1 **Synthesis of azoxystrobin transformation products and selection**
2 **of monoclonal antibodies for immunoassay development**

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20 **Abstract**

21 The use of agrochemicals for crop protection may result in the presence of toxic residues in soils and
22 aquatic environments, besides in foodstuffs. Most often just the parent compound is included in the
23 definition of pesticide residue, even though chemicals resulting from biotransformation and degradation
24 routes might also be of toxicological relevance. Azoxystrobin is a broad-spectrum systemic fungicide widely
25 used worldwide to combat pathogenic fungi affecting plants. We herein report the synthesis and detailed
26 chemical characterization of several of the most relevant metabolites and degradates of azoxystrobin.
27 These compounds were further employed as ligands for screening a collection of monoclonal antibodies to
28 azoxystrobin, which had been previously generated from haptens functionalized at different positions of
29 the target chemical. As a result, an antibody was identified capable of binding, with subnanomolar affinity,
30 not only azoxystrobin but also its main transformation products, such as the so-called acid and enol
31 derivatives, as well as the azoxystrobin (Z)-isomer. The selected binder was demonstrated as a useful
32 immunoreagent for the development of immunochemical assays as novel analytical tools for the qualitative
33 determination of azoxystrobin and its metabolites and degradates.

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37 **Keywords**

38 Metabolite; degradate; fungicide; strobilurins; immunoanalytical methods; generic antibody

39

1. Introduction

Modern agricultural practices strongly rely on the use of plant protection chemicals that can provide the required crop yields in order to feed an ever-growing human population with high quality and inexpensive foods. Even though pesticides are usually applied according to the rules of Good Agricultural Practices, residues are regularly detected in agricultural commodities and in aquatic environments. The presence of trace amounts of this sort of xenobiotics might result in adverse consequences to humans and to non-target organisms. Hence, these substances need to be strictly controlled to safeguard human health and to protect the environment. In addition, the presence of multiple pesticide residues, even at low concentrations, may induce higher toxicological and carcinogenic effects. Pesticide transformation products, both metabolites and degradates, are also an issue of concern because they may feature harmful properties comparable to or higher than those of the active parent substance, and they may cause synergic noxious effects. Nevertheless, breakdown compounds are only occasionally included in the definition of pesticide residue, either because they are of lower toxicity or they are not present in significant amounts, or just because reference standards are not easily available.

Among pesticides, the presence of fungicide residues in foodstuffs is increasingly becoming of greater relevance since a better protection of fruits and vegetables after harvesting must be ensured. The agrochemical industry is continuously pursuing the discovery of new biocides acting at novel molecular targets and simultaneously being less toxic to humans and the environment. One such novel group of synthetic chemicals is the strobilurin family of fungicides. Their discovery was inspired by the identification, in the mushrooms *Strobilurus tenacellus* and *Oudemansiella mucida*, of a group of active natural products displaying a potent antifungal activity (Anke et al., 1977; Sauter et al., 1999). Strobilurins exert their biological action by binding to the Qo site of the cytochrome b in the mitochondrial electron transport chain (Balba, 2007). The first patented strobilurin was azoxystrobin (**1**, Fig. 1), which is currently approved worldwide for disease control in most cereal, fruit, and vegetable crops. Over 4000 tones of this fungicide were used worldwide in 2009, with global annual sales above \$1 billion, which makes this substance the world's leading proprietary fungicide (Atkin, 2010). According to the European Pesticide Monitoring Programs (EFSA, 2009), azoxystrobin was among the most frequently found pesticides in foodstuffs; around 5% of the analyzed fruit and vegetable samples contained residues at or below the tolerance levels. Azoxystrobin has low acute and chronic toxicity to humans, birds, mammals, and bees (US EPA, 1997; Bartlett et al., 2002). However, it is considered toxic to freshwater fish, freshwater invertebrates, and estuarine/marine fish, and highly toxic to estuarine/marine invertebrates (PMRA, 2000; Gustafsson et al., 2010). Recently, some limited adverse effects have been observed on Atlantic salmon smolts (Olsvik et al., 2010), on *Daphnia magna* (Warming et al., 2009; Friber-Jensen et al., 2010), and on *Rana temporaria* spawn and tadpoles (Johansson et al., 2006).

Surprisingly, very few independent data exist, in the regular scientific literature, about azoxystrobin transformation products and their toxicity to different organisms. As a matter of fact, most of the available information comes from registration documents provided by the manufacturer (Adetutu et al., 2008). Likewise, novel analytical methods for azoxystrobin and its major metabolites and degradation products have hardly ever been reported (Kern et al., 2009). In order to unequivocally identify unknown compounds, combined MS and NMR data of the purified substances are required. Besides, analytical reference standards for the most relevant derivatives are needed so as to improve our understanding of the overall presence of those chemicals in the environment and their toxicological relevance. In this work, we describe the synthesis and the complete spectroscopic characterization of five compounds widely recognized as major degradates of azoxystrobin. Additionally, this group of chemicals was used to screen a collection of in-house developed monoclonal antibodies (mAbs) with the aim of finding biomolecules that could recognize with high affinity not only azoxystrobin but also its main transformation products. Antibodies with such features may be greatly valuable for the development of rapid screening methods, such as the competitive enzyme-linked immunosorbent assays (cELISA), which can provide high-throughput capacity and reliable results on the occurrence of this relevant biocide and its breakdown compounds in food, biological, and environmental samples.

