hexane–EtOAc (from 9:1 to 8:2) as eluent, afforded amide 11 (488 mg, 97%) as a colorless oil. IR (NaCl) $\nu_{\max}$ (cm⁻¹) 3076w, 2973w, 2939w, 1651s, 1578w, 1460m, 1414s, 1384s, 1316s, 1207s, 1174s, 1132s, 1102s, 986m, 885m, 741m, 692s; ¹H-NMR (300 MHz) δ (ppm) 8.03 (1H, br s, H-2), 7.91 (1H, br s, H-6), 7.85 (1H, br s, H-4), 3.55 (3H, s, OCH₃), 3.38 (3H, s, CH₃); ¹³C-NMR (75 MHz) δ (ppm) 166.58 (CO), 136.42 (C-1), 134.71 (C-2), 132.14 (C-5, q, *J* = 32.6 Hz), 130.36 (C-4, q, *J* = 3.6 Hz), 124.03 (C-6, q, *J* = 3.6 Hz), 122.78 (CF₃, q, *J* = 273.1 Hz), 122.36 (C-3), 61.39 (OCH₃), 33.25 (CH₃); MS (EI) *m/z* (%) 314 (M⁺+1 for ⁸¹Br, 10), 313 (M⁺ for ⁸¹Br, 95), 312 (M⁺+1 for ⁷⁹Br, 12), 311 (M⁺ for ⁷⁹Br, 98), 293 (2), 252 (94), 250 (100), 224 (23), 222 (23), 172 (3); HRMS *m/z* calcd. for C₁₀H₉F₃⁷⁹BrNO₂ 310.9769, found 310.9761.

1-(3-Bromo-5-(trifluoromethyl)phenyl)ethanone (12). A 3 M solution of ClMgCH₃ in THF (2.4 mL, 7.106 mmol) was drop wise added to a solution of amide 11 (443.4 mg, 1.421 mmol) in dry THF (8.6 mL) under nitrogen at -78 °C. The reaction mixture was allowed to warm to room temperature and stirred for 19 h, then cooled to 0 °C, treated with a 1 M HCl and worked up as usually. Chromatographic purification, using hexane–EtOAc (8:2) as eluent, gave methyl ketone 12 (351.5 mg, 93%) as a colorless oil. IR (NaCl) $\nu_{\max}$ (cm⁻¹) 3079w, 3006w, 1688s, 1604w, 1582w, 1361m, 1324s, 1239s, 1175s, 1134s, 1098m, 885m, 783m, 692s; ¹H-NMR (300 MHz) δ (ppm) 8.25 (1H, br s, H-2), 8.11 (1H, br s, H-6), 7.95 (1H, br s, H-4), 2.64 (3H, s, CH₃); ¹³C-NMR (75 MHz) δ (ppm) 195.13 (CO), 139.05 (C-1), 134.58 (C-2), 132.87 (C-5, q, *J* = 32.8 Hz), 132.53 (C-4, q, *J* = 3.8 Hz), 123.72 (C-6, q, *J* = 3.8 Hz), 123.38 (C-3), 122.70 (CF₃, q, *J* = 272.9), 26.63 (CH₃); MS (EI) *m/z* (%) 268 (M⁺ for ⁸¹Br, 32), 266 (M⁺ for ⁷⁹Br, 34), 253 (45), 250 (45), 225 (15), 144 (13), 97 (23), 85 (50), 71 (65), 57 (100); HRMS *m/z* calcd. for C₉H₆F₃⁷⁹BrO 265.9554, found 265.9542.

tert-Butyl 6-(3-acetyl-5-(trifluoromethyl)phenyl)hex-5-ynoate (13). The synthesis of this compound was performed following the same procedure reported for compound 7, starting from aryl bromide 12 (330 mg, 1.236 mmol), *tert*-butyl hex-5-ynoate (623 mg, 3.708 mmol), CuI (7.0 mg, 0.037 mmol), Pd(PPh₃)₂Cl₂ (26 mg, 0.037 mmol), DMF (5.3 mL), and Et₃N (2.7 mL), and 2 h of reaction time. The crude product was

purified by chromatography, using hexane–Et₂O (9:1) as eluent, to give alkyne 13 (371.9 mg, 85%) as an oil. IR (NaCl) $\nu_{\max}$ (cm⁻¹) 2978w, 2928w, 1728m, 1698m, 1599w, 1449w, 1369m, 1352m, 1251s, 1172m, 1134s; ¹H-NMR (300 MHz) δ (ppm) 8.11 (1H, br s, H-2'), 8.08 (1H, br s, H-4'), 7.82 (1H, br s, H-6'), 2.64 (3H, s, CH₃), 2.50 (2H, t, J = 7.2 Hz, H₂-4), 2.41 (2H, t, J = 7.2 Hz, H₂-2), 1.91 (2H, quint, J = 7.2 Hz, H₂-3), 1.46 (9H, s, C(CH₃)₃); ¹³C-NMR (75 MHz) δ (ppm) 196.07 (CO), 172.32(CO₂^tBu), 137.55 (C-3'), 134.38 (C-2'), 132.27 (C-6', q, J = 3.6 Hz), 131.99 (C-5', q, J = 32.6 Hz), 125.62 (C-1'), 123.77 (C-4', q, J = 3.6 Hz), 122.40 (CF₃, q, J = 272.2), 92.45 (C-5), 80.49 (C-6), 79.09 (C(CH₃)₃), 34.37 (C-2), 28.10 (C(CH₃)₃), 26.68 (CH₃), 23.79 (C-3), 18.75 (C-4); MS (EI) m/z (%) 354 (M⁺, 0.2), 298 (23), 281 (16), 256 (10), 238 (21), 225 (6), 205 (4), 182 (7), 57 (100); HRMS m/z calcd. for C₁₉H₂₁F₃O₃ 354.1443, found 354.1436.

tert-Butyl 6-(3-acetyl-5-(trifluoromethyl)phenyl)hexanoate (14). Alkyne 13 (166.1 mg, 0.469 mmol) was hydrogenated as described above for the preparation of 7 using Rh(PPh₃)₃Cl (13 mg, 0.003 mmol) and THF (4.3 mL). Purification of the crude product by chromatography with hexane–Et₂O (9:1) as eluent gave title compound 14 (151.1 mg, 90%) as an oil. IR (NaCl) $\nu_{\max}$ (cm⁻¹) 2978w, 2935w, 2863w, 1728m, 1694m, 1606w, 1458w, 1368m, 1348m, 1245s, 1166s, 1129s; ¹H-NMR (300 MHz) δ (ppm) 8.00 (1H, br s, H-4'), 7.94 (1H, br s, H-2'), 7.61 (1H, br s, H-6'), 2.73 (2H, t, J = 7.6 Hz, H₂-6), 2.64 (3H, s, CH₃), 2.21 (2H, t, J = 7.3 Hz, H₂-2), 1.73–1.56 (4H, m, H₂-3 and H₂-5), 1.42 (9H, s, C(CH₃)₃), 1.36 (2H, m, H₂-3); ¹³C-NMR (75 MHz) δ (ppm) 196.93 (CO), 173.01 (CO₂^tBu), 144.33 (C-1'), 137.59 (C-3'), 131.32 (C-2'), 131.13 (C-5', q, J = 32.5 Hz), 129.44 (C-6', q, J = 3.5 Hz), 122.77 (C-4', q, J = 3.5 Hz), 122.38 (CF₃, q, J = 272.2), 80.07 (C(CH₃)₃), 35.48 (C-6), 35.33 (C-2), 30.88 (C-5), 28.55 (C-3), 28.07 (C(CH₃)₃), 26.72 (CH₃), 24.77 (C-4); MS (EI) m/z (%) 359 (M⁺+1, 0.7), 303 (100), 285 (66), 200 (8), 165 (4), 153 (72), 137 (26), 134 (49); HRMS m/z calcd. for C₁₉H₂₅F₃O₃ 358.1756, found 358.1742.

(*E*)-*tert*-Butyl 6-(3-(1-(hydroxyimino)ethyl)-5-(trifluoromethyl)phenyl)hexanoate (15). Prepared as described above for the preparation of 3, starting from methyl ketone 14 (130.7 mg, 0.365 mmol), hydroxylamine chlorhydrate (101 mg, 1.460 mmol), and pyridine (2.5 mL). Chromatography of the crude product, using hexane–Et₂O (8:2) as eluent, afforded oxime 15 (122.5 mg, 90%) as a colorless oil. IR (NaCl) $\nu_{\max}$ (cm⁻¹) 3359 (broad), 2979w, 2934w, 2862w, 1728m, 1696m, 1604w, 1368m, 1319d, 1272s, 1165s, 1126s, 1008w, 882w, 836w, 703w; ¹H-NMR (300 MHz) δ (ppm) 7.77 (1H, br s, OH), 7.69 (1H, br s, H-4'), 7.62 (1H, br s, H-2'), 7.42 (1H, br s, H-6'), 2.68 (2H, t, J = 7.6 Hz, H₂-6), 2.29 (3H, s, CH₃), 2.21 (2H, t, J = 7.3 Hz, H₂-2), 1.72–1.55 (4H, m, H₂-3 and H₂-5), 1.43 (9H, s, C(CH₃)₃), 1.36 (2H, m, H₂-3); ¹³C-NMR (75 MHz) δ (ppm) 173.12 (CO₂^tBu), 155.12 (C=N), 143.76 (C-1'), 137.28 (C-3'), 130.95 (C-5', q, J = 32.5

Hz), 129.18 (C-2'), 125.68 (C-6', q, $J = 3.8$ Hz), 122.38 (CF₃, q, $J = 272.2$), 120.42 (C-4', q, $J = 3.8$ Hz), 80.08 (C(CH₃)₃), 35.59 (C-6), 35.39 (C-2), 30.92 (C-5), 28.60 (C-3), 28.07 (C(CH₃)₃), 24.83 (C-4), 11.95 (CH₃); MS (EI) m/z (%) 373 (M⁺, 0.6), 342 (1), 317 (37), 300 (44), 272 (4), 256 (4), 240 (4), 230 (23), 212 (12), 198 (2), 184 (3), 158 (4), 146 (2), 57 (100); HRMS m/z calcd. for C₁₉H₂₆F₃NO₃ 373.1865, found 373.1857.

