Tetrahedron 67 (2011) 624–635

Contents lists available at ScienceDirect

Tetrahedron

journal homepage: www.elsevier.com/locate/tet

Concise and modular synthesis of regioisomeric haptens for the production of high-affinity and stereoselective antibodies to the strobilurin azoxystrobin

Javier Parra [a], Josep V. Mercader [a], Consuelo Agulló [b], Antonio Abad-Fuentes [a,*], Antonio Abad-Somovilla [b,*]

[a] Department of Biotechnology, IATA–CSIC, Agustí Escardino 7, 46980 Paterna, València, Spain
[b] Department of Organic Chemistry, Universitat de València, Doctor Moliner 50, 46100 Burjassot, València, Spain

ARTICLE INFO

Article history:
Received 19 October 2010
Received in revised form 8 November 2010
Accepted 15 November 2010
Available online 24 November 2010

Keywords:
Hapten design
C–C coupling
Linker attachment site
Landsteiner
Molecular recognition
Immunochemistry

ABSTRACT

The immune response to regioisomeric haptens of azoxystrobin with varied derivatization sites was studied. Based on the Sonogashira and Suzuki–Miyaura couplings and following a straightforward modular design, we have synthesized four haptens with the same linker anchored through C–C bonds and located at different sites of the molecule. The most stereoselective antibodies were produced from immunogens with the spacer arm at a distal position from the β-methoxyacrylate moiety characteristic of strobilurins. Moreover, we observed that assay cross-reactivity was reliant on the functionalization site of the competitor derivative. Finally, the antibody binding site was explored using synthetic chemical analogues.

1. Introduction

The generation of antibodies against small organic molecules has a broad applicability in the medical, environmental and analytical sciences. During the past decades, antibodies have been raised and immunoassays have been developed for the detection and quantification of chemical substances of clinical interest, such as hormones[1] and drugs of abuse,[2] as well as antibiotic[3] and pesticide[4] residues in environmental and food samples.

In order to produce antibodies against a small organic molecule and for the further development of a sensitive immunoassay, it is necessary to covalently link the molecule (or a synthetic analogous hapten) to a carrier protein. This bioconjugate is capable of stimulating the immune system and producing antibodies against the target molecule. The usual way of coupling a hapten to a carrier antigen is to synthesize, first, a derivative that incorporates a spacer arm, generally consisting in a linear hydrocarbon chain of a certain length, most frequently between three and six carbon atoms, which is ended in a functional group that can be used for the subsequent conjugation to the carrier molecule. The design and synthesis of such functionalized derivatives of the parent compound is one of the most important steps in the process to produce high-affinity antibodies.[5] Hapten design must be guided by the principle of maximizing steric, electronic and geometric similarity with the analyte. An ideal or optimum immunizing hapten should maintain the integrity of the framework and functional groups of the parent molecule, incorporating the linker chain at the site that induces the lowest possible changes in the electronic and conformational properties of the target compound.

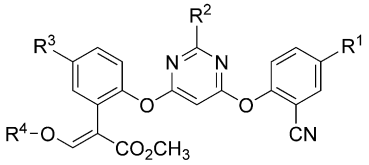
As originally stated by Karl Landsteiner,[6] the position of the spacer arm that connects the hapten to the carrier protein is critical in determining the specificity of the generated antibody, which is usually maximal for those sites distal to the point of conjugation. In accordance with this so-called Landsteiner's principle, 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. To our knowledge, however, very few studies have been carried out that allow an in-depth evaluation of the significance of the derivatization site over the affinity and specificity of the raised antibodies, and to what extent it is possible to modulate antibody specificity by using bioconjugates with different linker positions during immunization.[7]

Based on these principles, we designed four regioisomeric haptens for azoxystrobin as a model molecule (Fig. 1). Azoxystrobin was the first discovered and patented antifungal of a new class of

* Corresponding authors. Tel.: +34 963636301; fax: +34 963900022 (A.A.-F.); tel.: +34 963544509; fax: +34 963544328 (A.A.-S.); e-mail addresses: aabad@iata.csic.es (A. Abad-Fuentes), antonio.abad@uv.es (A. Abad-Somovilla).

0040-4020/$ – see front matter © 2010 Published by Elsevier Ltd.
doi:10.1016/j.tet.2010.11.054

agrochemicals, the strobilurins,[8] and it is currently the world's best selling proprietary fungicide, with global annual sales over $1 billion in 2008.[9] Each of the four haptens incorporated the same hydrocarbon spacer arm, but at different positions, which encompasses the entire azoxystrobin skeleton. All derivatives maintained the whole structure and characteristic groups of the target molecule, while the spacer arm was connected via a carbon–carbon single bond at a site where a carbon–hydrogen bond previously existed, thus introducing the least possible changes on the electronic properties of the tricyclic azoxystrobin framework.



Azoxystrobin (**1**): $R^1 = R^2 = R^3 = H$; $R^4 = CH_3$
Hapten Aza6 (**2**): $R^1 = R^2 = H$; $R^3 = (CH_2)_5CO_2H$; $R^4 = CH_3$
Hapten Azb6 (**3**): $R^2 = (CH_2)_5CO_2H$; $R^1 = R^3 = H$; $R^4 = CH_3$
Hapten Azc6 (**4**): $R^1 = (CH_2)_5CO_2H$; $R^2 = R^3 = H$; $R^4 = CH_3$
Hapten Azo6 (**5**): $R^1 = R^2 = R^3 = H$; $R^4 = (CH_2)_5CO_2H$

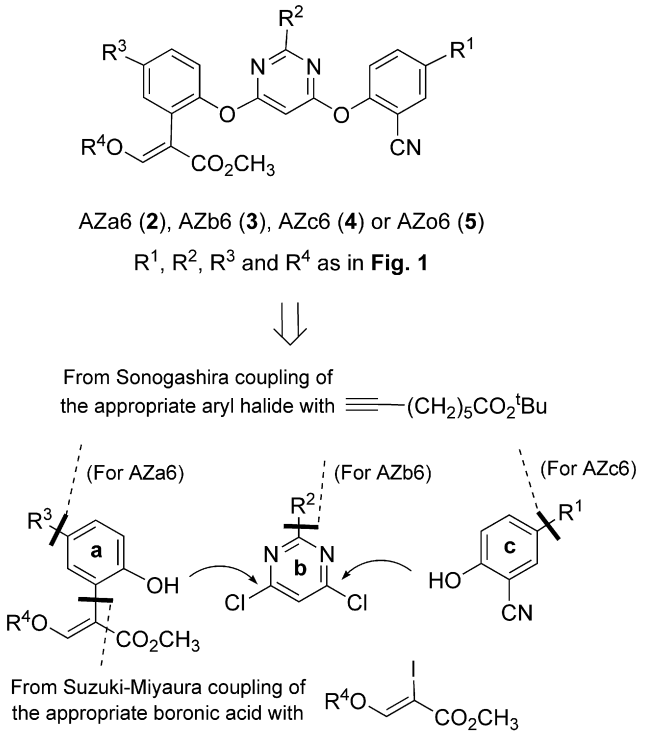
Fig. 1. Structure of azoxystrobin and regioisomeric haptenic derivatives.

This comprehensive approach, which has rarely been used before in the design of haptens for the production of antibodies against structurally complex non-rigid small organic molecules, allowed a broad diversity of orientations in which the antigen was presented to the immune system, thereby increasing the chance of producing antibodies with enhanced binding properties. Moreover, the regioisomeric nature of the synthesized immunizing haptens ensured that the differences in the binding affinity and/or specificity of the derived antibodies could be only attributed to the variation in the linker attachment site. Antisera, representing the unbiased whole-response of the animal immune system, were chosen as the source of antibodies, and competitive enzyme-linked immunosorbent assay (cELISA) was employed to evaluate the affinity and selectivity of the produced antibodies.

2. Results and discussion

2.1. Hapten synthesis

The preparation of each of the four regioisomeric haptens (AZa6, AZb6, AZc6 and AZo6) was undertaken via a concise, modular and convergent synthesis based on two consecutive nucleophilic aromatic substitutions of the chlorine atoms of a 4,6-dichloropyrimidine (ring b) with two conveniently functionalized phenols (rings a and c) (Scheme 1).[10] The saturated carboxylated alkyl chain that constitutes the spacer arm in each hapten is introduced at the appropriate position of the aromatic ring subunit by means of a Sonogashira coupling reaction between the suitable aryl halide and *tert*-butyl hex-5-ynoate, followed by hydrogenation of the acetylenic triple bond. The incorporation of the carboxylated spacer arm as its *tert*-butyl ester is made on the basis of its easy acid hydrolysis to the corresponding carboxylic acid in presence of the base sensitive β-methoxyacrylate moiety. On the other hand, the incorporation of the β-methoxyacrylate moiety to the phenolic a-ring synthon is carried out by means of a Suzuki–Miyaura cross-coupling reaction between the appropriate arylboronic acid and methyl (*Z*)-2-iodo-3-alkoxy-acrylate, a kind of reaction that has been successfully used for the elaboration of the β-methoxyacrylate moiety in related systems.[11]



AZa6 (**2**), AZb6 (**3**), AZc6 (**4**) or AZo6 (**5**)
R^1, R^2, R^3 and R^4 as in **Fig. 1**

From Sonogashira coupling of the appropriate aryl halide with $(CH_2)_5CO_2{}^tBu$

(For AZa6) (For AZb6) (For AZc6)

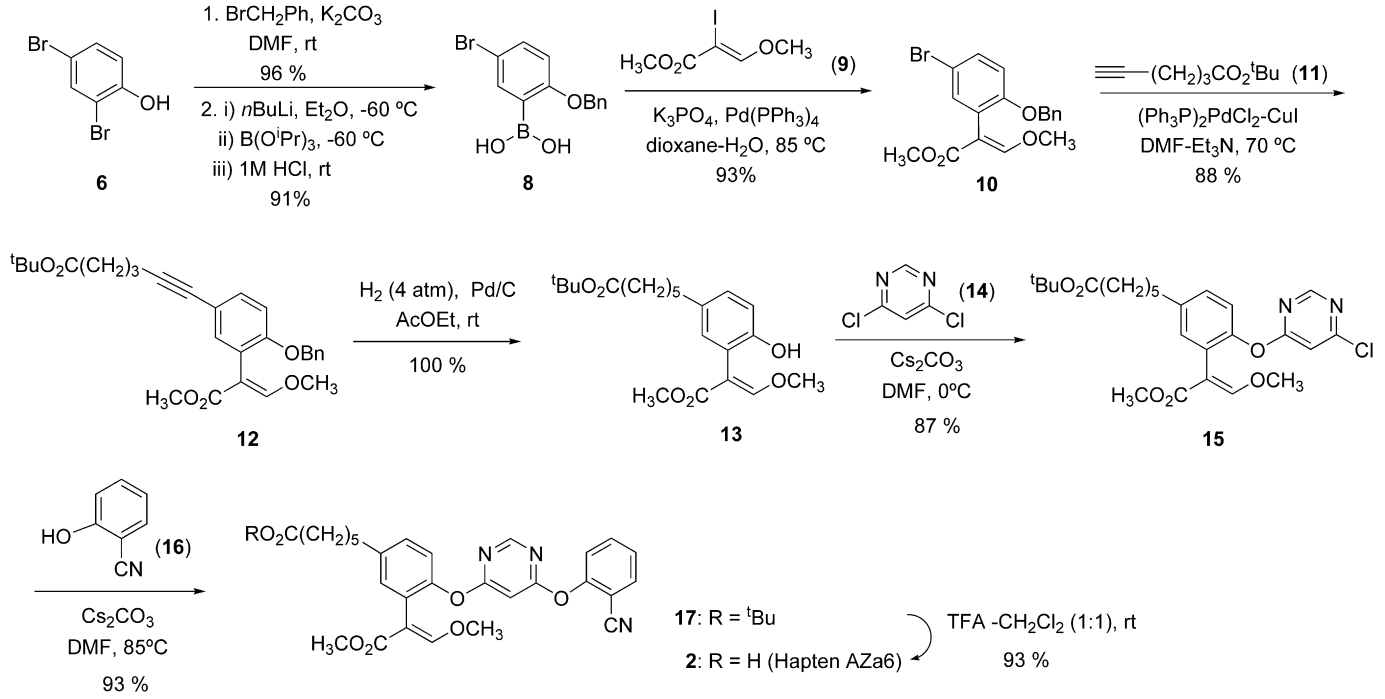
From Suzuki-Miyaura coupling of the appropriate boronic acid with

For AZa6: $R^1 = R^2 = H$; $R^3 = (CH_2)_5CO_2{}^tBu$; $R^4 = CH_3$
For AZb6: $R^1 = R^3 = H$; $R^2 = (CH_2)_5CO_2{}^tBu$; $R^4 = CH_3$
For AZc6: $R^1 = (CH_2)_5CO_2{}^tBu$; $R^2 = R^3 = H$; $R^4 = CH_3$
For AZo6: $R^1 = R^2 = R^3 = H$; $R^4 = (CH_2)_5CO_2{}^tBu$

Scheme 1. Modular synthetic approach (a→ab→abc) for the preparation of azoxystrobin haptens.

