JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Letter

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Immunoreagents and Competitive Assays to Fludioxonil

ABSTRACT: Fludioxonil is a new-generation fungicide widely used for postharvest fruit protection. The aim of this study was to produce hitherto unreported immunoreagents for Fludioxonil analysis by immunoassay. Derivatives of this agrochemical were synthesized with different linker tethering sites. Those functionalized haptens were activated, and the purified active esters were efficiently conjugated to different carrier proteins for immunogen and assay antigen preparation. Antibodies to Fludioxonil were raised in rabbits, and their selectivity and affinity were characterized, revealing the significance of the linker. Those antibodies were evaluated using homologous and heterologous conjugates by direct and indirect competitive ELISA formats. Finally, a pair of immunoreagents was identified showing an IC_{50} value for Fludioxonil of 5.7 μg/L.

KEYWORDS: *postharvest fungicide, hapten, active ester, immunoreagents, antibody, ELISA*

INTRODUCTION

Research by the agrochemical industry has often been focused on naturally occurring compounds to find novel active principles with adequate biocide activity and lower environmental and human health effects. With that aim, phenylpyrroles were investigated, and a synthetic derivative of pyrrolnitrin, an antibiotic produced by a number of *Pseudomonas* species, was launched as a last-generation fungicide.[1,2] This molecule was registered, in the mid 1990s, under the name Fludioxonil for postharvest treatment of stone fruit, pome fruit, pomegranate, kiwifruit, and citrus.[3,4] Despite its reduced toxicity to humans, the proximity between application and consumption makes exposure risks a concern. Chromatographic separation techniques coupled to mass spectrometry are commonly applied for Fludioxonil residue analysis.[5,6]

High-affinity antibody production for small chemicals, such as Fludioxonil, demands accurate hapten design to mimic the electronic distribution and molecular conformation of the target analyte.[7,8] Moreover, the main determinant chemical groups should be properly displayed to the immune system upon coupling to a carrier protein through a spacer arm with adequate length.[9] However, the optimum hapten derivatization site is still difficult to predict. The aim of the present study was to prepare different functionalized Fludioxonil derivatives for producing selective and high-affinity antibodies. Generated immunoglobulins and conjugates of Fludioxonil were evaluated by competitive enzyme-linked immunosorbent assay (cELISA) to select the best pair of immunoreagents for future assay development and sample analysis.

MATERIALS AND METHODS

Hapten Synthesis. Functionalized derivatives of Fludioxonil (haptens FD*c* and FD*n*) were prepared as schematized in Figures 1 and 2. Experimental details and spectroscopic characterization data of haptens and synthetic intermediates are provided in the Supporting Information.

Hapten Activation and Purification. Haptens were readily activated by formation of the corresponding *N*-hydroxysuccinimidyl ester (NHS-ester) using *N*,*N*′-disuccinimidyl carbonate (DSC) and triethylamine in dry acetonitrile as previously described.[10] The activated hapten was straightforwardly purified by column chromatography, using $CHCl_3$ as eluent, affording the nearly pure NHS esters as determined by the ^{1}H NMR spectra (see the Supporting Information).

Conjugate Preparation and Antibody Production. Conjugation was carried out following standard procedures as described in the Supporting Information. Hapten-to-protein molar ratios (MR) were calculated from absorbance values of the conjugates and the protein at 280 nm. Animal manipulation was performed in compliance with the laws and guidelines of the Spanish Ministry of Agriculture, Fisheries, and Food, and approved by the Ethics Committee of the Universitat de València. Antibodies were generated in rabbits and purified from the antisera following regular protocols as described in the Supporting Information.

Competitive ELISAs. Assays were performed following common procedures for the antibody-coated direct and the conjugate-coated indirect cELISA formats, as previously published.[11] Mean absorbance values (492 nm) were plotted versus the logarithm of analyte concentration, and assay sensitivity to Fludioxonil was estimated as the analyte concentration reducing by 50% (IC_{50}) the maximum absorbance (A_{max}).

RESULTS AND DISCUSSION

Hapten Synthesis. Two functionalized derivatives of Fludioxonil were prepared, each of them holding the spacer arm at different sites of the pyrrole ring. Hapten FD*n* was easily obtained (overall yield = 77%) from Fludioxonil in two steps, which involved N-alkylation reaction of the pyrrole nitrogen atom with *tert*-butyl 6-bromohexanoate (**1**), to form the N-alkylated Fluodioxonil derivative **2**, followed by acid hydrolysis of the *tert*-butyl ester group (Figure 1).

The synthesis of hapten FD*c* started with the transformation of the nitrile group of Fludioxonil into a carboxylic acid group, which was carried out in two steps. First, the nitrile group was reduced to yield aldehyde **3**, the subsequent oxidation of which provided carboxylic acid **4**. The carboxylated hydrocarbon chain that constituted the spacer arm was incorporated by coupling the carboxyl group of **4** with the amine group of **6**, after derivatization of the carboxyl group to the corresponding *N*-succinimidyl ester, for example, compound **5**. With amide **7** at hand, the synthesis of hapten FD*c* was readily finished by base-catalyzed hydrolysis of the methyl ester group. The complete synthetic route involved five steps and displayed an overall yield of 54% (Figure 2).

