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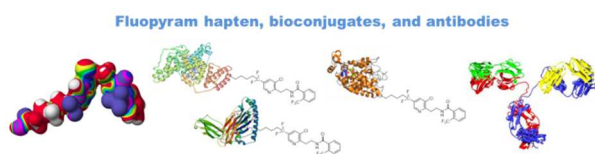


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Graphical abstract

A fluopyram mimicking hapten was designed, immunochemically active bioconjugates were produced and high-affinity and specific antibodies to fluopyram were generated.



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Rational design of a fluopyram hapten and preparation of bioconjugates and antibodies for immunoanalysis†

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E. Ceballos-Alcantarilla,^a C. Agulló,^a A. Abad-Fuentes,^b A. Abad-Somovilla,^{a,*} and J. V. Mercader^{b,*}

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A fluopyram hapten was designed in which insignificant electronic and structural modifications were foreseen and all potentially interacting chemical moieties were maintained. This hapten was prepared by total synthesis and three immunologically active bioconjugates were obtained and characterized. High-affinity and specific antibodies to fluopyram were raised.

Small organic chemicals constitute particular immunochemical targets because they are haptens from the immunological point of view. This means that a mimic of the analyte needs to be covalently coupled to a carrier macromolecule in order to generate antibodies by animal immunization. Most commonly, functionalized derivatives of the target compound holding a spacer arm are prepared in the laboratory because no reactive chemical groups are available in the molecule, or just because it is not advisable to modify potential antigenic determinants. The resulting synthetic hapten should preserve as much as possible the electronic and conformational properties of the target compound. Linear saturated aliphatic bridges with 3 to 6 carbon atoms are generally preferred for minimum physicochemical and antigenic interferences over the hapten or the immune system, respectively. Unfortunately, the optimum linker tethering site for immunizing bioconjugate preparation is difficult to predict.¹ Substitution of a C–H bond by a C–C bond in hapten synthesis has afforded excellent immunochemical binders,² though other approaches have also been successful.³ From our experience, the spacer should be better placed at the edge of molecular longitudinal axis, at distal positions from characteristic chemical moieties. On the contrary, positions next to highly antigenic groups or central

locations with respect to the molecular longitudinal axis seem to be detrimental for antibody affinity and specificity.⁴ Furthermore, introduction at the right position of a hydrocarbon arm holding a reactive group is not so straightforward for many compounds, thus total synthesis is usually compulsory in such cases. For coupling to carrier proteins, carboxyl is the preferred moiety because it is a relatively robust group, compatible with a variety of reaction conditions, and it is readily activated to react in aqueous media with primary amine groups which are usually abundant in proteins as lysine side chains. However, severe molecular charge changes could destabilize the resulting bioconjugate, so moderate hapten densities are recommended.

Succinate dehydrogenase, also called respiratory complex II, is a fundamental constituent of the mitochondrial electron transport chain and the tricarboxylic acid cycle of fungi. Since the late 1960s, succinate dehydrogenase inhibitors (SDHI) have been employed to fight fungal diseases.⁵ In recent years, new-generation SDHI fungicides with extended biocide properties have been developed. Novel SDHIs include fluopyram, penthiopyrad, fluxapyroxad, and boscalid which are characterized by a higher efficiency and a broader spectrum of anti-mycotic activity. Fluopyram was first commercially registered in the United States in 2012⁶ and approved in the

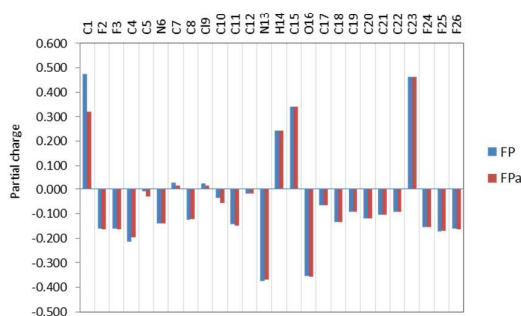
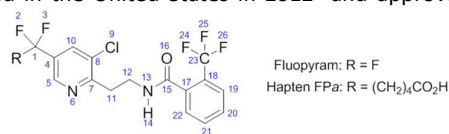


Fig. 1. Computed partial atomic charges of fluopyram and hapten FPα. Only those atoms that are common to both structures are depicted. Hydrogen atoms are not included with the exception of the amide hydrogen (H14). The numbering in the figure does not reflect the systematic IUPAC numbering.

^a Department of Organic Chemistry, Universitat de València, Doctor Moliner 50, 46100 Burjassot, València, Spain.

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

* Corresponding authors. Email: antonio.abad@uv.es (A. Abad-Somovilla) jmercader@iata.csic.es (J. V. Mercader).