*2.1.1. Synthesis of hapten AZa6 (**2**).* The synthesis of hapten AZa6 started with the preparation of the phenolic a-ring synthon (i.e., **13**) from 2,4-dibromophenol (Scheme 2). First, the phenolic hydroxyl group was protected with a conventional benzyl group using standard conditions. Regioselective lithium–bromide exchange by treatment of the obtained dibromobenzylether with BuLi at low temperature,[12] followed by quenching with triisopropyl borate afforded the corresponding diisopropyl arylboronate, which was then hydrolyzed in situ to the arylboronic acid **8** by treatment with hydrochloric acid at rt. The Suzuki–Miyaura cross-coupling reaction between the arylboronic acid **8** and the iodoacrylate **9**[13] that completed the incorporation of the β-methoxyacrylate moiety, was undertaken using similar reaction conditions to the optimized once developed by Heo's group for related couplings, i.e., $Pd(PPh)_4$ as the palladium catalyst and K_3PO_4 as the base in a THF/H_2O system at 85 °C.[14] Under these conditions, the coupling of **8** and **9** took place very efficiently to give the α-aryl-β-methoxyacrylate **10** in 81% overall yield from starting dibromophenol (**6**).

The incorporation of the hydrocarbon spacer arm was based on the Sonogashira cross-coupling reaction between the aryl bromide **10** and the terminal alkyne-ester **11**, which had been obtained from commercially available hex-5-ynoic acid.[15] The cross-coupling reaction was carried out efficiently using $Pd(OAc)_2$ as the source of the palladium catalyst and 1,4-diazabicyclo[2.2.2]octane (DABCO) as the base in acetonitrile at rt.[16] The next and last step that completed the preparation of the required arylic a-ring synthon involved the hydrogenation of the acetylenic compound **12**, using 10% palladium-on-carbon as the catalyst. Under these conditions, both complete hydrogenation of the triple bond and hydrogenolysis of



Scheme 2. Synthesis of hapten AZa6 (**2**).

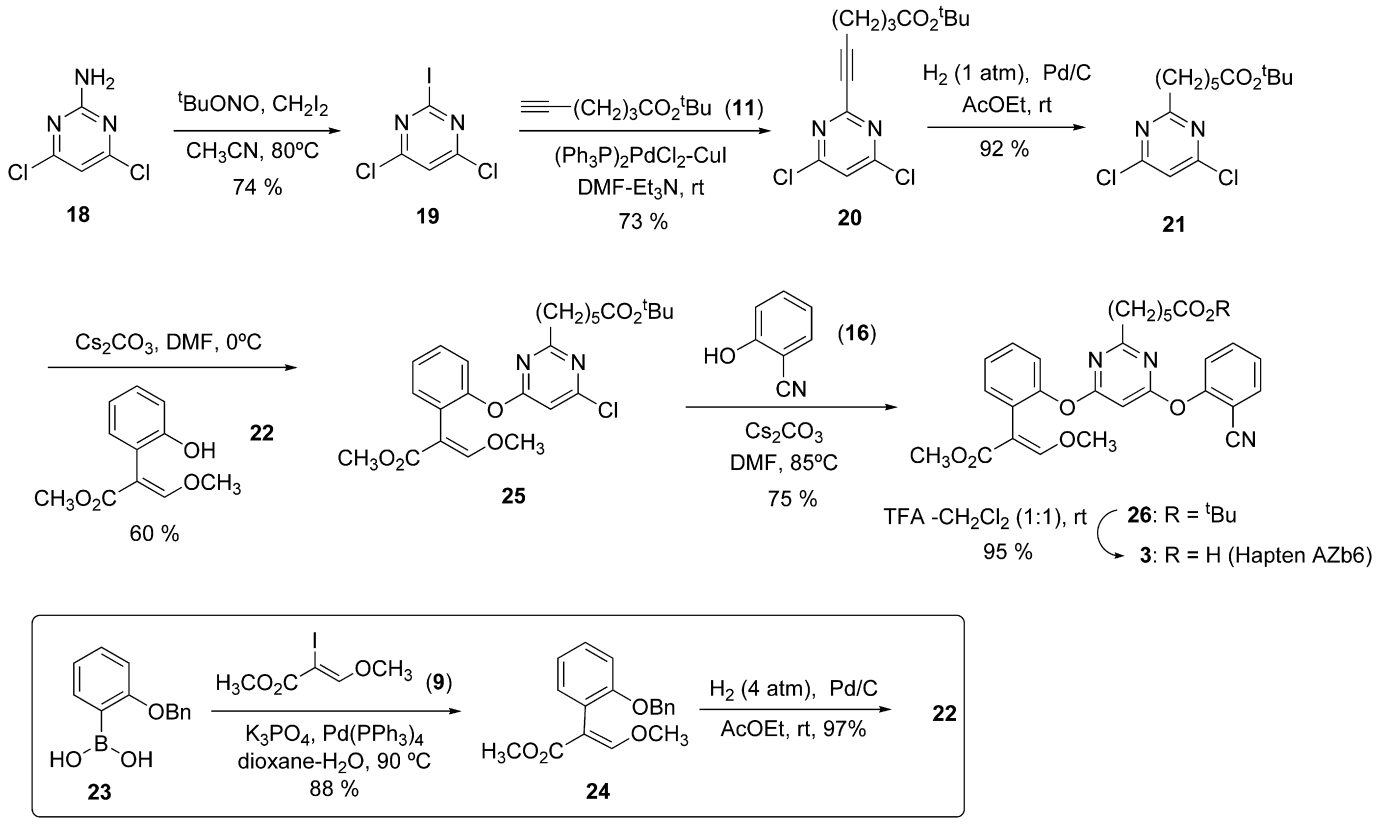
the benzyl protecting group took place to afford the desired phenol **13** in almost quantitative yield.

With compound **13** at hand, the synthesis of the hapten AZa6 was readily completed following the designed modular strategy. The phenol **13** was reacted with 4,6-dichloropyrimidine (**14**) using caesium carbonate as base in DMF at 0 °C. Nucleophilic displacement of the chlorine atom by the phenoxide moiety took place smoothly under these conditions to afford the corresponding substituted pyrimidine in high yield. It must be mentioned that both, the low temperature and the use of caesium carbonate as the base was essential to achieve a good yield for this reaction. Thus, higher temperatures or the use of other bases habitually used in this type of transformation, such as other alkaline carbonates, gave less clean reactions resulting in lower yields of compound **15**. In particular, substantial amounts of benzofurano-2(3*H*)-one and benzofurano-type derivatives, originated by intramolecular cyclization of the phenolic hydroxyl group of **13** with the β-methoxyacrylate moiety, were formed in the above conditions at rt or when K_2CO_3 or NaH were used as bases.

The tricyclic carbon skeletal framework of the target hapten was completed via another nucleophilic substitution reaction of the chlorine atom of chloropyrimidine **15** with 2-cyanophenol (**16**). In this case, this chloride substitution reaction was much more difficult than the former and required heating of the reaction mixture at 85 °C. Anyway, the reaction was also very efficient affording the *tert*-butyl ester of hapten AZa6 (**17**) in an excellent 93% yield. Finally, chemoselective trifluoroacetic acid-promoted hydrolysis of the *tert*-butyl ester moiety of **17** afforded the hapten AZa6 (**2**) in 93% yield after its chromatographic purification.

*2.1.2. Synthesis of hapten AZb6 (**3**).* Following the designed convergent synthetic route outlined in Scheme 1 for the preparation of the haptens, the synthesis of hapten AZb6 was accomplished with the previous preparation of the synthon corresponding to the pyrimidinyl b-ring, the 2-alkyl-4,6-dichloropyrimidine **21** (Scheme 3). A key step in the preparation of this synthon was the incorporation of the hydrocarbon spacer arm at the C-2 position of the pyrimidine ring. Several attempts to prepare **20** starting from 4,6-dichloropyrimidine, via hydride substitution at C-2 with a lithium acetylide reagent,[17] or 2-methyl-4,6-dichloropyrimidine, via alkylation reaction of the benzylic-type carbanion derived from abstraction of a proton from the methyl group,[18] were unsuccessful. Therefore, we used the alternative route based on a Sonogashira cross-coupling reaction starting from the commercially available 2-aminopyrimidine **18**. The 2-iodination of **18** was performed in good yield by the method of Nair and Fasbender,[19] using *tert*-butyl nitrite to generate the corresponding pyrimidin-2-yl radical and diiodomethane as the source of iodine. Subsequent Sonogashira cross-coupling reactions with the terminal alkyne **11** took place under very smooth conditions affording the 2-alkenyl pyrimidine derivative **20**. It should be mentioned that, in spite of the recognized much better ability of iodine, as compared with chlorine, as leaving group in this type of coupling, the reaction also led to variable amounts of the coupling products formed through the chlorinated positions. This circumstance limits the yield obtained for **20** and can be attributed to the intrinsically higher reactivity of the 4/6-pyrimidine positions towards the oxidative addition step.[20] Completion of the introduction of the hydrocarbon chain at the C-2 position of the pyrimidine nucleus was undertaken by hydrogenation of the triple bond of compound **20** under low hydrogen pressure and careful control of the progress of the reaction by TLC analysis to avoid excessive hydrogenolysis of the C–Cl bonds. The desired 2-alkyl pyrimidine **21** was thus obtained in about 68% yield from 2-iodopyrimidine **19**.

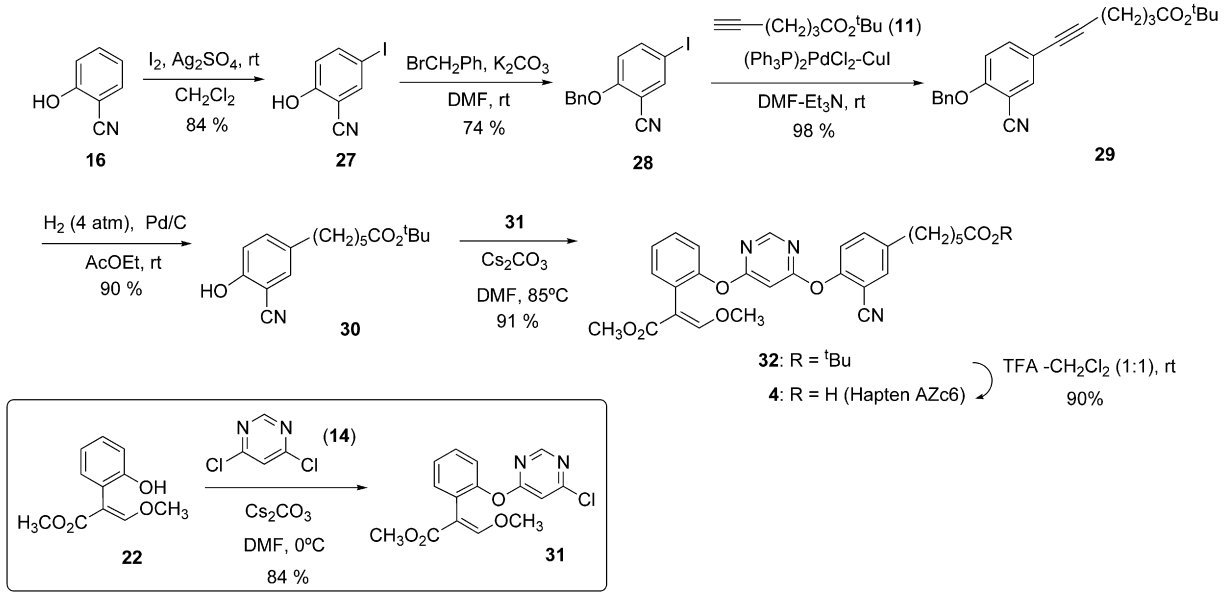
With **21** available, further elaboration of the oxygen-bridged tricyclic framework of hapten AZb6 was achieved, as before, through two consecutive nucleophilic aromatic substitution reactions. First, the dichloropyrimidine **21** was reacted under very smooth basic conditions with methyl (*E*)-2-(2-hydroxyphenyl)-3-methoxyacrylate (**22**) to afford the pyrimidinyl-aryl ether **25** in 60% yield. Although the preparation of α-aryl-β-methoxyacrylate **22** has already been described,[21] it was more easily obtained in this case from commercial boronic acid **23**, in only two steps and 85% overall yield, via Suzuki–Miyaura cross-coupling reaction with iodoacrylate **9** to give **24** and hydrogenolysis of the protective benzyl group. Completion of the tricyclic aromatic ring system was achieved through a second nucleophilic aromatic substitution reaction between chloropyrimidine derivative **25** and 2-



Scheme 3. Synthesis of hapten AZb6 (**3**).

cyanophenol (**16**) catalyzed by Cs_2CO_3 in DMF at 85 °C. Finally, the synthesis of the required hapten AZb6 (**3**) was completed very efficiently by trifluoroacetic acid-promoted hydrolysis of the *tert*-butyl ester moiety of **26**.

