Moiety and linker site heterologies for highly sensitive immunoanalysis of cyprodinil in fermented alcoholic drinks

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Abstract

Cyprodinil is a new-generation anilinopyrimidine fungicide widely used in crop protection and frequently found in fruits. In this study, novel derivatives of cyprodinil with linker site heterologies were synthesized and employed in order to produce antibodies with enhanced affinity. Moreover, moiety-heterologous haptens were designed and prepared for assay sensitivity improvement. Two competitive enzyme-linked immunosorbent assays for the analysis of this active substance were developed using direct and indirect formats, achieving IC_{50} values around 0.15 $\mu\text{g/L}$. Analytical figures of merit and usability of the optimized assays were evaluated with wine and cider as model food processed matrices. The obtained recoveries were from 90% to 120%, and the limit of quantification was in the 1 to 5 $\mu\text{g/L}$ range. Finally, a monitoring study ($n=150$) was performed to estimate the occurrence and the concentration of cyprodinil in commercial wine and cider products from different origins. We found that 28% of the analysed wine samples contained cyprodinil residues at levels higher than 5 $\mu\text{g/L}$.

Keywords

ELISA, Hapten design, Fungicide, Food analysis, Wine, Cider.

Chemical compounds studied in this article

Cyprodinil (PubChem [CID: 86367](#)); Pyrimethanil (PubChem [CID: 91650](#)); Mepanipyrim (PubChem [CID: 86296](#)); *N,N*-disuccinimidyl carbonate (PubChem [CID: 676246](#))

Abbreviations used

BSA: bovine serum albumin; CR: cross-reactivity; CB: coating buffer; cELISA: competitive enzyme-linked immunosorbent assay; DMF: *N,N*-dimethylformamide; GAR: goat anti-rabbit immunoglobulin; HRP: horseradish peroxidase; LOQ: limit of quantification; MR: molar ratio; OVA: ovalbumin; PB: phosphate buffer; PBS: phosphate buffered saline; PBST: PBS containing Tween 20.

1. Introduction

Anilinopyrimidines (cyprodinil, pyrimethanil, and mepanipyrim, Table 1) are new-generation and highly efficient compounds active against a broad-spectrum of fungal pests. They show a particular mode of action consisting in the inhibition of methionine biosynthesis, so they are frequently combined with other fungicides possessing a different target site. Cyprodinil was the first anilinopyrimidine active ingredient to be introduced in all European countries ([Pesticide Properties DataBase](#)). Nowadays, cyprodinil-based formulations are widely employed for plant and crop protection. According to different monitoring programs, cyprodinil is one of the most commonly found residues ([EFSA, 2013](#); [USDA, 2012](#)). In order to regulate the presence of toxic compounds in food commodities, the European Food Safety Authority (EFSA) has established maximum residue levels (MRLs) as the upper legal concentration of a chemical to guarantee the lowest possible consumer exposure whilst reaching adequate phytosanitary efficiency ([EU Pesticide Database](#)). Those MRLs generally refer to raw or baby food products, but the legal pesticide contents for most processed foodstuff are not regulated yet in the EU. Nevertheless, different studies have confirmed the persistence of pesticides after industrial processing, for example during fermentation of grape must, with common concentrations in wine at micrograms per litre level ([Cabras & Angioni, 2000](#); [Cabras, et al., 1997](#); [Edder, et al., 2009](#); [González-Rodríguez, Cancho-Grande, & Simal-Gándara, 2011](#)). In addition, the presence of more than one residue has been repeatedly found in such high added value goods ([Carpinteiro, Ramil, Rodríguez, & Cela, 2010](#); [Pesticide Action Network, 2008](#); [Trosken, Bittner, & Volkel, 2005](#)).

Several methods have been described for the detection of cyprodinil traces in wines, based on gas or liquid chromatography with mass spectrometry detection. A previous extraction step is usually employed to avoid matrix effects, such as solid-phase microextraction with divinylbenzene–carboxen–polydimethylsiloxane fibers ([Rial-Otero, Yagüe-Ruiz, Cancho-Grande, & Simal-Gándara, 2002](#)), liquid–liquid extraction ([Vaquero-Fernández, et al., 2008](#)), solid-phase extraction with Oasis HLB cartridges ([Fontana, Rodríguez, Ramil, Altamirano, & Cela, 2011](#)), dispersive liquid–liquid microextraction ([Rodríguez-Cabo, Rodríguez, Ramil, & Cela, 2011](#)), or the QuEChERS (quick, easy, cheap, effective, rugged, and safe) procedure ([Moeder, Bauer, Popp, van Pinxteren, &](#)

Reemtsma, 2012; Walorczyk, Drozdzyński, & Gnusowski, 2011). Also, immunoassays have been developed and validated in wines for the analysis of pesticides like benalaxyl (Rosso, Giraudi, Gamberini, Baggiani, & Vanni, 2000), tebufenozide (Irwin, Tolhurst, Jackson, & Gale, 2003), fenhexamid (Mercader & Abad-Fuentes, 2009), or bromopropylate (Ramon-Azcón, Sánchez-Baeza, Sanvicens, & Marco, 2009), showing excellent performance.

Immunoassays can be nowadays considered a mature technology for the analysis of chemical residues and contaminants in food and the environment, being the competitive enzyme-linked immunosorbent assay (cELISA) one of the most employed setups. This immunochemical method is rapid, simple, sensitive, and specific; nevertheless, when cELISAs are compared with standard chromatographic methods, the main advantages of the bioanalytical method are the high sample throughput and the minimum sample treatment. Immunoassays are strongly recommended for the analysis of a high number of samples with reduced costs (Meulenbergh, Mulder, & Stoks, 1995). In a previous study, polyclonal antibodies against cyprodinil were generated for the first time by our group (Esteve-Turrillas, Agulló, Abad-Fuentes, Abad-Somovilla, & Mercader, 2012). Now, with the aim of analysing traces of cyprodinil in fermented drinks by immunoassay, we have designed two novel regioisomeric immunizing haptens with linker site heterologies in order to produce higher-affinity antibodies. Moreover, assay conjugates were prepared with new haptens bearing moiety heterologies for assay sensitivity enhancement. With those novel immunoreagents, a direct and an indirect cELISA have been optimized for the analysis of cyprodinil in white, red, and sparkling wines and in cider samples. For further validation, the developed immunoassays were applied to the analysis of cyprodinil in a variety of commercial wines from different origins.

2. Materials and methods

2.1. Reagents and instrumentation

Cyprodinil (4-cyclopropyl-6-methyl-*N*-phenylpyrimidin-2-amine, CAS Registry 121552-61-2, Mw 225.29 g/mol) and other pesticide standards were purchased from Fluka/Riedel-de-Haën (Seelze, Germany) or Dr. Ehrenstorfer (Augsburg, Germany). Horseradish peroxidase (HRP), ovalbumin (OVA), and *o*-phenylenediamine were purchased from Sigma/Aldrich (Madrid, Spain).

Bovine serum albumin (BSA) was from Roche Applied Science (Mannheim, Germany). Sephadex G-25 HiTrap Desalting and HiTrap Protein G HP columns from GE Healthcare (Uppsala, Sweden) were used for conjugate purification. Polyclonal goat anti-rabbit immunoglobulin peroxidase conjugate (GAR- $\square$ HRP) was from Biorad (Hercules, CA, USA). Foetal bovine serum and Freund's adjuvants were from Sigma/Aldrich (Madrid, Spain). Costar flat-bottom high-binding polystyrene ELISA plates were from Corning (Corning, NY, USA). Ultraviolet-visible spectra and ELISA absorbances were read (in dual wavelength mode, 492-650 nm) with a PowerWave HT microplate reader from BioTek Instruments (Winooski, VT, USA). ELISA plates were washed with an ELx405 microplate washer also from BioTek Instruments. Most stable conformers were calculated using CONFLEX with MM3 molecular mechanics to systematically search for low-energy conformers, followed by geometry optimization in MOG using AM1 parameters and including aqueous solvation effects simulated by COSMO (CACHe WorkSystem Pro, version 7.5.0.85).

Different buffers were employed in this study. The composition, concentration, and pH of the employed buffers were: i) Phosphate buffer (PB), 100 mM sodium phosphate buffer (pH 7.4); ii) Phosphate buffered saline (PBS), 10 mM sodium phosphate buffer (pH 7.4) with 140 mM NaCl; iii) PBST, PBS containing 0.05% (v/v) Tween 20; iv) Carbonate buffer (CB), 50 mM carbonate-bicarbonate buffer (pH 9.6); v) Washing solution, 15 mM NaCl and 0.05% (v/v) Tween 20; vi) Developing buffer, 25 mM citrate and 62 mM sodium phosphate buffer (pH 5.4); and vii) PBST-2x, 20 mM sodium phosphate buffer (pH 7.4) with 280 mM NaCl and 0.05% (v/v) Tween 20.

2.2. Hapten synthesis and activation

Preparation details and characterization data of new immunizing haptens (CD*b* and CD*m*) and heterologous assay haptens (Cl*p* and Cl*m*) are described in the Supplementary Material file. Carboxyl functionalized haptens were activated using *N,N*-disuccinimidyl carbonate, and the corresponding NHS ester was purified by flash chromatography following a previously published procedure (Esteve-Turrillas, et al., 2010). Briefly, the hapten (0.1 mmol) and *N,N'*-disuccinimidyl carbonate (33.3 mg, 0.13 mmol, 1.3 equiv.) were dissolved in anhydrous CH₃CN (0.7 mL) under nitrogen atmosphere at 0 °C. Dry Et₃N (38.4 mg, 53 μ L, 0.38 mmol, 3.8 equiv.) was added and the

reaction mixture was stirred at 0 °C for 1 h and then at room temperature until TLC (CHCl₃/MeOH 9:1 as eluent) showed completion of the reaction (ca. 1–2 h). The solution was diluted with chloroform, washed with an aqueous saturated solution of NaHCO₃ and brine, and then dried over anhydrous Na₂SO₄. The residue that was left after evaporation of the solvent was purified by column chromatography, using chloroform as eluent, to give the corresponding active *N*-hydroxysuccinimidyl ester in high yields (80–90%).

2.3. Protein conjugate production

Conjugates were prepared by coupling the purified NHS-activated ester of the synthetic haptens with three different carrier proteins (BSA, HRP, and OVA). Hapten-to-protein molar ratios (MR) of the purified conjugates were calculated using the absorbance values of the conjugate at 280 nm, by assuming that the molar absorptions of the hapten and the protein were the same for the free and for the conjugated forms.

2.3.1. Immunizing conjugates

A 50 mM solution (200 µL) of CDb-NHS or CD*m*-NHS ester in DMF was added drop wise over 1.8 mL of a 15 mg/mL BSA solution in CB. The conjugation reaction was carried out during 4 h at room temperature with moderate stirring. Next, the conjugate was separated from uncoupled hapten by gel filtration on Sephadex G-25, using PB as eluent, diluted to 1 mg/mL with the same buffer, sterilized with 0.45 µm filters, and stored at –20 °C.