tert-Butyl 6-(3-((*E*)-1-(((2-((*E*)-2-methoxy-1-(methoxyimino)-2-oxoethyl)benzyl)oxy)imino)ethyl)-5-(trifluoromethyl)phenyl)hexanoate (17). This compound was prepared following the same procedure as for compound 6 starting from oxime 15 (58.3 mg, 0.156 mmol), benzyl bromide 16 (synthesized in the laboratory following a modification of literature procedures)^{5,6}, 44.7 mg, 0.156 mmol), and Cs₂CO₃ (76.4 mg, 0.234 mmol) in DMF (660 μ L). The crude product was purified by chromatography, using hexane–Et₂O (from 9:1 to 8.5:1.5) as eluent, to give title compound 17 (78.7 mg, 87%) as a colorless oil. IR (NaCl) $\nu_{\max}$ (cm⁻¹) 2984w, 2938w, 2848w, 1728s, 1598w, 1367m, 1279s, 1218m, 1164s, 1126s, 1069m, 1021m; ¹H-NMR (300 MHz) δ (ppm) 7.76 (1H, br s, H-4'), 7.57 (1H, br s, H-2'), 7.49 (1H, dd, $J = 7.4, 1.4$ Hz, H-3''), 7.46–7.34 (3H, m, H-5'', H-6' and H-4''), 7.18 (1H, dd, $J = 7.4, 1.3$ Hz, H-6''), 5.13 (2H, s, OCH₂), 4.03 (3H, s, NOCH₃), 3.82 (3H, s, CO₂CH₃), 2.66 (2H, t, $J = 7.7$, H₂-6), 2.25–2.17 (5H, m, H₂-2 and CH₃), 1.68–1.55 (4H, m, H₂-5 and H₂-3), 1.42 (9H, s, C(CH₃)₃), 1.35 (2H, m, H₂-3); ¹³C-NMR (75 MHz) δ (ppm) 173.05 (CO₂tBu), 163.27 (CO₂CH₃), 153.90 (CH₃CN), 149.50 (C=N), 143.61 (C-1'), 137.15 (C-1''), 135.90 (C-3'), 130.67 (C-5', q, $J = 31.9$ Hz), 129.75 (C-2''), 129.37 (C-4''), 129.24 (C-2'), 128.72 (C-3''), 128.48 (C-6''), 127.72 (C-5''), 125.49 (C-6', q, $J = 3.7$ Hz), 124.03 (CF₃, q, $J = 272.3$ Hz), 120.43 (C-4', q, $J = 3.7$ Hz), 80.01 (C(CH₃)₃), 74.82 (OCH₂), 63.77 (NOCH₃), 52.87 (CO₂CH₃), 35.56 (C-6), 35.36 (C-2), 30.91 (C-5), 28.61 (C-4), 28.04 (C(CH₃)₃), 24.81 (C-3), 12.66 (CH₃); MS (EI) m/z (%) 505 (12), 342 (4), 300 (8), 222 (47), 206 (60), 146 (14), 132 (56), 116 (100); FAB-HRMS m/z calcd. for C₃₀H₃₈F₃N₂O₆ [M+H]⁺ 579.2637, found 579.2643.

6-(3-((*E*)-1-(((2-((*E*)-2-Methoxy-1-(methoxyimino)-2-oxoethyl)benzyl)oxy)imino)ethyl)-5-(trifluoromethyl)phenyl)hexanoic acid (hapten TFb). Hydrolysis of the *tert*-butyl ester of 17 (78.7 mg, 0.136 mmol) with formic acid (2.8 mL) under the same conditions reported above for 8 afforded hapten TFb (65.9 mg, 93%) as a colorless oil. IR (NaCl) $\nu_{\max}$ (cm⁻¹) 3314 (broad), 2939m, 2859w, 1726s, 1598w, 1279s, 1218m, 1166m, 1124s, 1070m, 1020m, 956w, 886w, 703w; ¹H-NMR (300 MHz) δ (ppm) 7.66 (1H, br s, H-4'), 7.59 (1H, br s, H-2'), 7.50 (1H, dd, $J = 7.6, 1.5$ Hz, H-3''), 7.47–7.35 (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, OCH₂), 4.03 (3H, s, NOCH₃), 3.82 (3H, s, CO₂CH₃), 2.68 (2H, t, $J = 7.5$

1.4. Synthesis of hapten TFc

Allyl 3-oxo-3-(3-(trifluoromethyl)phenyl)propanoate (**19**). Methyl ketone **18** (1 g, 5.310 mmol) was drop wise added to a suspension of NaH (60 % dispersion in mineral oil, 532 mg, 13.27 mmol, prewashed with pentane) and diallyl carbonate (1.5 mL, 7.965 mmol) in dry benzene (5 mL) under nitrogen. The reaction mixture was heated at reflux for 5 h, and then cooled to 0 °C, cautiously treated with 3 M aqueous HCl solution (20 mL) and extracted with CH₂Cl₂. Work up in the usual way and purification by chromatography, using hexane–EtOAc (9:1) as eluent, afforded the β -keto ester **19** (952.5 mg, 66%) as a yellowish oil. IR (NaCl) $\nu_{\max}$ (cm⁻¹) 3038w, 2985w, 2943w, 1745s, 1696s, 1629m, 1440m, 1404m, 1328s, 1255s, 1170s, 1129s, 1098m, 1074s, 990m, 932m, 802m, 694m; ¹H-NMR (300 MHz) δ (ppm) 8.20 (1H, br s, H-2''), 8.13 (1H, d, J = 7.8 Hz, H-6''), 7.85 (1H, d, J = 7.8 Hz, H-4''), 7.64 (1H, br t, J = 7.8 Hz, H-5''), 5.88 (1H, m, H-2'), 5.36–5.21 (2H, m, H₂-3'), 4.66 (2H, dt, J = 5.7, 1.3 Hz, H₂-1'), 4.05 (2H, s, H₂-2); ¹³C-NMR (75 MHz) δ (ppm) 191.05 (CO), 166.61 (COO), 136.42 (C-1''), 131.65 (C-6''), 131.32 (C-2'), 131.50 (C-3''), q, J = 32.3 Hz, 130.17 (C-4''), q, J = 3.8 Hz, 129.53 (C-5''), 125.35 (C-2''), q, J = 3.8 Hz, 123.92 (CF₃, q, J = 272.0 Hz), 119.01 (C-3'), 66.26 (C-1'), 45.81 (C-2); MS (EI) m/z (%) 272 (M⁺, 4), 253 (2), 215 (8), 188 (35), 173 (100), 159 (4), 145 (30); HRMS m/z calcd. for C₁₃H₁₁F₃O₃ 272.0660, found 272.0668.

1-Allyl 7-tert-butyl 2-(3-(trifluoromethyl)benzoyl)heptanedioate (**20**). A solution of β -keto ester **19** (400 mg, 1.470 mmol) in dry DMF (2 mL) was drop wise added to a suspension of NaH (60% dispersion in mineral oil, 88 mg, 2.205 mmol, prewashed with pentane) in DMF (6 mL) at 0 °C under nitrogen. After about 30 min, when the evolution of hydrogen had ceased, *tert*-butyl 5-bromopentanoate (700 mg, 2.940 mmol) and KI (244 mg, 1.470 mmol) were added and the mixture was stirred at 50 °C for 18 h. The reaction mixture was poured into water and worked up in the usual manner extracting with EtOAc. Chromatographic purification, using hexane–Et₂O (from 8.5:1.5 to 8:2) as eluent, led to alkylated β -keto ester **20** (463 mg, 70%) as a yellow oil. IR (NaCl) $\nu_{\max}$ (cm⁻¹) 2980m, 2933w, 2863w, 1729s, 1695m, 1611d, 1367m, 1331s, 1254m, 1167s, 1127s, 1073m; ¹H-NMR (300 MHz) δ (ppm) 8.24 (1H, br s, H-2''), 8.16 (1H, d, J = 7.8 Hz, H-6''), 7.84 (1H, d, J = 7.8 Hz, H-4''), 7.63 (1H, br t, J = 7.8 Hz, H-5''), 5.87–5.72 (1H, m, H-2'), 5.27–5.12 (2H, m, H₂-3'), 4.58 (2H, br d, J = 5.8 Hz, H₂-1'), 4.32 (1H, t, J = 7.2 Hz, H-2), 2.21 (2H, t, J = 7.4 Hz, H₂-6), 2.05 (2H, m, H₂-4), 1.70–1.57 (4H, m, H₂-3 and H₂-5), 1.42 (9H, s, C(CH₃)₃); ¹³C-NMR (75 MHz) δ (ppm) 193.58 (CO), 172.74 (C-7), 169.08 (COO), 136.69 (C-1''), 131.65 (C-6''), 131.50 (C-3''), q, J = 32.5 Hz, 131.19 (C-2'), 129.88 (C-4''), q, J = 3.7 Hz, 129.45 (C-5''), 125.39 (C-2''), q, J = 3.9 Hz, 123.50 (CF₃, q, J = 272.4 Hz), 118.96 (C-3'), 80.12 (C(CH₃)₃), 66.08 (C-1'), 54.01 (C-2), 35.10 (C-6), 28.43 (C-3), 28.03

(C(CH₃)₃), 26.88 (C-4), 24.75 (C-5); MS (EI) *m/z* (%) 372 (4), 371 (5), 337 (7), 315 (4), 313 (7), 297 (8), 272 (17), 182 (27), 173 (100); FAB-HRMS *m/z* calcd. for C₂₂H₂₈F₃O₅ [M+H]⁺ 429.1889, found 429.1984.