*2.1.3. Synthesis of hapten AZc6 (**4**).* The synthesis of hapten AZc6, which incorporated the hydrocarbon spacer arm at the cyanophenoxy moiety, required the previous preparation of the synthon corresponding to the arylic c-ring, the phenol **30** (Scheme 4). The first step for the preparation of this intermediate was the iodination of 2-cyanophenol (**16**), necessary for the further incorporation of the hydrocarbon spacer arm at this aromatic system via a palladium-catalyzed cross-coupling process. This was achieved using the iodine-silver sulfate system in dichloromethane, which afforded regioselectively the iodo-phenol **27** in an excellent 84% yield. Several attempts to directly incorporate the hydrocarbon chain from the iodo-phenol **27**, via a Sonogashira reaction with *tert*-butyl hex-5-ynoate (**11**), failed, even under the conditions described previously for related transformations.[22] Therefore, the phenolic hydroxyl group of **27** was transformed into the corresponding benzyl ether by alkylation with benzyl bromide in basic medium. The resulting benzyl ether **28** underwent the Sonogashira palladium-catalyzed



Scheme 4. Synthesis of hapten AZc6 (**4**).

cross-coupling reaction with the alkyne-ester **11**, under similar conditions as those previously used in the preparation of hapten AZa6, to afford the aryl-alkyne **29** in almost quantitative yield. Further catalytic hydrogenation of the triple bond, with concomitant *O*-benzyl group hydrogenolysis, completed the preparation of the key phenol intermediate **30**.

With phenol intermediate **30** available, further elaboration of the whole framework of hapten AZc6 was straightforward following essentially the same strategy as that described above for the preparation of the other haptens. Thus, reaction of **30** with 4-phenyloxy-6-chloropyrimidine **31**, in the same conditions described above for the preparation of regioisomeric compounds **17** and **26**, afforded the *tert*-butyl ester of hapten AZc6 (**32**) in 91% yield after silica-gel column chromatographic purification. The 4-aryloxy-6-chloropyrimidine **31** was prepared in an improved way (84% yield) by modification of a previously described procedure,[23] involving the reaction between 4,6-dichloropyrimidine (**14**) and 2-(2-hydroxyphenyl)-3-methoxy acrylate (**22**) catalyzed by Cs_2CO_3 in DMF at 0 °C.

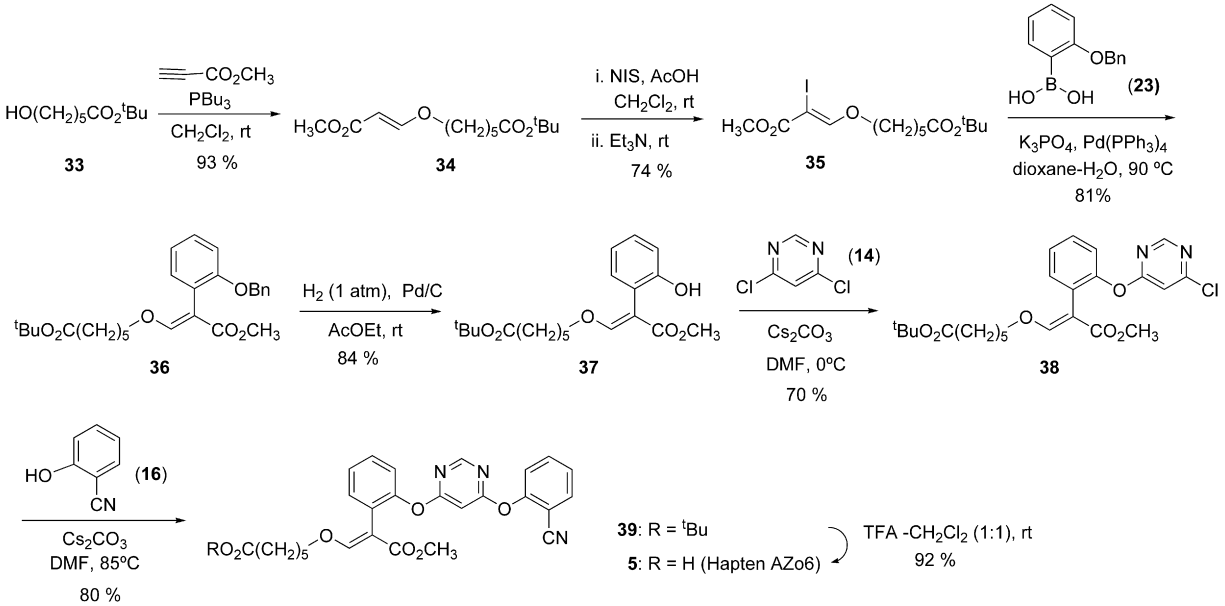
Finally, acid hydrolysis of the *tert*-butyl ester group of **32** furnishes the corresponding carboxylic acid, thus completing the synthesis of hapten AZc6 (**4**).

*2.1.4. Synthesis of hapten AZo6 (**5**).* The synthesis of the hapten AZo6 began with the previous elaboration of the aryl a-ring synthon that introduced the hydrocarbon spacer arm at the β-alkoxyl group of the acrylate moiety (Scheme 5). The preparation of this intermediate, the phenol **37**, was accomplished following a similar strategy to that previously used for the preparation of the related phenolic compound **22** (see Scheme 3). The synthesis of **37** began with the achievement of β-alkoxyacrylate **34**, which was undertaken in very high yield by the stereoselective reaction of *tert*-butyl 6-hydroxyhexanoate (**33**) with methyl propiolate in the presence of a catalytic amount of tri-*n*-butyl phosphine.[24] Subsequent acetoxy-iodination of the vinyl ether moiety of **34** with *N*-iodosuccinimide (NIS) and AcOH, followed by elimination of AcOH from the resulting mixture of diastereoisomeric iodoacetates using Et_3N,[25] afforded exclusively the methyl (*Z*)-α-iodo-β-alkoxyacrylate isomer **35** in good overall yield. The subsequent Suzuki–Miyaura cross-coupling reaction between the iodoacrylatye **35** and the commercial arylboronic acid **23** proceeded smoothly to afford the corresponding α-aryl-β-alkoxyacrylate **36**, which was transformed under hydrogenolysis conditions into the required phenol **37** in 68% overall yield. It must be noted that the hydrogenolysis reaction of the *O*-benzyl group of **36** resulted more problematic that it might be expected on the basis of the results previously obtained for the related reaction of benzyl ether **24**. In this case, the β-alkoxyacrylate moiety was partially hydrogenated (up to 50%) to the corresponding saturated system under the hydrogenation conditions previously used for **24**. This side reaction could be minimized using a low pressure of hydrogen and limiting the reaction time to about 1 h.

The final steps to complete the synthesis of hapten AZo6 were, as designed, the same as those previously used for the preparation of hapten AZa6. Thus, reaction of phenol **37** with 4,6-dichloropyrimidine (**14**) followed by the reaction with 2-cyanophenol (**16**) and the final acid-catalyzed hydrolysis of the *tert*-butyl ester moiety completed the synthesis of hapten AZo6 (**5**), with an overall yield of about 52% for the three steps.

2.2. Immunological response

For rabbit immunization, the synthesized haptens were converted to NHS-active esters and covalently linked to bovine serum albumin (BSA) to afford bioconjugates with similar hapten-to-protein molar ratios (15–20). After the fourth boost, all of the immunized animals gave rise to high titres (over 10^5) against the homologous ovalbumin (OVA) conjugate. The obtained antisera also bound tightly to free azoxystrobin, showing affinity values in the low nanomolar range (Table 1). Interestingly, rabbits that were immunized with BSA–AZb6 (the hapten that was functionalized at the central pyrimidine ring) produced the antisera with the lowest affinity to azoxystrobin in homologous assays, whereas the antisera derived from the three other immunogens showed a very similar binding to the free compound (Fig. 2). The IC_{50} values in competitive assays using AZb6-type sera could be lowered if heterologous conjugates were employed, but still these antisera provided the least sensitive assays, with IC_{50} values over 3 nM. The highest affinity was observed with AZo6-derived antisera in combination with the heterologous conjugate OVA–AZa6 (IC_{50}=0.3 nM), in which the derivatization site was at the aromatic ring located proximal to the methoxyacrylate moiety where the linker in AZo6 was located.



Scheme 5. Synthesis of hapten AZo6 (**5**).

Table 1
Antibody affinity for azoxystrobin by cELISA[a]

Antiserum[c]	Coating conjugate[b]			
	OVA–AZa6	OVA–AZb6	OVA–AZc6	OVA–AZo6
AZa6	1.64 ± 0.26	1.62 ± 0.02	0.94 ± 0.19	1.39 ± 0.16
AZb6	3.05 ± 0.29	19.04 ± 1.20	3.47 ± 0.23	3.32 ± 0.21
AZc6	1.01 ± 0.17	0.79 ± 0.09	1.85 ± 0.05	1.68 ± 0.38
AZo6	0.30 ± 0.04	1.40 ± 0.13	2.89 ± 0.37	0.93 ± 0.12

[a] Values are the mean of three independent experiments. Immunoreagents were used at limiting concentrations. A_{max} values were between 0.7 and 1.5. Affinity values are expressed in nM.
[b] Coating conjugate concentration was 0.1 μg/mL.
[c] Results are for one antiserum from each immunogen.

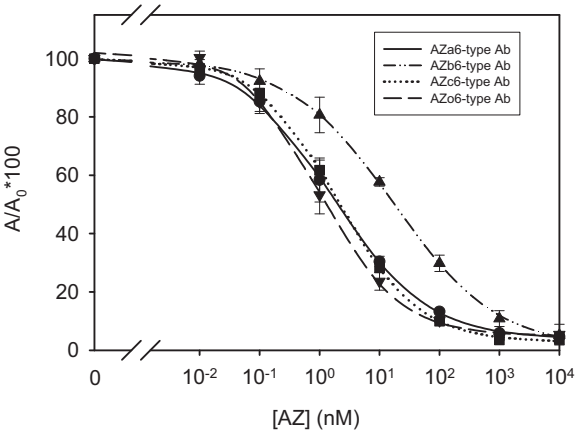


Fig. 2. Inhibition curves for azoxystrobin in homologous competitive assays using antibodies derived from AZa6 (circles), AZb6 (up triangles), AZc6 (squares) and AZo6 (down triangles).

Concerning selectivity, all of the generated antisera were highly specific to their target and none of them recognized any of the other strobilurin fungicides. In fact, this family of agrochemicals only shares the small β-methoxyacrylate group or a modification of this toxophore moiety. Instead, the bulk of the molecule is particular and distinct for each compound. Azoxystrobin exists in two stereoisomeric forms, although the fungicide activity strongly relies on the *E*-isomer, which accounts for up to 98% of the technical commercial product. On the other hand, the *Z*-isomer is the main degradation product, and it appears primarily by photochemical reaction.[26] During hapten synthesis, especial precaution was taken to preserve the *E*-conformation of the toxophore in all derivatives. Antibodies are biomolecules most often displaying exquisite specificity to their target, being able to discriminate even between chiral molecules and geometric isomers.[27] Accordingly, we thought it was worthwhile to challenge the generated antisera with the *Z*-isomer to further explore the fine specificity of their binding sites and to study the relationship between their stereoselectivity and the linker position in the parental hapten. The *Z*-isomer of azoxystrobin was prepared as described by Clough et al.,[28] and competitive assays with both isomers were run in parallel. Table 2 lists the cross-reactivity values for this isomer that were achieved by cELISA with homologous and heterologous coating conjugates. A dissimilar response was clearly found for the different antisera. Following Landsteiner's principle, the highest stereospecificity was obtained with AZc6-derived antisera, independently of the hapten conjugate that was employed in the assay. Interestingly, the Landsteiner's principle was also applicable to the coating conjugate because, for all sorts of antisera, the cross-reactivity values could be lowered below 10% if OVA–AZc6 was used for coating, independently of the employed antiserum. Therefore, not only AZc6-type antisera but also AZc6 conjugates, in which the linker was placed at the aromatic ring located distal to the β-methoxyacrylate group, clearly afforded the best stereospecificity. On the contrary, if plates were coated with OVA–AZb6, AZa6-derived antisera bound similarly both stereoisomers (cross-reactivity close to 90%).

Table 2
Cross-reactivities for the azoxystrobin *Z*-isomer (%)[a]

Antiserum[c]	Coating conjugate[b]			
	AZa6	AZb6	AZc6	AZo6
AZa6	16.0 ± 2.9	88.1 ± 4.2	1.7 ± 0.2	16.4 ± 1.3
AZb6	51.9 ± 6.8	15.7 ± 0.9	7.3 ± 0.8	45.5 ± 7.7
AZc6	8.0 ± 0.6	2.0 ± 0.1	2.4 ± 0.3	2.7 ± 0.3
AZo6	21.8 ± 5.1	20.8 ± 3.6	3.0 ± 1.0	11.9 ± 3.1

[a] Values are the mean of four independent experiments.
[b] Coating conjugate concentration was 0.1 μg/mL.
[c] Results are for one antiserum from each immunogen.

Finally, in order to further enquire into the specificity properties of the antibody binding site, the capacity of the produced antisera to distinguish between structurally quite similar molecules was investigated. With this aim, two structural analogues of azoxystrobin (compounds **40** and **41**, Fig. 3) were prepared in our laboratory following a similar strategy to that used for the preparation of the synthetic haptens (see the Supplementary data). Each of them contained just one modified moiety located at opposite sites, either at the cianophenyl ring or at the acrylate group. These minimal changes in the azoxystrobin structure drastically reduced the affinity of the antisera for such analogues (cross-reactivity values were lower than 1%), thus confirming the high selectivity of the produced antibodies. However, differential binding behaviours to compounds **40** and **41** could be observed for the four types of antisera. As shown in Fig. 3, AZo6- and AZa6-derived antisera displayed a lower affinity towards analogue **41** (the compound without the nitrile group) than to analogue **40** (the compound without the methoxymethylene group). On the contrary, AZc6-type antibodies bound worst to compound **40** than to compound **41**. In both cases, the affinity was lower towards the compound in which the modification was distal from the derivatization site of the corresponding immunizing hapten, demonstrating again that the specificity of the immune response was mainly directed towards the chemical moieties that were located opposite to the linker.