2.3.2. Tracer conjugates

A 10 mM solution (100 µL) of CDb-NHS, CD*m*-NHS, Clp-NHS, or Cl*m*-NHS in DMF was added drop wise over 0.9 mL of a 2.2 mg/mL HRP solution in CB. Reagents were allowed to couple during 4 h at room temperature with gentle stirring. The prepared conjugates were separated from uncoupled hapten by gel chromatography as described before. Purified conjugates were diluted 1:1 (v/v) with PBS containing 1% (w/v) BSA and 0.01% (w/v) thimerosal and stored at –20 °C in amber vials. For daily use, a working aliquot was kept at 4 °C.

2.3.3. Coating conjugates

A 100 mM solution (100 µL) of CDb-NHS, CD*m*-NHS, Clp-NHS, or Cl*m*-NHS in DMF was added drop wise over 1.9 mL of a 15 mg/mL OVA solution in CB. The reaction was carried out

during 4 h at room temperature with moderate stirring. Then, conjugates were purified by gel chromatography as described. Finally, conjugates were stored at $-20\text{ }^{\circ}\text{C}$ in amber vials at 1 mg/mL in PB containing 0.01% (w/v) thimerosal.

2.4. Antibody generation

Animal manipulation was performed in compliance with the laws and guidelines of the Spanish Ministry of Agriculture, Food, and Environment, and approved by the Ethics Committee of the Universitat de València. Two female New Zealand white rabbits weighing $1\pm 2\text{ kg}$ were immunized by subcutaneous injection with 0.3 mg of BSA-CD b or BSA-CD m conjugates in 1 mL of a 1:1 mixture of PB and complete Freund's adjuvant. Animals were boosted at 21-day intervals with the same immunogen using incomplete Freund's adjuvant. Whole blood was collected from the ear vein of the rabbits and by intracardiac puncture 10 days after the fourth injection. Blood samples were allowed to coagulate overnight at $4\text{ }^{\circ}\text{C}$. Then, the serum was separated by centrifugation, and a fraction was diluted 1/5 with PBS containing 0.01% thimerosal (w/v) and kept at $4\text{ }^{\circ}\text{C}$ for daily usage. The remainder of each anti serum was precipitated twice with 1 volume of saturated ammonium sulphate solution, and stored at $4\text{ }^{\circ}\text{C}$.

2.5. Antibody-coated direct competitive ELISA

Ninety-six-well polystyrene ELISA plates were coated with 100 μL of antiserum solution in CB, and plates were incubated overnight at room temperature. Coated plates were washed four times with washing solution and received, afterwards, 50 μL per well of analyte in PBS plus 50 μL per well of HRP tracer solution in PBST. For sample analysis, 50 μL per well of wine or cider conveniently diluted with Milli-Q water was mixed with 50 μL per well of HRP tracer solution in PBST-2x. The immunological reaction took place during 1 h at room temperature, and plates were washed again as before. Finally, 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) hydrogen peroxide in developing buffer. The enzymatic reaction was stopped after 10 min at room temperature by the addition of 100 μL per well of 1 M sulphuric acid, and then absorbances were immediately read at 492 nm with a reference wavelength at 650 nm.

2.6. Conjugate-coated indirect competitive ELISA

Microplates were coated with 100 μ L per well of OVA conjugate solution in CB by overnight incubation at room temperature. Coated plates were washed four times with washing solution, and then received 50 μ L per well of analyte in PBS plus 50 μ L per well of antiserum dilution in PBST. In the case of samples, 50 μ L per well of wine or cider conveniently diluted with Milli-Q water was mixed with 50 μ L per well of antibody solution in PBST-2x. The immunological reaction took place during 1 h at room temperature, and plates were washed again as described above. Next, 100 μ L per well of a $1/10^4$ dilution of GAR- $\square$ HRP conjugate in PBST containing 10% (v/v) foetal bovine serum was added, and plates were incubated 1 h at room temperature. Finally, the signal was produced as aforementioned.

2.7. ELISA data treatment

Eight-point standard curves, including a blank, were set by serial dilution from the most concentrated point. For screening purposes, curves beginning with a 2000 μ g/L cyprodinil solution in PBS were arranged by 10-fold serial dilution with the same buffer, and for sample analysis, a standard curve was prepared by 6-fold serial dilution in Milli-Q water of a 50 μ g/L cyprodinil solution also in Milli-Q water. Sigmoidal curves were mathematically fitted to a four-parameter logistic equation using the SigmaPlot software package from SPSS Inc. (Chicago, IL, USA). Assay sensitivity was estimated as the concentration of analyte at the inflection point of the sigmoidal curve, typically corresponding to a 50% inhibition (IC_{50}) of the maximum absorbance reached at zero dose of analyte (A_{max}) if the background signal approaches to zero.

3. Results and discussion

3.1. Hapten synthesis, activation, and conjugation

In a previous study, two cyprodinil derivatives (CD n and CD p) with different linker tethering sites were prepared (Esteve-Turrillas, et al., 2012). In this work, two novel regioisomeric immunizing haptens have been designed; one with the linker at the pyrimidine ring (CD b) in order to have a completely different orientation of the molecule, and one with the linker at the aniline ring

(CD m) of the cyprodinil molecule, in a contiguous position compared to hapten CD p (Table 1). A similar synthetic strategy was followed for the synthesis of the novel immunizing haptens, CD b and CD m , which involved a condensation reaction between an aryl guanidine and a 1,3-dicarbonyl compound as the key synthetic step to construct the suitably functionalized anilinopyrimidine framework. The synthesis of hapten CD b is schematized in Fig. 1. It comprised the prior preparation of the 1,3-dicarbonyl compound **6**, which bears the C5 carboxylated alkyl chain that constituted the spacer arm. Its preparation was carried out through the acylation reaction of allyl β -oxopropanoate **3**, obtained via Claisen condensation of cyclopropyl methyl ketone (**1**) with diallyl carbonate (**2**), with methyl 6-chloro-6-oxohexanoate (**4**), followed by palladium-catalyzed decarboxylative deallylation reaction. The thus obtained β -dicarbonylic compound **6**, which exists in solution as a mixture with the enol tautomer, was subsequently condensed with phenylguanidine nitrate (**7**) in basic media to form the complete skeleton of hapten CD b , the synthesis of which ended with the hydrolysis of the methyl ester moiety of **8** in basic aqueous medium.

The regioisomeric hapten CD m was prepared as illustrated in Fig. 2, and involved the preparation of the pyrimidine ring via a condensation reaction in basic medium of 1-cyclopropylbutane-1,3-dione (**9**) with the aryl guanidine **11**, which incorporated the C6 hydrocarbon chain that constituted the linker at the adequate position of the phenyl ring. This aryl-guanidine was readily prepared as the nitrate salt by nitric acid catalyzed reaction of methyl 6-(3-aminophenyl)hexanoate (**10**) with cyanamide in an ethanolic medium. Once completed the anilinopyrimidine framework, the synthesis of hapten CD m was accomplished by basic hydrolysis of the methyl ester moiety of **12**. Synthesis of the moiety heterologous haptens, Cl p and Cl m , was based on a strategy similar to that used for the preparation of haptens CD b and CD m .

All haptens were activated by the active ester method, and conjugation to proteins was efficiently carried out with the chromatographically purified *N*-succinimidyl ester of the corresponding hapten, prepared in high yield using *N,N*-disuccinimidyl carbonate as the activating reagent. Since no secondary reactions occurred by the followed coupling procedure, the same strategy could be employed for immunizing and assay conjugates. The MR of both BSA conjugates was 11. Regarding assay conjugates, the MRs were 3, 4, 4, and 3 for HRP-CD b , HRP-CD m ,

HRP-Clp, and HRP-Clm, respectively, and 6, 8, 11, and 8 for OVA-CDb, OVA-CDm, OVA-Clp, and OVA-Clm, respectively.

3.2. Antibody affinity

Four novel antibodies, named CDm#1, CDm#2, CDb#1, and CDb#2, were produced from immunogens containing regioisomeric haptens CDm and CDb, respectively. In addition, previously reported antisera – CDp#1, CDp#2, CDn#1, and CDn#2 – and assay conjugates (Esteve-Turrillas, et al., 2012) were included in this study for a broader checkerboard heterologous competitive analysis, using direct and indirect cELISA formats. All immunizing haptens held equivalent linker arms (Table 1). Besides, conjugates carrying moiety-heterologous haptens (Clm and Clp) were evaluated with all of the available antibodies in both assay formats. For direct assays, plates were coated with several dilutions of the antiserum (from 3×10^3 to 3×10^4), and next day a range of tracer conjugate concentrations (from 1 to 300 $\mu\text{g/L}$) was evaluated under competitive conditions. For indirect assays, plates were coated with a solution of the respective OVA conjugate at 10, 100, or 1000 ng/mL, and the competitive step was carried out using a range of antiserum dilutions (from 3×10^3 to 1×10^6). Thus, a set of inhibition curves was obtained for every immunoreagent combination in both cELISA formats. Table S1 lists a summary of the results of the checkerboard competitive study, showing the respective inhibition curve parameters for direct and indirect cELISAs achieved with all of the antibodies and the linker site-heterologous conjugates. The overall trend of these results indicated that sensitivity in the direct format was higher than with the indirect one, but a broader conjugate recognition pattern was seen with the latter. As formerly observed, the antibodies obtained from hapten CDn showed the lowest affinity, in line with the results described elsewhere with an equivalent derivative of mepanipyrin – another compound of the same family (Esteve-Turrillas, Mercader, Agulló, Abad-Somovilla, & Abad-Fuentes, 2013). By comparison of homologous assays, it was seen that the highest affinity to cyprodinil was achieved with antibodies that had been produced using the novel CDb immunizing hapten – IC₅₀ values were 1.31 and 0.81 $\mu\text{g/L}$ for antisera CDb#1 and CDb#2, respectively. Moreover, linker site-heterologies afforded improved IC₅₀ values, even with CDn-type antibodies. Overall, with several heterologous immunoreagent combinations, IC₅₀ values below 1.0 $\mu\text{g/L}$ were retrieved.