tert-Butyl 7-oxo-7-(3-(trifluoromethyl)phenyl)heptanoate (21). A mixture of allyl ester 20 (214.3 mg, 0.500 mmol), morpholine (87 μ L, 87.61 mg, 1.00 mmol), and Pd(PPh₃)₄ (32 mg, 0.027 mmol) in anhydrous THF (2.8 mL) was stirred at room temperature under nitrogen for 40 min. The reaction mixture was diluted with water and worked up as usually, extracting with EtOAc, and the crude product purified by chromatography, using hexane–Et₂O (9:1) as eluent, to give title compound 21 (103.3 mg, 60%) as a colorless oil. IR (NaCl) $\nu_{\max}$ (cm⁻¹) 3420m (broad), 2956w, 1739s, 1685s, 1601w, 1573w, 1457w, 1435w, 1317w, 1281w, 1179m, 1129w, 1002m, 724m, 666m; ¹H-NMR (300 MHz) δ (ppm) 8.20 (1H, br s, H-2'), 8.12 (1H, d, *J* = 7.8 Hz, H-6'), 7.80 (1H, d, *J* = 7.8 Hz, H-4'), 7.60 (1H, br t, *J* = 7.8 Hz, H-5'), 3.00 (2H, t, *J* = 7.2 Hz, H₂-6), 2.24 (2H, t, *J* = 7.3 Hz, H₂-2), 1.77 (2H, quint, *J* = 7.3 Hz, H₂-3), 1.63 (2H, quint, *J* = 7.2 Hz, H₂-5), 1.43 (9H, s, C(CH₃)₃), 1.41 (2H, m, H₂-4); ¹³C-NMR (75 MHz) δ (ppm) 198.70 (CO), 173.02 (COO), 137.44 (C-1'), 131.18 (C-3', *q*, *J* = 32.9 Hz), 131.13 (C-6'), 129.31 (C-4', *q*, *J* = 3.6 Hz), 129.25 (C-5'), 124.82 (C-2', *q*, *J* = 3.8 Hz), 123.65 (CF₃, *q*, *J* = 272.5 Hz), 80.06 (C(CH₃)₃), 38.39 (C-6), 35.31 (C-2), 28.61 (C-4), 28.07 (C(CH₃)₃), 24.82 (C-5), 23.62 (C-3); MS (EI) *m/z* (%) 288 (25), 271 (18), 251 (23), 225 (20), 188 (94), 173 (100), 145 (40); FAB-HRMS *m/z* calcd. for C₁₈H₂₃F₃O₃ 344.1599, found 344.1598.

(*E*)-*tert*-Butyl 7-(hydroxyimino)-7-(3-(trifluoromethyl)phenyl)heptanoate (22). Prepared as described above for the preparation of 3, starting from ketone 21 (98.4 mg, 0.286 mmol), hydroxylamine chlorhydrate (80 mg, 1.144 mmol), and pyridine (1.5 mL). Chromatography of the crude product, using hexane–Et₂O (8:2) as eluent, afforded oxime 22 (99.4 mg, 97%) as a colorless oil. IR (NaCl) $\nu_{\max}$ (cm⁻¹) 3365w (broad), 2978w, 2933w, 2866w, 1728m, 1334m, 1269m, 1166s, 1127s, 1074m, 698m, 666m; ¹H-NMR (300 MHz) δ (ppm) 7.95 (1H, br s, OH), 7.87 (1H, br s, H-2'), 7.77 (1H, d, *J* = 7.8 Hz, H-6'), 7.62 (1H, d, *J* = 7.8 Hz, H-4'), 7.50 (1H, br t, *J* = 7.8 Hz, H-5'), 2.82 (2H, t, *J* = 7.8 Hz, H₂-6), 2.20 (2H, t, *J* = 7.4 Hz, H₂-2), 1.65–1.55 (4H, m, H₂-5 and H₂-3), 1.42 (9H, s, C(CH₃)₃), 1.40 (2H, m, H₂-4); ¹³C-NMR (75 MHz) δ (ppm) 173.14 (CO₂^tBu), 158.58 (C=N), 136.52 (C-1'), 130.98 (C-3', *q*, *J* = 32.3 Hz), 129.43 (C-6'), 129.05 (C-5'), 125.73 (C-4', *q*, *J* = 3.6 Hz), 124.45 (CF₃, *q*, *J* = 272.4 Hz), 123.04 (C-2', *q*, *J* = 3.9 Hz), 80.10 (C(CH₃)₃), 35.34 (C-2), 29.04 (C-4), 28.06 (C(CH₃)₃), 25.89 (C-5), 25.63 (C-6), 24.71 (C-3); MS (EI) *m/z* (%) 359 (M⁺, 0.1), 303 (5), 286 (14), 240 (1), 203 (20), 187 (2), 178 (4), 145 (5), 97 (2), 83 (2), 71 (2), 69 (8), 57 (100); HRMS *m/z* calcd. for C₁₈H₂₄F₃NO₃ 359.1708, found 359.1717.

(*E*)-*tert*-Butyl 7-(((2-((*E*)-2-methoxy-1-(methoxyimino)-2-oxoethyl)benzyl)oxy)imino)-7-(3-(trifluoromethyl)phenyl)heptanoate (23). This compound was prepared following basically the procedure already described for compound 6 starting from oxime 22 (32.8 mg, 0.091 mmol), benzyl bromide 16 (31.2 mg, 0.109 mmol), and Cs₂CO₃ (44 mg, 0.136 mmol) in DMF (1.0 mL) and 4 h of reaction time. The crude product was purified by chromatography, using hexane–Et₂O (8:2) as eluent, to give title compound 23 (50.4 mg, 98%) as a colorless oil. IR (NaCl) $\nu_{\max}$ (cm⁻¹) 2980w, 2939m, 2869w, 1728s, 1344m, 1304m, 1276m, 1218m, 1127s, 1072s, 1019m; ¹H-NMR (300 MHz) δ (ppm) 7.88 (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''), 7.49 (1H, m, H-3' partially overlapped with H-5', H-4' and H-5''), 7.46 (1H, m, H-5' partially overlapped with H-5', H-4' and H-3') 7.40 (1H, m, H-4' partially overlapped with H-5', H-3' and H-5''), 7.38 (1H, m, H-5' partially overlapped with H-5', H-6' and H-5''), 7.18 (1H, dd, *J* = 7.3, 1.2 Hz, H-6'), 5.11 (2H, s, OCH₂), 4.04 (3H, s, NOCH₃), 3.81 (3H, s, CO₂CH₃), 2.74 (2H, t, *J* = 7.6 Hz, H₂-6), 2.17 (2H, t, *J* = 7.6 Hz, H₂-2), 1.50–1.40 (4H, m, H₂-5 and H₂-3), 1.42 (9H, s, C(CH₃)₃), 1.37 (2H, m, H₂-4); ¹³C-NMR (75 MHz) δ (ppm) 172.99 (CO₂^tBu), 163.26 (CO₂CH₃), 157.49 (CH₂CN), 149.35 (C=N), 136.39 (C-1'), 136.12 (C-1''), 130.81 (C-3'', q, *J* = 32.3 Hz), 129.50 (C-4'), 129.48 (C-2'), 129.42 (C-6''), 128.82 (C-3'), 128.54 (C-5''), 128.40 (C-6'), 127.61 (C-5'), 125.56 (C-4'', q, *J* = 3.7 Hz), 123.96 (CF₃, q, *J* = 272.3 Hz), 123.04 (C-2'', q, *J* = 3.9 Hz), 80.00 (C(CH₃)₃), 74.49 (OCH₂), 63.79 (NOCH₃), 52.89 (CO₂CH₃), 35.30 (C-2), 29.12 (C-4), 28.06 (C(CH₃)₃), 26.21 (C-6), 26.06 (C-3), 24.71 (C-5); MS (EI) *m/z* (%) 564 (M⁺, 1), 509 (1), 508 (5), 491 (8), 477 (1), 342 (1), 317 (2), 286 (16), 240 (5), 222 (45), 206 (100); HRMS *m/z* calcd. for C₂₉H₃₅F₃N₂O₆ 564.2447, found 564.2437.

(*E*)-7-(((2-((*E*)-2-Methoxy-1-(methoxyimino)-2-oxoethyl)benzyl)oxy)imino)-7-(3-(trifluoromethyl)phenyl)heptanoic acid (haptent TFc). Hydrolysis of the *tert*-butyl ester of 23 (58.8 mg, 0.104 mmol) with formic acid (2.1 mL) under the same conditions reported above for 8 afforded, after chromatographic purification with CHCl₃ as eluent, haptent TFc (49.7 mg, 94%) as a colorless oil. IR (NaCl) $\nu_{\max}$ (cm⁻¹) 3339 (broad), 2941s, 2853m, 2689w, 1728s, 1711s, 1607w, 1437s, 1340s, 1304s, 1277s, 1220s, 1168s, 1127s, 1071s, 1019s, 958m, 699m; 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''), 7.49–7.37 (4H, m, H-3', H-4', H-5', and H-5''), 7.19 (1H, dd, *J* = 7.7, 1.5 Hz, H-6'), 5.11 (2H, s, OCH₂), 4.04 (3H, s, NOCH₃), 3.81 (3H, s, CO₂CH₃), 2.74 (2H, t, *J* = 7.9 Hz, H-6), 2.31 (2H, t, *J* = 7.4 Hz, H-2), 1.63 (2H, quint, *J* = 7.5 Hz, H-3), 1.53 (2H, m, H-5), 1.39 (2H, m, H-4); ¹³C-NMR (75 MHz) δ (ppm) 179.1 (CO₂H), 163.3 (CO₂CH₃), 157.4 (CH₂C=N), 149.4 (C=N), 136.3 (C-1'), 136.1 (C-1''), 130.9 (C-3'', q, *J* = 32 Hz), 130.2 (C-2'), 129.5 (C-4'), 129.4 (C-6''), 128.9 (C-3'), 128.6 (C-5''), 128.4 (C-6') 127.7 (C-5'),

125.6 (C-4'', q, $J = 3.8$ Hz), 123.9 (CF₃, q, $J = 272$ Hz), 123.1 (C-2'', q, $J = 3.8$ Hz), 74.5 (OCH₂), 63.8 (NOCH₃), 52.9 (CO₂CH₃), 33.7 (C-2), 29.0 (C-4), 26.1 (C-6), 26.0 (C-5), 24.2 (C-3); MS (EI) m/z (%) 508 (M⁺, 1), 491 (2), 286 (8), 266 (3), 222 (35), 206 (88), 190 (7), 187 (9), 172 (6), 116 (100); HRMS m/z calcd for C₂₆H₂₇F₃N₂O₆ 508.1821, found 508.1807; UV (100 mM sodium phosphate buffer, pH 7.4) ϵ (280 nm) 3.36 mM⁻¹ cm⁻¹.