2. Materials and methods

2.1. Reagents and instrumentation

Analytical-grade azoxystrobin (methyl (E)-2-{2-[6-(2-cyanophenoxy)pyrimidin-4-yloxy]phenyl}-3-methoxyacrylate) (CAS number 131860-33-8, MW 403.4 g/mol) was kindly provided by Syngenta (Basel, Switzerland). Other reagents and solvents were acquired from commercial sources and were used without purification. The reactions were monitored with the aid of thin-layer chromatography (TLC) using 0.25 mm pre-coated silica gel plates. Visualization was carried out with UV light and aqueous ceric or ammonium molybdate solution at 50% (v/v). Chromatography refers to flash column chromatography and it was carried out with the indicated solvents on silica gel 60 (particle size 0.040–0.063 mm). All operations involving air-sensitive reagents were performed under an inert atmosphere of dry nitrogen using syringe and cannula techniques, oven-dried glassware, and freshly distilled and dried solvents. Melting points (Mp) were determined using a Kofler hot-stage apparatus or a Büchi melting point apparatus, and they are uncorrected. NMR spectra were recorded in CDCl₃ or CD₃OD at room temperature on a Bruker AC-300 spectrometer (300.13 MHz for ¹H and 75.47 MHz for ¹³C). The spectra were referenced to residual solvent protons in the ¹H NMR spectra (7.26 ppm for CDCl₃ and 4.84 and 3.31 ppm for CD₃OD) and to solvent carbons in the ¹³C NMR spectra (77.0 ppm for CDCl₃ and 49.05 ppm for CD₃OD). Carbon substitution

degrees were established by distortionless enhancement by polarization transfer (DEPT) pulse sequences. Complete assignment of ^1H and ^{13}C chemical shifts of all compounds was made on the basis of a combination of correlation spectroscopy (COSY) and heteronuclear single quantum coherence (HSQC) experiments. Infrared (IR) spectra were measured as thin films between NaCl plates for liquid compounds and as KBr pellets for solids using a Nicolet Avatar 320 spectrometer. The peak intensity is given as strong (s), medium (m) or weak (w). MS and high-resolution MS (HRMS) were run either by the electron impact (EI, 70 eV) or fast atom bombardment (FAB) mode, obtained with a Micromass VG Autospec spectrometer, or the electrospray (ES) mode, which was acquired with a Q-TOF premier mass spectrometer with an electrospray source (Waters, Manchester, UK).

The production of the mAbs that were used in this work was carried out in our laboratory following standard procedures for the generation of hybridoma cell lines (unpublished results), whereas the synthesis of the haptens that were employed for immunization (Fig. S1 in the [Supplementary Data File](#)) and their conjugation to carrier proteins have been previously reported (Parra et al., 2011a). *o*-Phenylenediamine was purchased from Sigma-Aldrich (Madrid, Spain) and polyclonal rabbit anti-mouse immunoglobulin peroxidase conjugate (RAM-HRP) was from Dako (Glostrup, Denmark). Costar flat-bottom high-binding polystyrene ELISA plates were from Corning (Corning, NY, USA). Microplates were washed with an ELx405 washer from BioTek Instruments (Winooski, VT, USA) and the ELISA absorbances were read in dual wavelength mode with a PowerWave HT also from BioTek Instruments.

The composition, concentration, and pH of the buffers employed in this study were as follows: i) CB, 50 mM carbonate–bicarbonate buffer, pH 9.6; ii) PBS, 10 mM sodium phosphate buffer, pH 7.4 with 140 mM NaCl; iii) PBST, PBS containing 0.05% (v/v) Tween 20; iv) Washing solution, 150 mM NaCl and 0.05% (v/v) Tween 20; and v) Enzyme substrate buffer, 25 mM sodium citrate and 62 mM sodium phosphate buffer, pH 5.4.

2.2. Synthesis of azoxystrobin transformation products

Spectroscopic characterization data of the synthesized compounds described below can be found in the [Supplementary Data File](#).

2.2.1. Preparation of (E)-2-(2-(6-(2-cyanophenoxy)pyrimidin-4-yloxy)phenyl)-3-methoxyacrylic acid (AZ-acid, **2**) and (Z)-methyl 2-(2-(6-(2-cyanophenoxy)pyrimidin-4-yloxy)phenyl)-3-hydroxyacrylate (AZ-enol, **3**)

Isopropanol (12.7 mL) and H_2O (6.1 mL) were successively added to a mixture of azoxystrobin (**1**, 513.8 mg, 1.27 mmol) and $\text{LiOH}\cdot\text{H}_2\text{O}$ (532 mg, 12.7 mmol); see Fig. 1A. The resulting yellowish suspension was stirred at room temperature (rt) for 3 h, then poured into water and extracted three times with ethyl ether.