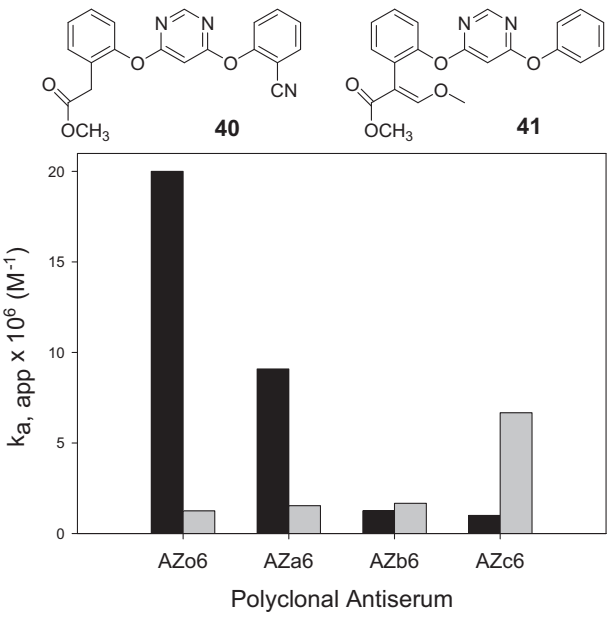


Fig. 3. Affinity of the different types of antisera towards two analogues of azoxystrobin. Black bars: compound **40**; grey bars: compound **41**.

3. Conclusion

A concise modular approach for the synthesis of regioisomeric haptens for azoxystrobin has been designed. Distinct functionalized haptens with the same linker located at varying positions of the azoxystrobin framework were prepared. Following this approach, we could generate antibodies showing superior specificity and higher affinity than the previously reported polyclonal antibodies for this compound.[29] It could also be concluded that the hapten in which the linker was placed at the central ring of azoxystrobin afforded antisera with lower affinity for the free molecule, probably because of a less efficient display of the chemical to the immune system. The prepared haptens provided expanded possibilities for tuning the assay sensitivity and specificity through the selection of optimum haptens for the preparation of solid surface-coating bioconjugates. Our study showed that the observed cross-reactivity for an antiserum can greatly depend on the employed coating conjugate. This observation is probably exclusive of polyclonal antibodies and it might be a result of the heterogeneity of this sort of immunoreagent. For comparison, further studies are being conducted to generate a collection of monoclonal antibodies using the described synthetic haptens. Finally, the produced antibodies and conjugates will be highly valuable reagents for the development of sensitive immunoassays applicable to monitoring programmes for the analysis of azoxystrobin in, for example, food and environmental samples.

4. Experimental

4.1. Hapten synthesis

General experimental details and full characterisation data for all of the described compounds are given as Supplementary data.

*4.1.1. Synthesis of hapten AZa6 (**2**).*

*4.1.1.1. 1-(Benzyloxy)-2,4-dibromobenzene (**7**).* Benzyl bromide (592 μL, 4.983 mmol) was added to a solution of 2,4-dibromobenzene (1.142 g, 4.53 mmol) and anhydrous K_2CO_3 (688.7 mg, 4.982 mmol) in dry DMF (9.5 mL) under nitrogen. The mixture was stirred at rt for 1.30 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/EtOAc (from 9:1 to 8:2) as eluent, afforded the benzyl ether **7** (1.475 g, 96%) as a white solid. Mp 65–67 °C (from hexane). ^{1}H NMR (300 MHz, $CDCl_3$): δ=7.69 (d, J=2.2 Hz, 1H), 7.40 (m, 5H), 7.33 (dd, J=8.8, 2.2 Hz, 1H), 6.80 (d, J=8.8 Hz, 1H), 5.14 (s, 2H). HRMS (EI): calcd for $C_{13}H_{10}{}^{79}Br_2O$ 339.9098, found 339.9097.

*4.1.1.2. 2-(Benzyloxy)-5-bromophenylboronic acid (**8**).* A 1.43 M solution of BuLi in hexane (1.4 mL, 2 mmol) was added drop wise into a white slurry of benzyl ether **7** (685 mg, 2 mmol) in anhydrous Et_2O (7.5 mL) at −60 °C. The reaction solution soon turned into a clear yellowish solution that was stirred at the same temperature for 30 min and then treated with $B(O^iPr)_3$ (462 μL, 2 mmol). The white slurry formed was stirred for 30 min at −60 °C and then for 1 h at rt. The reaction mixture was quenched with 1 M aqueous HCl solution (5 mL), then stirred for 45 min and poured into water. Work up as usual, using EtOAc to extract, yielded a solid residue, which was purified by chromatography with hexane/EtOAc (from 9:1 to 8:2) as eluent, to yield the arylboronic acid **8** (557 mg, 91%) as a white solid. Mp 99–100 °C (from hexane/MeOH). ^{1}H NMR (300 MHz, $CHCl_3$): δ=7.97 (d, J=2.6 Hz, 1H), 7.51 (dd, J=8.8, 2.6 Hz, 1H), 7.41 (m, 5H), 6.86 (d, J=8.8 Hz, 1H), 6.05 (s, 2H), 5.12 (s, 2H). HRMS (ES): calcd for $C_{13}H_{12}B^{79}BrNaO_3$ $[M^+ + Na]^+$ 328.9961, found 328.9974.

*4.1.1.3. (E)-Methyl 2-(2-(benzyloxy)-5-bromophenyl)-3-methoxyacrylate (**10**).* A mixture of iodoacrylate **9** (214 mg, 0.886 mmol), arylboronic acid **8** (272 mg, 0.886 mmol), K_3PO_4 (564.2 mg, 2.658 mmol) and $Pd(PPh_3)_4$ (51 mg, 0.044 mmol) in a mixture of dioxane (1.15 mL) and water (233 μL), previously degasified by bubbling nitrogen under ultrasonic irradiation for a period of 10 min, was stirred under nitrogen at 85 °C for 4.45 h. After this time, the reaction mixture was cooled down, poured into water and worked up using EtOAc as extraction solvent. Column chromatography, eluting with hexane/EtOAc (from 9:1 to 8:2) as eluent, gave methyl acrylate derivative **10** (312 mg, 93%) as a solid. Mp 121–122 °C (from hexane/EtOAc). ^{1}H NMR (300 MHz, $CHCl_3$): δ=7.50 (s, 1H), 7.342 (m, 5H), 7.341 (dd, J=8.8, 2.5 Hz, 1H), 7.32 (d, J=2.5 Hz, 1H), 6.81 (d, J=8.8 Hz, 1H), 5.04 (s, 2H), 3.81 (s, 3H), 3.66 (s, 3H) ppm. HRMS (ES): calcd for $C_{18}H_{17}{}^{79}BrNaO_4$ $[M^+ + Na]^+$ 399.0208, found 399.0207.

*4.1.1.4. tert-Butyl 6-(3-((E)-1-(methoxycarbonyl)-2-methoxyvinyl)-4-(benzyloxy)phenyl)hex-5-ynoate (**12**).* A solution of *tert*-butyl hex-5-ynoate (**11**, 194 mg, 1.154 mmol) in anhydrous degassed DMF (1.03 mL) was added to a mixture of aryl bromide **10** (152.3 mg, 0.40 mmol), CuI (12 mg, 0.063 mmol) and $(Ph_3P)_2PdCl_2$ (38 mg, 0.054 mmol) followed anhydrous Et_3N (1.03 mL) under nitrogen atmosphere. The reaction mixture, initially yellow that turned deep-orange after a few minutes, was stirred in an oil bath at 70 °C for 22 h. It was then cooled down to rt, poured into water and worked up in the usual manner. Chromatography of the crude product with hexane/EtOAc (from 9:1 to 7:3) as eluent afforded alkyne **12** (162 mg, 88%) as a solid. Mp 94–96 °C (from hexane). ^{1}H NMR (300 MHz, $CHCl_3$): δ=7.50 (s, 1H), 7.34 (m, 5H), 7.31 (dd, J=8.5, 2.0 Hz, 1H), 7.25 (d, J=2.0 Hz, 1H), 6.84 (d, J=8.5 Hz, 1H), 5.06 (s, OCH_2, 2H), 3.80 (s, 3H), 3.64 (s, 3H), 2.43 (t, J=7.2 Hz, 2H), 2.39 (t, J=7.2 Hz, 2H), 1.86 (quint, J=7.2 Hz, 2H), 1.45 (s, 9H) ppm. HRMS (EI): calcd for $C_{28}H_{32}O_6$ 464.2199, found 464.2196.

*4.1.1.5. tert-Butyl 6-(3-((E)-1-(methoxycarbonyl)-2-methoxyvinyl)-4-(benzyloxy)phenyl)hexanoate (**13**).* A mixture of alkyne **12** (90.3 mg, 0.195 mmol) and 10% Pd/C in EtOAc (2.4 mL) was stirred under an atmosphere of hydrogen at 4 atm overnight. The reaction mixture was filtered through a short silica-gel column, using EtOAc as the eluent. The filtrate was concentrated to dryness to give pure the title compound **13** (73.5 mg, 100%) as a colourless oil. ^{1}H NMR (300 MHz, $CHCl_3$): δ=7.61 (s, 1H), 7.02 (dd, J=8.2, 2.1 Hz, 1H), 6.94 (d, J=2.1 Hz, 1H), 6.88 (d, J=8.2 Hz, 1H), 6.15 (br s, 1H), 3.88 (s, 3H), 3.76 (s, 3H), 2.53 (t, J=7.6 Hz, 2H), 2.20 (t, J=7.6 Hz, 2H), 1.60 (quint, J=7.6 Hz, 2H), 1.59 (quint, J=7.6 Hz, 2H,), 1.43 (s, 9H), 1.34 (quint, J=7.6 Hz, 2H) ppm. HRMS (EI): calcd for $C_{21}H_{30}O_6$ 378.2042, found 378.2031.

*4.1.1.6. tert-Butyl 6-(4-(6-chloropyrimidin-4-yloxy)-3-((E)-1-(methoxycarbonyl)-2-methoxyvinyl) phenyl)hexanoate (**15**).* A mixture of phenol **13** (60.8 mg, 0.16 mmol), Cs_2CO_3 (104.5 mg, 0.32 mmol) and 4,6-dichloropyrimidine (49.3 mg, 0.32 mmol) was dissolved in dry DMF (1.2 mL) at 0 °C under nitrogen. The mixture was stirred at this temperature for 2½ h, poured into water and worked up as usual using EtOAc as the extraction solvent. Chromatographic purification, eluting with hexane/EtOAc (9:1), afforded the chloropyrimidine **15** (68.4 mg, 87%) as a slightly coloured oil. ^{1}H NMR (300 MHz, $CHCl_3$): δ=8.56 (s, 1H), 7.42 (s, 1H), 7.18 (dd, J=8.3, 2.0 Hz, 1H), 7.11 (d, J=2.0 Hz, 1H), 7.05 (d, J=8.3 Hz, 1H), 6.75 (s, 1H), 3.72 (s, 3H), 3.58 (s, 3H), 2.63 (t, J=7.6 Hz, 2H), 2.21 (t, J=7.6 Hz, 2H), 1.66 (quint, J=7.6 Hz, 2H), 1.62 (quint, J=7.6 Hz, 2H), 1.43 (s, 9H), 1.25 (quint, J=7.6 Hz, 2H) ppm. HRMS (EI): calcd for $C_{25}H_{31}{}^{35}ClN_2O_6$ 490.1871, found 490.1860.

*4.1.1.7. tert-Butyl 6-(4-(6-(2-cyanophenoxy)pyrimidin-4-yloxy)-3-((E)-1-(methoxycarbonyl)-2-methoxyvinyl)phenyl)hexanoate (**17**).* A mixture of chloropyrimidine **15** (61.8 mg, 0.126 mmol), Cs_2CO_3

(82 mg, 0.252 mmol) and 2-cyanophenol (**16**, 60 mg, 0.504 mmol) in dry DMF (0.87 mL) was stirred at 85 °C under nitrogen for 2½ h. The resulting orange suspension was cooled to rt, diluted with water and worked up as usual using EtOAc as the extraction solvent. Chromatographic purification, eluting with hexane/EtOAc (from 8:2 to 7:3), yielded the *tert*-butyl ester**17** (67.2 mg, 93%) as an oil. ^{1}H NMR (300 MHz, $CHCl_3$): δ=8.40 (d, J=0.8 Hz, 1H), 7.71 (dd, J=7.7, 1.7 Hz, 1H), 7.66 (ddd, J=7.7, 8.3, 1.7 Hz, 1H), 7.47 (s, 1H), 7.36 (td, J=7.7, 1.0 Hz, 1H), 7.29 (dd, J=8.3, 1.0 Hz, 1H), 7.20 (dd, J=8.2, 2.3 Hz, 1H), 7.13 (d, J=2.3 Hz, 1H), 7.11 (d, J=8.2 Hz, 1H), 6.40 (d, J=0.8 Hz, 1H), 3.74 (s, 3H), 3.63 (s, 3H), 2.64 (t, J=7.7 Hz, 2H), 2.22 (t, J=7.4 Hz, 2H), 1.67 (quint, J=7.7 Hz, 2H), 1.64 (quint, J=7.4 Hz, 2H), 1.44 (s, 9H), 1.40 (m, 2H) ppm. HRMS (EI): calcd for $C_{32}H_{35}N_3O_7$ 573.2475, found 573.2481.