1.5. Synthesis of hapten TFe.

Figure S4. Synthetic sequence for the preparation of hapten TFe.

(E)-2-(Methoxyimino)-2-(2-(((*E*)-(1-(3-(trifluoromethyl)phenyl)ethylidene)amino)oxy)methyl)phenyl)acetic acid (**24**). A solution of trifloxystrobin (5.0 g, 12.24 mmol) in a mixture of 5 M aqueous sodium hydroxide (12.5 mL) and methanol (70 mL) was stirred under reflux for 5 h. The methanol evaporated and the residue obtained diluted with water and extracted with EtOAc. The aqueous layer was acidified to pH 2–3 with 5 M aqueous HCl solution and extracted with EtOAc. Work up as usual and purification by crystallization from hexane–acetone afforded acid **24** (3.52 g, 73%) as a white solid. The physical and spectroscopic properties of this compound have been previously described.^{7,8}

tert-Butyl 6-((*E*)-2-(methoxyimino)-2-(2-(((*E*)-(1-(3-(trifluoromethyl)phenyl)ethylidene)amino)oxy)methyl)phenyl)acetoxyl)hexanoate (**25**). A mixture of acid **24** (100 mg, 0.253 mmol), *tert*-butyl 6-bromohexanoate (76.2 mg, 0.304 mmol), and anhydrous K₂CO₃ (42 mg, 0.304 mmol) in dry DMF (10 mL) was stirred at room temperature for 24 h. The reaction mixture was poured into water and worked up in the usual manner extracting with EtOAc. Purification of the crude product by chromatography, using hexane–Et₂O (from 9:1 to

7:3) as eluent, afforded title compound 25 as a colorless oil (100 mg, 70%). IR (NaCl) $\nu_{\max}$ (cm⁻¹) 2984w, 2940m, 2859w, 1725s, 1618w, 1459w, 1368m, 1341m, 1298m, 1275s, 1216m, 1167s, 1127s, 1071s, 1014m; ¹H-NMR (300 MHz) δ (ppm) 7.86 (1H, br s, H-2''), 7.79 (1H, d, J = 7.9 Hz, H-6''), 7.59 (1H, d, J = 7.9 Hz, H-4''), 7.48 (1H, m, H-6' partially overlapped with H-4', H-5' and H-5''), 7.46 (1H, m, H-5'' partially overlapped with H-4', H-5' and H-6') 7.42 (1H, m, H-5' partially overlapped with H-4', H-6' and H-5''), 7.37 (1H, m, H-4' partially overlapped with H-5', H-6' and H-5''), 7.18 (1H, dd, J = 7.5, 1.8 Hz, H-3'), 5.14 (2H, s, OCH₂), 4.20 (2H, t, J = 6.8 Hz, H₂-6), 4.02 (3H, s, NOCH₃), 2.23 (3H, s, CH₃), 2.15 (2H, t, J = 7.4 Hz, H₂-2), 1.65 (2H, quint, J = 7.3 Hz, H₂-5), 1.54 (2H, quint, J = 7.3 Hz, H₂-3), 1.43 (9H, s, C(CH₃)₃), 1.30 (2H, m, H₂-4); ¹³C-NMR (75 MHz) δ (ppm) 172.86 (CO₂tBu), 162.87 (CO₂CH₂), 151.90 (CH₃CN), 149.68 (C=N), 137.18 (C-2'), 135.90 (C-1''), 129.80 (C-3'', q, J = 32.1 Hz), 129.28 (C-5' and C-6''), 128.81 (C-6'), 128.61 (C-5''), 128.49 (C-3'), 127.68 (C-4'), 127.32 (C-1'), 125.62 (C-4'', q, J = 3.8 Hz), 123.96 (CF₃, q, J = 272.6 Hz), 122.88 (C-2'', q, J = 3.8 Hz), 80.07 (C(CH₃)₃), 74.89 (C-6), 65.77 (OCH₂), 63.73 (NOCH₃), 35.28 (C-2), 28.16 (C-5), 28.08 (C(CH₃)₃), 25.23 (C-4), 24.37 (C-3), 12.57 (CH₃); MS (EI) m/z (%) 564 (M⁺, 0.2), 533 (2), 509 (1), 491 (8), 322 (35), 192 (79), 186 (42), 172 (5), 145 (12), 132 (8), 116 (94), 115 (43), 97 (19), 69 (32), 57 (100); HRMS m/z calcd. for C₂₉H₃₅F₃N₂O 564.2447, found 564.2447.

6-((*E*)-2-(Methoxyimino)-2-(2-((((*E*)-(1-(3-(trifluoromethyl)phenyl)ethylidene)amino)oxy)methyl)phenyl)acetoxyl)hexanoic acid (hapten TFe). Hydrolysis of the *tert*-butyl ester of 25 (32.7 mg, 0.079 mmol) with formic acid (1.6 mL) under the same conditions reported above for 8 afforded, after chromatographic purification with CHCl₃ as eluent, hapten TFe (30.3 mg, 93%) as a colorless oil. IR (NaCl) $\nu_{\max}$ (cm⁻¹) 3347 (broad), 3063w, 2941m, 1713s, 1608w, 1586w, 1341s, 1299s, 1276s, 1168s, 1126s, 1071s, 1014s, 738m, 698m; ¹H-NMR (300 MHz) δ (ppm) 7.86 (1H, br s, H-2''), 7.79 (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 = 7.6, 1.7 Hz, H-3'), 5.15 (2H, s, OCH₂), 4.21 (2H, t, J = 6.7 Hz, H-6), 4.02 (3H, s, NOCH₃), 2.31 (2H, t, J = 7.3 Hz, H-2) 2.23 (3H, s, CH₃), 1.72–1.52 (4H, m, H-3 and H-5), 1.31 (2H, m, H-4); ¹³C-NMR (75 MHz) δ (ppm) 179.3 (CO₂H), 162.8 (CO₂CH₂), 153.66 (CH₃C=N), 149.65 (C=N), 137.1 (C-2'), 135.9 (C-1''), 130.7 (C-3'', q, J = 32 Hz), 129.9 (C-1'), 129.3 (C-5' and C-6''), 128.8 (C-6'), 128.6 (C-5''), 128.5 (C-3'), 127.7 (C-4'), 125.6 (C-4'', q, J = 3.8 Hz), 123.9 (CF₃, q, J = 272 Hz), 122.8 (C-2'', q, J = 3.8 Hz), 74.9 (C-6), 65.6 (OCH₂), 63.7 (NOCH₃), 33.7 (C-2), 28.0 (C-5), 25.2 (C-4), 24.1 (C-3), 12.6 (CH₃); MS (EI) m/z (%) 444 (2), 286 (5%), 192 (36), 121 (21), 116 (100), 69 (46); FAB-HRMS m/z calcd for C₂₅H₂₈F₃N₂O₆ [M+H]⁺ 509.1900, found 509.1901; UV (100 mM sodium phosphate buffer, pH 7.4) ϵ (280 nm) 2.80 mM⁻¹ cm⁻¹.

1.6. Synthesis of hapten TFo

Figure S5. Synthetic sequence for the preparation of hapten TFo.

(*E*)-*tert*-Butyl 6-(((2-methoxy-2-oxo-1-(*o*-tolyl)ethylidene)amino)oxy)hexanoate (**27**). A mixture of oxime **26** (synthesized in the laboratory following the procedure described by Li et al.,^{9,10} 90 mg, 0.466 mmol), *tert*-butyl 6-bromohexanoate (117 mg, 0.466 mmol), and anhydrous Cs_2CO_3 (228 mg, 0.699 mmol) in dry DMF (1.7 mL) was stirred under nitrogen for 4 h, poured into water and worked up as usual using EtOAc to extract. Chromatography of the crude product, using hexane–EtOAc (9:1) as eluent, afforded the corresponding O-alkylated oxime **27** (141 mg, 83%) as a colorless oil. IR (NaCl) ν_{max} (cm^{-1}) 2973m, 2937m, 2858m, 1728s, 1655w, 1367m, 1321m, 1279d, 1219m, 1152m, 1068m, 998m, 755m; ^1H -NMR (300 MHz) δ (ppm) 7.30 (1H, td, $J = 7.6, 7.0, 1.1$ Hz, H-5'), 7.18 (2H, m, H-4' and H-6'), 7.09 (1H, dd, $J = 7.3, 1.7$ Hz, H-3'), 4.25 (2H, t, $J = 6.8$ Hz, H₂-6), 3.86 (3H, s, CO_2CH_3), 2.18 (3H, s, CH_3), 2.17 (2H, t, $J = 7.4$ Hz, H₂-2), 1.68 (2H, quint, $J = 7.1$ Hz, H₂-5), 1.56 (2H, quint, $J = 7.4$ Hz, H₂-3), 1.43 (9H, s, $\text{C}(\text{CH}_3)_3$), 1.29 (2H, m, H₂-4); ^{13}C -NMR (75 MHz) δ (ppm) 172.90 (CO_2^tBu), 163.72 (CO_2CH_3), 149.79 ($\text{C}=\text{N}$), 135.95 (C-2'), 130.60 (C-1'), 129.86 (C-6'), 129.15 (C-5'), 127.91 (C-3'), 125.42 (C-4'), 80.01 ($\text{C}(\text{CH}_3)_3$), 76.05 (C-6), 52.86 (CO_2CH_3), 35.38 (C-2), 28.74 (C-5), 28.09 ($\text{C}(\text{CH}_3)_3$), 25.16 (C-4), 24.74 (C-3), 19.53 (CH_3); MS (EI) m/z (%) 363 (M^+ , 0.2), 308 (0.4), 276 (4), 234 (19), 233 (15), 218 (6), 176 (65), 132 (29), 118 (22), 117 (34), 57 (100); HRMS m/z calcd. for $\text{C}_{20}\text{H}_{29}\text{NO}_5$ 363.2046, found 363.2044.