In addition, two moiety-heterologous haptens (Clp and Clm) were synthesized with the aim of further improving IC₅₀ values (Ahn et al., 2012; Shen et al., 2012; Suárez-Pantaleón, Mercader, Agulló, Abad-Somovilla, & Abad-Fuentes, 2011). These haptens held the linker at different positions of the aniline ring and contained a distal heterologous chemical group – a chlorine atom – at the pyrimidine ring replacing the cyclopropyl moiety of the cyprodinil molecule (Table 1). All of the available antibodies were challenged with these heterologous conjugates by checkerboard cELISA in both formats (Table S2). Interestingly, it was observed that CDp-type and CDm-type antibodies perfectly recognized both heterologous conjugates – signals higher than 1.0 were obtained at usual immunoreagent concentrations. Thus, the chlorine atom seemed to mimic fairly well the cyclopropyl moiety. On the contrary, with CDb-type antibodies – which were produced with a hapten carrying the linker at the pyrimidine ring – high immunoreagent concentrations were required to observe binding in the indirect format, whereas low or no binding was seen in the direct format, probably due to the opposite location of the spacer arm in haptens Clp and Clm if compared to the homologous hapten CDb. As expected, no signal was obtained with CDn-type antibodies, with the only rare exception of antibody CDn#1 with conjugate OVA-Clm, even though high antibody and conjugate concentrations were also needed. In general terms, the sensitivity of most assays was improved using these new competitive haptens, with many antibody/conjugate pairs showing IC₅₀ values in the submicrogram per litre level. The most sensitive assay (IC₅₀ = 0.13 µg/L) was obtained with antibody CDp#2 in the direct format using tracer HRP-Clm. Fig. 3 shows the standard inhibition curves of the selected cELISAs obtained using antibody CDp#2 in the direct and indirect assay formats in combination with the moiety-heterologous tracer HRP-Clm and the linker site-heterologous conjugate OVA-CDb, respectively.

3.3. Antibody selectivity

Antibody selectivity towards anilinopyrimidines was assessed by cross-reactivity (CR) studies as the percentage value of the quotient between the IC₅₀ value for cyprodinil and the IC₅₀ value for pyrimethanil or mepanipyrim (see Table 1 for the structures of these two compounds). Calibration curves were prepared up to 10 µM for every compound in PBS and measured by direct and indirect homologous cELISA. Results for the eight antibodies in the direct format are depicted

in Fig. 4a as radar line plots. The corresponding plot for the indirect assay is shown in the Supplementary Material file (Fig. S1). It was observed that the selectivity pattern was similar by direct and indirect cELISA, indicating a minute influence of the assay format. Interestingly, CD*n*-type antibodies showed CR values around 100% for all three anilinopyrimidine fungicides, suggesting that the central derivatization position of hapten CD*n* (Fig. 4b) determined the generation of antibodies with a variety of preferred orientations of the molecule. On the contrary, the selectivity of all of the other antisera was generally good – CR values were generally between 1% and 10% – no matter of the ring where the spacer arm was located at; either the aniline ring (CD*p* and CD*m*) or the pyrimidine ring (CD*b*). Moreover, mepanipyrim – characterized by a 1-propenyl group – was less recognized than pyrimethanil – with a methyl moiety – by most of the antibodies, particularly by CD*m*-type antisera. Surprisingly, the most selective antibody was CD*b*#1 – CRs around 1% for both compounds –, whose immunizing hapten (CD*b*) held both the linker and the specific cyclopropyl group at the same ring (Table 1). These results show that for such small molecules, the moieties located at a proximal ring also played a determinant role for selectivity, and in the case of hapten CD*b*, the cyclopropyl moiety could be properly displayed as presumed from the most stable conformer of cyprodinil (Fig. 4b).

Selectivity was also evaluated for the appointed cELISAs using antibody CD*p*#2 with a moiety-heterologous tracer (HRP-Cl*m*) for the direct immunoassay and a linker site-heterologous conjugate (OVA-CD*b*) for the indirect assay. In both cases, a slight increase of CR was observed when compared to the homologous assays – CR values to pyrimethanil and mepanipyrim were between 9% and 18%. As discussed above, the distal position of those heterologies helped to improve immunoassay sensitivity to cyprodinil; conversely, regarding selectivity, both distal heterologies accentuated the binding to other anilinopyrimidines. Thus, when using distal heterologies, antibody molecules with specificity towards the common aniline ring seemed to prevail in the competitive reaction.

3.4. Assay robustness

Sample constituents may affect the cELISA response, so several potential interferents were evaluated in order to establish the robustness of the proposed immunoassays for the analysis of cyprodinil traces. White, red, and sparkling wines and apple cider were chosen as target samples.

3.4.1. Alcohol tolerance

Solvent contents in the sample often change the analytical parameters of an immunoassay (He et al., 2014; Mercader & Abad-Fuentes, 2009). Fermented drinks like wine or cider may contain important concentrations of ethanol, so the influence of alcohol over the inhibition curve of the proposed cELISAs needs to be evaluated. Cyprodinil standards were prepared in water containing different concentrations of ethanol and analyzed by the selected direct and indirect assays. Methanol was also included in the study for comparison. Fig. 5 shows the effect of those solvents over the $A_{\max}$ and IC_{50} values of the cyprodinil inhibition curves. It was seen that both immunoassays tolerated reasonably well the presence of a 5% (v/v) solvent concentration.

3.4.2. Matrix effects

Water dilution is a simple and common strategy to avoid interferences of the sample matrix over immunoassay analytical performance. Thus, cyprodinil standard curves were prepared using several dilutions (1/5, 1/15, and 1/50) of every evaluated matrix in water and analysed using the proposed direct and indirect cELISAs. Fig. S2 shows the variation of the inhibition curves at every sample dilution for white, red, and sparkling wines and for cider. A 1/15 sample dilution was considered appropriate to avoid matrix interferences, with the exception of red wine, for which a 1/50 dilution was proposed.

3.4.3. Fungicide interferences

Cyprodinil commercial products are usually coformulated with other fungicides, like fludioxonil or picoxystrobin. Moreover, other active principles may be present in the sample. Thus, CR studies were performed with those and other commonly used fungicides such as: azoxystrobin, pyraclostrobin, kresoxim-methyl, trifloxystrobin, fenhexamid, boscalid, imidacloprid, procimidone, cyazofamid, tolylfluanid, tebuconazole, fenamidone, vinclozolin, proquinazid, epoxiconazol, and propiconazol. From this study, it was seen that no antibody recognized any of the listed

compounds – CR values were lower than 0.1% –, so no interferences from these substances with the proposed cELISAs can be expected.

3.5. Recovery studies

Trueness of the selected assays was evaluated by spiking cyprodinil-free wine and cider samples with known amounts of this fungicide from 1 to 1000 µg/L. After adequate water dilution, fortified samples were analysed by the developed cELISAs. Recoveries (Table 2) ranged from 97% to 120% and from 90% to 116% for direct and indirect assays, respectively, and relative standard deviation remained mostly lower than 15%. From this study, the high sensitivity of the proposed cELISAs was evidenced. The limit of quantification (LOQ) of the method is defined as the lowest validated spiked level meeting the method performance acceptability criteria – mean recoveries in the range 70-120%, with RSD values $\leq 20\%$ (DG SANCO, 2007) – was 1 µg/L for the direct assay, except for red wine, and 5 µg/L with the indirect cELISA, except for white wine. Thus, the optimized immunoassays are suitable for the determination of very small traces of cyprodinil in alcoholic fermented drinks.

3.6. Sampling study

The usefulness of the developed method to identify and quantify fungicide residues in wine was assessed using commercial samples acquired in local supermarkets. A total of 150 samples of commercial wine and cider samples encompassing 84 red, 57 white, and 6 sparkling wines and 3 cider samples were screened for the analysis of cyprodinil residues. Samples from different origins (Spain, Germany, Italy, France, Portugal, USA, and South Africa) were included in the survey, and they were analysed by the indirect cELISA. We found that 28% of the wine samples contained residues of cyprodinil, considering the LOQ for this method was 5 µg/L. No residues were detected in cider samples. In detail, 44% of red wines contained detectable residues of cyprodinil, while lower percentages were obtained for white and sparkling wines (7 and 17%, respectively). Remarkably, the presence of cyprodinil in positive samples was confirmed by a reference procedure based in a QuEChERS extraction (Walorczyk, et al., 2011) and UPLC/MS-MS determinations.

The maximum concentration found for every type of wine was 74, 11, and 18 µg/L for red, white, and sparkling wines, respectively. The obtained results are in accordance to literature data regarding other wine monitoring studies using chromatographic analytical methods. For example, the Pesticide Action Network encountered concentrations of cyprodinil in wine from 1 to 15 µg/L (Pesticide Action Network, 2008), Rial-Otero et al. found cyprodinil between 0.9 and 25 µg/L (Rial-Otero, et al., 2002), cyprodinil contents observed by Čuš et al. were from 10 to 440 µg/L (Cus, Cesnik, Bolta, & Gregorcic, 2010), and Rodríguez-Cabo et al. (2011) and Fontana et al. (2011) reported cyprodinil levels up to 43 µg/L.

4. Conclusions

Traces of pesticides have been found in fermented alcoholic drinks such as wine. With the aim of developing rapid and sensitive analytical methods for cyprodinil residue control, two novel immunizing haptens were designed and high-affinity antibodies were produced. In addition, two moiety-heterologous compounds were synthesized for competitive assay sensitivity improvement. Enhanced assay sensitivity was achieved by employing linker site and moiety heterologies, with IC₅₀ values of 0.13 and 0.17 µg/L for the direct and the indirect competitive format, respectively. We observed that, for very small molecules such as anilinopyrimidines, proximal moieties to the linker site of the immunizing hapten can actively participate in the antibody-antigen interaction, as demonstrated by cross-reactivity studies with the polyclonal antibodies described herein. The optimized assays were validated for the analysis of cyprodinil residues in fortified wine and cider samples, reaching LOQ values between 1 and 5 µg/L, recoveries from 90 to 120%, and RSD values lower than 17%. A monitoring study conducted with the developed indirect cELISA demonstrated the presence of cyprodinil residues in 28% of commercial wine samples, proving the high applicability of this analytical tool for pesticide surveillance studies in such high added value products.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at

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Figure captions

Fig. 1. Synthesis pathway to prepare hapten CD*b*.

Fig. 2. Synthesis pathway to prepare hapten CD*m*.

Fig. 3. Standard inhibition curves of the selected direct and indirect cELISAs using antibody CD*p*#2 with tracer HRP-CD*m* and conjugate OVA-CD*b*, respectively.

Fig. 4. A) Radar line plots of cross-reactivity values (%) for pyrimethanil (grey) and mepanipyrin (ochre) by homologous direct cELISA obtained with each pair of antibodies generated from every immunizing hapten. B) Cyprodinil most stable conformer. Elements are presented as follows: carbon, grey; hydrogen, white; nitrogen, blue. Arrows point out the spacer site of the four synthetic immunizing haptens.

Fig. 5. Variation of $A_{\max}$ and IC_{50} values of the cyprodinil standard inhibition curve of the selected cELISAs (direct: solid lines; indirect: dashed lines) at different ethanol (ochre lines) and methanol (grey lines) concentrations in the assay buffer.