138 The aqueous layer was reserved and the combined organic layers were washed with brine and dried over
139 anhydrous Na₂SO₄. Filtration and evaporation of the solvent under reduced pressure afforded a solid
140 corresponding to unreacted azoxystrobin (210 mg, 41%). The aqueous phase was cooled in an ice bath and
141 acidified with stirring to pH 3 by the addition of solid KHSO₄. The resulting yellow suspension was extracted
142 with ethyl ether and the combined organic layers were washed with brine and dried over anhydrous
143 Na₂SO₄. The residue remaining after evaporation of the solvent was purified by chromatography, using
144 mixtures of increasing polarity of CHCl₃–MeOH (from 99:1 to 90:10) as eluent, to give, in order of elution,
145 AZ-enol (**3**, 198 mg) as a yellowish oil and AZ-acid (**2**, 43 mg) as a white solid (mp 85–87 °C crystallized from
146 CH₂Cl₂–hexane). These amounts correspond to yields of 68% and 15%, respectively, based on recovered
147 starting material.

148

149 *2.2.2. Preparation of (Z)-Methyl 2-(2-(6-(2-cyanophenoxy)pyrimidin-4-yloxy)phenyl)-3-methoxyacrylate*
150 *(AZ-Z, 5)*

151 The photoisomerization product of azoxystrobin, Z-stereoisomer **5**, was prepared from azoxystrobin (**1**)
152 as shown in Fig. 1A following a reported procedure (Clough et al., 1992). Spectroscopic data, not previously
153 described in the literature, allowing a complete and unequivocal structural characterization of this
154 compound are given in the [Supplementary Data File](#).

155

156 *2.2.3. Preparation of 2-(6-hydroxypyrimidin-4-yloxy)benzonitrile (AZ-pyOH, 8)*

157 *2.2.3.1. Synthesis of 4-tert-butoxy-6-chloropyrimidine (6)*

158 A 1 M solution of potassium *tert*-butoxide in tetrahydrofuran (THF) was dropwise added (2.18 mL, 2.18
159 mmol) under nitrogen into a solution of 4,6-dichloropyrimidine (325.7 mg, 2.18 mmol) in anhydrous THF
160 cooled to 0 °C (Fig. 1B). The resulting orange mixture was stirred at 0 °C for 90 min, then poured into water
161 and extracted with EtOAc. The combined organic layers were washed with brine and dried over anhydrous
162 Na₂SO₄. The solvent was carefully eliminated in the rotavapor (the compound formed is relatively volatile),
163 and the residue left was purified by chromatography, using hexane–EtOAc 9:1 as eluent, to give the *tert*-
164 butoxypyrimidine **6** (315 mg, 75%) as a colorless oil.

165

166 2.2.3.2. *Synthesis of 2-(6-tert-butoxypyrimidin-4-yloxy)benzonitrile (7)*

167 Anhydrous N,N-dimethylformamide (DMF, 2.5 mL) was added to a mixture of *tert*-butoxypyrimidine **6**
168 (123.5 mg, 0.662 mmol), 2-cyanophenol (94.63 mg, 0.794 mmol), and Cs₂CO₃ (323.53 mg, 0.993 mmol)
169 under nitrogen. The mixture was stirred at 110 °C for 18 h, then cooled to rt, poured into water, and
170 extracted with EtOAc. The organic layer was washed consecutively with 10% aqueous NaOH, water, and
171 brine and dried over anhydrous Na₂SO₄. Evaporation of the solvent afforded compound **7** (166 mg, 99%) as
172 a white solid (mp 88–90 °C crystallized from CH₂Cl₂–hexane) that was pure enough, as judged by ¹H NMR,
173 to be used in the next step without further purification.

174

175 2.2.3.3. *Synthesis of 2-(6-hydroxypyrimidin-4-yloxy)benzonitrile (8)*

176 Trifluoroacetic acid (1 mL) was dropwise added under nitrogen to a stirred solution of *tert*-
177 butoxypyrimidine **7** (57.3 mg, 0.212 mmol) in anhydrous CH₂Cl₂ (1 mL) cooled at 0 °C (Fig. 1B). The mixture
178 was stirred at rt for 20 min, then diluted with benzene and concentrated to dryness under reduced
179 pressure to give a nearly quantitative yield of AZ-pyOH (**8**, 45 mg) as a pure yellowish solid. Mp 200–202 °C
180 (crystallized from CH₂Cl₂–MeOH).

181

182 2.2.4. *Preparation of 2-(6-(2-cyanophenoxy)pyrimidin-4-yloxy)benzoic acid (AZ-benzoic, 11)*

183 2.2.4.1. *Synthesis of 2-(6-chloropyrimidin-4-yloxy)benzonitrile (9)*

184 A mixture of 2-cyanophenol (262 mg, 2.19 mmol), 4,6-dichloropyrimidine (326 mg, 2.19 mmol), and
185 Cs₂CO₃ (1.07 g, 3.29 mmol) in anhydrous DMF (7.5 mL) was stirred at rt under nitrogen for 3 h. The mixture
186 was poured into water, extracted with EtOAc, and the combined extracts washed with brine, dried over
187 anhydrous Na₂SO₄, and concentrated under vacuum to leave a yellowish oil of nearly pure compound **9**
188 (505 mg, 99%), which was used in the next step without further purification.