(*E*)-*tert*-Butyl 6-(((1-(2-(bromomethyl)phenyl)-2-methoxy-2-oxoethylidene)amino)oxy)hexanoate (28). A solution of O-alkyloxime 27 (123 mg, 0.339 mmol), NBS (60.3 mg, 0.339 mmol), and AIBN (3.3 mg, 0.020 mmol) in anhydrous benzene (1.5 mL) was degassed by nitrogen bubbling and ultrasound for 15 min and then heated at reflux overnight. The reaction mixture was allowed to cool room temperature, diluted with EtOAc and worked up as usual to afford the crude product as a pale brown oil. Purification by column chromatography, using hexane–Et₂O (from 9:1 to 8:2) as eluent, gave benzyl bromide 28 (137.8, 92%) as a brownish oil. IR (NaCl) $\nu_{\max}$ (cm⁻¹) 2970w, 2938w, 1728s, 1588w, 1367m, 1219m, 1152s, 1066m, 1001m; ¹H-NMR (300 MHz) δ (ppm) 7.48 (1H, dd, *J* = 7.4, 1.6 Hz, H-6'), 7.40 (1H, td, *J* = 7.4, 7.3, 1.7 Hz, H-5') 7.35 (1H, td, *J* = 7.3, 7.3, 1.6 Hz, H-4'), 7.13 (1H, dd, *J* = 7.3, 1.7 Hz, H-3'), 4.33 (2H, s, CH₂Br), 4.27 (2H, t, *J* = 6.9 Hz, H₂-6), 3.87 (3H, s, CO₂CH₃), 2.16 (2H, t, *J* = 7.3 Hz, H₂-2), 1.68 (2H, quint, *J* = 7.1 Hz, H₂-5), 1.54 (2H, quint, *J* = 7.3 Hz, H₂-3), 1.42 (9H, s, C(CH₃)₃), 1.28 (2H, m, H₂-4); ¹³C-NMR (75 MHz) δ (ppm) 172.90 (CO₂^tBu), 163.77 (CO₂CH₃), 149.57 (C=N), 138.22 (C-2'), 130.58 (C-1'), 130.11 (C-6'), 129.72 (C-5'), 128.67 (C-3'), 128.38 (C-4'), 80.04 (C(CH₃)₃), 76.34 (C-6), 53.07 (CO₂CH₃), 35.34 (C-2), 30.88 (CH₂Br), 28.69 (C-5), 28.08 (C(CH₃)₃), 25.13 (C-4), 24.70 (C-3); MS (EI) *m/z* (%) 384 (10), 354 (11), 307 (33), 256 (32), 175 (55), 146 (60), 116 (100); FAB-HRMS *m/z* calcd. for C₂₀H₂₉⁷⁹BrNO₅ [M+H]⁺ 442.1229, found 442.1181.

tert-Butyl 6-(((*E*)-(2-methoxy-2-oxo-1-(2-(((*E*)-(1-(3-(trifluoromethyl)phenyl)ethylidene)amino)oxy)methyl)phenyl)ethylidene)amino)oxy)hexanoate (29). This compound was prepared following the procedure described above for compound 6 starting from benzyl bromide 28 (52.2 mg, 0.118 mmol), oxime 5 (25.6 mg, 0.118 mmol), and Cs₂CO₃ (57.6 mg, 0.177 mmol) in DMF (0.5 mL) and 6 h of reaction time. The crude product was purified by chromatography, using hexane–Et₂O (from 9:1 to 8:2) as eluent, to give title compound 29 (62.6 mg, 92%) as a colorless oil. (NaCl) $\nu_{\max}$ (cm⁻¹) 2974m, 2938m, 1723s, 1608d, 1590w, 1437m, 1368m, 1341s, 1299m, 1275s, 1219m, 1167s, 1127s, 1072s, 1000m, 698m, 672m; ¹H-NMR (300 MHz) δ (ppm) 7.86 (1H, br s, H-2''), 7.79 (1H, d, *J* = 7.9 Hz, H-6''), 7.59 (1H, d, *J* = 7.9 Hz, H-4''), 7.48 (1H, m, H-6' partially overlapped with H-4', H-5' and H-5''), 7.46 (1H, m, H-5'' partially overlapped with H-4', H-5' and H-6') 7.42 (1H, m, H-5' partially overlapped with H-4', H-6' and H-5''), 7.38 (1H, m, H-4' partially overlapped with H-5', H-6' and H-5''), 7.18 (1H, dd, *J* = 7.1, 1.7 Hz, H-3'), 5.14 (2H, s, OCH₂), 4.23 (2H, t, *J* = 6.9 Hz, H₂-6), 3.80 (3H, s, CO₂CH₃), 2.22 (3H, s, CH₃), 2.11 (2H, t, *J* = 7.4 Hz, H₂-2), 1.66 (2H, quint, *J* = 7.2 Hz, H₂-5), 1.54 (2H, quint, *J* = 7.4 Hz, H₂-3), 1.42 (9H, s, C(CH₃)₃), 1.27 (2H, m, H₂-4); ¹³C-NMR (75 MHz) δ (ppm) 172.89 (CO₂^tBu), 163.45 (CO₂CH₃), 153.54 (CH₃CN), 149.24 (C=N), 137.17 (C-2'), 135.87

(C-1''), 130.70 (C-3'', q, $J = 32.0$ Hz), 129.99 (C-1'), 129.23 (C-6''), 129.22 (C-5'), 128.79 (C-6'), 128.64 (C-5''), 128.49 (C-3'), 127.69 (C-4'), 125.56 (C-4'', q, $J = 3.8$ Hz), 123.90 (CF₃, q, $J = 272.0$ Hz), 122.82 (C-2'', q, $J = 3.8$ Hz), 79.99 (C(CH₃)₃), 76.13 (C-6), 74.91 (OCH₂), 52.80 (CO₂CH₃), 35.32 (C-2), 28.68 (C-5), 28.05 (C(CH₃)₃), 25.11 (C-4), 24.70 (C-3), 12.50 (CH₃); MS (EI) m/z (%) 377 (4), 362 (4), 323 (12), 322 (70), 319 (6), 307 (7), 306 (39), 304 (18), 192 (61), 186 (87), 172 (12), 160 (12), 148 (32), 132 (12), 116 (100); FAB-HRMS m/z calcd. for C₂₉H₃₆F₃N₂O₆ [M+H]⁺ 565.2525, found 565.2526.

6-(((E)-(2-Methoxy-2-oxo-1-(2-(((E)-(1-(3-(trifluoromethyl)phenyl)ethylidene)amino)oxy)methyl)phenyl)ethylidene)amino)oxy)hexanoic acid (hapten TFO). Hydrolysis of the *tert*-butyl ester of 29 (88.8 mg, 0.154 mmol) with formic acid (3.2 mL) under the same conditions reported above for 8 afforded, after chromatographic purification with CHCl₃–MeOH (99:1) as eluent, hapten TFO (73.6 mg, 92%) as a colorless oil. IR (NaCl) $\nu_{\max}$ (cm⁻¹) 3314 (broad), 2948m, 2880m, 1727s, 1709s, 1608w, 1586w, 1341s, 1275s, 1167s, 1120s, 1072s, 1001s, 737m, 698m; ¹H-NMR (300 MHz) δ (ppm) 7.86 (1H, br s, H-2''), 7.79 (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'), 7.17 (1H, dd, $J = 7.4, 1.5$ Hz, H-3'), 5.14 (2H, s, OCH₂), 4.24 (2H, t, $J = 6.7$ Hz, H-6), 3.81 (3H, s, CO₂CH₃), 2.29 (2H, t, $J = 7.4$ Hz, H-2), 2.22 (3H, s, CH₃), 1.67 (2H, quint, $J = 7.4$ Hz, H-5), 1.59 (2H, quint, $J = 7.4$ Hz, H-3), 1.30 (2H, m, H-4); ¹³C-NMR (75 MHz) δ (ppm) 179.4 (CO₂H), 163.5 (CO₂CH₃), 153.6 (CH₃C=N), 149.3 (C=N), 137.1 (C-2'), 135.8 (C-1''), 130.7 (C-3'', q, $J = 32$ Hz), 129.9 (C-1'), 129.3 (C-5' and C-6''), 128.8 (C-6'), 128.6 (C-5''), 128.5 (C-3'), 127.7 (C-4'), 125.59 (C-4'', q, $J = 3.8$ Hz), 124.0 (CF₃, q, $J = 272$ Hz), 122.8 (C-2'', q, $J = 3.8$ Hz), 75.97 (C-6), 74.9 (OCH₂), 52.8 (CO₂CH₃), 33.7 (C-2), 28.6 (C-5), 25.1 (C-4), 24.2 (C-3), 12.5 (CH₃); FAB-HRMS m/z calcd for C₂₅H₂₈F₃N₂O₆ [M+H]⁺ 509.1899, found 509.1912; UV (100 mM sodium phosphate buffer, pH 7.4) ϵ (280 nm) 3.47 mM⁻¹ cm⁻¹.