Table 1
Structures of anilinopyrimidine fungicides and synthetic haptens.

The chemical structure shows a pyrimidine ring with substituents R¹, R², and R³. The pyrimidine ring is connected to an aniline ring, which has substituents R⁴ and R⁵.

	R ¹	R ²	R ³	R ⁴	R ⁵
Anilinopyrimidine					
Cyprodinil	CHCH ₂ CH ₂	CH ₃	H	H	H
Pyrimethanil	CH ₃	CH ₃	H	H	H
Mepanipyrim	CCCH ₃	CH ₃	H	H	H
Immunizing haptens					
CD _p	CHCH ₂ CH ₂	CH ₃	H	H	(CH ₂) ₅ COOH
CD _m	CHCH ₂ CH ₂	CH ₃	H	(CH ₂) ₅ COOH	H
CD _n	CHCH ₂ CH ₂	CH ₃	(CH ₂) ₄ COOH	H	H
CD _b	CHCH ₂ CH ₂	(CH ₂) ₄ COOH	H	H	H
Moiety-heterologous haptens					
Cl _p	Cl	CH ₃	H	H	(CH ₂) ₅ COOH
Cl _m	Cl	CH ₃	H	(CH ₂) ₅ COOH	H

Table 2

Recoveries from spiked alcoholic fermented drinks measured by the developed cELISAs.

cELISA	Spiked ($\mu\text{g/L}$)	Recovery (% $\pm$ s, n=3) ^a			
		White wine	Red wine	Sparkling wine	Cider
Direct	1	120 $\pm$ 9	_{-b}	97 $\pm$ 11	113 $\pm$ 15
	5	114 $\pm$ 15	115 $\pm$ 2	110 $\pm$ 4	104 $\pm$ 9
	10	120 $\pm$ 10	110 $\pm$ 9	102 $\pm$ 3	102 $\pm$ 2
	50	105 $\pm$ 9	109 $\pm$ 14	101 $\pm$ 5	111 $\pm$ 15
	100	108 $\pm$ 12	106 $\pm$ 17	104 $\pm$ 3	99 $\pm$ 1
	500	103 $\pm$ 4	102 $\pm$ 10	97 $\pm$ 4	97 $\pm$ 1
	1000	99 $\pm$ 1	105 $\pm$ 9	105 $\pm$ 8	98 $\pm$ 6
Indirect	1	116 $\pm$ 11	_{-b}	_{-b}	_{-b}
	5	116 $\pm$ 9	94 $\pm$ 14	108 $\pm$ 8	106 $\pm$ 11
	10	109 $\pm$ 6	90 $\pm$ 2	109 $\pm$ 15	109 $\pm$ 10
	50	106 $\pm$ 10	109 $\pm$ 12	98 $\pm$ 9	103 $\pm$ 4
	100	111 $\pm$ 6	103 $\pm$ 12	95 $\pm$ 12	111 $\pm$ 3
	500	105 $\pm$ 15	100 $\pm$ 10	100 $\pm$ 1	111 $\pm$ 12
	1000	102 $\pm$ 2	100 $\pm$ 6	96 $\pm$ 7	110 $\pm$ 11

^a Samples spiked at 1-10 $\mu\text{g/L}$ were diluted in water 1/50 for red wine and 1/15 for the other drinks, whereas for samples spiked at 50-1000 $\mu\text{g/L}$ higher dilutions were employed. ^b Inconsistent results.

Figure 1
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Figure 2
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Figure 3
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Figure 5
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SUPPLEMENTARY MATERIAL

Moiety and linker site heterologies for highly sensitive immunoanalysis of cyprodinil in fermented alcoholic drinks

Francesc A. Esteve-Turrillas, Josep V. Mercader, Consuelo Agulló, Antonio Abad-Somovilla, Antonio Abad-Fuentes

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General Information

Phenylguanidine nitrate salt (**7**) was prepared from reaction of aniline, cyanamide and nitric acid following a literature procedure.¹ 1-cyclopropylbutane-1,3-dione (**9**) was prepared from cyclopropyl methyl ketone, EtOAc and potassium *tert*-butoxide according to a procedure previously described.² 6-(3-Aminophenyl)hexanoate (**10**) was prepared by esterification of the corresponding acid³ with MeOH and catalytic SOCl₂.⁴ 4-Chloro-6-methyl-2-(methylsulfonyl)pyrimidine (**13**) was prepared from commercially available 4-hydroxy-2-mercapto-6-methylpyrimidine following a previously described three steps synthetic route.⁵ *N*-(4-iodophenyl)formamide (**14**) and *N*-(4-iodophenyl)formamide (**20**) were obtained by *N*-formylation of the corresponding iodophenylaniline.^{6,7} Other reagents were acquired from commercial sources and used without purification. Reactions were monitored with the aid of thin layer chromatography using 0.25 mm precoated silica gel plates. Visualization was carried out with UV light and a 50% (v/v) aqueous ceric ammonium molybdate solution. 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 melting points were determined using a Kofler hot-stage apparatus and are uncorrected. NMR spectra were recorded on a Bruker AC-300 spectrometer (300.13 MHz for ¹H and 75.47 MHz for ¹³C), and they were referenced to residual solvent protons in the ¹H NMR spectra (7.26, 3.58/1.73 and 2.50 ppm for CDCl₃, THF-d₈ and DMSO-d₆, respectively) and to solvent carbons in the ¹³C NMR spectra (77.0, 67.6 and 39.43 ppm for CDCl₃, THF-d₈ and DMSO-d₆, respectively). Carbon substitution degrees were established by distortionless enhancement by polarization transfer (DEPT) pulse sequences. Infrared (IR) spectra were measured using a Nicolet Avatar 320 spectrometer. Mass spectra (MS) and high-resolution mass spectra (HRMS) were run either by the electron impact (EI, 70 eV), obtained with

¹ N-Phenyl-4-pyrazolo[1,5-b]pyridazin-3-ylpyrimidin-2-amines as Potent and Selective Inhibitors of Glycogen Synthase Kinase 3 with Good Cellular Efficacy. F. X. Tavares; J. A. Boucheron; S. H. Dickerson; R. J. Griffin; F. Preugschat; S. A. Thomson; T. Y. Wang; H. Q. Zhou. *J. Med. Chem.* 2004, 47, 4716-4730.

² Process for the preparation of 1-cyclopropylalkane-1, 3-diones. J. Muhr, Patent Number US5545762. Date of Patent: Aug. 13, 1996.

³ Synthesis and Polyclonal Antibody-Based Immunoassay Development for the Analysis of Forchlorfenuron in Kiwifruit. C. Suarez-Pantaleon, J. V. Mercader; C. Agullo; A. Abad-Somovilla; A. Abad-Fuentes. *J. Agric. Food Chem.*, 2010, 58, 8502-8511.

⁴ Design, Synthesis, and Pharmacological Evaluation of Thapsigargin Analogues for Targeting Apoptosis to Prostatic Cancer Cells. C. M. Jakobsen; S. R. Denmeade; J. T. Isaacs; A. Gady; C. E. Olsen; S. B. Christensen. *J. Med. Chem.* 2001, 44, 4696-4703.

⁵ Design, synthesis, and activity of 2-imidazol-1-ylpyrimidine derived inducible nitric oxide synthase dimerization inhibitors. D. D. Davey; M. Adler; D. Arnaiz; K. Eagen; S. Erickson; W. Guilford; M. Kenrick; M. M. Morrissey; M. Ohlmeyer; G. Pan; V. M. Paradkar; J. Parkinson; M. Polokoff; K. Saionz; C. Santos; B. Subramanyam; R. Vergona; R. G. Wei; M. Whitlow; B. Ye; Z. Zhao; J. J. Devlin; G. Phillips. *J. Med. Chem.* 2007, 50, 1146-1157.

⁶ Synthesis of 3,1-benzothiazines by cyclization of 2-thioformylaminodiphenylacetylenes. M. A. Fernandes; D. H. Reid. *Synlett*, 2003, 2231-2233.

⁷ Understanding the Anchoring Group Effect of Molecular Diodes on Rectification. Y. Lee; B. Carsten; L. Yu. *Langmuir*, 2009, 25, 1495-1499.

a Micromass VG Autospec spectrometer, or the electrospray (ES) mode, which was obtained with a Q-TOF premier mass spectrometer with an electrospray source (Waters, Manchester, UK).

Hapten Synthesis

a) Synthesis of Hapten CD*b*

Preparation of allyl 3-cyclopropyl-3-oxopropanoate (3). Diallyl carbonate (**2**, 1.0 g, 2.55 ml, 17.82 mmol, 1.5 equiv.) and 1-cyclopropylethanone (**1**, 1.17 mL, 11.88 mmol, 1 equiv.) were successively added into a stirred suspension of HNa (60% dispersion in mineral oil, 1.19 g, 29.75 mmol, 2.5 equiv.) in anhydrous benzene (5 mL) at rt under nitrogen and the mixture was refluxed for 5 h. The reaction mixture was cooled to 0 °C, cautiously treated with 3 M HCl solution to acidic pH and extracted with CH₂Cl₂. The organic extracts were washed with brine, dried over anhydrous MgSO₄ and concentrated to dryness. The residue was chromatographed, using hexane-Et₂O 9:1 as eluent, to afford the β-ketoester **3** as an oil (1.36 g, 60%). ¹H NMR (CDCl₃) δ (ppm) 5.88 (1H, ddt, *J* = 17.2, 10.4, 5.7 Hz, H-2 allyl), 5.32 (1H, ddt, *J* = 17.2, 1.5, 1.5 Hz, H-3 allyl), 5.23 (1H, ddt, *J* = 10.4, 1.5, 1.4 Hz, H'-3 allyl), 4.62 (2H, dt, *J* = 5.7, 1.4 Hz, H-1 allyl), 3.58 (2H, s, H-2), 2.02 (1H, tt, *J* = 4.5, 4.4 Hz, CH-Cy), 1.09 and 0.95 (2H each, each m, CH₂CH₂-Cy); ¹³C NMR (CDCl₃) δ (ppm) 202.4 (C-1), 166.6 (C-3), 131.5 (C-2 allyl), 118.4 (C-3 allyl), 65.6 (C-1 allyl), 49.6 (C-2), 20.6 (CH-Cy), 11.5 (CH₂CH₂-Cy); IR (NaCl) $\nu_{\text{max}}/\text{cm}^{-1}$ 3088, 3012, 2943, 1736, 1707, 1649, 1442, 1385, 1312, 1271, 1151, 1074, 992, 933, 735. HRMS *m/z* calcd. for C₉H₁₂O₃ 168.07864, found 168.07884.