189 2.2.4.2. *Synthesis of methyl 2-(6-(2-cyanophenoxy)pyrimidin-4-yloxy)benzoate (10)*

190 A solution of the chloropyrimidine **9** (91.2 mg, 0.393 mmol), methyl salicilate (92.7 mg, 0.609 mmol),
191 and Cs₂CO₃ (256 mg, 0.786 mmol) in anhydrous DMF (2 mL) was stirred at 100 °C for 90 min under nitrogen.
192 The mixture was cooled to rt, poured into water and extracted with EtOAc. The acetate extracts were
193 washed with a cooled 10% aqueous solution of NaOH to remove the excess of phenol, washed with brine,

194 dried over anhydrous Na₂SO₄, and concentrated under vacuum to yield the methyl benzoate **10** (136 mg,
195 99%) as a colourless oil.

196

197 2.2.4.3. Synthesis of 2-(6-(2-cyanophenoxy)pyrimidin-4-yloxy)benzoic acid (**11**)

198 A suspension of **10** (49.6 mg, 0.142 mmol) and LiOH·H₂O (59.58 mg, 1.42 mmol) in a mixture of
199 isopropanol (0.75 mL) and water (0.68 mL) was stirred at rt for 45 min (Fig. 1B). The resulting clear solution
200 was poured into water and extracted with EtOAc to recover unreacted starting material. The aqueous
201 phase was acidified with KHSO₄ to pH 2–3 and the precipitated acid was extracted with EtOAc. The organic
202 layer was washed with brine, dried over anhydrous Na₂SO₄, and evaporated to dryness to obtain AZ-
203 benzoic (**11**, 41 mg, 87%) as a white solid. Mp 128–130 °C (crystallized from CH₂Cl₂–MeOH).

204

205 2.3. ELISA procedure

206 Conjugate-coated indirect cELISAs were employed for metabolite recognition experiments by mAbs.
207 Ninety-six-well polystyrene ELISA plates were coated by overnight incubation at rt with 100 µL solutions of
208 OVA–hapten conjugates. Coated plates were washed four times with washing solution and received,
209 afterwards, 50 µL per well of analyte standard (or metabolite) in PBS plus 50 µL per well of mAb solution in
210 PBST. After 1 h at rt of immunological reaction, plates were washed as described before. Next, 100 µL per
211 well of a 1/2000 dilution of RAM–HRP conjugate in PBST was added and incubated again 1 h at rt. After
212 washing, the signal was obtained by addition of 100 µL per well of freshly prepared 2 mg/mL
213 o-phenylenediamine solution containing 0.012% (v/v) H₂O₂ in enzyme substrate buffer. The enzymatic
214 reaction was stopped after 10 min at rt with 100 µL per well of 2.5 M sulfuric acid. The absorbance was
215 immediately read at 492 nm with a reference wavelength at 650 nm.

216 2.4. Standards and data treatment

217 Eight-point standard curves of azoxystrobin and transformation products were prepared from
218 concentrated stock solutions in anhydrous DMF by serial dilution in PBS, including a blank point.
219 Experimental values were fitted to a four-parameter logistic equation using the SigmaPlot software package
220 from SPSS Inc. (Chicago, IL, USA). Assay sensitivity was defined as the concentration of analyte at the
221 inflection point of the fitted curve, typically corresponding to a 50% inhibition (IC₅₀) of the maximum
222 absorbance (A_{max}) if the background signal approaches to zero. Cross-reactivity was calculated as the

percentage of the ratio between the IC₅₀ value for azoxystrobin and the IC₅₀ value for the corresponding transformation product.

2.5. Buffer studies

The influence of ionic strength, pH, and Tween 20 concentration over the antibody–antigen interaction was evaluated following a multiparametric approach. The selected model was based on a central composite design. Briefly, a full factorial design was performed with 3 factors that included 8 cube, 6 axial, and 6 central points, with 3 replicates each located at random positions, involving a total of 15 buffers. Ionic strength values ranging from 50 to 300 mM, pH values from 5.5 to 9.5, and Tween 20 concentrations from 0.00 to 0.05% (v/v) were used as axial points. Buffer characteristics used in this study are shown in [Table S1](#) of the [Supplementary Data File](#) and they were prepared as follows. First, a 40 mM trisodium citrate, 40 mM disodium hydrogen phosphate, and 40 mM Tris solution (pH 9.9) was prepared, and known volumes of 5 M HCl were added in order to achieve the desired pH in each case. Then, the ionic strength of each solution was calculated, considering the initial solution and the employed volume of HCl, and the appropriate volume of a 2 M NaCl solution was added to every buffer aliquot in order to achieve the required ionic strength. Finally, Tween 20 was included at the corresponding concentration before the final volume was achieved by addition of deionized water. The concentration of Tween 20 and the ionic strength of the buffers were twice the final values in the assays. For the evaluated antibody (mAb AZa6#210), OVA–haptan coated microplates were prepared (OVA–AZa6 at 0.1 µg/mL) and cELISAs were carried out using azoxystrobin standard curves in water, whereas mAb solutions (80 ng/mL) were prepared in every evaluated buffer. Changes in the inhibition curve parameters were fitted by a multiple regression equation, including curvature and interaction terms, using Minitab 14.1 software (Minitab Inc., State College, PA, USA).