† Electronic supplementary information (ESI) available: Materials and instruments; hapten synthesis, activation, and coupling protocols; spectroscopic data; MALDI spectra of bioconjugates; antibody generation and competitive ELISA procedures; and copies of the ¹H NMR spectra. See DOI: 10.1039/x0xx00000x

European Union in 2014.⁷ Structurally, it is characterized by two trifluoromethyl substituents and a diaryl aromatic system connected by a propanamide bridge (Fig. 1). Since no active chemical groups are available for conjugation to carrier macromolecules, introduction of a functional spacer arm is compulsory in order to prepare immunogenic and antigenic fluopyram bioconjugates.

The aim of the present study was to evaluate the capacity of a fluopyram derivative, obtained by total synthesis, to elicit high-affinity and specific antibodies. Conformational studies together with electronic analysis were carried out for hapten suitability assessment. Immunochemically active biomolecules were prepared using the purified active ester of the hapten in order to better modulate the final hapten-to-protein molar ratio. The immune response to a synthetic bioconjugate was assessed from rabbit antisera collected at different stages of immunization. Polyclonal antibodies were partially purified and the affinity and specificity to fluopyram was estimated by competitive enzyme-linked immunosorbent assay (cELISA), using the antibody-coated direct and the conjugate-coated indirect formats.

Molecular modelling techniques have been explored by several research groups for hapten design.⁸ In order to introduce minimum electronic and structural modifications to the fluopyram framework, a novel approach was envisaged, *i.e.*, substitution of a C–F bond by a C–C bond. An analogous molecule was designed (hapten FP α , Fig. 1), wherein the hydrocarbon chain constituting the spacer arm was incorporated via formal replacement of a trifluoromethyl fluorine atom. Such modification of the trifluoromethyl moiety had a limited effect on the electronic distribution on the fluopyram molecule – only a significant alteration on the directly involved carbon atom (C1) was observed (Fig. 1). Moreover, the linker was located at the edge of the molecular longitudinal axis, such that, presumably, it did not hamper the fluopyram framework in the hapten to cover the same conformational space as the fluopyram molecule itself (Fig. 2). In principle, other aspects that are fundamental for the generation of the antibody binding pocket, which are hydrophobic interactions and low-energy interactions such as

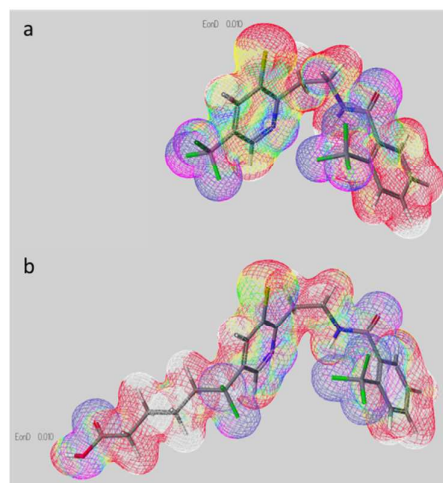


Fig. 2. a) Electron density surface colored by electrostatic potential of the most stable conformation of fluopyram. Color alteration from red to blue describes the shift from an electron-poor region to an electron-rich one. The energy values (in atomic units, au) at each color interface are: white-red, +0.09 au; red-yellow, +0.02 au; yellow-green, +0.01 au; green-light blue, 0.00 au; light blue-dark blue, -0.01 au; dark blue-pink, -0.03 au; pink-violet, -0.06 au; where 1 au = 627.503 kcal mol⁻¹. Calculations were performed using Molecular Mechanics (MM3) as implemented in the CACHE program [CACHE WorkSystem Pro software, version 7.5.0.85 (Fujitsu Ltd., Tokyo, Japan)]. A systematic conformational search was performed (all rotatable bonds were rotated by 24 degree steps) and the geometry of the generated conformers was refined by performing an optimized geometry calculation in MOPAC using PM3 parameters. b) Equivalent conformer of hapten FP α .

hydrogen bonding, are minimally affected by this structural modification, since the remaining difluoromethyl group (CF₂) shows a high lipophilicity,⁹ similar to the CF₃ group, and it is also able to act as a weak hydrogen acceptor through hydrogen bonding.¹⁰

The synthesis of hapten FP α is outlined in Scheme 1. It was based on the initial preparation of a dichloropyridine bearing a lineal keto-carboxylate chain at the C-3 position of the pyridine ring (compound **4**). Its preparation was carried out by a Pd-catalyzed cross-coupling reaction¹¹ between nicotinoyl chloride **1** and the functionalized alkylzinc reagent **3**, which was readily prepared immediately before use by reaction of methyl 5-iodopentanoate¹² and activated zinc dust in the presence of 1,3-dimethyl-2-imidazolinone. Next, the keto-carbonyl group of **4** was transformed into the corresponding