Preparation of (E)-1-allyl 8-methyl 2-(cyclopropanecarbonyl)-3-hydroxyoct-2-enedioate (5). β-Ketoester **3** (288.3 mg, 1.5 mmol) was added to a stirred suspension of anhydrous MgCl₂ (142.8 mg, 1.5 mmol) in dry CH₂Cl₂ (2 mL) at rt under nitrogen. The reaction mixture was cooled to 0 °C, dry pyridine (243 μL, 3 mmol) was added and the mixture was stirred at this temperature for 15 min. Then, methyl adipoyl chloride (**4**, 234 μL, 1.5 mmol) was added and the mixture was stirred at 0 °C for 15 min. and then at rt for an additional 1 h. After the addition of cold 6 M HCl (3

mL) to the white slurry formed, the mixture was extracted with Et₂O, the extracts were washed with brine and then dried over MgSO₄. Evaporation of the solvent afforded the crude product that was purified by chromatography, using hexane-EtOAc 9:1 as eluent, to yield a yellowish oil corresponding to the expected β -diketone, which exists exclusively in the keto-enol tautomeric form **5** (457.5 mg, 98%). ¹H NMR (CDCl₃) δ (ppm) 5.98 (1H, ddt, J =17.2, 10.4, 5.9 Hz, H-2 allyl), 5.32 (1H, ddt, J = 17.2, 1.5, 1.5 Hz, H-3 allyl), 5.23 (1H, ddt, J = 10.4, 1.3, 1.3 Hz, H'-3 allyl), 4.72 (2H, dt, J = 5.9, 1.3 Hz, H-1 allyl), 2.60 (2H, m, H-7), 2.31 (3H, m, H-4 and CH-Cy), 1.66 (4H, m, H-5 and H-6), 1.22 and 0.99 (2H each, each m, CH₂CH₂-Cy). ¹³C NMR (CDCl₃) δ (ppm) 199.2 (CO-Cy), 195.0 (C-3), 173.8 (CO₂Me), 167.4 (C-1), 131.7 (C-2 allyl), 119.1 (C-3 allyl), 108.6 (C-2), 65.7 (C-1 allyl), 51.5 (CO₂Me), 36.6 (C-4), 33.7 (C-7), 25.4 (C-6), 24.5 (C-5), 16.6 (CH-Cy), 12.1 (CH₂CH₂-Cy); IR (NaCl) $\nu_{\max}/\text{cm}^{-1}$ 3087, 3014, 2952, 2873, 1735, 1560, 1437, 1271, 1208, 1101, 1062, 936, 737; MS (EI) m/z (%) 310 (M⁺, 0.7), 282 (0.6), 252 (1), 253 (0.5), 251 (0.4), 226 (2.5), 220 (0.4), 195 (4), 143 (6.4), 126 (10), 111 (30), 69 (100); HRMS m/z calcd. for C₁₆H₂₂O₆ 310.14164, found 310.14148.

Preparation of (Z)-methyl 8-cyclopropyl-6-hydroxy-8-oxooct-6-enoate (6a) and methyl 8-cyclopropyl-6,8-dioxooctanoate (6b). To a solution of allyl ester **5** (431 mg, 1.388 mmol) in THF (5.8 mL) at 0 °C were added Pd(PPh₃)₄ (105.6 mg, 0.091 mmol, 0.06 equiv.) and dropwise morpholine (254 μ L, 2.916 mmol, 2 equiv.). The reaction mixture was allowed to warm to rt and stirred at this temperature for 40 min. The solvent was removed under reduced pressure and the remaining residue was purified by silica gel column chromatography, using hexane-Et₂O 9:1 as eluent, to afford the keto-enol **6a** (285.8 mg, 91%), which exists in CDCl₃ solution as a mixture with the 6,8-diketo tautomer [ca. a 4:1 mixture of **6a** and **6b**]. ¹H NMR (CDCl₃) δ (ppm) 15.63 (0.8H, s, OH, keto-enol form), 5.60 (0.8H, s, H-7 keto-enol form), 3.67 (3H, s, CO₂Me keto-enol+diketo forms), 3.66 (0.4H, s, H-7 diketo form), 2.51 (0.4H, m, H-5 keto form), 2.34 (2H, t, J = 7.1 Hz, H-2 keto-enol+diketo forms), 2.26 (1.6H, t, J = 7.1 Hz, H-5 keto-enol form), 2.0

(0.2H, m, CH-Cy diketo form), 1.55-1.51 (4.8H, m, H-3 and H-4 keto-enol+diketo forms and CH-Cy keto-enol form), 1.09 and 0.92 (2H each, each m, CH₂CH₂-Cy keto-enol+diketo forms); ¹³C NMR (CDCl₃) (only the signals of the major keto-enol tautomer **6a** are given) δ (ppm) 199.0 (C-8), 187.3 (C-6), 173.7 (CO₂Me), 98.8 (C-7), 51.5 (CO₂Me) 36.3 (C-5), 33.6 (C-2), 25.3 (C-3), 24.3 (C-4), 18.5 (CH-Cy), 10.2 (CH₂CH₂-Cy); IR (NaCl) $\nu_{\max}/\text{cm}^{-1}$ 3008, 2945, 2863, 1735, 1609, 1440, 1377, 932, 777.

*Preparation of methyl 5-(6-cyclopropyl-2-(phenylamino)pyrimidin-4-yl)pentanoate (**8**).* A mixture of phenylguanidine nitrate salt (**7**, 289.1 mg, 1.459 mmol, 1.6 equiv.), Na₂CO₃ (76.3 mg, 0.72 mmol, 0.8 equiv.) and keto-enol **6** (203.6 mg, 0.90 mmol) in MeOH (2.5 mL) contained in a sealed ampoule was heated with stirring at 80 °C for 24 h. The reaction mixture was poured into water and extracted with EtOAc. The organic extracts were washed with brine, dried over anhydrous Na₂SO₄ and concentrated to give an oily residue that was purified by chromatography, using hexane-EtOAc 9:1, to afford the pyrimidine derivative **8** (231.3 mg, 79%) as an oil. ¹H NMR (CDCl₃) δ (ppm) 7.62 (2H, apparent dd, part AA' of an AA'BB'C system, *J* = 7.5, 0.9 Hz, H-2/H-6 Ph), 7.30 (2H, apparent t, part BB' of an AA'BB'C system, *J* = 7.5 Hz, H-3/H-5 Ph), 7.01 (1H, br s, NH), 6.97 (1H, apparent tt, part C of an AA'BB'C system, *J* = 7.5, 0.9 Hz, H-4 Ph), 6.49 (1H, s, H-5 Pym), 3.67 (3H, s, CO₂Me), 2.59 (2H, t, *J* = 7.2 Hz, H-5), 2.36 (2H, t, *J* = 7.2 Hz, H-2), 1.86 (1H, m, CH-Cy), 1.72 (4H, m, H-3 and H-4), 1.15 and 0.98 (2H each, each m, CH₂CH₂-Cy); ¹³C NMR (CDCl₃) δ (ppm) 173.9 (CO₂Me), 172.5 (C-4 Pym), 169.8 (C-2 Pym), 159.8 (C-6 Pym), 140.1 (C-1 Ph), 128.7 (C-3/C-5 Ph), 121.6 (C-4 Ph), 118.4 (C-2/C-6 Ph), 109.5 (C-5 Pym), 51.5 (CO₂Me), 37.1 (C-5), 33.8 (C-2), 27.9 (C-4), 24.5 (C-3), 16.8 (CH-Cy), 10.3 (CH₂CH₂-Cy); IR (NaCl) $\nu_{\max}/\text{cm}^{-1}$ 3356, 3002, 2939, 2856, 1730, 1584, 1443, 1249, 1026, 754; MS (EI) *m/z* (%) 325 (M⁺, 17), 324 (4), 295 (2), 294 (8), 266 (4), 252 (13), 226 (16), 225 (100), 224 (11), 178 (3); HRMS *m/z* calcd. for C₁₉H₂₃N₃O₂ 325.17903, found 325.17933.

Preparation of 5-(6-cyclopropyl-2-(phenylamino)pyrimidin-4-yl)pentanoic acid (Hapten CDb). A solution of methyl ester **8** (159 mg, 0.488 mmol) in MeOH (5.3 mL) and aqueous 2 M NaOH (0.98 mL, 1.96 mmol, 4 equiv.) was stirred at 60 °C for 1.5 h. The solvent was evaporated under reduced pressure, the resulting residue was dissolved in the minimum amount of formic acid and the solution again concentrated to dryness in vacuum. The white solid obtained was purified by chromatography, using CHCl₃-EtOAc 9:1 as eluent, to give pure hapten CDb (129.3 mg, 85%) as a white solid. Mp 158-160 °C (crystallized from benzene); ¹H NMR (DMSO-d₆) δ (ppm) 12.02 (1H, s, COOH), 9.32 (1H, s, NH), 7.74 (2H, apparent dd, part AA' of an AA'BB'C system, *J* = 7.7, 0.9 Hz, H-2/H-6 Ph), 7.23 (2H, apparent t, part BB' of an AA'BB'C system, *J* = 7.7 Hz, H-3/H-5 Ph), 6.88 (1H, apparent tt, part C of an AA'BB'C system, *J* = 7.3, 0.9 Hz, H-4 Ph), 6.67 (1H, s, H-5 Pym), 2.54 (2H, t, *J* = 7.3 Hz, H-5), 2.25 (2H, t, *J* = 7.2 Hz, H-2), 1.95 (1H, m, CH-Cy), 1.67 (2H, m, H-4), 1.54 (2H, m, H-3), 1.03-0.97 (4H, m, CH₂CH₂-Cy); ¹³C NMR (DMSO-d₆) δ (ppm) 174.3 (CO₂H), 171.6 (C-4 Pym), 169.7 (C-2 Pym), 159.8 (C-6 Pym), 140.9 (C-1 Ph), 128.3 (C-3/C-5 Ph), 120.7 (C-4 Ph), 118.3 (C-2/C-6 Ph), 108.8 (C-5 Pym), 36.4 (C-5), 33.4 (C-2), 27.5 (C-4), 24.1 (C-3), 16.4 (CH-Cy), 9.9 (CH₂CH₂-Cy); IR (KBr) *v*_{max}/cm⁻¹ 3290, 3138, 2936, 2360, 1684, 1590, 1558, 1499, 1386, 1250, 970, 756, 689; MS (EI) *m/z* (%) 311 (M⁺, 24), 310(4), 309 (2), 266 (4), 265 (6), 264 (14), 250 (4), 238 (11), 226 (14), 224 (16), 225 (100), 210 (4.5); HRMS *m/z* calcd. for C₁₈H₂₁N₃O₂ 311.16338, found 311.16254.