3. Results and discussion

3.1. Preparation of azoxystrobin transformation products

The major metabolite and degradation compound of azoxystrobin in aerobic soils, anaerobic soils, and water-sediment systems was reported to result from hydrolysis of the methyl ester moiety to form the β-methoxyacrylic acid (AZ-acid, **2**) ([Ghosh and Singh, 2009](#); [Singh et al., 2010](#)). Because AZ-acid (**2**) is known to be very soluble in water (860 mg/L), this compound will likely leach to groundwater. Other minor degradation products identified under environmental conditions include AZ-pyOH (**8**) and AZ-benzoic (**11**) ([Joseph, 1999](#); [Boudina et al., 2007](#)). Likewise, although azoxystrobin is a relatively stable pesticide with regard to photodegradation under natural sunlight conditions, the (Z)-isomer of azoxystrobin (AZ-Z, **5**) was identified as the main product of photochemical transformation under UV irradiation ([Boudina et al., 2007](#)).

Other breakdown products tentatively identified by HPLC–MS in the same study included, but were not restricted to, AZ-enol (**3**) and compounds **8** and **11**. The presence of trace levels of the biocide, and even AZ-acid (**2**), in natural aquatic environments has also been reported (Kern et al., 2009; Warming et al., 2009).

Azoxystrobin is extensively metabolized in animals and plants. The major metabolite in mammals is AZ-acid (**2**), which subsequently undergoes conjugation to form the corresponding glucuronide. Other identified metabolic pathways include hydroxylation on the cyanophenyl ring followed by conjugation, and the cleavage of the ether linkage between aromatic rings to give AZ-pyOH (**8**) (Joseph, 1999; Laird et al., 2003; JMPR, 2008). Most animal metabolites have been also found in plants, with the remarkable exception of AZ-Z (**5**), which was identified in plants but not in animals (Joseph, 1999), besides arising from photochemical transformation, as described above.

Based on those precedents, relevant azoxystrobin transformation products intended for antibody recognition studies were prepared from readily available starting materials. Compounds **2**, **3**, and **5**, which preserve most of the molecular framework, were obtained from technical-grade fungicide (Fig. 1A). The preparation of AZ-acid (**2**), the major metabolite of azoxystrobin, was more difficult than initially expected. Under smooth acidic conditions, the original molecule was recovered practically unchanged, but under more severe settings (HCl in isopropanol–water, 100 °C) the major observed reactions involved hydrolytic ether cleavage between the aromatic rings and hydrolysis of the nitrile group. On the other hand, complex reaction mixtures were obtained under most of the basic tested conditions. From a synthetic point of view, the best results were obtained by treatment of azoxystrobin with LiOH in a mixture of isopropanol–water at rt for a short period of time. Unlike previous results reported by Kern et al. (2009), who under similar hydrolytic conditions observed a single peak by HPLC that they ascribed to AZ-acid (**2**), we detected by TLC the formation of two major compounds. Both products, which could be readily separated by flash column chromatography on silica gel, were analyzed by MS and HRMS, showing the same molecular mass (389.1 g/mol) but clearly different fragmentation patterns (Fig. 2). In order to definitely identify each transformation product, a detailed ¹H and ¹³C NMR spectroscopy study was carried out (see the Supplementary Data File). As a result, the most abundant hydrolytic compound was unambiguously identified as the AZ-enol **3** (68% yield based on recovered unreacted starting material) while the other substance could be identified as the acid metabolite **2** (15% yield). The preference for the formation of the enol derivative under basic hydrolysis conditions was also confirmed with picoxystrobin, a strobiluring fungicide bearing in its structure the same methyl β-methoxyacrylate moiety than azoxystrobin (Parra et al., 2011b). Finally, it is worthy to note that enol **3** exists, at least in CDCl₃ solution, with the (Z)-geometry of the double bond, which seems to be stabilized by formation of an intramolecular hydrogen bond between the hydroxyl proton and the carbonyl oxygen.

289 The synthesis of the azoxystrobin photoproduct, the (Z)-isomer **5**, was also accomplished from
290 azoxystrobin (Fig. 1A) following a literature procedure (Clough et al., 1992). This strategy involved the
291 oxidative cleavage of the acrylate double bond to give keto-ester **4**, from which the methoxymethylene
292 moiety was regenerated by means of a Wittig reaction. The last reaction is not stereoselective, leading to a
293 nearly equimolecular mixture of (E)- and (Z)-isomers, from which compound **5** could be separated, in about
294 22% yield based on recovered starting material, and spectroscopically characterized.