*4.1.1.8. 6-(4-(6-(2-Cyanophenoxy)pyrimidin-4-yloxy)-3-((E)-1-(methoxycarbonyl)-2-methoxyvinyl) phenyl)hexanoic acid (hapten AZa6, **2**).* A solution of *tert*-butyl ester**17** (57.7 mg, 0.10 mmol) in dry CH_2Cl_2 (700 μL) was treated with CF_3CO_2H (700 μL) under nitrogen. The mixture was stirred at rt for 20 min, then diluted with benzene and concentrated to dryness in vacuum; the residue was purified by chromatography, using $CHCl_3$/MeOH (95:5) as eluent, to give hapten AZa6 (**2**, 48 mg, 93%) as a viscous oil. IR (NaCl): ν_{max}=3219 (m), 2230 (m), 1709 (s), 1636 (s), 1589 (s), 1571 (s) cm^{-1}. ^{1}H NMR (300 MHz, $CHCl_3$): δ=8.41 (s, 1H, H-2 pyrim), 7.71 (dd, J=7.8, 1.6 Hz, 1H, H-3 C˜N−Ph), 7.66 (td, J=8.0, 1.6 Hz, 1H, H-5 CN−Ph), 7.48 (s, 1H, H-2′), 7.36 (br t, J=7.8 Hz, 1H, H-4 CN−Ph), 7.30 (br d, J=8.0, Hz, 1H, H-6 CN−Ph), 7.20 (dd, J=8.2, 2.1 Hz, 1H, H-6 Ph), 7.13 (d, J=2.1 Hz, 1H, H-2Ph), 7.11 (d, J=8.2 Hz, 1H, H-5 Ph), 6.41 (s, 1H, H-5 pyrim), 3.74 (s, 3H, OCH_3), 3.64 (s, 3H, CO_2CH_3), 2.65 (t, J=7.5 Hz, 2H, H_2-6), 2.36 (t, J=7.5 Hz, 2H, H_2-2), 1.69 (quint, J=7.5 Hz, 4H, H_2-5 and H_2-3), 1.43 (m, 2H, H_2-4) ppm. ^{13}C NMR (75 MHz, $CHCl_3$): δ=171.93 (C-6 pyrim), 171.89 (COOH), 170.02 (C-4 pyrim), 167.56 (*C*O$_2$Me), 160.69 (C-2′), 157.91 (C-2 pyrim), 154.10 (C-1 CN−Ph), 148.08 (C-4 Ph), 139.93 (C-1 Ph), 134.18 (C-5 CN−Ph), 133.56 (C-3 CN−Ph), 132.48 (C-2 Ph), 129.09 (C-6 Ph), 126.05 (C-4 CN−Ph), 125.46 (C-3 Ph), 123.03 (C-6 CN−Ph), 121.67 (C-5 Ph), 115.19 (CN), 107.25 (C-2 CN−Ph), 106.99 (C-1′), 92.33 (C-5 pyrim), 61.97 (OCH_3), 51.61 (CO$_2$*Me*), 35.02 (C-6), 33.82 (C-2), 30.67 (C-5), 28.59 (C-4), 24.45 (C-3) ppm. MS (EI): *m*/*z* (%)=517 (M^+, 18), 502 (35), 486 (18), 474 (10), 467 (4), 458 (100), 398 (13), 305 (12), 229 (40), 189 (22), 119 (8), 75 (25). HRMS (EI): calcd for $C_{28}H_{27}N_3O_7$ 517.1849, found 517.1857.

*4.1.2. Synthesis of hapten AZb6 (**3**).*

*4.1.2.1. 4,6-Dichloro-2-iodopyrimidine (**19**).* ^{t}BuONO (431 μL, 3.625 mmol) was added to a solution of aminopyrimidine **18** (129.3 mg, 0.788 mmol) and CH_2I_2 (3.27 mL) in anhydrous CH_3CN (820 μL) under nitrogen. The reaction mixture was heated in an oil bath at 80 °C for 3½ h, then cooled to rt and concentrated under reduced pressure. The residue was directly chromatographed on silica gel, eluting with hexane, to give the iodopyrimidine **19** (160 mg, 74%) as a white solid. Mp 97–98 °C (from hexane). ^{1}H NMR (300 MHz, $CHCl_3$): δ=7.40 (s, 1H) ppm. HRMS (EI): calcd for $C_4H^{35}Cl_2IN_2$ 273.8561, found 273.8557.

*4.1.2.2. tert-Butyl 6-(4,6-dichloropyrimidin-2-yl)hex-5-ynoate (**20**).* A solution of alkyne **20** (86 mg, 0.512 mmol) in anhydrous DMF (611 μL) and Et_3N (611 μL) were added sequentially via syringe to a mixture of iodopyrimidine **19** (127.7 mg, 0.464 mmol), CuI (6.3 mg, 0.033 mmol) and $(Ph_3P)_2PdCl_2$ (7.8 mg, 0.011 mmol) under nitrogen. The deep-orange mixture formed was stirred at rt for 2½ h, then poured into water and worked up using EtOAc to extract. Chromatographic purification, using hexane/EtOAc (from 9.5:0.5 to 8:2) as eluent, yielded the alkynylpyrimidine derivative **20** (106 mg, 73%) as a yellowish oil. ^{1}H NMR (300 MHz, $CHCl_3$): δ=7.31 (s, 1H), 2.53 (t, J=7.2 Hz, 2H), 2.38 (t, J=7.2 Hz, 2H), 1.91 (quint, J=7.2 Hz, 2H), 1.43 (s, 9H) ppm. HRMS (ES): calcd for $C_{14}H_{16}{}^{35}Cl_2N_2NaO_2$ $[M^+ + Na]^+$ 337.0487, found 337.0490.

*4.1.2.3. tert-Butyl 6-(4,6-dichloropyrimidin-2-yl)hexanoate (**21**).* To a solution of alkynylpyrimidine **20** (101.6 mg, 0.31 mmol) in EtOAc (4 mL) was added 10% palladium-on-carbon (39.2 mg) and the stirred mixture was hydrogenated at rt under balloon pressure of hydrogen. After 18 h, the mixture was filtered through a pad of silica gel, washing with EtOAc. The filtrate was concentrated under reduced pressure and the residue was chromatographed on silica gel, using hexane/EtOAc (9:1) as eluent, to yield alkylpyrimidine **21** (93.8 mg, 92%) as a colourless oil. ^{1}H NMR (300 MHz, $CHCl_3$): δ=7.23 (s, 1H), 2.89 (t, J=7.7 Hz, 2H), 2.20 (t, J=7.7 Hz, 2H), 1.80 (quint, J=7.7 Hz, 2H), 1.61 (quint, J=7.7 Hz, 2H), 1.41 (s, 9H), 1.38 (m, 2H) ppm. HRMS (EI): calcd for $C_{14}H_{20}{}^{35}Cl_2N_2O_2$ 318.0902, found 318.0909.

*4.1.2.4. Methyl (E)-2-(2-hydroxyphenyl)-3-methoxyacrylate (**22**).* A mixture of iodoacrylate **9** (634 mg, 2.62 mmol), arylboronic acid **23** (896.2 mg, 3.93 mmol), K_3PO_4 (1.668 g, 7.86 mmol) and $Pd(PPh_3)_4$ (121.1 mg, 0.105 mmol) in a previously degasified mixture of dioxane (13 mL) and water (2.62 mL) was stirred under nitrogen at 90 °C for 10 h. After this time, the reaction mixture was cooled down, diluted with ether and worked up. Column chromatography, eluting first with $CHCl_3$ to separate the excess of boronic acid and then with hexane/EtOAc (from 9:1 to 8:2), gave (*E*)-methyl 2-(2-(benzyloxy)phenyl)-3-methoxyacrylate (**24**, 687 mg, 88%) as an oil, which had physical and spectroscopic properties identical with those described in the literature.[30]

A mixture of **24** (690 mg, 0.065 mmol) and 10% Pd/C (120 mg) in EtOAc (6.3 mL) was stirred at rt under a pressure of hydrogen of 4 atm for 6 h. The mixture was filtered through a short column of silica gel, eluting with EtOAc, to afford pure compound **22** (467 mg, 97%) as an amorphous solid. The physical and spectroscopic properties of **22** were also identical to those described previously in the literature for this compound.[31]

*4.1.2.5. tert-Butyl 6-(4-chloro-6-(2-((E)-1-(methoxycarbonyl)-2-methoxyvinyl)phenoxy)pyrimidin-2-yl)hexanoate (**25**).* A mixture of 4,6-dichloro pyrimidine **21** (117 mg, 0.366 mmol), Cs_2CO_3 (118.9 mg, 0.366 mmol) and phenol **22** (38.2 mg, 0.184 mmol) was dissolved in dry DMF (1.4 mL) at 0 °C under nitrogen. The mixture was stirred at this temperature for 2½ h. After an additional 1 h at rt, the mixture was poured into water and worked up as usual using EtOAc as the extraction solvent. Chromatographic purification, eluting with hexane/EtOAc (from 95:5 to 8:2), afforded the chloropyrimidine **25** (54.1 mg, 60%) as a yellowish oil. ^{1}H NMR (300 MHz, $CHCl_3$): δ=7.45 (s, 1H), 7.39 (ddd, J=6.2, 8.0, 2.4 Hz, 1H), 7.33 (dd, J=6.2, 2.4 Hz, 1H), 7.29 (td, J=6.2, 1.2 Hz, 1H), 7.16 (dd, J=8.0, 1.2 Hz, 1H), 6.48 (s, 1H), 3.73 (s, 3H), 3.59 (s, 3H), 2.79 (t, J=7.7 Hz, 2H), 2.19 (t, J=7.6 Hz, 2H), 1.76 (quint, J=7.7 Hz, 2H), 1.59 (quint, J=7.6 Hz, 2H), 1.43 (s, 9H), 1.36 (m, 2H) ppm. HRMS (EI): calcd for $C_{25}H_{31}{}^{35}ClN_2O_6$ 490.1871, found 490.1881.

*4.1.2.6. tert-Butyl 6-(4-(2-cyanophenoxy)-6-(2-((E)-1-(methoxycarbonyl)-2-methoxyvinyl)phenoxy) pyrimidin-2-yl)hexanoate (**26**).* A mixture of chloropyrimidine **25** (67.8 mg, 0.138 mmol), Cs_2CO_3 (89.9 mg, 0.276 mmol) and 2-cyanophenol (**16**, 65.7 mg, 0.552 mmol) in dry DMF (0.96 mL) was stirred at 85 °C under nitrogen for 4 h. The reaction mixture was cooled to rt, diluted with water and worked up as usual using EtOAc as the extraction solvent. Chromatographic purification, eluting with hexane/EtOAc (from 9:1 to 7:3) as eluent, afforded the *tert*-butyl ester **26** (59.4 mg, 75%) as a yellowish oil. ^{1}H NMR (300 MHz, $CHCl_3$): δ=7.68 (dd, J=7.7, 1.5 Hz, 1H), 7.63 (ddd, J=7.7, 8.2, 1.5 Hz, 1H), 7.49 (s, 1H), 7.39 (ddd,

J=6.8, 8.0, 2.3 Hz, 1H), 7.33 (dd, *J*=6.8, 2.3 Hz, 1H), 7.32 (td, *J*=7.7, 1.4 Hz, 1H), 7.28 (td, *J*=6.8, 1.2 Hz, 1H), 7.26 (br d, *J*=8.2 Hz, 1H), 7.20 (dd, *J*=8.0, 1.2 Hz, 1H), 6.08 (s, 1H), 3.75 (s, 3H), 3.62 (s, 3H), 2.65 (t, *J*=7.7 Hz, 2H), 2.14 (t, *J*=7.7 Hz, 2H), 1.63 (quint, *J*=7.7 Hz, 2H), 1.52 (quint, *J*=7.7 Hz, 2H), 1.43 (s, 9H), 1.27 (m, 2H) ppm. HRMS (EI): calcd for $C_{32}H_{35}N_3O_7$ 573.2475, found 573.2471.