b) Synthesis of Hapten CDm

Preparation of methyl 6-(3-guanidinophenyl)hexanoate (11). A mixture of methyl 6-(3-aminophenyl)hexanoate (**10**, 188 mg, 0.850 mmol), 50% aqueous cyanamide solution (132 μL, 1.70 mmol, 2 equiv.) and concentrated HNO₃ (70% w/v, 84 μL, 1.13 mmol, 1.3 equiv.) in EtOH (1.6 mL) was placed in a dark glass ampoule under nitrogen. The ampoule was sealed under vacuum and then heated with stirring at 78 °C overnight. The content of the ampoule was transferred into a flask with the aid of EtOH, concentrated to dryness under vacuum and the residue purified by chromatography, using CHCl₃-MeOH 9:1 as eluent, to give the aryl guanidine **11** (242 mg, 87%). ¹H NMR (300 MHz, CDCl₃) δ (ppm) 9.62 (1H, s, NH-C(NH)-NH₂), 7.92 (2H, br

s, NH-C(NH)-NH₂), 6.60 (1H, br s, NH-C(NH)-NH₂), 7.33 (1H, dd, *J* = 8.0, 8.0 Hz, H-5 Ph), 7.14 (1H, d, *J* = 8.0 Hz, H-6 Ph), 7.07 (2H, m, H-2 and H-4 Ph), 3.64 (3H, s, CO₂Me), 2.62 (2H, t, *J* = 7.6 Hz, H-6), 2.30 (2H, t, *J* = 7.4 Hz, H-2), 1.64 (4H, m, H-3 and H-5), 1.35 (2H, m, H-4); ¹³C NMR (75 MHz, CDCl₃) δ (ppm) 174.3 (CO₂Me), 156.4 (NH-C(NH)-NH₂), 145.1 (C-3 Ph), 134.0 (C-1 Ph), 130.0 (C-5 Ph), 128.0 (C-4 Ph), 125.2 (C-6 Ph), 122.5 (C-2 Ph), 51.5 (CO₂Me), 35.3 (C-6), 33.8 (C-2), 30.6 (C-3), 28.5 (C-5), 24.5 (C-4); IR (NaCl) *v*_{max}/cm⁻¹ 3338, 3200, 2940, 1724, 1674, 1600, 1352, 1267, 736; MS (EI) *m/z* (%) 263 (M⁺, 100), 262 (13), 246 (4), 233 (3), 232 (18), 221 (17), 190 (5), 170 (12), 148 (8), 132 (38); HRMS *m/z* calcd. for C₁₄H₂₁N₃O₂ 263.16338, found 263.16271.

Preparation of methyl 6-(3-((4-cyclopropyl-6-methylpyrimidin-2-yl)amino)phenyl)hexanoate (12). A mixture of aryl guanidine **11** (143.1 mg, 0.438 mmol), 1-cyclopropylbutane-1,3-dione (**9**, 110.5 mg, 0.876 mmol, 3 equiv.), Na₂CO₃ (23.2 mg, 0.219 mmol, 0.5 equiv.) and MeOH (1 mL) contained in a sealed glass ampoule was heated with stirring at 78 °C for 48 h. The ampoule was opened and the solvent was removed under vacuum, giving a residue that was chromatographed on silica gel, using CHCl₃ as eluent, to give the pyrimidine derivative **12** (96.5 mg, 62%) as an oil. ¹H NMR (300 MHz, CDCl₃) δ (ppm) 7.50 (1H, dd, *J* = 1.6, 1.6 Hz, H-2 Ph), 7.39 (1H, ddd, *J* = 7.7, 1.8, 1.6 Hz, H-4 Ph), 7.19 (1H, dd, *J* = 7.7, 7.7 Hz, H-5 Ph), 6.96 (1H, br s, H-NH), 6.80 (1H, ddd, *J* = 7.7, 1.6, 1.6 Hz, H-6 Ph), 6.51 (1H, s, H-5 Pym), 3.66 (3H, s, CO₂Me), 2.60 (2H, t, *J* = 7.5 Hz, H-6), 2.34 (3H, s, Me), 2.31 (2H, t, *J* = 7.7 Hz, H-2), 1.85 (1H, m, CH-Cy), 1.67 (4H, m, H-3 and H-5), 1.39 (2H, m, H-4), 1.15 and 0.99 (2H each, each m, CH₂CH₂-Cy); ¹³C NMR (75 MHz, CDCl₃) δ (ppm) 174.2 (CO₂Me), 172.4 (C-2 Pym), 166.6 (C-6 Pym), 159.8 (C-4 Pym), 143.1 (C-3 Ph), 140.1 (C-1 Ph), 128.6 (C-5 Ph), 121.9 (C-4 Ph), 118.6 (C-6 Ph), 116.0 (C-2 Ph), 110.0 (C-5 Pym), 51.4 (CO₂Me), 35.9 (C-6), 34.0 (C-2), 31.0 (C-3), 28.7 (C-5), 24.8 (C-4), 23.8 (Me), 16.8 (CH-Cy), 10.3 (CH₂CH₂-Cy); IR (NaCl) *v*_{max}/cm⁻¹ 3356, 3009, 2932, 2850, 1736, 1588, 1541, 1445, 1171, 789, 695; MS (EI) *m/z* (%) 353 (M⁺, 97), 352 (28), 323 (49), 322 (15), 294 (12), 280 (17), 266 (11), 252 (51), 250 (87), 240 (17), 239 (100), 238 (23), 221 (20); HRMS *m/z* calcd. for C₂₁H₂₇N₃O₂ 353.21033, found 353.21022.

Preparation of 6-(3-((4-cyclopropyl-6-methylpyrimidin-2-yl)amino)phenyl)hexanoic acid (Hapten CDm). A solution of methyl ester **12** (82.3 mg, 0.233 mmol) in a mixture of MeOH (2 mL) and 2M aqueous NaOH (0.47 mL, 0.932 mmol, 4 equiv.) was heated at reflux with stirring for 2 h. After removal of the solvent at reduced pressure, the solid residue obtained was dissolved in the minimum amount of formic acid and the resulting solution was stirred while treated dropwise with water until the apparition of a white precipitate. The mixture was cooled in the refrigerator for a few hours and filtered. The crystals were washed with cool water and dried overnight in the vacuum desiccator yielding hapten CDm (57.7 mg, 73%), practically pure by NMR analysis. Mp 170-173 °C (crystallized from DMSO-H₂O); ¹H NMR (300 MHz, DMSO-d₆) δ (ppm) ¹³C NMR (75 MHz, DMSO-d₆) δ (ppm) 174.4 (CO₂H), 171.4 (C-2 Pym), 166.2 (C-6 Pym), 159.8 (C-4 Pym), 142.1 (C-3 Ph), 140.8 (C-1 Ph), 128.1 (C-5 Ph), 120.9 (C-4 Ph), 118.2 (C-6 Ph), 115.8 (C-2 Ph), 109.4 (C-5 Pym), 35.3 (C-6), 33.5 (C-2), 30.5 (C-5), 28.2 (C-4), 24.3 (C-3), 23.3 (Me), 16.2 (CH-Cy), 9.8 (CH₂CH₂-Cy); IR (KBr) ν_{max}/cm⁻¹ 3292, 3213, 3101, 3014, 2932, 2853, 1700, 1633, 1604, 1558, 1487, 1452, 1404, 1264, 960, 784; HRMS (TOF MS ES+) *m/z* calcd. for C₂₀H₂₅N₃NaO₂ [M+Na]⁺ 362.18445, found 362.18380.

Synthesis of Moiety Heterologous Haptens

Heterologous haptens Clm and Clp were prepared by construction of the complete framework as detailed in Schemes S1 and S2. Their synthesis is based on the preliminary preparation of a conveniently functionalized phenylaminopyrimidine moiety followed by incorporation of the C6 hydrocarbon chain that constitutes the spacer arm at the required position of the phenyl ring by a strategy based on the Sonogashira cross-coupling reaction. A detailed description of each synthetic step as well as the complete spectroscopic characterization data of the heterologous haptens and of all the intermediates of their synthesis are reported below.

a) Synthesis of Hapten Clm

Scheme S1. Synthetic pathway to prepare hapten Clm

Preparation of N-(4-chloro-6-methylpyrimidin-2-yl)-N-(3-iodophenyl)formamide (15). A solution of aryl iodide **14** (244 mg, 0.988 mmol) in anhydrous THF (2.2 mL) was dropwise added to a stirred suspension of NaH (60% dispersion in mineral oil, 37 mg, 0.93 mmol, 1.1 equiv., prewashed with dry pentane) in THF (2.2 mL) under nitrogen. After the evolution of hydrogen ceased, about 30 min, a solution of pyrimidine **13** (170 mg, 0.823 mmol) in anhydrous THF (3 mL) was added and the resulting mixture was stirred overnight, then poured into water and extracted with EtOAc. The combined organic layers were washed with brine and then dried over anhydrous Na₂SO₄. The residue left after evaporation of the solvent was chromatographed over silica gel, using hexane-EtOAc from 9:1 to 8:2 as eluent, to afford compound **15** (181 mg, 59%) as a solid. Mp 178.5-179.5 °C (crystallized from cold hexane-Et₂O). ¹H NMR (300 MHz, CDCl₃) δ (ppm) 9.91 (1H, s, CHO), 7.75 (1H, dt, *J* = 7.0, 1.8 Hz, H-4 Ph), 7.55 (1H, m, H-2 Ph), 7.25-7.16 (2H, m, H-5 and H-

6 Ph), 6.98 (1H, br s, H-Pym), 2.42 (3H, br s, Me-Pym); ^{13}C NMR (75 MHz, CDCl_3) δ (ppm) 170.4 (C-6 Pym), 162.7 (CHO), 161.6 (C-2 Pym), 159.0 (C-4 Pym), 137.6 and 137.5 (C-4 and C-5 Ph), 136.8 (C-1 Ph), 130.6 (C-2 Ph), 128.2 (C-6 Ph), 116.7 (C-5 Pym), 93.8 (C-3 Ph), 24.0 (Me-Pym); IR (KBr) $\nu_{\text{max}}/\text{cm}^{-1}$ 3442, 3085, 1686, 1573, 1542, 1362, 1279, 1120, 754; HRMS (TOF MS ES+) m/z calcd. for $\text{C}_{12}\text{H}_{10}\text{ClIN}_3\text{O}_2$ $[\text{M}+\text{H}]^+$ 373.9557, found 373.9566.