295 The synthesis of the two other intended transformation products of azoxystrobin (compounds **8** and **11**)
296 was undertaken starting from 4,6-dichloropyrimidine (Fig. 1B) via nucleophilic aromatic substitution of the
297 chlorine atoms by the required oxygenated moieties (Parra et al., 2011a). For the synthesis of **8**, all
298 attempts to directly introduce the hydroxyl group failed, so it was incorporated in the form of a *tert*-
299 butoxide group and ultimately released in due course by acid hydrolysis. Thus, treatment of
300 4,6-dichloropyrimidine with an equivalent of potassium *tert*-butoxide in THF at rt (Kofink and Knochel,
301 2006) afforded the *tert*-butoxide derivative **6**, which was subjected to another nucleophilic substitution
302 reaction of the chlorine atom with 2-cyanophenol, using in this case more vigorous conditions, to give **7**.
303 The final step that led to pyrimidinol **8** involved acid-promoted cleavage of the *tert*-butyl ether moiety,
304 which took place very efficiently by treatment of intermediate **7** with trifluoroacetic acid at rt. The synthesis
305 of the benzoic acid derivative **11** was achieved, as before, through two consecutive nucleophilic aromatic
306 substitution reactions. First, 4,6-dichloropyrimidine was reacted under very smooth basic conditions with
307 2-cyanophenol to afford pyrimidinyl-aryl ether **9**, which then reacted with methyl 2-hydroxybenzoate to
308 give compound **10** under conditions similar to those previously used for the transformation of **6** into **7**.
309 Finally, base-catalyzed hydrolysis of the methyl ester moiety of **10** provided AZ-benzoic (**11**) in very high
310 overall yield from 4,6-dichloropyrimidine.

311 All the synthesized products were characterized using widely available instrumentation instead of
312 sophisticated equipment in order to facilitate the identification of azoxystrobin transformation products
313 under less demanding situations. To this purpose, degradates were analyzed by HPLC-DAD employing two
314 different gradient systems, *i.e.*, mixtures of MeOH–H₂O and MeCN–H₂O (Table 1). Under both conditions,
315 the two azoxystrobin stereoisomers eluted with clearly different retention times. However, none of the
316 assayed eluents was able to properly resolve the structural isomers AZ-acid and AZ-enol; indeed, with
317 mixtures of MeCN–H₂O also AZ-benzoic coeluted with AZ-acid and AZ-enol. On the other hand, with the
318 MeOH–H₂O gradient, AZ-benzoic separated from these two isomers but coeluted with AZ-pyOH. These
319 results emphasize the actual complexity of distinguishing between AZ-acid and AZ-enol, given their similar
320 chromatographic behaviour and identical mass. Fortunately, all five transformation products migrated
321 separately by TLC using a CHCl₃–MeOH mixture (98:2, v/v). As a matter of fact, AZ-acid and AZ-enol clearly
322 separated from each other by TLC (R_f values of 0.21 and 0.68, respectively), which makes this analytical

323 procedure a simple method to differentiate between these two azoxystrobin degradates ([Table 1](#)). The
324 product with the lowest R_f value (0.10) was AZ-benzoic. This result contrasts with that obtained by Singh et
325 al. ([2010](#)), who reported an R_f value for AZ-benzoic of 0.62, higher than the value they gave for AZ-acid
326 (0.175) and very similar to that of azoxystrobin itself (0.71).

327 3.2. Recognition of azoxystrobin transformation products by a collection of mAbs

328 Antibodies are binding biomolecules most often exhibiting outstanding specificity to their target
329 antigen. In the case of mAbs against low molecular mass analytes, the recognition profile to structurally
330 related compounds is however greatly influenced by the structure of the immunizing hapten ([Suárez-
331 Pantaleón et al., 2011](#)). With this idea in mind, we decided to test a collection of anti-azoxystrobin mAbs for
332 their ability to also recognize, with high affinity, the transformation products described above. The assayed
333 panel of mAbs was produced in our laboratory following standard cell culture techniques from mice
334 immunized with haptens bearing, as reactive chemical group for protein coupling, a carboxylic linker
335 tethered at different positions of the azoxystrobin molecular framework ([Fig. S1](#)).

336 The binding properties of our antibody library were examined by performing inhibition experiments in
337 the homologous indirect cELISA format with standard solutions of the azoxystrobin transformation
338 products (from 10⁵ to 10⁻² nM, plus a blank). First, competitive checkerboard experiments were run in
339 order to determine the adequate concentration of each immunoreagent combination for an optimal
340 competition. Our aim was to identify an antibody capable of equally recognizing azoxystrobin and most of
341 its breakdown compounds. To this purpose, IC₅₀ values for each mAb/degrade pair were obtained and
342 used to calculate cross-reactivities as described in the Materials and Methods section ([Table 2](#)). Not
343 surprisingly, no mAb was able to noticeably recognize AZ-pyOH, most likely because this compound lacks
344 one of the three aromatic rings that constitute the whole azoxystrobin molecule. Similarly, most mAbs did
345 not bind AZ-benzoic, thus emphasizing the importance of the acrylate moiety in the antibody interaction
346 with the fungicide. Only two mAbs, AZa6#26 and AZa6#210, bound this degradate to a slight degree (4.69%
347 and 6.19%, respectively). Concerning recognition of compounds AZ-Z, AZ-acid, and AZ-enol, several mAbs
348 could clearly accommodate them at their binding sites. These antibodies were mainly found among those
349 sets deriving from immunizing haptens AZa6 and AZc6 ([Fig. S1](#)). In particular, five out of eight mAbs from
350 these two groups bound AZ-Z with sound affinity. This result is particularly relevant because both
351 azoxystrobin stereoisomers are included in the residue definition of the regulations laid down by countries
352 such as Canada ([PMRA, 2000](#)), United States ([US EPA, 2008](#)), and New Zealand ([NZFSA, 2011](#)), which makes
353 those mAbs potentially valuable reagents for analytical applications. The main metabolite, AZ-acid, was
354 tightly recognized by three out of four mAbs coming from the immunizing hapten AZa6 (CR = 20–30%). This
355 was also the case of AZ-enol, which was recognized even better than AZ-Z (CR = 25–85%). In fact, the IC₅₀