*4.1.2.7. 6-(4-(2-Cyanophenoxy)-6-(2-((E)-1-(methoxycarbonyl)-2-methoxyvinyl)phenoxy)pyrimidin-2-yl)hexanoic acid (hapten AZb6, **3**).* Trifluoroacetic acid (522 μL) was added to a solution of *tert*-butyl ester **26** (43 mg, 0.075 mmol) in dry CH_2Cl_2 (522 μL) at 0 °C. The mixture was stirred at rt for 20 min and then diluted with benzene. The mixture was concentrated under vacuum without heating and the residue obtained was purified by silica gel chromatography, using $CHCl_3$/MeOH (95:5) as eluent, to give hapten AZb6 (**3**, 37 mg, 95%) as a yellowish viscous oil. IR (NaCl): ν_{max}=3213 (m), 3017 (m), 2233 (m), 1710 (s), 1634 (m), 1588 (s), 1562 (s) cm^{-1}. ^1H NMR (300 MHz, $CHCl_3$): δ=7.68 (dd, *J*=7.7, 1.6 Hz, 1H, H-3 CN−Ph), 7.62 (td, *J*=7.7, 1.6 Hz, 1H, H-5 CN−Ph), 7.51 (s, 1H, H-2′), 7.39 (ddd, *J*=6.8, 7.9, 2.3 Hz, 1H, H-5 Ph), 7.33 (dd, *J*=6.8, 2.3 Hz, 1H, H-3 Ph), 7.32 (td, *J*=7.7, 2.1 Hz, 1H, H-4 CN−Ph), 7.29 (td, *J*=6.8, 1.3 Hz, 1H, H-4 Ph), 7.25 (br d, *J*=7.7 Hz, 1H, H-6 CN−Ph), 7.21 (dd, *J*=7.9, 1.3 Hz, 1H, H-6 Ph), 6.08 (s, 1H, H-5 pyrim), 3.75 (s, 3H, OCH_3), 3.62 (s, 3H, CO_2CH_3), 2.66 (t, *J*=7.4 Hz, 2H, H_2-6), 2.28 (t, *J*=7.7 Hz, 2H, H_2-2), 1.64 (quint, *J*=7.4 Hz, 2H, H_2-5), 1.57 (quint, *J*=7.7 Hz, 2H, H_2-3), 1.30 (m, 2H, H_2-4) ppm. ^{13}C NMR (75 MHz, $CHCl_3$): δ=179.06 (COOH), 171.95 (C-4 pyrim), 171.36 (C-6 pyrim), 169.96 (C-2 pyrim), 167.61 (CO_2Me), 160.92 (C-2′), 154.38 (C-1 CN−Ph), 150.36 (C-1 Ph), 133.95 (C-5 CN−Ph), 133.38 (C-3 CN−Ph), 132.74 (C-3 Ph), 129.15 (C-5 Ph), 126.05 (C-2 Ph), 125.70 (C-4 CN−Ph), 125.63 (C-4 Ph), 122.85 (C-6 CN−Ph), 121.88 (C-6 Ph), 115.40 (CN), 107.11 (C-2 CN−Ph), 106.86 (C-1′), 88.76 (C-5 pyrim), 62.01 (OCH_3), 51.65 (CO_2Me), 38.18 (C-6), 33.77 (C-2), 28.31 (C-4), 27.06 (C-5), 24.40 (C-3) ppm. MS (EI): *m*/*z* (%)=518 (M^++1, 24), 517 (M^+, 89), 502 (37), 486 (98), 458 (52), 430 (41), 402 (77), 398 (83), 386 (95), 359 (57), 326 (46), 267 (20), 223 (37), 191 (47), 176 (45), 145 (47), 119 (63), 102 (65), 91 (100), 75 (68). HRMS (EI): calcd for $C_{28}H_{27}N_3O_7$ 517.1849, found 517.1843.

*4.1.3. Synthesis of hapten AZc6 (**4**).*

*4.1.3.1. 2-Hydroxy-5-iodobenzonitrile (**27**).* Ag_2SO_4 (4.978 g, 15.967 mmol) was added in small portions to a stirred solution of 2-cyanophenol (**16**, 1.902 g, 15.967 mmol) and I_2 (4.457 g, 17.547 mmol) in dry CH_2Cl_2 (40.5 mL). The reaction mixture was stirred for 2 days at rt and filtered to removed the solids. The filtrate and washings ($CHCl_3$) were successively washed with a 5% aqueous solution of sodium thiosulfate and brine, and dried over anhydrous Na_2SO_4. Evaporation of the solvent under reduced pressure gave almost pure the iodo-phenol **27** (3.258 g, 84%) as a white solid, which showed the same physical and spectroscopic data as those previously reported.[32]

*4.1.3.2. 2-(Benzyloxy)-5-iodobenzonitrile (**28**).* Benzyl bromide (1.4 mL, 12.013 mmol) was added to a solution of anhydrous K_2CO_3 (1.66 g, 12.013 mmol) and phenol **27** (2.676 g, 10.921 mmol) in dry DMF (20 mL). The mixture was stirred for 4 h at rt, then poured into water and work up as usual with hexane. The residue was purified by silica-gel column, eluting with hexane/EtOAc (9:1), to afford benzyl ether **28** (2.77 g, 74%) as a white solid. Mp 75–77°C (from benzene). ^1H NMR (300 MHz, $CHCl_3$): δ=7.84 (d, *J*=2.2 Hz, 1H), 7.74 (dd, *J*=8.9, 2.2 Hz, 1H), 7.38 (m, 5H), 6.77 (d, *J*=8.9 Hz, 1H), 5.20 (s, 2H) ppm. HRMS (EI): calcd for $C_{14}H_{10}INO$ 334.9807, found 334.9801.

*4.1.3.3. tert-Butyl 6-(4-(benzyloxy)-3-cyanophenyl)hex-5-ynoate (**29**).* A solution of alkyne **11** (670 mg, 3.99 mmol) in anhydrous DMF (4.2 mL) and Et_3N (4.2 mL) were consecutively added to a mixture of aryl iodide **28** (1.08 g, 3.2 mmol), CuI (80 mg, 0.42 mmol) and $(Ph_3P)_2PdCl_2$ (82 mg, 0.12 mmol) under nitrogen. The reaction mixture, originally yellowish that turned deep-orange after a few minutes, was stirred at rt for 5½ h, poured into water and worked up with EtOAc. Chromatographic purification, using hexane/EtOAc (9.5:0.5) as eluent, afforded the aryl-alkyne **29** (1.18 g, 98%) as a white solid. Mp 86–88 °C (from benzene). ^1H NMR (300 MHz, $CHCl_3$): δ=7.60 (d, *J*=2.1 Hz, 1H), 7.49 (dd, *J*=8.8, 2.1 Hz, 1H), 7.38 (m, 5H), 6.91 (d, *J*=8.8 Hz, 1H), 5.21 (s, 2H), 2.44 (t, *J*=7.1 Hz, 2H), 2.38 (t, *J*=7.1 Hz, 2H), 1.86 (quint, *J*=7.1 Hz, 2H), 1.45 (s, 9H) ppm. HRMS (EI): calcd for $C_{24}H_{25}NO_3$ 375.1834, found 375.1828.

*4.1.3.4. tert-Butyl 6-(3-cyano-4-hydroxyphenyl)hexanoate (**30**).* A mixture of aryl-alkyne **29** (315.6 mg, 0.841 mmol) and 10% Pd/C (33.4 mg) in EtOAc (4.3 mL) was purged with nitrogen, hydrogen bubbled through the solution and the mixture kept under hydrogen at atmospheric pressure (balloon) during 7 h at rt. The reaction mixture was filtered through a short pad of Celite and the residue left after evaporation of the solvent was chromatographed on silica gel with hexane/Et_2O (9:1) as eluent to give the phenol **30** (218.7 mg, 90%) as colourless oil. ^1H NMR (300 MHz, $CHCl_3$): δ=7.27 (d, *J*=2.1 Hz, 1H), 7.24 (dd, *J*=8.5, 2.1 Hz, 1H), 6.90 (d, *J*=8.5 Hz, 1H), 6.76 (br s, 1H), 2.53 (t, *J*=7.5 Hz, 2H), 2.22 (t, *J*=7.4 Hz, 2H), 1.60 (quint, *J*=7.4 Hz, 2H), 1.57 (quint, *J*=7.5 Hz, 2H), 1.44 (s, 9H), 1.31 (m, 2H) ppm. HRMS calcd for $C_{17}H_{23}NO_3$ 289.1678, found 289.1667.

*4.1.3.5. tert-Butyl 6-(3-cyano-4-(6-(2-((E)-1-(methoxycarbonyl)-2-methoxyvinyl)phenoxy) pyrimidin-4-yloxy)phenyl)hexanoate (**32**).* A mixture of 6-chloropyrimidine **31** (122.4 mg, 0.382 mmol), Cs_2CO_3 (134.2 mg, 0.412 mmol) and phenol **30** (119.4 mg, 0.412 mmol) was dissolved in dry DMF (2.1 mL) at 85 °C under nitrogen. The mixture was stirred at this temperature for 3 h, then cooled down to rt, poured into water and worked up as usual using EtOAc as the extraction solvent. Chromatographic purification, eluting with hexane/EtOAc (8:2), afforded the compound **32** (199 mg, 91%) as a yellowish oil. ^1H NMR (300 MHz, $CHCl_3$): δ=8.40 (s, 1H), 7.49 (br s, 2H), 7.45 (dd, *J*=8.6, 2.2 Hz, 1H), 7.41 (ddd, *J*=7.7, 8.4, 2.3 Hz, 1H), 7.35 (dd, *J*=7.7, 2.3 Hz, 1H), 7.30 (td, *J*=7.7, 1.0 Hz, 1H), 7.22 (dd, *J*=8.4, 1.0 Hz, 1H), 7.18 (d, *J*=8.6 Hz, 1H), 6.39 (s, 1H), 3.74 (s, 3H), 3.63 (s, 3H), 2.65 (t, *J*=7.6 Hz, 2H), 2.22 (t, *J*=7.6 Hz, 2H), 1.65 (quint, *J*=7.6 Hz, 2H), 1.63 (quint, *J*=7.6 Hz, 2H), 1.44 (s, 9H), 1.37 (m, 2H) ppm. HRMS (EI): calcd for $C_{32}H_{35}N_3O_7$ 573.2475, found 573.2485.

*4.1.3.6. 6-(3-Cyano-4-(6-(2-((E)-1-(methoxycarbonyl)-2-methoxyvinyl)phenoxy)pyrimidin-4-yloxy) phenyl)hexanoic acid (hapten AZc6, **4**).* A solution of *tert*-butyl ester **32** (32 mg, 0.055 mmol) in dry CH_2Cl_2 (160 μL) was treated with CF_3CO_2H (160 μL) under nitrogen. The mixture was stirred at rt for 10 min and concentrated to dryness in vacuum; the residue was purified by chromatography, using $CHCl_3$/MeOH (95:5) as eluent, to give hapten AZc6 (**4**, 25.9 mg, 90%) as a viscous oil. IR (NaCl) ν_{max}=3213 (m), 3019 (m), 2234 (m), 1709 (s), 1629 (m), 1591 (s), 1570 (s) cm^{-1}. ^1H NMR (300 MHz, $CHCl_3$): δ=8.41 (s, 1H, H-2 pyrim), 7.50 (s, 1H, H-2′), 7.49 (d, *J*=2.3 Hz, 1H, H-2 CN−Ph), 7.45 (dd, *J*=8.7, 2.3 Hz, 1H, H-6 CN−Ph), 7.41 (ddd, *J*=7.7, 8.2, 2.3 Hz, 1H, H-5 Ph), 7.35 (dd, *J*=7.7, 2.3 Hz, 1H, H-3 Ph), 7.30 (td, *J*=7.7, 1.0 Hz, 1H, H-4 Ph), 7.21 (dd, *J*=8.2, 1.0 Hz, 1H, H-6 Ph), 7.18 (d, *J*=8.7 Hz, 1H, H-5 CN−Ph), 6.91 (br s, 1H, COOH), 6.38 (s, 1H, H-5 pyrim), 3.75 (s, 3H, OCH_3), 3.63 (s, 3H, CO_2CH_3), 2.66 (t, *J*=7.6 Hz, 2H, H_2-6), 2.36 (t, *J*=7.4 Hz, 2H, H_2-2), 1.68 (quint, *J*=7.4 Hz, 2H, H_2-3), 1.67 (quint, *J*=7.6 Hz, 2H, H_2-5), 1.40 (m, 2H, H_2-4) ppm. ^{13}C NMR (75 MHz, $CHCl_3$): δ=178.81 (COOH), 171.71 (C-4 pyrim), 170.27 (C-6 pyrim), 167.52 (CO_2Me), 160.84 (C-2′), 157.89 (C-2 pyrim), 152.00 (C-4 CN−Ph), 150.10 (C-1 Ph), 140.81 (C-1 CN−Ph), 134.36 (C-6 CN−Ph), 133.06 (C-2 CN−Ph), 132.74 (C-3 Ph), 129.18 (C-5 Ph), 125.98 (C-2 Ph), 125.92 (C-4 Ph), 122.83 (C-5 CN−Ph), 122.01 (C-6 Ph), 115.34 (CN), 106.92 (C-3 CN−Ph), 106.85 (C-1′), 92.27 (C-5 pyrim), 61.02 (OCH_3),

51.65 (CO_2Me), 34.63 (C-6), 33.66 (C-2), 30.51 (C-5), 28.33 (C-4), 24.38 (C-3) ppm. MS (EI): *m*/*z* (%)=518 (M^++1, 6), 517 (M^+, 20), 502 (42), 486 (15), 458 (100), 440 (14), 414 (4), 343 (3), 145 (4), 75 (5). HRMS (EI): calcd for $C_{28}H_{27}N_3O_7$ 517.1849, found 517.1849.