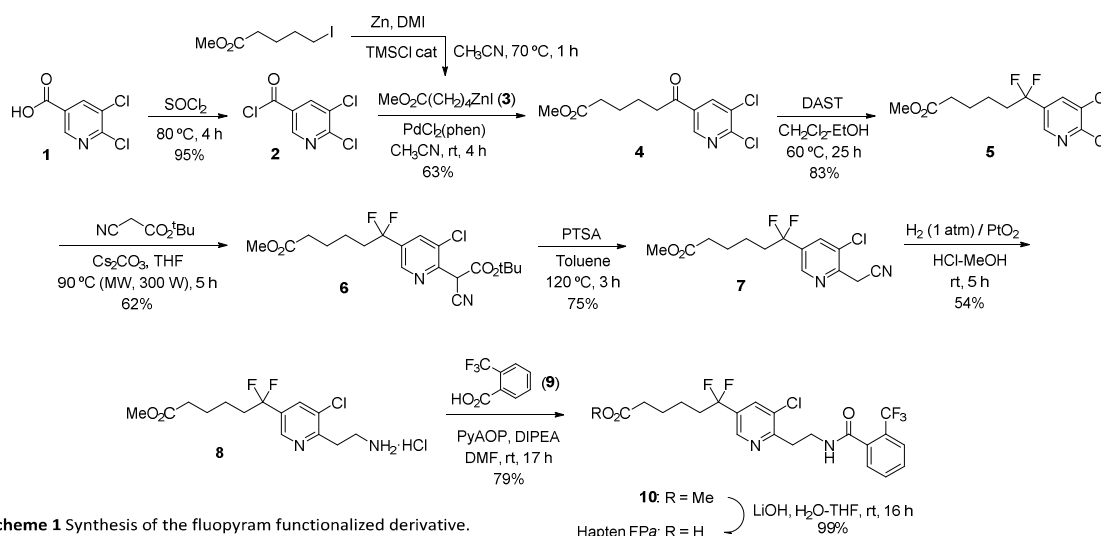


Table 1 Checkerboard cELISA with antisera collected after the third and the fourth injection.

Rabbit	Third injection						Fourth injection					
	Direct			Indirect			Direct			Indirect		
	[As] ^a	[HRP] ^b	IC ₅₀ ^c	[As]	[OVA]	IC ₅₀	[As]	[HRP]	IC ₅₀	[As]	[OVA]	IC ₅₀
#1	10	300	38.8	30	100	103.0	10	100	6.5	30	100	26.0
#2	10	100	6.1	10	100	22.7	10	30	3.4	30	100	12.0

^a Antiserum dilution $\times 10^3$. ^b Assay bioconjugate concentration in ng mL^{-1} . ^c Values are in nM.

gem-difluoro group using the electrophilic fluorinating agent diethylaminosulfur trifluoride, thus completing the hapten linking arm. Microwave-promoted regioselective aromatic nucleophilic substitution at the C-6 position of dichloropyridine **5** with *tert*-butyl cyanoacetate in the presence of cesium carbonate, followed by decarboxylation under acidic conditions, afforded the substituted pyridin-acetonitrile **7** that was transformed into the amino hydrochloride **8** via catalytic hydrogenation of the nitrile group in methanolic HCl. The entire carbon framework of the target hapten was completed by a reaction of amidation between the amino group of **8** and the benzoic acid **9** mediated by the phosphonium salt coupling reagent PyAOP.¹³ The synthesis of hapten FP α was finished by hydrolysis of the methyl ester moiety of **10** to the corresponding acid under standard basic conditions. Overall, the synthesis of hapten FP α proceeded in 8 steps with a total yield of ca. 10%. Full experimental synthetic details and spectroscopic data can be found in the ESI[†].