356 values for these three compounds with mAbs AZa6#26, AZa6#210, and AZa6#31 were around 1 nM, a
357 remarkable affinity for such small organic chemicals. The most notable antibody was mAb AZa6#210, which
358 displayed the highest recognition for AZ-acid and AZ-enol, and almost the same affinity towards the two
359 azoxystrobin estereoisomers (see Fig. 3 for a visual comparison with a more specific antibody). Accordingly,
360 this mAb was the subject of further studies.

361 3.3. Influence of buffer composition over the interaction of mAb AZa6#210 and azoxystrobin

362 A multiparametric approach was carried out to evaluate the influence of pH and ionic strength over the
363 antibody–analyte interaction. Three buffer systems, *i.e.* citrate ($\text{pK}_{\text{a}2} = 4.8$, $\text{pK}_{\text{a}3} = 6.4$), phosphate ($\text{pK}_{\text{a}2} =$
364 7.2), and Tris ($\text{pK}_{\text{a}} = 8.1$) were employed to adjust pH over a wide range of values, together with NaCl for
365 ionic strength correction, as previously described (Suárez-Pantaleón et al., 2010). Also, several
366 concentrations of Tween 20, a detergent usually employed in immunoassays to reduce unspecific
367 interactions, were evaluated. A full factorial design, including 15 different buffers, was employed, fixing the
368 center point conditions at 10 mM phosphate, 140 mM NaCl, pH 7.5, 0.025% (v/v) Tween 20. Parameters
369 A_{max} and IC_{50} were taken as response values and fitted using the Minitab software as described.

370 Little influence over the inhibition curve parameters were generally observed upon buffer composition
371 (see the respective contour plots in Fig. S2). The A_{max} value remained stable at most of the pH, ionic
372 strength, and Tween 20 evaluated conditions. Regarding the IC_{50} , low pH and ionic strength values together
373 with high Tween 20 concentrations seemed to be counterproductive. Tween 20 was well tolerated through
374 a wide range of concentrations if conditions equivalent to those of PBS were maintained. For a better
375 comprehension of the influence of pH and ionic strength, the degree of modification of both the A_{max} and
376 IC_{50} was calculated taking as 100% those parameters obtained at the center point. When the contour plots
377 of the percentage of variation of A_{max} and IC_{50} were overlaid (Fig. 4), a wide area of pH and ionic strength
378 variations was clearly defined in which changes in those parameters remained tolerable; that is, between
379 80 and 120%. A low dependence on the ionic strength conditions was revealed, whereas assay sensitivity
380 slightly decreased at acidic pHs. Therefore, mAb AZa6#210 withstands modifications in the immunoreaction
381 conditions, so it is a suitable candidate for the development of generic competitive immunoassays adapted
382 to the analysis of azoxystrobin residues. If acidic samples were to be analyzed, assays could be run in 100
383 mM phosphate, pH between 7.5 and 8.0, together with Tween 20 at a usual concentration (0.025%).

384

385 3.4. Analysis of mixtures of azoxystrobin stereoisomers

386 As a proof-of-concept for the development of a generic rapid immunochemical assay, mixtures of
387 azoxystrobin and AZ-Z in water were determined by cELISA using mAb AZa6#210 as bioreceptor. For
388 comparison, the same samples were simultaneously determined using mAb AZo6#49, an antibody that
389 displayed a CR value for AZ-Z of just 1%. Deionized water aliquots were spiked with the same total amount
390 of fungicide (1000 ng mL^{-1}) but at different proportions of the two isomers. When the sample just
391 contained AZ-Z (Fig. 5, mixture A), only the mAb AZa6#210 was able to detect the analyte. Otherwise, in
392 samples containing 100% azoxystrobin (*E* isomer) the same experimental concentration was obtained with
393 both antibodies (Fig. 5, mixture G). Briefly, a clearly different output was obtained by both antibodies in
394 mixtures containing less than 75% of AZ-E. This simple experiment clearly proved the feasibility of mAb
395 AZa6#210 to determine samples containing both stereoisomers as a whole, which is a requirement in some
396 international regulations.

397 In summary, five relevant azoxystrobin transformation products were synthesized and characterized in
398 detail by a number of different techniques, including MS, NMR, IR, HPLC-DAD, and TLC. With these
399 degradates available, a collection of mAbs derived from haptens functionalized at selected sites was
400 screened in order to find binders able to recognize the synthesized compounds. This approach allowed us
401 to identify a mAb (AZa6#210) displaying high affinity values to azoxystrobin and to three important
402 breakdown products; that is, AZ-Z, AZ-enol, and AZ-acid. The described antibody could be considered as a
403 generic receptor and therefore suitable to be implemented in a number of different immunoanalytical
404 platforms, such as ELISA, immunochromatographic strips, affinity columns, and biosensors, in order to
405 analyze the total azoxystrobin content, including major metabolites and degradation products.