1.7. Synthesis of haptens TFf and TFt

Figure S6. Synthetic sequence for the preparation of haptens TFf and TFt.

tert-Butyl 6-(3-acetylphenyl)hex-5-ynoate (**31**). The synthesis of this compound was performed following the same procedure described above for compound **7**, starting from 1-(3-bromophenyl)ethanone (**30**, 360.3 mg, 1.810 mmol), *tert*-butyl hex-5-ynoate (912.7 mg, 4.430 mmol), CuI (10.3 mg, 0.054 mmol), $\text{Pd}(\text{PPh}_3)_2\text{Cl}_2$ (38 mg, 0.044 mmol), DMF (5.5 mL), and Et_3N (3.0 mL), and 6 h of reaction time. The crude product was purified by chromatography, using hexane– Et_2O (9:1) as eluent, to give alkyne **31** (414.2 mg, 80%) as yellowish oil. IR (NaCl) ν_{max} (cm^{-1}) 2977m, 2934m, 2231w, 1727s, 1687s, 1597w, 1574w, 1424m, 1366s, 1219s, 1148s, 800m, 688m; ^1H -NMR (300 MHz) δ (ppm) 7.96 (1H, t, $J = 1.6$ Hz, H-2'), 7.85 (1H,

ddd, $J = 7.7, 1.6, 1.6$ Hz, H-6'), 7.56 (1H, ddd, $J = 7.7, 1.6, 1.3$ Hz, H-4'), 7.37 (1H, t, $J = 7.7$ Hz, H-5'), 2.59 (3H, s, CH₃), 2.47 (2H, t, $J = 7.1$ Hz, H-4), 2.40 (2H, t, $J = 7.2$ Hz, H-2), 1.89 (2H, quint, $J = 7.2$ Hz, H-3), 1.45 (9H, s, C(CH₃)₃); ¹³C-NMR (75 MHz) δ (ppm) 197.45 (CO), 172.40 (CO₂tBu), 137.07 (C-3'), 135.86 (C-4'), 131.54 (C-2'), 128.52 (C-5'), 127.22 (C-6'), 124.41 (C-1'), 90.36 (C-5), 80.32 (C-6), 80.27 (C(CH₃)₃), 34.45 (C-2), 28.06 (C(CH₃)₃), 26.59 (CH₃), 23.97 (C-3), 18.81 (C-4); ESI-HRMS m/z calcd. for C₁₈H₂₂NaO₃ [M+Na]⁺ 309.1467, found 309.1460.

(*E*)-*tert*-Butyl 6-(3-(1-(hydroxyimino)ethyl)phenyl)hex-5-ynoate (32). Obtained as described above for the preparation of 3, starting from methyl ketone 31 (56.4 mg, 0.197 mmol), hydroxylamine chlorhydrate (55 mg, 0.788 mmol), and dry pyridine (1.3 mL). Chromatography of the crude product, using hexane–Et₂O (from 9:1 to 8:2) as eluent, afforded oxime 32 (54.9 mg, 92%) as an oil. IR (NaCl) $\nu_{\max}$ (cm⁻¹) 3406m, 2975m, 2933m, 2355w, 2219w, 1725s, 1594w, 1368m, 1313m, 1247m, 1149s, 1003m, 911w, 843w, 796w; ¹H-NMR (300 MHz) δ (ppm) 8.26 (1H, br s, OH), 7.64 (1H, t, $J = 1.5$ Hz, H-2'), 7.55 (1H, ddd, $J = 7.7, 1.5, 1.5$ Hz, H-6'), 7.39 (1H, ddd, $J = 7.7, 1.5, 1.5$ Hz, H-4'), 7.29 (1H, t, $J = 7.7$ Hz, H-5'), 2.47 (2H, t, $J = 7.1$ Hz, H-4), 2.41 (2H, t, $J = 7.2$ Hz, H-2), 2.27 (3H, s, CH₃), 1.89 (2H, quint, $J = 7.1$ Hz, H-3), 1.45 (9H, s, C(CH₃)₃); ¹³C-NMR (75 MHz) δ (ppm) 172.36 (CO₂tBu), 155.51 (C=N), 136.60 (C-3'), 132.20 (C-4'), 129.30 (C-2'), 128.38 (C-5'), 125.21 (C-6'), 124.11 (C-1'), 89.58 (C-5), 80.85 (C-6), 80.35 (C(CH₃)₃), 34.45 (C-2), 28.13 (C(CH₃)₃), 24.12 (C-3), 18.84 (C-4), 11.96 (CH₃); ESI-HRMS m/z calcd. for C₁₈H₂₃NNaO₃ [M+Na]⁺ 324.1576, found 324.1580.

tert-Butyl 6-(3-((*E*)-1-(((*E*)-2-methoxy-1-(methoxyimino)-2-oxoethyl)benzyl)oxy)imino)ethyl)phenyl)hex-5-ynoate (33). This compound was prepared following the procedure described above for compound 6 starting from oxime 32 (40.3 mg, 0.134 mmol), benzyl bromide 16 (57.4 mg, 0.201 mmol), and Cs₂CO₃ (65.5 mg, 0.201 mmol) in DMF (0.9 mL) and 15 h of reaction time. Chromatographic purification of the crude product, using hexane–Et₂O (from 9:1 to 8:2) as eluent, afforded title compound 33 (57.3 mg, 84%) as a colorless oil. IR (NaCl) $\nu_{\max}$ (cm⁻¹) 2972w, 2938w, 2339w, 1727s, 1590w, 1367m, 1219m, 1147m, 1069s, 1019m, 946w, 861w, 787w; ¹H-NMR (300 MHz) δ (ppm) 7.62 (1H, t, $J = 1.5$ Hz, H-2'), 7.55–7.47 (2H, m, H-6' and H-3''), 7.46–7.33 (3H, m, H-5'', H-4'' and H-4'), 7.25 (1H, t, $J = 7.7$ Hz, H-5'), 7.18 (1H, dd, $J = 7.3, 1.4$ Hz, H-6''), 5.12 (2H, s, OCH₂), 4.03 (3H, s, NOCH₃), 3.82 (3H, s, CO₂CH₃), 2.46 (2H, t, $J = 7.1$ Hz, H-4), 2.40 (2H, t, $J = 7.1$ Hz, H-2), 2.18 (3H, s, CH₃), 1.89 (2H, quint, $J = 7.2$ Hz, H-3), 1.46 (9H, s, C(CH₃)₃); ¹³C-NMR (75 MHz) δ (ppm) 172.49 (CO₂tBu), 163.28 (CO₂CH₃), 154.39 (CH₃CN), 149.77 (C=N), 136.51 (C-1'), 136.17 (C-3'), 132.12 (C-6'), 129.36 (C-2'), 128.71 (C-4'), 128.60 (C-5'), 128.50 (C-6''),

128.24 (C-5'), 127.63 (C-4''), 125.34 (C-3'' and C-2''), 123.92 (C-1'), 89.43 (C-5), 80.92 (C-6), 80.29 (C(CH₃)₃), 74.67 (OCH₂), 63.77 (NOCH₃), 52.87 (CO₂CH₃), 34.44 (C-2), 28.10 (C(CH₃)₃), 24.05 (C-3), 18.86 (C-4), 12.63 (CH₃); ESI-HRMS (*m/z* calcd. for C₂₉H₃₄N₂NaO₆ [M+Na]⁺ 529.2315, found 529.2319.

6-(3-((*E*)-1-(((2-((*E*)-2-Methoxy-1-(methoxyimino)-2-oxoethyl)benzyl)oxy)imino)ethyl) phenyl)hex-5-ynoic acid (hapten TFt). Hydrolysis of the *tert*-butyl ester of 33 (49.8 mg, 0.098 mmol) with formic acid (2.0 mL) under the same conditions reported above for 8 afforded, after chromatographic purification with CHCl₃ as eluent, hapten TFt (40.8 mg, 92%) as a colorless oil. IR (NaCl) $\nu_{\max}$ (cm⁻¹) 3500-2700s (broad) 2942m, 2246w, 1726s, 1590w, 1437m, 1318m, 1069s, 1019s, 910m, 732s; ¹H-NMR (300 MHz) δ (ppm) 7.62 (1H, t, *J* = 1.5 Hz, H-2'), 7.55–7.47 (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* = 7.7 Hz, H-5'), 7.18 (1H, dd, *J* = 7.3, 1.4 Hz, H-6''), 5.12 (2H, s, OCH₂), 4.03 (3H, s, NOCH₃), 3.81 (3H, s, CO₂CH₃), 2.56 (2H, t, *J* = 7.1 Hz, H-4), 2.51 (2H, t, *J* = 7.2 Hz, H-2), 2.18 (3H, s, CH₃), 1.94 (2H, quint, *J* = 7.2 Hz, H-3); ¹³C-NMR (75 MHz) δ (ppm) 178.4 (CO₂H), 163.3 (CO₂CH₃), 154.4 (CH₃C=N), 149.6 (C=N), 136.5 (C-1'), 136.2 (C-3'), 132.1 (C-6'), 129.7 (C-2''), 129.4 (C-2'), 129.2 (C-5''), 128.7 (C-4'), 128.5 (C-6''), 128.2 (C-5'), 127.6 (C-4''), 125.4 (C-3''), 123.8 (C-1'), 89.9 (C-5), 81.3 (C-6), 74.7 (OCH₂), 63.9 (NOCH₃), 52.9 (CO₂CH₃), 32.7 (C-2), 23.5 (C-3), 18.8 (C-4), 12.6 (CH₃); ESI-HRMS *m/z* calcd for C₂₅H₂₆N₂NaO₆ [M+Na]⁺ 473.1689, found 473.1687; UV (100 mM sodium phosphate buffer, pH 7.4) ϵ (280 nm) 2.67 mM⁻¹ cm⁻¹.