Prior to protein coupling, the synthetic hapten was straightforward activated and readily purified. Active ester formation was carried out with *N,N*-disuccinimidyl carbonate during 4 h at room temperature. This scarcely used approach in immunochemistry¹⁴ affords high synthetic yields and the corresponding ester can be easily purified due to the absence of relevant by-products. An adapted procedure of previous studies was followed.¹⁵ The reaction mixture was cleaned-up by liquid-liquid extraction affording the *N*-hydroxysuccinimide ester of hapten FP α in nearly quantitative yield. Activation protocol and spectra of the corresponding succinimidyl active ester can be found in the ESI[†]. As a consequence of using pure activated hapten, high-yield coupling reactions to proteins with minute amounts of hapten could be performed at room temperature in just 2 h. Moreover, no secondary undesirable reactions occurred, thus the same hapten activation strategy could be applied for preparing all bioconjugates. As carrier proteins, bovine serum albumin (BSA), ovalbumin (OVA), and horseradish peroxidase (HRP) for immunogen, coating antigen, and enzyme tracer preparation were respectively employed. The resulting bioconjugates were purified by size exclusion chromatography. Highly suitable hapten-to-protein molar ratios of ca. 16, 4, and 1 for BSA-FP α , OVA-FP α , and HRP-FP α conjugates, respectively, were obtained, as determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Complete coupling procedures and MALDI mass spectrometry analysis (Fig. S1) of the prepared biomolecules are included in the ESI[†].

Two rabbits were immunized with 300 μg of BSA bioconjugate and antisera were obtained following established procedures¹⁶ in compliance with the laws and guidelines of the Spanish

Ministry of Agriculture, Food, and Environment, and approved by the Ethics Committee of the University of Valencia (permit number: A1329731961154). Blood samples were taken from the ear vein after the third injection, and total sera were collected 10 days after the final boost. Immunoglobulins were partially purified by double salting out with saturated ammonium sulphate. Immunization was confirmed and antibodies were characterized by checkerboard antibody-coated direct and antigen-coated indirect cELISA (see immunoassay protocols in the ESI[†]). Standards, including a blank, were prepared in borosilicate glass vials by serially diluting fluopyram in 10 mM phosphate buffer, pH 7.4, containing 140 mM NaCl, starting from a 10 μM fluopyram solution in the same buffer. Coated microplates at 3×10^3 -, 1×10^4 -, or 3×10^4 -fold antibody dilution and 100 or 1000 ng mL^{-1} bioconjugate concentration were prepared. A standard curve was run in each plate column, and increasing tracer concentrations (from 3 to 300 ng mL^{-1}) or antibody dilutions (from 3×10^3 -fold to 3×10^5 -fold) were evaluated for direct and indirect cELISA, respectively, in different plate columns. Therefore, a series of inhibition curves with varying maximum absorbance values at zero dose of analyte (A_{max}) were obtained for each antibody in every assay format. Mean absorbance values were plotted against the logarithm of fluopyram concentration and experimental data were fitted to a four-parameter logistic equation using the SigmaPlot software, version 12.5 (Systat Software Inc., Chicago, IL, USA). The concentration of fluopyram affording a 50% inhibition of the maximum signal (IC₅₀) was taken as an estimation of antibody affinity.

The immunogenic capacity of the prepared immunoreagents was assessed by checkerboard cELISA employing bioconjugates OVA-FP α and HRP-FP α as counterpart antigen, depending on the assay format as described above. Antisera were sampled after the third injection, and titres and affinities were determined. At this stage, regular antisera titres ($1/10^4$ or $1/3 \times 10^4$) were found in order to achieve A_{max} values between 0.8 and 1.5 at zero dose of analyte, and IC₅₀ values were acceptable, or even better than usual for antiserum #2 with the direct cELISA (Table 1). Besides, the immune response was evaluated after the fourth injection. As expected, titres were similar to those of third-injection blood samples but the affinity of both antisera had significantly increased. Now, the generated antisera showed IC₅₀ values in the low nanomolar range, particularly with the direct immunoassay format. Antibody FP α #2 showed the highest affinity to fluopyram (IC₅₀ = 3.4 nM). The inhibition curve for fluopyram using antibody FP α #2 and tracer HRP-FP α obtained by direct cELISA can be seen in Fig. S2. Moreover, specificity of both antisera was

studied and no cross-reactivity was observed with other fungicides of the same family (penthiopyrad and flupyroxad), fungicides with structural similarities (fluopicolide and trifloxystrobin), and the main metabolite, *i.e.* 2-(trifluoromethyl)-benzamide, commonly referred to as fluopyram-benzamide or M25.¹⁷

Conclusions

As far as we know, these are the first ever reported bioconjugates and antibodies for the new-generation fungicide fluopyram. Rational hapten design and total synthesis was carried out in order to obtain a suitable fluopyram derivative. A single, straightforward, and high-yield coupling strategy using pure active ester was applied for immunogenic and antigenic biomolecule preparation. Our results confirm the adequacy of the hapten functionalization strategy by substitution of a C–F bond by a C–C bond, and the competence of the produced bioconjugates for high-affinity and specific antibody generation – as predicted by computational evaluation and MALDI–TOF–MS analysis – as well as for antigenic application. As preliminary results, the immunosensing capability of the obtained antibodies has been studied.

Acknowledgements

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