*4.1.4. Synthesis of hapten AZo6 (**5**).*

*4.1.4.1. tert-Butyl 6-((E)-2-(methoxycarbonyl)vinyloxy)hexanoate (**34**).* PBu_3 (72 μL, 0.291 mmol) was added to a solution of *tert*-butyl 6-hydroxyhexanoate[33] (**33**; 331.5 mg, 1.763 mmol) in anhydrous CH_2Cl_2 (19.3 mL) under nitrogen. The mixture was cooled to 0 °C and methyl propiolate (162 μL, 1.938 mmol) was then added drop wise via a syringe over 5 min. The reaction mixture was allowed to reach rt and stirred for 30 min, during which time the initially colourless solution turned to yellow and finally to deep red. The reaction mixture was exposed to the air and stirred for 20 min in order to oxidize and facilitate the separation of the PBu_3. The resulting black mixture was concentrated at reduced pressure and the residue was purified by chromatography, using hexane/EtOAc (8:2) as eluent, to give acrylate derivative **34** (445 mg, 93%) as an oil. ^{1}H NMR (300 MHz, $CHCl_3$): δ=7.56 (d, *J*=12.6 Hz, 1H), 5.16 (d, *J*=12.6 Hz, 1H), 3.81 (t, *J*=6.4 Hz, 2H), 3.67 (s, 3H), 2.20 (t, *J*=7.3 Hz, 2H), 1.69 (quint, *J*=6.4 Hz, 2H), 1.59 (quint, *J*=7.3 Hz, 2H), 1.41 (s, 9H), 1.38 (m, 2H) ppm. HRMS (ES): calcd for $C_{14}H_{24}NaO_5$ $[M+Na]^+$ 295.1521, found 295.1527.

*4.1.4.2. tert-Butyl 6-((Z)-2-(methoxycarbonyl)-2-iodovinyloxy) hexanoate (**35**).* A solution of **34** (211 mg, 0.77 mmol) in CH_2Cl (2 mL) was added to *N*-iodosuccinimide (235 mg, 1.044 mmol) at rt under nitrogen. Then AcOH (80 μL, 1.386 mmol) was added and the mixture was stirred for 17 h. After this time, the red-wine solution formed was treated with Et_3N (347 μL, 2.49 mmol) and then stirred for 22 h, poured into water, and extracted with EtOAc. The combined organic extracts were washed with 10% aqueous solution of $Na_2S_2O_3$ and brine, and dried over anhydrous Na_2SO_4. Chromatographic purification of the residue left after evaporation of the solvent, using hexane/EtOAc (from 9:1 to 8:2) as eluent, afforded, in order of elution, first the vinyl iodide **35** (172 mg, 74% based on recovered starting material) as a yellowish oil, followed by the starting compound **34** (54 mg). Spectral data for **35**: ^{1}H NMR (300 MHz, $CHCl_3$): δ=7.71 (s, 1H), 4.16 (t, *J*=6.5 Hz, 2H), 3.77 (s, 3H), 2.23 (t, *J*=7.3 Hz, 2H), 1.74 (quint, *J*=6.5 Hz, 2H), 1.62 (quint, *J*=7.3 Hz, 2H), 1.43 (s, 9H), 1.42 (m, 2H) ppm. HRMS (ES): calcd for $C_{14}H_{23}INaO_5$ $[M+Na]^+$ 421.0488, found 421.0496.

*4.1.4.3. tert-Butyl 6-((E)-2-(methoxycarbonyl)-2-(2-(benzyloxy) phenyl)vinyloxy)hexanoate (**36**).* A mixture of vinyl iodide 35 (89 mg, 0.223 mmol), 2-(benzyloxy)phenylboronic acid (**23**, 79.7 mg, 0.349 mmol), K_3PO_4 (148.38 mg, 0.699 mmol) and Pd $(PPh_3)_4$ (17 mg, 0.015 mmol) in a previously degassed mixture of dioxane (1.15 mL) and water (233 μL) was heated at 90 °C with stirring under nitrogen for 7 h. The cooled mixture was poured into water and worked up with EtOAc in the usual manner to yield an oily residue. Purification by chromatography gave compound **36** (82.1 mg, 81%) as an oil. ^{1}H NMR (300 MHz, $CHCl_3$): δ=7.56 (s, 1H), 7.32 (m, 5H), 7.23 (ddd, *J*=7.5, 8.1, 1.7 Hz, 1H), 7.21 (dd, *J*=7.5, 1.7 Hz, 1H), 6.97 (td, *J*=7.5, 1.0 Hz, 1H), 6.94 (dd, *J*=8.1, 1.0 Hz, 1H), 5.07 (s, 2H), 3.97 (t, *J*=6.7 Hz, 2H), 3.64 (s, 3H), 2.16 (t, *J*=7.5 Hz, 2H), 1.63 (quint, *J*=6.7 Hz, 2H), 1.54 (quint, *J*=7.5 Hz, 2H), 1.43 (s, 9H), 1.30 (m, 2H) ppm. HRMS (ES): calcd for $C_{27}H_{34}O_6$ 454.2355, found 454.2377.

*4.1.4.4. tert-Butyl 6-((E)-2-(methoxycarbonyl)-2-(2-hydroxyphenyl)vinyloxy)hexanoate (**37**).* Benzyl ether **36** (548 mg, 1.205 mmol) was dissolved in EtOAc (5.3 mL), 10% Pd/C (26 mg) was added to the solution and the mixture was hydrogenated at 1 atm of H_2 pressure (balloon) for 1 h. The catalyst was remove by filtration though a pad of Celite and rinsed with EtOAc. The solvent was removed under reduced pressure to give a clear yellow oil. Purification by column chromatography using hexane/EtOAc (from 9:1 to 7:3) gave the phenol **37** (368 mg, 84%) as an oil. ^{1}H NMR (300 MHz, $CHCl_3$): δ=7.67 (s, 1H), 7.20 (ddd, *J*=7.4, 8.4, 1.7 Hz, 1H), 7.14 (dd, *J*=7.4, 1.7 Hz, 1H), 6.95 (dd, *J*=8.4, 1.2 Hz, 1H), 6.90 (td, *J*=7.4, 1.2 Hz, 1H), 6.43 (br s, 1H), 4.05 (t, *J*=6.5 Hz, 2H), 3.75 (s, 3H), 2.20 (t, *J*=7.4 Hz, 2H), 1.68 (quint, *J*=6.5 Hz, 2H), 1.58 (quint, *J*=7.4 Hz, 2H), 1.43 (s, 9H), 1.34 (m, 2H) ppm. HRMS (ES): calcd for $C_{20}H_{28}O_6$ 364.1886, found 364.1877.

*4.1.4.5. tert-Butyl 6-((E)-2-(methoxycarbonyl)-2-(2-(6-chloropyrimidin-4-yloxy)phenyl)vinyloxy) hexanoate (**38**).* A solution of phenol **37** (94 mg, 0.257 mmol) in anhydrous DMF (1 mL) was added to a solution of 4,6-dichoropyrimidine (38.5 mg, 0.257 mmol) and Cs_2CO_3 (84 mg, 0.257 mmol) in DMF (1 mL) at 0 °C under nitrogen. The mixture was stirred at 0 °C for 45 min, then poured into water and worked up as usual with EtOAc. Chromatographic purification of the residue obtained, using hexane/EtOAc (9:1) as eluent, gave the chloropyrimidine **38** (85.5 mg, 70%) as a yellowish oil. ^{1}H NMR (300 MHz, $CHCl_3$): δ=8.57 (d, *J*=0.6 Hz, 1H), 7.50 (s, 1H), 7.40 (ddd, *J*=7.2, 8.0, 3.0 Hz, 1H), 7.34 (dd, *J*=7.2, 3.0 Hz, 1H), 7.31 (td, *J*=7.2, 1.2 Hz, 1H), 7.16 (br d, *J*=8.0 Hz, 1H), 6.75 (d, *J*=0.6 Hz, 1H), 3.92 (t, *J*=6.9 Hz, 2H), 3.58 (s, 3H), 2.19 (t, *J*=7.4 Hz, 2H), 1.59 (quint, *J*=6.9 Hz, 2H), 1.56 (quint, *J*=7.4 Hz, 2H), 1.43 (s, 9H), 1.29 (m, 2H) ppm. HRMS (ES): calcd for $C_{24}H_{29}{}^{35}ClN_2O_6$ 476.1714, found 476.1702.

*4.1.4.6. tert-Butyl 6-((E)-2-(methoxycarbonyl)-2-(2-(6-(2-cyanophenoxy)pyrimidin-4-yloxy)phenyl) vinyloxy)hexanoate (**39**).* A solution of chloropyrimidine **38** (85.5 mg, 0.180 mmol), Cs_2CO_3 (58.4 mg, 0.18 mmol) and 2-cyanophenol (31.3 mg, 0.18 mmol) in dry DMF (1.2 mL) was stirred at 85 °C for 5 h under nitrogen. The mixture was cooled down to rt and worked up as usual with EtOAc. Purification by chromatography eluting with hexane/EtOAc (from 8:2 to 7:3) afforded the compound **39** (77.5 mg, 80%) as an oil. ^{1}H NMR (300 MHz, $CHCl_3$): δ=8.38 (s, 1H), 7.71 (dd, *J*=7.8, 1.6 Hz, 1H), 7.66 (td, *J*=7.8, 1.6 Hz, 1H), 7.55 (s, 1H), 7.40 (ddd, *J*=6.9, 8.0, 2.3 Hz, 1H), 7.36 (td, *J*=7.8, 1.1 Hz, 1H), 7.34 (dd, *J*=6.9, 2.3 Hz, 1H), 7.293 (br d, *J*=7.8 Hz, 1H), 7.290 (td, *J*=6.9, 1.1 Hz, 1H), 7.21 (dd, *J*=8.0, 1.1 Hz, 1H), 6.391 (s, 1H), 3.95 (t, *J*=6.7 Hz, 2H), 3.61 (s, 3H), 2.18 (t, *J*=7.3 Hz, 2H), 1.61 (quint, *J*=6.7 Hz, 2H), 1.56 (quint, *J*=7.3 Hz, 2H), 1.42 (s, 9H), 1.31 (m, 2H) ppm. HRMS (ES): calcd for $C_{31}H_{33}N_3O_7$ 559.2319, found 559.2313.

*4.1.4.7. 6-((E)-2-(Methoxycarbonyl)-2-(2-(6-(2-cyanophenoxy) pyrimidin-4-yloxy)phenyl)vinyloxy) hexanoic acid (hapten AZo5, **5**).* Trifluoroacetic acid (0.5 mL) was added to a solution of *tert*-butyl ester **39** (40 mg, 0.072 mmol) in dry CH_2Cl_2 (0.5 mL) at 0 °C. The mixture was stirred at rt for 20 min under nitrogen, the mixture was diluted with benzene and then concentrated under vacuum without heating. The residue obtained was purified by silica gel chromatography, using $CHCl_3$/MeOH (90:1) as eluent, to give hapten AZo6 (**5**, 33.1 mg, 92%) as a yellowish viscous oil. IR (NaCl) ν_{max}=3200 (m), 3071 (m), 1708 (s), 1632 (s), 1591 (s), 1567 (s) cm^{-1}. ^{1}H NMR (300 MHz, $CHCl_3$): δ=8.40 (s, 1H, H-2 pyrim), 7.71 (dd, *J*=7.8, 1.6 Hz, 1H, H-3 CN−Ph), 7.66 (td, *J*=7.8, 1.6 Hz, 1H, H-5 CN−Ph), 7.56 (s, 1H, C-1′), 7.41 (ddd, *J*=7.7, 7.8, 2.7 Hz, 1H, H-4 Ph), 7.36 (td, *J*=7.8, 1.0 Hz, 1H, H-4 CN−Ph), 7.34 (dd, *J*=7.7, 2.7 Hz, 1H, H-6 Ph), 7.29 (br d, *J*=7.8 Hz, 1H, H-6 CN−Ph), 7.30 (td, *J*=7.7, 0.8 Hz, 1H, H-5 Ph), 7.21 (dd, *J*=7.8, 0.8 Hz, 1H, H-3 Ph), 6.38 (s, 1H, H-5 pyrim), 3.97 (t, *J*=7.2 Hz, 2H, H_2-6), 3.62 (s, 3H, CO_2CH_3), 2.33 (t, *J*=7.2 Hz, 2H, H_2-2), 1.62 (quint, *J*=7.2 Hz, 4H, H_2-5, H_2-3), 1.36 (m, 2H, H_2-4) ppm. ^{13}C NMR (75 MHz, $CHCl_3$): δ=177.92 (COOH), 171.82 (C-6 pyrim), 170.12 (C-4 pyrim), 167.62 (CO_2Me), 159.86 (C-1′), 157.88 (C-2 pyrim), 154.08 (C-1 CN−Ph), 150.21 (C-2, Ph), 134.19 (C-5 CN−Ph), 133.59 (C-3 CN−Ph), 132.78 (C-6 Ph), 129.18 (C-4 Ph),

126.25 (C-1 Ph), 126.10 (C-4 CN–Ph), 125.93 (C-5 Ph), 123.03 (C-6 CN–Ph), 121.83 (C-3 Ph), 115.18 (CN), 107.27 (C-2 CN–Ph), 106.54 (C-2′), 92.44 (C-5 pyrim), 74.95 (C-6), 51.60 (CO_2Me), 33.68 (C-2), 29.23 (C-5), 24.76 (C-4), 24.19 (C-3) ppm. MS (EI): *m*/*z* (%)=504 (M^++1, 5), 503 (M^+, 16), 444 (58), 388 (100), 329 (62), 301 (20), 176 (19), 172 (17), 145 (11), 102 (8), 69 (25), 55 (19). HRMS (ES): calcd for $C_{27}H_{25}N_3O_7$ 503.1693, found 503.1718.

4.2. Immunochemistry

Procedure details and equipment for protein conjugation and immunoassay performance are given as Supplementary data.