tert-Butyl 6-(3-((*E*)-1-(((2-((*E*)-2-methoxy-1-(methoxyimino)-2-oxoethyl)benzyl)oxy)imino)ethyl)phenyl)hexanoate (34). Alkyne 33 (62.5 mg, 0.123 mmol) was hydrogenated as described above for the preparation of 7 using Rh(PPh₃)₃Cl (5.7 mg, 0.006 mmol) and THF (2.7 mL). Purification of the crude product by chromatography with hexane–Et₂O (8:2) as eluent gave title compound 34 (57.1 mg, 91%) as a colorless oil. IR (NaCl) $\nu_{\max}$ (cm⁻¹) 2976s, 2936s, 2858s, 1731s, 1592w, 1485m, 1437s, 1391m, 1367s, 1218s, 1155s, 1069s, 1020s, 957s, 898m, 790m; ¹H-NMR (300 MHz) δ (ppm) 7.51 (1H, dd, *J* = 7.4, 1.3 Hz, H-3''), 7.46–7.33 (4H, m, H-2', H-4', H-4'' and H-5''), 7.25 (1H, t, *J* = 7.8 Hz, H-5'), 7.21–7.12 (2H, m, H-6'' and H-6'), 5.13 (2H, s, OCH₂), 4.03 (3H, s, NOCH₃), 3.81 (3H, s, CO₂CH₃), 2.62 (2H, t, *J* = 7.5 Hz, H-6), 2.21 (2H, t, *J* = 7.5 Hz, H-2), 2.20 (3H, s, CH₃), 1.69–1.55 (4H, m, H-3 and H-5), 1.43 (9H, s, C(CH₃)₃), 1.41–1.32 (2H, m, H-4); ¹³C-NMR (75 MHz) δ (ppm) 173.14 (CO₂^tBu), 163.28 (CO₂CH₃), 155.29 (CH₃CN), 149.58 (C=N), 142.63 (C-1'), 136.46 (C-1''), 136.24 (C-3'), 129.73 (C-2''), 129.38 (C-6'), 129.16 (C-6''), 128.67 (C-3''), 128.49 (C-5''), 128.23 (C-5'), 127.53 (C-4''), 126.07 (C-2'), 123.58 (C-4'), 79.92 (C(CH₃)₃), 74.52 (OCH₂), 63.74 (NOCH₃), 52.82 (CO₂CH₃), 35.62 (C-6), 35.50 (C-2), 31.06 (C-5), 28.68 (C-4), 28.05

(C(CH₃)₃), 24.93 (C-3), 12.86 (CH₃); ESI-HRMS *m/z* calcd. for C₂₉H₃₈N₂NaO₆ [M+Na]⁺ 533.2628, found 533.2630.

6-(3-((*E*)-1-(((2-((*E*)-2-Methoxy-1-(methoxyimino)-2-oxoethyl)benzyl)oxy)imino)ethyl)phenyl) hexanoic acid (hapten TFf). Hydrolysis of the *tert*-butyl ester of 34 (26.5 mg, 0.052 mmol) with formic acid (1.0 mL) under the same conditions reported above for 8 afforded, after chromatographic purification with CHCl₃ as eluent, hapten TFf (21.4 mg, 91%) as a white solid. Mp 85–87 °C (crystallized from hexane–benzene); IR (KBr) $\nu_{\max}$ (cm⁻¹) 3500-2700 (broad), 3060m, 2933s, 1717s, 1595w, 1436m, 1298s, 1066s, 1010s, 932s; ¹H-NMR (300 MHz) δ (ppm) 7.50 (1H, dd, *J* = 7.4, 1.3 Hz, H-3''), 7.46–7.33 (4H, m, H-2', H-4', H-4'' and H-5''), 7.25 (1H, t, *J* = 7.7 Hz, H-5'), 7.21–7.14 (2H, m, H-6'' and H-6'), 5.13 (2H, s, OCH₂), 4.03 (3H, s, NOCH₃), 3.81 (3H, s, CO₂CH₃), 2.62 (2H, t, *J* = 7.5 Hz, H-6), 2.35 (2H, t, *J* = 7.5 Hz, H-2), 2.20 (3H, s, CH₃), 1.73–1.58 (4H, m, H-3 and H-5), 1.43–1.33 (2H, m, H-4); ¹³C-NMR (75 MHz) δ (ppm) 178.9 (CO₂H), 163.3 (CO₂CH₃), 155.3 (CH₃C=N), 149.6 (C=N), 142.5 (C-1'), 136.5 (C-1''), 136.2 (C-3'), 129.7 (C-2''), 129.3 (C-6'), 129.2 (C-6''), 128.7 (C-3''), 128.5 (C-5''), 128.3 (C-5'), 127.6 (C-4''), 126.1 (C-2'), 123.7 (C-4'), 74.6 (OCH₂), 63.8 (NOCH₃), 52.9 (CO₂CH₃), 35.6 (C-6), 33.8 (C-2), 31.0 (C-5), 28.6 (C-4), 24.5 (C-3), 12.9 (CH₃); ESI-HRMS *m/z* calcd for C₂₅H₃₈N₂NaO₆ [M+Na]⁺ 477.2002, found 477.2005; UV (100 mM sodium phosphate buffer, pH 7.4) ϵ (280 nm) 2.85 mM⁻¹ cm⁻¹.

2. Synthesis of trifloxystrobin analogues

Oxime	analogue	R ¹	R ²	yield
35	TFc-H	CF ₃	H	90%
36	TFc-Et	CF ₃	C ₂ H ₅	98%
37	TFf-H	H	CH ₃	64%
38	TFf-NO₂	NO ₂	CH ₃	84%

Figure S7. Synthesis of trifloxystrobin analogues

2.1. Synthesis of TFc-H, TFc-Et, TFf-H and TFf-NO₂.

General procedure. A mixture of the oxime 35-38 (1 mmol), benzyl bromide **16** (1 mmol), and anhydrous Cs₂CO₃ (1.5 mmol) in anhydrous DMF (3.8 mL) was stirred at room temperature under nitrogen until TLC showed completion of reaction (approx. 3-4 hours) at which time the reaction mixture was poured into water and worked up as usual using EtOAc to extract. Chromatography of the residue left after evaporation of the solvent, using hexane–Et₂O (9:1 to 8:2) as eluent, afforded the corresponding trifloxystrobin analogue.

Physical and spectroscopic data of analogue TFc-H. An oil [Lit.¹¹ no data given]; IR (NaCl) ν_{max} (cm⁻¹) 3065w, 2943m, 2820w, 1728s, 1585w, 1437m, 1350m, 1322s, 1278m, 1219s, 1127s, 1079s, 1019s, 957m, 766m, 668m; ¹H NMR (300 MHz) δ 8.06 (1H, s, HC=), 7.81 (1H, s, H-2'), 7.71 (1H, d, *J* = 7.8 Hz, H-6'), 7.60 (1H, d, *J* = 7.8 Hz, H-4'), 7.51–7.35 (4H, m, H-6, H-5', H-5 and H-4), 7.19 (1H, dd, *J* = 7.3, 1.5 Hz, H-3), 5.12 (2H, s, OCH₂), 4.02 (3H, s, NOCH₃), 3.84 (3H, s, CO₂CH₃); ¹³C NMR (75 MHz) δ 163.31 (CO₂CH₃), 149.60 (HCN), 147.70 (C=N), 135.60 (C-2), 132.93 (C-1'), 130.22 (C-3', *c*, *J* = 32.5 Hz), 130.21 (C-6'), 130.20 (C-1), 129.43 (C-6), 129.13 (C-5'), 128.79 (C-5), 128.54 (C-3), 127.89 (C-4),

126.30 (C-4', c, $J = 3.8$ Hz), 123.70 (C-2', c, $J = 3.8$ Hz), 115.12 (CF₃, c, $J = 272.0$ Hz), 74.89 (OCH₂), 63.78 (NOCH₃), 52.90 (CO₂CH₃); ESI-HRMS m/z calcd. for C₁₉H₁₈F₃N₂O₄ [M+H]⁺ 395.1219, found 395.1222.

Physical and spectroscopic data of analogue TFc-Et. IR (NaCl) $\nu_{\max}$ (cm⁻¹) 2978w, 2934w, 1722m, 1630w, 1558w, 1432w, 1323m, 1295m, 1213m, 1170m, 1126s, 1071s, 1017s, 957w; ¹H NMR (300 MHz) δ 7.86 (1H, s, H-2'), 7.77 (1H, d, $J = 7.5$ Hz, H-6'), 7.59 (1H, d, $J = 7.5$ Hz, H-4'), 7.51 (1H, dd, $J = 7.6, 1.2$ Hz, H-6), 7.47 (1H, t, $J = 7.5$ Hz, H-5'), 7.43 (1H, ddd, $J = 7.6, 7.5, 1.5$ Hz, H-5), 7.38 (1H, ddd, $J = 7.5, 7.5, 1.2$ Hz, H-4), 7.19 (1H, dd, $J = 7.5, 1.5$ Hz, H-3), 5.15 (2H, s, OCH₂), 4.04 (3H, s, NOCH₃), 3.82 (3H, s, CO₂CH₃), 2.74 (2H, c, $J = 7.6$ Hz, CH₂), 1.11 (3H, t, $J = 7.6$ Hz, CH₃); ¹³C NMR (75 MHz) δ 163.29 (CO₂CH₃), 158.64 (CH₂CN), 149.42 (C=N), 136.20 (C-2), 136.01 (C-1'), 130.83 (C-3', c, $J = 32.5$ Hz), 129.68 (C-1), 129.50 (C-6'), 129.37 (C-6), 128.87 (C-5'), 128.70 (C-5), 128.46 (C-3), 127.70 (C-4), 125.50 (C-4', c, $J = 3.8$ Hz), 123.89 (CF₃, c, $J = 272.2$ Hz), 123.03 (C-2', c, $J = 3.8$ Hz), 74.70 (OCH₂), 63.80 (NOCH₃), 52.89 (CO₂CH₃), 19.81 (CH₂), 10.97 (CH₃); ESI-HRMS m/z calcd. for C₂₁H₂₁F₃N₂NaO₄ [M+Na]⁺ 445.1351, found 445.1354.