Hapten Activation and Conjugation. Hapten succinimidyl esters were readily prepared and purified in high yields (92 and 93% for FD*c*−NHS and FD*n*−NHS, respectively) using

Received: January 13, 2014
Revised: March 14, 2014
Accepted: March 17, 2014
Published: March 17, 2014

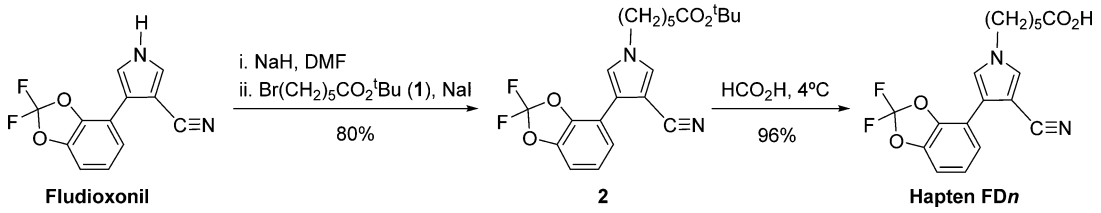

Figure 1. Synthesis of hapten FD*n*.

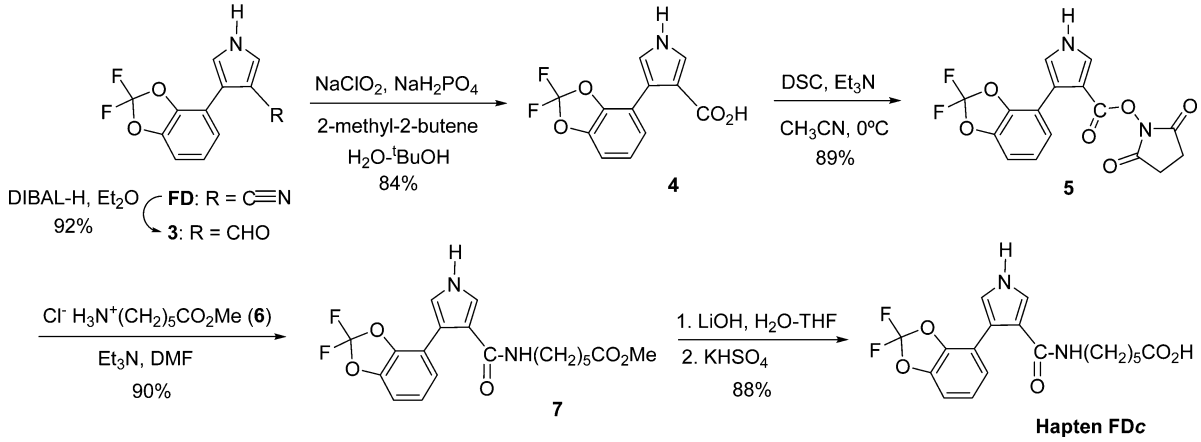

Figure 2. Synthesis of hapten FD*c*. FD, Fludioxonil.

Table 1. Assay Sensitivity in the Direct cELISA Format

	tracer conjugate							
	HRP−FD*c*				HRP−FD*n*			
Ab	[Ab][a]	[C][b]	A_{max}	IC_{50}[c]	[Ab]	[C]	A_{max}	IC_{50}
FD*c*#1	30	3	1.0	1366.4	3	100	0.8	92.0
FD*c*#2	3	10	1.8	1542.6	3	100	nd[d]	
FD*n*#1	3	100	ns		10	3	0.8	22.9
FD*n*#2	3	100	ns		30	10	1.2	129.5

[a]Antibody dilution (×10[3]). [b]Conjugate concentration in ng/mL. [c]Values in nM. [d]Signal was below 0.5.

Table 2. Assay Sensitivity in the Indirect cELISA Format

	coating conjugate							
	OVA−FD*c*				OVA−FD*n*			
Ab	[Ab][a]	[C][b]	A_{max}	IC_{50}[c]	[Ab]	[C]	A_{max}	IC_{50}
FD*c*#1	300	100	1.1	1405.7	10	1000	0.9	88.1
FD*c*#2	100	100	0.8	1329.4	10	1000	0.8	
FD*n*#1	3	1000	ns[d]		30	100	1.5	75.1
FD*n*#2	3	1000	ns		100	100	1.0	372.8

[a]Antibody dilution (×10[3]). [b]Conjugate concentration in ng/mL. [c]Values in nM. [d]Signal was below 0.5.