4.2.1. Bioconjugate preparation. Immunogens were prepared by coupling the haptens to BSA. In a first step, the carboxylate group of haptens was activated in DMF using either *N*-hydroxysuccinimide and *N*,*N*-dicyclohexylcarbodiimide or *N*,*N*′-disuccinimidyl carbonate. Next, the coupling reaction was performed in buffer at a hapten-to-protein molar ratio of 44:1. On the other hand, assay coating conjugates were prepared using OVA as carrier. In this case, the activation of the carboxylate was accomplished using tributylamine and isobutyl chloroformate. The initial molar ratio in the coupling reaction with this protein was 13:1. A brief description of the followed coupling procedures has been included as Supplementary data.

4.2.2. Polyclonal antibody production. Animal manipulation was performed in compliance with the laws and guidelines of the Spanish Ministry of Science and Innovation (RD 1201/2005 and law 32/2007) and according to the European Directive 2003/65/EC concerning the protection of animals used for experimental and other scientific purposes. New Zealand white rabbits were immunized at three-week intervals with 300 μg of BSA–hapten conjugate (see the Supplementary data) following described standard protocols.[34] 10 days after the fourth boost the animals were exsanguinated, the antisera were separated by centrifugation and precipitated twice with an ammonium sulfate saturated solution.

4.2.3. Competitive ELISAs. 96-well polystyrene ELISA plates were coated with 100 μL per well of OVA conjugate solution at 1.0 or 0.1 μg/mL in 50 mM carbonate–bicarbonate buffer, pH 9.6 by overnight incubation at rt. Plates were washed four times with a solution containing 150 mM NaCl and 0.05% (v/v) Tween 20. Bidimensional competitive assays were carried out in order to find the limiting concentrations of the immunoreagents. A serial dilution of azoxystrobin was prepared, in borosilicate glass tubes, with 10 mM phosphate buffer, pH 7.4 containing 140 mM NaCl, and the antisera were also serially diluted in the same buffer containing 0.05% (v/v) Tween 20. Each plate column received a complete standard curve of the analyte (50 μL per well) followed by a different dilution per column of a given antiserum (50 μL per well). The same distribution of the reagents was repeated for each plate with a different coating conjugate concentration. The immunological reaction took place during 1 h at rt, and plates were washed again as described. Next, 100 μL per well of a 1/10,000 dilution of goat anti-rabbit immunoglobulin peroxidase conjugate in phosphate buffer with Tween 20 was added, and plates were incubated 1 h at rt. After washing, retained peroxidase activity was determined by addition of 100 μL per well of freshly prepared 2 mg/mL *o*-phenylenediamine and 0.012% (v/v) H_2O_2 solution in 25 mM citrate and 62 mM sodium phosphate buffer, pH 5.4. The enzymatic reaction was stopped after 10 min with 100 μL per well of 2.5 M H_2SO_4.

The absorbance was immediately read at 492 nm with a reference wavelength at 650 nm. These simultaneous titration and competitive assays resulted in one inhibition curve per column corresponding to a certain combination of concentrations of the immobilized immunoreagent (OVA conjugate) and the immunoreagent in solution (antiserum). Sigmoidal curves were mathematically fitted to a four-parameter logistic equation using the SigmaPlot software package from SPSS Inc. (Chicago, IL). The antiserum titre was defined as the reciprocal of the dilution that results in a maximum absorbance value (A_{max}) around 1.0 reached at the zero dose of analyte. Antibody affinity or IC_{50} was estimated as the concentration of analyte at the inflection point of the fitted curve, typically corresponding to a 50% reduction of the A_{max} if the background signal approaches to zero. Cross-reactivity (CR) was calculated according to the formula:

$$CR = [IC_{50}(AZ_{E\ \text{isomer}})/IC_{50}(AZ_{Z\ \text{isomer}})] \times 100$$

Acknowledgements

This work was supported by Ministerio de Educación y Ciencia (AGL2006-12750-C02-01/02/ALI) and cofinanced by FEDER funds. J.P. and J.V.M. were hired by the CSIC, the former under a predoctoral I3P contract and the latter under a Ramón y Cajal postdoctoral contract, both of them financed by Ministerio de Ciencia e Innovación and the European Social Fund.

Limited amounts of the newly described immunoreagents are available upon request for evaluation.

Supplementary data

Supplementary data associated with this article can be found in the online version, at This Supplementary data contains general details, experimental biochemical procedures, full spectral data and assignments for all intermediates of the synthesis of haptens **2**–**5**, preparation and characterisation data of compounds **40** and **41** and copies of the ^{1}H NMR spectra of haptens **2**–**5**. Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.tet.2010.11.054. These data include MOL files and InChiKeys of the most important compounds described in this article.

References and notes

1. (a) Review: Toldrá, F.; Reig, M. *Trends Food Sci. Technol.* **2006**, *17*, 482–489; (b) Salvador, P.; Sánchez-Baeza, F.; Marco, M.-P. *Anal. Chem.* **2007**, *79*, 3734–3740.
2. (a) Pozharski, E.; Moulin, A.; Hewagama, A.; Shanafelt, A. B.; Petsko, G. A.; Ringe, D. *J. Mol. Biol.* **2005**, *349*, 570–582; (b) Carrera, M. R. A.; Ashley, J. A.; Parsons, L. H.; Wirsching, P.; Koob, G. F.; Janda, K. D. *Nature* **1995**, *378*, 727–730.
3. (a) For example: Adrián, J.; Font, H.; Diserens, J.-M.; Sánchez-Baeza, F.; Marco, M.-P. *J. Agric. Food Chem.* **2009**, *57*, 385–394; (b) Wang, Z.; Zhu, Y.; Ding, S.; He, F.; Beier, R. C.; Li, J.; Jiang, H.; Feng, C.; Wan, Y.; Zhang, S.; Kai, Z.; Yang, X.; Shen, J. *Anal. Chem.* **2007**, *79*, 4471–4483.
4. Shan, G.; Lipton, C.; Gee, S.; Hammock, B. D. In *Handbook of Residue Analytical Methods for Agrochemicals*; Lee, P. W., Ed.; John Wiley: Chichester, UK, 2002; pp 623–679.
5. (a) Matsushita, M.; Hoffman, T. Z.; Ashley, J. A.; Zhou, B.; Wirshing, P.; Janda, K. D. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 87–90; (b) Peterson, E. C.; Gunnell, M.; Che, Y.; Goforth, R. L.; Carroll, F. I.; Henry, R.; Liu, H.; Owens, S. M. *J. Pharmacol. Exp. Ther.* **2007**, *322*, 30–39.
6. Landsteiner, K. In *The Specificity of Serological Reactions (Revised Edition)*; Dover Publications: New York, NY, 1962.
7. (a) Ballesteros, B.; Barceló, D.; Sánchez-Baeza, F.; Camps, F.; Marco, M.-P. *Anal. Chem.* **1998**, *70*, 4004–4014; (b) Galve, R.; Camps, F.; Sánchez-Baeza, F.; Marco, M.-P. *Anal. Chem.* **2000**, *72*, 2237–2246; (c) Shelver, W. L.; Keum, Y. S.; Kim, H. J.; Rutherford, D.; Hakk, H. H.; Bergman, A.; Li, Q. X. *J. Agric. Food Chem.* **2005**, *53*, 3840–3847.
8. Bartlett, D. W.; Clough, J. M.; Godwin, J. R.; Hall, A. A.; Hamer, M.; Parr-Dobrzanski, B. *Pest Manage. Sci.* **2002**, *58*, 649–662.
9. For azoxystrobin sales visit the Syngenta web site at: http://www2.syngenta.com/en/investor_relations/index.html.
10. (a) Clough, J.M.; Godfrey, C.R.A. European Patent 0382375, 1990; (b) Wood, W.W.; Cuccia, S.J.; Brigance, J. European Patent 0794177, 1997; (c) Gayer, H.; Gerdes, P.; Schallner, O.; Dutzmann, S. U.S. Patent 05,773,445, 1998; (d) Jones, R.V.; Ewins, R.; Fleming, I.G.; McNeish, S.; Whitton, A.J. WO Patent 09818767, 1998.
11. (a) Brayer, J.L.; Hodgson, D.M.; Richards, I.C.; Witherington, J. WO Patent 9424085, 1994; (b) Kim, B.T.; Min, Y.K.; Lee, Y.S.; Heo, J.; Lee, H.; Park, N.K.; Kim, J.-K.; Kim, S.-W.; Ko, S.-Y. WO Patent 2005123054, 2005.
12. Espinet, P. *Chem.—Eur. J.* **2006**, *12*, 9346–9352.

13. Methyl (*Z*)-2-iodo-3-methoxyacrylate (**9**) was prepared according to: Coleman, R. S.; Lu, X. *Chem. Commun.* **2006**, 423–425.
14. Kim, H. H.; Lee, C. H.; Song, Y. S.; Park, N. K.; Kim, B. T.; Heo, J. N. *Bull. Korean Chem. Soc.* **2006**, *27*, 191–192.
15. *tert*-Butyl hex-5-ynoate (**11**) was prepared by esterification of commercial 2-hex-5-ynoic acid, see: Bartoli, G.; Bosco, M.; Carlone, A.; Dalpozzo, R.; Marcantoni, E.; Melchiorre, P.; Sambri, L. *Synthesis* **2007**, 3489–3496.
16. Li, J.-H.; Liang, Y.; Xie, Y.-X. *J. Org. Chem.* **2005**, *70*, 4393–4396.
17. Harden, D. B.; Mokrosz, M. J.; Strekowski, L. *J. Org. Chem.* **1998**, *53*, 4137–4140.
18. Mathieu, R.; Baurand, A.; Schmitt, M.; Gachet, C.; Bourguignon, J.-J. *Bioorg. Med. Chem.* **2004**, *12*, 1769–1779.
19. (a) Nair, V.; Fasbender, A. J. *Tetrahedron* **1993**, *49*, 2169–2184; (b) Nair, V.; Richardson, S. G. *Synthesis* **1982**, 670–672; (c) Nair, V.; Richardson, S. G. *J. Org. Chem.* **1980**, *45*, 3969–3974.
20. (a) Achelle, S.; Ramondenc, Y.; Dupas, G.; Plé, N. *Tetrahedron* **2008**, *64*, 2783–2791; (b) Solberg, J.; Undheim, K. *Acta Chem. Scand.* **1989**, *43*, 62–68; (c) Kondo, Y.; Watanabe, R.; Sakamoto, T.; Yamanaka, H. *Chem. Pharm. Bull.* **1989**, *37*, 2814–2816.
21. Clough, J.M.; Godfrey, C.R.A. U.S. Patent 04,870,075, 1989.
22. Ahmed, M.; Mori, A. *Tetrahedron* **2004**, *60*, 9977–9982.
23. Sivasankaran, R.; Rao, K.S.; Ratnam, K.R.; Mithyantha, M.S. Indian Patent 179039, 1997.
24. (a) Inanaga, J.; Baba, Y.; Hanamoto, T. *Chem. Lett.* **1993**, 241–244; (b) Wende, M.; Gladysz, J. A. *J. Am. Chem. Soc.* **2003**, *125*, 5861–5872.
25. (a) Adinolfi, M.; Parrilli, M.; Barone, G.; Laonigro, G.; Mangoni, L. *Tetrahedron Lett.* **1976**, 3661–3662; (b) Coleman, R. S.; Lu, X. *Chem. Commun.* **2006**, 423–425.
26. (a) Boudina, A.; Emmelin, C.; Baaliouamer, A.; Païssé, O.; Chovelon, J. M. *Chemosphere* **2007**, *68*, 1280–1288; (b) For more complete information, see the technical report for azoxystrobin of the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) at the site: http://www.fao.org/fileadmin/templates/agphome/documents/Pests_Pesticides/JMPR/Evaluation08/Azoxystrobin.pdf.
27. (a) Abad, A.; Manclús, J. J.; Mojarrad, F.; Mercader, J. V.; Miranda, M. A.; Primo, J.; Guardiola, V.; Montoya, A. *J. Agric. Food Chem.* **1997**, *45*, 3694–3702; (b) Shelver, W. L.; Smith, D. J.; Berry, E. S. *J. Agric. Food Chem.* **2000**, *48*, 4020–4026; (c) Jana, C. K.; Ali, E. *Steroids* **1999**, *64*, 228–232.
28. Clough, J.M.; Godfrey, C.R.A.; Eshelby, J.J.G.B. Patent 2248615(A), 1992.
29. Furzer, G. S.; Veldhuis, L.; Hall, J. C. *J. Agric. Food Chem.* **2006**, *54*, 688–693.
30. Bushell, M.J.; Beautement, K.; Glough, J.M. European Patent 0178826, 1986.
31. Clough, J.M.; Godfrey, C.R.A. European Patent 0260794, 1988.
32. (a) Rivault, F.; Schons, V.; Liebert, C. *Tetrahedron* **2006**, *62*, 2247–2254; (b) Talekar, R. S.; Chen, G. S. *J. Org. Chem.* **2005**, *70*, 8590–8593.
33. Larock, R. C.; Leach, D. R. *J. Org. Chem.* **1984**, *49*, 2144–2148.
34. Suárez-Pantaleón, C.; Mercader, J. V.; Agulló, C.; Abad-Somovilla, A.; Abad-Fuentes, A. *J. Agric. Food Chem.* **2010**, *58*, 8502–8511.