Physical and spectroscopic data of analogue TFf-H. Mp 82–83 °C (Crystallized from hexane–benzene) [Lit.¹² 69–71°C]; IR (KBr) $\nu_{\max}$ (cm⁻¹) 3049w, 2940w, 1739s, 1612w, 1585m, 1492m, 1438s, 1368m, 1302s, 1226s, 1208m, 1115w, 1067s, 1034s, 1009s, 928s, 898s, 696m; ¹H NMR (300 MHz) δ 7.62–7.56 (2H, m, H-6' and H-2'), 7.50 (1H, dd, $J = 7.4, 1.3$ Hz, H-6), 7.46–7.31 (5H, m, H-4, H-5, H-4', H-5' and H-3'), 7.19 (1H, dd, $J = 7.4, 1.3$ Hz, H-3), 5.12 (2H, s, OCH₂), 4.03 (3H, s, NOCH₃), 3.81 (3H, s, CO₂CH₃), 2.21 (3H, s, CH₃); ¹³C NMR (75 MHz) δ 161.25 (CO₂CH₃), 152.39 (CH₃CN), 149.61 (C=N), 136.48 (C-2), 136.28 (C-1'), 129.36 (C-4'), 128.75 (C-6), 128.50 (C-4), 128.33 (C-3' and C-5'), 128.21 (C-1), 127.60 (C-5), 126.13 (C-2' and C-6'), 124.05 (C-3), 74.68 (OCH₂), 63.77 (NOCH₃), 52.86 (CO₂CH₃), 12.73 (CH₃); ESI-HRMS m/z calcd. for C₁₉H₂₀N₂NaO₄ [M+Na]⁺ 363.1321, found 363.1321.

Physical and spectroscopic data of analogue TFfNO₂. Mp 126–128 °C (Crystallized from hexane–benzene) [Lit.¹³ solid, no mp given]; IR (KBr) $\nu_{\max}$ (cm⁻¹) 2956w, 2934w, 2863w, 1733s, 1612w, 1530s, 1435m, 1350s, 1301m, 1222m, 1070s, 1003s, 916m, 774m, 737m, 681m; ¹H NMR (300 MHz) δ 8.45 (1H, t, $J = 2.0$ Hz, H-2'), 8.18 (1H, ddd, $J = 8.0, 2.0, 1.0$ Hz, H-4'), 7.95 (1H, ddd, $J = 8.0, 2.0, 1.0$ Hz, H-6'), 7.51 (1H, t, $J = 8.0$ Hz, H-5'), 7.49 (1H, dd, $J = 7.5, 1.5$ Hz, H-6), 7.45 (1H, ddd, $J = 7.5, 7.4, 1.2$ Hz, H-5), 7.39 (1H, ddd, $J = 7.5, 7.4, 1.5$ Hz, H-4), 7.19 (1H, dd, $J = 7.4, 1.2$ Hz, H-3), 5.16 (2H, s, OCH₂), 4.04 (3H, s,

NOCH₃), 3.83 (3H, s, CO₂CH₃), 2.25 (3H, s, CH₃); ¹³C NMR (75 MHz) δ 163.29 (CO₂CH₃), 152.75 (CH₃CN), 149.51 (C=N), 148.34 (C-3'), 138.08 (C-2), 135.76 (C-1'), 131.70 (C-6'), 129.85 (C-1), 129.42 (C-5'), 129.26 (C-6), 128.82 (C-5), 128.55 (C-3), 127.87 (C-4), 123.63 (C-4'), 120.96 (C-2'), 75.13 (OCH₂), 63.82 (NOCH₃), 52.92 (CO₂CH₃), 12.40 (CH₃); ESI-HRMS *m/z* calcd. for C₁₉H₂₁N₃NaO₆ [M+Na]⁺ 408.1172, found 408.1171.

2.2. Synthesis of TFf-NH₂.

A mixture of compound TFf-NO₂ (24 mg, 0.062 mmol) and SnCl₂ (59 mg, 0.310 mmol) in EtOAc (2 mL) was stirred under nitrogen at 50°C overnight. The mixture was then cooled to room temperature, diluted with water and extracted with EtOAc. The combined organic layers were washed with 10% aqueous NaOH solution and brine and dried over anhydrous Na₂SO₄. Chromatographic purification of the residue left after evaporation of the solvent, using hexane-EtOAc 8:2 as eluent, led to compound TFf-NH₂ (19.1 mg, 87%) as an oil. IR (NaCl) ν_{max} (cm⁻¹) 3459w, 3374w, 2938w, 1727s, 1624w, 1580w, 1439w, 1368m, 1219m, 1069s, 1017s, 957w, 861w, 781m; ¹H NMR (300 MHz) δ 7.51 (1H, dd, *J* = 7.5, 1.3 Hz, H-6), 7.42 (1H, ddd, *J* = 7.5, 7.5, 1.5 Hz, H-5), 7.38 (1H, ddd, *J* = 7.5, 7.5, 1.3 Hz, H-4), 7.19 (1H, dd, *J* = 7.5, 1.5 Hz, H-3), 7.12 (1H, t, *J* = 8.0 Hz, H-5'), 6.97–6.92 (2H, m, H-2' and H-6'), 6.67 (1H, ddd, *J* = 8.0, 2.3, 1.0 Hz, H-4'), 5.10 (2H, s, OCH₂), 4.03 (3H, s, NOCH₃), 3.81 (3H, s, CO₂CH₃), 2.17 (3H, s, CH₃); ¹³C NMR (75 MHz) δ 163.72 (CO₂CH₃), 155.32 (CH₃CN), 149.78 (C=N), 146.78 (C-3'), 137.92 (C-2), 136.76 (C-1'), 129.38 (C-1), 129.35 (C-6), 129.22 (C-5'), 128.73 (C-5), 128.45 (C-3), 127.51 (C-4), 116.68 (C-6'), 115.95 (C-4'), 112.53 (C-2'), 74.47 (OCH₂), 63.72 (NOCH₃), 52.85 (CO₂CH₃), 12.82 (CH₃); ESI-HRMS *m/z* calcd. for C₁₉H₂₂N₃O₄ [M+H]⁺ 356.1610, found 356.1615.

Table S1
Cell fusion results.

Immunizing conjugate	Fusion	Fusion efficiency (%) ^a	Number of wells			Number of stabilized hybridomas
			Seeded ^b	Positive ^c	Competitive ^d	
BSA-TFa	#1 ^e	53	576	6	1	1
	#2 ^e	78	960	13	6	3
BSA-TFb	#1	98	864	23	6	1
	#2	73	480	4	0	0
	#3	65	960	56	3	0
	#4	100	672	23	4	1
BSA-TFc	#1 ^f	95	960	200	10	3
BSA-TFe	#1	46	672	16	3	0
	#2	49	672	31	7	2
	#3	100	672	107	3	2
	#4	69	960	453	6	0
BSA-TFo	#1 ^e	100	1152	57	14	4
	#2 ^e	59	864	5	0	0
BSA-TFf	#1 ^e	67	1344	38	5	1
	#2 ^e	59	1056	30	8	2
BSA-TFt	#1	61	768	120	10	1
	#2	64	864	635	4	3
	#3 ^e	78	1440	644	10	4

^a Percentage of seeded wells showing hybrid cell growth by visual inspection 11 days after fusion. ^b 96-well culture plates were seeded at 1.5×10^5 splenocytes per well. ^c The supernatant afforded A_{max} values higher than 0.5 in a control experiment without free analyte. ^d A_{max} was reduced more than 50% with 100 nM trifloxystrobin. ^e This cell fusion was carried with a pool of mixed splenocytes from two mice. ^f One mouse died and the splenocytes of the other three mice were mixed and processed together.

4. Copies of ^1H NMR spectra of activated haptens

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5. Supporting Information References

- (1) *Purification of Laboratory Chemicals 4th Ed.* (Ed.: W. L. F. Armarego and D. D. Perrin), Butterworth Heinemann Press: Oxford, 1996.
- (2) J.V. Mercader, C. Suárez-Pantaleón, C. Agulló, A. Abad-Somovilla, A. Abad-Fuentes, J. Agric. Food Chem. 56 (2008) 7682–7690.
- (3) S.R. Neufeldt, M.S. Sanford, Org. Lett. 12 (2010) 532–535.
- (4) E. Marzi, A. Spitaleri, F. Mongin, M. Schlosser, Eur. J. Org. Chem. (2002) 2508–2517.
- (5) I.-C. Hwang, J.-K. Kim, H.-H. Kim, S.-H. Kyung, Bull. Korean Chem. Soc. 30 (2009) 1475–1480.
- (6) B. Wenderoth, H. Sauter, H. Wingert, M. Hepp, S. Brand, T. Kuekenhoehner, F. Roehl, E. Ammermann, G. Lorenz, Patent N° US 5145980. Sep 8, 1992.
- (7) K. Banerjee, A.P. Ligon, M. Schuermann, H. Preut, M. Spiteller, Acta Cryst. E61 (2005) o1528–o1529.
- (8) J.V. Mercader, C. Suárez-Pantaleón, C. Agulló, A. Abad-Somovilla, A. Abad-Fuentes, J. Agric. Food Chem. 56 (2008) 2581–2588.
- (9) Y.; Li, H.-G. Zhang, J. Liu, X.-P. Yang, Z.-J. Liu, J. Agric. Food Chem. 54 (2006) 3636–3640.
- (10) Y. Li, J. Liu, H. Zhang, X. Yang, Z. Liu, Bioorg. Med. Chem. Lett. 16 (2006) 2278–2282.
- (11) S. Brand, U. Kardorff, R. Kirstgen, B. Mueller, K. Oberdorf, H. Sauter, G. Lorenz, E. Ammermann, C. Kuenast, A. Harreus, Patent EP463488, Jan 2, 1992.
- (12) H. Ziegler, D. Neff, W. Stutz, Patent WO9520569, Aug 3, 1995.
- (13) H.P. Isenring, B. Weiss, Patent EP460575, Dec 11, 1991.