N,*N*′-disuccinimidyl carbonate. Activated purified haptens were coupled to bovine serum albumin (BSA), ovalbumin (OVA), and horseradish peroxidase (HRP). The amount of employed activated hapten was adjusted according to the desired final hapten density. Thus, high MRs for BSA conjugates (15 and 17 for FD*c* and FD*n*, respectively) and low MRs for OVA conjugates (2 for both haptens) were obtained as intended. On the other hand, calculated hapten densities of HRP conjugates were 6 and 4 for FD*c* and FD*n*, respectively. Unrealistically high MR values for HRP conjugates (this enzyme contains 3−4 available lysine residues) are probably due to modified molar extinction coefficients of the protein and/or the hapten after conjugation. However, adequate enzymatic activity and hapten coupling was demonstrated by competitive assay.

Antibody Affinity and Selectivity. Competitive assays were performed with FD*c*-type antibodies (obtained with BSA−FD*c* as immunizing conjugate) and FD*n*-type antibodies (from BSA−FD*n* immunized animals), using homologous conjugates (same hapten as the immunizing conjugate) and following the direct and indirect cELISA procedures. Antibody affinity was assessed by checkerboard competitive assays, running a standard curve of Fludioxonil in each microplate column (from 10 pM to 10 μM plus a blank) and assaying diverse antibody and antigen concentrations simultaneously as described in the Supporting Information. The lowest IC_{50} values with homologous

dx.doi.org/10.1021/jf5001716 | J. Agric. Food Chem. 2014, 62, 2742−2744

conjugates were obtained with antibodies from FD*n*-immunized rabbits in both cELISA formats (Tables 1 and 2). Thus, hapten FD*n* behaved better as immunogen than hapten FD*c*, so it was a better mimic of Fludioxonil. Hapten FD*n* keeps unmodified all moieties of the target molecule, and only an N−H bond is substituted by an N−C bond. Comparatively, in hapten FD*c*, the cyano group modification *probably* alters more significantly the electronic properties of the pyrrole moiety (Figures 1 and 2). Moreover, the linker in hapten FD*n* is located further to the bulky aryl moiety than in hapten FD*c*, which probably favors the aromatic portion of this hapten adopting similar conformations to those adopted by the common framework of parent Fludioxonil (see Figure S1 of Supporting Information).

With regard to selectivity, binding of the four antibodies to additional new-generation fungicides that are commonly formulated together with Fludioxonil was assessed, such as cyprodinil, pyrimethanil, mepanipyrim, azoxystrobin, boscalid, and fenhexamid. As expected, no inhibition was observed by any of the studied compounds at a concentration of 10^4 nM with any of the described antibodies.

Competitive Immunoassays. Checkerboard assays were performed with both FD*c*- and FD*n*-type antibodies using also the heterologous conjugates. Concerning the direct cELISAs, antibody FD*c*#1 bound the heterologous tracer HRP−FD*n*, and a moderate IC_{50} value to Fludioxonil was found in this case, improving the IC_{50} value that was obtained with the homologous combination. However, FD*n*-type antibodies recognized only the homologous tracer. Parallel results were observed with the indirect format (Table 2). For a particular antibody/antigen combination, the IC_{50} values stayed in the same order of magnitude in both cELISA formats. Overall, the most sensitive assay was achieved with antibody FD*n*#1 using the homologous combination. The inhibition curve of such an assay is depicted in Figure S2 in the Supporting Information, showing an IC_{50} value for Fludioxonil of 22.9 nM (equivalent to 5.7 μg/L) and an A_{max} of 0.8 absorbance unit.

Antonio Abad-Fuentes[†]
Consuelo Agulló[§]
Francesc A. Esteve-Turrillas[†]
Antonio Abad-Somovilla[§]
Josep V. Mercader*[,†]

[†]Department of Biotechnology, Institute of Agrochemistry and Food Technology, Consejo Superior de Investigaciones Científicas (IATA-CSIC), Agustí Escardino 7, 46980 Paterna, València, Spain

[§]Department of Organic Chemistry, Universitat de València, Doctor Moliner 50, 46100 Burjassot, València, Spain

■ ASSOCIATED CONTENT

Supporting Information

Synthesis details and characterization data of intermediates, haptens, and active esters; equipment, immunoreagent production, ELISA procedures, Fludioxonil most-stable conformations, and inhibition curve. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*(J.V.M.) Phone: +34-963900022. Fax: +34-963636301. E-mail: jvmercader@iata.csic.es.

Funding

This work was supported by the Spanish Ministerio de Ciencia e Innovación (MICINN) (AGL2009-12940-C02-01/02/ALI) and cofinanced by FEDER funds. F.A.E.-T. and J.V.M. were hired by CSIC with postdoctoral contracts, the former under the JAE-doc program and the latter under the Ramón y Cajal program, both cofinanced by MICINN and by the European Social Fund (ESF).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Ana Izquierdo-Gil, Laura López-Sánchez, and Laura Vila-Dasí for excellent technical assistance.

■ ABBREVIATIONS USED

BSA, bovine serum albumin; cELISA, competitive enzyme-linked immunosorbent assay; DSC, *N,N′*-disuccinimidyl carbonate; HRP, horseradish peroxidase; MR, molar ratios; OVA, ovalbumin

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