Preparation of tert-butyl 6-(3-(N-(4-chloro-6-methylpyrimidin-2-yl)formamido)phenyl)hex-5-ynoate (17). A mixture of CuI (0.7 mg, 0.0037 mmol), $(\text{Ph}_3\text{P})_2\text{PdCl}_2$ (7.8 mg, 0.011 mmol), aryl iodide **15** (137 mg, 0.37 mmol) and *tert*-butyl hex-5-ynoate (**16**, 92.9 mg, 0.55 mmol) in anhydrous DMF (1.3 mL) was purged by three cycles of vacuum and nitrogen. Then, anhydrous Et_3N (1.3 mL) was added and the resulting brownish solution obtained was stirred at room temperature for 4 hours, then poured into water and extracted with EtOAc. The combined organic layers were washed with an aqueous solution of LiCl and brine, dried over anhydrous Na_2SO_4 and concentrated to dryness. Chromatography of the crude product, using hexane-EtOAc from 9:1 to 8:2 as eluent, afforded aryl-alkyne **17** (111 mg, 73%) as an oil. ^1H NMR (300 MHz, CDCl_3) δ (ppm) 9.93 (1H, s, CHO), 7.44 (1H, dt, $J = 7.5, 1.8$ Hz, H-4 Ph), 7.39 (1H, ddd, $J = 7.5, 7.5, 0.7$ Hz, H-5 Ph), 7.23 (1H, br t, $J = 1.8$ Hz, H-2 Ph), 7.11 (1H, dt, $J = 7.5, 1.8$ Hz, H-6 Ph), 6.96 (1H, br s, H-Pym), 2.45 (2H, $J = 7.3$ Hz, H-4), 2.41 (3H, br s, Me-Pym), 2.39 (2H, t, $J = 7.5$ Hz, H-2), 1.87 (2H, quint, $J = 7.5$ Hz, H-3), 1.45 (9H, s, CMe_3); ^{13}C NMR (75 MHz, CDCl_3) δ (ppm) 172.5 (CO_2), 170.3 (C-6 Pym), 162.8 (CHO), 161.5 (C-2 Pym), 159.2 (C-4 Pym), 135.7 (C-3 Ph), 131.8 (C-4 Ph), 131.6 (C-5 Ph), 129.1 (C-6 Ph), 128.1 (C-2 Ph), 125.2 (C-1 Ph), 116.5 (C-5 Pym), 90.2 (C-5), 80.5 (C-6), 80.3 (CMe_3), 34.4 (C-2), 28.1 (CMe_3), 24.0 (Me-Pym), 23.9 (C-3), 18.8 (C-4); IR (KBr) $\nu_{\text{max}}/\text{cm}^{-1}$ 2977, 2361, 1707, 1570, 1546, 1369, 1232, 1150, 1110; HRMS (TOF MS ES+) m/z calcd. for $\text{C}_{22}\text{H}_{24}\text{ClIN}_3\text{NaO}_2$ $[\text{M}+\text{Na}]^+$ 436.1404, found 436.1411.

Preparation of tert-butyl 6-(3-((4-chloro-6-methylpyrimidin-2-yl)amino)phenyl)hexanoate (18). A mixture of aryl-alkyne **17** (107 mg, 0.26 mmol) and PtO_2 (10 mg) in EtOH (3.8 mL) was stirred under an atmosphere of hydrogen at 45-50 psi during 16 hours. The reaction mixture was filtered through a short silica-gel column using EtOAc to wash the column and the residue left after evaporation of the solvent was chromatographed on silica gel, using hexane-EtOAc from 9:1 to 8:2 as eluent, to give compound **18** (81.1 mg, 75%) as an oil, followed by the corresponding *N*-formylated derivative **19** (7.6 mg, 7%) as a semisolid. Data for compound **18**: ^1H NMR (300 MHz, CDCl_3) δ (ppm) 7.49 (1H, br dd, $J = 7.8, 1.2$ Hz, H-4 Ph), 7.36 (1H, br t, $J = 1.8$ Hz, H-2 Ph), 7.24 (1H, dd, $J = 7.8, 7.8$ Hz, H-5 Ph), 7.12 (1H, br s, NH), 6.87 (1H, br d, $J = 7.8$ Hz, H-6 Ph), 6.63 (1H, s, H-Pym), 2.61 (2H, $J = 7.5$ Hz, H-6), 2.39 (3H, s, Me-Pym), 2.21 (2H, t, $J = 7.5$ Hz, H-2), 1.65 (4H, m, H-3 and H-5), 1.43 (9H, s, CMe_3), 1.38 (2H, m, H-4); ^{13}C NMR (75 MHz, CDCl_3) δ (ppm) 173.2 (CO_2), 169.7 (C-6 Pym), 161.0 and 159.5 (C-2 and C-4 Pym), 143.4 (C-3 Ph), 138.7 (C-1 Ph), 128.8 (C-5 Ph), 123.2 (C-6 Ph), 119.3 (C-4 Ph), 116.8 (C-2 Ph), 111.2 (C-5 Pym), 79.9 (CMe_3), 35.7 and 35.5 (C-6 and C-2), 30.9 (C-3), 28.6 (C-5), 28.1 (CMe_3), 24.9 (C-4),

23.9 (Me-Pym); IR (KBr) $\nu_{\text{max}}/\text{cm}^{-1}$ 3434, 2933, 1729, 1689, 1576, 1544, 1366, 1280, 1155, 771; HRMS (TOF MS ES+) m/z calcd. for $\text{C}_{21}\text{H}_{29}\text{ClN}_3\text{O}_2$ $[\text{M}+\text{H}]^+$ 390.1948, found 390.1951.

Preparation of 6-(3-((4-chloro-6-methylpyrimidin-2-yl)amino)phenyl)hexanoic acid (Hapten Clm). A solution of *tert*-butyl ester **18** (29 mg, 0.074 mmol) in formic acid (1 mL) was stirred at rt for 6 hours and then the excess of formic acid was removed using a rotary evaporator. The residue was dissolved in benzene and concentrated again to obtain hapten Clm (24.5 mg, 98.7%) as a solid. Mp 165.4-165.8 °C (crystallized from MeOH); ^1H NMR (300 MHz, THF- d_8) δ (ppm) 8.95 (1H, br s, NH), 7.64 (1H, br d, J = 7.8 Hz, H-4 Ph), 7.52 (1H, br t, J = 1.8 Hz, H-2 Ph), 7.15 (1H, dd, J = 7.8, 7.8 Hz, H-5 Ph), 6.80 (1H, br d, J = 7.8 Hz, H-6 Ph), 6.66 (1H, s, H-Pym), 2.60 (2H, J = 7.5 Hz, H-6), 2.34 (3H, s, Me-Pym), 2.22 (2H, t, J = 7.5 Hz, H-2), 1.64 (4H, m, H-3 and H-5), 1.39 (2H, m, H-4); ^{13}C NMR (75 MHz, THF- d_8) δ (ppm) 174.6 (CO_2H), 170.1 (C-6 Pym), 161.5 and 161.1 (C-2 and C-4 Pym), 143.8 (C-3 Ph), 141.1 (C-1 Ph), 129.2 (C-5 Ph), 123.1 (C-6 Ph), 120.1 (C-4 Ph), 117.5 (C-2 Ph), 111.0 (C-5 Pym), 36.8 (C-6), 34.3 (C-2), 32.1 (C-5), 29.8 (C-4), 25.8 (C-3), 23.9 (Me-Pym); IR (KBr) $\nu_{\text{max}}/\text{cm}^{-1}$ 3443, 3279, 3109, 2918, 2847, 1701, 1629, 1552, 1491, 1293, 897, 777; HRMS (TOF MS ES+) m/z calcd. for $\text{C}_{17}\text{H}_{21}\text{ClN}_3\text{O}_2$ $[\text{M}+\text{H}]^+$ 334.1322, found 334.1327.

b) Synthesis of Hapten Clp

Scheme S2. Synthetic pathway to prepare hapten Clp

Preparation of N-(4-chloro-6-methylpyrimidin-2-yl)-N-(4-iodophenyl)formamide (21). Compound **21** was synthesized by the same procedure as that used for **15**. Starting from NaH (60% in mineral oil, 42.7 mg, 1.07 mmol, 1.1 equiv.), aryl iodide **20** (287 mg, 1.16 mmol) and **13** (200 mg,

0.97 mmol), compound **21** was obtained as a solid (242 mg, 67%). Mp 178.0-178.8 °C (crystallized from cold hexane-Et₂O). ¹H NMR (300 MHz, CDCl₃) δ (ppm) 9.93 (1H, CHO), 7.80 (2H, apparent dt, part AA' of an AA'BB' system, *J* = 8.7, 2.7 Hz, H-3/H-5 Ph), 6.97 (2H, apparent dt, part BB' of an AA'BB' system, *J* = 8.7, 2.7 Hz, H-2/H-6 Ph), 6.97 (1H, s, H-5 Pym), 2.42 (3H, s, Me-Pym); ¹³C NMR (75 MHz, CDCl₃) δ (ppm) 170.4 (C-6 Pym), 162.7 (CHO), 161.5 (C-2 Pym), 159.0 (C-4 Pym), 138.5 (C-2/C-6 Ph), 135.5 (C-1 Ph), 130.6 (C-3/C-5 Ph), 116.6 (C-5 Pym), 94.0 (C-4 Ph), 24.0 (Me-Pym); IR (KBr) $\nu_{\text{max}}/\text{cm}^{-1}$ 3426, 3076, 1693, 1572, 1543, 1365, 2191, 1120, 1012, 763; HRMS (TOF MS ES+) *m/z* calcd. for C₁₂H₉ClIN₃NaO₂ [M+Na]⁺ 395.9377, found 395.9388.

Preparation of tert-butyl 6-(4-(N-(4-chloro-6-methylpyrimidin-2-yl)formamido)phenyl)hex-5-ynoate (22). This compound was prepared following the same procedure as described for compound **17**. Starting from Cul (0.8 mg, 0.0042 mmol), (Ph₃P)₂PdCl₂ (8.8 mg, 0.012 mmol), aryl iodide **21** (160 mg, 0.428 mmol), *tert*-butyl hex-5-ynoate (**16**, 108 mg, 0.640 mmol), DMF (1.5 mL) and Et₃N (1.5 mL), aryl alkyne **22** (139 mg, 78%) was obtained as a solid. Mp 89.2-90.4 °C (crystallized from cold hexane-Et₂O). ¹H NMR (300 MHz, CDCl₃) δ (ppm) 9.93 (1H, CHO), 7.49 (2H, apparent dt, part AA' of an AA'BB' system, *J* = 8.7, 2.7 Hz, H-3/H-5 Ph), 7.12 (2H, apparent dt, part BB' of an AA'BB' system, *J* = 8.7, 2.7 Hz, H-2/H-6 Ph), 6.96 (1H, s, H-5 Pym), 2.48 (2H, *J* = 7.2 Hz, H-4), 2.41 (2H, t, *J* = 7.5 Hz, H-2), 2.40 (3H, s, Me-Pym), 1.89 (2H, quint, *J* = 7.5 Hz, H-3), 1.46 (9H, s, CMe₃); ¹³C NMR (75 MHz, CDCl₃) δ (ppm) 172.5 (CO₂), 170.3 (C-6 Pym), 162.8 (CHO), 161.5 (C-2 Pym), 159.2 (C-4 Pym), 135.0 (C-4 Ph), 132.5 (C-2/C-6 Ph), 128.5 (C-3/C-5 Ph), 124.0 (C-1 Ph), 116.5 (C-5 Pym), 90.1 (C-5), 80.7 (C-6), 80.3 (CMe₃), 34.4 (C-2), 28.1 (CMe₃), 24.1 (C-3), 23.9 (Me-Pym), 18.8 (C-4); IR (KBr) $\nu_{\text{max}}/\text{cm}^{-1}$ 2979, 2932, 2360, 1710, 1570, 1548, 1369, 1159, 1279, 1116, 775; HRMS (TOF MS ES+) *m/z* calcd. for C₂₂H₂₄ClN₃NaO₂ [M+Na]⁺ 436.1404, found 436.1407.