406

407

408 **Conflict of interest statement**

409 The authors declare that there are no conflicts of interest

410

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416 *Ministerio de Ciencia e Innovación* and the European Social Fund.

417 Limited amounts of the compounds and immunoreagents described in this paper are available upon
418 request for evaluation.

419 **Appendix A. Supplementary data**

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

421

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493

494 Figure legends

495

496 **Fig. 1.** Synthesis scheme of azoxystrobin metabolites and degradation products

497

498 **Fig. 2.** Mass spectra of azoxystrobin (a) and its transformation products, AZ-acid (b) and AZ-enol (c),
499 showing the tentative fragmentation peak assignment. The mass spectra were measured in electron
500 impact mode. A comparison of the high-mass regions of the mass spectra clearly shows the similar
501 electron impact-induced fragmentation patterns showed by azoxystrobin and AZ-acid.

502

503 **Fig. 3.** Inhibition curves for a generic mAb (AZa6#210) and for a more specific antibody (AZo6#49). The solid
504 line with open symbols shows the azoxystrobin standard curve. Dashed lines are used for the curves of
505 metabolites and degradation products: (▲) AZ-Z, (■) AZ-acid, (◆) AZ-enol, (▼) AZ-benzoic, and (●) AZ-
506 pyOH.

507

508 **Fig. 4.** Overlaid contour plots of the $A_{\max}$ and IC_{50} variation (%) as a function of buffer pH and ionic strength
509 (I). The white area sets the limits of acceptable pH and I conditions.

510

511 **Fig. 5.** Analysis of mixtures of azoxystrobin and its (Z)-isomer using either the competitive assay with the
512 generic (mAb AZa6#210, grey) or the specific (mAb AZo6#49, black) antibody. The sum of the
513 concentrations of the two stereoisomers was kept constant to 1000 ng/mL. The percentage of
514 azoxystrobin (*E* isomer) in each mixture was: A, 0%; B, 10%; C, 25%; D, 50%; E, 75%; F, 90%; G, 100%.

515

Table 1

HPLC-DAD retention times (min) and TLC R_f values of azoxystrobin transformation products

Compound	HPLC–DAD ^a		TLC ^d
	MeOH–H ₂ O ^b	MeCN–H ₂ O ^c	CHCl ₃ –MeOH ^e
AZ-benzoic (11)	12.5	9.2	0.10
AZ-pyOH (8)	12.5	3.7	0.15
AZ-acid (2)	17.5	9.2	0.21
AZ-enol (3)	17.5	9.2	0.68
AZ-Z (5)	18.6	11.4	0.75
Azoxystrobin (1)	19.1	12.7	0.86

^a A Hitachi (Tokyo, Japan) L-2130 HPLC system, equipped with a Hitachi L-4500 diode array detector and a Merck KGaA (Darmstadt, Germany) LiChroCART RP-18 column (250 mm x 4 mm, 5 μm) was employed for chromatographic separations.

^b From 10% MeOH and 90% H₂O, both containing 0.1% HCOOH, to 95% MeOH and 5% H₂O in 20 min. Flow rate: 1 mL/min

^c From 40% MeCN and 60% H₂O, both containing 0.1% HCOOH, to 75% MeCN and 25% H₂O in 12 min, then from 75% MeCN and 25% H₂O to 100% MeCN and 0% H₂O in 8 min. Flow rate: 1 mL/min

^d TLC was carried out on Pre-coated Merck Silica gel 60 F 254 TLC plates. The plates were visualized under UV light.

^e Mixture 98:2 (v/v).

Table 2
Recognition of azoxystrobin transformation products by the collection of mAbs (%)^a

mAb	AZ-Z	AZ-acid	AZ-enol	AZ-benzoic	AZ-pyOH
AZa6#21	0.97	0.01	2.70	<0.01	<0.01
AZa6#26	63.60	26.80	72.00	4.69	0.70
AZa6#210	96.71	28.45	82.93	6.19	1.21
AZa6#31	45.03	20.43	27.65	0.37	0.11
AZb6#22	4.39	6.36	0.92	0.01	<0.01
AZb6#24	3.82	5.61	0.79	0.01	<0.01
AZb6#38	0.99	0.03	1.94	<0.01	0.03
AZb6#43	3.97	0.69	3.05	<0.01	<0.01
AZc6#22	1.22	0.01	0.19	<0.01	0.01
AZc6#25	15.17	0.02	0.07	<0.01	<0.01
AZc6#26	38.23	0.06	8.89	<0.01	<0.02
AZc6#27	3.30	0.03	3.58	<0.01	<0.01
AZo6#41	1.02	0.03	1.61	0.01	<0.01
AZo6#43	1.03	0.02	2.08	0.01	<0.01
AZo6#45	2.95	0.06	73.03	<0.01	<0.01
AZo6#49	1.01	0.02	1.69	<0.01	<0.01

^a Values correspond to cross-reactivities referred to the IC₅₀ (nM) of azoxystrobin, which is considered 100%. Assays were carried out in the homologous indirect cELISA format, using for every combination of mAb and conjugate those concentrations resulting in the lowest IC₅₀ value for azoxystrobin with an A_{max} higher than 1.0.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

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