Preparation of tert-butyl 6-(4-((4-chloro-6-methylpyrimidin-2-yl)amino)phenyl)hexanoate (23). The transformation of aryl alkyne **22** (105 mg, 0.253 mmol) into **23** (76 mg, 77%) was undertaken following the procedure described for the transformation of **17** into **18** using PtO₂ (9 mg) and EtOH (3.7 mL). A small amount of the *N*-formylated derivative **24** (7 mg, 7%) was also obtained from the same reaction. Data for compound **23**: Mp 70.7-71.8 °C (crystallized from cold hexane-Et₂O). ¹H NMR (300 MHz, CDCl₃) δ (ppm) 7.50 (2H, br d, *J* = 8.5 Hz, H-3/H-5 Ph), 7.13 (2H, br d, *J* = 8.5 Hz, H-2/H-6 Ph), 7.06 (1H, br s, NH), 6.61 (1H, s, H-5 Pym), 2.58 (2H, *J* = 7.5 Hz, H-6), 2.39 (3H, s, Me-Pym), 2.20 (2H, t, *J* = 7.5 Hz, H-2), 1.61 (4H, m, H-3 and H-5), 1.43 (9H, s, CMe₃), 1.35 (2H, m, H-4); ¹³C NMR (75 MHz, CDCl₃) δ (ppm) 173.2 (CO₂), 169.7 (C-6 Pym), 161.0 (C-2), 159.5 (C-4 Pym), 137.4 (C-1 Ph), 136.4 (C-4 Ph), 128.8 (C-2/C-6 Ph), 119.4 (C-3/C-5 Ph), 111.1 (C-5 Pym), 80.0 (CMe₃), 35.5 (C-6), 35.1 (C-2), 31.2 (C-3), 28.6 (C-5), 28.1 (CMe₃), 24.9 (C-4), 23.9 (Me-Pym); IR (KBr) $\nu_{\text{max}}/\text{cm}^{-1}$ 3421, 3356, 2971, 2923, 1719, 1529, 1413,

13676, 1151, 1121, 796; HRMS (TOF MS ES+) m/z calcd. for $C_{21}H_{29}ClN_3O_2$ $[M+H]^+$ 390.1948, found 390.1950.

Preparation of 6-(4-((4-chloro-6-methylpyrimidin-2-yl)amino)phenyl)hexanoic acid (Hapten Clp). Hydrolysis of the *tert*-butyl ester moiety of **23** (31.5 mg, 0.081 mmol) to give hapten Clp (25.6 mg, 95%) was realized as described above for the same transformation of **18** into hapten Clm. Data for hapten Clp: Mp 156-158 °C (crystallized from MeOH). 1H NMR (300 MHz, THF- d_8) δ (ppm) 8.94 (1H, br s, NH), 7.62 (2H, br d, $J = 8.4$ Hz, H-3/H-5 Ph), 7.09 (2H, br d, $J = 8.4$ Hz, H-2/H-6 Ph), 6.64 (1H, s, H-5 Pym), 2.57 (2H, $J = 7.5$ Hz, H-6), 2.33 (3H, s, Me-Pym), 2.22 (2H, t, $J = 7.5$ Hz, H-2), 1.62 (4H, m, H-3 and H-5), 1.37 (2H, m, H-4); ^{13}C NMR (75 MHz, THF- d_8) δ (ppm) 174.6 (CO_2), 170.6 (C-6 Pym), 161.5 and 161.1 (C-2 and C-4 Pym), 138.8 (C-1 Ph), 137.2 (C-4 Ph), 129.2 (C-2/C-6 Ph), 120.1 (C-3/C-5 Ph), 110.9 (C-5 Pym), 36.1 (C-6), 34.3 (C-2), 32.4 (C-5), 29.8 (C-4), 25.8 (C-3), 23.9 (Me-Pym); IR (KBr) ν_{max}/cm^{-1} 3431, 3290, 3118, 2922, 1701, 1613, 1559, 1509, 1295, 900, 720; HRMS (TOF MS ES+) m/z calcd. for $C_{17}H_{21}ClN_3O_2$ $[M+H]^+$ 334.1322, found 334.1327.

Table S1

Assay parameters obtained from the checkerboard characterization of cyprodinil antibodies using homologous and linker site-heterologous conjugates.

Ab	Assay hapten	Direct cELISA					Indirect cELISA				
		Ab dilution ($\times 10^3$)	[HRP-hapten] ($\mu\text{g/L}$)	A_{max}	Slope	IC_{50} ($\mu\text{g/L}$)	Ab dilution ($\times 10^3$)	[OVA-hapten] ($\mu\text{g/L}$)	A_{max}	Slope	IC_{50} ($\mu\text{g/L}$)
CDp#1	CDp	30	10	1.68	0.55	4.03	10	10	0.93	0.58	3.88
	CDm	30	10	1.74	0.56	0.68	30	100	1.45	0.69	3.36
	CDn	10	300	--- ^a			3	1000	---		
	CDb	10	300	---			10	1000	1.31	0.65	5.25
CDp#2	CDp	30	3	0.92	0.64	1.76	10	10	1.10	0.64	3.30
	CDm	30	3	1.03	0.72	0.38	100	100	1.12	0.64	1.96
	CDn	10	300	---			3	1000	---		
	CDb	10	300	---			10	100	1.09	0.95	0.17
CDm#1	CDp	30	10	1.65	0.49	2.52	100	100	1.26	0.48	8.20
	CDm	30	10	1.53	0.52	2.93	300	100	0.84	0.48	13.54
	CDn	3	300	---			3	1000	---		
	CDb	30	100	0.73	0.58	0.90	100	1000	0.92	0.47	3.99
CDm#2	CDp	3	10	1.52	0.67	2.82	100	100	1.24	0.56	6.80
	CDm	30	3	0.81	0.62	2.01	300	100	0.96	0.52	5.75
	CDn	10	300	---			3	1000	---		
	CDb	30	300	1.39	0.45	0.99	100	1000	0.90	0.51	9.91
CDn#1	CDp	10	300	---			3	1000	1.37	0.40	n.i. ^b
	CDm	3	300	---			3	1000	0.52	1.12	n.i.
	CDn	30	10	1.30	0.59	58.92	30	10	1.32	0.39	n.i.
	CDb	3	300	1.15	0.60	17.39	3	100	1.11	0.66	7.91
CDn#2	CDp	30	300	---			3	1000	1.25	0.63	n.i.
	CDm	3	300	---			3	1000	0.68	1.02	83.81
	CDn	30	10	1.23	0.69	62.75	30	10	1.48	0.88	n.i.
	CDb	3	300	---			3	100	1.15	0.67	14.96
CDb#1	CDp	3	10	1.41	0.78	1.04	10	100	1.16	0.73	2.07
	CDm	10	10	1.36	0.94	0.65	10	100	1.04	0.62	2.59
	CDn	10	3	0.84	0.80	1.13	3	1000	0.92	0.72	6.94
	CDb	3	3	1.25	0.66	1.31	100	100	1.49	0.55	5.95
CDb#2	CDp	10	300	---			10	1000	0.84	0.58	11.99
	CDm	3	300	---			3	1000	1.05	0.54	50.35
	CDn	30	300	---			3	100	0.87	0.98	2.48
	CDb	3	10	1.39	0.73	0.81	10	10	1.04	0.63	7.07

^a Absorbance lower than 0.5 units. ^b IC_{50} value was higher than 100 $\mu\text{g/L}$.

Table S2

Assay parameters obtained for the checkerboard characterization of cyprodinil antibodies using moiety-heterologous conjugates.

Ab	Assay hapten	Direct cELISA					Indirect cELISA				
		Ab dilution ($\times 10^3$)	[HRP-hapten] ($\mu\text{g/L}$)	A_{max}	Slope	IC_{50} ($\mu\text{g/L}$)	Ab dilution ($\times 10^3$)	[OVA-hapten] ($\mu\text{g/L}$)	A_{max}	Slope	IC_{50} ($\mu\text{g/L}$)
CDp#1	Clp	10	10	1.21	0.52	1.22	10	10	1.41	0.58	2.66
CDp#2		30	100	1.36	0.88	2.01	30	10	1.18	0.59	0.68
CDm#1		10	30	1.82	0.67	0.92	10	10	1.11	0.54	0.74
CDm#2		10	10	0.84	0.73	1.98	10	10	1.51	0.65	0.59
CDn#1		3	300	---			3	1000	---		
CDn#2		3	300	---			3	1000	---		
CDb#1		3	300	0.71	0.72	2.66	3	100	1.70	0.93	12.32
CDb#2		3	300	---			10	1000	0.83	0.60	6.85
CDp#1	Clm	10	30	0.95	0.55	0.29	30	100	1.25	0.62	0.99
CDp#2		10	30	1.40	0.85	0.13	30	100	1.85	0.69	0.59
CDm#1		10	10	1.03	0.75	1.80	100	100	1.60	0.50	15.07
CDm#2		30	30	1.35	1.02	3.60	30	10	1.05	0.63	2.57
CDn#1		3	300	---			3	1000	1.31	0.86	10.54
CDn#2		3	300	---			3	1000	---		
CDb#1		3	300	---			10	100	1.08	0.31	0.88
CDb#2		3	300	---			10	1000	0.53	0.80	7.75

^a Absorbance lower than 0.5 units.

Fig. S1. Radar line plots of cross-reactivity values (%) for pyrimethanil (grey) and mepanipyrim (ochre) by homologous indirect cELISA obtained with each pair of antibodies generated from every immunizing hapten.

Fig. S2. Inhibition curves of the proposed direct and indirect cELISAs obtained with different dilutions of white (A), red (B), and sparkling (C) wine, and cider (D).

Copies of ^1H NMR spectra of haptens

^1H NMR spectrum of hapten CD*b* (300 MHz, DMSO- d_6)

^1H NMR spectrum of hapten CDm (300 MHz, DMSO- d_6)

^1H NMR spectrum of hapten Clm (300 MHz, THF- d_8)

¹H NMR spectrum of hapten Clp (300 MHz, THF-d₈)